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Agathe Pirog

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Agathe Pirog. Structure génétique des populations et biologie de la reproduction chez le requin bouledogue *Carcharhinus leucas* et le requin tigre *Galeocerdo cuvier*. Ecologie, Environnement. Université de la Réunion, 2018. Français. NNT : 2018LARE0008 . tel-01879924

HAL Id: tel-01879924

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Submitted on 24 Sep 2018

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Université de La Réunion

Ecole doctorale Sciences, Technologies, Santé E.D. n°542

UMR Ecologie marine TROPicale des océans Pacifique et IndiEn (ENTROPIE)

THÈSE

Présentée pour l'obtention du grade de docteur de l'Université de La Réunion

Discipline : Écologie marine

Structure génétique des populations et biologie de la reproduction chez le requin bouledogue *Carcharhinus leucas* et le requin tigre *Galeocerdo cuvier*

Agathe PIROG

Soutenue le 29 juin 2018 devant le jury composé de :

Aletta E. BESTER-VAN DER MERWE	<i>Senior Lecturer</i> University of Stellenbosch	Rapporteur
Stefano MONA	<i>Maitre de Conférences HDR</i> Ecole Pratique des Hautes Etudes	Rapporteur
Henrich BRUGGEMANN	<i>Professeur</i> Université de La Réunion	Examineur
Claudie DOUMS	<i>Professeur</i> Ecole Pratique des Hautes Etudes	Examineur
Sébastien JAQUEMET	<i>Professeur</i> Université de La Réunion	Co-directeur de thèse
Hélène MAGALON	<i>Maitre de Conférences HDR</i> Université de La Réunion	Co-directeur de thèse

Résumé

Les grands requins jouent, en tant que prédateurs apicaux, un rôle important dans la structuration et le fonctionnement des communautés marines. La compréhension de la structure et de la dynamique de leurs populations reste limitée alors qu'ils sont particulièrement vulnérables aux pressions anthropiques. Ils peuvent par ailleurs interagir avec les activités humaines en milieu littoral, conduisant parfois à des attaques, ce qui est notamment le cas à La Réunion, particulièrement depuis 2011. Il est donc primordial d'améliorer nos connaissances sur ces espèces afin de définir des plans de gestion durable.

Cette thèse porte ainsi sur deux espèces de grands requins de la famille des Carcharhinidés, le requin bouledogue *Carcharhinus leucas* et le requin tigre *Galeocerdo cuvier*. Ainsi, les objectifs de cette thèse sont d'étudier (1) la structure et la connectivité génétique de leurs populations, (2) la diversité génétique et la taille efficace des populations ainsi identifiées et (3) les modes de reproduction de ces deux espèces. Les deux premiers objectifs ont été abordés grâce au cadre théorique de la génétique des populations (tests d'assignement, indices de différenciation génétique, réseaux d'haplotypes mitochondriaux, calcul bayésien approché) couplé à un échantillonnage dans deux régions : l'Ouest de l'océan Indien et l'Ouest du Pacifique. Le dernier objectif a consisté en l'analyse des traits biologiques de requins pêchés à La Réunion, afin de décrire certains paramètres de la reproduction pour chaque espèce.

Une différenciation génétique importante a ainsi été identifiée entre les populations de requin bouledogue des deux régions étudiées, reflétant soit une absence totale de flux de gènes contemporains, soit des flux de gènes uniquement assurés par les mâles. À l'inverse, les populations de requin tigre de ces deux régions sont homogènes génétiquement, avec des flux de gènes importants. Par ailleurs, au sein de chacune de ces régions, aucune différenciation génétique n'a été identifiée pour ces deux espèces. En outre, une plus faible diversité génétique a été identifiée chez le requin tigre que chez le requin bouledogue, qui pourrait être liée à une diminution forte des effectifs datant de moins de 3 000 ans. Pour le requin bouledogue, la taille efficace estimée des populations délimitées (6 000 à 10 000 individus) est similaire à celles trouvées pour d'autres espèces de requin. Autour de La Réunion, les populations des requins bouledogue et tigre suivent des dynamiques bien différentes, liées à leurs modes de reproduction. Chez le requin bouledogue, les individus des deux sexes semblent fidèles à des zones côtières particulières (philopatrie) pour s'accoupler et/ou mettre bas, et les portées sont fréquemment issues de plusieurs pères (polyandrie). À l'inverse, les zones d'accouplement et de mise bas du requin tigre restent mal connues, et cette espèce semble exclusivement monoandre, caractéristiques liées à sa nature semi-océanique.

Les travaux de cette thèse montrent les capacités de dispersion importantes de ces deux espèces. En outre, leurs populations présentent des dynamiques différentes qui induisent une vulnérabilité différente aux pressions anthropiques. Ces résultats pointent la nécessité d'adopter des mesures de gestion spécifiques à chaque espèce.

Mots clés : structure des populations, taille efficace, requin bouledogue, requin tigre, *Carcharhinus leucas*, *Galeocerdo cuvier*, cycles de reproduction, polyandrie, philopatrie

Abstract

As apex predators, large sharks play key roles in the structuring and functioning of marine communities. Knowledge on the structure and dynamics of their populations remains limited, while they are particularly sensitive to anthropogenic pressures. Furthermore they may interact with human activities in coastal areas, sometimes leading to attacks, as it is the case in Reunion Island notably since 2011. Improving the knowledge on these species is thus essential to define efficient management plans.

This PhD thesis focuses on two large shark species belonging to the Carcharhinidae family, the bull shark *Carcharhinus leucas* and the tiger shark *Galeocerdo cuvier*. The aims are to study (1) the genetic structuring and connectivity of their populations, (2) the genetic diversity and effective population size of the delimited populations and (3) the reproductive modes of both species. The two first objectives were addressed thanks to the theoretical framework of population genetics (assignment tests, genetic differentiation indices, mitochondrial haplotype networks, approximate Bayesian computation) coupled with samples collected within two regions: the Western Indian Ocean and the Western Pacific. For the last objective, biological traits of sharks fished in Reunion Island were analysed to describe some of the reproductive parameters for each species.

A strong genetic differentiation was thus highlighted between bull shark populations from both regions, due to either an absence of contemporary gene flow or to an absence of female gene flow only. On the opposite, tiger shark populations seem genetically homogenous, with important genetic connectivity between both regions. Within each region, no genetic differentiation among localities was highlighted for both species. Furthermore a weaker genetic diversity was identified for the tiger shark, probably linked to the occurrence of a recent bottleneck occurring less than 3,000 years ago. Concerning the bull shark, effective population sizes estimated for each delimited population (6,000 to 10,000 individuals) were in the same range than those estimated for other shark species. Finally, around Reunion Island, bull and tiger shark populations present different dynamics, linked to their reproductive modes. Bull shark individuals from both sexes seem to exhibit some fidelity to specific coastal sites (philopatry) to mate and/or deliver embryos, and litters are frequently issued from several fathers (polyandry). On the opposite, mating and pupping areas of the tiger shark remain poorly known, and this species seems exclusively monoandrous, probably linked to its semi-oceanic nature, lowering encounter probabilities of the mates.

This work thus highlights the high dispersal abilities of both species. Besides their populations present different dynamics, leading to different sensitivities to anthropogenic pressures. These results point out the need to adopt management plans specific to each species.

Keywords: population structure, effective population size, bull shark, tiger shark, *Carcharhinus leucas*, *Galeocerdo cuvier*, reproductive cycles, polyandry, philopatry

Remerciements

Je tiens tout d'abord à remercier les rapporteurs et membres du jury, Aletta E. Bester-Van Der Merwe, Stefano Mona, Henrich Bruggemann et Claudie Doums, d'avoir accepté d'évaluer cette thèse. Je voudrais également remercier la région Réunion pour m'avoir permis de réaliser ce travail en m'attribuant une Allocation de Recherche.

Un grand merci à mes deux directeurs de thèse, Sébastien et Hélène. Merci de m'avoir fait confiance et proposé cette thèse, mais surtout, d'avoir tous les deux été des directeurs présents et toujours disponibles. Seb, j'espère qu'on ne t'aura pas trop embêté avec notre génétique (mais si c'est super intéressant !), nos propositions de titres d'articles (et encore, on est restées soft !) et notre envie de sauver tous les poissons, oiseaux, chiens, chats, concombres de mer et autres consorts. Hélène, merci de m'avoir acceptée en stage, j'espère avoir réussi à te prouver que non, je ne suis pas dilettante. Tu m'auras appris quantité de choses, et bien d'autres que je n'ai pas réussi à assimiler. J'ai beaucoup pleuré, toi aussi, alors ça compense. Merci pour tous les bons moments passés ensemble pendant ces quatre années à La Réunion, et de m'avoir permis de découvrir cette belle île.

Merci aux autres membres de l'UMR Entropie, pour vos conseils et votre aide au cours de ces années. Merci aux habitants du couloir de l'IRD, pour les discussions et les encouragements. Merci également à Virginie Ravigné et Adrien Rieux pour les moments passés au Cirad. Ça a vraiment été une super expérience de travailler avec vous, et vous m'avez fait gagner un temps fou sur toutes ces analyses génétiques. Merci également aux personnes qui m'ont permis de donner des cours, ce fut extrêmement enrichissant. Je pense que c'est aussi grâce à la façon dont vous m'avez soutenue et encadrée que j'y ai pris du plaisir (Johanna, Laurence, Mathilde, Hélène). Merci aussi aux stagiaires (Grégoire, Stéphane, Clément, Nicolas, Solène), nos petits esclaves à nous, vous avez été tellement valorisants pour notre amour propre (j'inclus Pauline dans ce nous, je sais qu'elle l'a vécu de la même façon). Grégoire et Stéphane, nos excursions dans le lagon pour aller chercher nos concombres de mer resteront mes meilleurs souvenirs du terrain. Un seul regret, avoir raté le jour de l'abandon des échantillons sur le toit de la voiture et la tête d'Hélène quand elle l'a su. Nicolas, bonne chance pour la suite, et j'espère que tu pourras la faire cette thèse.

Merci à tous ceux qui m'ont soutenue, à Sandrine, pour les papotages de pause dans la journée, à Thomas, pour avoir accepté de sentir mauvais à ma place (mmmh ce doux fumet de requin pêché depuis 3 jours...). Armelle, tes encouragements du matin ont été des bouffées d'air frais. Merci aussi aux autres doctorants, soit partis vers d'autres horizons, soit qui vont passer par cette étape : Baudisse, Alexis, Clément, Simon, Isa, Florian.

Big up à mes géologues préférés, ma petite Mezon et Marco, vous êtes ouf les gars ! Hé, finalement, ça a eu du bon les doctoriales, hahaha ! Merci pour le soutien indéfectible, until the end !

Popo, ma Popo. On en aura passé du temps dans ce bureau à papoter (non mais pas tant que ça Hélène, on t'assure) de tout, de rien, mais aussi beaucoup de génétique, de ces foutues analyses qui

ne veulent rien dire. Ça aurait été une autre thèse sans toi, et merci d'avoir continué à m'épauler même à distance.

Merci aux autres copains de La Réunion, Yohan et Alina, Anthony, Bhavani, Alicia (ouhla, ça fait beaucoup de cailloux tout ça) et à ceux qui sont déjà partis, Alex, mon petit Jérèm, Chacha, vous êtes loin maintenant mais on se retrouve bientôt ! J'aurais plein de temps pour vous ! Merci aussi aux potes de métropole, vous avez eu des vacances pendant 4 ans, but we will be back soon ! Et pareil, j'aurais plein de temps pour vous aussi !

Merci à la famille, toujours là, même à 11 000 km, et ça c'est beau quand même. Bon, Papa, je pense que tu peux aussi me dire merci pour t'avoir trouvé ta nouvelle île de vie !

Last but not least, merci à mon homme, tu as même failli avoir le droit à une nouvelle page pour toi tout seul (j'ai ajouté d'autres personnes entre temps, désolée) (et même plutôt beaucoup de personnes). Merci d'être un homme moderne, promis à partir de maintenant je refais la vaisselle, les lessives et la bouffe (tu vois, je l'écris même noir sur blanc). Enfin, après la soutenance quoi... Merci d'être là les bons comme les mauvais jours, de prendre sur toi quand je dis « et si on adoptait un chat ? », et de ne même plus rouspéter quand je dis « on peut en prendre un deuxième pour occuper le premier ? ». Je me fatigue moi-même des fois ! Bref, merci, je n'en dirais pas plus.
coin coin

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Avant-propos

Les approches génétiques fournissent un outil unique pour l'étude des populations marines, notamment des Chondrichthyens. Elles permettent notamment, grâce au cadre théorique de la génétique de populations, d'estimer la structure et la connectivité des populations, leurs tailles, mais aussi certains comportements de reproduction. Or, chez les Chondrichthyens en général, et chez les grands requins en particulier, ces paramètres restent encore très méconnus. S'ils ont pu être estimés pour certaines espèces, il semble de plus en plus évident que généraliser ces résultats n'a pas de sens : la diversité de stratégies d'utilisation de l'habitat, de comportements de migration et de reproduction influencent la structure et la taille des populations. Ces animaux présentent de la variabilité individuelle et des spécificités régionales, qui limitent la généralisation des observations réalisées sur des populations ou individus particuliers. Par ailleurs, il est maintenant avéré que ces grands prédateurs jouent des rôles majeurs dans la dynamique des écosystèmes marins, et le déclin de leurs populations dû à des pressions anthropiques pourrait avoir des conséquences encore mal estimées sur la structure et le fonctionnement de ces écosystèmes. Bien que des plans de conservation adaptés doivent donc être mis en place, la gestion de ces grands requins reste difficile, particulièrement pour ceux responsables d'attaques sur l'Homme.

Le but de cette thèse est donc d'améliorer, grâce à l'utilisation d'outils moléculaires notamment, les connaissances sur la biologie du requin bouledogue *Carcharhinus leucas* et du requin tigre *Galeocerdo cuvier*, deux grands requins dont la structure des populations et les comportements de reproduction restent largement méconnus. En effet, les deux espèces sont régulièrement impliquées dans des attaques sur l'Homme à La Réunion, aux conséquences socio-économiques dramatiques pour un territoire insulaire qui doit naturellement se tourner vers le milieu marin pour son développement. Dans ce contexte, il s'agissait de contribuer à l'amélioration des connaissances dans le cadre d'un plan gouvernemental de gestion durable du risque, dont la ligne directrice est « mieux connaître pour mieux gérer ». Ainsi mes recherches s'intègrent à un effort global qui vise à assurer sur le long terme la conservation des espèces et des fonctions écologiques qui leurs sont associées pour le maintien de l'état de santé des écosystèmes marins, tout en permettant une gestion efficace du « risque requin », en apportant des informations d'aide à la décision. Ce travail a été rédigé sous forme d'articles scientifiques, qui constituent les différents chapitres de ce mémoire. Ils possèdent chacun leurs références bibliographiques, leurs annexes, et leurs figures et tableaux

dont la numérotation est unique à chaque chapitre. Les références bibliographiques autres que celles des articles scientifiques se situent avant les annexes. Les **Annexes** se situent à la fin du manuscrit et comprennent les travaux publiés parallèlement à ce travail de thèse.

Chapitre 1. Ce chapitre décrit l'apport des analyses moléculaires à l'étude de la structure et de la démographie des populations. Il définit le cadre théorique de la génétique des populations ainsi que les outils et analyses moléculaires incluses dans ce cadre. Par ailleurs, ce chapitre décrit le développement de marqueurs moléculaires spécifiques à l'étude de la génétique des populations du requin bouledogue et du requin tigre, marqueurs qui n'étaient pas encore disponibles dans la littérature au début de cette thèse. Le développement de ces marqueurs a fait l'objet de deux publications scientifiques (Pirog et al., 2015a; Pirog et al., 2016). Ces marqueurs sont désormais disponibles dans la littérature pour l'ensemble de la communauté scientifique et pourront ainsi être utilisés pour des études de génétique des populations de ces espèces.

Chapitre 2. Dans ce chapitre, nous avons caractérisé la structure des populations de requin bouledogue, leur degré de connectivité au sein d'une même région océanique et entre régions océaniques différentes, ainsi que leur histoire démographique. Ceci a été réalisé grâce à un échantillonnage conséquent dans les océans Indien et Pacifique (via la contribution d'un réseau de collaborateurs), auquel s'ajoutent des échantillons de l'océan Atlantique, et à l'utilisation d'un nombre important de marqueurs moléculaires nucléaires et mitochondriaux. Nous nous sommes particulièrement intéressés aux différents patrons de structuration retrouvés par ces deux types de marqueurs, et avons tenté d'identifier les processus biologiques dont ils pourraient résulter. Ces éléments sont indispensables pour comprendre la structure et le fonctionnement de la population de requins bouledogue présente à La Réunion, en particulier dans le cadre d'un programme de pêche visant à réguler la population pour diminuer le risque. Ces travaux font l'objet d'une publication soumise à *Molecular Ecology* (Pirog et al., soumis-a).

Chapitre 3. Le chapitre précédent ayant permis de délimiter et caractériser génétiquement la population de requin bouledogue de La Réunion dans un contexte global et régional, les données issues des programmes de régulation menés à La Réunion peuvent être utilisées pour estimer les paramètres biologiques régissant la dynamique de cette population, notamment les tailles à maturité sexuelle ainsi que les cycles de reproduction. Les marqueurs microsatellites développés au Chapitre 1 ont également été utilisés pour estimer la fréquence de polyandrie dans ces populations,

et estimer son impact sur la diversité génétique des populations. Les résultats de ce chapitre ont été publiés pour une partie (Pirog et al., 2015b) et sont soumis pour publication pour une autre partie dans *Plos One* (Pirog et al., soumis-b).

Chapitre 4. Par une approche similaire à celle utilisée pour étudier la génétique des populations du requin bouledogue, nous avons également caractérisé la structure des populations du requin tigre dans les océans Indien et Pacifique pour mieux comprendre comment les requins tigre de La Réunion s'intègrent dans ce schéma global. Grâce à un échantillonnage plus complet par rapport aux études précédentes, réalisé dans l'Ouest de l'océan Indien et dans l'Ouest de l'océan Pacifique, nous comparons les patrons de structuration identifiés dans cette étude avec ceux des études de Bernard et al. (2016) et Holmes et al. (2017), et estimons pour la première fois la taille efficace des populations délimitées. Une publication est en préparation (Pirog et al., en préparation-a).

Chapitre 5. Une fois les populations de requin tigre délimitées, les données issues des campagnes de régulation menées à La Réunion ont également été utilisées pour caractériser les tailles à maturité sexuelle, les cycles et comportements de reproductions influant sur la dynamique de ces populations, dans une approche similaire à celle réalisée pour le requin bouledogue. Une publication est en préparation (Pirog et al., en préparation-b).

Preamble

Molecular approaches represent an invaluable tool to study marine populations, notably Chondrichthyans. They allow, thanks to the theoretical framework of population genetics, to estimate population structure and connectivity, as well as their size, but also to investigate reproductive behaviours. In Chondrichthyans, and especially large sharks, these characteristics remain poorly known. While they have been studied in some species, generalizing the results seems difficult: the diversity of habitat use strategies as well as migrating and reproductive patterns demonstrated by these species influence their population size and structure patterns. Sharks indeed present individual variability as well as regional specificities, hereby limiting the generalisation of observations realised for specific populations or individuals. Furthermore, it is now established that these large predators play some important roles in marine ecosystem dynamics, and the decline of their populations due to anthropogenic pressures may have misunderstood consequences on the structure and functioning of these ecosystems. While adapted conservation plans thus need to be settled, management of these large sharks remains difficult, particularly for coastal species responsible of attacks on humans.

The aim of this PhD thesis is thus to improve, thanks mostly to molecular markers, knowledge on the biology of the bull shark *Carcharhinus leucas* and the tiger shark *Galeocerdo cuvier*, two large sharks for which population structure and reproductive behaviours remain poorly documented. Indeed, these two species are regularly responsible of attacks on humans in Reunion Island, inducing disastrous socio-economic consequences for this insular territory that naturally interacts with the ocean for its development. In this context, this work aims to improve knowledge in the framework of a governmental plan for risk management, for which the guiding principle is “better knowledge for better management”. Thus our researches are a part of a global effort to insure long-term conservation of sharks to maintain healthy marine ecosystems, along with the settlement of effective management plans against shark attacks. This work is presented as a suite of seven scientific publications, constituting the different chapters of the manuscript. Each article includes its own references, annexes, figures and tables, which are numbered independently for each chapter. Additional references not included in the scientific publications are listed before the Annexes. The **Annexes** located at the end of the main text includes scientific articles published alongside the thesis.

Chapter 1. This chapter describes how molecular approaches may be useful to study the structure and demographic history of populations. It defines the theoretical framework of population genetics as well as the molecular tools and analyses included in this framework. Furthermore, this chapter describes the isolation and development of specific microsatellite markers to study population genetics of the bull and tiger sharks, markers that were not available in the scientific literature at the beginning of this thesis. The characterisation of these markers led to the publication of two scientific articles (Pirog et al., 2015a; Pirog et al., 2016). These markers are thus now available in the literature for the scientific community and may be used in other population genetics studies on these species.

Chapter 2. In this chapter, we investigated population structure of the bull shark, their degree of connectivity between and within oceanic regions, as well as their demographic history. This was possible thanks to a consequent sampling in the Western Indian Ocean and in the Western Pacific (thanks to a network of collaborators), to which were added samples from the Western Atlantic, analysed with an important number of nuclear and mitochondrial markers. We particularly investigated the discrepancy highlighted between genetic differentiation indices estimated with mitochondrial and nuclear loci, and tried to identify the biological processes responsible of this discrepancy. This is essential to understand the structure and functioning of the bull shark population located near Reunion Island, especially in the frame of a fishing program set up to regulate the population and thereby to diminish the risk of attacks. This work has been submitted to *Molecular Ecology* (Pirog et al., submitted-a).

Chapter 3. The precedent chapter having allowed to delineate and characterised genetically the bull shark population around Reunion Island in a global and regional context, data from the regulation programs settled in Reunion Island may be used to estimate the biological parameters characterising the dynamics of this population, notably size at sexual maturity as well as the reproductive cycle. The microsatellite markers developed in Chapter 1 were also used to estimate the frequency of multiple paternity in this population. The results of this chapter are in part already published (Pirog et al., 2015b) and have been submitted to *Plos One* (Pirog et al., submitted-b).

Chapter 4. Using a similar approach than the one used to study population genetics of the bull shark, we also characterised population structure of the tiger shark in the Western Indian Ocean and the Western Pacific, in order to better understand how tiger sharks found around Reunion

Island integrate in this global picture. Thanks to a more intensive sampling in the Western Indian Ocean compared to previous studies, and adding samples from the Western Pacific, we compared structuring patterns identified in this studied to those already reported in Bernard et al. (2016) and Holmes et al. (2017). We also estimated for the first time the effective population size of the delineated populations. One publication is in preparation (Pirog et al., in prep-a).

Chapter 5. Once tiger shark populations were delineated, data from regulation fishing programs taking place in Reunion Island were also used to characterise the size at sexual maturity, the reproductive cycle and behaviours influencing dynamics of these populations, in a similar approach as for the bull shark. One publication is in preparation (Pirog et al., in prep-b).

INTRODUCTION

1. Statut des requins dans le monde, conservation et implications pour les pêcheries

1.1. Diversité des requins

Les Chondrichthyens représentent la classe taxonomique des poissons cartilagineux, qui se divise en deux sous-classes, les Élasmobranches (requins et raies), représentant 96 % des Chondrichthyens, et les Holocéphales (chimères) (Bigelow & Schroeder, 1948; Compagno, 1999a). Parmi les Élasmobranches, les requins représentent à ce jour huit ordres, 34 familles, 105 genres et 509 espèces (Compagno, 1999b, 2005; Weigmann, 2016 ; Figure 1), et auraient vu le jour il y a quelques 350 millions d'années (Ma), au cours de l'ère du Paléozoïque, avec une diversification jusqu'à la fin du Crétacé (66 Ma) (Heinicke et al., 2009). Ils sont caractérisés morphologiquement par un squelette cartilagineux et la présence de cinq à sept fentes branchiales, tandis que leur peau est recouverte d'écailles placoides en forme de dents, connues aussi sous le nom de denticules cutanées. Chez les requins, l'ordre des Carcharhiniformes est le plus représenté, comprenant 284 espèces, soit 56 % des espèces de requins, suivi par les Squaliformes (119 espèces). Les deux autres ordres majeurs sont les Orectolobiformes et les Lamniformes tandis que les Hexanchiformes, les Squatiniformes, les Heterodontiformes et les Pristiophoriformes sont constitués d'un nombre beaucoup plus réduit d'espèces (Compagno, 1990; Weigmann, 2016). Les différentes phylogénies réalisées à partir d'outils moléculaires ont rejeté l'hypothèse émise par de Carvalho (1996) plaçant les Batoides (raies) au sein de requins, et confirmé les requins comme un groupe monophylétique (Heinicke et al., 2009; Naylor et al., 2005). Néanmoins, jusqu'à récemment, la plupart des phylogénies moléculaires établies n'étaient bien définies qu'au niveau de l'ordre ou de la famille (Human et al., 2006; Iglésias et al., 2005; López et al., 2006; Naylor et al., 2005). Les études au niveau spécifique n'incluaient que peu d'espèces (Human et al., 2006) et ne concernaient que quelques familles ou genres (Corrigan & Beheregaray, 2009; Dosay-Akbulut, 2008; Eitner, 1995; Stelbrink et al., 2010). La phylogénie moléculaire la plus complète établie à ce jour confirme la monophylie de la majorité des ordres, tandis que les familles définies sur critères morphologiques semblent en majeure partie paraphylétiques et restent mal définies (Vélez-Zuazo & Agnarsson, 2011).



Figure 1. Quelques espèces représentatives des huit ordres définis chez les requins. **A** Carcharhiniformes (requin tigre *Galeocerdo cuvier*) ; **B** Orectolobiformes (requin baleine *Rhincodon typus*) ; **C** Squaliformes (aiguillat commun *Squalus acanthias*) ; **D** Lamniformes (requin blanc *Carcharodon carcharias*) ; **E** Hexanchiformes (requin perlon *Hepranchias perlo*) ; **F** Squatiniformes (requin ange *Squatina australis*) ; **G** Heterodontiformes (requin dormeur nekozame *Heterodontus japonicus*) ; **H** Pristiophoriformes (poisson-scie tident *Pristis pectinata*).

Les requins constituent donc un groupe très diversifié et occupent une grande variabilité d'habitats, aussi bien tropicaux que tempérés, des milieux fluviaux et lacustres aux milieux océaniques, mais aussi les estuaires et milieux côtiers. Ainsi, si 95 % des requins vivent en milieu marin, certaines espèces sont trouvées exclusivement en eau douce, comme les espèces du genre *Glyphis*, tandis que d'autres sont euryhalines, trouvées communément en milieu marin côtier mais remontant fréquemment les estuaires et fleuves, comme le requin bouledogue *Carcharhinus leucas* (Compagno, 1984a, 1990; Martin, 2005). Les espèces marines utilisent également des habitats différents, la majorité d'entre elles étant trouvées le long des plateaux continentaux à moins de 200 m de profondeur (par exemple, le requin taureau *Carcharhias taurus*). D'autres utilisent des

zones plus profondes, mais restent proches des talus continentaux, entre 200 et 2 000 m de profondeur, comme le requin noronhai *Odontaspis noronhai* (Compagno, 1990). Seules quelques espèces sont considérées comme exclusivement océaniques, tels le requin peau bleue *Prionace glauca* (Aires-da-Silva & Gallucci, 2007; Veríssimo et al., 2017) ou le requin pèlerin *Cetorhinus maximus* (Hoelzel et al., 2006).

Malgré cette incroyable diversité, six régions constituent les points chauds de la diversité mondiale en Chondrichthyens : l’Australie, le Japon, l’Indonésie, l’Afrique australe, l’Atlantique tropical occidental et Taiwan (Carpenter, 2002; Ebert, 2013; Ebert & van Hees, 2015; Last & White, 2011 ; Figure 2). Ces régions correspondent à des points chauds de biodiversité mondiale ou *hotspots*, qui combinent biodiversité extraordinaire et vulnérabilité élevée des espèces du fait des activités humaines (Myers et al., 2000; Weigmann, 2016).

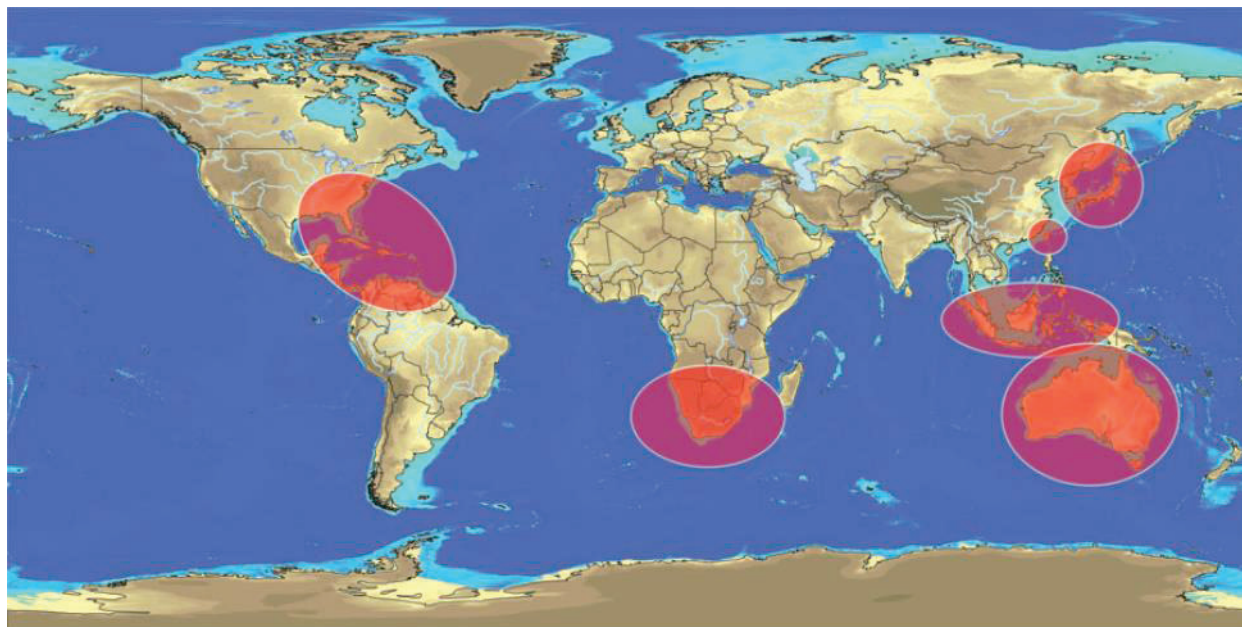


Figure 2. Points chauds de la biodiversité des Chondrichthyens. D’après Weigmann (2016).

Confirmant ce qui avait déjà été noté dans de précédentes études (Last et al., 2014; Myers et al., 2000; Roberts et al., 2002; Wafar et al., 2011; White & Sommerville, 2010), Weigmann (2016) a montré que le Sud-Ouest de l’océan Indien ainsi que la région regroupant l’Est de l’océan Indien et le Pacifique Ouest présentent une concentration particulière de ces points chauds, avec certainement une variété d’espèces encore non décrites.

Malgré l'incroyable diversité d'habitats et de formes des requins, ce sont des prédateurs, jouant ainsi un rôle important dans les écosystèmes marins, même si celui-ci reste encore mal compris.

1.2. Rôles dans les écosystèmes marins

1.2.1. Places des requins dans les communautés marines

Les requins sont tous des prédateurs : aucune espèce n'est herbivore, même parmi les plus petites (par exemple, *Etmopterus perryi* ou *Apristurus sibogae*, espèces ne mesurant pas plus de 25 cm de long à l'âge adulte ; Compagno, 1984a, b). Néanmoins, alors qu'auparavant ils étaient tous considérés comme appartenant à un seul groupe apical, ils présentent des régimes alimentaires variés et ne peuvent être rassemblés en un seul groupe fonctionnel (Bozec et al., 2004). En effet, certaines espèces présentent plutôt des comportements charognards, comme le requin tigre *Galeocerdo cuvier* (Lowe et al., 1996), ou d'autres, parfois parmi les plus grandes (par exemple, le requin pèlerin *C. maximus* et le requin baleine *Rhincodon typus*, mesurant jusqu'à 14 m de long ; Compagno, 1984b, 2001), se nourrissent de plancton ou de petits poissons par filtration. Ainsi, si certains occupent bien la place de prédateur apical dans les réseaux trophiques marins, d'autres requins jouent plutôt le rôle de méso-prédateur, occupant un haut niveau dans les réseaux trophiques mais demeurant sensibles à la prédation inter- et intra-spécifiques, notamment par des requins de plus grande taille (Frisch et al., 2016; Roff et al., 2016).

Par leur position de prédateurs, notamment apicaux, ils influencent donc les réseaux trophiques. Des suivis réalisés entre 1972 et 2002 dans le Golfe du Mexique ont mis en évidence un déclin de l'abondance de 11 espèces de grands requins, dû aux pêcheries industrielles de crevette qui utilisent le chalut de fond et dont les Élasmobranches sont une prise accessoire fréquente (par exemple, 90 % de déclin de l'abondance de requin marteau *Sphyrna lewini* ; Shepherd & Myers, 2005). Ce déclin des prédateurs supérieurs aurait entraîné une augmentation d'espèces de niveaux trophiques intermédiaires (par exemple, l'ange de mer de sable *Squatina dumeril* ou l'émissole douce *Mustelus canis*), dont l'abondance a été multipliée par six, voire 13 pour certaines espèces, sur la période d'étude (Shepherd & Myers, 2005). De même, dans le Pacifique, le suivi des pêcheries palangrières tropicales entre 1950 et 2000 a montré une diminution par dix des prises de 12 espèces de grands prédateurs océaniques (requins, thons et marlins), alors que les prises de méso-prédateurs de plus

petite taille (notamment de la raie *Dasyatis violacea*) ont, sur la même période, été multipliées par dix, voire par cent (Ward & Myers, 2005). Par ailleurs, une étude modélisant les réseaux trophiques d'un écosystème marin subtropical situé le long de la partie sud du Brésil a montré que cinq espèces de requins (*G. cuvier*, *Carcharhinus obscurus*, *C. taurus*, *Sphyrna zygaena* et *S. lewini*) jouent un rôle clé dans cet écosystème, contrôlant les niveaux inférieurs de la chaîne alimentaire par la prédation (Bornatowski et al., 2014). Néanmoins, le rôle des requins et leur influence sur les écosystèmes varient fortement d'une communauté à l'autre. Ainsi, Kitchell et al. (2002) n'ont pas identifié de rôle majeur des requins en analysant les interactions trophiques dans le centre du Pacifique, et les conséquences des déclin des populations de requins restent encore méconnues (Ferretti et al., 2010; Heithaus et al., 2008; Heupel et al., 2014). Ceci est notamment dû au fait que les études essayant d'établir l'existence de cascades trophiques induites par la disparition des requins disposaient souvent de peu de connaissances sur le régime alimentaire des espèces étudiées et sous-estiment donc la complexité des réseaux trophiques.

En outre, les requins jouent d'autres rôles écologiques dans les communautés qu'ils occupent, et n'influent pas uniquement le fonctionnement des écosystèmes par la prédation (effet direct).

1.2.2. Rôles indirects des requins dans les communautés marines

Les espèces prédatrices de grande taille, apicales en particulier, peuvent induire des modifications comportementales de leurs proies et ceci constitue un mécanisme indirect de régulation des flux trophiques au sein de l'écosystème. En effet, les proies évitant les habitats ou les périodes temporelles où leurs prédateurs sont présents, réduisent leur effort d'alimentation au détriment d'une vigilance anti-prédateur et diminuent ainsi la pression de prédation sur les niveaux trophiques inférieurs. Ainsi, des études menées en Australie, à Shark Bay, ont estimé l'influence du requin tigre *G. cuvier* sur les distributions de grands herbivores et méso-prédateurs (notamment le dugong *Dugong dugon*, la tortue verte *Chelonia mydas* ou encore le grand dauphin *Tursiops aduncus*) au sein de deux habitats (bancs de sable peu profonds et herbiers, ou eaux profondes et pentes externes) (Heithaus, 2005; Heithaus & Dill, 2006; Heithaus et al., 2007a; Wirsing et al., 2007a, b). Lors des périodes saisonnières d'abondance des requins tigre, les auteurs ont pu mettre en évidence que les méso-prédateurs et herbivores étudiés fréquentaient moins les herbiers ou bancs de sable peu profonds qui produisent plus de ressources mais qui rendent la fuite plus difficile en cas

d'attaque, privilégiant des zones plus sûres telles que les eaux plus profondes ou les pentes externes des récifs qui offrent davantage de possibilités de s'échapper. En modifiant leur comportement, les proies subissent un coût énergétique (vigilance anti-prédateur dans leur budget d'activité) et n'utilisent pas complètement les ressources du milieu, ce qui peut limiter leur croissance et leur taux de fécondité (Creel et al., 2007; Creel & Christianson, 2008; Walters & Juanes, 1993), mais favorise la production de biomasse végétale par l'écosystème.

Par ailleurs, plusieurs études considérant plus spécifiquement les écosystèmes coralliens ont mis en évidence divers autres rôles écologiques des requins, notamment dans le cycle des nutriments, la prédation sur les individus faibles ou malades, des perturbations de l'habitat ou le contrôle d'espèces invasives (Figure 3). En effet, les mouvements des petits requins récifaux (méso-prédateurs) permettraient un flux de nutriments entre des habitats récifaux et océaniques adjacents, tandis que les plus grands requins capables de parcourir de grandes distances (>1 000 km) permettraient un flux entre les milieux côtiers et océaniques (Heupel & Simpfendorfer, 2015; McCauley et al., 2012; Meyer et al., 2010; Schmitz et al., 2010; Williams et al., 2018). Par ailleurs, par prédation opportuniste, les requins maintiennent de faibles densités chez les populations de proies (Pongsiri et al., 2009) et éliminent aussi les individus faibles ou malades (Lowe et al., 1996; Lucifora et al., 2008), pouvant ainsi réduire la propagation de maladies. En outre, certaines espèces de requins sont également des charognards (Dudley et al., 2000), améliorant la stabilité des réseaux trophiques en augmentant le nombre de liens dans ces réseaux (augmentation du nombre d'espèces proies) et créant un lien direct entre prédateurs et détritiques (Wilson & Wolkovich, 2011). Les requins méso-prédateurs exercent également des contrôles descendants sur des espèces invasives, comme il a été montré dans les Caraïbes, où la rascasse volante *Pterois volitans* a envahi les écosystèmes récifaux : les requins de récif étant leurs seuls prédateurs, eux seuls peuvent limiter naturellement cette invasion (Albins & Hixon, 2013; Wallach et al., 2015). Enfin, des auteurs ont également mis en évidence que les requins récifaux peuvent abîmer des colonies coralliennes lorsqu'ils chassent, étant ainsi une source de perturbation permettant le maintien de la dynamique du récif et de la construction du corail, en ouvrant le milieu (Begg et al., 2003; Jiménez-Centeno, 1997).

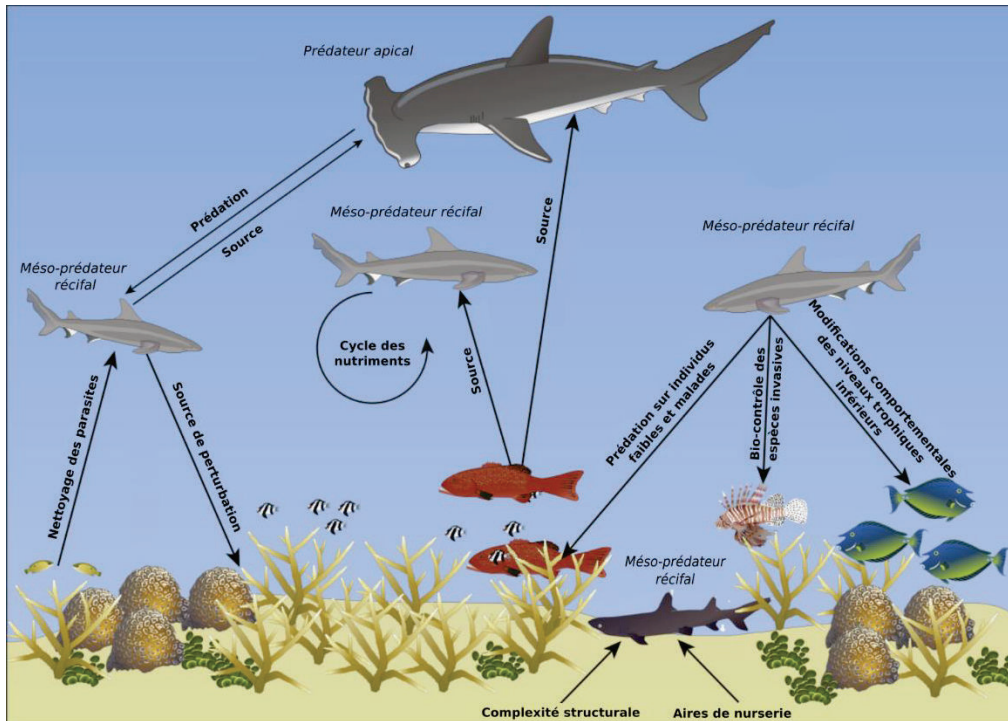


Figure 3. Rôles écologiques des requins dans les écosystèmes coralliens, d'après Roff et al. (2016).

Les requins jouent donc des rôles variés dans les écosystèmes marins qui, s'ils sont mieux connus aujourd'hui, restent encore vraisemblablement sous-évalués. Ceci est notamment dû au fait que leur biologie reste également mal comprise en comparaison d'autres espèces marines. Les connaissances de leurs traits d'histoire de vie, de leur reproduction ou de la dynamique de leurs populations sont encore restreintes à quelques espèces, notamment celles présentant une valeur économique et étant ciblées par les pêcheries.

1.3. Traits d'histoire de vie et modes de reproduction

Les traits d'histoire de vie d'un organisme sont les processus biologiques caractérisant son cycle de vie (par exemple, la fécondité, le taux de croissance, le recrutement, la mortalité) et les stratégies qui vont influencer sa survie et sa reproduction. La taille et la croissance d'une population peuvent être évaluées grâce à l'estimation de ces paramètres. Les espèces de requins dont l'âge et la croissance ont pu être estimés présentent des traits d'histoire de vie similaires. Ce sont des animaux qui, comparativement aux Téléostéens (poissons osseux), sont (1) de grande taille (taille moyenne

d'1,50 m, toutes espèces confondues ; Compagno, 1981), (2) vivent longtemps (jusqu'à 70 ans pour l'aiguillat commun *Squalus acanthias* ; Ketchen, 1975; Nammack et al., 1985), (3) présentent des taux de survie importants pour toutes les classes d'âge, (4) présentent des âges à maturité sexuelle tardifs (20-25 ans chez le requin requiem des sables *C. obscurus* ; Natanson et al., 1995), (5) ont des temps de gestation longs (jusqu'à 24 mois pour *S. acanthias* ; Compagno, 1984b; Nammack et al., 1985) et (6) présentent de faibles taux de fécondité (Compagno, 1999b). En tant que prédateurs supérieurs ayant peu d'ennemis naturels, les requins n'ont pas besoin de produire beaucoup de juvéniles pour maintenir leurs populations, ce qui peut expliquer ces traits d'histoire de vie caractéristiques d'une stratégie de reproduction K. Néanmoins, ces caractéristiques, qui limitent le potentiel de reproduction et la croissance des populations (Pratt & Casey, 1990), rendent ces espèces sensibles à la surexploitation ou à d'autres perturbations d'origine humaine (Holden, 1974).

Par ailleurs, même s'ils semblent présenter des stratégies de reproduction similaires, les requins arborent une diversité de modes de reproduction sexuée. Ces modes de reproduction ont été classés selon le mode de développement du fœtus (vivipare : au sein de la mère ou ovipare : ponte d'œufs) ainsi que son mode de nutrition (lécithotrophe : assuré par des réserves vitellines ou matrotrophe : assuré par la mère) (Blackburn, 1992; Wourms, 1981). Ainsi, la lécithotrophie a été observée pour deux formes d'oviparité et une forme de viviparité tandis que la matrotrophie a été observée pour quatre formes de viviparité définies selon le lien des embryons à la mère (Hamlett et al., 2005; Musick & Ellis, 2005; Wourms, 1981 ; Tableau 1). Par ailleurs, des femelles de plusieurs espèces de requin retenues en captivité ont montré la capacité à se reproduire de manière asexuée, par parthénogenèse. Cette capacité a été mise en évidence chez des espèces vivipares, notamment le requin marteau *Sphyrna tiburo* (Chapman et al., 2007) ou le requin bordé *Carcharhinus limbatus* (Chapman et al., 2008), et est soupçonnée chez plusieurs espèces ovipares (Heist, 2004). Les requins pourraient donc se reproduire de manière asexuée en cas d'absence de partenaires (Chapman et al., 2007; Chapman et al., 2008). Néanmoins, cette capacité n'a été démontrée que pour des individus en captivité et, dans certains cas, pourrait avoir résulté d'un stockage de sperme particulièrement long chez les femelles, bien que l'hypothèse semble peu probable pour certains de ces individus (Chapman et al., 2007; Chapman et al., 2008).

Suivant les espèces, le nombre de fœtus par portée peut varier de 2 individus (par exemple, le requin taureau *C. taurus* ; Gilmore, 1993) jusqu'à 300 (par exemple, le requin baleine *R. typus* ; Chang et al., 1997), mais est majoritairement compris entre 2 et 20 petits (Fowler et al., 2005). Le nombre de jeunes produits peut dépendre notamment du mode de nutrition des embryons. Par ailleurs, chez certaines espèces, il existe du cannibalisme intra-utérin qui entraîne la production d'un jeune par utérus uniquement (Gilmore, 1993). Les temps de gestation restent encore méconnus pour beaucoup d'espèces, la plupart étant estimés entre 10 et 12 mois et les cycles de reproduction ne sont pas toujours annuels. Ceci est dû à la durée de la gestation et/ou à une période de « repos » des femelles (parfois au moins un an) chez certaines espèces (Branstetter, 1990; Pratt & Casey, 1990). Les femelles investissent ainsi beaucoup d'énergie à la production périodique de quelques juvéniles, le plus souvent complètement développés et présentant des taux de survie relativement importants. Après cet investissement, la plupart des requins mettent bas dans des estuaires ou des zones côtières appelées nurseries, distincts des zones d'habitat des adultes (Knip et al., 2010; Speed et al., 2010), où la prédation (majoritairement par d'autres requins) est réduite et où les apports nutritifs sont plus importants (Branstetter, 1990; Castro, 1993; Heupel et al., 2007; Springer, 1967).

Ces traits d'histoire de vie et la tendance des requins à se regrouper par âge, sexe ou stade de maturité les rendent particulièrement sensibles aux pêcheries. Ils adoptent en effet des stratégies et modes de reproduction très différents de ceux des Téléostéens, qui sont la cible principale de la plupart des pêcheries et dont les traits d'histoire de vie sont donc considérés pour définir la plupart des stratégies de régulation (Hillborn & Walters, 1992).

Tableau 1. Définition des différents modes de reproduction identifiés chez les requins. D’après Hamlett et al. (2005), Musick & Ellis (2005), Musick (2010) et Wourms (1981).

mode de développement	mode de nutrition	mode de reproduction	description	espèces concernées
ovipare	lécithotrophe	oviparité simple	ponte d'œufs, embryons se nourrissant des réserves vitellines; développement majoritairement hors de la mère	Heterodontiformes et la plupart des Scyliorhinidae
		oviparité multiple	accumulation des œufs dans chaque oviducte et rétention plusieurs mois avant la ponte	quelques Scyliorhinidae
vivipare	lécithotrophe	viviparité lécithotrophe	développement des embryons dans la mère; reliés à un sac vitellin représentant leur unique source de nutriments	tous les ordres sauf les Heterodontiformes et Lamniformes (<i>Galeocerdo cuvier</i>)
		viviparité aplacentaire; mucoïde histotrophie	embryons nourris à partir d'un sac vitellin mais recevant des nutriments supplémentaires en ingérant du mucus produit par l'utérus	détectée chez certains Squaliformes
	matrotrophe	viviparité aplacentaire; oophagie	maintien de l'ovulation chez la mère après fécondation; œufs non-fécondés servant de source de nutriments aux fœtus	Lamniformes
		viviparité aplacentaire; adelphophagie	développement du premier embryon dans chaque corne utérine se nourrissant des autres fœtus en développement	<i>Carcharias taurus</i>
		viviparité placentaire	chaque embryon se nourrit d'un sac vitellin relié au placenta	certains Carcharhiniformes (<i>Carcharhinus leucas</i>)

1.4. Pêcheries et impacts

Jusque récemment, les Chondrichthyens n'étaient pas considérés comme une ressource d'intérêt, présentant une faible valeur économique et une faible abondance (Bonfil, 1994). Les statistiques de l'Organisation des Nations Unies pour l'Alimentation et l'Agriculture (FAO) estiment que les poissons cartilagineux représentent entre 1 et 2 % des pêcheries commerciales mondiales (Figure 4A), comprenant environ 50 % des espèces de requins (Figure 4B) (Bonfil, 1994; Camhi et al., 1998; Compagno, 1990; Worm et al., 2013). Les requins sont notamment pêchés ponctuellement pour leur chair ou pour l'huile de leur foie, riche en vitamine A et en squalène, et utilisée pour fabriquer des lubrifiants ou des produits cosmétiques, mais aussi pour leurs nageoires ou uniquement, pour certaines espèces, pour leurs mâchoires et dents, vendues comme souvenirs touristiques. Par ailleurs, ces espèces sont souvent ciblées lors de la déplétion de stocks d'espèces plus importantes économiquement (Dulvy et al., 2008; Stevens et al., 2000). Comme d'autres espèces de la mégafaune marine (cétacés, tortues et oiseaux marins), elles sont également régulièrement capturées par des pêcheries qui ne les ciblent pas, et représentent un volume non négligeable de prises accessoires des pêcheries palangrières et au filet (Bonfil, 1994). Enfin, l'augmentation rapide de la demande en ailerons, considérés comme un met de choix dans les pays asiatiques, a donné une valeur disproportionnée à une petite partie du corps de l'animal, menant à la découpe de cette partie uniquement (*shark finning*), le reste de l'animal étant rejeté à la mer, le condamnant à une mort certaine. Bien que certains pays, tels le Canada, les Etats-Unis, l'Australie ou les pays européens, aient progressivement légiféré contre cette pratique ces 20 dernières années, elle reste encore répandue dans de nombreux pays (Fowler et al., 2005).

Ainsi, les rapports de captures d'Élasmobranches par la FAO ont augmenté d'environ 2 % par an entre 1985 et le début des années 2000, et sont depuis décroissantes (Figure 4B) (Fowler et al., 2005; Worm et al., 2013). Depuis 1950, les captures de requins ont été multipliées par 3,4 (de 120 677 t en 1950 à 414 345 t en 1997), et ont ensuite déclinées de 7,5 %, atteignant 383 549 t en 2010. En comparaison, les captures de raies et chimères ont été multipliées par 3,6 sur la même période, atteignant 556 470 t en 2003, mais ont ensuite décliné de 26,5 %, atteignant 353 549 t en 2010. Les pêches de poissons cartilagineux ont ainsi suivi la même tendance que celles des poissons osseux (Figure 4A), même si les captures de Chondrichthyens ont atteint un pic plus tardif, pour ensuite diminuer plus fortement (Worm et al., 2013). En découpant les captures par origine géographique, Worm et al. (2013) ont pu mettre en évidence une évolution plutôt constante depuis 1990 du nombre de captures en Europe, Amérique et

Océanie, alors que les captures ont augmenté en Afrique et fortement diminué en Asie, cette dernière région représentant environ 52 % du nombre global de captures de Chondrichthyens en 2010 (Figure 4C). Alors que les captures sont restées stables ou ont décliné depuis la fin des années 1980, le volume d'ailerons de requin trouvés dans le commerce a augmenté de manière significative, aucun déclin n'étant mis en évidence dans l'import (Figure 4D) ou l'export (Figure 4E) d'ailerons de requin depuis les interdictions de *shark finning*. Cette observation semble être corroborée par les calculs d'importations d'ailerons de requin au marché de Hong-Kong (principal marché concernant ces produits), qui ne montrent pas de décroissance (Figure 4F), et pourrait donc être liée à des captures illégales ou non reportées.

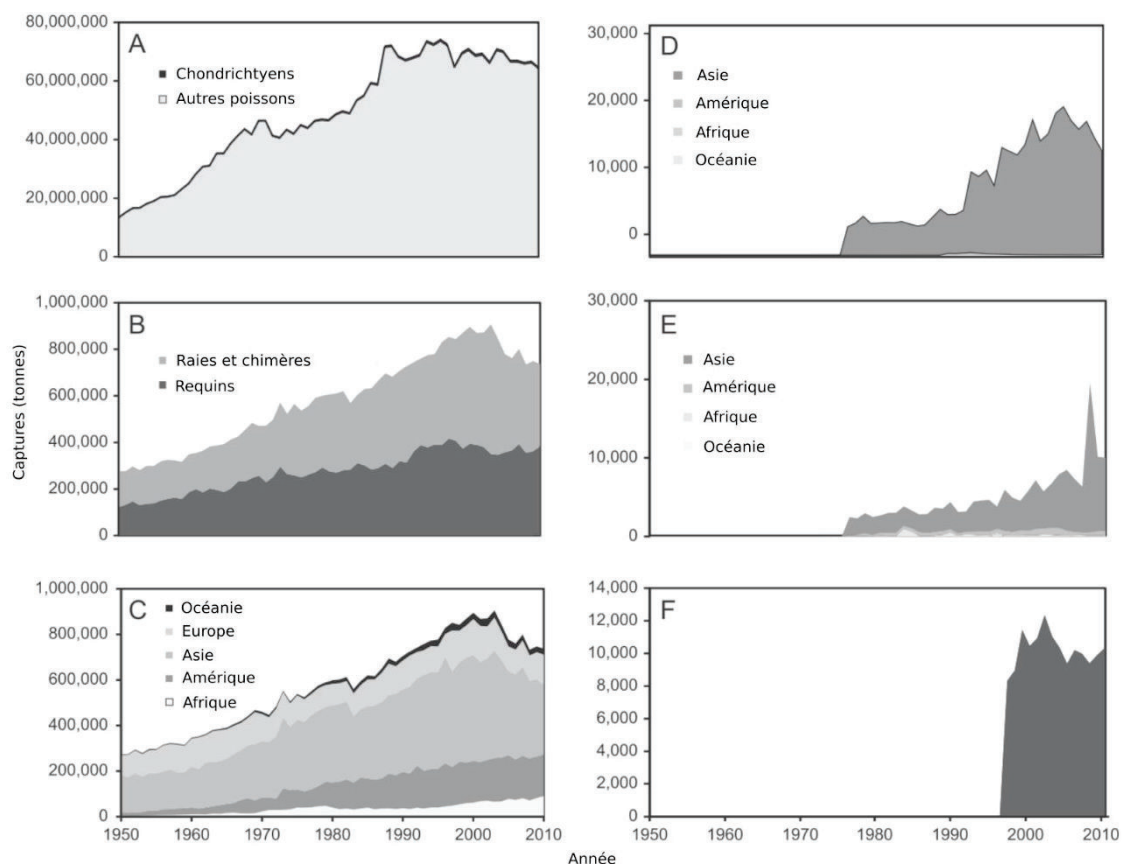


Figure 4. Tendances mondiales de captures, d'après les données de la FAO et du Département de l'Agriculture et des Pêches du Gouvernement de Hong-Kong. **A** Captures de poissons osseux et Chondrichthyens ; **B** Captures de requins comparées aux autres Chondrichthyens (raies et chimères) ; **C** Captures de Chondrichthyens par région ; **D** Imports et **E** exports d'ailerons de requin ; **F** Imports d'ailerons de requin au marché de Hong-Kong. D'après Worm et al. (2013).

En effet, ces données ne concernent que celles rapportées par la FAO et restent incomplètes. Les requins capturés sont régulièrement rejetés directement en mer (Clarke et al., 2006; Stevens et al., 2000), et ne sont pas toujours comptabilisés dans les rapports fournis aux institutions nationales et internationales de suivi des pêches (à moins que des observateurs formés ne soient

embarqués), ce qui est notamment le cas pour le *shark finning* (Clarke et al., 2006). Dès lors, il est fort probable que les estimations de la FAO ne représentent qu'une fraction du nombre réel de requins pêchés. Les données issues du Département de l'Agriculture et des Pêches du Gouvernement de Hong-Kong pour l'année 2000 par exemple, reportent des importations (en volume de requins pêchés) quatre fois supérieures à celles de la FAO (Clarke et al., 2006). En faisant quelques hypothèses sur le nombre de requins pêchés illégalement, dérivées de données de différents articles scientifiques, Worm et al. (2013) ont estimé, pour l'année 2000, une mortalité totale pouvant varier de 63 à 273 millions de requins, avec une moyenne de 100 millions de requins. Ce calcul a été dérivé d'une estimation de 1 445 000 t de requins tués, dont seulement 392 000 t (27 %) ont été comptabilisées par la FAO (Figure 5).

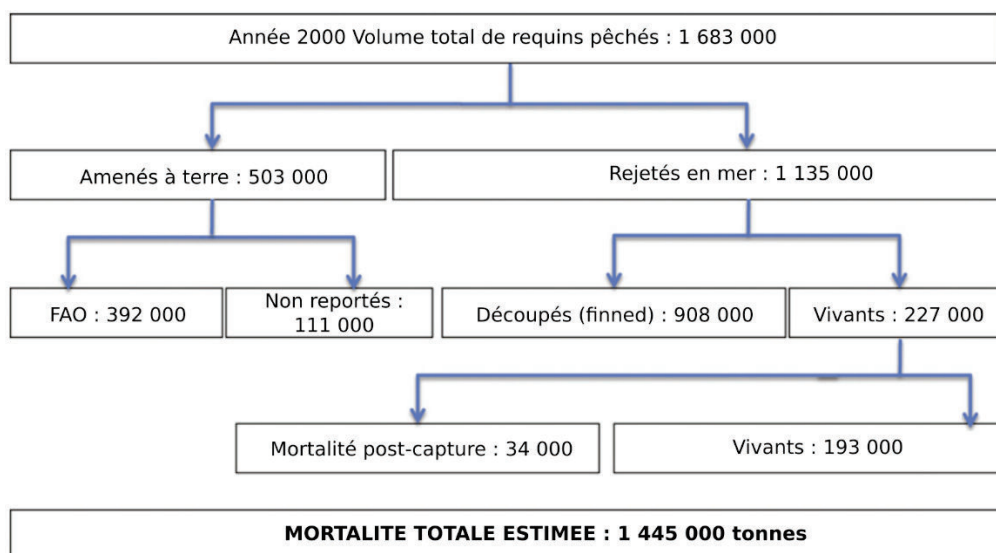


Figure 5. Estimation de la mortalité totale de requins pour l'année 2000, incluant les données de la FAO et celles des captures illégales et non reportées. La mortalité totale a été calculée comme la différence du volume total de captures moins le volume de requins survivants après capture. Les unités sont des tonnes. D'après Worm et al. (2013).

L'augmentation de la pression de pêche depuis les années 1950 sur ces espèces à faible taux de fécondité et à maturité sexuelle tardive a entraîné le rapide déclin de nombreuses populations de requins, reflété notamment par la baisse des captures depuis le début des années 2000. Ainsi, sur les 465 espèces de requins étudiées par l'Union Internationale pour la Conservation de la Nature (IUCN), 74 (15,9 %) sont considérées menacées : 11 (1,1 %) en danger critique, 15 (1,4 %) en danger et 48 (4,6 %) vulnérables, tandis que 209 espèces (20,1 %) sont considérées comme présentant des données insuffisantes (Dulvy et al., 2014). Les grandes espèces de requins côtiers sont particulièrement touchées, comme les requins marteau *S. lewini* ou

S. zygaena, ou les requins requiem comme *Carcharhinus plumbeus*, *C. limbatus*, *C. obscurus*, ou encore *C. leucas*, avec des déclinés de populations estimés à plus de 75 % (Baum et al., 2003; Dulvy et al., 2014; Ferretti et al., 2010; Myers et al., 2007). Par ailleurs, sur 19 aires de pêches identifiées prioritaires pour la gestion de la conservation des requins, avec (1) des pressions de pêche importantes, (2) un grand nombre d'espèces endémiques et (3) un grand nombre d'espèces avec le statut UICN « données insuffisantes », les six majeures sont situées dans les océans Indien et Pacifique (Dulvy et al., 2014).

Les efforts de conservation s'orientent vers la mise en place de plans de gestion durable des stocks plutôt que l'interdiction totale de l'exploitation (Dulvy et al., 2017; Simpfendorfer & Dulvy, 2017). Ceci nécessite de pouvoir délimiter les stocks qui peuvent être gérés indépendamment, d'estimer le nombre d'individus qui les composent, mais aussi le nombre d'individus migrant entre ces unités délimitées, des données notamment obtenues grâce au cadre théorique de la génétique des populations couplé aux outils moléculaires (Frankham, 2010; Schwartz et al., 2007).

1.5. Biogéographie et modes de reproduction des populations de requins

Comparé aux Mammifères et Téléostéens, les études s'intéressant à la phylogéographie des requins restent moins nombreuses (Beheregaray, 2008) mais ont produit, pour les espèces étudiées, des analyses détaillées de la structure régionale des populations, avec des implications majeures pour la conservation et la gestion des pêcheries. Des patrons de structure des populations ont ainsi pu être mis en évidence suivant l'habitat utilisé par les espèces, notamment selon qu'elles soient côtières ou océaniques. Les espèces de grands requins côtiers, bien que présentant des capacités de dispersion importantes, montrent souvent de l'hétérogénéité génétique entre bassins océaniques. Ainsi, les requins gris *C. plumbeus* (Portnoy et al., 2010), soyeux *Carcharhinus falciformis* (Clarke et al., 2015) ou encore le requin marteau *S. lewini* (Duncan et al., 2006; Nance et al., 2011) par exemple, ne présentent que de faibles (voire nuls) taux de migration entre les bassins Atlantique, Indien et Pacifique. Les espèces côtières plus petites, telles le requin à pointes noires *Carcharhinus melanopterus* (Vignaud et al., 2014b) ou le requin à pointes blanches *Triaenodon obesus* (Whitney et al., 2012) présentent également de la structuration entre récifs à l'intérieur des bassins océaniques, suggérant de la fidélité à certains sites spécifiques. Ces patrons de structuration seraient dus à des barrières biogéographiques, mises en évidence également pour d'autres espèces marines (Briggs, 1995).

A l'opposé, les espèces océaniques présentent une homogénéité importante à l'échelle mondiale. Ainsi, les populations du requin pèlerin *C. maximus* (Hoelzel et al., 2006) ou du requin peau bleue *Prionace glauca* (Veríssimo et al., 2017) notamment, ne semblent pas structurées entre les différents bassins océaniques. Les grandes barrières géographiques traditionnelles (par exemple, la barrière du Pacifique Est, *Eastern Pacific Barrier* en anglais, séparant le Pacifique Ouest et le Pacifique Est) ne semblent donc pas avoir d'impact sur ces espèces, qui restent néanmoins des cas particuliers. Une étude récente sur le requin peau bleue discute de la possibilité que cette absence de structuration à l'échelle mondiale ne reflète pas la panmixie de la population, mais pourrait être due à un décalage dans le temps des signaux retrouvés par les méthodes génétiques d'analyses traditionnelles, qui ne peuvent détecter des changements démographiques qu'après une longue période (Bailleul et al., 2018).

Par ailleurs, les études de structuration des populations, mais aussi celles s'intéressant aux comportements de reproduction des requins, ont montré chez de nombreuses espèces des comportements de philopatrie, c.-à-d. le retour de l'individu à un site particulier, comme son site de naissance (Hueter et al., 2005; Speed et al., 2010). Si certains de ces comportements semblent liés à des activités de chasse, comme chez le requin tigre *G. cuvier* (Lowe et al., 2006; Meyer et al., 2010) ou le grand requin blanc *Carcharodon carcharias* (Domeier & Nasby-Lucas, 2008; Jorgensen et al., 2009), beaucoup semblent liés à la reproduction. En effet, les femelles présentent de la fidélité aux zones de nurserie, qui sont parfois celles où elles sont nées (Heupel et al., 2007; Hueter et al., 2005), ce qui limite ainsi les flux de gènes. Les mâles n'étant pas philopatrics à ces zones, cette dispersion biaisée par le sexe est reflétée par des niveaux de différenciation génétique plus importants au niveau des marqueurs mitochondriaux (ADN mitochondrial hérité de la mère uniquement) que celle retrouvée par les marqueurs nucléaires (ADN nucléaire hérité des deux parents). Ces différences de différenciation génétique ont ainsi été observées, et imputées à des migrations différenciées entre sexe chez de nombreuses espèces de requin, telles le requin bouledogue *C. leucas* (Karl et al., 2011; Tillett et al., 2012), le requin bordé *C. limbatus* (Keeney et al., 2005), le requin gris *C. plumbeus* (Portnoy et al., 2010), le requin tigre *G. cuvier* (Bernard et al., 2016), le grand requin blanc *C. carcharias* (Pardini et al., 2001) ou le requin nez noir *Carcharhinus acronotus* (Portnoy et al., 2014). Cependant, ces résultats doivent être considérés avec précaution, du fait de la plus petite taille efficace de l'ADN mitochondrial, ce qui peut induire des niveaux de différenciation génétique différents entre marqueurs mitochondriaux et nucléaires malgré des flux de migration équivalents entre mâles et femelles (Buonaccorsi et al., 2001). La philopatrie des femelles a toutefois été

confirmée par d'autres méthodes, reposant sur la reconstruction des génotypes parentaux grâce à l'échantillonnage sur plusieurs années et génotypage, avec des marqueurs microsatellites, de juvéniles trouvés dans des nurseries. Ceci a été fait chez le requin citron *Negaprion brevirostris*, aux Bahamas, où des échantillonnages effectués sur presque 20 ans ont montré que les femelles revenaient fidèlement, parfois 14 à 17 ans plus tard, au même site pour mettre bas (Feldheim et al., 2004; Feldheim et al., 2013). Ces études ont également pu confirmer un comportement de reproduction déjà observé directement, la polyandrie, ou paternité multiple.

Traditionnellement, les connaissances sur les systèmes de reproduction des Élasmobranches, et plus particulièrement des requins, ont été acquises par l'observation directe d'accouplements, mais ce chez quelques espèces uniquement (Pratt & Carrier, 2001). Par exemple, les premières recherches sur les requins nourrice *Ginglymostoma cirratum* ont montré que les mâles présentaient des comportements agressifs envers les femelles lors de l'accouplement, et que plusieurs mâles s'accouplaient avec une même femelle (Carrier et al., 1994). Cependant accouplement ne signifie pas fécondation. Utilisant par la suite des méthodes moléculaires, il a pu être montré que ce comportement avait également un impact génétique sur les portées, avec cinq à sept mâles contribuant à chacune des trois portées de 29 à 39 embryons étudiées (Heist et al., 2011). Alors que le nombre de mâles contribuant à ces trois portées demeure le plus important à ce jour, beaucoup d'espèces pour lesquelles la présence de paternité multiple a été testée ont montré de la polyandrie (voir Byrne & Avise, 2012). Cette constatation est d'autant plus intéressante que les observations d'accouplements dans la nature sont rares, et la présence de polyandrie dans la majorité des espèces suggère que les comportements observés chez le requin nourrice ou le requin à pointes blanches *T. obesus* (Whitney et al., 2004) sont probablement fréquents. Par ailleurs, il est à noter que la fréquence de polyandrie et le nombre de mâles contribuant à chaque portée varient énormément entre les espèces. Par exemple, la totalité des portées de requin tigre *G. cuvier* étudiées par Holmes et al. (2018) étaient monoandres, ainsi que la plupart des portées étudiées de requin marteau *S. tiburo* (Chapman et al., 2004), de l'aiguillat épinette *Squalus mitsukurii* (Daly-Engel et al., 2010) et de l'aiguillat commun *S. acanthias* (Lage et al., 2008; Verissimo et al., 2011) (respectivement 17, 11, 30 et 17 % de paternité multiple détectée dans chaque étude). A l'opposé, la majorité des portées étudiées de requin citron *N. brevirostris* (Feldheim et al., 2004), de requin gris *C. plumbeus* (Portnoy et al., 2007) ou de requin gris de récif *Carcharhinus amblyrhynchos* (Green et al., 2017) étaient polyandres (respectivement 86, 85 et 66 % des portées étudiées dans chaque étude). Par ailleurs, la fréquence de polyandrie peut également varier entre les populations

d'une même espèce. Ainsi, si plus de 80 % des portées de requin gris *C. plumbeus* de l'Atlantique Nord sont polyandres (Portnoy et al., 2007), seulement 40 % de celles étudiées à Hawaï sont issues de plusieurs pères (Daly-Engel et al., 2007). Les avantages de ce comportement restent encore mal compris. Plusieurs études n'ont pas mis en évidence de bénéfices génétiques indirects (comme des portées plus nombreuses, des embryons plus grands à la naissance, ou une survie plus importante des juvéniles), notamment chez le requin gris *C. plumbeus* (Portnoy et al., 2007) ou le requin citron *N. brevirostris* (Dibattista et al., 2008). Étant donné les coûts induits par l'accouplement pour les femelles, qui présentent des marques de morsures et de traumatismes internes de l'appareil reproducteur (Pratt & Carrier, 2001), plusieurs auteurs ont conclu que la polyandrie chez les requins est une polyandrie dite « de confort » (*convenience polyandry*; Daly-Engel et al., 2010; Dibattista et al., 2008; Portnoy et al., 2007). Les femelles augmenteraient leur valeur adaptative en acceptant des accouplements multiples, évitant ainsi les coûts associés à la résistance contre l'accouplement (Arnqvist & Nilsson, 2000; Holman & Kokko, 2013; Wolff & Macdonald, 2004). Même si cette hypothèse est séduisante, elle est difficile à valider empiriquement et n'est pas exclusive, la possibilité de bénéfices cryptiques, notamment en augmentant la diversité génétique de la population et évitant la dépression de consanguinité, restant envisageable (Chapman et al., 2004; Jennions & Petrie, 2000; Sugg & Chesser, 1994). Un autre avantage possible est de limiter l'infanticide par effet de dilution. Chez plusieurs espèces de Vertébrés, les mâles tuent de façon sélective les jeunes qui ne sont pas de leur descendance pour augmenter leurs propres perspectives de reproduction. Les femelles peuvent alors développer des stratégies pour lutter contre ce phénomène, en particulier en pratiquant l'accouplement multiple (Lukas & Huchard, 2014). Reste à savoir si une telle stratégie est plausible chez des animaux comme les requins.

Ainsi, les analyses génétiques ont été appliquées avec succès à diverses espèces de requins, révélant des patrons de structuration et des modes de reproduction que les méthodes directes, seules, n'ont pas pu identifier. Néanmoins, il reste encore de nombreuses espèces pour lesquelles les caractéristiques biologiques et la structuration des populations sont encore mal connues. C'est notamment le cas du requin tigre *G. cuvier*, seule espèce représentative de son genre, et du requin bouledogue *C. leucas*, une des seules espèces de requin euryhaline. Ces deux espèces sont considérées comme « quasiment menacées » sur la liste rouge de l'IUCN (Simpfendorfer, 2009; Simpfendorfer & Burgess, 2009), et les études génétiques les concernant étaient soit non existantes lors du début de cette thèse, soit n'étaient menées qu'à une échelle régionale dans certaines parties de leurs aires de distribution, avec un nombre réduit de

marqueurs moléculaires. Par ailleurs, ces deux espèces sont régulièrement responsables d'attaques sur les populations humaines, et sont notamment incriminées dans ce qui a été appelé la « crise requin » à La Réunion, suite à une série d'attaques sur des surfeurs principalement, et qui a débuté en 2011.

2. Modèles d'étude, le requin bouledogue *Carcharhinus leucas* (Müller & Henle 1839) et le requin tigre *Galeocerdo cuvier* (Péron & Lesueur 1822)

2.1. État actuel des connaissances sur la biologie du requin bouledogue *Carcharhinus leucas*

2.1.1. Statut du requin bouledogue *Carcharhinus leucas*

Le requin bouledogue *Carcharhinus leucas* (Müller & Henle 1839) (Figure 6) est un requin de la famille des Carcharhinidés, trouvé mondialement dans les eaux tropicales et subtropicales (Compagno, 1984b, 1990).

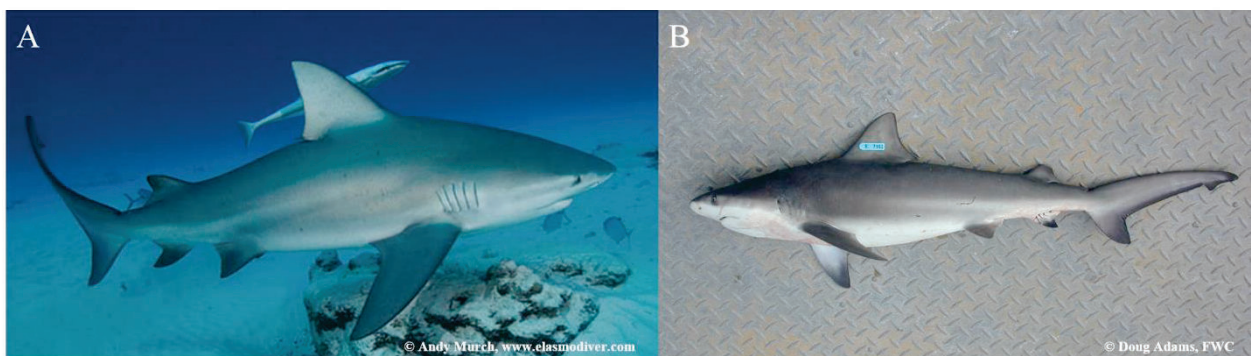


Figure 6. Requin bouledogue *Carcharhinus leucas*. **A** adulte ; **B** juvénile.

Ce grand requin peut mesurer jusqu'à 4 m de long et se nourrit majoritairement de Téléostéens, occupant une place supérieure dans les réseaux trophiques et jouant ainsi un rôle important dans la structuration des écosystèmes marins (Daly et al., 2013; Heupel et al., 2014; Matich et al., 2011; Trystram et al., 2017). En effet, cette espèce présente une niche trophique beaucoup plus large que celles des grands prédateurs téléostéens (tels le thazard *Acanthocybium solandri*, la carangue *Caranx sexfasciatus* ou la dorade coryphène *Coryphaena hippurus*), probablement car ces requins utilisent une aire géographique plus grande que ces espèces, se nourrissant à la fois dans les écosystèmes côtiers et plus profonds (Daly et al., 2013). Par ailleurs, une transition de niche écologique semble avoir lieu au passage à l'âge adulte. En effet, le requin bouledogue pouvant ajuster la pression osmotique de ses fluides internes grâce à des adaptations particulières (Pillans et al., 2006), les juvéniles sont majoritairement trouvés dans les estuaires

et grands fleuves (Jenson, 1976; Thorson et al., 1966; Thorson, 1976), zones protégées des prédateurs, notamment des adultes, et riches en proies (Heupel et al., 2007; Heupel & Simpfendorfer, 2011). Leur régime alimentaire serait donc représentatif d'une niche trophique moins haute et moins large que celle des adultes (Daly et al., 2013; Werry et al., 2011). En grandissant et développant leur capacités natatoires, les juvéniles utilisent davantage des habitats plus risqués mais plus productifs au sein des estuaires, continuant à utiliser les zones plus en amont pour se protéger des prédateurs côtiers, pour enfin utiliser le milieu marin côtier à l'âge adulte (Curtis et al., 2011; Simpfendorfer et al., 2005; Werry et al., 2011). En outre, ce changement ontogénique de niche au niveau populationnel présente une variabilité interindividuelle relativement importante due à des facteurs intrinsèques encore mal connus (Matich et al., 2011; Matich & Heithaus, 2015). Néanmoins, cette variabilité est retrouvée très tôt, pour les classes d'âges les plus jeunes : certains individus utilisent spécifiquement une zone restreinte sur le long terme tandis que d'autres présentent des mouvements plus variés en lien avec des interactions trophiques également plus diverses (Matich & Heithaus, 2015). Le changement ontogénétique et une variabilité individuelle d'écologie trophique a été également suggéré à La Réunion pour cette espèce (Trystram et al., 2017).

L'utilisation prédominante des zones côtières et même estuariennes rend le requin bouledogue particulièrement sensible aux pressions anthropiques. L'altération des milieux côtiers naturels par l'Homme le pousse vers des habitats plus artificiels, en particulier les embouchures de canaux et ravines utilisées pour le rejet d'eaux et de déchets, le rapprochant des zones urbanisées et augmentant ainsi le risque d'attaques sur l'Homme (Lagabrielle et al., 2018; Werry et al., 2012). Ils sont ainsi la cible de divers programmes de régulation, notamment en Afrique du Sud (Cliff & Dudley, 1991; Dudley, 1997), en Australie (Collins, 1972; Reid & Krogh, 1992; Reid et al., 2011), ainsi qu'à La Réunion. Par ailleurs, ils sont parfois ciblés par la pêche sportive, et sont également capturés en tant que prises accessoires de pêcheries commerciales sur toute leur aire de répartition (Aguilar et al., 2014; Almeida et al., 2001; Bonfil, 1997; Branstetter & Stiles, 1987; Clarke et al., 2006; Doukakis et al., 2010). Les impacts de ces prélèvements sur les populations restent encore mal estimés, mais semblent entraîner un déclin des populations. En effet, utilisant les données collectées par les pêcheries pélagiques de l'Atlantique Nord-Ouest sur plus de 30 ans (1970-2005), Myers et al. (2007) ont montré un déclin de plus de 99 % des populations de requin bouledogue de cette région. Sur une période similaire, des déclin significatifs des captures de cette espèce, bien que plus faibles, ont également été observés en Afrique du Sud (Dudley & Simpfendorfer, 2006). Par ailleurs, les

captures étaient déjà faibles au début de la période étudiée, les pêcheries des années 1960 semblant avoir déjà eu un impact important sur les populations de la région (Cliff & Dudley, 1992; Dudley & Simpfendorfer, 2006). En Australie, bien que non identifiés systématiquement au niveau de l'espèce, les captures de requins du genre *Carcharhinus* ont également diminué sur la période 1990-2010. Les identifications moléculaires réalisées depuis 1998 ont montré que les requins bouledogue représentaient 5 % des requins du genre *Carcharhinus* les plus souvent capturés (Reid et al., 2011). Néanmoins, les estimations génétiques de la taille efficace des populations, donc des estimations à long terme, sont de l'ordre de plusieurs milliers à plusieurs centaines de milliers d'individus suivant les études (Karl et al., 2011; Testerman, 2014; Tillett et al., 2012), ce qui est représentatif d'une importante diversité génétique comparativement aux estimations réalisées pour d'autres espèces de requin (Hoelzel et al., 2006; Schultz et al., 2008), suggérant soit que les populations ne sont pas trop touchées par la pêche, soit que l'impact de la pêche est trop récent pour être détecté par ces estimations. Il semble donc important de mieux connaître les caractéristiques biologiques et la structure des populations de cette espèce, afin d'établir des programmes de gestion durables et efficaces.

2.1.2. Structure des populations

Très peu d'études se sont intéressées à la structure des populations de requin bouledogue. Au début de cette thèse, deux articles avaient été publiés dans des revues scientifiques internationales, chacun étudiant une partie réduite de l'aire de distribution du requin bouledogue : Atlantique Nord-Ouest et Sud-Ouest (Karl et al., 2011) ou Nord de l'Australie (Tillett et al., 2012). La seule étude ayant travaillé sur la structure à l'échelle mondiale des populations de requin bouledogue est une thèse de doctorat (Testerman, 2014), et n'a pas abouti à ce jour à la rédaction d'un article sur le sujet dans une revue internationale à comité de lecture. Par ailleurs, toutes ces études ne disposaient que d'un nombre limité de marqueurs moléculaires : 5 marqueurs microsatellites et 1 marqueur mitochondrial pour la première, 3 marqueurs microsatellites et 2 marqueurs mitochondriaux pour la deuxième, et 11 marqueurs microsatellites pour la dernière. Bien que disposant donc d'une résolution réduite, ces études ont néanmoins mis en évidence certaines caractéristiques des populations de requin bouledogue. Ainsi, à l'échelle mondiale, les populations de l'Atlantique Nord-Ouest, des Fidji et de l'océan Indien/Pacifique Ouest semblent structurées génétiquement (Testerman, 2014). Au sein de chacune de ces régions, aucune différenciation génétique nucléaire significative n'a été observée, suggérant un niveau de connectivité génétique élevé entre populations, suffisant

pour assurer l'homogénéisation des fréquences alléliques. Par ailleurs, des flux de gènes semblent avoir également lieu entre l'Atlantique Ouest et le Pacifique Est, malgré un faible échantillonnage dans cette dernière région ($n = 5$), possiblement par la traversée de l'Isthme de Panama (Testerman, 2014). L'absence d'échantillons provenant de l'Atlantique Sud-Ouest et de l'Atlantique Est n'a pas permis d'estimer la structure et la connectivité entre les populations de ces régions et des autres régions. Néanmoins, l'étude de Karl et al. (2011) a montré des patrons de différenciation génétique différents entre marqueurs nucléaires et mitochondriaux entre l'Atlantique Nord-Ouest et Sud-Ouest. En effet, malgré une absence de différenciation génétique au niveau des marqueurs nucléaires, des indices de différenciation mitochondriaux significativement non nuls et élevés ont été estimés entre ces deux régions (Karl et al., 2011). Les auteurs ont émis l'hypothèse que cette observation était liée à la philopatrie des femelles à des sites de nurserie spécifiques. En effet, les femelles étant les seuls individus matures régulièrement trouvés dans les zones estuariennes ou dans les fleuves (Last & White, 2011; Montoya & Thorson, 1982; Snelson et al., 1984), elles remonteraient les fleuves afin de mettre bas dans ces zones privilégiées pour le développement des juvéniles, et choisiraient les mêmes zones que celles où elles sont nées, produisant ainsi une structuration pouvant être retrouvée par les marqueurs mitochondriaux (Karl et al., 2011; Tillett et al., 2012). Un patron similaire a également été identifié le long de la côte nord de l'Australie (Tillett et al., 2012), bien que la différenciation mitochondriale soit très ténue, trop peut-être pour représenter une vraie caractéristique biologique. Ainsi, l'absence de structuration mise en évidence par Testerman (2014) au sein de l'océan Indien et du Pacifique Ouest grâce aux marqueurs microsatellites pourrait bien cacher une structuration plus fine, ne pouvant être retrouvée qu'en utilisant d'autres types de marqueurs, notamment mitochondriaux.

Les données obtenues par des approches génétiques indirectes semblent être corroborées par les quelques études de suivis acoustique et satellite. En effet, les marquages de requins bouledogue ont montré qu'ils pouvaient parcourir de grandes distances le long des côtes, de l'ordre de plusieurs milliers de kilomètres, pouvant également réaliser des incursions dans le milieu océanique, mais aucun mouvement entre bassins océaniques ou à travers de grandes étendues d'eau profonde n'a été observé pour le moment. Ainsi, des requins marqués par satellite dans le Golfe du Mexique (Carlson et al., 2010), en Australie (Espinoza et al., 2015; Espinoza et al., 2016; Heupel & Simpfendorfer, 2015), ou le long de la côte est-africaine (Daly et al., 2014) pour des périodes variables (de quelques mois à plusieurs années), ont effectué des migrations allant de quelques kilomètres à 1 770 km. Par ailleurs, deux requins marqués à La

Réunion ont effectué des mouvements de plusieurs centaines de kilomètres en eaux profondes (Soria et al., 2015). Toutes ces études ont également mis en évidence des différences inter-individuelles, avec des individus bougeant moins que d'autres, et des périodes plus ou moins longues de fidélité au site. Enfin, si la philopatrie des femelles à des nurseries spécifiques n'a pas été mise en évidence directement, une femelle gravide marquée aux Seychelles a effectué une migration de plus de 2 000 km, traversant des eaux profondes, jusqu'à la côte Est de Madagascar, où elle a résidé pendant quelques jours en eaux peu profondes, avant de retourner aux Seychelles, et ce non gravide (Lea et al., 2015a). Il semblerait donc que cette femelle a effectué une longue migration afin de mettre bas dans une zone protégée.

Ainsi, beaucoup de questions encore non résolues demeurent concernant la structuration et le fonctionnement des populations de requin bouledogue. Par ailleurs, la dynamique de ces populations reste encore mal connue, notamment les cycles et comportements de reproduction.

2.1.3. Dynamique des populations

Le requin bouledogue se reproduit par viviparité placentaire (Compagno, 1984a; Parsons et al., 2008). La taille des portées varie de 1 (Jenson, 1976) à 14 embryons (Nevill et al., 2014), avec une moyenne aux alentours de 6-8 (Compagno, 1984b; Fowler et al., 2005; Jenson, 1976), les embryons mesurant à la naissance entre 50 et 80 cm (Branstetter & Stiles, 1987; Cliff & Dudley, 1991; Compagno, 1984a; Jenson, 1976; Snelson et al., 1984). La taille à maturité a été estimée pour des requins étudiés dans différentes régions, et varie selon ces régions. En effet, dans le Golfe du Mexique, les mâles semblent devenir matures à partir de 195-215 cm et les femelles autour de 204-226 cm (Branstetter & Stiles, 1987; Cruz-Martinez et al., 2005) tandis qu'en Afrique du Sud, la maturité semble être atteinte autour de 235 cm pour les mâles et autour de 240 cm pour les femelles (Cliff & Dudley, 1991). Les courbes de croissance établies à l'aide de l'analyse de vertèbres de requins bouledogue du Golfe du Mexique estiment que ces tailles à maturité correspondraient à un âge oscillant entre 10 et 18 ans pour les deux sexes, les plus vieux individus échantillonnés mesurant entre 240 et 270 cm, soit âgés de 21 à 28 ans (Branstetter & Stiles, 1987; Cruz-Martinez et al., 2005). Les tailles plus grandes mesurées en Afrique du Sud semblent correspondre à des âges plus avancés, l'âge à maturité sexuelle déterminé par Wintner et al. (2002) étant de 20-21 ans (246 et 250 cm, respectivement), et les individus les plus grands étant âgés de 29 et 32 ans (278 et 284 cm, respectivement).

Par ailleurs, les données sur les cycles de reproduction, notamment les périodes d'accouplements et de mises bas, sont encore très rares. Certaines études pointent vers une période de parturition pendant la saison chaude, entre novembre et février dans l'Hémisphère Sud (Bass et al., 1973; Brunnschweiler & Baensch, 2011; Sadowsky, 1971), et autour des mois de avril-juin dans l'Hémisphère Nord (Clark & von Schmidt, 1965). A l'inverse, d'autres études n'ont pas observé de périodes spécifiques d'accouplements ou de mise bas (Cliff & Dudley, 1991; Jenson, 1976). En outre, le faible nombre de femelles gravides étudiées et l'absence de suivi de gestation en aquarium n'ont pas permis d'estimer la durée exacte de la période de gestation. Se basant sur la présence de nouveau-nés dans les estuaires en avril-mai, et sur une période d'accouplements en juin-juillet, Clark et von Schmidt (1965) ont estimé un temps de gestation de 10-11 mois. Par ailleurs, le suivi photographique et filmographique régulier d'individus fidélisés à un site de nourrissage aux Fidji a mené à l'hypothèse d'un cycle bisannuel, avec une période de « repos » d'un an entre deux gestations (Brunnschweiler & Baensch, 2011). En effet, les individus présentaient des cicatrices dues à l'accouplement seulement sur la période allant de décembre à février, tandis que, sur la même période, les femelles gravides quittaient le site pour quelques semaines, revenant après la mise bas (Brunnschweiler & Baensch, 2011). Ce cycle, incluant une période de repos entre deux gestations, a déjà été supposé chez d'autres espèces de Carcharhinidés (Castro, 1993; Clark & von Schmidt, 1965), tels le requin milandre *Galeorhinus galeus* (Peres & Vooren, 1991) ou le requin requiem de sable *C. obscurus* (Musick et al., 1993), et serait donc possible chez le requin bouledogue. Néanmoins, les données pour estimer ces caractéristiques restent rares et peuvent mener à de fausses conclusions. Par ailleurs, il est connu que les femelles de certains Carcharhinidés peuvent stocker le sperme dans leur glande nidamentaire pour des périodes plus ou moins longues (Conrath et al., 2012; Pratt, 1993), ce qui peut biaiser les estimations des temps de gestation. Enfin, la présence de polyandrie chez le requin bouledogue n'avait pas encore été testée au début de cette thèse, et pourrait potentiellement induire des conséquences directes sur le comportement des requins en période d'accouplements (agrégation), mais aussi des conséquences indirectes sur la diversité génétique des populations.

Ainsi, il existe encore beaucoup d'inconnues concernant la biologie du requin bouledogue, qui pourraient être levées grâce à des études utilisant à la fois des méthodes directes et indirectes, amenant de nouvelles connaissances pour des plans de gestion adaptés à la taille et à l'état de santé des populations à travers l'aire de répartition de cette espèce. C'est également le cas pour

le requin tigre *G. cuvier*, une espèce appartenant à la même famille que le requin bouledogue, et, qui bien qu'ayant des caractéristiques communes (grand requin, prédateur apical), présente pourtant des comportements et des caractéristiques biologiques différentes.

2.2. Etat actuel des connaissances sur la biologie du requin tigre *Galeocerdo cuvier*

2.2.1. Statut du requin tigre *Galeocerdo cuvier*

Le requin tigre *Galeocerdo cuvier* (Péron & Lesueur 1822) (Figure 7) est également un requin de la famille des Carcharhinidés trouvé mondialement dans les eaux tropicales et subtropicales (Compagno, 1984b, 1990).

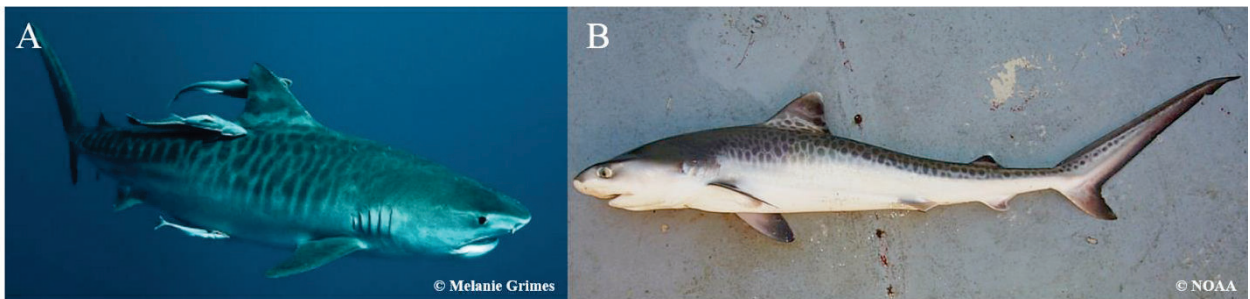


Figure 7. Requin tigre *Galeocerdo cuvier*. **A** adulte ; **B** juvénile.

Ce requin, qui peut faire jusque 5,5 m de long (Meyer et al., 2014), est l'une des plus grandes espèces avec le grand requin blanc *C. carcharias* et le requin baleine *R. typus*. Cette espèce est très opportuniste (Lowe et al., 1996), présentant un régime alimentaire très varié et se nourrissant même parfois de charognes (Dudley et al., 2000), ce qui se traduit par de grandes variations de régime alimentaire entre régions, dues probablement à la disponibilité en proies (Bornatowski et al., 2014; Heithaus, 2001; Lowe et al., 1996; Simpfendorfer, 1992; Simpfendorfer et al., 2001; Trystram et al., 2017). Par ailleurs, comme discuté précédemment, il a été démontré qu'il exerçait un rôle important dans les écosystèmes marins en influençant la façon dont ses proies utilisent l'habitat, modifiant ainsi la structure des communautés des producteurs primaires, la biomasse et la composition en nutriments des écosystèmes (Burkholder et al., 2013; Heithaus et al., 2008; Wirsing et al., 2007b). Bien qu'il ne soit pas visé spécifiquement par des pêcheries, le requin tigre est une prise accessoire régulière des pêcheries océaniques à la palangre horizontale dans le Pacifique Ouest (Polovina & Lau, 1993), dans l'Atlantique Nord-Ouest (Baum et al., 2003) et l'Atlantique Sud (Afonso & Hazin, 2014).

Il est estimé qu'environ 400 000-500 000 requins tigre sont retrouvés par an sur le marché mondial des ailerons de requins (Clarke et al., 2006). Tout comme le requin bouledogue, cette espèce est impliquée dans des attaques sur l'Homme et est visée par des programmes de régulation en Australie (Holmes et al., 2012; Reid & Krogh, 1992; Simpfendorfer, 1992), en Afrique du Sud (Cliff & Dudley, 1991; Dudley, 1997; Sumpton et al., 2011), à Hawaii (Wetherbee et al., 1994) et à La Réunion. Ainsi, même s'il n'existe pas de suivi de captures sur le long terme provenant des pêcheries commerciales, les programmes de régulation, qui sont menés depuis plusieurs décennies, permettent de suivre les populations grâce à des données de captures par des méthodes de pêche standardisées. L'analyse de ces données a notamment mis en évidence des déclinés de taux de captures en Australie [Queensland, Holmes et al. (2012) et New South Wales, Reid et al. (2011)], et dans l'Atlantique Nord (Baum et al., 2003; Myers et al., 2007), déclinés probablement liés à des déclinés de populations. La seule région où les captures de requin tigre ont augmenté est le Kwazulu-Natal, en Afrique du Sud (Dudley & Simpfendorfer, 2006). L'impact des pêcheries sur ce requin reste donc encore mal estimé, et encore aucune étude génétique n'a estimé la taille efficace des populations de requins tigre, dont la structure n'a été étudiée que très récemment.

2.2.2. Structure des populations

Les études de marquage-recapture ou de suivi satellite ayant décrit les mouvements des requins tigre ont montré une grande hétérogénéité interindividuelle de l'utilisation de l'habitat, rendant difficile de faire des conclusions sur la taille, en terme de surface géographique, des populations. En effet, certains individus ont montré des capacités de migration très importantes (Ferreira et al., 2015; Hammerschlag et al., 2012; Holmes et al., 2014; Lea et al., 2015b; Werry et al., 2014), avec notamment deux requins ayant traversé l'Atlantique, de l'Amérique (Sud ou Nord) jusqu'à la côte est-africaine, une distance représentant plusieurs milliers de kilomètres (Afonso et al., 2017; Kohler et al., 1998; Kohler & Turner, 2001). Par ailleurs, un autre individu a été suivi lors de sa traversée de l'océan Indien, de l'Australie jusqu'en Afrique du Sud, traversant plus de 8 000 km en 99 jours (Heithaus et al., 2007b). Ainsi, si ce requin était majoritairement considéré comme une espèce inféodée aux milieux côtiers, il semble capable de faire des incursions longues et fréquentes en milieu océanique, plus que d'autres espèces côtières telles le requin bouledogue par exemple. Il est ainsi de plus en plus considéré comme une espèce semi-océanique plutôt que strictement côtière (Domingo et al., 2016; Polovina & Lau, 1993). A l'opposé, d'autres études ont montré des patrons de fidélité importants, certains

individus utilisant des aires géographiques spécifiques, certes grandes, mais retournant régulièrement à des sites précis (Ferreira et al., 2015; Fitzpatrick et al., 2012; Heithaus, 2001; Holland et al., 1999; Lowe et al., 2006). Ces comportements de fidélité au site semblent liés à des facteurs intrinsèques, comme la taille et le sexe des individus, mais aussi à des facteurs extrinsèques, notamment l'abondance en proies ou la température de l'eau. Ainsi, comme le requin bouledogue, les requins plus jeunes ne sont pas trouvés dans les mêmes habitats que les adultes, utilisant majoritairement les milieux côtiers plus riches en proies et évitant également la prédation par des requins plus grands (Lowe et al., 1996). En outre, les femelles sont plus régulièrement trouvées dans des eaux moins profondes que les mâles, et, même si cette différence est encore mal expliquée (Heithaus et al., 2006), elle pourrait être due à la reproduction, les femelles évitant ainsi le harcèlement des mâles (Sulikowski et al., 2016) ou choisissant des lieux particuliers pour mettre bas (Papastamatiou et al., 2013). Enfin, la variabilité d'habitats et de comportements de migration des requins tigre semble également dépendante de leurs comportements de chasse. En effet, ces requins, au fur et à mesure qu'ils grandissent, parcourent leur habitat par des mouvements exploratoires particuliers afin de découvrir de nouveaux territoires de chasse, expliquant probablement les variations interindividuelles de régime alimentaire (Heithaus et al., 2006; Holland et al., 1999; Meyer et al., 2009a; Meyer et al., 2010; Papastamatiou et al., 2011). Par ailleurs, des migrations saisonnières ont également été observées et mises en lien avec des comportements de prédation, notamment à Hawaii. En effet, plusieurs requins tigre ont été observés effectuant des migrations vers un atoll pendant la période d'envol des jeunes albatros, lors de la saison chaude, avant de parcourir plusieurs milliers de kilomètres le long de l'archipel hawaïen, revenant à cet atoll la saison d'après (Lowe et al., 2006; Meyer et al., 2010). Ces migrations saisonnières peuvent également être liées à des changements de température de l'eau, ce qui a été observé en Australie, où les requins tigre marqués par des émetteurs acoustiques sont moins détectés pendant la saison froide (juin-août), quand les températures sont inférieures à 19-20°C (Heithaus, 2001; Wirsing et al., 2006). Par ailleurs, les captures diminuent également à cette période (Holmes et al., 2014). Ainsi, toutes ces études montrent la complexité des patrons de migration et de l'utilisation de l'habitat du requin tigre, même si elles permettent également de tirer quelques conclusions. Ainsi, il semble que cette espèce peut effectuer des migrations importantes et bouger sur des grandes aires géographiques, bien que présentant de la fidélité à certains sites, due à une combinaison de facteurs intrinsèques et extrinsèques. Les conséquences de ces comportements sur la structure et la connectivité des populations n'ont été étudiées que très récemment, depuis les années 2010, à l'aide de marqueurs moléculaires.

En effet, la première étude de génétique des populations du requin tigre était initialement conçue pour faire de l'identification d'espèces, reposant donc sur un échantillonnage faible (29 individus échantillonnés à travers l'aire de répartition de l'espèce) et un seul marqueur mitochondrial (Naylor et al., 2012). Elle a néanmoins permis d'identifier la présence de deux clades monophylétiques dans les océans Indien/Pacifique et Atlantique ne présentant aucun haplotype partagé, pouvant potentiellement constituer deux sous-espèces. Cette hypothèse a été par la suite réfutée par Bernard et al. (2016), qui ont réalisé un échantillonnage spécifique (380 échantillons provenant de 10 localités réparties dans les trois bassins océaniques) et utilisé 10 marqueurs microsatellites et deux marqueurs mitochondriaux. Ils ont en effet mis en évidence un isolement génétique ancien entre les populations de requin tigre de l'Atlantique et des océans Indien/Pacifique, mais ont identifié des haplotypes partagés entre ces régions, ce qui est incompatible avec l'hypothèse des deux sous-espèces. Par ailleurs, les échantillons collectés à Hawaii semblaient génétiquement différenciés de ceux prélevés dans les autres localités (Bernard et al., 2016), possiblement dû à une fidélité des requins tigre de cette région à l'archipel hawaïen (Meyer et al., 2010; Papastamatiou et al., 2013). Enfin, les auteurs ont également identifié une différenciation mitochondriale légèrement plus importante que la différenciation nucléaire au sein des océans Indien et Pacifique, même entre des localités des côtes Est et Ouest de l'Australie. Ils ont supposé que cela pouvait être dû à de la philopatrie des femelles à des sites de nurseries. Une deuxième étude parue peu de temps après s'est focalisée sur la structure des populations des océans Indien et Pacifique, avec un échantillonnage plus spécifique à ces deux bassins (347 échantillons, majoritairement prélevés en Australie, mais aussi à Hawaii), mais n'utilisant que neuf marqueurs microsatellites (Holmes et al., 2017). Utilisant huit échantillons provenant du Brésil en tant que groupe externe ou *outgroup*, les auteurs ont confirmé l'isolement génétique entre les populations de l'Atlantique et celles des océans Indien et Pacifique, ainsi que l'importante connectivité autour de l'Australie. Néanmoins, aucune différenciation génétique n'a été identifiée entre les échantillons prélevés en Australie et ceux d'Hawaii, contredisant les résultats de Bernard et al. (2016). Par ailleurs, ils n'ont pu infirmer ou confirmer la philopatrie des femelles à des zones de nurseries, n'utilisant pas de marqueurs mitochondriaux.

Ainsi, s'il semble maintenant bien établi que les populations de requin tigre de l'Atlantique sont génétiquement différenciées de celles des océans Indien et Pacifique, la structure des populations au sein de ces deux derniers bassins océaniques reste controversée. Par ailleurs, si la structure des populations de requin tigre reste méconnue, des études se sont intéressées à la

dynamique de ces populations, mettant notamment en évidence certaines caractéristiques biologiques de la reproduction de ces requins.

2.2.3. Dynamique des populations

Le requin tigre est la seule espèce de la famille des Carcharhinidés à se reproduire par viviparité lécithotrophe, les embryons étant reliés à un sac vitellin représentant leur unique source de nutriments (Compagno, 1984a; Parsons et al., 2008). Les portées comprennent généralement 30 à 50 embryons (Bigelow & Schroeder, 1948; Clark & von Schmidt, 1965; Simpfendorfer, 1992; Varghese et al., 2017; Whitney & Crow, 2007), même si ces nombres ne sont pas toujours vérifiés, des portées de trois embryons ayant déjà été collectées (Whitney & Crow, 2007) ainsi que des portées bien plus grandes, allant jusqu'à 82 embryons (Bigelow & Schroeder, 1948). À la naissance, les juvéniles mesurent entre 50 et 90 cm (Compagno, 1984a; Randall, 1992; Simpfendorfer, 1992; Whitney & Crow, 2007), ce qui représente une variabilité assez importante. Par ailleurs, les tailles à maturité reportées présentent également des variations, notamment entre régions, variant de 286 cm (Mer d'Arabie ; Varghese et al., 2017) à 318 cm (Afrique du Sud ; Dicken et al., 2016) pour les mâles, et de 300 cm (Golfe du Mexique ; Clark & von Schmidt, 1965) à 359 cm (Afrique du Sud ; Dicken et al., 2016) pour les femelles. Selon les estimations réalisées grâce aux analyses de vertèbres, ces tailles correspondraient à des âges compris entre 7 et 10 ans (Branstetter et al., 1987; Holmes et al., 2015; Kneebone et al., 2008; Wintner & Dudley, 2000), l'âge maximal estimé étant de 27-29 ans (Kneebone et al., 2008).

En outre, contrairement à la majorité des espèces de requins étudiées, il semblerait qu'il n'existe pas de polyandrie chez le requin tigre (Holmes et al., 2018). Bien que seules quatre portées aient été étudiées à ce jour, et que des analyses supplémentaires soient nécessaires pour confirmer cette observation, cela pourrait être lié aux capacités de migration de cette espèce. En effet, les espèces de requins avec de grandes populations et une connectivité importante, telles les espèces océaniques et semi-océaniques, sont supposées présenter des fréquences de polyandrie plus faibles que les espèces présentant des populations plus structurées, telles les espèces côtières (Chapman et al., 2004). Le requin tigre serait donc une espèce exclusivement, ou du moins majoritairement, monoandre, ce qui pourrait le rendre plus vulnérable à des pertes de diversité génétique, et le nombre de femelles reproductrices constituerait une contrainte majeure de la taille efficace de ses populations (Holmes et al., 2018; Sugg & Chesser, 1994).

Enfin, cette espèce présenterait des cycles de reproduction saisonniers, la parturition ayant lieu plutôt durant la saison chaude, pendant l'été austral dans l'Hémisphère Sud (Bass et al., 1975; Dicken et al., 2016; Fourmanoir, 1961; Jaquemet et al., 2013) et pendant les mois de mai-octobre dans l'Hémisphère Nord (Varghese et al., 2017; Whitney & Crow, 2007). Grâce aux données issues des programmes de régulation, Whitney et Crow (2007) ont pu étudier plus précisément le cycle du requin tigre, par rapport à ce qui a pu être fait pour le requin bouledogue. Ainsi, le requin tigre se reproduirait selon un cycle trisannuel, avec une période de gestation d'une quinzaine de mois avant laquelle le sperme serait stocké dans la glande nidamentaire pendant 4-5 mois, et suivie d'une période de repos d'un an (Whitney & Crow, 2007). Ce cycle doit être confirmé par des études complémentaires, notamment menées dans d'autres régions de l'aire de répartition de ce requin, afin d'identifier une potentielle variabilité régionale.

Ainsi, les études sur le requin bouledogue *Carcharhinus leucas* et le requin tigre *Galeocerdo cuvier* sont encore rares, ce qui induit un manque de connaissance sur la biologie de ces espèces. C'est suite à cette observation, et à une recrudescence des attaques sur les humains à La Réunion, qu'a été initiée le projet de thèse.

2.3. Contexte réunionnais

Depuis 2011, 25 attaques de requin ont eu lieu à La Réunion, dont neuf mortelles (Lagabrielle et al., 2018). Bien que des attaques aient toujours eu lieu à La Réunion, qui est une île dont la quasi-absence de plateau insulaire et la géomorphologie des fonds côtiers prédisposent à la présence immédiate de requins près des côtes, leur fréquence semble avoir augmenté depuis 2011. Ces attaques, en plus des conséquences humaines, ont eu un impact négatif sur la dynamique économique de l'île, entraînant notamment une baisse du tourisme et des activités nautiques, ce qui a été qualifié de « crise requin ». Face à cette crise aux dimensions sociales, économiques et écologiques, et au manque de connaissances sur les deux espèces impliquées dans les attaques, le requin bouledogue et le requin tigre, un plan gouvernemental a été mis en place. Un de ses objectifs est notamment d'apporter des connaissances sur ces espèces, afin d'apporter des éléments d'aide à la gestion du risque, tout en préservant la biodiversité et les écosystèmes marins. Ainsi, un premier programme a été financé, le programme CHARC (Connaissance de l'écologie et de l'HAbitat de deux espèces de Requins Côtiers sur la côte Ouest de La Réunion), visant à étudier la biologie, l'écologie et le comportement de ces deux

espèces à La Réunion. Ce programme a permis de mieux comprendre les déplacements des individus le long de la côte Ouest de La Réunion, ainsi que leur écologie trophique (Blaison et al., 2015; Soria et al., 2015; Trystram et al., 2017). Les individus pêchés étaient marqués avec un émetteur acoustique (disposé dans la cavité interne de l'animal) et des récepteurs ont été déployés tout le long de la côte Ouest de La Réunion afin de suivre les mouvements des individus marqués. Sur chaque individu marqué, un morceau d'aile a été prélevé (1 cm ; méthode non invasive et non destructrice) pour les premières études de génétique des populations. En parallèle de ce programme, un programme expérimental de pêche ciblée a également été mis en place, le programme Caprequins. Ce programme avait pour objectif de réduire les populations de requins bouledogue et tigre aux alentours de La Réunion et ainsi réduire le risque d'attaques, sans savoir réellement les conséquences de cette pêche sur l'occurrence des attaques. En effet, des études réalisées à Hawaii concernant le programme de régulation mis en place pour limiter les attaques de requin tigre n'ont pas mis en évidence de réelles diminutions des attaques suite à la pêche de régulation (Wetherbee et al., 1994). Des études similaires réalisées sur les programmes de régulation menés en Australie et en Afrique du Sud n'ont également pas été concluantes sur l'efficacité de la pêche pour réduire les attaques, mais ont montré, en revanche, que ces programmes de régulation pouvaient avoir des impacts non négligeables sur les écosystèmes marins, en pêchant des espèces vulnérables non ciblées, comme des tortues marines, des raies, des cétacés, ou d'autres espèces de requins (Cliff & Dudley, 2011; Gibbs & Warren, 2015; Sumpton et al., 2011). Par ailleurs, ces programmes n'auraient qu'un impact limité et ponctuel si les populations de requin sont ouvertes, c.-à-d. si de nouveaux individus peuvent venir régulièrement de l'extérieur des zones ciblées par ces pêches (Dudley, 1997; Reid et al., 2011). Le programme ECoReCo-Run a ensuite été mis en place pour continuer à approfondir les connaissances sur ces deux espèces de requin, en proposant une valorisation scientifique des requins pêchés dans le cadre du programme Caprequins. Il vise notamment à améliorer les connaissances sur le régime alimentaire de chaque espèce, en prenant en compte une potentielle variation saisonnière et/ou selon le sexe, à mieux comprendre les cycles de reproduction de ces deux espèces et à estimer les paramètres biologiques de dynamique de ces populations (tels l'âge à maturité sexuelle, ou l'établissement de relations taille-poids). C'est dans ce cadre que cette thèse s'est inscrite.

3. Problématique

Ainsi, il s'agira au cours de ce travail de répondre à plusieurs questions concernant la biologie du requin bouledogue *Carcharhinus leucas* et du requin tigre *Galeocerdo cuvier*, afin d'assurer leur conservation et conserver l'équilibre des écosystèmes marins, tout en apportant des connaissances pour une gestion efficace du risque requin, à La Réunion, mais aussi à travers l'aire de répartition de ces espèces. Concernant le requin bouledogue, la mise en évidence à une échelle géographique restreinte de différents patrons de différenciation génétique entre marqueurs nucléaires et mitochondriaux pose la question de savoir si ces patrons sont également identifiables à une échelle plus grande, et quelles peuvent être leurs causes et conséquences (**Chapitre 2**). Une fois cette question résolue, il reste à délimiter les différentes populations : Existe-il des événements de dispersion efficace entre l'océan Indien et l'océan Pacifique ? Et au sein de chacune de ces zones ? Concernant le requin tigre, les deux études existantes de génétique des populations mettent en évidence des patrons discordants au sein des océans Indien et Pacifique, et il s'agira de se demander si les individus de ces deux régions peuvent être considérés comme appartenant à une même population ou non (**Chapitre 4**). Par ailleurs, il reste encore beaucoup d'interrogations sur les comportements de reproduction de ces deux espèces (**Chapitre 3** et **Chapitre 5**): quels sont les cycles de reproduction ? Quelle est la durée de la période de gestation ? Ces espèces présentent-elles de la polyandrie, et à quelle fréquence ? Plus globalement, les populations des deux espèces présentent-elles les mêmes caractéristiques ? Cette question est fondamentale pour envisager des stratégies de gestion du risque différentes selon les sites, si les populations fonctionnent différemment. Des marqueurs moléculaires développés spécifiquement pour chaque espèce (**Chapitre 1**) permettront notamment d'apporter des éléments de réponses à ces questions.

La comparaison des résultats obtenus pour chacune de ces espèces pourra également mettre en évidence le besoin de mesures de gestion adaptées à chacune, et la difficulté à généraliser des mesures pour des espèces présentant des similarités (grande taille, prédateur apical), mais aussi des caractéristiques biologiques différentes.

CHAPITRE 1. Apport de la génétique des populations et de la génétique mendélienne à l'étude de la structure et de la démographie des populations

Les approches génétiques fournissent un outil unique pour l'étude des populations marines, notamment des Chondrichthyens (Dudgeon et al., 2012; Ovenden, 2013; Portnoy & Heist, 2012). Les méthodes directes communément utilisées pour estimer la structure des stocks et leur démographie sont en effet difficilement applicables sur ces espèces ne remontant pas à la surface pour respirer. Parmi les méthodes directes, les méthodes de capture-marquage-recapture ne permettent pas d'obtenir des informations entre la capture et la recapture de l'animal (Thorsteinsson, 2002) et sous-estiment généralement les véritables migrations des individus (Ng et al., 2007). Par ailleurs, l'utilisation de ces données pour estimer la taille des populations dépend de plusieurs hypothèses irréalistes pour ces espèces discrètes (Schwartz et al., 2007). D'autres difficultés sont rencontrées lors de l'utilisation de suivis télémétriques ou acoustiques. Ces méthodes étant onéreuses, elles ne permettent de suivre qu'un faible nombre d'individus (Grothues, 2009).

Bien que présentant aussi des inconvénients (par exemple, ne représente pas les mouvements actuels et observables des individus), les approches moléculaires sont non invasives et non létales, ne nécessitant qu'un fragment de tissu de quelques millimètres, et permettent d'obtenir l'intégralité de son génome nucléaire (hérité des deux parents) et mitochondrial (hérité de la mère). Elles permettent notamment, grâce au cadre théorique de la génétique de populations, d'estimer la structure et la connectivité des populations, leurs tailles, mais aussi certains comportements de reproduction.

1. Génétique des populations marines

1.1. Cadre théorique de la génétique des populations

La génétique des populations, discipline initiée dans les années 1920, vise à étudier la fréquence des allèles et des génotypes, ainsi que les forces susceptibles de modifier ces fréquences au cours des générations successives. Cette évolution peut être simplifiée par l'utilisation de modèles mathématiques. Cette discipline repose sur une loi de référence, qui définit l'évolution des fréquences alléliques et génotypiques, la loi de Hardy-Weinberg. Cette loi énonce que, dans

une population théorique idéale, les fréquences alléliques et génotypiques restent stables de génération en génération. Pour que cette loi soit vérifiée, il faut que les populations répondent à plusieurs caractéristiques : (1) elles sont de taille infinie, (2) les individus sont diploïdes et la reproduction est sexuée, (3) le croisement des individus se fait aléatoirement (pas de choix des partenaires et pas de structuration spatiale, c.-à-d., panmixie), de même que l'association des gamètes (pangamie), (4) les générations ne sont pas chevauchantes, et (5) elles ne sont pas soumises aux forces évolutives. Ces forces modulent la diversité génétique des populations et sont au nombre de quatre : la mutation, la sélection naturelle, la migration et la dérive génétique (Wright, 1931).

La diversité génétique est le résultat de mutations, des modifications aléatoires de l'ADN pouvant mener à l'apparition de nouveaux allèles, qui pourront ensuite se propager dans les populations. Ces mutations peuvent être délétères, neutres ou avantageuses. Alors que les mutations neutres sont conservées ou éliminées aléatoirement, les mutations délétères ou avantageuses vont être soit éliminées, soit fixées par la sélection naturelle. Cette dernière induit le maintien des individus les plus aptes à survivre ou à se reproduire, quelle que soit la raison pour laquelle ils possèdent une telle aptitude. Plusieurs types de sélection sont distingués : (1) directionnelle (si un génotype présente un avantage évolutif), (2) balancée (s'il existe plusieurs optimums phénotypiques) ou (3) stabilisatrice (si elle élimine les génotypes extrêmes en maintenant les génotypes intermédiaires). La dérive génétique, quant à elle, est un phénomène aléatoire d'échantillonnage de certains allèles à chaque génération, ce qui peut causer la fixation ou la perte de ces allèles au cours du temps. Ce phénomène est d'autant plus prononcé que la taille efficace (nombre d'individus participant « efficacement » à la génération suivante) de la population est petite. Ces deux forces accentuent la différenciation des populations en fixant, aléatoirement ou non, des allèles. À l'opposé, la migration d'individus d'une population à l'autre permet, à condition que les individus se reproduisent dans la population d'arrivée, l'homogénéisation des fréquences alléliques et limite ainsi la différenciation des populations (Hartl & Clark, 1997).

Ainsi, la génétique des populations permet le calcul d'estimateurs permettant de mesurer à la fois la structure et la différenciation génétique des populations, les taux de migrations entre ces populations, ainsi que leur taille efficace, reconstruisant ainsi les histoires démographiques des populations. Néanmoins, la génétique des populations est basée sur des modèles mathématiques et ne permet pas toujours de traduire la complexité de la réalité, et donc d'obtenir une réponse claire et univoque, notamment lorsque l'on cherche à répondre à des questions de délimitation

de stocks, ce qui est souvent recherché par les gestionnaires des pêcheries (Sagarin et al., 2009; Waples et al., 2008).

1.2. Estimation de la différenciation génétique des populations marines

Les populations marines, en particulier des Chondrichthyens et Téléostéens, sont généralement très grandes, mobiles et distribuées sur de grandes aires géographiques. Ainsi, même le meilleur échantillonnage possible sera intrinsèquement moins représentatif que ceux pouvant être réalisés pour des espèces terrestres ou d'eau douce. Par ailleurs, les rapides avancées technologiques dans le domaine des biotechnologies et en bio-informatique ont mis à la disposition des scientifiques un large panel de marqueurs moléculaires, permettant même d'obtenir l'intégralité du génome d'une espèce, ainsi que des méthodes d'analyses de plus en plus sophistiquées. Il devient alors parfois difficile pour les gestionnaires des pêches et décisionnaires de savoir quel outil choisir.

Par ailleurs, la grande taille de ces populations présente un problème qu'il est fondamental de comprendre pour bien appréhender les limites des approches génétiques pour caractériser la démographie des stocks exploités. En génétique, on définit la taille efficace de la population, N_e , qui représente la taille de la population idéale ayant le même taux de dérive génétique et de consanguinité que la population considérée (Wright, 1931), et qui est parfois considérée comme une approximation du nombre d'individus reproducteurs ayant effectivement contribué à la génération suivante. Le F_{ST} , indice permettant d'estimer le degré de différenciation entre les populations, est lié à N_e et au taux de migration m , par la relation $F_{ST} = 1/(1 + 4N_e m)$. Cet indice permet d'exprimer la proportion de variation allélique entre les populations (Wright, 1965). En absence de mutations ou de migrations, le F_{ST} est une mesure approximative de l'impact de la dérive génétique entre deux populations, qui est inversement proportionnelle à N_e et augmente dans le temps d'après l'équation $F_{ST} = 1 - (1 - 1/2N_e)^t$, où t représente le nombre de générations. Dans les populations marines, N_e tend à être assez grand (>1 000) pour que la dérive génétique soit faible. Ainsi, même des taux de migration très différents (entre 0,05 et 0,2) ne provoquent pas des variations de F_{ST} importantes quand N_e est grand (Figure 8A). Au contraire, sur des périodes temporelles courtes, des variations de F_{ST} non négligeables peuvent être détectées pour des N_e faibles et en absence de migration (Figure 8B), situations probablement rares et restreintes au cas de colonisations récentes et d'effet fondateur.

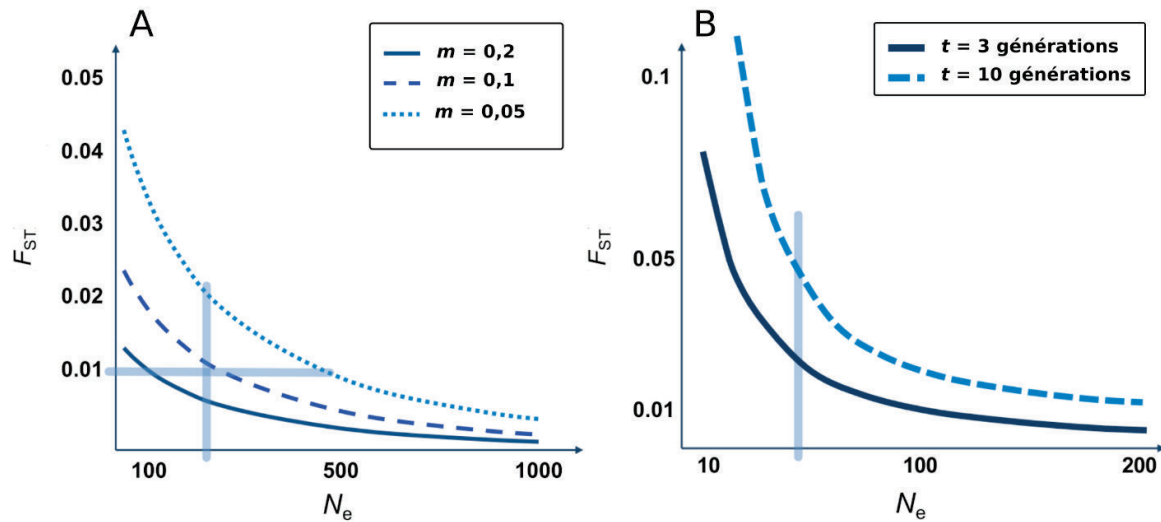


Figure 8. Relations entre la taille efficace N_e et l'indice de différenciation F_{ST} pour **A** trois taux de migrations m différents et **B** absence de migration et deux intervalles de temps t différents. En **A**, la barre horizontale à $F_{ST} = 0,01$ montre que cette valeur peut être détectée pour des populations de $N_e \approx 100$ et connectées par $m = 0,2$ mais aussi pour $N_e \approx 500$ et $m = 0,05$. En **B**, la barre verticale montre que, pour des populations de petite taille ($N_e \approx 50$), la dérive génétique seule peut produire des valeurs de F_{ST} supérieures à 0,02 après seulement trois générations, et supérieures à 0,05 après 10 générations. D'après Mariani et Bekkevold (2014).

Les points discutés ci-dessus sont applicables aux marqueurs moléculaires uniquement sujets aux forces évolutives « neutres », comme la migration et la dérive génétique, ce qui est une hypothèse traditionnellement émise pour la plupart des applications en biologie des populations de poissons (Ihssen et al., 1981). Néanmoins, cette hypothèse de neutralité n'est pas réaliste pour les régions fonctionnelles du génome, codant pour les protéines notamment. Ainsi, les marqueurs neutres, comme les marqueurs microsatellites (Wright & Bentzen, 1995) et l'ADN mitochondrial, ont ainsi été largement utilisés ces 20 dernières années pour étudier les stocks marins exploités, permettant de définir les limites de nombreux stocks de par les océans (Hauser & Carvalho, 2008; Paris et al., 2018).

1.3. Outils d'analyses de la génétique des populations marines: les marqueurs moléculaires

1.3.1. Marqueurs microsatellites

Les marqueurs microsatellites, aussi appelés « *short tandem repeats* » (STR) ou « *simple sequence repeats* » (SSR) sont de courts (de quelques dizaines à quelques centaines de paires

de bases) segments d'ADN répétés, non-codants, trouvés dans tous les génomes eucaryotes. Ils contiennent généralement des motifs répétitifs constitués de deux (par exemple, ...[CG]_n...) à six (...[CGATTG]_n...) paires de bases (pb), avec n le nombre de répétitions trouvées pour un allèle donné. Ce sont des marqueurs extrêmement polymorphes, dits hypervariables, tant du point de vue du nombre de répétitions que de la taille du motif répété, et peuvent être trouvés par milliers, voire centaines de milliers, dans les génomes. La longueur du fragment amplifié définissant les différents allèles du marqueur, on parle de polymorphisme de longueur.

Les taux de mutation de ces marqueurs sont relativement élevés, variant de 10^{-6} à 10^{-2} suivant les espèces, alors que le taux d'apparition des mutations ponctuelles est plutôt de l'ordre de 10^{-9} à 10^{-10} (Ellegren, 2000; Estoup & Angers, 1998; Jarne & Lagoda, 1996). Plusieurs théories ont été développées pour définir le modèle d'évolution de ces marqueurs. Le modèle « pas à pas » (*Stepwise Mutation Model*, SMM ; Ohta & Kimura, 1973) suppose que chaque mutation va induire la perte ou le gain d'un seul motif de répétition, avec une fréquence μ (Shriver et al., 1993). Ainsi, les fréquences alléliques du marqueur considéré devraient présenter une distribution uni-modale, les longueurs extrêmes étant moins souvent représentées. Néanmoins, il est rapidement apparu que le SMM ne reflétait pas toujours les distributions alléliques observées (Di Rienzo et al., 1994). Le modèle de mutation par « allèles infinis » (*Infinite Allele Model*, IAM ; Takezaki & Nei, 1996), modèle plus classique, ne semble pas non plus approprié pour définir l'évolution de ces marqueurs. Dans ce modèle, chaque mutation crée un nouvel allèle avec une fréquence μ , par ajout ou suppression d'une ou plusieurs unités de répétition (Kimura & Crow, 1964), chaque nouvel allèle ayant la même probabilité d'apparaître. Ainsi, un autre modèle intermédiaire aux deux précédents a été défini, le modèle à « deux phases » (*Two Phase Model*, TPM ; Di Rienzo et al., 1994), dans lequel l'allèle muté change de X répétitions, enlevées ou soustraites avec une probabilité égale pour que le modèle soit symétrique. X est égal à 1 avec une probabilité p et, si X est différent de 1 (probabilité $1-p$), alors il suit une loi géométrique ($P(X = x) = \alpha(1 - \alpha)^{x-1}$) de variance $\sigma^2 = (1 - \alpha)/\alpha^2$. Ce modèle dérivé du SMM permet donc d'autoriser des pas de mutations supérieurs à une répétition. Une version simplifiée de ce modèle a également été décrite, le modèle par « pas généralisé » (*Generalized Stepwise Mutation model*, GSM), avec $p = 0$. La plupart des études montrent que ces deux derniers modèles sont les plus réalistes pour modéliser les mutations des marqueurs microsatellites [pour une revue, voir Ellegren (2000), Estoup & Cornuet (1999) et Schlötterer (2000)], même s'il est maintenant admis qu'il existe beaucoup de variations selon les marqueurs et les espèces (Ellegren, 2000; Schlötterer, 2000).

Enfin, même si les marqueurs microsatellites présentent les avantages d'être hypervariables et *a priori* neutres, leurs mécanismes mutationnels et le type de polymorphisme qu'ils génèrent peuvent faire apparaître de l'homoplasie de taille (Estoup et al., 1995). En effet, deux allèles identiques car présentant le même nombre de répétitions peuvent être dus à des mutations convergentes (sous le SMM par exemple, l'allèle (GT)₉ peut provenir d'une mutation d'une répétition de l'allèle (GT)₈ ou de l'allèle (GT)₁₀), et donc présenter des histoires évolutives différentes indétectables. De même, deux allèles présentant la même longueur peuvent présenter des séquences différentes. Ainsi, en génétique des populations, ce phénomène peut mener à des sous-estimations de la distance génétique entre deux individus, ou mal représenter les relations de descendance entre haplotypes (Estoup et al., 2002). Même si l'homoplasie de taille peut être détectée dans certains cas, notamment lorsque des mutations dans la séquence du marqueur microsatellite ne font pas varier la taille, mais peuvent être décelables en séquençant le fragment d'ADN, elle ne peut pas toujours être mise en évidence par des techniques moléculaires (Estoup et al., 2002). Il faut alors tenter de minimiser son impact en utilisant un grand nombre de marqueurs microsatellites, tant que possible utiliser les modèles mutationnels adaptés aux marqueurs étudiés, et prêter particulièrement attention aux espèces impliquant des taux de mutations et des tailles de populations importantes, couplés à des fortes contraintes de tailles alléliques (Estoup et al., 2002).

1.3.2. ADN mitochondrial

L'ADN mitochondrial est présent en nombreuses copies dans les mitochondries des cellules, et est totalement distinct de l'ADN nucléaire, bien que le fonctionnement des mitochondries dépende de ce dernier. Suivant le type cellulaire, des milliers de copies peuvent être trouvées et, suivant les espèces, peuvent être circulaires ou linéaires, double ou simple brin, et de taille variable. Chez les animaux plus complexes, l'ADN mitochondrial mesure environ 16 000 pb, mais peut mesurer plus de 39 000 pb chez les pétoncles (Snyder et al., 1987). En terme de gènes, chez les organismes plus complexes et notamment les Vertébrés, l'ADN mitochondrial est relativement stable : il contient généralement 13 gènes codant pour des protéines, 22 codant pour des ARN de transfert (ARN_t), quelques gènes codant pour des ARN ribosomiques (ARN_r) et une région non-codante, la région contrôle (appelée D-loop chez les Vertébrés). L'ordre de ces gènes peut changer légèrement au sein des Vertébrés, notamment par des translocations des gènes codant pour les ARN_t, mais celui des poissons au sens large présente le même ordre que celui trouvé dans la majorité des espèces de Vertébrés. Cet ADN est considéré comme « un

exemple extrême d'économie génétique » (Attardi, 1985), car il n'existe pas d'introns entre les gènes, très peu de séquences répétitives, et quasiment pas de séquences intercalées entre les gènes.

Bien que plus faibles que ceux des marqueurs microsatellites, l'ADN mitochondrial est aussi caractérisé par des taux de mutation relativement importants par rapport à l'ADN nucléaire. Ceci a d'abord été mis en évidence chez les Primates (Brown et al., 1979), et induit des niveaux de polymorphisme et de divergence plus importants par rapport à l'ADN nucléaire (Awise, 1987, 2009). Cela permet à l'ADN mitochondrial d'être très informatif pour étudier la structure génétique des populations et leur histoire évolutive, mais aussi pour étudier les relations phylogénétiques entre espèces apparentées. Ce taux de mutation important peut être dû à plusieurs facteurs, comme (1) peu de contraintes fonctionnelles car l'ADN mitochondrial ne code que peu de polypeptides et ne produit pas de protéines directement impliquées dans sa réplication, transcription, ou traduction ; (2) le fait qu'il ne soit pas lié fortement aux histones, elles-mêmes conservatives et induisant des taux d'évolution contraints ; et (3) les effets délétères du stress oxydatif et les processus inefficaces de réparation produisant continuellement de nouveaux allèles mitochondriaux (Awise, 2009; Rand, 2001). Bien que semblant contre-intuitif du fait de la conservation de la structure des gènes mitochondriaux sur un temps évolutif long, la séquence nucléotidique mitochondriale évolue rapidement. Le taux d'évolution de l'ADN mitochondrial a longtemps été considéré comme étant constant (théorie de l'horloge moléculaire), le taux de divergence correspondant donc au temps de divergence. Néanmoins, en s'intéressant à des divergences récentes, Nabholz et al. (2008, 2009) ont montré que les taux de substitution mitochondriaux pouvaient varier d'un facteur 30 chez les oiseaux, et même d'un facteur 100 chez les mammifères. Par ailleurs, le taux de substitution moyen de la troisième position des codons peut varier d'une substitution tous les 100 millions d'années chez les baleines à une substitution tous les millions d'années chez les gerbilles, rendant peu convaincante l'hypothèse de l'horloge moléculaire constante (même si les temps de générations entre espèces varient grandement). Des différences importantes ont aussi été montrées entre les taux d'évolution de plusieurs lignées d'Élasmobranches, ainsi qu'au sein des requins, avec le taux d'évolution de la séquence du gène codant pour le cytochrome b (cytb) variant d'un facteur 6,5 entre les genres (Martin, 1995; Winchell et al., 2004).

Par ailleurs, l'ADN mitochondrial des animaux est généralement considéré non-recombinant et hérité uniquement de la mère. En effet, les mitochondries sont des organites cytoplasmiques, et

le cytoplasme du zygote provient uniquement de l'ovule. Les mitochondries paternelles contenues dans la pièce intermédiaire des spermatozoïdes, et qui peuvent éventuellement pénétrer dans le zygote pendant la fécondation, sont activement dégradées peu après. Malgré quelques mentions de « fuites » d'ADN mitochondrial paternel dans la descendance (voir Avise (2012) et références incluses), il semble que l'héritabilité strictement maternelle de l'ADN mitochondrial, ainsi que le fait qu'il soit non-recombinant, induit une histoire évolutive de transmission maternelle des génotypes mitochondriaux. Par ailleurs, elle induit aussi que l'information génétique mitochondriale est haploïde. Dès lors, pour les deux copies (une du père et une de la mère) de chaque gène nucléaire, il n'existe qu'une copie de chaque gène mitochondrial (Castellana et al., 2011). Ainsi, du fait de son haploïdie et de son héritabilité maternelle, il est considéré que l'ADN mitochondrial présente une taille efficace N_e quatre fois plus réduite que celle du génome nucléaire (Lynch, 1997), ce qui le rend plus sensible à la dérive génétique et aux goulots d'étranglement ou *bottleneck* (réduction temporaire de la taille de la population provoquant une perte de diversité génétique) (Birky et al., 1983). Néanmoins, cela n'est pas toujours vérifié, notamment à cause de la variabilité importante du succès reproducteur des mâles par rapport aux femelles, particulièrement dans les systèmes polygames, ce qui peut réduire le N_e du génome nucléaire sans altérer celui du génome mitochondrial (Ballard & Whitlock, 2004).

Ainsi, les marqueurs moléculaires présentés permettent d'estimer la diversité génétique des populations pour procéder par la suite à des analyses de génétique des populations.

1.4. Méthodes d'analyse de la structure et de la démographie des populations

Comme vu précédemment, l'évolution de la diversité génétique se fait sous l'effet de forces évolutives et du système de reproduction. Le terme « population » peut être défini de plusieurs manières (Waples & Gaggiotti, 2006). Selon la plus utilisée, et celle que nous utiliserons tout au long de ce manuscrit, la population est l'ensemble des individus d'une même espèce présents dans une aire géographique suffisamment restreinte pour que chaque membre du groupe puisse se reproduire avec n'importe quel autre individu avec la même probabilité (Hartl & Clark, 1997). Dès lors, il est possible de définir des groupes correspondant à cette définition, qui peuvent être connectés entre eux par des événements de migration fréquents et réguliers, pouvant ainsi constituer des métapopulations. La différenciation des populations peut alors être

représentée le long d'un continuum, l'isolement complet correspondant à un extrême de ce continuum (Figure 9A), et la panmixie représentant l'autre (Figure 9D).

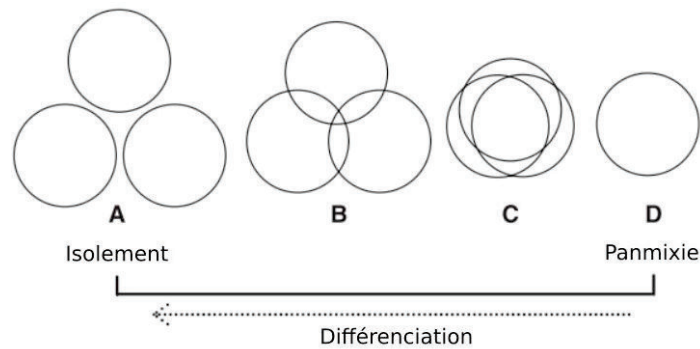


Figure 9. Le continuum de la différenciation des populations. Chaque groupe de cercles représente un groupe de populations avec différents degrés de connectivité. **A** Isolement complet, **B** Faible connectivité, **C** Connectivité importante, **D** Panmixie : les populations sont totalement similaires. D'après Waples et Gaggiotti (2006).

La plupart des populations réelles sont dans des situations intermédiaires (Figures 9B et 9C) à ces deux extrêmes. Il faut ainsi définir, dans ce continuum, un taux de migration seuil m au-dessous duquel on considère les sous-unités d'une métapopulation comme des populations. En biologie de la conservation, la règle d'un migrant par génération est souvent utilisée pour définir les unités de conservation, avec $m = 1/N_e$ (Mills & Allendorf, 1996; Wang, 2004). Une fois ce seuil défini, on peut donc délimiter les sous-unités constituant les populations, notamment grâce à des tests d'assignement et des mesures de différenciation génétique.

1.4.1. Tests d'assignement et mesure de la différenciation génétique

Les tests d'assignements sont des méthodes qui ont été développées afin d'identifier et de différencier les groupes génétiques auxquels appartiennent les individus échantillonnés. Plusieurs analyses peuvent être utilisées, et diffèrent de par les hypothèses à vérifier afin de les appliquer. La première de ces méthodes (STRUCTURE ; Pritchard et al., 2000), utilise une approche bayésienne supposant un modèle avec K populations (où K peut être inconnu), chacune caractérisée par un certain nombre de fréquences alléliques à chaque marqueur utilisé. Cette méthode calcule, pour le nombre K de groupes génétiques définis, les probabilités d'assignement des individus en minimisant les écarts à la loi de Hardy-Weinberg au sein de chaque groupe et en maximisant le déséquilibre de liaison entre ces groupes (Pritchard et al., 2000). La seconde méthode communément utilisée est l'analyse discriminante en composantes principales (DAPC ; Jombart et al., 2010). Cette approche permet de réduire significativement

les temps de calculs, et vise à partitionner la variabilité génétique observée en deux composantes, une composante intra-groupe et une composante inter-groupe, en minimisant la première et maximisant la seconde (Jombart et al., 2010). Cette méthode assigne donc également les individus à des groupes génétiques, mais ne fait aucune hypothèse sur les groupes génétiques auxquels les individus sont assignés.

Par ailleurs, la différenciation génétique des populations est estimée communément par les « statistiques F de Wright » (Wright, 1949, 1965). Ces indices prennent en compte la corrélation des allèles au sein des individus et décrit leur association non-aléatoire au niveau intra-populationnel (F_{IS}), au niveau inter-populationnel (F_{ST}) et entre groupes de populations (F_{SC}). Le plus utilisé est le F_{ST} , aussi appelé indice de fixation, qui exprime la diminution de l'hétérozygotie d'une sous-population et permet de mesurer l'écart à la loi de Hardy-Weinberg dû à la différenciation des sous-populations par rapport à une population totale panmictique (Weir & Cockerham, 1984). Cet indice varie entre 0 (panmixie) et 1 (isolement total) et représente donc le continuum de différenciation illustré précédemment (Figure 9). Sa significativité se calcule par des tests de permutations testant l'hypothèse nulle de panmixie. En outre, l'analyse de variance moléculaire (AMOVA), fondée sur l'analyse des variances de fréquences alléliques, permet de définir le niveau d'organisation (entre groupes de populations, inter-populationnel et intra-populationnel) pour lequel la variabilité génétique est la plus importante (Excoffier et al., 1992).

Ces méthodes peuvent présenter des limites, car elles ne prennent pas en compte l'aspect stochastique des données génétiques et démographiques, notamment les variations de taille efficace, qui peuvent jouer sur la dérive génétique. Cela peut augmenter la différenciation génétique entre les populations et brouiller le signal. Par ailleurs, ces méthodes supposent un échantillonnage exhaustif, ce qui est rarement le cas pour les espèces marines, ou certaines populations peuvent ne pas être échantillonnées (population fantôme). Enfin, elles ne prennent pas en compte l'histoire démographique des populations, contrairement au calcul bayésien approché.

1.4.2. Reconstruction de l'histoire démographique des populations : Apport du calcul bayésien approché

Le calcul bayésien approché, ou *Approximate Bayesian Computation* (ABC), est une méthode récente qui s'est avérée être un outil puissant pour répondre à de nombreuses questions de

génétique des populations (Beaumont et al., 2002). En effet, il permet de simuler des données synthétiques (d'où le terme « approché ») pour lesquelles il est impossible de déterminer formellement la probabilité des observations, permettant ainsi d'étudier l'origine des populations et leurs voies d'expansion. Cette approche permet notamment de comparer différents scénarios démographiques et d'estimer *a posteriori* la probabilité de ces scénarios, mais aussi d'inférer des valeurs des paramètres démographiques et évolutifs une fois le scénario connu. Pour cela, les données simulées sont comparées aux données observées (variabilité et caractéristiques génétiques des populations échantillonnées), et sont acceptées ou rejetées suivant leur degré de proximité, calculé grâce à une distance, généralement euclidienne normalisée. Cette ressemblance est mesurée à l'aide d'un certain nombre de statistiques résumées représentant la diversité et la différenciation génétique des populations, telles le nombre moyen d'allèles par locus, l'hétérozygotie, le F_{ST} ... Cette méthode, bien que très puissante, présente deux inconvénients majeurs : (1) elle nécessite, pour être fiable, un nombre de simulations très important (usuellement 1 million de données simulées par scénario testé), et (2) la calibration de l'algorithme ABC (c.-à-d. le niveau de tolérance indiquant le seuil des simulations acceptées par rapport aux rejetées) est une étape critique qui influe sur la distribution postérieure (Blum et al., 2013; Marin et al., 2012). Par ailleurs, le choix des statistiques résumées utilisées est primordial, un nombre trop faible ou trop grand (espace à explorer trop grand) pouvant diminuer l'information disponible.

Ainsi, une nouvelle méthode a été développée très récemment, qui repose sur la méthode des forêts aléatoires, ou *random forest* (RF) (Breiman, 2001). Appliquée aux algorithmes ABC (Pudlo et al., 2016; Raynal et al., 2017), la méthode RF est une méthode permettant de sélectionner *a posteriori* le scénario le plus probable en construisant une classification par apprentissage automatique (« *machine learning* ») des simulations suivant leur distribution *a priori*. Comparée aux anciennes méthodes ABC, l'ABC-RF (1) permet de distinguer plus efficacement les différents scénarios proposés, (2) dépend beaucoup moins du choix des statistiques résumées, et (3) offre un temps de calcul très réduit (100 000 simulations peuvent suffire pour estimer les distributions postérieures des paramètres) (Pudlo et al., 2016; Raynal et al., 2017).

Même si le choix des priors des scénarios à tester reste un point délicat dans toute analyse ABC, surtout lorsque peu d'informations sont disponibles sur la biologie de l'espèce (Templeton, 2009, 2010), ces nouvelles méthodes, couplées aux autres méthodes plus classiques décrites, s'avèrent très efficaces pour explorer la démographie des populations.

Afin de pouvoir réaliser des analyses de génétique de populations, il faut donc disposer de marqueurs moléculaires adaptés aux espèces étudiées. Dans le cas du requin bouledogue *Carcharhinus leucas*, aucun marqueur microsatellite spécifiquement développé pour cette espèce n'était disponible dans la littérature. Les quelques études ayant mené des analyses de génétique des populations sur cette espèce ne disposaient que d'un faible nombre de marqueurs microsatellites (trois à 11 marqueurs), développés pour d'autres espèces de requins de la famille des Carcharhinidés, et qui amplifiaient chez le requin bouledogue (Karl et al., 2011; Testerman, 2014; Tillett et al., 2012). Concernant le requin tigre *Galeocerdo cuvier*, aucune étude de génétique des populations n'avait été publiée au commencement de cette thèse. Par ailleurs, les marqueurs développés pour d'autres espèces et amplifiant sur le requin tigre sont généralement moins variables que ceux spécifiquement caractérisés pour l'espèce étudiée.

2. Développement de marqueurs microsatellites spécifiques au requin bouledogue *Carcharhinus leucas*

Résumé

Au début de cette thèse, nous avons donc développé une banque microsatellite spécifique au requin bouledogue *Carcharhinus leucas*, et testé les amplifications croisées de ces marqueurs sur plusieurs autres espèces de requins. Cette banque a été développée à partir d'échantillons prélevés sur 11 requins adultes pêchés à La Réunion, et le polymorphisme a été testé sur un échantillonnage populationnel provenant de La Réunion, comprenant 41 individus. Ainsi, 20 marqueurs microsatellites ont pu être isolés pour le requin bouledogue, dont trois amplifiant également le requin tigre, 11 amplifiant le requin gris *Carcharhinus plumbeus* et 19 le requin requiem des sables *Carcharhinus obscurus*.

Ces résultats ont permis la publication d'un article dans *Conservation Genetic Resources*.

Isolation and characterization of 20 microsatellite markers from *Carcharhinus leucas* (bull shark) and cross-amplification in *Galeocerdo cuvier* (tiger shark), *Carcharhinus obscurus* (dusky shark) and *Carcharhinus plumbeus* (sandbar shark)

Agathe Pirog · Antonin Blaison · Sébastien Jaquemet · Marc Soria · Hélène Magalon

Received: 4 August 2014 / Accepted: 19 August 2014
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Abstract With the development of genetics methods, it becomes possible to study the population structure and some aspects of the reproductive behaviour of endangered sharks. Here we describe the isolation of 20 polymorphic microsatellite markers in the bull shark *Carcharhinus leucas* (Carcharhinidae) and their characteristics. Two to 10 alleles per locus were detected. Observed and expected heterozygosities ranged from 0.00 to 0.78 and from 0.05 to 0.80, respectively. Four markers showed deviations from Hardy–Weinberg equilibrium; among them, three showed presence of null alleles. No linkage disequilibrium was detected among any of the loci. Moreover, four, 11 and 19 of these 20 markers successfully cross-amplified in the tiger shark *Galeocerdo cuvier*, the sandbar shark *Carcharhinus plumbeus* and the dusky shark *Carcharhinus obscurus*, respectively.

Keywords Bull shark · *Carcharhinus leucas* · Microsatellite · *Galeocerdo cuvier* · *Carcharhinus plumbeus* · *Carcharhinus obscurus*

A. Pirog · A. Blaison · S. Jaquemet · H. Magalon (✉)
Laboratoire ECOMAR-FRE3560 CNRS, Université de la Réunion, 15 Avenue René Cassin, CS 92003, 97744 St Denis Cedex 09, La Réunion, France
e-mail: helene.magalon@univ-reunion.fr

A. Blaison · M. Soria
UMR EME, IRD Réunion, Parc Technologique Universitaire, 2 rue Joseph Wetzell, CS 41095, 97495 Ste Clotilde Cedex, La Réunion, France

S. Jaquemet · H. Magalon
Laboratory of Excellence CORAIL, BP 1013, Moorea 98729, French Polynesia

Bull sharks (*Carcharhinus leucas*) are apex predators in tropical and subtropical seas and are unique among Elasmobranchs for their capacity to inhabit coastal freshwater systems for prolonged periods of time (Compagno 1990). They are classified as Near Threatened in the International Union of Conservation for Nature Red List (IUCN 2013) and are submitted to recreational and commercial pressures (Karl et al. 2011).

Samples of muscle and fin tissues from 11 adults (both female and male) of *C. leucas* caught from Reunion Island were used for DNA extraction. Total genomic DNA was isolated using Qiagen DNeasy Blood and Tissue Kit and sent to GenoScreen, Lille, France (www.genoscreen.fr). One µg was used for the development of microsatellites libraries through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries as described in Malausa et al. (2011). A total of 7,556 sequences containing microsatellite motif were identified. Sequences were analysed using the software QDD (Meglecz et al. 2010) and 20 microsatellite markers were finally selected and characterized for *C. leucas*.

Thus genotyping of these 20 markers was conducted on 41 adult individuals of Reunion Island population. Each amplification reaction contained 10 µL of PCR product. Microsatellite loci developed in this study were directly fluorochrome labelled, and the reaction mixture contained 5 µL of MasterMix Applied 2x (Applied Biosystems), 1.5 µL of demineralised water, 0.5 µL of each primer (10 µM) and 2.5 µL of genomic DNA (10 ng/µL). The thermocycling program was as follow: an initial denaturing step at 94 °C for 5 min, seven cycles including a step at 94 °C for 30 s, a step at 62 °C (−1 °C at each cycle) for 30 s and a step at 72 °C for 30 s. These seven cycles are followed by 35 cycles including a step at 94 °C for 30 s, a step at 55 °C for 30 s and a step at 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min. Allelic sizes

Table 1 Microsatellite loci developed for *Carcharhinus leucas* and their primers sequences (F Forward, R Reverse)

Name	Primer sequences (5'-3')	GenBank accession number	Repeat motif	Size (bp)	N _a	N	H _O	H _E	F _{IS}	r
CI01	F: GACACAATGCACAGGCAATC R: GCGCACAGACATATAATCTGAA	KJ916104	(AC) ₆	114–116	2	41	0.000	0.347	1.000***	0.352 yes
CI02	F: ACAGGCAAAGACAATGCAAAAC R: GCTTCCTGTGCTGGAGGGT	KJ916105	(AC) ₆	141–143	2	41	0.195	0.253	0.231 NS	0.090
CI03	F: GAGGCAGTTGCTGGCTCTAT R: AAAGCTCCACACCCAGACAC	KJ916106	(TC) ₇	106–108	2	41	0.610	0.505	-0.211 NS	-0.118 no
CI04	F: GTGCAGAAAACAGTCGGACA R: GGCTGCTCTAGTCCAGCTT	KJ916107	(GA) ₇	124–130	2	41	0.683	0.455	-0.510**	-0.437 no
CI05	F: AGGCCCTATCCCCATAACC R: TGTGTGAGTGAAAAGTGAGCG	KJ916108	(CA) ₇	137–141	3	41	0.415	0.409	-0.015 NS	-0.014 no
CI06	F: ATGGGGATAGGCAGATGG R: TTGTTTATCTCTGTGTATGTTTCATGT	KJ916109	(AC) ₇	144–150	3	41	0.049	0.048	-0.013 NS	-0.025 no
CI07	F: TTACCAGCTGATATCTACTCACACTC R: CTCACTGCCCTGGACGATTC	KJ916110	(CA) ₇	143–151	5	41	0.463	0.409	-0.136 NS	-0.081 no
CI08	F: GAAAGGAAAACTCGAAGAAATTCAC R: AGTACTGGAGCAGACCCGCAT	KJ916111	(AG) ₇	144–154	6	41	0.683	0.671	-0.018 NS	-0.015 no
CI09	F: GGCTGGTGACATTAITGAGTG R: TGAGGAGCTCAATCAGATCTTT	KJ916112	(AG) ₈	107–115	4	41	0.415	0.369	-0.127 NS	-0.079 no
CI10	F: GCATCAGCTTGGAAACAACAC R: TTCAAAAGACTTCCAAAGTTGGGT	KJ916113	(CT) ₈	123–129	3	40	0.075	0.162	0.539*	0.159 yes
CI11	F: AGATTACTGTGACTTTTGTCTGTGTTTT R: TCTCTATTCTTGTGCTGCAGAGGT	KJ916114	(AG) ₈	232–238	3	41	0.341	0.301	-0.135 NS	-0.179 no
CI12	F: TGCTCTTTATCACATTTCTTCACA R: ACCATGGTGCAGTCTACCAA	KJ916115	(TC) ₉	105–111	3	41	0.171	0.158	-0.081 NS	-0.089 no
CI13	F: CCCAAAACCCTCGAGTAACA R: CGTTGGAGTTTGTAACAAGGTGA	KJ916116	(AC) ₉	94–126	10	41	0.683	0.633	-0.080 NS	-0.070 no
CI14	F: TGCTCAGGATGTGTAGTGC R: GAGGATAGCTGCCACCGATA	KJ916117	(GT) ₉	211–219	6	41	0.220	0.203	-0.081 NS	-0.113 no
CI15	F: TGACCTGCAATATTTTCCTGC R: TCGCTGACAGGCAAGGTTAT	KJ916118	(GT) ₉	290–300	4	41	0.098	0.095	-0.029 NS	-0.050 no
CI16	F: TCTTTGCAAAACATCCCAGC R: TTGAAAATACTCAGAGACGCAC	KJ916119	(TC) ₁₂	103–115	6	41	0.780	0.732	-0.033 NS	-0.026 no
CI17	F: TGCAGACGCTATTACTTCCG R: TTGTTCTCTCACTCAACGCCAA	KJ916120	(TG) ₁₂	175–183	5	41	0.561	0.568	0.013 NS	-0.003 no
CI18	F: TCTTCGGAGTGACTAACGCA R: AGTCGTGGTGTCTTCTAACGA	KJ916121	(CA) ₁₂	209–219	6	41	0.390	0.432	0.097 NS	0.039 no

Table 1 continued

Name	Primer sequences (5'-3')	GenBank accession number	Repeat motif	Size (bp)	N _a	N	H _O	H _E	F _{IS}	r
CI19	F: ACAAGCTGCAAAGATCCTCAA R: TGGGGTTACCTGGACACTTT	KJ916122	(CCA) ₇	198–216	6	41	0.415	0.408	-0.016 NS	-0.058 no
CI20	F: ACGAGGATGACAACAACAACTGG R: CTTTCCCTCCTTTCCCAATC	KJ916123	(AGG) ₉	134–167	10	41	0.634	0.797	0.207*	0.092 yes

Accessibility numbers in GenBank are indicated

N_a number of alleles per locus, N number of individuals, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} inbreeding coefficient

Concerning the F_{IS}, is indicated the significance of the P-values for deviation to Hardy-Weinberg equilibrium. NS: non significant, * P < 0.05, ** P < 0.01, *** P < 0.001

Null allele frequencies (r) are issued from MicroChecker v 2.2.3 (Van Oosterhout et al. 2004), and presence or absence is indicated below (yes or no)

Annealing temperature T_a = 55 °C

Table 2 Cross-amplification for 20 microsatellite markers designed for *Carcharhinus leucas* across three carcharhinidae species: *Carcharhinus obscurus* (n = 2), *Carcharhinus plumbeus* (n = 3) and *Galeocerdo cuvier* (n = 41) (+, amplified, +P, polymorphic; -, no amplification). Size ranges in base pairs and number of alleles (in brackets) were also indicated

Locus Name	<i>C. obscurus</i> (n = 2)	<i>C. plumbeus</i> (n = 3)	<i>G. cuvier</i> (n = 41)
CI01	+ 114 (1)	+P 114–134 (2)	-
CI02	+ 141 (1)	-	-
CI03	+ 106 (1)	+ 106 (1)	-
CI04	+ 124 (1)	+P 122–126 (3)	-
CI05	+ 141 (1)	-	-
CI06	+ 144 (1)	-	-
CI07	+ 143 (1)	+ 143(1)	-
CI08	+ 144 (1)	+P 144–146 (2)	-
CI09	+P 111–113 (2)	-	+ 107 (1)
CI10	+ 123 (1)	-	-
CI11	+P 232–238 (3)	+P 106–116 (5)	-
CI12	+ 111 (1)	+P 103–105 (2)	+P 105–115 (2)
CI13	+P 94–102 (2)	+ 96 (1)	-
CI14	+P 209–215 (2)	+ 216 (1)	+P 184–210 (2)
CI15	+ 300 (1)	-	-
CI16	+P 103–111 (3)	-	-
CI17	+ 179 (1)	+P 171–179 (3)	+P 167–169 (2)
CI18	-	-	-
CI19	+ 198 (1)	-	-
CI20	+ 143 (1)	+ 149 (1)	-

were determined using Genemapper v 4.0 (Applied Biosystems).

Diversity indices, Hardy–Weinberg equilibrium and linkage disequilibrium were assessed using Arlequin v 3.5.1.2. All loci were polymorphic, the number of alleles ranged from 2 to 10, observed heterozygosities ranged from 0.00 to 0.78 and expected heterozygosities from 0.05 to 0.80 (Table 1).

Linkage disequilibrium was observed for some loci at a significance level of 0.05 but none were still significant after Bonferroni correction for multiple testing. Four loci were found to deviate from Hardy–Weinberg equilibrium: three of them probably due to the presence of null alleles assessed using MicroChecker v 2.2.3 (Van Oosterhout et al. 2004) (Table 1).

The markers developed were also tested on *Galeocerdo cuvier*, *Carcharhinus plumbeus* and *Carcharhinus obscurus*: 4, 11 and 19 markers successfully cross-amplified, respectively (Table 2).

The development of these markers will be very useful in studying sharks ecology, which remains poorly documented, above all in assessing population structure, effective population size and phylopatry.

Acknowledgments The authors would like to thank T. Gazzo and C. Perry as fishermen, B. Reche (veterinary), D. Guyomard (Comité Régional des Pêches Maritimes et des Elevages Marins de La Réunion) and all the participants who took part in collecting samples. This study was carried out under the scientific program CHARC (Connaissances de l'écologie et de l'habitat de deux espèces de requins côtiers à la Réunion) financially supported by the Commission of the European Communities (FEDER fund), the French Government and the Regional Council of Reunion Island. We thank Stéphanie Duthoy (Genoscreen, Lille, France) for her assistance in screening the sequences and helpful discussions.

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3. Développement de marqueurs microsatellites spécifiques au requin tigre *Galeocerdo cuvier*

Résumé

Nous avons développé une banque microsatellite spécifique au requin tigre *Galeocerdo cuvier*, et testé les amplifications croisées de ces marqueurs sur plusieurs autres espèces de requins. Cette banque a été développée à partir d'échantillons prélevés sur 11 requins adultes pêchés à La Réunion, et le polymorphisme a été testé sur un échantillonnage populationnel provenant de La Réunion, comprenant 101 individus. Par ailleurs, des amorces spécifiques à la région contrôle de l'ADN mitochondrial (ou D-loop) ont également été dessinées. Ainsi, huit marqueurs ont pu être caractérisés dont un amplifiant le requin bouledogue *Carcharhinus leucas*, quatre le requin tisserand *Carcharhinus brevipinna*, cinq le requin gris *Carcharhinus plumbeus* et deux le requin marteau *Sphyrna lewini*.

Ces résultats ont permis la publication d'un article dans *Peer J*.

Isolation and characterization of eight microsatellite loci from *Galeocerdo cuvier* (tiger shark) and cross-amplification in *Carcharhinus leucas*, *Carcharhinus brevipinna*, *Carcharhinus plumbeus* and *Sphyrna lewini*

Agathe Pirog¹, Sébastien Jaquemet¹, Antonin Blaison^{1,2}, Marc Soria² and Hélène Magalon^{1,3}

¹UMR ENTROPIE, Université de la Réunion, Saint Denis, La Réunion

²UMR MARBEC, IRD Réunion, Saint Denis, La Réunion

³Laboratory of Excellence CORAIL, France

ABSTRACT

The tiger shark *Galeocerdo cuvier* (Carcharhinidae) is a large elasmobranch suspected to have, as other apex predators, a keystone function in marine ecosystems and is currently considered Near Threatened (Red list IUCN). Knowledge on its ecology, which is crucial to design proper conservation and management plans, is very scarce. Here we describe the isolation of eight polymorphic microsatellite loci using 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. Their characteristics were tested on a population of tiger shark ($n = 101$) from Reunion Island (South-Western Indian Ocean). All loci were polymorphic with a number of alleles ranging from two to eight. No null alleles were detected and no linkage disequilibrium was detected after Bonferroni correction. Observed and expected heterozygosities ranged from 0.03 to 0.76 and from 0.03 to 0.77, respectively. No locus deviated from Hardy-Weinberg equilibrium and the global F_{IS} of the population was of 0.04^{NS} . Some of the eight loci developed here successfully cross-amplified in the bull shark *Carcharhinus leucas* (one locus), the spinner shark *Carcharhinus brevipinna* (four loci), the sandbar shark *Carcharhinus plumbeus* (five loci) and the scalloped hammerhead shark *Sphyrna lewini* (two loci). We also designed primers to amplify and sequence a mitochondrial marker, the control region. We sequenced 862 bp and found a low genetic diversity, with four polymorphic sites, a haplotype diversity of 0.15 and a nucleotide diversity of 2×10^{-4} .

Subjects Fisheries and Fish Science, Conservation Biology, Genetics, Marine Biology, Molecular Biology

Keywords Carcharhiniform, Microsatellites, Control region, Population Genetics

INTRODUCTION

The tiger shark *Galeocerdo cuvier* is a large carcharhinid (up to 5.5 m in total length for the largest females), which lives in warm temperate, tropical and subtropical waters ([Compagno, 1984](#); [Randall, 1992](#)). This species is opportunistic and feeds on a very large range of preys

Submitted 4 March 2016

Accepted 25 April 2016

Published 17 May 2016

Corresponding author

Agathe Pirog, agathe.p2609@yahoo.fr

Academic editor

David Coltman

Additional Information and
Declarations can be found on
page 6

DOI [10.7717/peerj.2041](https://doi.org/10.7717/peerj.2041)

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according to their availability in the environment. It is considered as a generalist forager (Lowe *et al.*, 1996; Simpfendorfer, Goodreid & McAuley, 2001). Different studies estimated the age of sexual maturity around seven to ten years for both sexes (Branstetter, Musick & Colvocoresses, 1987; Natanson *et al.*, 1999; Kneebone *et al.*, 2008), and a lifespan between 27 and 29 years for males and females, respectively (Kneebone *et al.*, 2008). The species favours coastal habitats (Heithaus *et al.*, 2006; Papastamatiou *et al.*, 2013) and can be highly reef associated (Meyer, Papastamatiou & Holland, 2010) even if transoceanic movements are regularly observed (Rooney *et al.*, 2006; Heithaus *et al.*, 2007; Lea *et al.*, 2015). Tiger sharks occupy defined but very large home ranges (Holland *et al.*, 1999), which renders difficult the implementation of adapted conservation measures. *Galeocerdo cuvier* is classified as Near Threatened by the International Union for Conservation of Nature (IUCN) Red List of Endangered Species (Simpfendorfer, 2009). Although it is one of the largest marine predators, little is known about the ecology of this species, which has mostly been studied using *in situ* and direct observations. To our knowledge, only one study has focused on population genetics of *G. cuvier*, in Hawaii (Bernard, Feldheim & Shivji, 2015), and characterized nine microsatellite loci for this species. Another study (Chen *et al.*, 2014) mapped the entire mitogenome for this species but did not test for mitochondrial markers that would be useful for population genetics studies.

Here, we developed a supplementary set of eight microsatellite loci for the tiger shark, polymorphic at the population scale. Until now, 12 microsatellite loci were available for the tiger shark, including the nine developed in Bernard, Feldheim & Shivji (2015) and the three loci characterized from *Carcharhinus leucas* that are polymorphic in *G. cuvier* (Pirog *et al.*, 2015). With these eight new loci, 20 microsatellite loci will now be available to study this species, which will be very useful when studying the genetic structure of tiger shark populations worldwide. We described their characteristics by genotyping 101 *G. cuvier* individuals caught at Reunion Island, South-Western Indian Ocean, and tested cross-amplification in four other carcharhiniform species. Furthermore, we designed primers to amplify and sequence the mitochondrial control region (also called D-loop).

MATERIAL AND METHODS

The samples were collected on tiger sharks caught during the scientific program CHARC (French acronym for “Knowledge on the ecology and the habitat of two coastal shark species”) off the west coast of Reunion Island (21°06’S, 55°36’E), South Western Indian Ocean, 700 km east from Madagascar between October 2011 and May 2013 (Blaison *et al.*, 2015). The program CHARC was approved by Reunion Island Ethic Committee, the local representative of the French national ethic committee. For sharks tagged during an acoustic study (Blaison *et al.*, 2015), a piece of fin tissue was biopsied on living animals. Pieces of muscle were also collected from professional fishermen by-catches. A total of 101 adults (46 males and 55 females) were sampled.

Total genomic DNA was extracted using Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) from small pieces of tissues (fin or muscle). The microsatellite library was developed using 11 individuals (six females and five males). Biggest tagged individuals

(320–390 cm total length) were chosen to decrease the probability of sampling related individuals and thus, to increase genetic variability. Indeed, choosing both small and big individuals increases the probability to use related individuals (parents and their offspring) to construct the library. Total genomic DNA was sent to GenoScreen, Lille, France (www.genoscreen.fr). One μg was used for the development of the microsatellites library through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries as described in [Malauza et al. \(2011\)](#). Briefly, total DNA was mechanically fragmented and enriched for AG, AC, AAC, AAG, AGG, ACG, ACAT and ATCT repeat motifs. Enriched fragments were subsequently amplified. PCR products were purified, quantified and GsFLX libraries were then carried out following manufacturer's protocols and sequenced on a GsFLX PTP. Sequences of the microsatellite-enriched library were analysed using the software QDD ([Meglecz et al., 2010](#)) and primer pairs were selected depending on the motif (di-, tri-, tetra-, hexanucleotide), the number of repeats (≥ 5) and the product size (≥ 100 bp) and tested on agarose gel for amplification. Then, depending on the putative allele number, the polymorphism was verified by genotyping 11 *G. cuvier* individuals on an ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA, USA).

The developed set of microsatellite loci was then used to genotype our sampling of 101 *G. cuvier* individuals. Each amplification reaction contained 10 μL of PCR product. Microsatellite loci developed in this study were directly fluorochrome labelled (using 6-FAM, PET, VIC or NED), and the reaction mixture contained 5 μL of MasterMix Applied 2 \times (Applied Biosystems, Foster city, CA, USA), 1.5 μL of demineralized water, 0.5 μL of each primer (10 μM) and 2.5 μL of genomic DNA (10 ng/ μL). The thermocycling program was: an initial denaturing step at 94 °C for 5 min, 7 cycles of 94 °C for 30 s, 62 °C (–1 °C at each cycle) for 30 s, 72 °C for 30 s, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min. Allelic sizes were determined using Genemapper v 4.0 (Applied Biosystems, Foster city, CA).

The transferability of these loci was checked on the bull shark *Carcharhinus leucas* ($n = 41$), the spinner shark *Carcharhinus brevipinna* ($n = 2$), the sandbar shark *Carcharhinus plumbeus* ($n = 3$) and the scalloped hammerhead shark *Sphyrna lewini* ($n = 4$). These samples have been collected from professional fishermen by-catches caught at Reunion Island. Extractions and genotyping were conducted following the same protocol as above.

From the *G. cuvier* mitochondrion sequence available in GenBank (KF111728.1), we designed primers to amplify the control region (D-loop) using PRIMER3 v 4.0.0 ([Rozen & Skaletsky, 2000](#)): the forward primer Gc-CR-F (5'-CCC AAA GCC AAG ATT CTG CC-3') and the reverse primer Gc-CR-R (5'-CGA GAC CAA CCA TGT ATA TTA AGG G-3'). These primers were used for both amplification and direct sequencing. PCR reactions were performed in a total volume of 25 μL containing 12.5 μL of Applied MasterMix 2x (Applied Biosystems, Foster city, CA), 7.5 μL of demineralized water, 1 μL of each primer (10 μM) and 3 μL of genomic DNA (10 ng/ μL). The thermocycling program contained an initial denaturing step at 94 °C for 5 min, 35 cycles of (94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min 30 s), and a final extension step at 72 °C for 5 min. The sequencing of the mitochondrial DNA was realised by GenoScreen, Lille, France (www.genoscreen.fr).

Table 1 Characterization of the eight microsatellite loci developed for *Galeocerdo cuvier* and their primer sequences (F: forward; R: reverse). No locus deviated from Hardy-Weinberg equilibrium. Annealing temperature $T_a = 55$ °C.

Locus name	Repeat motif	Forward primer (5'–3')	Reverse primer (5'–3')	Allele size (bp)	N_a	H_O	H_E	F_{IS}
Gc01	(GA) ₆	AGGTGTGGTGGCTCTCCTC	GGACGCAAAATCCAACAGAG	143–147	2	0.03	0.03	– 0.01
Gc02	(AG) ₇	GAGAGGGAGAAGCAAGTCAACATA	GTTTCTCTTCTGTCTCTTCCA	93–105	4	0.15	0.15	0.01
Gc03	(TC) ₈	TTGATTTCTACCTGGTCGGC	TCAGAGCAAAGAGCTCCAGA	121–129	3	0.56	0.58	0.04
Gc04	(TC) ₉	CCCCAGGGAAATAATCTAAGG	CAGGGGGACGACTAGTCAAG	195–199	2	0.36	0.38	0.07
Gc05	(CT) ₁₁	CTGGGTGGCAGCAAATTAGA	TGAGCCTTCTCACCCAGAGT	117–123	2	0.45	0.50	0.10
Gc06	(AC) ₁₁	CATGACGTTTCGCCACAATA	TTTCTCCACAGTCCAAAG	116–122	3	0.13	0.14	0.07
Gc07	(CA) ₁₄	ATTGCAATCTGTGCCATCAA	TTTGTGAGAGTGTCTGTATGTTTG	114–130	8	0.76	0.77	0.02
Gc08	(AGTG) ₆	GTGCAGGGAGGAATGTGAGT	TTGTCAAGAGTCCACGTGTCTT	207–219	3	0.49	0.49	0.02

Notes.

Diversity indices are issued from FSTAT v2.9.3.2;

bp, Base pairs; N_a , Number of alleles per locus; H_O , Observed heterozygosity; H_E , Expected heterozygosity; F_{IS} , Inbreeding coefficient.

Concerning microsatellite loci, presence and frequencies of null alleles, which may be responsible for an excess of homozygotes, were assessed using MicroChecker v 2.2.3 (Van Oosterhout et al., 2004). Tests of linkage disequilibrium were performed using Arlequin v 3.5.1.2 (Excoffier & Lischer, 2010). Diversity indices such as the number of alleles per locus N_a , the observed and expected heterozygosities (H_O and H_E), the inbreeding coefficient F_{IS} (Cockerham & Weir, 1984) were assessed, and Hardy-Weinberg equilibrium was tested using FSTAT v 2.9.3.2 (Goudet, 1995).

Mitochondrial sequences were edited and aligned using Geneious v 6.1.7 created by Biomatters (available from <http://www.geneious.com/>). Haplotype and nucleotide diversities were calculated using DnaSP v 5.10.1 (Librado & Rozas, 2009).

RESULTS

Sequencing of the microsatellite-enriched library yielded 20,303 reads. A total of 6,982 sequences (34%) containing microsatellite motif were identified. After QDD analysis, 103 primer pairs were recognized. Among these, 95 primer pairs were selected depending on our criteria: 36 (37.9%) successfully amplified. Then, depending on the putative allele number, the polymorphism of 32 primer pairs was verified by genotyping 11 *G. cuvier* individuals on an ABI 3730 XL sequencer. A total of eight microsatellite loci (GenBank accession numbers: KP300805, KP300806, KP300807, KP300808, KP300809, KP300810, KP300811, KP300812) were finally selected and characterized for *G. cuvier* (Table 1).

The eight loci developed in *G. cuvier* were then used to genotype 101 tiger sharks caught at Reunion Island between 2011 and 2013. All loci were polymorphic with a number of alleles ranging from two to eight. No null alleles were detected and no linkage disequilibrium was detected after Bonferroni nor FDR corrections. Observed and expected heterozygosities ranged from 0.03 to 0.76 and from 0.03 to 0.77, respectively (Table 1). No locus was found to deviate from Hardy-Weinberg equilibrium and the global F_{IS} of the population was of 0.04^{NS}, following Hardy-Weinberg proportions.

Table 2 Cross-amplification for eight microsatellite loci designed for *Galeocerdo cuvier* across four Carcharhiniformes: *Carcharhinus leucas* ($n = 41$), *Carcharhinus brevipinna* ($n = 2$), *Carcharhinus plumbeus* ($n = 3$) and *Sphryna lewini* ($n = 4$).

Locus name	<i>C. leucas</i> ($n = 41$)	<i>C. brevipinna</i> ($n = 2$)	<i>C. plumbeus</i> ($n = 3$)	<i>S. lewini</i> ($n = 4$)
Gc01	+P 136-142 (3) 41/41	+139(1) 2/2	+135(1) 3/3	+P 152-156 (3) 4/4
Gc02	–	–	+P 99-101 (2) 2/3	–
Gc03	–	–	–	–
Gc04	–	+198(1) 2/2	+P 194-200 (2) 2/3	–
Gc05	–	+159(1) 2/2	+105(1) 2/3	–
Gc06	–	–	+P 112-116 (3) 3/3	–
Gc07	–	–	–	+P 173-185 (4) 4/4
Gc08	–	+222(1) 2/2	–	–

Notes.

+, Amplified; +P, Polymorphic; –, No amplification.

Size ranges in base pairs and number of alleles (in parentheses) are also indicated. Numbers of amplifications observed are indicated in bold.

Among these eight loci, one (12.5%) successfully cross-amplified in *C. leucas*, four (50%) in *C. brevipinna*, five (62.5%) in *C. plumbeus*, and two (25%) in *S. lewini* (Table 2). The number of alleles ranged from one to four depending on the species (Table 2).

The 101 *G. cuvier* adults were sequenced, and sequences were edited and aligned over 862 bp. All sequences were of high quality and easily readable without ambiguities. We observed four polymorphic sites (over 862 bp), distributed among only five haplotypes, which were deposited in GenBank (accession numbers: [KP317128](#), [KP317129](#), [KP317130](#), [KP317131](#), [KP317132](#),). The haplotype diversity (h) was 0.15 ± 0.048 (sd) and the nucleotide diversity (π) $2 \times 10^{-4} \pm 7 \times 10^{-5}$ (sd). Among these haplotypes, one was over-represented ($n = 93$; 92% of the individuals sequenced).

DISCUSSION

The development of these loci (nuclear and mitochondrial), added to those previously described in [Bernard, Feldheim & Shivji \(2015\)](#) and in [Pirog et al. \(2015\)](#) (three loci characterized from *C. leucas* polymorphic for *G. cuvier*), will be very useful in studying tiger shark ecology, which remains poorly documented, especially in assessing population structure and patterns of migration, effective population size and some aspects of their reproductive behaviour. Furthermore, the mitochondrial control region highlighted low genetic diversity in the tiger shark and should be useful to study the evolution of tiger shark populations.

These loci may also be used when studying other carcharhiniform species. Indeed, most shark species being heavily exploited by both artisanal and industrial fisheries, including in the Western Indian Ocean (*Campana & Ferretti, 2016*), it is relevant and useful to possess genetic tools to conduct population genetics analyses.

ACKNOWLEDGEMENTS

The authors thank fishermen T Gazzo and C Perry, veterinaries B Reche and J Robert, D Guyomard (Comité Régional des Pêches Maritimes et des Elevages Marins de La Réunion), G Vangrevelinghe (Squal'Idées) and all the participants who took part in collecting samples. This study was carried out under the scientific program CHARC (Connaissances de l'écologie et de l'habitat de deux espèces de requins côtiers à La Réunion ; FEDER fund Convention 2011 Presage No 33021). We thank S Duthoy (Genoscreen, Lille, France) for her assistance and helpful discussions.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

A Pirog benefited from European Social Funds from the EU and attributed by the Reunion Island regional council to conduct her PhD. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
European Social Funds.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Agathe Pirog performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Sébastien Jaquemet and Marc Soria conceived and designed the experiments, performed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Antonin Blaison performed the experiments, reviewed drafts of the paper.
- Hélène Magalon conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.

Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The program CHARC was approved by Reunion Island Ethic Committee, the local representative of the French national ethic committee (approval letter).

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

For microsatellite sequences, GenBank accession numbers: [KP300805](#), [KP300806](#), [KP300807](#), [KP300808](#), [KP300809](#), [KP300810](#), [KP300811](#), [KP300812](#).

For mitochondrial sequences, Genbank accession numbers: [KP317128](#), [KP317129](#), [KP317130](#), [KP317131](#), [KP317132](#).

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as [Data S1](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.2041#supplemental-information>.

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CHAPITRE 2. Structure des populations et histoire démographique du requin bouledogue *Carcharhinus leucas*

Résumé

Le cadre théorique de la génétique des populations et les marqueurs moléculaires sont de plus en plus utilisés pour étudier la démographie d'espèces élusives et non accessibles avec des méthodes de suivis directes (par exemple, marquage-recapture, suivi acoustique ou télémétrique). L'incorporation de la migration dans des modèles de simulations, combinée à de nouveaux algorithmes de calcul bayésien approché reposant sur la méthode des forêts aléatoires, ou *random forest* (c.-à-d. l'ABC-RF) permet de comparer et de sélectionner des modèles démographiques représentant au mieux les données observées. Ces méthodes ont été utilisées afin d'étudier une espèce dont la structure génétique, la connectivité et la taille efficace des populations restent méconnues, et qui est pourtant emblématique, jouant des rôles importants et variés dans les écosystèmes marins, et de plus en plus soumise à des pressions anthropiques : le requin bouledogue *Carcharhinus leucas*.

À ce jour, peu d'études ont étudié la structure génétique des populations de requin bouledogue, et disposaient (1) d'un échantillonnage global mais d'un seul type de marqueurs moléculaires, ou (2) d'un échantillonnage local (restreint à l'Atlantique Ouest ou au Nord de l'Australie) et d'un faible nombre de marqueurs moléculaires (3 à 5 marqueurs microsatellites ainsi que 1 ou 2 gènes mitochondriaux). Combinant ici les informations de 25 marqueurs microsatellites et de 3 gènes mitochondriaux, nous avons analysé la variation génétique de 370 échantillons de requin bouledogue provenant de 11 localités de l'Ouest de l'océan Indien, de l'Ouest du Pacifique et de l'Ouest de l'Atlantique, situées le long des côtes mais aussi provenant d'îles océaniques. Une importante discordance a été ainsi mise en évidence entre les indices de différenciation génétique calculés avec chaque type de marqueurs (nucléaires ou mitochondriaux), entre l'Ouest de l'océan Indien et l'Ouest du Pacifique. Couplant l'information des marqueurs microsatellites et mitochondriaux grâce à l'ABC-RF, cette discordance pourrait être due à (1) une absence complète de flux de gènes contemporains ou (2) une absence de migration efficace des femelles, mais des flux de gènes contemporains assurés par les mâles. Les tailles efficaces des différentes populations délimitées (c.-à-d. l'Ouest de l'océan Indien, l'Ouest du Pacifique et l'Atlantique Ouest) ont aussi été estimées pour la première fois, pointant vers des populations probablement impactées, mais faiblement, par les pressions anthropiques auxquelles elles sont soumises.

Cette publication est soumise à *Molecular Ecology*.

Population structure, connectivity and demographic history of an apex marine predator, the bull shark *Carcharhinus leucas*

Agathe Pirog¹, Virginie Ravigné², Michaël C. Fontaine³, Adrien Rieux², Aude Gilibert², Jeremy Cliff^{4,5}, Eric Clua^{6,13}, Ryan Daly^{7,8}, Michael R. Heithaus⁹, Jeremy J. Kiszka⁹, Philip Match⁹, John E. G. Nevill¹⁰, Amy F. Smoothey¹¹, Andrew J. Temple¹², Sébastien Jaquemet¹, Hélène Magalon^{1,13}

¹ UMR ENTROPIE (Université de La Réunion/IRD/CNRS), Université de La Réunion, Saint Denis, La Réunion, France

² UMR PVBMT, CIRAD, St Pierre, La Réunion, France

³ Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, Groningen, The Netherlands

⁴ KwaZulu-Natal Sharks Board, Umhlanga Rocks, South Africa

⁵ School of Life Sciences, University of KwaZulu-Natal, Durban, South Africa

⁶ PSL Research University, EPHE, CNRS UPVD, USR 3278 CRILOBE, Perpignan, France

⁷ Save Our Seas Foundation, D'Arros Research Centre (SOSF-DRC), Rue Philippe Plantamour 20, 1201 Genève, Switzerland

⁸ Port Elizabeth Museum at Bayworld, Port Elizabeth, Eastern Cape, South Africa

⁹ Department of Biological Sciences, Florida International University, North Miami, FL33181, USA

¹⁰ Environment Seychelles, P.O. Box 1299, Victoria, Seychelles

¹¹ NSW Department of Primary Industries, Sydney Institute of Marine Science, Mosman, NSW, Australia

¹² School of Marine Science & Technology, Newcastle University, Newcastle-upon-Tyne, United Kingdom

¹³ Laboratoire d'Excellence CORAIL, Perpignan, France, www.labex-corail.fr

Keywords: ABC-RF, bull shark, microsatellite DNA, mitochondrial DNA, mito-nuclear discordance, population genetics

Corresponding Author:

Magalon Hélène

UMR ENTROPIE, Université de La Réunion, 15 Avenue René Cassin, CS 92003, 97744 Saint Denis Cedex 09, La Réunion, France

Email address: helene.magalon@univ-reunion.fr

Orcid number: 0000-0002-7061-955X

Running title: Population genetics of the bull shark

Abstract

Knowledge on population structure, connectivity and effective population size remains limited for many marine apex predators, like the bull shark *Carcharhinus leucas*. This large-bodied coastal shark, distributed worldwide in warm temperate and tropical waters, uses estuaries and rivers as nurseries. Due to its ability to consume and threaten large preys, bull sharks could play important ecological roles, but is at risk from fisheries. We investigated the global population structure and demographic history by analysing the genetic diversity of 370 individuals using 25 microsatellite loci and three mitochondrial genes (*CR*, *nd4*, *cytb*). Both types of markers revealed clear clustering between sharks from the Western Atlantic and those from the Western Pacific and the Western Indian Ocean, with no contemporary gene flow. Between the Western Indian Ocean and the Western Pacific, microsatellite data suggested low differentiation while a substantial one was found with mitochondrial DNA. Integrating information from both types of markers and using Bayesian computation with a random forest procedure (ABC-RF), this discordance was found to be due to either a complete lack of contemporary gene flow, or a complete lack of female gene flow along with some contemporary male effective dispersal. Within the Western Indian Ocean and within the Western Pacific, important effective dispersal was found. Together, these results suggest more structuring of bull shark populations than previously thought for this apex predator, highlighting the need for management and conservation plans at more restricted scales.

Introduction

Delineating populations and their connectivity by gene flow (i.e., effective dispersal) is of primary importance for the conservation and management of endangered and/or exploited species (Begg, Friedland, & Pearce, 1999; Moritz, 1994; Palsbøll, Bérubé, & Allendorf, 2007). In marine species, it allows defining stocks, assessing exploitation status, and preserving the population genetic diversity underlying recruitment potential and species adaptability (Begg et al., 1999; Hilborn, Quinn, Schindler, & Rogers, 2003; Palumbi, 2003). Once genetically distinct groups (i.e., populations) that may be managed independently are identified, estimating their number of individuals and the number of individuals effectively exchanged among them is needed to assess their viability and resilience (Frankham, 2010; Schwartz, Luikart, & Waples, 2007). Among highly mobile, wide-ranging species, like marine megafauna (e.g., marine mammals, seabirds, turtles, sharks and rays) and large-bodied teleosts, such studies are particularly important because of exposure to anthropogenic pressures (Halpern et al., 2008; Payne, Bush, Heim, Knope, & McCauley, 2016) and the key roles many play within food webs (Bowen, 1997; Estes, 1979; Heithaus, Frid, Wirsing, & Worm, 2008; Katona & Whitehead, 1988).

Studies of population structure and connectivity are challenging because direct approaches (mark-recapture, satellite and acoustic tracking) commonly used are often difficult to apply in marine pelagic species. This leads to small sample sizes (Grothues, 2009) and an underestimation of individual movements (Ng, Able, & Grothues, 2007; Thorsteinsson, 2002). Therefore, indirect methods based on the conceptual framework of population genetics have been increasingly used to address ecological and evolutionary questions in such species. First, such an approach allows the assessment of population structure, resulting from evolutionary forces shaping allele frequencies within and among populations (mutation, genetic drift, migration and selection) (Wright, 1931). At neutral loci, while gene flow homogenizes allele frequencies and limits population differentiation, genetic drift promotes population differentiation by randomly fixing alleles (Hartl & Clark, 1997). Second, these methods can provide estimates of the effective population size (N_e) (Wright, 1931). This parameter represents the size of an idealized Wright-Fisher population affected by genetic drift at the same rate per generation found in the population of interest. Combined with the mutation rate (μ), it provides an estimate of population genetic diversity ($4N_e\mu$ for the diploid autosomal part of the genome and $N_e\mu$ for the haploid mitochondrial genome). It is also related to the number of

breeders per generation (Waples, Antao, & Luikart, 2014), and has been shown to correlate with a population ability to adapt to environmental changes (Hare et al., 2011).

Marine species characterized by large populations commonly show weak genetic structuring at neutral loci. In large populations, even a low dispersal rate can lead to a weak population genetic structure because the number of migrants is not negligible. Also, genetic drift is limited in these species due to their large population sizes. It may result from the existence of large isolated populations or conversely, the existence of one large panmictic population. Identifying which situation is operating can be challenging but recent developments are providing the necessary analytical resolution. Incorporation of migration into simulation models combined with new Approximate Bayesian Computation algorithm relying on Random Forest (i.e., ABC-RF) allows comparisons and selection of alternative demographic models that best fit the observed dataset (Pudlo et al., 2016; Raynal et al., 2017). ABC-RF provides estimates of the posterior probability of the selected model and the parameters of interests, such as migration rates between populations and effective population size (Pudlo et al., 2016; Raynal et al., 2017). For both model choice and parameter estimates, it is more accurate and requires a smaller number of simulated datasets than previous ABC methods (Fraitout et al., 2017; Pudlo et al., 2016; Raynal et al., 2017), which is advantageous for marine megafauna that can occur at low relative abundances.

Many large sharks face considerable exploitation and populations have declined globally in recent decades (Dulvy et al., 2014). The bull shark *Carcharhinus leucas* is caught in recreational, subsistence and targeted commercial fisheries, as well as bycatch, throughout its range (Aguilar et al., 2014; Almeida, McGrath, & Ruffino, 2001; Bonfil, 1997; Branstetter & Stiles, 1987; Clarke, Magnussen, Abercrombie, McAllister, & Shivji, 2006; Doukakis et al., 2010; Temple et al., 2018). In several locations, it has been the subject of lethal risk reduction programs due to attacks on humans (Dudley, 1997; Dudley & Simpfendorfer, 2006; Lagabriele et al., 2018). This high-trophic level predator inhabits tropical and subtropical waters worldwide, and could play an important role in coastal and estuarine ecosystems (Daly, Froneman, & Smale, 2013; Heupel, Knip, Simpfendorfer, & Dulvy, 2014; Matich, Heithaus, & Layman, 2011; Smoothey et al., 2016; Trystram, Rogers, Soria, & Jaquemet, 2017). Therefore, stock assessments and evaluation of genetic structure is a priority for this species.

Population structuring and connectivity in large sharks vary in relation to environmental features, movement ecology and habitat preferences (Dudgeon et al., 2012; Heist, 2005). Oceanic species generally exhibit high levels of genetic connectivity including across ocean

basins (e.g., basking shark *Cetorhinus maximus*; Hoelzel, Shivji, Magnussen, & Francis, 2006), while coastal species tend to exhibit more structure [e.g., blacktip reef shark *Carcharhinus melanopterus* (Mourier & Planes, 2013; Vignaud et al., 2014) and scalloped hammerhead shark *Sphyrna lewini* (Duncan, Martin, Bowen, & De Couet, 2006)]. Despite the bull shark being able to undergo long distance migrations (Brunnschweiler, Queiroz, & Sims, 2010; Daly, Smale, Cowley, & Froneman, 2014; Kohler & Turner, 2001; Lea, Humphries, Clarke, & Sims, 2015; Simpfendorfer, Freitas, Wiley, & Heupel, 2005; Yeiser, Heupel, & Simpfendorfer, 2008), its dispersal may be restricted as suggested by the high genetic differentiation observed between Fiji, the Atlantic and Indo-West Pacific Oceans (Testerman, 2014). However, no genetic subdivision has been identified between populations within a continental basin (Karl, Castro, Lopez, Charvet, & Burgess, 2011; Testerman, 2014; Tillett, Meekan, Field, Thorburn, & Ovenden, 2012). This low connectivity has been suggested to result from (i) oceanic waters acting as a barrier and (ii) possible female philopatry to natal nurseries.

Many sharks tend to exhibit philopatry, returning either to specific feeding areas (e.g., the tiger shark *Galeocerdo cuvier*; Meyer, Clark, Papastamatiou, Whitney, & Holland, 2009; Meyer, Papastamatiou, & Holland, 2010) or nursery grounds (Hueter, Heupel, Heist, & Keeney, 2005; Portnoy & Heist, 2012; Speed, Field, Meekan, & Bradshaw, 2010). These behaviours may be sex-specific and, for many coastal sharks, result in population structure at smaller geographic scales than would be expected based on locomotive abilities (Chapman, Feldheim, Papastamatiou, & Hueter, 2015). Bull sharks use estuaries and rivers for nurseries (Heupel, Yeiser, Collins, Ortega, & Simpfendorfer, 2010; Ortega, Heupel, Van Beynen, & Motta, 2009; Snelson, Mulligan, & Williams, 1984), making female philopatry likely throughout their range (Karl et al., 2011; Tillett et al., 2012).

Estimates of long term effective population size of *C. leucas* vary among studies and locations, but is likely on the order of 100,000 individuals (Karl et al., 2011; Testerman, 2014), which is representative of an important genetic diversity compared to other shark species (Hoelzel et al., 2006; Schultz et al., 2008). This may suggest that (i) bull shark populations are not severely depleted and/or (ii) that fishery pressures are too recent to be detected through genetic analyses (Karl et al., 2011; Testerman, 2014).

To date, few studies have investigated bull shark genetic structure and have relied either on (i) extensive sampling on a global scale using only nuclear markers (Testerman, 2014), or (ii) a

locally intensive sampling (either restricted to the Atlantic or Northern Australia), using relatively few nuclear and mitochondrial markers (3 to 5 microsatellites along with 1 or 2 mitochondrial genes) (Karl et al., 2011; Tillett et al., 2012). Thus, improving our understanding of bull shark population structuring and connectivity across ocean basins is needed. Combining the information from two types of molecular markers [25 microsatellite loci and three mitochondrial genes (*CR*, *nd4*, *cytb*)], we analysed the genetic variation based on 370 bull shark samples from 11 locations in the Western Indian Ocean, the Western Pacific and the Western Atlantic, including both continental coasts and oceanic islands (Figure 1). By including new locations and increasing the number of markers presenting different modes of evolution, our objective was to combine classical population genetic analyses with coalescent-based approximate Bayesian computation approaches (Beaumont, 2010; Csilléry, Blum, Gaggiotti, & François, 2010) to delineate bull shark populations and assess their demographic history and connectivity using model selection analyses to refine the evolutionary history of this species. Specifically, we aimed to:

- (1) expand our understanding of the genetic structure previously documented by Testerman (2014) in order to delineate genetic clusters at different scales (e.g., within vs among ocean basins) that should be managed separately;
- (2) decipher whether contemporary migration occurs among defined clusters;
- (3) estimate the effective population sizes of these clusters.

Materials and Methods

Sampling

Tissue samples were collected in the Western Indian Ocean (WIO), the Western Pacific (WP) and the Western Atlantic (WA) (Figure 1). In the WIO, samples came from continental coasts and oceanic islands: Zanzibar (ZAN), $n = 13$; Mozambique (MOZ), $n = 18$; South Africa (SAF), $n = 32$; the Seychelles (SEY), $n = 39$; Madagascar (MAD), $n = 25$; Reunion Island (RUN), $n = 126$ and Rodrigues Island (ROD), $n = 6$. Samples from the Western Pacific were collected in two regions along the east coast of Australia [Clarence River (AUS1; $n = 44$) and Sydney Harbour (AUS2; $n = 26$), New South Wales] and in New Caledonia (NCA, $n = 10$). Most of the samples came from biopsies made on individuals caught in the wild for commercial, risk reduction or scientific purposes. Samples from Madagascar came from carcharhinid jaws or teeth found in markets and *a posteriori* confirmed as belonging to *C. leucas* by sequencing

the mtDNA control region (*CR*). Finally, in the Western Atlantic, samples of young-of-the-year and juveniles were collected in the Shark River estuary in the Florida Coastal Everglades (Florida, US) (FLO; $n = 31$). In total, 370 samples were collected and preserved in 90% ethanol until laboratory analyses (Figure 1).

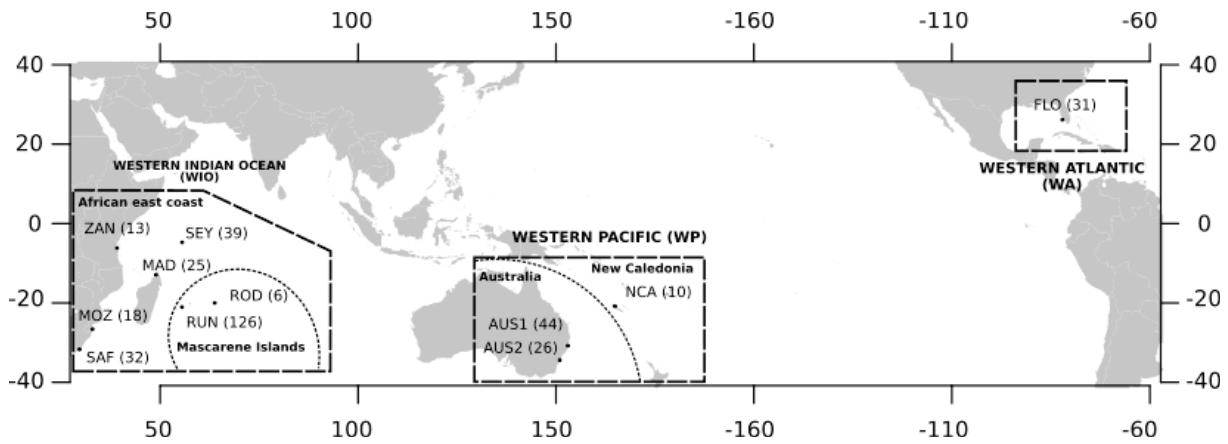


Figure 1 Map of bull shark (*Carcharhinus leucas*) sampling locations (ZAN: Zanzibar; SEY: Seychelles; MOZ: Mozambique; SAF: South Africa; MAD: Madagascar; RUN: Reunion Island; ROD: Rodrigues Island; AUS1: Clarence River, Australia; AUS2: Sydney Harbour, Australia; NCA: New Caledonia; FLO: Florida). Sample sizes are in brackets. Boxes indicate ocean basins and dotted lines delineate regions.

Laboratory procedures

Genomic DNA was extracted using Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following manufacturer instructions.

Each sample was genotyped at 25 microsatellite loci. Twenty of them were species-specific (Cl01 to Cl20; Pirog, Blaison, Jaquemet, Soria, & Magalon, 2015) and were analysed following the procedure described in Pirog et al. (2015). The remaining five microsatellite loci were originally developed for the tiger shark *G. cuvier* [Gc01 (Pirog, Jaquemet, Blaison, Soria, & Magalon, 2016); TIG10 (Mendes et al., 2016)], the sandbar shark *Carcharhinus plumbeus* (Cpl166; Portnoy, Mcdowell, Thompson, Musick, & Graves, 2006), the Australian blacktip shark *Carcharhinus tilsoni* (Ct05; Ovenden, Street, & Broderick, 2006) and the lemon shark *Negaprion brevirostris* (Ls24; Feldheim, Gruber, & Ashley, 2001) and successfully cross-amplified in the bull shark. These loci were indirectly labelled using 6-FAM, PET, VIC or NED fluorochromes and PCR reactions were carried out following G elin, Postaire, Fauvelot, and Magalon (2017). The 25 loci were multiplexed post-PCR in five panels (Supplementary Material S1). The allelic sizes of the PCR products were separated on an ABI 3730XL capillary

sequencer at the Plateforme Gentyane (INRA, Clermont-Ferrand, France) and scored with GENE Mapper v.4.0 (Applied Biosystems) using the Genescan LIZ-500 size standard (Applied Biosystems). Some samples were analysed twice to check the consistency of the results.

The mtDNA control region (*CR*) was PCR amplified using the set of primers GWF (Pardini et al., 2001) and CL2 (Tillett et al., 2012), the nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit 4 (*nd4*) using primers *nd4* (Arevalo, Davis, & Sites, 1994) and H12293_LEU (Inoue, Miya, Tsukamoto, & Nishida, 2001), and the cytochrome b (*cytb*) with primers GluDG and C61121H (Naylor, Ryburn, Fedrigo, & Lopez, 2005). This was performed for subsets of the sampling: 266 individuals for *CR*, 255 individuals for *nd4* and 227 for *cytb*.

PCR reactions were performed in a total volume of 25 μ L: 1X of MasterMix (Applied Biosystems), 0.3 μ M of forward and reverse primers and 1.6 ng/ μ L of genomic DNA. The thermocycling program for *CR* contained an initial denaturing step at 94°C for 5 min, 35 cycles \times (94°C for 30 s, 56°C for 30 s, 72°C for 1 min 30 s), and a final extension step at 72°C for 5 min. For *cytb*, the same program was used, except that the annealing temperature was set to 53°C. For *nd4*, the annealing temperature was 50°C and the elongation step was 45 s. Amplicons were sequenced directly with primers used for PCR on a capillary sequencer ABI 3730XL (Applied Biosystems) by Genoscreen (Lille, France).

Genetic diversity analysis

In Madagascar, 12 out of 25 samples were kept for data analyses, as the remaining samples, extracted from teeth, exhibited high amount of missing data (more than 50%) due to low-quality DNA.

Null alleles were assessed with MICROCHECKER v.2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004). Linkage disequilibrium (LD) between pairs of loci was tested using a likelihood-ratio test with 10,000 permutations in ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010). Diversity indices such as the number of alleles per locus N_a , observed and expected heterozygosities (H_O and H_E), and inbreeding coefficient F_{IS} (Weir & Cockerham, 1984) were estimated using FSTAT v.2.9.3.2 (Goudet, 1995). Departure from Hardy-Weinberg equilibrium (HWE) at each microsatellite locus was tested using 5,000 permutations in FSTAT v.2.9.3.2 (Goudet, 1995). The mean allelic richness A_r and the mean private allelic richness A_{rp} were calculated using a rarefaction method, as implemented in HP-RARE v.1.0 (Kalinowski, 2005).

This method accounts for differences in sample size by standardizing A_r and A_{rp} values across sampled locations by resampling the lowest number of genotypes available (i.e., 12 haploid gene copies or six diploid genotypes in Rodrigues Island) in each location.

Mitochondrial sequences were checked and aligned using GENEIOUS v.8.1.2 (Kearse et al., 2012). Alignments were performed using the MAFFT method (Kato, Misawa, Kuma, & Miyata, 2002) for each marker separately first and then for the concatenated sequence (*CR-nd4-cytb*). Diversity indices (i.e., number of haplotypes, number of segregating sites, haplotype (h) and nucleotide (π) diversities) were calculated for the concatenated alignment and for each marker separately using DNASP v.5.10.1 (Librado & Rozas, 2009).

Detection of partitioning schemes within the concatenated sequence *CR-nd4-cytb* and of substitution models was performed using PARTITIONFINDER v.2.1.1 (Guindon et al., 2010; Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017). We used BEAST v.1.8.4 (Drummond, Suchard, Xie, & Rambaut, 2012) to reconstruct phylogenetic relationships and infer divergence times on the mitochondrial concatenated sequence *CR-nd4-cytb*. Bayesian Markov chain Monte Carlo (MCMC) analyses were performed assuming a HKY85+I model of substitution as the latter was shown to best fit the data. The rate of variation among sites was modeled with a discrete gamma distribution with four rate categories. We assumed an uncorrelated lognormal relaxed clock to account for rate variation among lineages. To minimize prior assumptions about demographic history, we adopted an extended Bayesian skyline plot (EBSP) approach in order to integrate data over different demographic histories. Trees were calibrated using two methods. First, an analysis was performed adding a sequence of *S. lewini* (mitochondrion available in GenBank; accession number JX827259), and the tree was calibrated using the divergence date between *Carcharhinus* and *Sphyrna* genera, 38 millions years ago (Mya), estimated from fossil data (Maisey, 1984). Second, the tree was calibrated using the closure of the Isthmus of Panama as the divergence time of bull shark populations from the Western Atlantic and the Indo-Pacific, 3.1-3.5 Mya (Coates, Collins, Aubry, & Berggren, 2004; Coates et al., 1992). For each analysis, a normal prior distribution was thus set for the calibrated node (mean \pm SD: 38 ± 7 and 3.5 ± 0.4 , respectively). Evolutionary model parameters were then estimated, with samples drawn from the posterior every 10^5 MCMC steps over a total of 10^8 steps from five independent runs. The first 10^7 steps were discarded as burn-in. Good mixing and convergence were assessed using TRACER v.1.6 (Rambaut, Suchard, Xie, & Drummond, 2014) and the best tree was selected using the maximum clade credibility option with

TREEANNOTATOR v.1.8.4 (Drummond et al., 2012) and viewed with FIGTREE v.1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>). To further evaluate phylogenetic relationships among haplotypes, a TCS statistical parsimony network (Clement, Posada, & Crandall, 2000) was constructed using POPART v.1.7 (Leigh & Bryant, 2015).

Population genetic structure

Two complementary clustering methods were used to investigate population structure in the bull shark. First, Bayesian clustering analyses were performed using STRUCTURE v.2.3.4 (Falush, Stephens, & Pritchard, 2003; Pritchard, Stephens, & Donnelly, 2000). For any given number of clusters (K) between 1 and 10, individual assignment probabilities to each cluster were determined so as to minimize departures from HWE within clusters and maximize LD between them. Two analyses were performed, with and without the LOCPRIOR model, which uses prior sampling location information in the Bayesian clustering, to detect genetic population structure that might be weaker (Hubisz, Falush, Stephens, & Pritchard, 2009). Conditions were set to 10^6 chain length after a burn-in of 5×10^5 and 10 chains were run for each K , assuming correlated allele frequencies and the admixture model. For a given K , distinct modes were identified and, for each mode and each individual, the assignment probabilities to each cluster were averaged using CLUMPAK (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015). Secondly, a Discriminant Analysis of Principal Components (DAPC Jombart, Devillard, & Balloux, 2010), that does not rely on HWE or LD contrary to STRUCTURE, was performed to check consistency between clustering methods based on different algorithms. This method transforms genotypes using PCA as a prior step to a discriminant analysis and defines clusters such as to minimize variations within them but maximize differentiation between them. DAPC was applied using the *adegenet* package (Jombart, 2008) for R (R Core Team 2017). Methods traditionally used to detect the most likely number of clusters within a dataset might provide different outputs. To cope with these inconsistencies, we chose to consider the highest number of clusters and the individual assignments that were retrieved by both analyses. Moreover, in a hierarchical approach, these analyses were repeated on each cluster found separately. Commonly, using STRUCTURE and DAPC, when the finest level of structuring is reached, adding a supplementary cluster leads to inconclusive assignments with individuals assigned to several clusters in the same proportions.

For each locus, the microsatellite allelic frequency distributions were plotted for each cluster identified and F_{ST} between pairs of clusters (Weir & Cockerham, 1984) were also assessed using FSTAT v.2.9.3.2 (Goudet, 1995) to detect loci contributing to genetic differentiation. Then,

analyses of molecular variance (AMOVA) (Cockerham, 1969, 1973) were performed to estimate the genetic variation due to the partitioning in clusters (identified with the TCS haplotype network for the mitochondrial data and with STRUCTURE and DAPC for microsatellite data), the variation within clusters among sampling locations, and the variation within sampling locations. AMOVAs were performed with ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010) and significance of fixation indices were tested using a non-parametric approach with 10,000 permutations (Excoffier, Smouse, & Quattro, 1992).

Assessing population differentiation between pairs of sampling locations, F_{ST} (Weir & Cockerham, 1984) and D_{est} (Jost, 2008) were estimated for the microsatellites using ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010) and *DEMEtics* v.0.8-7 (Gerlach, Jueterbock, Kraemer, Deppermann, & Harmand, 2010) respectively. Contrary to F_{ST} , which depends on within-population diversity and is affected by migration rates and effective population sizes, D_{est} , based on the effective number of alleles strictly reflects the genetic distance between populations. For the mitochondrial dataset, the Φ_{ST} (Slatkin, 1995) was estimated using ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010). Significance of pairwise population differentiation indices was tested using 10,000 permutations.

Demographic history and variations of effective population sizes

To test for departures from a constant population size (Ramos-Onsins & Rozas, 2000), the summary statistics Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) were estimated from the concatenated mitochondrial dataset with ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010), with significance tested implementing 10^5 simulated samples.

Following results of clustering and variation of population size and combining the information given by both types of markers (microsatellites and mtDNA), historical relationships among regions (WIO, WP and WA) were inferred in a Bayesian framework, using random forests to identify the best model and estimate the model parameters (ABC-RF; Pudlo et al., 2016; Raynal et al., 2017). To limit the number of possible evolutionary scenarios, each region was represented by the sampling location with the highest number of individuals [Florida (FLO), Reunion Island (RUN) and Eastern Australia (Clarence River, AUS1)], each considered as a population, and only scenarios considering two populations at a time were analysed. For each pair of populations, four demographic scenarios were built: (1) split followed by bi-directional recurrent migration, (2) split followed by a period of bi-directional recurrent migration, then

followed by isolation, (3) split followed by isolation and (4) split followed by a period of isolation, then followed by bi-directional recurrent migration (Figure 2). For each scenario, we simulated 100,000 microsatellite and mitochondrial datasets using FASTSIMCOAL (Laval & Excoffier, 2004). We calculated 19 summary statistics describing the genetic polymorphism of both types of loci using ARLSUMSTAT (Excoffier & Lischer, 2010). The scenarios which best fitted the data were identified using a random forest procedure (abcrf R package; Marin, Raynal, Pudlo, Robert, & Estoup, 2017) using 20,000 of the simulated datasets. The linear discriminant analysis (LDA) axes were added to the 19 summary statistics mentioned earlier to summarize the datasets, as it has been shown to improve the discrimination between scenarios (Pudlo et al., 2016). When the analyses failed to clearly discriminate scenarios, groups of scenarios were compared, notably to test the presence (Scenarios 1 and 4) or absence (Scenarios 2 and 3) of contemporary migration. Parameters of the selected scenarios were subsequently estimated using ABC random forests as developed by Raynal et al. (2017), using the whole 100,000 simulated datasets. ABC-RF methodology showing variability due to the use of simulations and the training on bootstrap samples (Raynal et al., 2017), model choice analyses and parameter inference analyses were replicated ten times to ensure consistency. Details on the procedure used for approximate Bayesian computation are provided in Supplementary Material S2.

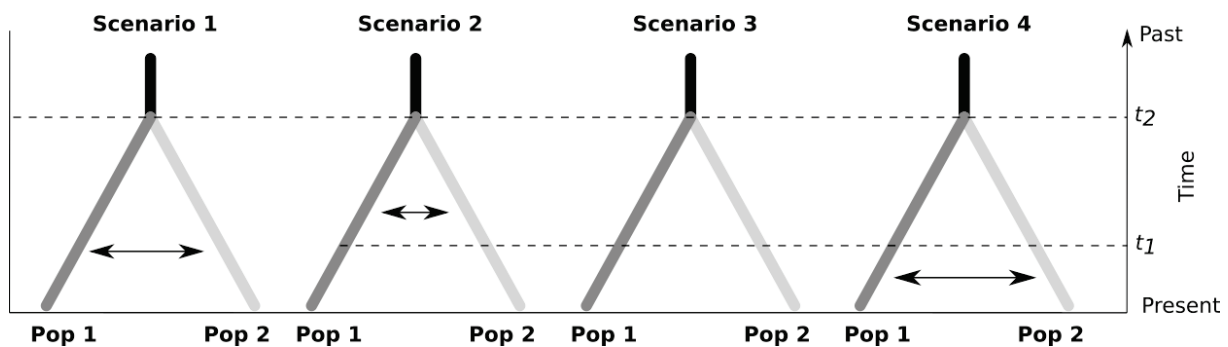


Figure 2 Graphical representations of the four scenarios depicting possible divergence histories for each pair of *Carcharhinus leucas* populations: FLO-RUN, FLO-AUS1 and RUN-AUS1. The time was measured backward in generations before present. In black, is represented the ancestral population of effective population size N_{anc} ; in dark grey, population 1 of effective population size N_1 and in light grey, population 2 of effective population size N_2 . Double arrows represent bi-directional migration events. t_2 , time of divergence; t_1 , start and end of the isolation period for Scenario 2 and Scenario 4, respectively.

Results

Genetic diversity analysis

Null alleles were detected for several loci in several sampling locations but were not constant among locations and were not correlated with significant deviations from HWE. All loci were thus kept for further analyses. For microsatellite loci, a global significant LD was detected for only four of 3,300 tests after FDR correction (0.12%, $P < 0.05$). So all loci were considered independent. The average number of alleles (\pm standard error SE) per location ranged from 2.88 ± 0.45 in New Caledonia and 2.88 ± 0.66 in Rodrigues Island to 4.56 ± 0.19 in Reunion Island. Mean allelic richness corrected by a standardized sample size of 6 diploid individuals remained relatively constant among sampling locations, varying from 2.56 ± 0.34 in Florida to 2.88 ± 0.66 in Rodrigues Island. H_E and H_O varied from 0.42 ± 0.05 in Australia (AUS2) to 0.54 ± 0.09 in Rodrigues Island and from 0.37 ± 0.05 in Florida to 0.56 ± 0.10 in Rodrigues Island, respectively (Table 1). Significant deviation from HWE was observed only for Florida ($F_{IS} = 0.17$, $P < 0.01$), which could be linked to sampling within a single nursery (sampling of relatives). The mean private allelic richness varied from 0.01 ± 0.01 in Zanzibar to 0.15 ± 0.13 in Rodrigues Island in the WIO and the WP, and was of 0.67 ± 0.29 in Florida (Table 1). Summary statistics for each mitochondrial gene are presented in Supplementary Material S3 (GenBank Accession numbers XXXXXXXX-XXXXXXX).

We obtained sequences of 923 bp for *CR*, 672 pb for *nd4* and 921 bp for *cytb* and resolved 19, 13, 17 haplotypes with 18, 22 and 23 polymorphic sites, respectively. Total haplotype diversities (h) were of the same order for each gene, varying from 0.80 ± 00 for *CR* and *cytb* to 0.86 ± 0.00 for *nd4*. Total nucleotide diversity (π) was higher for *nd4* (0.00834 ± 0.00003) than for *CR* and *cytb* (0.00448 ± 0.00001 and 0.00426 ± 0.00002 , respectively).

The concatenated sequences *CR-nd4-cytb* ($N = 218$, fragment of 2,516 bp) resolved 36 haplotypes with an overall haplotype diversity of 0.93 ± 0.00 and a nucleotide diversity of 0.00551 ± 0.00002 (GenBank Accession numbers XXXXXXXX-XXXXXXX). No partitioning scheme was detected within the concatenated sequence, and the HKY85+I model of substitution was selected with the BIC criterion. For both calibration strategies, Bayesian analyses of the concatenated mitochondrial sequence *CR-nd4-cytb* produced topologies with high support at most internal nodes and showed good convergence and mixing, with ESS above 200

(Supplementary Material S4). For each analysis, similar lineages were strongly supported, with a first splitting event between the WA and both the WIO and WP populations, a second splitting event between the WIO and WP populations and a third splitting event in two lineages within the WIO (Figure 3).

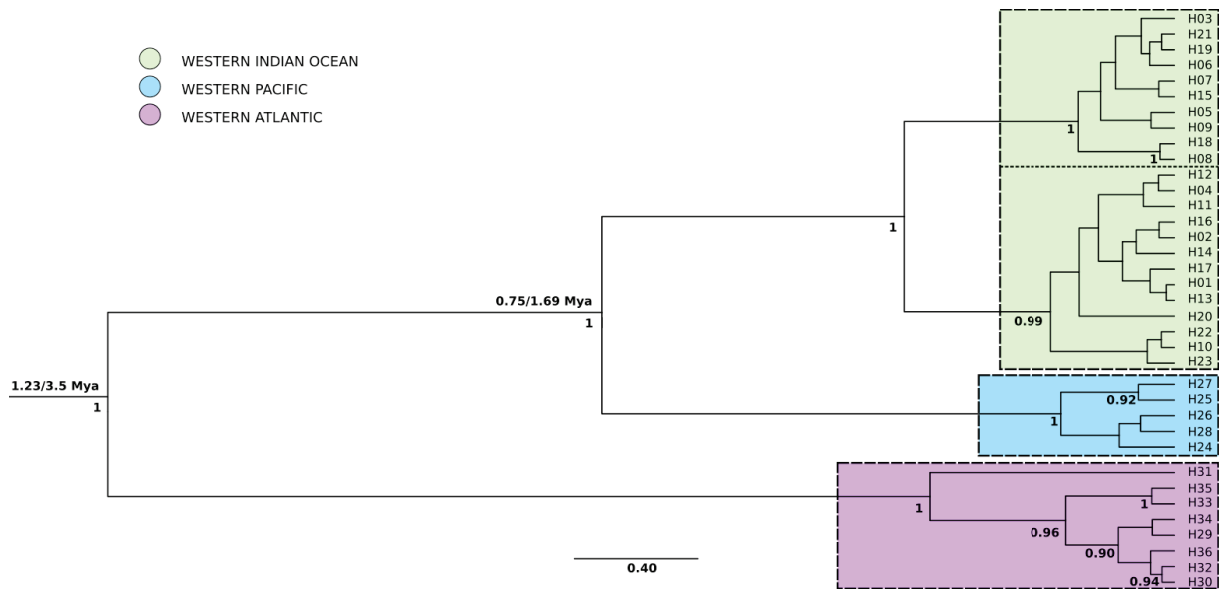


Figure 3 Maximum clade credibility tree of the mitochondrial concatenated sequence *CR-nd4-cytb* for the bull shark *Carcharhinus leucas*. Only the different haplotypes are represented. Boxes delineate lineages discussed in the text. Below branches, are indicated node supports above 0.90; above branches, are indicated the mean divergence dates (in millions years ago; Mya) retrieved using either the time of divergence between *Carcharhinus* and *Sphyrna* genera (left) or the closure of the Isthmus of Panama separating Atlantic and Pacific populations (right).

The calibration of the tree with the divergence date between *Sphyrna* and *Carcharhinus* genera, 38 Mya, provided a divergence rate between lineages per million years of 0.61% (95% confidence interval = [0.12, 1.23]). Using this calibration, populations from WA and both the WIO and WP diverged 1.23 Mya [0.22, 4.27], while WIO and WP populations diverged 0.75 Mya [0.05, 1.22]. The calibration of the tree with the date of closure of the Isthmus of Panama, 3.1 - 3.5 Mya, provided a divergence rate between lineages per million years of 0.24% [0.14, 0.36] and a divergence date of 1.69 Mya [0.75, 2.69] between WIO and WP populations. The mean of the two divergence rates was estimated, providing a mean substitution rate per site per year of 4.23×10^{-9} [1.14×10^{-9} , 1.17×10^{-8}].

The TCS statistical parsimony network built from the *CR-nd4-cytb* dataset retrieved the same lineages as the phylogenetic tree, and highlighted the absence of shared haplotypes among

lineages retrieved in each region, with 23 haplotypes identified in the WIO, five in the WP and eight in the WA (Figure 4). Furthermore, the two lineages retrieved in the WIO seemed to correspond to the locations sampled along or near the African east coast (i.e., WIO1: Zanzibar, Seychelles, Mozambique, South Africa, Madagascar) and to the Mascarene Islands (i.e., WIO2: Reunion Island and Rodrigues Island), despite some shared haplotypes.

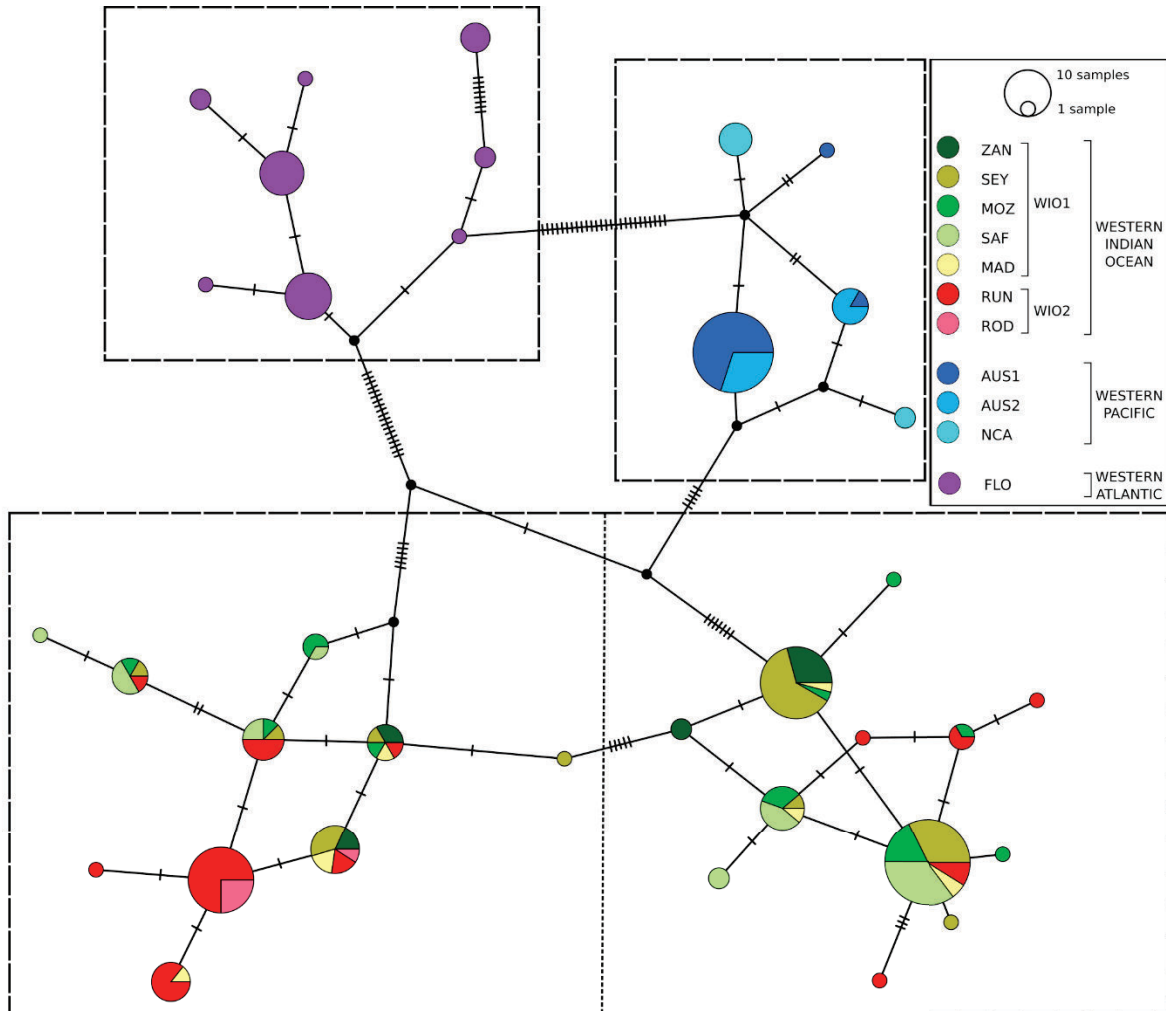


Figure 4 TCS statistical parsimony network of 36 bull sharks *Carcharhinus leucas* mitochondrial concatenated sequence *CR-nd4-cytb* haplotypes. Each circle represents a haplotype and each trait, a mutation. Boxes and the dotted line separating the Western Indian Ocean in two groups demarcate lineages discussed in the text (WIO1/WIO2). Circle size is proportional to the number of individuals harbouring each haplotype and colours correspond to sampling locations (WIO1: ZAN, Zanzibar; SEY, Seychelles; MOZ, Mozambique; SAF, South Africa; MAD, Madagascar; WIO2: RUN, Reunion Island; ROD, Rodrigues Island; AUS1, Clarence River, Australia; AUS2, Sydney Harbour, Australia; NCA, New Caledonia; FLO, Florida)

Haplotype and nucleotide diversities were globally weaker in the WP ($h = 0.51 \pm 0.01$ and $\pi = 0.00056 \pm 0.00002$) than in the WIO and in the WA (WIO: $h = 0.88 \pm 0.00$ and

$\pi = 0.00191 \pm 0.00000$; WA: $h = 0.80 \pm 0.01$ and $\pi = 0.00131 \pm 0.00005$). Within the WIO, h ranged from 0.33 ± 0.01 to 0.93 ± 0.03 and π from 0.00013 ± 0.00007 to 0.00212 ± 0.00046 , both for Rodrigues Island and Madagascar, respectively. Within the WP, Clarence River (AUS1) showed the lowest values ($h = 0.17 \pm 0.02$ and $\pi = 0.00024 \pm 0.00005$) and Sydney Harbour (AUS2), the highest ($h = 0.49 \pm 0.02$ and $\pi = 0.00059 \pm 0.00011$; Table 1). Geographic distributions of all haplotypes are indicated in Supplementary Material S5.

Table 1 Summary statistics for each *Carcharhinus leucas* sampling location averaged across 25 microsatellite loci or for the 2,516 bp concatenated mitochondrial sequence *CR-nd4-cytb*. In brackets are indicated standard error values.

ZAN, Zanzibar; SEY, Seychelles; MOZ, Mozambique; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; ROD, Rodrigues Island; AUS1, Clarence River, Australia; AUS2, Sydney Harbour, Australia; NCA, New Caledonia; FLO, Florida

<i>Microsatellite</i>	ZAN	SEY	MOZ	SAF	MAD	RUN	ROD	AUS1	AUS2	NCA	FLO
<i>N</i>	13	39	18	32	12	126	6	44	26	10	31
<i>N_a</i>	3.12 (0.48)	4.08 (0.38)	3.52 (0.39)	3.84 (0.34)	3.44 (0.48)	4.56 (0.19)	2.88 (0.66)	4.04 (0.38)	3.76 (0.38)	2.88 (0.45)	3.88 (0.83)
<i>A_r</i>	2.63 (0.33)	2.75 (0.18)	2.75 (0.29)	2.77 (0.21)	2.82 (0.35)	2.78 (0.10)	2.88 (0.66)	2.77 (0.19)	2.70 (0.24)	2.58 (0.39)	2.56 (0.34)
<i>A_{rp}</i>	0.01 (0.01)	0.07 (0.02)	0.03 (0.02)	0.07 (0.01)	0.06 (0.04)	0.05 (0.00)	0.15 (0.13)	0.06 (0.01)	0.06 (0.02)	0.03 (0.03)	0.67 (0.29)
<i>H_O</i>	0.45 (0.06)	0.44 (0.03)	0.44 (0.06)	0.43 (0.04)	0.46 (0.07)	0.42 (0.02)	0.56 (0.10)	0.47 (0.03)	0.39 (0.05)	0.47 (0.08)	0.37 (0.05)
<i>H_E</i>	0.48 (0.06)	0.45 (0.03)	0.43 (0.06)	0.45 (0.04)	0.44 (0.06)	0.44 (0.02)	0.54 (0.09)	0.47 (0.03)	0.42 (0.05)	0.47 (0.07)	0.44 (0.05)
<i>F_{IS}</i>	0.06	0.02	-0.02	0.03	-0.03	0.03	-0.04	0.00	0.06	-0.01	0.17**
<i>CR-nd4-cytb</i>	ZAN	SEY	MOZ	SAF	MAD	RUN	ROD	AUS1	AUS2	NCA	FLO
<i>N_s</i>	13	36	18	25	8	38	6	23	14	7	30
<i>H</i>	4	9	10	8	6	12	2	3	2	2	8
<i>h</i>	0.69 (0.03)	0.74 (0.01)	0.88 (0.01)	0.81 (0.01)	0.93 (0.03)	0.81 (0.01)	0.33 (0.09)	0.17 (0.02)	0.49 (0.02)	0.48 (0.06)	0.80 (0.01)
<i>S</i>	8	12	13	12	10	17	1	6	3	5	13
<i>π</i>	0.00143 (0.00024)	0.00133 (0.00013)	0.00154 (0.00021)	0.00159 (0.00018)	0.00212 (0.00046)	0.0015 (0.00014)	0.00013 (0.00007)	0.00024 (0.00005)	0.00059 (0.00011)	0.00095 (0.00025)	0.00131 (0.00014)

N, number of individuals genotyped with less than 50% missing data

N_a, number of alleles averaged across loci

A_r, mean rarified allelic richness based on a standardized sample size of 6 diploid individuals (ROD)

A_{rp}, mean rarified private allelic richness based on a standardized sample size of 6 diploid individuals (ROD)

H_O, mean observed heterozygosity

H_E, mean expected heterozygosity

F_{IS}, inbreeding coefficient and significant deviations from Hardy-Weinberg equilibrium (**: $P < 0.01$)

N_s, number of individuals sequenced

H, number of haplotypes

h, haplotype diversity

S, number of polymorphic sites

π, nucleotide diversity.

Genetic clustering

Structure clustering analysis suggested that the genetic structure is best explained by two clusters. For the microsatellite dataset without the LOCPRIOR model, for $K = 2$, a single mode including the 10 runs was found (averaged log likelihood of observing the data across 10 runs \pm standard deviation: $-14,785.03 \pm 0.09$) with a clear clustering observed between samples from the WA and those from both the WIO and WP (Figure 5). For $K = 3$, two modes were identified, one regrouping six runs and the other one including four runs. Both modes presented similar averaged log likelihoods of observing the data ($-14,648.23 \pm 1.24$ and $-14,648.48 \pm 0.57$). For both modes, one cluster corresponded to WA samples, while the other two were represented in equal proportion in each individual from the WIO and the WP (averaged individual memberships proportions of 52% and 47% for each cluster, both in the major and minor modes). With increasing K values, similar patterns were found, with one cluster in the WA, and subsequent clusters were represented in similar proportions in each individual from the WIO and the WP (Figure 5). When removing samples from the WA, for $K = 2$, two modes were retrieved, one grouping seven runs and the other three runs, with the major mode presenting the highest overall log likelihood of observing the data ($-13,897.81 \pm 71.50$ vs $-13,971.1 \pm 136.52$). For both modes, each individual was equally assigned to both clusters, with probabilities of memberships surrounding 0.50, suggesting the presence of only one genetic cluster (Figure 5).

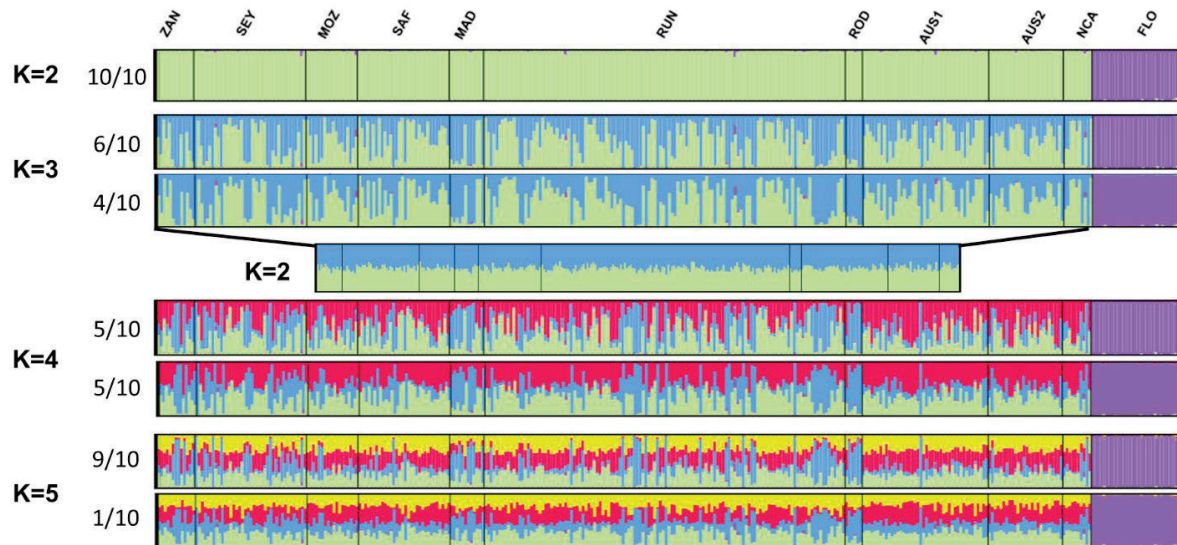


Figure 5 Average probability of membership (*y* axis) of *Carcharhinus leucas* individuals ($N = 357$, *x* axis) using microsatellites and no *a priori* sampling location information, and assuming correlated allele frequencies and admixture as performed by STRUCTURE. Major and minor modes as well as the number of times they were retrieved in the 10 replicates performed for each *K* (from 2 to 5) are presented. ZAN, Zanzibar; SEY, Seychelles; MOZ, Mozambique; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; ROD, Rodrigues Island; AUS1, Clarence River, Australia; AUS2, Sydney Harbour, Australia; NCA, New Caledonia; FLO, Florida.

Using the LOCPRIOR model on the microsatellite dataset, all samples included, similar results were retrieved for $K = 2$, with a unique mode regrouping the 10 runs (averaged log likelihood of observing the data $-14,783.61 \pm 0.85$), identifying a cluster in both the WIO and the WP and the other one in the WA (Supplementary Material S6a). For increasing *K*, each new identified cluster was found to be largely uninformative, with individual membership proportions in new clusters low (respectively $8.46\% \pm 9.32\%$, $3.06\% \pm 5.81\%$ and $4.71\% \pm 7.86\%$ for major modes of $K = 3$, $K = 4$ and $K = 5$). Similar results were retrieved for analyses using the microsatellite and mitochondrial datasets, both without and with the LOCPRIOR model (Supplementary Material S6b and S6c).

The DAPC performed on microsatellites confirmed the clear clustering between the WA and both the WIO and WP with the first axis explaining 49.87% of total inertia. Locations from the WIO and the WP were not tightly grouped, with the second axis explaining 10.29% of total inertia, and ellipses for each location still overlapped (Figure 6a). When removing samples from Florida, ellipses of each location remained overlapped, the first axis explaining 31.26% and the second 20.77% of total inertia (Figure 6b).

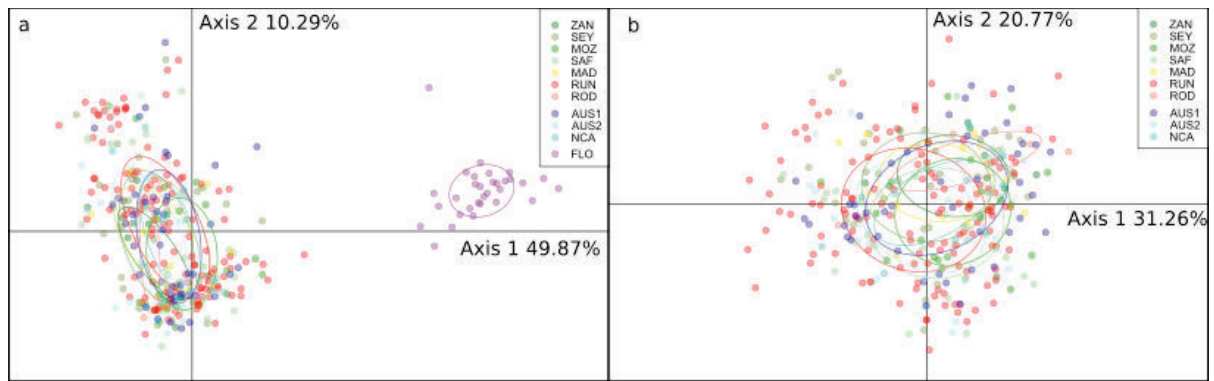


Figure 6 *Carcharhinus leucas* scatterplot output from a DAPC from microsatellites using the first and second components (**a**) all sampling locations kept, (**b**) removing FLO. Dots represent individuals with sampling locations in colors (ZAN, Zanzibar; SEY, Seychelles; MOZ, Mozambique; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; ROD, Rodrigues Island; AUS1, Clarence River, Australia; AUS2, Sydney Harbour, Australia; NCA, New Caledonia; FLO, Florida).

Furthermore, for each locus separately, allelic frequencies were very similar in the WIO and in the WP, even for loci presenting no dominant alleles (Supplementary Material S7). Global F_{ST} for each locus between the WIO and the WP were very weak, with none significantly different from zero (Supplementary Material S7).

Genetic differentiation

AMOVAs were conducted with the previously obtained clusters (microsatellites: WA and WIO/WP; mtDNA: WIO1, WIO2, WP and WA) as first level of structuration. Percentages of variation associated with clusters were 26.35% and 81.61% for the microsatellite and the mitochondrial datasets, respectively. The weakest level of differentiation was observed among locations within clusters, with percentages of variation of 0.54% and 1.68% for the microsatellites and mtDNA, respectively (Supplementary Material S8). Pairwise F_{ST} and D_{est} among locations from the WIO and the WP were weak ($F_{ST} = [0.000, 0.047]$ and $D_{est} = [0.000, 0.039]$; higher values found for Rodrigues Island may be biased by low sample size) while the ones between all locations and WA were high ($F_{ST} = [0.252, 0.335]$, all $P < 0.001$ after FDR correction and $D_{est} = [0.313, 0.360]$, all $P < 0.01$ after FDR correction; Table 2). Similarly, pairwise Φ_{ST} values for the mitochondrial concatenated dataset were high among locations from the three regions ($\Phi_{ST} = [0.776, 0.929]$, all $P < 0.001$ after FDR correction; Table 2) and, within the WIO, pairwise Φ_{ST} values were higher between locations from the Mascarene Islands

(Reunion Island and Rodrigues Island) and the other locations that are along or near the African east coast (Zanzibar, Mozambique, South Africa, Seychelles), with values varying from 0.346 (South Africa/Reunion Island) to 0.623 (Seychelles/Rodrigues Island) (all $P < 0.001$ after FDR correction; Table 2). Within the WP, pairwise Φ_{ST} values varied from 0.193 to 0.509 and were all significantly different from zero after FDR correction (Table 2).

Table 2 *Carcharhinus leucas* genetic differentiation between sampling locations (ZAN, Zanzibar; SEY, Seychelles; MOZ, Mozambique; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; ROD, Rodrigues Island; AUS1, Clarence River, Australia; AUS2, Sydney Harbour, Australia; NCA, New Caledonia; FLO, Florida) estimated for (a) microsatellite loci with Weir and Cockerham's F_{ST} (lower-left matrix) and Jost's D_{est} . (upper-right matrix) estimates and (b) the mitochondrial dataset *CR-nd4-cytb* with Weir and Cockerham's Φ_{ST} (lower-left matrix). Test significances were assessed after FDR correction and values significantly different from zero are indicated in bold; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. The number of individuals used for the analyses are indicated in parentheses.

(a) Microsatellites	ZAN	SEY	MOZ	SAF	MAD	RUN	ROD	AUS1	AUS2	NCA	FLO
ZAN (13)	-	0.000	0.006	0.004	0.000	0.006	0.039*	0.000	0.002	0.006	0.359**
SEY (39)	0.000	-	0.005	0.006	0.000	0.003	0.026	0.006	0.006	0.016	0.350**
MOZ (18)	0.013	0.010	-	0.000	0.008	0.006	0.02	0.008	0.001	0.000	0.313**
SAF (32)	0.011	0.009*	0.000	-	0.005	0.003	0.031	0.002	0.005	0.008	0.320**
MAD (12)	0.000	0.000	0.010	0.007	-	0.000	0.014	0.001	0.007	0.019	0.360**
RUN (126)	0.010	0.004	0.010*	0.004	0.000	-	0.032*	0.005*	0.004	0.017	0.329**
ROD (6)	0.035	0.025	0.023	0.030*	0.009	0.030*	-	0.034*	0.031	0.056	0.357**
AUS1 (44)	0.004	0.008*	0.008	0.001	0.007	0.008**	0.034*	-	0.005	0.015	0.340**
AUS2 (26)	0.009	0.010*	0.001	0.005	0.015	0.006	0.033	0.004	-	0.015	0.351**
NCA (10)	0.005	0.009	0.000	0.001	0.012	0.009	0.047*	0.005	0.009	-	0.333**
FLO (31)	0.317***	0.285***	0.272***	0.265***	0.300***	0.252***	0.335***	0.271***	0.297***	0.287***	-
(b) <i>CR-nd4-cytb</i>	ZAN	SEY	MOZ	SAF	MAD	RUN	ROD	AUS1	AUS2	NCA	FLO
ZAN (13)											
SEY (36)	0.027										
MOZ (18)	0.119	0.022									
SAF (25)	0.184*	0.090*	0.000								
MAD (8)	0.031	0.105	0.058	0.091							
RUN (38)	0.396***	0.435***	0.354***	0.346***	0.108						
ROD (6)	0.618***	0.623***	0.581***	0.551***	0.342*	0.057					
AUS1 (23)	0.887***	0.850***	0.870***	0.856***	0.890***	0.868***	0.973***				
AUS2 (14)	0.836***	0.816***	0.823***	0.815***	0.829***	0.840***	0.943***	0.193*			
NCA (7)	0.804***	0.805***	0.797***	0.794***	0.776***	0.821***	0.928***	0.509***	0.234*		
FLO (30)	0.883***	0.887***	0.882***	0.881***	0.874***	0.883***	0.907***	0.929***	0.909***	0.895***	

Demographic history retrieved with Bayesian analyses using both microsatellite and mtDNA data

Considering the concatenated mitochondrial dataset, no evidence of any historical population expansions or contractions were found with tests of selective neutrality, either by considering all locations separately or by grouping them in the clusters identified (all Tajima's D and Fu's F_S not significantly different from zero; Supplementary Material S9).

Combining results from both microsatellites and mtDNA, for both pairs of populations FLO-AUS1 and FLO-RUN, the model choice procedure (Principal Component Analyses in Supplementary Material S10 and variable contribution in Supplementary Material S11) showed that Scenario 3 (no migration; Figure 2) had the highest mean percentage of votes ($64.33 \pm 0.89\%$ and $73.54 \pm 1.03\%$, respectively), with mean posterior probabilities of 0.79 ± 0.01 and 0.81 ± 0.01 , and mean prior error rates of 0.42 ± 0.00 and 0.40 ± 0.00 , respectively (Table 3). For the population pair RUN-AUS1, it was not possible to discriminate Scenario 2 (migration followed by isolation) from Scenario 3 (Figure 2), with mean percentages of votes of $33.71 \pm 1.08\%$ and $37.39 \pm 0.69\%$, respectively (Table 3). Scenario 2 was the preferred scenario in two of the 10 replicates performed, while Scenario 3 was preferred in the eight other replicates. Scenario 4 also presented a high percentage of votes (20.27 ± 0.36). Grouping Scenarios 2 and 3 and Scenarios 1 and 4, the two former scenarios with no contemporary migration were preferred compared to Scenarios 1 and 4 with contemporary migration, with a mean percentage of votes of $60.47 \pm 0.58\%$, a mean posterior probability of 0.73 ± 0.01 and a mean prior error rate of 0.39 ± 0.00 (Table 3). For pairs of populations FLO-AUS1 and FLO-RUN, parameters were thus estimated using data simulated only under Scenario 3 while for the pair RUN-AUS1, we used data simulated under Scenarios 2 and 3.

Table 3 Model choice procedure of the ABC random forest method used to compare demographic scenarios of *Carcharhinus leucas* populations. FLO, Florida; AUS1, Clarence River, Australia; RUN, Reunion Island. Values are averaged over 10 replicate analyses and in parentheses are the standard errors. In bold is the best scenario selected for each pair of populations

	Votes (%)	Posterior Probability	Prior error rate
FLO_AUS1			
Scenario 1	0.13 (0.05)		
Scenario 2	24.91 (0.70)		
Scenario 3	64.33 (0.89)	0.79 (0.01)	0.42 (0.00)
Scenario 4	10.63 (0.76)		
FLO_RUN			
Scenario 1	0.05 (0.02)		
Scenario 2	22.62 (0.78)		
Scenario 3	73.54 (1.03)	0.81 (0.01)	0.40 (0.00)
Scenario 4	3.79 (0.33)		
RUN_AUS1			
Scenario 1	8.56 (0.51)		
Scenario 2	33.71 (1.08)	0.55 (0.02)¹	0.39 (0.00)¹
Scenario 3	37.39 (0.69)	0.57 (0.01)¹	0.39 (0.00)¹
Scenario 4	20.27 (0.36)		
Scenarios 1,4	39.53 (0.58)		
Scenarios 2,3	60.47 (0.58)	0.73 (0.01)	0.18 (0.00)

¹ The posterior probabilities of Scenario 2 and Scenario 3 as best models were averaged over 2 and 8 replicates, respectively.

Using 1,000 pseudo-observed datasets, we found that the effective population sizes of the estimation procedure had very low bias and good precision over the whole prior range with low NMSE values (especially for population pairs FLO-AUS1 and FLO-RUN), ranging from 0.01 to 0.10 (Table 4 and Supplementary Material S12). Using these estimations, effective population sizes varied from 5,623 for RUN to 9,772 for FLO. Other parameters were more difficult to infer (Supplementary Material S13), and values will not be interpreted because they were not the parameters of interest here.

Table 4 Characteristics of posterior distributions of *Carcharhinus leucas* effective population size (N_e) of contemporary populations estimated with ABC random forest method. NMSE: Normalized mean square error. NMAE: Normalized mean absolute error. CI: 95% confidence interval.

	FLO-AUS1		FLO-RUN		RUN-AUS1	
	$\log_{10}(N_{eFLO})$	$\log_{10}(N_{eAUS1})$	$\log_{10}(N_{eFLO})$	$\log_{10}(N_{eRUN})$	$\log_{10}(N_{eRUN})$	$\log_{10}(N_{eAUS1})$
Expectation	3.98 (0.01)	3.89 (0.01)	4.03 (0.01)	3.78 (0.01)	5.25 (0.02)	3.61 (0.03)
Median	3.89 (0.01)	3.87 (0.01)	3.99 (0.01)	3.75 (0.01)	5.15 (0.05)	3.58 (0.03)
Variance	0.10 (0.01)	0.03 (0.00)	0.06 (0.00)	0.04 (0.00)	0.56 (0.03)	0.40 (0.01)
2.5% quantile	3.45 (0.01)	3.49 (0.01)	3.63 (0.01)	3.44 (0.00)	3.60 (0.02)	2.22 (0.04)
97.5% quantile	5.09 (0.13)	4.43 (0.02)	4.67 (0.04)	4.33 (0.03)	7.74 (0.04)	6.39 (0.16)
Out of bag mean square error	0.27 (0.00)	0.04 (0.00)	0.27 (0.00)	0.04 (0.00)	0.20 (0.00)	0.56 (0.00)
NMSE	0.05	0.01	0.05	0.01	0.04	0.10
NMAE	0.07	0.04	0.07	0.04	0.06	0.10
Mean relative CI	0.35	0.19	0.36	0.19	0.35	0.52
Median relative CI	0.32	0.16	0.32	0.16	0.24	0.42

Discussion

Using a combination of markers representing different modes of evolution and different inference methods along with hierarchical sampling, we were able to refine the genetic structuring and genetic connectivity of bull shark populations and estimate their effective population size. We detected strong genetic differentiation and no contemporary migration among the bull shark *Carcharhinus leucas* populations from the Western Atlantic and both the Western Indian Ocean and the Western Pacific (hereafter designated by Western Indian Ocean/Western Pacific). Between the Western Indian Ocean/Western Pacific samples, a discordant pattern of structuring was detected according to the markers considered: low differentiation inferred from microsatellite data while high with mtDNA with no haplotypes shared between both regions. Within the Western Indian Ocean and the Western Pacific separately, this contrast was considerably less, suggesting some connectivity and/or high effective population sizes within each of these regions.

No migration between the Atlantic and the Western Indian Ocean/Western Pacific

Combining both types of markers, the absence of gene flow between the Western Atlantic and the Western Indian Ocean/Western Pacific since their divergence confirmed the results of previous research on bull shark using microsatellites, which identified three isolated genetic clusters, one in

Indo-Australia, one in Fiji and one in the Atlantic Ocean (Testerman, 2014). This strong genetic divergence could be correlated to biological differences between Atlantic Ocean bull shark populations on one side, and those of the Indian and Pacific Oceans (Indian/Pacific Oceans), on the other. In the Indian/Pacific Oceans, individuals are larger, both in terms of maximum length (Blaison et al., 2015) and size at maturity (Cliff & Dudley, 1991) than those from the Gulf of Mexico (Branstetter & Stiles, 1987; Cruz-Martinez, Chiappa-Carrara, & Arenas-Fuentes, 2005).

Divergence times were inferred based on a molecular clock estimate and should thus be regarded as qualitative indicators, rather than precise values. The use of the divergence between *Sphyrna* and *Carcharhinus* genera, or of the Isthmus of Panama closure as the divergence date between the Atlantic and the Indian/Pacific bull shark populations, yielded mutation rates similar to what has been observed in other shark species using several different fossil records (Duncan et al., 2006; Gubili et al., 2014; Karl, Castro, & Garla, 2012; Schultz et al., 2008). Using two different calibration dates, we estimated the divergence time of the Atlantic and the Indian/Pacific populations to be *ca.* 1.23 Mya [0.22 Mya-4.27 Mya], between the end of the Pliocene and the beginning of the Pleistocene. Divergence between these bull shark populations may be due to two biogeographical events: (1) the closure of the Isthmus of Panama, which occurred 3.1-3.5 Mya, and was important in shaping the current distribution of many species and genera by closing the link between the Eastern Pacific and the Western Atlantic (Briggs, 1995; Coates et al., 2004), and (2) the formation of the Benguela Upwelling System (~ 2 Mya), a cold water oceanographic system running along the west coast of South Africa and Namibia (Briggs, 1995) that restricts the mixing of tropical species populations between the Atlantic and the Indian Oceans via the southern tip of Africa (see Gaither, Bowen, Rocha, and Briggs (2016) for a review). Nevertheless, despite a small sample size in the Eastern Pacific ($n = 5$), Testerman (2014) identified only one cluster which grouped bull shark samples from the Eastern Pacific and the Western Atlantic, suggesting that bull shark migration might have occurred after the Isthmus of Panama closure through the Panama Canal. Such a scenario is possible since bull sharks are known to travel many hundreds of kilometres upstream in freshwater rivers and lakes (Heupel & Simpfendorfer, 2008; Thorson, 1976). The lack of samples from the Eastern Pacific did not allow us to test this hypothesis or the presence of any relationships between animals from the Eastern and the Western Pacific. Yet, populations from these two regions might be genetically structured because of the East Pacific Barrier, in place since 65 Mya (Grigg & Hey, 1992). This biogeographical barrier is characterized

by depths over 5,000 m over a wide oceanic distance (~ 7,000 km), limiting latitudinal dispersal across the Pacific Ocean (Briggs, 1995). Nevertheless some gene flow among these three regions may have occurred after the formation of the East Pacific Barrier, via the southern tip of Africa, before the formation of the Benguela Upwelling System.

The Benguela Upwelling System may be more constraining than the closure of the Isthmus of Panama for the bull shark, which is more sensitive to cold temperatures than species for which some gene flow after the formation of this current has been highlighted [e.g., tiger shark *Galeocerdo cuvier* (Bernard et al., 2016), dusky shark *Carcharhinus obscurus* (Benavides et al., 2011), or scalloped hammerhead shark *Sphyrna lewini* (Duncan et al., 2006)]. Bull sharks remain in warmer waters, favoring temperatures of 25-26°C and found less frequently in waters less than 18°C (Brunnschweiler et al., 2010; Carlson, Ribera, Conrath, Heupel, & Burgess, 2010; Lea et al., 2015; Matich & Heithaus, 2012). The formation of the Benguela Upwelling System may thus have disrupted the migratory behavior of bull sharks, and led to the divergence of the Atlantic and Indian Ocean populations. Additional samples from the Eastern Pacific and the Southern Atlantic (both Eastern and Western) are needed to further investigate the worldwide phylogeography of the bull shark.

Barrier to gene flow between Western Indian Ocean and Western Pacific or sex-biased dispersal?

Previous studies did not detect the variation in patterns of microsatellite and mitochondrial datasets between the Western Indian and the Western Pacific Oceans that we observed. This is because Testerman (2014) only used nuclear information and Tillett et al. (2012) only sampled Northern Australia, despite using both types of markers (albeit only 3 microsatellites and 2 mitochondrial genes). Importantly the weak differentiation we detected with the microsatellites is not due to homoplasy (see Supplementary Material S7). Similar to our results, low nuclear and higher mitochondrial differentiations were found between bull shark populations from the North-Western and the South-Western Atlantic (Karl et al., 2011). Such a pattern is common (reviewed in Toews & Brelsford, 2012) because the two types of markers are characterized by different modes of evolution (mutation models, mutation rates, inheritance and ploidy level) leading to difficulty in interpretation. While the quasi-absence of differentiation at nuclear loci is suggestive of gene flow

or large population sizes, the mitochondrial analyses provide complementary information, with a pronounced structuring. Here we propose two possible explanations.

Coupling both types of markers, the ABC random forest procedure, regarded as one of the most precise Bayesian methods to identify demographic histories (Fraimout et al., 2017; Pudlo et al., 2016; Raynal et al., 2017), revealed that scenarios with no contemporary gene flow between the Western Indian Ocean and the Western Pacific (Scenarios 2 and 3) best explained the observed data. Additionally, mitochondrial analyses indicated a divergence date of 0.75-1.69 Mya between Western Indian and Western Pacific bull shark populations. With as many as 20 glacial periods during the Pleistocene, each lasting approximately 100,000 years, followed by shorter interglacial periods of about 10,000 years (Dawson, 1992; Martinson et al., 1987), fluctuations in sea levels were as great as 100 m (Shackleton, 1987). These fluctuations may have changed the distribution of shallow, near-shore habitats used by bull sharks and modified their movement patterns along the coasts, especially in Indonesia, possibly explaining the historical migration (Scenario 2). Indeed, several studies on chondrichthyan species have shown greater population subdivision between Indonesia and Northern Australia than within Australian waters (Dudgeon, Broderick, & Ovenden, 2009; Ovenden, Kashiwagi, Broderick, Giles, & Salini, 2009). It is possible that the deep waters of the Timor Trench (2,000-3,000 m) and the strong Indonesian through-flow current along the Makassar and Lombok Straits induced the genetic subdivisions observed between Indonesian and Australian waters (Dudgeon et al., 2012; Dudgeon et al., 2009; Ovenden et al., 2009), and thus, limits contemporary gene flow between the Indian and Pacific Oceans.

Alternatively, occasional contemporary male migration combined with female philopatry could explain both the strong mitochondrial and low nuclear differentiation levels (Prugnolle & de Meeus, 2002), even if disregarding other processes such as variations in mutation rates and drift intensity on these two sets of markers (Chesser & Baker, 1996; Prugnolle & de Meeus, 2002). Indeed, for populations of large sizes ($N_e > 10^3$), rare effective dispersal events may be sufficient to homogenize allelic frequencies leading to F_{ST} estimates non-significantly different from zero (in the order of 10^{-3}) while maintaining high mitochondrial differentiation (Hauser & Carvalho, 2008; Mariani & Bekkevold, 2014). The sea level variations that occurred during the Pleistocene may also explain bull shark sex-biased dispersal between the Western Indian Ocean and the Western Pacific, changing the distribution of shallow near-shore habitats used by bull sharks as nurseries,

and modifying female movement patterns along coasts, while impacting male movements minimally. This pattern has been hypothesized to explain structuring of the sandbar shark *Carcharhinus plumbeus* in the Indian/Pacific populations (Portnoy, McDowell, Heist, Musick, & Graves, 2010). Unfortunately, this last scenario could not be tested as the available simulation software do not allow sex-biased dispersal when using bi-parentally inherited markers, such as microsatellites. Female philopatry to nursery areas has been demonstrated in the lemon shark *Negaprion brevirostris* in the Bahamas by reconstructing parental genotypes (microsatellites) through sampling juveniles in specific nurseries over several decades: some females returned to the nursery to give birth, sometimes 14 to 17 years after being born (Feldheim et al., 2013). To increase juvenile survival, females may exhibit high-fidelity to their breeding areas and nurseries which are typically good foraging areas and offer protection from large predators (Branstetter, 1990; Castro, 1993; Heupel, Carlson, & Simpfendorfer, 2007; Springer, 1967). These breeding sites are sometimes the same as the natal places of females, as these latter represent suitable habitats for parturition (Heupel et al., 2007; Hueter et al., 2005). In contrast, males may exhibit roaming behaviours and undertake migration, possibly to avoid inbreeding depression, demographic and environmental stochasticity, especially in polygynous systems (Henry, Coulon, & Travis, 2016), as may occur for the bull shark (AP, personal communication).

Currently, no direct evidence of bull sharks moving between the Western Indian Ocean and the Western Pacific has been documented, possibly due to the very low number of satellite-tracked individuals, but possibly also due to the lack of contemporary gene flow retrieved by the ABC analyses. Further studies using coalescent simulations with sex-biased dispersal, as well as genome-wide analyses, are required.

Gene flow within the Western Indian Ocean and within the Western Pacific

Low genetic differentiation was shown both within the Western Indian Ocean and within the Western Pacific, regardless of the markers used (microsatellites or mtDNA). To date, a limited number of tracking studies have explored long-distance movements of adult bull sharks, but each has highlighted the capability of bull sharks to undertake long-distance coast-wise migrations (up to 1,770 km) (Carlson et al., 2010; Daly et al., 2014; Espinoza, Heupel, Tobin, & Simpfendorfer, 2016; Espinoza, Lédée, Simpfendorfer, Tobin, & Heupel, 2015; Heupel et al., 2015) and across

hundreds of kilometres of open ocean (Soria et al., 2015). As such, long-distance migration of adult bull sharks may genetically link ecosystems within these regions. Each movement study also highlights the fidelity of bull sharks to specific sites at discrete times, as shown in Reunion Island (Blaison et al., 2015), in New Caledonia (Werry & Clua, 2013), in Fiji and in the Bahamas (Brunnschweiler & Baensch, 2011; Brunnschweiler et al., 2010). Thus, previous tracking studies and the low genetic differentiation from the present study suggest that individuals may disperse on a regular basis among locations within each of these regions. Nevertheless, slightly higher mitochondrial differentiation values were retrieved among locations separated by deep-water expanses, such as the Mascarene Islands (WIO1) and locations along the Eastern African coast (WIO2), or among locations of the Eastern Australian coast and New Caledonia. These higher values may reflect some level of female philopatry to nursery areas at the described spatial scale. Indeed, even if some mitochondrial haplotypes are shared between these locations, as samples analysed in this study were taken from sharks fished or caught opportunistically, the geographic location assigned to each individual does not reflect necessarily its nursery or natal site. Hence, the shared haplotypes potentially reflect female (and also male) movements between two stays (possibly lengthy ones) in their birthing and/or natal nurseries. As an illustration, a gravid female bull shark satellite-tagged in the Seychelles travelled 2,000 km, to the southeast coast of Madagascar, where it remained in shallow waters for several days, before returning, no longer in a gravid condition to the Seychelles (Lea et al., 2015), suggesting this female may have given birth in Madagascar (perhaps its natal site) and therefore undertakes long distance movements between Madagascar and the Seychelles. However, no direct evidence of female philopatry to nursery sites has been documented for bull sharks. This would require the sampling of juveniles in nurseries for parentage analyses as direct observation of parturition is highly unlikely, especially for tagged females, due to the turbid nature of estuaries and frequency of occurrence.

Effective population sizes

Changes in population size were not detected with neutrality tests performed with mitochondrial data. Estimates of effective population sizes (N_e) from our study with both nuclear and mitochondrial data were *ca.* 6,000-10,000 for each of the three regions investigated (i.e., the Western Indian Ocean, the Western Pacific and the Western Atlantic). Using the mismatch

distribution of the mitochondrial control region, Tillett et al. (2012) estimated long-term N_e of bull shark populations of Northern Australia (Western Pacific) to be within the same range as presented here, with a θ -value of 0.293 corresponding to an effective population size of 11,000-13,000. Comparatively, using 11 microsatellite loci, Testerman (2014) estimated long-term N_e of populations from the Western Atlantic, the Indo-Pacific and Fiji to be *ca.* 100,000. Karl et al. (2011) found similar estimates using the mitochondrial control region and five microsatellite loci separately for populations of the Northern and South-Western Atlantic, with long-term N_e ranging from 148,000 to 214,200. The discrepancy between our estimates and those of previous studies may be due to the higher number of loci we used, 25 vs 11 and 5, with the accuracy in the estimate of θ being proportional to the number of loci (Felsenstein, 2006; Pluzhnikov & Donnelly, 1996).

Estimates of effective population size using genetic markers are increasingly used for fisheries stock assessments (Ovenden et al., 2016). The ability of a population to adapt to environmental changes is postulated to require an N_e of at least 500 (Frankham, Briscoe, & Ballou, 2010) although others estimate that at least 5,000 breeding individuals may be required (Lande, 1995). Avoiding deleterious allele accumulation may require an N_e above 1,000 (Frankham et al., 2010; Palstra & Ruzzante, 2008) and inbreeding depression may occur if N_e falls below 50 (Frankham et al., 2010). Our estimates (6,000-10,000) are similar to what has been found in the basking shark *Cetorhinus maximus* (i.e., 8,200; Hoelzel et al., 2006), but lower than estimates for the lemon shark *N. brevirostris* (26,000 to 52,000 in the Atlantic) and the sicklefin lemon shark *Negaprion acutidens* (34,000 to 52,000 in the Western Pacific) (Schultz et al., 2008), and much lower than for the tope shark, *Galeorhinus galeus* (138,000; Chabot & Allen, 2009). All of these species are considered either (i) globally Vulnerable on the IUCN Red List or (ii) subjected to a loss of genetic diversity due to a bottleneck (for *C. maximus*), which may potentially be the case for bull sharks, despite our current estimates do not suggest that populations are extremely depleted.

Conclusion

Here we highlight several key findings for the bull shark *Carcharhinus leucas* to inform management and conservation issues:

(1) The genetic isolation between bull shark populations from the Western Atlantic and from the Western Indian Ocean/Western Pacific imply that the Western Atlantic populations should be managed separately.

(2) Between the Western Indian Ocean and the Western Pacific, the strong discrepancy in population differentiation between microsatellites and mtDNA may be due to: (i) a lack of contemporary migration between both regions, or (ii) occasional contemporary effective dispersal of males but not females, these latter being philopatric to their natal site for mating and/or breeding. Understanding that low nuclear differentiation is not a guarantee of extant gene flow may have important implications for population management.

(3) Within the Western Indian Ocean and within the Western Pacific, males and females are capable of undergoing long-distance movements at this scale, with either (i) both sexes contributing to effective dispersal (i.e., gene flow) or (ii) males contributing to effective dispersal and females exhibiting philopatry to their natal sites for mating and/or breeding. Thus, conservation and management programs (for example, post-attack culling programs) may be ineffective if implemented at a localized local scale.

(4) Estimates of the effective population size for bull shark populations are comparable to other shark species and whilst they do not indicate extremely depleted populations, caution should be taken when implementing fisheries guidelines for this species.

Acknowledgments

Samples from Zanzibar were collected under the Western Indian Ocean Marine Science Association funded BYCAM project. Samples collected from New South Wales, Australia, were done under research permits from New South Wales Fisheries and animal ethics approvals Ref 07/08. The Operations staff of the KwaZulu-Natal Sharks Board provided the bull sharks from

South Africa and the research staff collected and catalogued the tissue samples. We acknowledge the Plateforme Gentyane of the Institut National de la Recherche Agronomique (INRA, Clermont-Ferrand, France) for guidance and technical support. Agathe Pirog was supported by a doctoral fellowship from the region Reunion.

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Data accessibility

Microsatellite genotypes: Dryad doi:XXX.

Mitochondrial sequences: GenBank Accession Numbers XX-XX

Author contributions

MCF, GC, EC, RD, MRH, JJK, PM, JEGN, AFS, AJT, and SJ provided samples.

AP, HM and MCF did lab work.

AP, HM, VR, MCF, AR, AG analysed the data.

All authors contributed to the writing of the manuscript.

HM and SJ designed research

Supplementary Material

Supplementary Material S1. Panels used to multiplex loci post-PCR and allelic ranges (in base pairs, without M13-tail).

Locus name	Panel	Fluorochrome	Allelic range	Reference
CI03	1	VIC	104-112	Pirog et al. 2015
CI01	1	6-FAM	114-116	Pirog et al. 2015
Gc01	1	VIC	136-146	Pirog et al. 2016
CI06	1	PET	148-150	Pirog et al. 2015
CI14	1	VIC	211-213	Pirog et al. 2015
CI11	1	NED	232-238	Pirog et al. 2015
CI12	2	6-FAM	103-113	Pirog et al. 2015
CI10	2	PET	123-133	Pirog et al. 2015
CI02	2	NED	141-145	Pirog et al. 2015
CI08	2	VIC	146-158	Pirog et al. 2015
CI17	2	6-FAM	167-183	Pirog et al. 2015
Cpl166	2	PET	218-352	Portnoy et al. 2006
CI13	3	VIC	106-140	Pirog et al. 2015
CI09	3	PET	107-115	Pirog et al. 2015
CI05	3	NED	137-141	Pirog et al. 2015
CI07	3	6-FAM	143-151	Pirog et al. 2015
CI18	3	6-FAM	201-221	Pirog et al. 2015
CI16	4	NED	103-121	Pirog et al. 2015
CI04	4	6-FAM	124-130	Pirog et al. 2015
CI20	4	VIC	134-182	Pirog et al. 2015
CI19	4	NED	198-219	Pirog et al. 2015
CI15	4	PET	290-298	Pirog et al. 2015
Ct05	5	NED	226-250	Ovenden et al. 2006
Ls24	5	6-FAM	249-275	Feldheim et al. 2001
TIG10	5	PET	257-265	Mendes et al. 2016

Supplementary Material S2. Inference of demographic history using Approximate Bayesian Computation (ABC) random forest.

Scenarios

Demographic scenarios were defined according to the results of the clustering analyses with the aim to detect the occurrence of migrations among the three potential distinct populations, *i.e.*, the Western Indian Ocean, the Western Pacific and the Western Atlantic. To simplify the number of possible evolutionary scenarios, we investigated relations between pairs of populations, each population being represented by samples from one location (as recommended by Lombaert et al., 2014), RUN for the Western Indian Ocean, AUS1 for the Western Pacific and FLO for the Western Atlantic. For each pair of populations, four models were tested: (1) split followed by bi-directional recurrent migration, (2) split followed by a period of bi-directional recurrent migration, then followed by isolation, (3) split followed by isolation and (4) split followed by a period of isolation, then followed by bi-directional recurrent migration (Figure 2).

Prior distributions

Parameters were drawn in the prior distributions described in Table S1. Because of the lack of knowledge on effective sizes and historical divergences of populations of *C. leucas*, ranges were chosen broad. For population sizes, uniform distributions with large interval were chosen, for both the sampled populations and the ancestral population. A log-uniform distribution on interval (220-350,000) was chosen for the divergence time. Parameters for microsatellite mutation models were set by default following a generalized stepwise mutation model.

Table S1. Priors for the demographic parameters

$\log_{10}(N)$	unif [2-8]
t_1	logunif [1-350000]
t_2	logunif [220-350000]
μ_{seq}	unif [10^{-8} - 10^{-6}]
μ_{sat}	unif [10^{-5} - 10^{-4}]
m	unif [0.01-0.2]
Constraint on parameters	$t_1 < t_2$

N , effective population sizes of all populations; t_1 , start and end of the isolation period; t_2 , time of divergence; μ_{seq} , sequence mutation rate; μ_{sat} , microsatellite mutation rate; unif, uniform distribution; logunif, log-uniform distribution; in brackets are minimal and maximal values

Summary statistics

Genetic variation within and between populations was summarized using a set of 19 summary statistics. For each sampled population, for both the microsatellite and mitochondrial markers, we used the mean number of alleles over loci K , as well as the mean of Nei's gene diversity H . For microsatellite loci, the mean over loci of the modified Garza-Williamson index NGW was also computed for each population, as well as the pairwise F_{ST} and the mean delta mu-square $DMUSQR$ (square difference in mean microsatellite allele length between pairs of populations) between the sampled populations. For the mitochondrial marker, the mean number of pairwise differences P_i , Tajima's D and Fu's F_s were computed for each population, as well as pairwise F_{ST} between the populations. Summary statistics of the observed datasets are indicated in Table S2.

Table S2. Summary statistics of the observed datasets estimated for the ABC analyses. For microsatellites: K , mean number of alleles over loci; H , mean of Nei's gene diversity; NGW , mean over loci of the modified Garza-Williamson index; F_{ST} , pairwise microsatellite F_{ST} ; $DMUSQR$, mean delta mu-square (square difference in mean microsatellite allele length between pairs of populations). For the mitochondrial marker: $seqK$, mean number of alleles over loci; $seqH$, mean of Nei's gene diversity; P_i , mean number of pairwise differences; D , Tajima's D ; F_s , Fu's F_s ; $seqF_{ST}$, pairwise F_{ST} . For each pair of populations, 1 refers to the first population of the pair; 2 refers to the second population of the pair

Microsatellites	FLO-AUS1	FLO-RUN	RUN-AUS1
K_1	3.88	3.88	4.56
K_2	4.04	4.56	4.04
H_1	0.35	0.35	0.44
H_2	0.43	0.44	0.43
NGW_1	0.36	0.31	0.46
NGW_2	0.47	0.46	0.39
F_{ST}	0.28	0.27	0.01
$DMUSQR$	62.10	61.42	0.23
Mitochondrial			
$seqK_1$	8.00	8.00	12.00
$seqK_2$	3.00	12.00	3.00
$seqH_1$	0.80	0.80	0.81
$seqH_2$	0.17	0.81	0.17
D_1	0.02	0.02	-0.07
D_2	-1.82	-0.07	-1.82
F_{s1}	0.40	0.40	-1.37
F_{s2}	0.24	-1.37	0.24
P_{i1}	3.30	3.30	3.78
P_{i2}	0.60	3.78	0.60
$seqF_{ST}$	0.93	0.88	0.87

Model choice

The scenario best fitting the data was selected using the ABC-RF algorithm (Breiman, 2001; Pudlo et al., 2016). Compared to standard model choice procedures (Cornuet, Ravigné, & Estoup, 2010; Wegmann, Leuenberger, Neuenschwander, & Excoffier, 2010), ABC-RF presents a larger discriminative power, is more robust to the choice and number of summary statistics, is less time-consuming, and provides a more reliable approximation of the posterior probability of the selected scenario (Pudlo et al., 2016). For each pair of populations, 100,000 datasets were simulated for each scenario using FASTSIMCOAL 2.6 (Laval & Excoffier, 2004). For each dataset, summary statistics were computed using ARLSUMSTAT (Excoffier & Lischer, 2010), and the 100,000 sets of parameter values and corresponding summary statistics were assembled using *ad hoc* bash scripts (available on request) in a reference table. Then, using 20,000 simulated datasets for each model, functions *abcrf* and *abcrf.predict* of the package *abcrf* were used to provide a classification vote representing the number of times a scenario is selected as the best one among n trees in the constructed random forest. The scenario with the highest number of classification vote was selected as the best scenario among a total of 1,000 trees (Breiman, 2001; Pudlo et al., 2016). The linear discriminant analysis (LDA) axes were added during the ABC-RF procedure to the 19 summary statistics mentioned earlier to summarize the datasets, as it has been shown to improve the discrimination between scenarios (Pudlo et al., 2016). Model choice consistency was evaluated by replicating the whole ABC-RF analysis 10 times. Posterior probabilities and prior error rates (i.e., the probability of choosing a wrong model when drawing model index and parameter values into priors; Pudlo et al., 2016) of the best scenario were averaged over the replicate analyses (Fraitout et al., 2017). To further test for contrasted hypotheses, sets of scenarios were compared to each other following the same procedure.

Parameter estimation

Parameter estimation was conducted using the ABC-RF procedure, as described in Raynal et al. (2017). For each parameter, 100,000 datasets simulated under the selected scenario(s) were used and analyses were replicated 10 times. A regression random forest with 500 trees was performed to predict posterior expectations, variances and quantiles of each parameter. Contributions of the 19 summary statistics and the out-of-bag mean squared errors associated with each parameter were plotted. To test the performance of the method in estimating parameters, we used 1,000 pseudo-

observed datasets on which the estimation procedure was applied to measure the precision of the estimation procedure. From these values, the 95% confidence interval (CI) and the normalized-mean-square error NMSE were then computed in R and averaged over the 10 replicates.

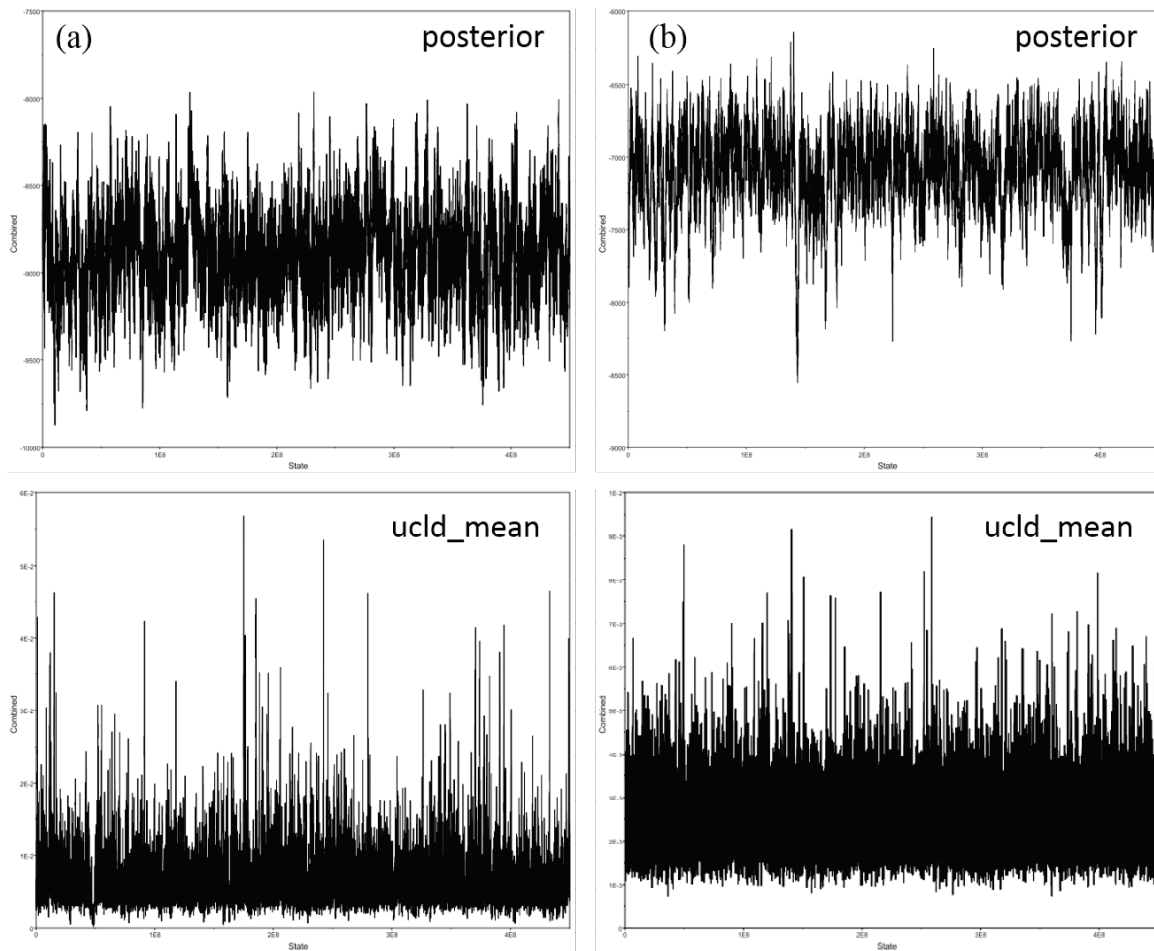
Supplementary Material S3. Summary statistics for each sampling location for the three mitochondrial markers used, the control region *CR* (923 bp), *nd4* (672 pb) and *cytb* (921 bp). ZAN, Zanzibar; SEY, Seychelles; MOZ, Mozambique; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; ROD, Rodrigues Island; AUS1, Clarence River, Australia; AUS2, Sydney Harbour, Australia; NCA, New Caledonia; FLO, Florida. N_s , number of individuals sequenced; H , number of haplotypes, h , haplotype diversity; S , number of polymorphic sites; π , nucleotide diversity. In parentheses, are indicated standard errors.

CR	ZAN	SEY	MOZ	SAF	MAD	RUN	ROD	AUS1	AUS2	NCA	FLO	TOT
N_s	13	36	18	29	13	66	6	31	15	9	31	267
H	3	4	5	4	3	5	1	3	2	2	6	19
h	0.64 (0.03)	0.41 (0.01)	0.66 (0.02)	0.70 (0.01)	0.62 (0.02)	0.37 (0.01)	0.00 (0.00)	0.18 (0.02)	0.51 (0.02)	0.50 (0.04)	0.74 (0.01)	0.80 (0.00)
S	5	5	5	5	5	9	0	4	1	3	6	18
π	0.00259 (0.00047)	0.00193 (0.00021)	0.00235 (0.00036)	0.00236 (0.00028)	0.00292 (0.00052)	0.00184 (0.00015)	0.00000 (0.00000)	0.00041 (0.00008)	0.00056 (0.00014)	0.00163 (0.00041)	0.00208 (0.00024)	0.00448 (0.00001)
nd4	ZAN	SEY	MOZ	SAF	MAD	RUN	ROD	AUS1	AUS2	NCA	FLO	TOT
N_s	13	39	18	30	15	48	6	31	14	10	31	255
H	2	6	7	5	4	6	2	2	2	2	1	13
h	0.46 (0.03)	0.71 (0.01)	0.74 (0.02)	0.54 (0.02)	0.76 (0.02)	0.63 (0.01)	0.33 (0.09)	0.12 (0.01)	0.49 (0.02)	0.47 (0.04)	0.00 (0.00)	0.86 (0.00)
S	1	5	6	4	3	6	1	1	1	1	0	22
π	0.00069 (0.00020)	0.00152 (0.00019)	0.00173 (0.00031)	0.00166 (0.00023)	0.00193 (0.00037)	0.00131 (0.00015)	0.0005 (0.00027)	0.00019 (0.00006)	0.00074 (0.0002)	0.00069 (0.00236)	0.00000 (0.00000)	0.00834 (0.00003)
cytb	ZAN	SEY	MOZ	SAF	MAD	RUN	ROD	AUS1	AUS2	NCA	FLO	TOT
N_s	13	39	18	27	9	40	6	23	15	7	30	227
H	3	3	3	4	3	5	1	2	2	2	5	15
h	0.51 (0.04)	0.39 (0.01)	0.50 (0.02)	0.56 (0.02)	0.64 (0.04)	0.57 (0.01)	0.00 (0.00)	0.09 (0.02)	0.51 (0.02)	0.48 (0.06)	0.54 (0.02)	0.80 (0.00)
S	2	2	2	3	2	3	0	1	1	1	7	23
π	0.00081 (0.0002)	0.00060 (0.00009)	0.00058 (0.00013)	0.00078 (0.00013)	0.00115 (0.00032)	0.00095 (0.00012)	0.00000 (0.00000)	0.00009 (0.00004)	0.00056 (0.00014)	0.00052 (0.00022)	0.00165 (0.00021)	0.00426 (0.00002)

Supplementary Material S4. Posterior trace files of the BEAST analyses calibrated (a) using the divergence date between *Carcharhinus* and *Sphyrna* genera, 38 Mya; (b) using the formation of

ESS	(a)	(b)
Posterior distribution	373	333
Mean rate of substitution	1901	7182

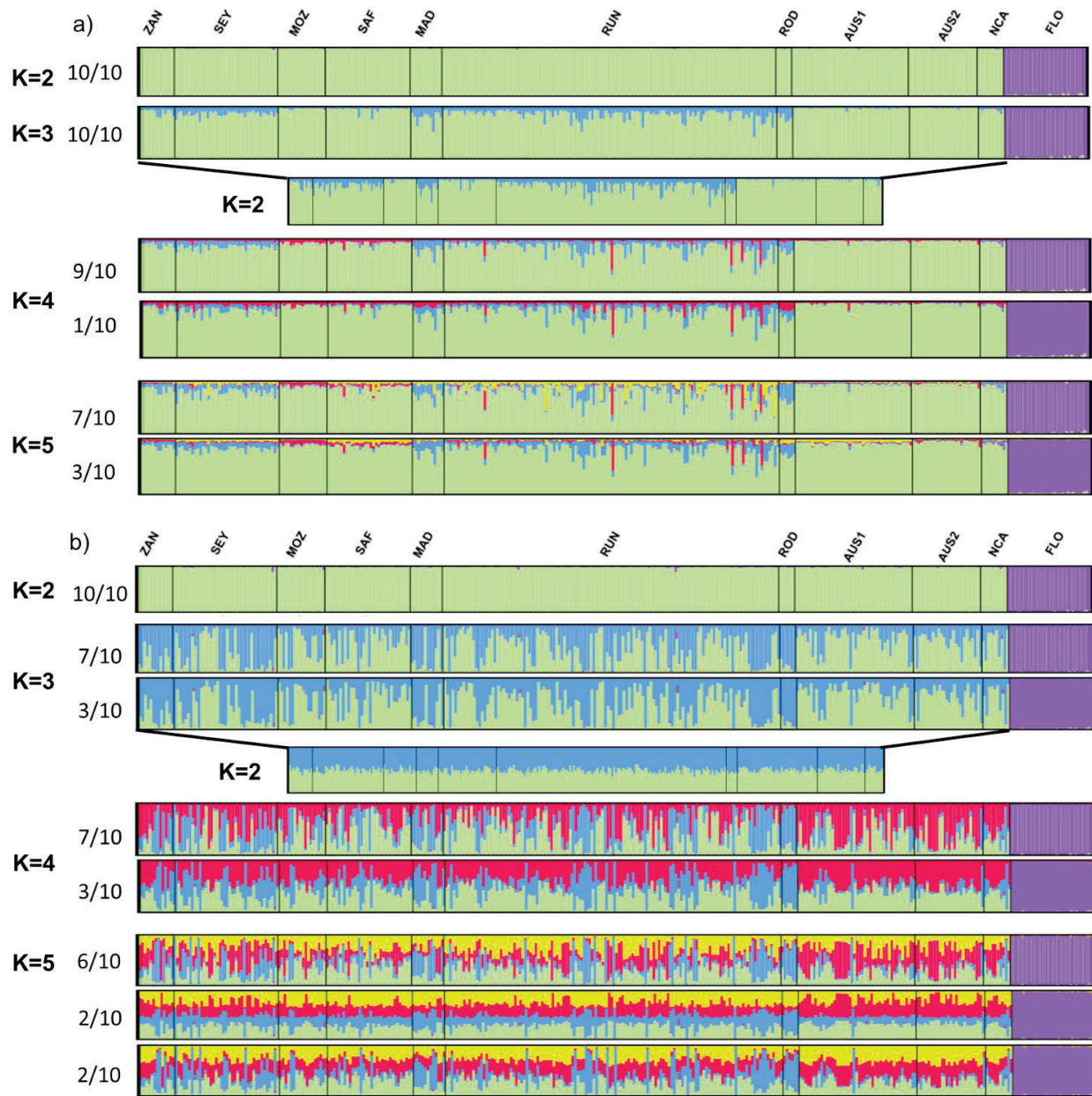
the Isthmus of Panama 3.1-3.5 Mya. Concatenated runs of the five analyses performed for each calibration are shown, as well as the Effective Sample Sizes (ESS) of the posterior distribution and of the mean rate of substitution.

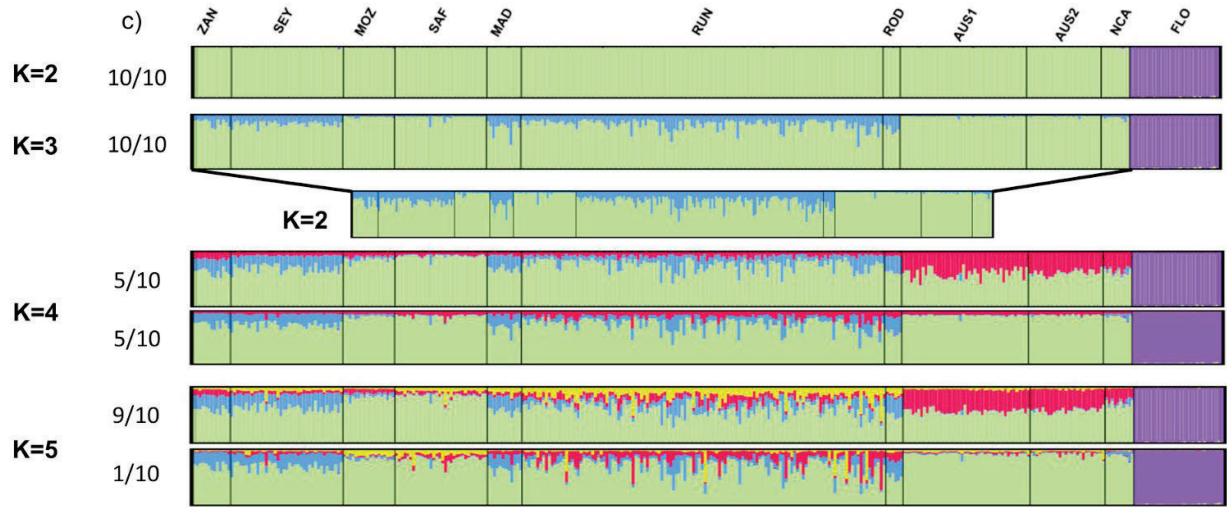


Supplementary Material S5. Geographic distribution of mitochondrial concatenated sequence *CR-nd4-cytb* haplotypes found in bull sharks from the Western Indian Ocean (WIO), the Western Pacific (WP) and the Western Atlantic (WA). ZAN, Zanzibar; SEY, Seychelles; MOZ, Mozambique; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; ROD, Rodrigues Island; AUS1, Clarence River, Australia; AUS2, Sydney Harbour, Australia; NCA, New Caledonia; FLO, Florida.

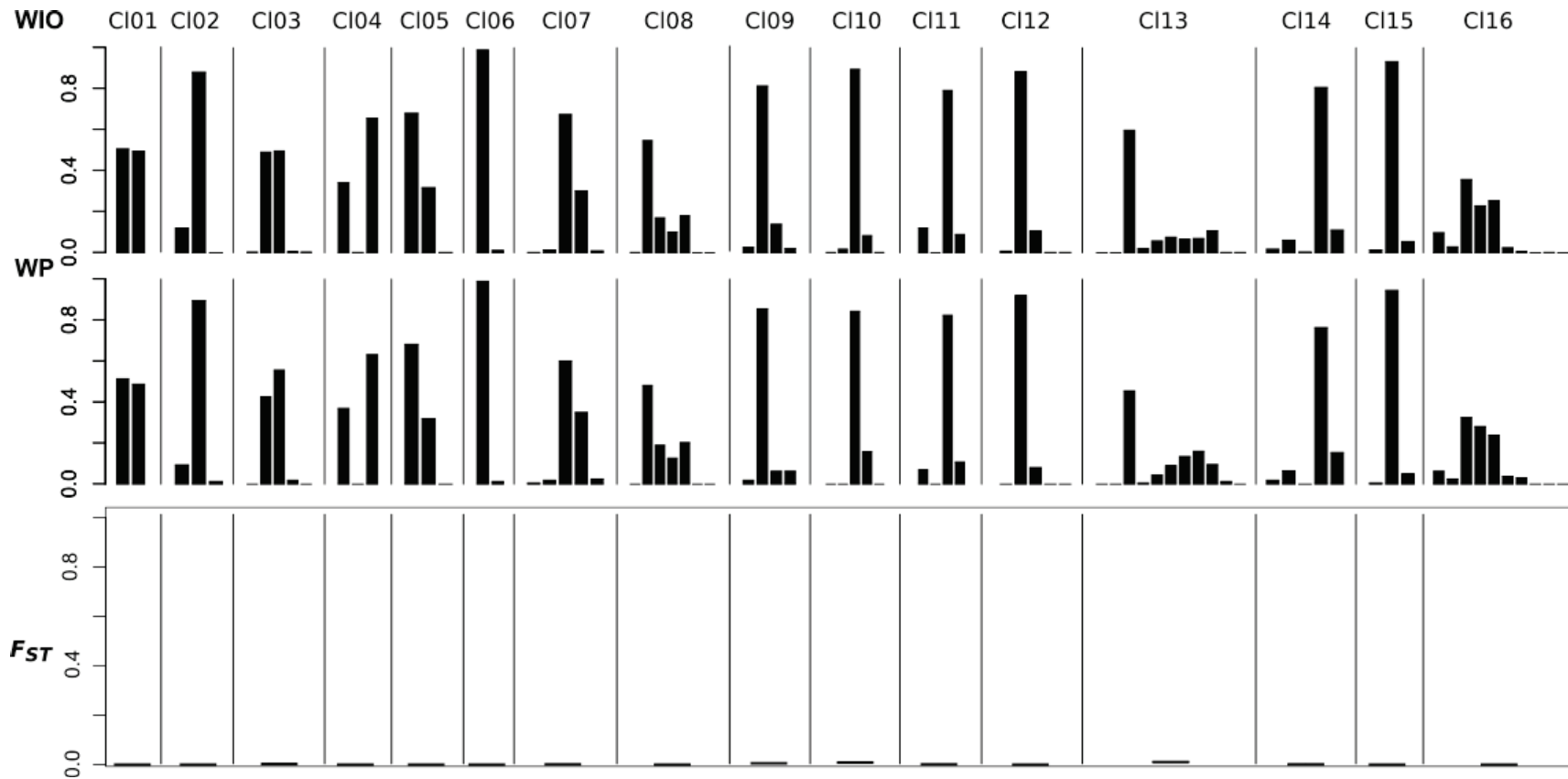
Haplotypes	WIO							WP			WA	TOT
	ZAN	SEY	MOZ	SAF	MAD	RUN	ROD	AUS1	AUS2	NCA	FLOR	
H01		11	6	10	2	3						32
H02	7	15	1		1							24
H03	2	4			2	2	1					11
H04		1	3	4	1							9
H05		1	1	2		4						8
H06					1	6						7
H07	2	1	1		1	1						6
H08		1	1	3		1						6
H09			2	1								3
H10			1			2						3
H11	2											2
H12				2								2
H13				2								2
H14		1										1
H15		1										1
H16			1									1
H17			1									1
H18				1								1
H19						15	5					20
H20						1						1
H21						1						1
H22						1						1
H23						1						1
H24								21	9			30
H25								1	5			6
H26										5		5
H27										2		2
H28								1				1
H29											10	10
H30											9	9
H31											4	4
H32											2	2
H33											2	2
H34											1	1
H35											1	1
H36											1	1
TOT	13	36	18	25	8	38	6	23	14	7	30	218

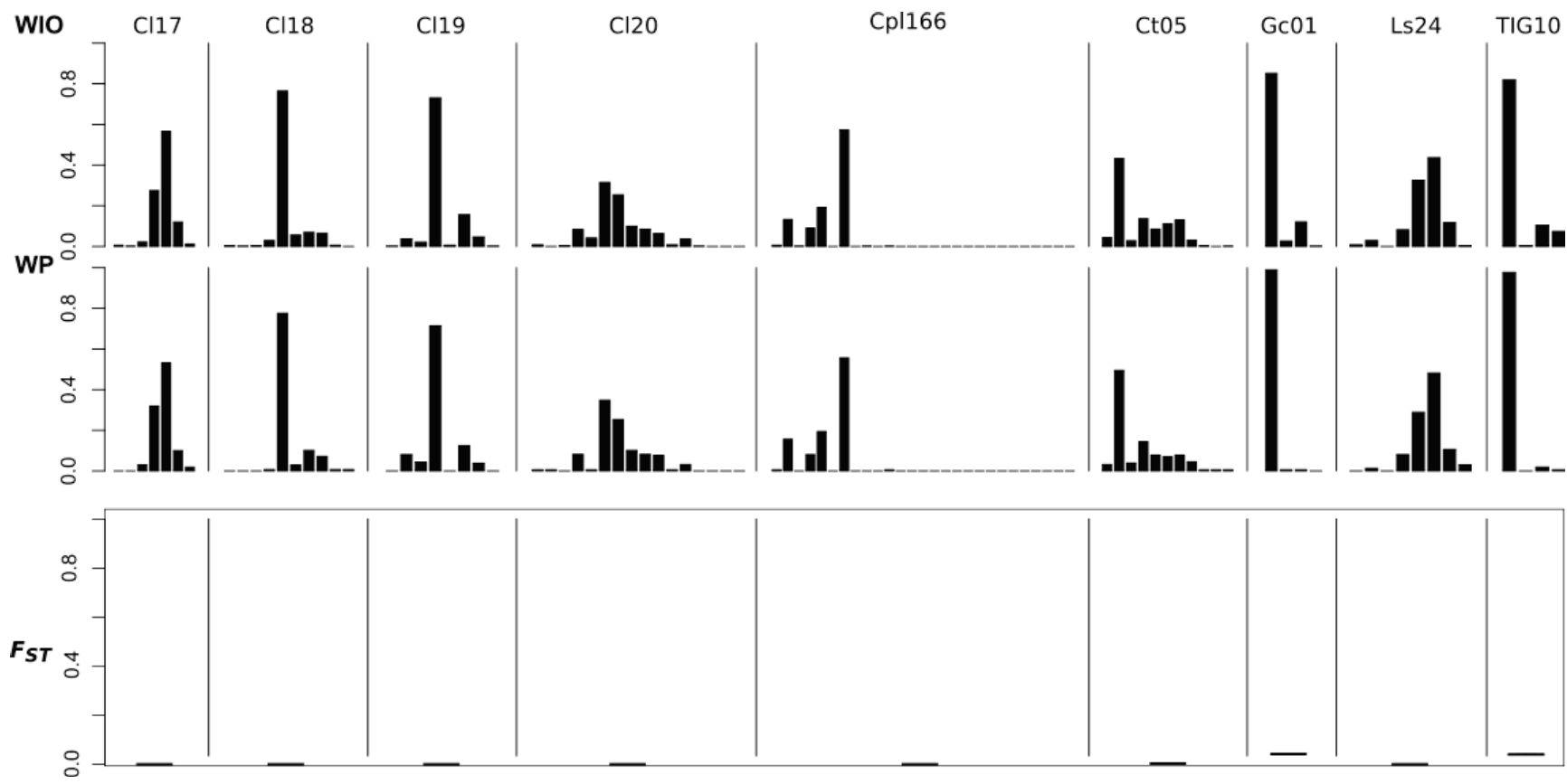
Supplementary Material S6. Average probability of membership (y axis) of *Carcharhinus leucas* individuals ($N = 357$, x axis) to putative clusters assuming correlated allele frequencies and admixture as performed by STRUCTURE. Major and minor modes as well as the number of times they were retrieved in the 10 replicates performed for each K (from 2 to 5) are presented for (a) 25 microsatellites and the LOCPRIOR model, (b) 25 microsatellites and the concatenated mitochondrial sequence *CR-nd4-cytb*, no *a priori* sampling location information, (c) 25 microsatellites and the concatenated mitochondrial sequence *CR-nd4-cytb*, with the LOCPRIOR model. ZAN, Zanzibar; SEY, Seychelles; MOZ, Mozambique; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; ROD, Rodrigues Island; AUS1, Clarence River, Australia; AUS2, Sydney Harbour, Australia; NCA, New Caledonia; FLO, Florida.





Supplementary Material S7. Allelic frequencies at the 25 microsatellite studied retrieved in the Western Indian Ocean (WIO) and the Western Pacific (WP) and global F_{ST} for each locus between these two regions





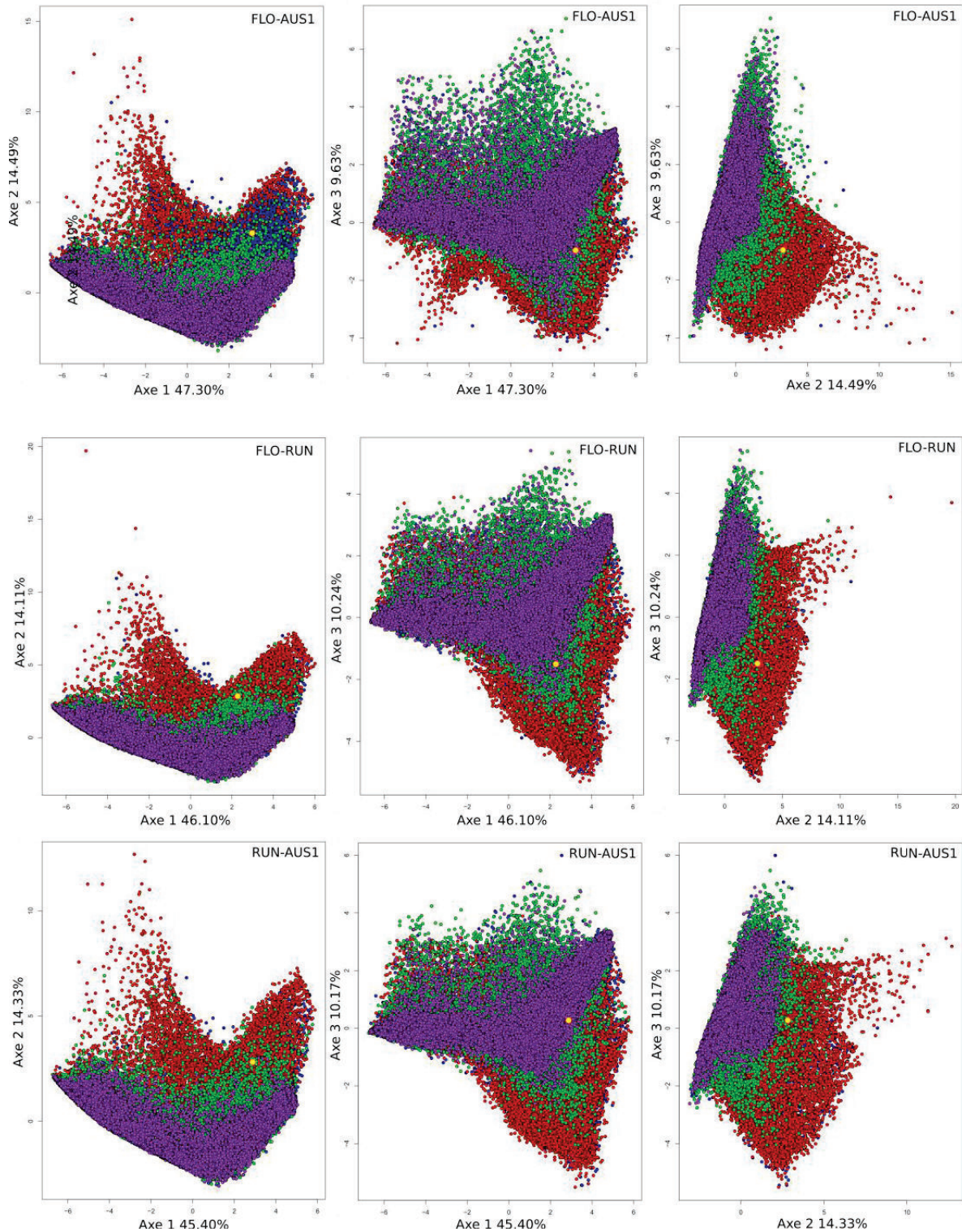
Supplementary Material S8. Analyses of molecular variance performed for 25 microsatellite loci and three mitochondrial loci concatenated (*CR-nd4-cytb*). **: $P < 0.01$; ***: $P < 0.001$.

Microsatellites		Variance components	Percentage of variation	Φ
Among genetic clusters (WIO/WP vs WA)	FCT	1.91	26.35	0.26***
Among sampling locations within genetic clusters	FSC	0.04	0.54	0.01***
Within sampling locations	FST	5.31	73.12	0.27***
Total		7.26		
<i>CR-nd4-cytb</i>		Variance components	Percentage of variation	Φ
Among genetic clusters (WIO1-WIO2-WP-WA)	FCT	7.59	81.61	0.82***
Among sampling locations within genetic clusters	FSC	0.16	1.68	0.09***
Within sampling locations	FST	1.55	16.71	0.83***
Total		9.3		

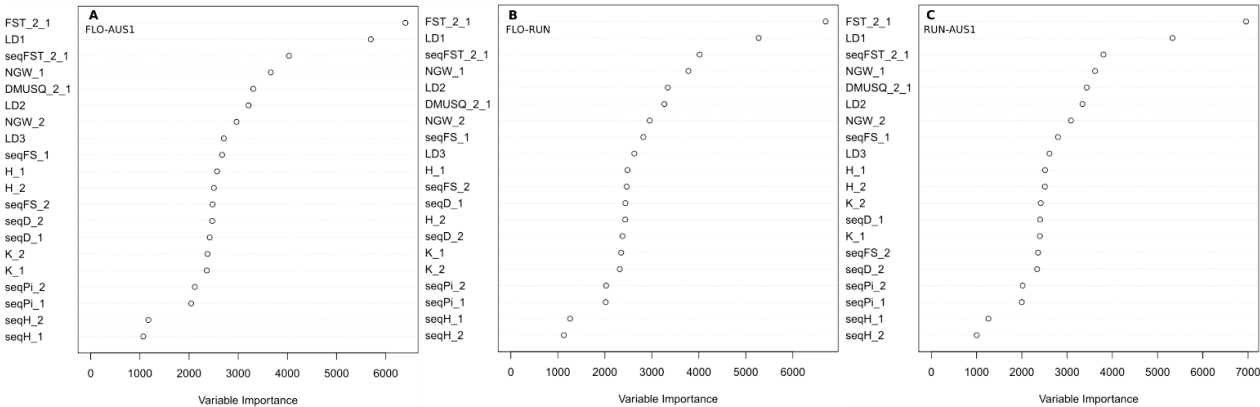
Supplementary Material S9. *Carcharhinus leucas* demography statistics (Tajima's D and Fu's F_S) for the concatenated mitochondrial sequence *CR-nd4-cytb* (ZAN, Zanzibar; SEY, Seychelles; MOZ, Mozambique; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; ROD, Rodrigues Island; AUS1, Clarence River, Australia; AUS2, Sydney Harbour, Australia; NCA, New Caledonia; FLO, Florida). All values were not significantly different from zero ($P > 0.05$).

	D	F_S
Western Indian Ocean	0.740	- 2.725
<i>African east coast (WIO1)</i>	0.575	- 2.235
ZAN	1.520	2.737
SEY	0.502	0.116
MOZ	0.089	- 2.181
MAD	1.880	- 0.355
SAF	1.059	0.623
<i>Mascarene Islands (WIO2)</i>	-0.261	- 1.409
RUN	-0.068	- 1.373
ROD	-0.933	- 0.003
Western Pacific	-0.352	0.816
AUS1	-1.821	0.244
AUS2	1.753	3.641
NCA	0.826	3.754
Western Atlantic		
FLO	0.020	0.402

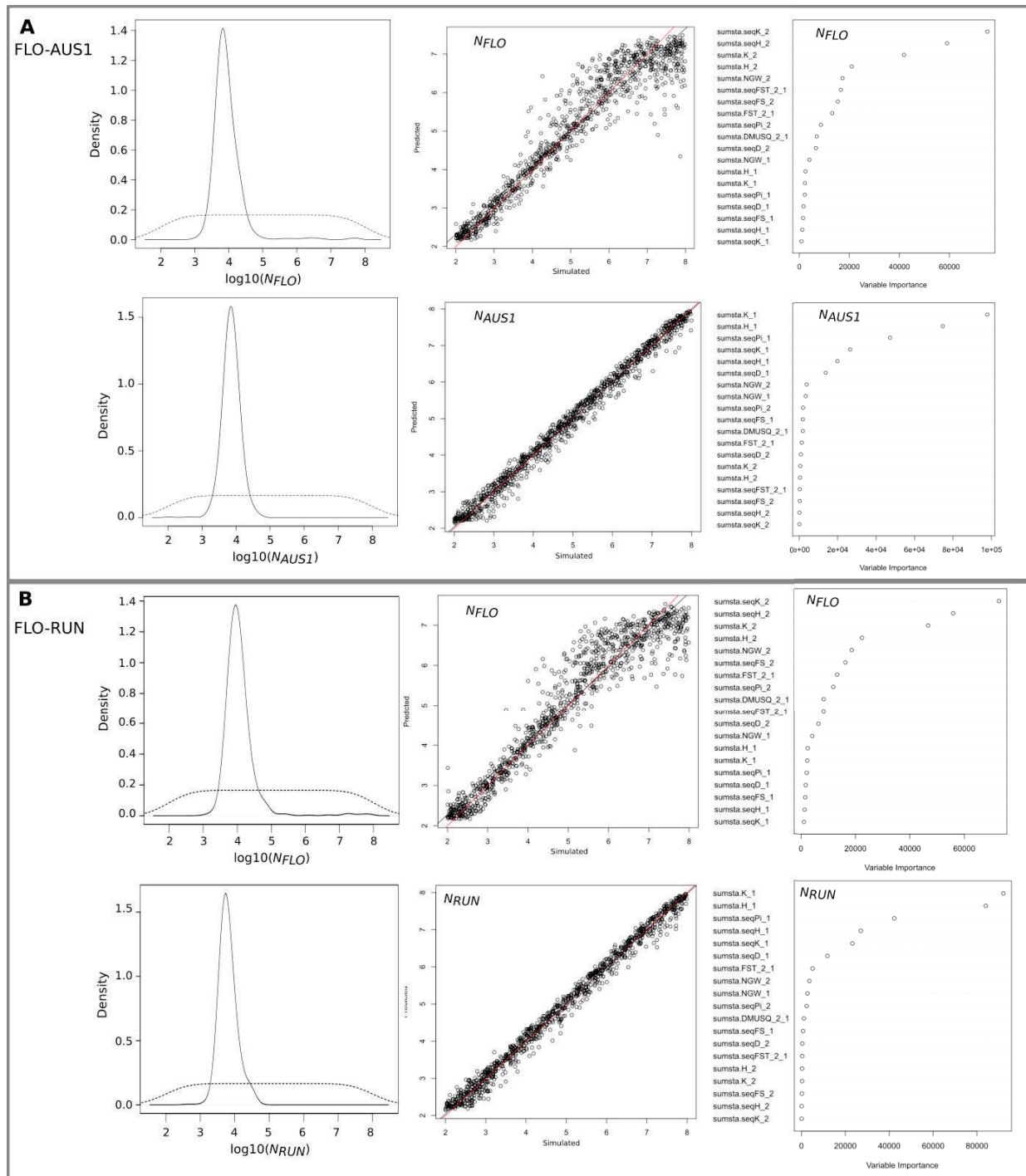
Supplementary Material S10. Principal Component Analyses of summary statistics simulated under the four demographic models for each pair of bull shark populations: FLO-AUS1, FLO-RUN and RUN-AUS1. In purple are simulations of Scenario 1; in blue, simulations of Scenario 2; in red, simulations of Scenario 3 and in green, simulations of Scenario 4. The observed dataset is represented by the yellow point.

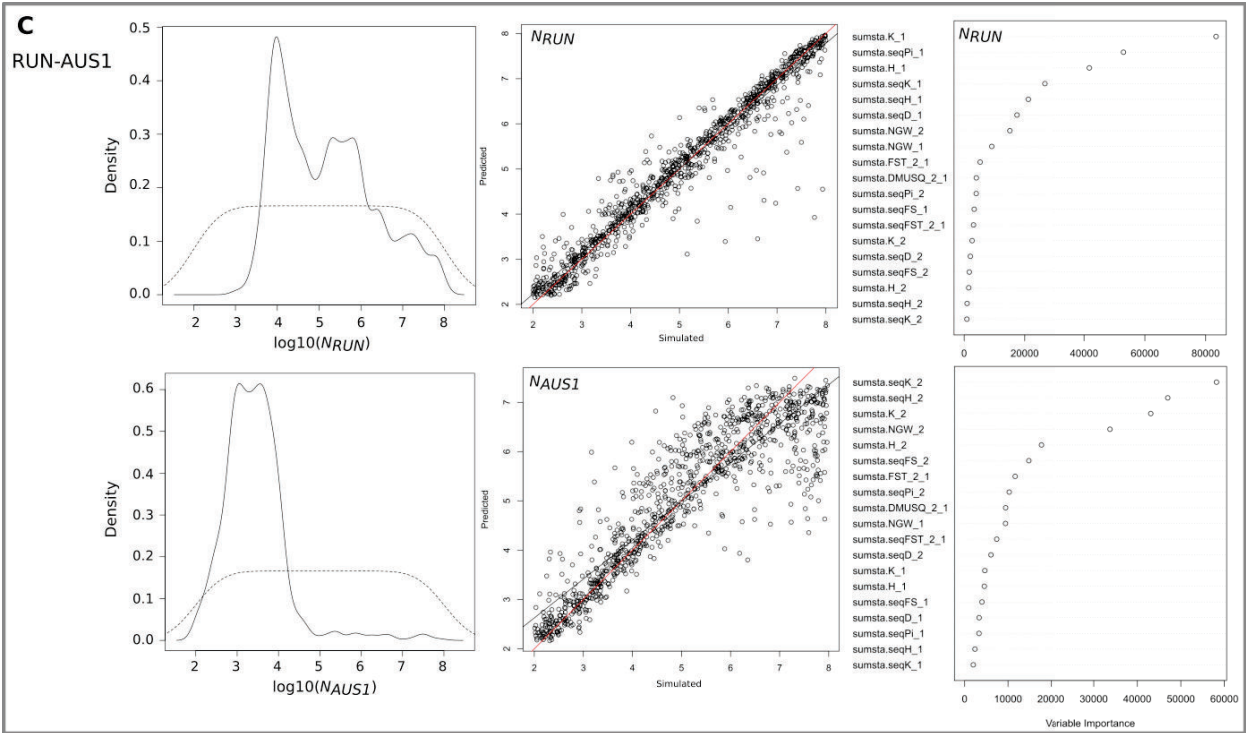


Supplementary Material S11. Contribution of each summary statistics to model choice for each pair of bull shark populations (A: FLO-AUS1, B: FLO-RUN and C: RUN-AUS1).



Supplementary Material S12. Effective population size estimates under the best scenario for each pair of populations (A: FLO-AUS1, B: FLO-RUN and C: RUN-AUS1). Left column: Prior (dotted line) and posterior (plain line) distributions of the effective population size of the contemporary populations for each pair of populations studied. Central column: Correlation between effective population sizes simulated in the 1,000 pseudo-observed datasets and their corresponding estimates using the fitted model. Right column: Contribution of each summary statistics to parameter estimation.





Supplementary Material S13. Characteristics of posterior distributions estimated with ABC-RF for all parameters studied and each pair of populations investigated. *NMSE*, normalized mean squared error; *NMAE*, normalized mean absolute error; *CI*, 95% confidence interval; $t_{composite} = t_1/\text{mean}(N_1+N_2)$.

FLO_AUS1	Expectation	Median	Variance	2.5% quantile	97.5% quantile	Out of bag Mean Square Error	NMSE	NMAE	Mean relative CI	Median relative CI
$\log_{10}(N_{AUS1})$	3.89 (0.01)	3.87 (0.01)	0.03 (0.00)	3.49 (0.01)	4.43 (0.02)	0.04 (0.00)	0.01	0.04	0.19	0.16
$\log_{10}(N_{FLO})$	3.98 (0.01)	3.89 (0.01)	0.10 (0.01)	3.45 (0.01)	5.09 (0.13)	0.27 (0.00)	0.05	0.07	0.35	0.32
$\log_{10}(N_{anc})$	5.01 (0.06)	5.01 (0.11)	3.35 (0.07)	2.15 (0.01)	7.86 (0.01)	3.02 (0.00)	0.78	0.37	1.32	1.17
t_2 (Mya)	3.01 (0.43)	2.97 (0.06)	6,088.14 (225.52)	1.15 (0.04)	5.03 (0.03)	2,652.32 (77.96)	117191.75	3.02	18.09	3.72
μ_{seq} ($\times 10^{-7}$)	1.13 (0.02)	0.85 (0.02)	6.08×10^{-8} (2.29×10^{-9})	0.16 (5.24×10^{-3})	3.98 (0.15)	3.37×10^{-7} (4.15×10^{-10})	0.00	0.48	2.16	1.45
μ_{sat} ($\times 10^{-5}$)	7.49 (0.08)	7.86 (0.11)	3.20×10^{-5} (8.04×10^{-7})	3.40 (0.13)	9.89 (0.01)	3.85×10^{-5} (5.42×10^{-8})	0.00	0.40	1.70	1.35
$\log_{10}(\theta_{sat_{FLO}})$	-1.82 (0.01)	-1.84 (0.00)	0.03 (0.00)	-2.18 (0.01)	-1.06 (0.10)	0.07 (0.00)	-0.59	-0.97	-2.55	-0.63
$\log_{10}(\theta_{sat_{AUS1}})$	-1.87 (0.00)	-1.85 (0.00)	0.01 (0.00)	-2.19 (0.02)	-1.69 (0.01)	0.01 (0.00)	-0.01	-0.07	-0.40	-0.28
$\log_{10}(\theta_{sat_{ANC}})$	-1.36 (0.03)	-1.35 (0.05)	0.68 (0.03)	-2.68 (0.02)	-0.07 (0.01)	0.6 (0.00)	-1.81	-1.49	-3.89	-1.64
$\log_{10}(\theta_{seq_{FLO}})$	-5.29 (0.01)	-5.32 (0.01)	0.07 (0.01)	-5.87 (0.01)	-4.38 (0.12)	0.07 (0.00)	-0.02	-0.05	-0.27	-0.25
$\log_{10}(\theta_{seq_{AUS1}})$	-5.6 (0.01)	-5.58 (0.01)	0.04 (0.00)	-6.17 (0.01)	-5.04 (0.01)	0.02 (0.00)	0.00	-0.03	-0.15	-0.14
$\log_{10}(\theta_{seq_{ANC}})$	-4.95 (0.04)	-4.99 (0.05)	0.73 (0.03)	-6.54 (0.04)	-3.36 (0.02)	0.61 (0.00)	-0.15	-0.16	-0.66	-0.63
$t_{composite}$	204.27 (2.75)	200.27 (4.02)	4,300.43 (144.61)	79.29 (3.35)	340.88 (1.62)	1,820.01 (5.36)	117.06	3.00	18.17	3.69
$\log_{10}(t_{composite})$	2.22 (0.00)	2.26 (0.01)	0.03 (0.00)	1.63 (0.04)	2.53 (0.00)	0.14 (0.00)	-0.10	-0.55	-4.11	0.79
FLO_RUN										
$\log_{10}(N_{RUN})$	3.78 (0.01)	3.75 (0.01)	0.04 (0.00)	3.44 (0.00)	4.33 (0.03)	0.04 (0.00)	0.01	0.04	0.19	0.16
$\log_{10}(N_{FLO})$	4.03 (0.01)	3.99 (0.01)	0.06 (0.00)	3.63 (0.01)	4.67 (0.04)	0.27 (0.00)	0.05	0.07	0.36	0.32
$\log_{10}(N_{anc})$	5.08 (0.07)	5.13 (0.11)	3.21 (0.04)	2.16 (0.01)	7.85 (0.02)	3.02 (0.00)	0.80	0.38	1.34	1.17

<i>t</i> ₂ (Mya)	3.04 (0.03)	2.97 (0.05)	6,280.04 (290.08)	1.13 (0.04)	5.03 (0.02)	2,551.43 (528.89)	97,529. 81	2.78	17.96	3.71
<i>μ</i> _{seq} (x10 ⁻⁷)	1.28 (0.02)	1.02 (0.02)	6.19x10 ⁻⁸ (2.14x10 ⁻⁹)	0.21 (0.01)	4.03 (0.13)	3.30x10 ⁻⁷ (4.37x10 ⁻¹⁰)	0.00	0.46	2.18	1.48
<i>μ</i> _{sat} (x10 ⁻⁵)	7.48 (0.07)	7.79 (0.09)	3.18x10 ⁻⁵ (9.24x10 ⁻⁷)	3.46 (0.10)	9.87 (0.01)	3.78x10 ⁻⁵ (4.11x10 ⁻⁸)	0.00	0.39	1.67	1.32
log10(θ_{sat}_{FLO})	-1.79 (0.00)	-1.79 (0.00)	0.02 (0.00)	-2.08 (0.01)	-1.41 (0.04)	0.07 (0.00)	-0.45	-1.53	-5.64	-0.60
log10(θ_{sat}_{RUN})	-1.93 (0.00)	-1.90 (0.00)	0.01 (0.00)	-2.22 (0.01)	-1.77 (0.00)	0.01 (0.00)	-0.01	-0.07	-0.36	-0.27
log10(θ_{sat}_{ANC})	-1.28 (0.03)	-1.22 (0.05)	0.63 (0.02)	-2.64 (0.02)	-0.07 (0.01)	0.60 (0.00)	-0.62	-0.69	-2.46	-1.65
log10(θ_{seq}_{FLO})	-5.29 (0.02)	-5.31 (0.01)	0.05 (0.00)	-5.81 (0.02)	-4.57 (0.09)	0.07 (0.00)	-0.02	-0.05	-0.26	-0.24
log10(θ_{seq}_{RUN})	-5.20 (0.01)	-5.19 (0.01)	0.03 (0.00)	-5.69 (0.02)	-4.79 (0.01)	0.02 (0.00)	0.00	-0.02	-0.14	-0.14
log10(θ_{seq}_{ANC})	-4.81 (0.02)	-4.81 (0.03)	0.65 (0.01)	-6.34 (0.04)	-3.30 (0.02)	0.61 (0.00)	-0.15	-0.16	-0.65	-0.63
<i>t</i> _{composite}	202.82 (2.55)	195.88 (3.34)	4,232.42 (199.54)	70.88 (2.20)	339.68 (1.74)	1,746.93 (4.12)	97.63	2.76	17.86	3.75
log10(<i>t</i>_{composite})	2.20 (0.01)	2.26 (0.01)	0.03 (0.00)	1.52 (0.03)	2.53 (0.00)	0.13 (0.00)	-1.22	-1.02	-1.75	0.86
RUN_AUS1										
log10(<i>N</i>_{RUN})	5.25 (0.02)	5.15 (0.05)	0.56 (0.03)	3.6 (0.02)	7.74 (0.04)	0.20 (0.00)	0.04	0.06	0.35	0.24
log10(<i>N</i>_{AUS1})	3.61 (0.03)	3.58 (0.03)	0.40 (0.01)	2.22 (0.04)	6.39 (0.16)	0.56 (0.00)	0.10	0.10	0.52	0.42
log10(<i>N</i>_{anc})	4.94 (0.04)	4.89 (0.07)	3.31 (0.09)	2.12 (0.01)	7.87 (0.01)	3.05 (0.00)	0.75	0.37	1.31	1.15
<i>t</i> ₁ (Mya)	0.07 (0.00)	0.02 (0.00)	1,135.73 (232.18)	0.00 (0.00)	0.52 (0.04)	1,770.61 (11.39)	83,459. 14	32.57	239.92	8.63
<i>t</i> ₂ (Mya)	0.97 (0.03)	0.25 (0.01)	124,351.5 (5,308.75)	0.01 (0.00)	4.68 (0.04)	65,182.5 (161.94)	788,86 3.03	17.47	119.17	14.97
<i>μ</i> _{seq} (x10 ⁻⁷)	1.40 (0.06)	0.92 (0.05)	1.23x10 ⁻⁷ (1.26x10 ⁻⁸)	0.14 (0.01)	5.75 (0.30)	3.54x10 ⁻⁷ (4.26x10 ⁻¹⁰)	0.00	0.51	2.31	1.49
<i>μ</i> _{sat} (x10 ⁻⁵)	6.40 (0.06)	6.73 (0.10)	6.11x10 ⁻⁵ (1.09x10 ⁻⁶)	1.53 (0.06)	9.87 (0.01)	4.05x10 ⁻⁵ (7.47x10 ⁻⁸)	0.00	0.43	1.77	1.37
<i>m</i> _{RUN-AUS1}	0.103 (0.002)	0.105 (0.003)	0.003 (0.000)	0.006 (0.001)	0.195 (0.001)	0.003 (0.000)	0.14	1.89	5.30	1.86
<i>m</i> _{AUS1-RUN}	0.098 (0.001)	0.096 (0.003)	0.003 (0.000)	0.005 (0.001)	0.196 (0.000)	0.003 (0.000)	0.13	1.63	4.54	1.82

log10($\theta_{sat_{AUSI}}$)	-2.02 (0.01)	-2.03 (0.01)	0.09 (0.00)	-2.75 (0.03)	-0.74 (0.05)	0.12 (0.00)	-0.14	-0.24	-1.21	-0.96
log10($\theta_{sat_{RUN}}$)	-1.35 (0.02)	-1.40 (0.03)	0.13 (0.01)	-2.18 (0.03)	-0.22 (0.03)	0.05 (0.00)	-0.05	-0.14	-0.71	-0.44
log10($\theta_{sat_{ANC}}$)	-1.47 (0.02)	-1.49 (0.03)	0.69 (0.01)	-2.87 (0.02)	-0.09 (0.01)	0.61 (0.00)	-0.66	-0.71	-2.50	-1.69
log10($\theta_{seq_{AUSI}}$)	-5.57 (0.01)	-5.59 (0.01)	0.13 (0.00)	-6.4 (0.02)	-4.43 (0.06)	0.13 (0.00)	-0.04	-0.07	-0.34	-0.35
log10($\theta_{seq_{RUN}}$)	-4.64 (0.03)	-4.82 (0.04)	0.22 (0.01)	-5.75 (0.03)	-3.08 (0.04)	0.06 (0.00)	-0.01	-0.04	-0.22	-0.17
log10($\theta_{seq_{ANC}}$)	-5.00 (0.04)	-5.02 (0.05)	0.74 (0.02)	-6.63 (0.04)	-3.33 (0.04)	0.62 (0.00)	-0.15	-0.17	-0.67	-0.64
log10($N_{msat_{RUN}}$)	-2.47 (0.02)	-2.45 (0.03)	0.27 (0.01)	-4.11 (0.06)	-1.14 (0.02)	0.22 (0.00)	-0.08	-0.14	-0.77	-0.69
log10($N_{msat_{AUSI}}$)	-3.33 (0.01)	-3.28 (0.01)	0.28 (0.01)	-4.63 (0.05)	-2.16 (0.12)	0.32 (0.00)	-0.16	-0.20	-0.99	-0.91
log10($N_{mseq_{RUN}}$)	-5.60 (0.02)	-5.58 (0.02)	0.35 (0.01)	-7.43 (0.05)	-4.01 (0.04)	0.24 (0.00)	-0.04	-0.07	-0.38	-0.36
log10($N_{mseq_{AUSI}}$)	-6.72 (0.02)	-6.66 (0.02)	0.31 (0.01)	-8.1 (0.05)	-5.62 (0.03)	0.33 (0.00)	-0.07	-0.09	-0.45	-0.45
$t_{composite}$	0.00 (0.00)	0.00 (0.00)	0.12 (0.01)	0.00 (0.00)	0.00 (0.00)	0.07 (0.00)	802.81	17.50	119.29	15.02
log10 ($t_{composite}$)	0.88 (0.02)	0.76 (0.03)	0.60 (0.01)	-0.53 (0.01)	2.47 (0.01)	0.45 (0.00)	-0.56	-0.45	-0.40	1.36

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CHAPITRE 3. Modes de reproduction du requin bouledogue

Carcharhinus leucas

Résumé

Le requin bouledogue *Carcharhinus leucas* est un grand requin de la famille des Carcharhinidés. Il présente la spécificité d'être euryhalin, utilisant les estuaires, les fleuves ou les lacs comme nurseries. Ce prédateur apical joue ainsi un rôle important dans les écosystèmes côtiers, où il interagit avec l'Homme, et est parfois responsable d'attaques fatales. Ainsi, cette espèce est souvent ciblée par des programmes de régulation des populations de requins, ainsi que par les pêcheries, commerciales ou sportives. Malgré ces nombreuses interactions avec l'Homme, les données sur la biologie de la reproduction de cette espèce restent rares. Ici, nous avons étudié les modes de reproduction de ce requin en deux temps. Dans un premier temps, nous avons utilisé les marqueurs microsatellites spécifiques développés pour cette espèce afin d'estimer la présence de polyandrie chez quatre portées pêchées le long de la côte Ouest de La Réunion (Ouest de l'océan Indien). Ce comportement a été ainsi mis en évidence dans deux de ces portées. Ces résultats sont publiés dans *Marine and Freshwater Research*.

Dans un second temps, nous avons analysé certains paramètres reproducteurs de 118 requins bouledogue pêchés le long de la côte Ouest de La Réunion (Ouest de l'océan Indien), comprenant 16 femelles gravides. Nous avons également estimé la fréquence de polyandrie dans ces 16 portées, ce qui nous a permis d'établir l'image la plus complète à ce jour des comportements reproducteurs du requin bouledogue. Les mâles et les femelles atteignent la maturité sexuelle pour des tailles d'environ 234 cm et 257 cm (longueur totale), respectivement. L'analyse des 16 portées, comportant 5 à 14 embryons par portée, et qui ont été collectées à divers mois de l'année, indique que la parturition a lieu entre octobre et décembre, et que la taille à la naissance est de 60-80 cm. La gestation dure probablement 12 mois, ce qui, avec une période de repos d'un an et un stockage du sperme d'environ 4-5 mois entre l'accouplement (en juin-septembre) et la fécondation, mène à un cycle de reproduction bisannuel. Enfin, au moins 56,25 % des portées étaient polyandres, provenant de 2 à 5 pères différents. Plusieurs mâles ont été identifiés comme pères de différentes portées, conçues soit lors d'une même saison de reproduction, soit lors de saisons de reproduction différentes. Cela suggère à la fois des agrégations de requins bouledogue pendant la saison d'accouplement et de la philopatrie des mâles aux sites d'accouplements. Ces résultats représentent des informations importantes pour la gestion durable des populations de requin bouledogue de l'Ouest de l'océan Indien.

Cette publication a été soumise à *Plos One*.

1. Polyandrie chez le requin bouledogue *Carcharhinus leucas*

Résumé

Le requin bouledogue *Carcharhinus leucas* est un grand requin de la famille des Carcharhinidés. Il présente la spécificité d'être euryhalin, utilisant les estuaires, les fleuves ou les lacs comme nurseries. Ce prédateur apical joue ainsi un rôle important dans les écosystèmes côtiers, où il interagit avec l'Homme, et est parfois responsable d'attaques fatales. Ainsi, cette espèce est souvent ciblée par des programmes de régulation des populations de requins, ainsi que par les pêcheries, commerciales ou sportives. Malgré ces nombreuses interactions avec l'Homme, les données sur la biologie de la reproduction de cette espèce restent rares.

Nous avons utilisé les marqueurs microsatellites spécifiques développés pour cette espèce afin d'estimer la présence de polyandrie chez quatre portées pêchées le long de la côte Ouest de La Réunion (Ouest de l'océan Indien). Ce comportement a été ainsi mis en évidence dans deux de ces portées.

Ces résultats sont publiés dans *Marine and Freshwater Research*.

First evidence of multiple paternity in the bull shark (*Carcharhinus leucas*)

Agathe Pirog^A, Sébastien Jaquetmet^{A,B}, Marc Soria^C and Hélène Magalon^{A,B,D}

^AUniversité de La Réunion, UMR 9220 ENTROPIE (Université de La Réunion/IRD/CNRS), 15 Avenue René Cassin, CS 92003, F-97744 Saint Denis Cedex 09, La Réunion, France.

^BLaboratory of Excellence CORAIL, 58, Avenue Paul Alduy, F-66860 Perpignan Cedex, France.

^CIRD Réunion, UMR 248 MARBEC, CS 41095 2 rue Joseph Wetzell, F-97492 Sainte-Clotilde, La Réunion, France.

^DCorresponding author. Email: helene.magalon@univ-reunion.fr

Abstract. The present study assessed the occurrence of multiple paternity in four litters of bull shark *Carcharhinus leucas* ($n = 5, 8, 9$ and 11 embryos) sampled at Reunion Island in the Western Indian Ocean. Using 21 microsatellite loci, we revealed that two litters were generated from two sires each, demonstrating for the first time multiple paternity for this species. We also reported a high paternal skew ($10:1$ in Litter 1 and $7:1$ in Litter 3), which may be because of post-copulatory or post-zygotic selection processes. These results contribute to a better understanding of the reproductive behaviour of the bull shark, which remains poorly documented. The present study must be expanded to assess the frequency of multiple paternity in this species, and to test for genetic or cryptic benefits (convenience polyandry), which is important for long-term conservation and management plans.

Additional keywords: microsatellite, paternity test, polyandry.

Received 6 July 2015, accepted 24 August 2015, published online 6 November 2015

Introduction

Compared with teleosts, elasmobranchs are usually characterised by low fecundity, low growth rates and late sexual maturity (Myers *et al.* 1999; Frisk *et al.* 2001; Dulvy *et al.* 2008) and are therefore very sensitive to overexploitation (Musick *et al.* 2000; Ferretti *et al.* 2010; Dulvy *et al.* 2014). Their populations are also slow to rebound from depletion (Smith *et al.* 1998; Myers and Worm 2005). Consequently, knowledge of their reproductive behaviour is important for developing management and conservation plans (Neff and Pitcher 2002; Rowe and Hutchings 2003). A wide variety of reproductive systems in elasmobranchs has been described, ranging from viviparity to oviparity (Compagno 1990, 2001; Conrath *et al.* 2012), and their mating systems are similarly diverse, with monoandrous and polyandrous species, as well as parthenogenesis being recorded occasionally for captive individuals (Chapman *et al.* 2007, 2008; Portnoy *et al.* 2014). Overall, mating systems in sharks remain poorly documented, because they are difficult to study. Although direct observations suggested multiple matings in the nurse shark (*Ginglymostoma cirratum*) (Pratt and Carrier 2001) and in the whitetip reef shark (*Triaenodon obesus*) (Whitney *et al.* 2004), only genetic tools can confirm multiple paternity, identifying full and half-siblings in the same litter. To date, genetic studies have shown multiple paternity in several elasmobranch species, including the small-spotted cat shark (*Scyliorhinus canicula*) (Griffiths *et al.* 2012), lemon shark

(*Negaprion brevirostris*), sandbar shark (*Carcharhinus plumbeus*), bonnet head shark (*Sphyrna tiburo*) and brown smooth hound (*Mustelus henlei*) (for a review, see Byrne and Avise 2012). Although multiple paternity is common in sharks, variability is observed at both inter- and intraspecific scales (Chapman *et al.* 2004; Feldheim *et al.* 2004; Daly-Engel *et al.* 2007; Portnoy *et al.* 2007; DiBattista *et al.* 2008b; Chabot and Haggin 2014) and several hypotheses have been proposed to explain this process, such as indirect benefits or convenience polyandry (Zeh and Zeh 2001; Griffiths *et al.* 2012).

The bull shark (*Carcharhinus leucas*) is a large shark species (up to 3.4 m long) that is widespread in tropical and subtropical coastal waters. Its diet is very diverse, including high trophic-level prey, which suggests that bull sharks are apex predators playing an important role in structuring marine communities (Daly *et al.* 2013) through top-down processes (Myers *et al.* 2007; Heithaus *et al.* 2008). Typical of most elasmobranchs, bull sharks have a long lifespan, around 39 years (Wintner *et al.* 2002), and delayed sexual maturity, estimated between 14 and 18 years (Branstetter and Stiles 1987). Thus, bull sharks are sensitive to both recreational and commercial fishing pressures to which they are subjected and, as a result, are classified as Near Threatened in the International Union of Conservation for Nature's Red List (Simpfendorfer and Burgess 2009; Worm *et al.* 2013). This species exhibits placental viviparity, and each embryo is initially dependent on a yolk sac, which elongates and

attaches to the uterine wall of the mother after a few weeks, forming a yolk sac placenta. For most of gestation, the mother provides nutrients to the embryo via this yolk sac placenta (Jenson 1976; Compagno 1984; Parsons *et al.* 2008). Litter sizes range from one to 13 embryos (Compagno 1984), with most litters between six and eight (Pattillo *et al.* 1997). Recently Nevill *et al.* (2014) reported a litter size of 14 late stage embryos in the Seychelles, which represents the largest litter size recorded to date for *C. leucas*. No sperm storage in the oviductal gland has been identified in this species yet, but it has been demonstrated in other carcharhinid species (Pratt and Carrier 2001; Parsons *et al.* 2008; Fitzpatrick *et al.* 2012). The bull shark is euryhaline and is able to inhabit both marine and freshwater habitats, including lakes, rivers and estuaries (Compagno 1984). The ability of bull sharks to penetrate freshwater seems mostly related to reproduction, when pregnant females migrate to estuarine areas to give birth (Castro 2011). Despite their ability to travel long distances (Kohler and Turner 2001; Simpfendorfer *et al.* 2005; Daly *et al.* 2013; Heupel *et al.* 2015), several studies have highlighted site fidelity in bull shark populations of Florida (Hueter *et al.* 2005; Karl *et al.* 2011), Australia (Tillett *et al.* 2012) and Fiji (Brunnschweiler and Baensch 2011), and even female reproductive philopatry (Karl *et al.* 2011). Nevertheless, to our knowledge, polyandry has never been demonstrated for the species.

Herein, using 20 microsatellite loci newly developed for this species (Pirog *et al.* 2015) and one microsatellite locus developed for the tiger shark *Galeocerdo cuvier* that cross-amplifies in the bull shark (A. Pirog, unpubl. data), we document the occurrence of multiple paternity in four litters of bull sharks from Reunion Island. Possible implications of this polyandry for population dynamics and management of the species are discussed.

Materials and methods

Samples were collected from four gravid bull shark females, as well as from unborn pups contained in whole uteri. These specimens were caught by local fishermen between June 2013 and June 2015 on the west coast of Reunion Island (21°6'S, 55°36'E). Samples consisted of a piece of muscle biopsied on the mother and on each pup ($n = 11, 5, 8$ and 9 pups per brood) and preserved in 95% ethanol. Total length (TL) was measured for each individual and the sex of each pup was recorded.

Total genomic DNA was extracted from small pieces of tissues using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). Genotyping was performed using the 20 microsatellite loci recently developed for *C. leucas* (Pirog *et al.* 2015). Moreover, we tested microsatellite loci developed for other shark species and that cross-amplified in the bull shark, namely five developed for *Carcharhinus limbatus* (Cli-007, Cli-106, Cli-107, Cli-108 and Cli-112; Keeney and Heist 2003) and one developed for *G. cuvier* (Gc01; A. Pirog, unpubl. data). Polymerase chain reactions (PCR) were performed following the conditions published by Keeney and Heist (2003) for the five loci isolated from *C. limbatus* (Cli) and Pirog *et al.* (2015) for the others. Amplicons were run on an ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA, USA) and allele size was determined using Genemapper v 4.0 (Applied Biosystems, Foster City, CA, USA).

The five loci isolated from *C. limbatus* were not used in further analyses because of important stuttering bands and potential scoring errors. The other microsatellite loci amplified were polymorphic for a population of bull shark from Reunion Island ($n = 41$). In all, 21 microsatellite loci were used in subsequent analyses.

The probability of detecting multiple paternity in a litter depends on the polymorphism of the microsatellite loci used (allele frequencies), the number of loci, the number of offspring and putative fathers. This probability was calculated using PrDM (Neff and Pitcher 2002) with six different scenarios. These scenarios were defined according to the number of pups observed in the present study ($n = 5-11$) and the number of fathers recorded in other shark species ($n = 2-4$) with and without paternal skew (number of embryos within a litter sired by each male). Multiple paternity was considered when more than two non-maternal alleles were found in the brood for at least two microsatellite loci. Moreover, full and half-siblings in the litter and putative fathers were inferred using Colony ver. 2.0.4.5 software (Jones and Wang 2010) and a maximum likelihood approach. A polygamous mating system was assumed for both sexes to allow the assignment of half-siblings. A long-run with medium likelihood precision and a genotyping error rate of 1% was performed.

Results

In the four litters studied, the number of embryos ranged from 5 to 11 and the sex ratio was biased towards female predominance (M : F = 5 : 6; 1 : 4; 2 : 6 and 3 : 6). In each litter, all pups were approximately the same size (Table 1), but the mean size of pups from each litter differed significantly (all $P < 0.05$, pairwise Wilcoxon test with Bonferroni correction), except between Litters 1 and 4 ($P = 0.17$, pairwise Wilcoxon test with Bonferroni correction). This may be due to the different gestation stages at which each female was caught and may reflect seasonality in the reproduction of the bull shark. Indeed, it is of note that the mother of Litter 2, with the smallest embryos (as well as the smallest number of embryos), was caught in April, the mothers of Litters 1 and 4 were both caught at the beginning of June, and the mother of Litter 3, with the biggest embryos, was caught at the end of August.

The probability of detecting multiple paternity was high (ranging from 0.75 to 1 according to the number of sires) when broods were comprised of more than eight embryos (Table 2). When broods were comprised of five embryos sired by two males, this probability was lower, especially with paternal skews (0.55 to 0.72; Table 2). Consequently it is possible that for litters with few embryos the microsatellite loci used in the present study may have led to underestimation of the number of sires.

For two litters, more than two paternal alleles were observed for at least two microsatellite loci (Table 3), with three to four paternal alleles recorded. Therefore, at least two fathers were identified for Litters 1 and 3. For Litters 2 and 4, only one sire was identified, because the criterion of more than two paternal alleles was not recovered for any of the loci. Nevertheless, for Litter 2, it is possible that the number of fathers was underestimated because of the low number of embryos and the

Table 1. Characteristics of the four litters of bull shark (*Carcharhinus leucas*) studied from Reunion Island

The mean total length (TL) \pm s.e. is given for each litter (mother not included). Data for mothers are shown in bold

	Date of capture	Sex	TL (cm)
Litter 1			
CIRUN100	06/06/2013	F	285
CIRUN101	06/06/2013	F	48
CIRUN102	06/06/2013	M	47
CIRUN103	06/06/2013	M	48
CIRUN104	06/06/2013	F	45
CIRUN105	06/06/2013	M	45
CIRUN106	06/06/2013	M	50
CIRUN107	06/06/2013	F	46
CIRUN108	06/06/2013	F	46
CIRUN109	06/06/2013	F	46
CIRUN110	06/06/2013	M	48
CIRUN111	06/06/2013	F	46
Mean \pm s.e.			46.8 \pm 0.5
Litter 2			
CIRUN200	28/04/2014	F	301
CIRUN201	28/04/2014	M	35
CIRUN202	28/04/2014	F	36
CIRUN203	28/04/2014	F	32
CIRUN204	28/04/2014	F	32
CIRUN205	28/04/2014	F	32
Mean \pm s.e.			33.4 \pm 0.9
Litter 3			
CIRUN300	26/08/2014	F	295
CIRUN301	26/08/2014	M	66
CIRUN302	26/08/2014	F	66
CIRUN303	26/08/2014	F	64
CIRUN304	26/08/2014	F	67
CIRUN305	26/08/2014	F	67
CIRUN306	26/08/2014	M	68
CIRUN307	26/08/2014	F	67
CIRUN308	26/08/2014	F	68
Mean \pm s.e.			66.6 \pm 0.5
Litter 4			
CIRUN400	02/06/2015	F	272
CIRUN401	02/06/2015	M	45
CIRUN402	02/06/2015	F	46
CIRUN403	02/06/2015	F	46
CIRUN404	02/06/2015	F	46
CIRUN405	02/06/2015	M	44
CIRUN406	02/06/2015	F	47
CIRUN407	02/06/2015	F	47
CIRUN408	02/06/2015	F	47
CIRUN409	02/06/2015	M	46
Mean \pm s.e.			46.0 \pm 0.3

characteristics of the microsatellite loci. For Litter 4, one embryo (CIRUN409) did not express the maternal allele for the locus CII6, because both the mother and embryo were homozygous, expressing alleles 109 and 107 respectively. Because genotyping and extraction of DNA were performed independently several times (twice for extraction and three for genotyping), it seems reasonable to reject the hypothesis of genotyping errors or sample confusion. Moreover, embryos

Table 2. Probability of detecting multiple paternity for the 21 microsatellite loci used under six scenarios varying in number of sires and paternal skews

	Number of embryos			
	5 (Litter 2)	8 (Litter 3)	9 (Litter 4)	11 (Litter 1)
Paternal skews				
Two males (50 : 50)	0.79	0.94	0.96	0.97
Two males (66.7 : 33.3)	0.72	0.9	0.92	0.95
Two males (80 : 20)	0.55	0.75	0.79	0.85
Three males (33.3 : 33.3 : 33.4)	0.91	0.99	0.99	1
Three males (57 : 28.5 : 14.5)	0.84	0.96	0.97	0.99
Four males (25 : 25 : 25 : 25)	0.95	1	1	1

Table 3. Allelic evidence of multiple paternity for two litters of bull shark (*Carcharhinus leucas*) at three microsatellite loci (CI08, CI13 and CI16) for which more than two paternal alleles were detected

Allele sizes are in base pairs. Paternal alleles shown in bold. Asterisks indicate one of two alleles from the sire

	CI08	CI13	CI16
Litter 1			
CIRUN100		112, 116	107, 111
CIRUN101		112, 112	103 , 111
CIRUN102		112* , 116*	107* , 111*
CIRUN103		112, 122	107* , 111*
CIRUN104		116, 122	107* , 111*
CIRUN105		112, 122	107, 107
CIRUN106		112* , 116*	103 , 111
CIRUN107		116, 120	107, 109
CIRUN108		112* , 116*	107* , 111*
CIRUN109		116, 122	107* , 111*
CIRUN110		112* , 116*	103 , 107
CIRUN111		112, 122	103 , 111
Paternal alleles		112 , 116* , 120 , 122	103 , 107 , 109 , 111*
Litter 3			
CIRUN300	148, 148	112, 112	
CIRUN301	148, 154	112, 116	
CIRUN302	148, 152	112, 122	
CIRUN303	148, 154	112, 122	
CIRUN304	148, 152	112, 116	
CIRUN305	148, 152	112, 116	
CIRUN306	148, 148	112, 112	
CIRUN307	148, 154	112, 122	
CIRUN308	148, 154	112, 122	
Paternal alleles	148 , 152 , 154	112 , 116 , 122	

were dissected separately from any other individual, avoiding DNA contamination. Consequently, this singularity seems real.

Finally, for each litter, full and half-siblings were inferred. For Litter 4, to perform the analysis, CIRUN409 was considered to have missing data at the CI16 locus. In the two litters with multiple paternity, only one pup (CIRUN107 for Litter 1 and CIRUN306 for Litter 3) was a half-sib with all other pups

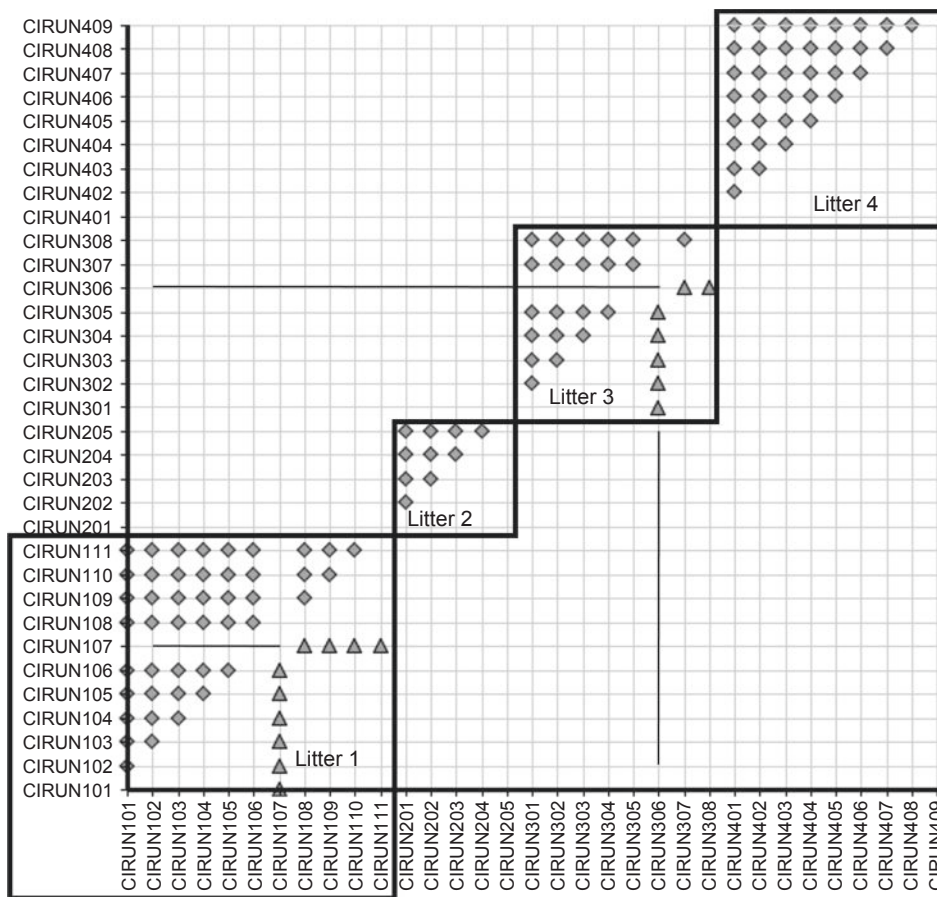


Fig. 1. Sibship relationships (sibships) in the four litters studied. Squares indicate group pups from the same litter. Diamonds (upper diagonal) indicate full siblings; triangles (lower diagonal) indicate half-siblings.

(Fig. 1), confirming that Litters 1 and 3 were fertilised by exactly two males with a high paternal skew (10 : 1 in Litter 1 and 7 : 1 in Litter 3).

Discussion

Herein, we provide the first evidence of multiple paternity in *C. leucas*, with two of four litters being fertilised by two males. Identifying multiple paternity in species is of paramount importance for conservation because it may help maintain genetic diversity in populations (Avise et al. 2002; Frankham et al. 2010) and may increase estimates of effective population size (Sugg and Chesser 1994; Martinez et al. 2000).

Multiple paternity may lead to genetic benefits (for a review, see Thonhauser et al. 2014), including fertility assurance, the good gene hypothesis or the genetic diversity hypothesis (Zeh and Zeh 2001; Thonhauser et al. 2014). An offspring showing higher genetic diversity would express a better fitness and heterozygosity (DiBattista et al. 2008a) and thus these genetic benefits may be detected if multiple paternity is correlated with litter size, heterozygosity or body mass (Zeh and Zeh 2001). Polyandrous litters may be larger than monoandrous litters, as demonstrated for the hammerhead shark (*S. tiburo*) (Chapman et al. 2004); however, for several shark species, such as the

lemon shark (*N. brevirostris*) (DiBattista et al. 2008a), the brown smooth hound shark (*M. henlei*) (Byrne and Avise 2012) and the leopard shark *Triakis semifasciata* (Nosal et al. 2013), no genetic benefits have been demonstrated. In the present study, the sample size was too small to test the correlation between multiple paternity and characteristics of the litters, but it is interesting to note that multiple paternity was not detected in two of four litters, one containing five embryos and the other containing nine embryos. This may suggest that polyandry in the bull shark would not lead to genetic benefits in terms of offspring production, because a smaller litter (eight embryos) was found polyandrous. Nevertheless, a larger sample is required to confirm this observation. Overall, the size of the litters in the present study was in accordance with the average litter size observed in other localities (Compagno 1984; Pattillo et al. 1997).

Post-copulatory mechanisms, such as cryptic female sperm choice, are important and may lead to paternal skews in litters (Jennions and Petrie 2000; Fitzpatrick et al. 2012; Marino et al. 2015). These skews may also be due to post-zygotic processes, as found in the mouse (*Mus musculus*) (Zeh and Zeh 1997), in which females are able to relocate nutrients between defective and viable embryos. Competition between siblings may also occur, such as intrauterine cannibalism described in the fire

salamander (*Salamandra salamandra*) (Dopazo and Alberch 1994) or in the sand tiger shark (*Carcharias taurus*) (Chapman *et al.* 2013). In the present study, an important paternal skew was found in the two broods exhibiting multiple paternity (10 : 1 in Litter 1 and 7 : 1 in Litter 3), which may infer post-copulatory or post-zygotic processes. On the basis of the data of the present study, because the bigger litters were also the most advanced in terms of gestation, it seems unlikely that intrauterine cannibalism occurs, but resource relocation between embryos at a very early stage of gestation may occur. Furthermore, intrauterine cannibalism in sharks has only been reported for *C. taurus* (Chapman *et al.* 2013), a species exhibiting size variation within a brood, whereas in the present study the pups within a litter were of a similar size.

Multiple paternity does not always involve genetic benefits and may be the result of convenience polyandry (Wolff and Macdonald 2004; Holman and Kokko 2013), as demonstrated, for example, in the water strider (*Gerris buenoi*) (Rowe 1992) or in the rock shrimp (*Rhynchocinetes typus*) (Thiel and Hinojosa 2003). Mating of sharks can be violent for females, which show marks and wounds during the mating season (Brunnschweiler and Baensch 2011) resulting from the males grasping females during copulation (Parsons *et al.* 2008). In addition, females also exhibit cloacal lesions because of penetration of the male's clasper (Pratt and Carrier 2001). Avoiding mating could imply a cost for the female, which could be higher than accepting the mating. Under these conditions, females will mate with several males, leading to convenience polyandry (DiBattista *et al.* 2008a; Griffiths *et al.* 2012). This may be the case for the bull shark, because the data of the present study do not infer genetic benefits. If this species aggregates seasonally at specific places to reproduce, then convenience polyandry could be favoured to minimise harassment because the probability of mating should be higher. In this case, polyandry would not lead to any genetic benefit, but would limit the decrease in female fitness resulting from wounds inflicted by males during mating (Holman and Kokko 2013).

Interestingly, one embryo (CIRUN409) did not express the maternal allele for the locus CI16, a dinucleotide (TC) microsatellite (Pirog *et al.* 2015). The mother was homozygous, expressing the allele 109 for this locus, and embryos of the litter were either heterozygous with alleles 107 and 109, or homozygous 109, except for CIRUN409, which was homozygous with the allele 107 (see Table S1, available as Supplementary material to this paper). We can infer from the other embryos of the litter that the father expressed alleles 107 and 109 for this locus. This anomaly may be due to a mutation that occurred in the mother's germ cells (Ellegren 2004; Ortego *et al.* 2008), the presence of a null allele (Dakin and Avise 2004; Chapuis and Estoup 2007) or to meiotic errors (e.g. loss of a chromosome; Engel 1980).

In conclusion, the present study provides new insights into the reproductive behaviour of *C. leucas*, which remains poorly documented. The sampling was insufficient to assess the frequency of multiple paternity in the population of bull sharks of Reunion Island and to test the presence of genetic benefits, but the results tend to support the hypothesis of convenience polyandry. It will be important to expand on this study using non-destructive methods in order to better assess the effects

of polyandry on the genetic diversity and estimates of the effective population size of bull sharks in Reunion Island. Finally, in the context of both shark-attack management and conservation of ecological processes associated with bull sharks, efforts need to continue to understand the full reproductive cycle of the species locally and investigate whether reproduction leads to behavioural changes that could partially explain attacks that happen during the mating period, which is, to date, poorly documented in the western Indian Ocean.

Acknowledgements

The authors thank fishermen T. Gazzo and C. Perry, veterinarians B. Reche and J. Robert, D. Guyomard (Comité Régional des Pêches Maritimes et des Elevages Marins de La Réunion) and all those who helped with sample collection. This study was performed under the scientific program CHARC (Connaissances de l'écologie et de l'habitat de deux espèces de requins côtiers à La Réunion).

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Supplementary material

First evidence of multiple paternity in the bull shark (*Carcharhinus leucas*)

Agathe Pirog^A, Sébastien Jaquemet^{A,B}, Marc Soria^C and Hélène Magalon^{A,B,D}

^AUniversité de La Réunion, UMR 9220 ENTROPIE (Université de La Réunion/IRD/CNRS),
15 Avenue René Cassin, CS 92003, F-97744 Saint Denis Cedex 09, La Réunion, France.

^BLaboratory of Excellence CORAIL, 58, Avenue Paul Alduy, F-66860 Perpignan Cedex, France.

^CIRD Réunion, UMR 248 MARBEC, CS 41095 2 rue Joseph Wetzell, F-97492 Sainte-Clotilde,
La Réunion, France.

^DCorresponding author. Email: helene.magalon@univ-reunion.fr

Table S1. Multi-locus genotypes of the embryos and the mothers of four litters of bull shark from Reunion Island

Data for mothers are shown in bold. TL, total length (cm); F, Female; M, Male

Litter	Date of capture	Location	Sex	TL (cm)	CI01	CI01	CI02	CI02	CI03	CI03	CI04	CI04	CI05	CI05	CI06	CI06	CI07	CI07	CI08	CI08	CI09	CI09	CI10	CI10	CI11	
Litter 1																										
CIRUN100	6/06/2013	Reunion Island	F	285	114	116	143	143	106	108	130	130	137	137	148	148	147	149	148	148	111	113	127	127	236	
CIRUN101	6/06/2013	Reunion Island	F	48	114	116	143	143	106	108	130	130	137	137	148	148	147	149	148	148	111	113	127	127	238	
CIRUN102	6/06/2013	Reunion Island	M	47	114	114	143	143	108	108	130	130	137	137	148	148	147	149	148	152	111	111	127	127	236	
CIRUN103	6/06/2013	Reunion Island	M	48	114	116	143	143	108	108	130	130	137	137	148	148	147	147	148	152	111	111	127	127	236	
CIRUN104	6/06/2013	Reunion Island	F	45	114	114	143	143	106	108	130	130	137	137	148	148	147	147	148	148	111	111	127	127	236	
CIRUN105	6/06/2013	Reunion Island	M	45	114	116	143	143	108	108	130	130	137	137	148	148	147	149	148	148	111	111	127	127	236	
CIRUN106	6/06/2013	Reunion Island	M	50	114	114	143	143	108	108	130	130	137	137	148	148	147	149	148	148	111	113	127	127	236	
CIRUN107	6/06/2013	Reunion Island	F	46	114	116	143	143	106	108	130	130	137	137	148	148	147	149	148	148	111	113	127	127	236	
CIRUN108	6/06/2013	Reunion Island	F	46	116	116	143	143	106	108	130	130	137	137	148	148	147	149	148	152	111	111	127	127	236	
CIRUN109	6/06/2013	Reunion Island	F	46	116	116	143	143	108	108	130	130	137	137	148	148	147	149	148	148	111	111	127	127	236	
CIRUN110	6/06/2013	Reunion Island	M	48	114	114	143	143	106	108	130	130	137	137	148	148	147	149	148	152	111	113	127	127	236	
CIRUN111	6/06/2013	Reunion Island	F	46	114	116	143	143	106	108	130	130	137	137	148	148	147	149	148	152	111	111	127	127	236	
Litter 2																										
CIRUN200	28/04/2014	Reunion Island	F	301	114	116	141	143	106	108	130	130	137	139	148	148	149	149	148	150	107	111	127	127	232	
CIRUN201	28/04/2014	Reunion Island	M	35	116	116	141	143	106	106	130	130	137	137	148	148	149	149	148	150	111	111	127	127	236	
CIRUN202	28/04/2014	Reunion Island	F	36	116	116	143	143	106	108	130	130	137	139	148	148	149	151	148	150	111	111	127	127	232	
CIRUN203	28/04/2014	Reunion Island	F	32	116	116	143	143	106	108	130	130	137	137	148	148	149	149	150	152	107	111	127	127	236	
CIRUN204	28/04/2014	Reunion Island	F	32	114	116	141	143	106	106	130	130	137	137	148	148	149	149	150	150	107	111	127	127	236	
CIRUN205	28/04/2014	Reunion Island	F	32	114	116	141	143	106	106	130	130	137	139	148	148	149	149	150	152	107	111	127	127	236	
Litter 3																										
CIRUN300	26/08/2014	Reunion Island	F	295	114	114	141	143	106	106	124	130	137	139	148	148	147	147	148	148	111	111	127	127	232	
CIRUN301	26/08/2014	Reunion Island	M	66	114	114	141	143	106	108	124	130	139	139	148	148	147	149	148	154	111	111	127	127	232	
CIRUN302	26/08/2014	Reunion Island	F	66	114	116	143	143	106	108	124	130	137	139	148	148	147	147	148	152	111	111	127	129	232	
CIRUN303	26/08/2014	Reunion Island	F	64	114	116	141	143	106	108	124	130	139	139	148	148	147	149	148	154	111	111	127	127	232	
CIRUN304	26/08/2014	Reunion Island	F	67	114	114	141	143	106	108	124	130	139	139	148	148	147	149	148	152	111	111	127	127	232	
CIRUN305	26/08/2014	Reunion Island	F	67	114	114	143	143	106	108	124	130	137	139	148	148	147	147	148	152	111	111	127	127	236	
CIRUN306	26/08/2014	Reunion Island	M	68	114	114	141	143	106	106	124	130	137	137	148	150	147	147	148	148	111	111	127	127	232	
CIRUN307	26/08/2014	Reunion Island	F	67	114	116	141	143	106	108	124	130	137	139	148	148	147	149	148	154	111	111	127	127	236	
CIRUN308	26/08/2014	Reunion Island	F	68	114	114	143	143	106	108	124	130	139	139	148	148	147	147	148	154	111	111	127	127	232	
Litter 4																										
CIRUN400	2/06/2015	Reunion Island	F	272	114	116	143	143	106	106	124	130	137	139	148	148	147	147	148	148	111	111	127	127	236	
CIRUN401	2/06/2015	Reunion Island	M	45	114	116	143	143	106	108	124	130	137	139	148	148	147	147	148	148	111	111	127	127	236	
CIRUN402	2/06/2015	Reunion Island	F	46	116	116	143	143	106	108	124	130	137	139	148	148	147	147	148	148	111	113	127	127	236	
CIRUN403	2/06/2015	Reunion Island	F	46	116	116	143	143	106	108	124	130	137	137	148	148	147	147	148	150	111	111	127	127	236	
CIRUN404	2/06/2015	Reunion Island	F	46	116	116	143	143	106	108	124	130	137	137	148	148	147	147	148	150	111	111	127	127	236	
CIRUN405	2/06/2015	Reunion Island	M	44	114	116	143	143	106	108	124	130	137	139	148	148	147	147	148	148	111	111	127	127	236	
CIRUN406	2/06/2015	Reunion Island	F	47	116	116	143	143	106	108	124	130	137	139	148	148	147	147	148	148	111	113	127	127	236	
CIRUN407	2/06/2015	Reunion Island	F	47	116	116	143	143	106	108	124	130	137	137	148	148	147	147	148	148	111	111	127	127	236	
CIRUN408	2/06/2015	Reunion Island	F	47	116	116	143	143	106	108	124	130	137	137	148	148	147	147	148	150	111	113	127	127	236	
CIRUN409	2/06/2015	Reunion Island	M	46	116	116	143	143	106	108	124	130	137	139	148	148	147	147	148	148	111	111	127	127	236	

Table S1. (Cont.)

Litter Sample name	Date of capture	Location	Sex	TL (cm)	CI11	CI12	CI12	CI13	CI13	CI14	CI14	CI15	CI15	CI16	CI16	CI17	CI17	CI18	CI18	CI19	CI19	CI20	CI20	Gc01	Gc01	
Litter 1																										
CIRUN100	6/06/2013	Reunion Island	F	285	238	105	105	112	116	217	217	296	298	107	111	177	177	211	215	207	207	158	161	136	140	
CIRUN101	6/06/2013	Reunion Island	F	48	238	105	105	112	116	217	217	296	298	107	111	177	177	211	215	207	207	158	161	136	140	
CIRUN102	6/06/2013	Reunion Island	M	47	238	105	105	112	112	217	217	296	298	103	111	177	179	215	217	207	207	161	161	136	136	
CIRUN103	6/06/2013	Reunion Island	M	48	236	105	105	112	116	217	217	296	296	107	111	177	177	211	217	207	207	158	161	136	136	
CIRUN104	6/06/2013	Reunion Island	F	45	236	105	105	112	122	217	217	296	296	107	111	177	177	211	217	207	207	152	161	136	136	
CIRUN105	6/06/2013	Reunion Island	M	45	238	105	105	116	122	217	217	296	296	107	111	177	177	215	217	207	207	152	158	136	136	
CIRUN106	6/06/2013	Reunion Island	M	50	236	105	105	112	122	217	217	296	296	107	107	177	177	211	217	207	207	161	161	136	136	
CIRUN107	6/06/2013	Reunion Island	F	46	238	105	105	112	116	217	217	296	298	103	111	177	179	211	217	207	207	161	161	136	136	
CIRUN108	6/06/2013	Reunion Island	F	46	236	105	105	116	120	217	217	296	298	107	109	177	179	211	215	207	207	149	158	136	136	
CIRUN109	6/06/2013	Reunion Island	F	46	238	105	105	112	116	217	217	296	296	107	111	177	177	215	217	207	207	152	158	136	136	
CIRUN110	6/06/2013	Reunion Island	M	48	238	105	105	116	122	217	217	296	296	107	111	177	179	211	217	207	207	161	161	136	136	
CIRUN111	6/06/2013	Reunion Island	F	46	236	105	105	112	116	217	217	296	298	103	107	177	177	215	217	207	207	152	161	136	136	
Litter 2																										
CIRUN200	28/04/2014	Reunion Island	F	301	238	105	105	112	122	217	217	296	298	103	111	177	177	211	217	207	207	161	161	136	136	
CIRUN201	28/04/2014	Reunion Island	M	35	236	105	105	112	116	217	217	296	296	107	109	177	179	211	211	207	213	152	155	136	136	
CIRUN202	28/04/2014	Reunion Island	F	36	236	105	105	112	116	217	217	296	296	109	111	177	177	211	211	207	213	155	170	136	136	
CIRUN203	28/04/2014	Reunion Island	F	32	236	105	105	112	116	217	217	296	296	107	111	177	179	211	217	207	213	155	170	136	136	
CIRUN204	28/04/2014	Reunion Island	F	32	236	105	105	112	112	217	217	296	296	107	111	177	177	211	217	213	213	155	170	136	136	
CIRUN205	28/04/2014	Reunion Island	F	32	236	105	105	112	112	217	217	296	296	109	111	177	179	211	217	207	207	152	170	136	136	
Litter 3																										
CIRUN300	26/08/2014	Reunion Island	F	295	236	105	105	112	112	217	217	296	296	109	111	177	179	211	211	207	213	155	155	136	136	
CIRUN301	26/08/2014	Reunion Island	M	66	236	105	107	112	112	217	217	296	296	107	107	179	179	211	211	207	213	161	164	136	142	
CIRUN302	26/08/2014	Reunion Island	F	66	232	107	107	112	116	217	217	296	296	107	111	179	181	211	211	207	207	164	164	136	136	
CIRUN303	26/08/2014	Reunion Island	F	64	236	107	107	112	122	217	217	296	296	107	111	179	179	211	211	207	207	161	164	136	136	
CIRUN304	26/08/2014	Reunion Island	F	67	236	107	107	112	122	217	217	296	296	107	111	179	179	211	211	207	207	161	164	136	136	
CIRUN305	26/08/2014	Reunion Island	F	67	232	107	107	112	116	217	217	296	296	107	111	179	181	211	211	207	213	161	164	136	136	
CIRUN306	26/08/2014	Reunion Island	M	68	236	107	107	112	116	217	217	296	296	107	111	179	181	211	211	207	207	164	164	136	136	
CIRUN307	26/08/2014	Reunion Island	F	67	236	105	105	112	112	217	217	296	296	107	111	179	179	211	211	207	207	161	161	136	136	
CIRUN308	26/08/2014	Reunion Island	F	68	236	105	107	112	122	217	217	296	296	107	111	179	179	211	211	207	207	161	161	136	136	
Litter 4																										
CIRUN400	2/06/2015	Reunion Island	F	272	232	107	107	112	122	217	217	296	296	107	111	179	181	211	211	207	213	161	161	136	136	
CIRUN401	2/06/2015	Reunion Island	M	45	236	105	105	112	112	217	217	296	296	109	109	179	179	211	213	207	213	152	155	136	136	
CIRUN402	2/06/2015	Reunion Island	F	46	236	105	105	112	124	213	217	296	296	107	109	177	179	211	211	207	207	152	161	136	136	
CIRUN403	2/06/2015	Reunion Island	F	46	236	105	105	112	116	217	217	296	296	109	109	177	179	211	211	207	207	155	161	136	136	
CIRUN404	2/06/2015	Reunion Island	F	46	236	105	105	112	124	217	217	296	296	109	109	179	183	211	211	207	207	152	152	136	136	
CIRUN405	2/06/2015	Reunion Island	M	44	236	105	105	112	124	213	217	296	296	107	109	177	179	211	213	207	213	152	152	136	136	
CIRUN406	2/06/2015	Reunion Island	F	47	236	105	105	112	124	217	217	296	296	109	109	177	179	211	211	207	213	152	152	136	136	
CIRUN407	2/06/2015	Reunion Island	F	47	236	105	105	112	116	217	217	296	296	109	109	179	183	211	211	207	207	152	155	136	136	
CIRUN408	2/06/2015	Reunion Island	F	47	236	105	105	112	124	217	217	296	296	109	109	177	179	211	213	207	213	152	152	136	136	
CIRUN409	2/06/2015	Reunion Island	M	46	236	105	105	112	116	217	217	296	296	107	109	177	179	211	211	207	207	152	155	136	136	

1 2. Biologie de la reproduction du requin bouledogue *Carcharhinus leucas*

2

3 **Résumé**

4 Afin d'étudier les modes de reproduction du requin bouledogue *Carcharhinus leucas*, nous avons
5 analysé certains paramètres reproducteurs de 118 requins pêchés le long de la côte Ouest de La
6 Réunion (Ouest de l'océan Indien), comprenant 16 femelles gravides. Nous avons également
7 estimé la fréquence de polyandrie dans ces 16 portées, ce qui nous a permis d'établir l'image la
8 plus complète à ce jour des comportements reproducteurs du requin bouledogue. Les mâles et les
9 femelles atteignent la maturité sexuelle pour des tailles d'environ 234 cm et 257 cm (longueur
10 totale), respectivement. L'analyse des 16 portées, comportant 5 à 14 embryons par portée, et qui
11 ont été collectées à divers mois de l'année, indique que la parturition a lieu entre octobre et
12 décembre, et que la taille à la naissance est de 60-80 cm. La gestation dure probablement 12 mois,
13 ce qui, avec une période de repos d'un an et un stockage du sperme d'environ 4-5 mois entre
14 l'accouplement (en juin-septembre) et la fécondation, mène à un cycle de reproduction bisannuel.
15 Enfin, au moins 56,25 % des portées étaient polyandres, provenant de 2 à 5 pères différents.
16 Plusieurs mâles ont été identifiés comme pères de différentes portées, conçues soit lors d'une même
17 saison de reproduction, soit lors de saisons de reproduction différentes. Cela suggère à la fois des
18 agrégations de requins bouledogue pendant la saison d'accouplement et de la philopatrie des mâles
19 aux sites d'accouplements. Ces résultats représentent des informations importantes pour la gestion
20 durable des populations de requin bouledogue de l'Ouest de l'océan Indien.

21 Cette publication a été soumise à *Plos One*.

22

23

25 **Everything you always wanted to know about sex* (*but were afraid to ask): lessons from**
26 **the bull shark *Carcharhinus leucas* from Reunion Island (Western Indian Ocean)**

27 Agathe Pirog¹, H el ene Magalon^{1,2}, Thomas Poirout¹, S ebastien Jaquemet^{1*}

28

29 ¹ UMR ENTROPIE (Universit e de La R eunion/IRD/CNRS), Universit e de La R eunion, Saint
30 Denis, La R eunion, France

31 ² Laboratoire d'Excellence CORAIL, Perpignan, France, www.labex-corail.fr

32

33 * Corresponding author

34 E-mail: sebastien.jaquemet@univ-reunion.fr

35 **Abstract**

36 The bull shark *Carcharhinus leucas* is a large coastal carcharhinid, the only one being euryhaline,
37 utilising estuaries, large rivers and lakes as nurseries. This apex predator plays an important role in
38 coastal ecosystems where it can interact with human activities, sometimes leading to injuries and
39 fatalities. Consequently, the species is often targeted by shark control programs in addition to
40 recreational and commercial fishing activities. Despite these numerous interactions with human
41 activities in coastal zones, knowledge on bull shark reproductive biology remains scant. Here we
42 analysed reproductive traits from 118 bull sharks caught along Reunion Island coasts (Western
43 Indian Ocean), including 16 gravid females. Specific microsatellite loci were also used to
44 investigate the frequency of multiple paternity. Males and females reached maturity at
45 approximately 234 cm and 257 cm (total length), respectively, and litter sizes ranged from 5 to 14
46 embryos. Analysis of the 16 litters collected in various months of the year indicated that parturition
47 occurs between October and December, with a size at birth around 60-80 cm, and that the gestation
48 period is likely around 12 months. Assuming a one-year resting period and a period of sperm
49 storage (4-5 months) between mating (in June-September) and fertilization, the reproductive cycle
50 of bull sharks in Reunion Island would be biennial. Finally, at least 56.25% of the litters
51 investigated were polyandrous, sired by two to five males. Several males that each sired several
52 litters conceived during the same or distinct mating seasons were detected, suggesting both a
53 seasonal aggregation of sharks to mate and some male fidelity to mating site. Altogether, these
54 findings provide valuable information for both shark risk management and conservation of the
55 species in the Western Indian Ocean.

56 Keywords: Chondrichthyes; Elasmobranch; Carcharhinidae; life history traits; reproductive cycle;
57 polyandry

58 **Introduction**

59 While more and more evidence accumulate to suggest both the importance of large-bodied sharks
60 in marine ecosystems functioning [1-6] as well as the worldwide decline of their populations [7,
61 8], their reproductive behaviours and life history traits remain largely unknown [9]. Most species
62 present specific *K*-strategy characteristics, with slow growth rates, low fecundities and late ages to
63 sexual maturity [10, 11], rendering them particularly sensitive to fishing and environmental pressures
64 [12], to which they are more and more exposed [13, 14]. Knowledge on life history traits and
65 reproductive behaviours of these species are thus of particular importance, notably to perform stock
66 assessment or to manage their populations in human-shark interactions in coastal areas.

67 The bull shark *Carcharhinus leucas* is a large coastal carcharhinid that inhabits tropical and warm
68 temperate waters worldwide [15]. This species reproduces by placental viviparity, providing
69 nutrients during most of the gestation through a yolk-sac placenta, a direct connection between
70 mother and embryos [15, 16]. Litter sizes range from 1 [17] to 14 [18] embryos, with typical litter
71 size being 6 to 8 embryos [17, 19-21] with sizes (total length *TL*) at birth ranging between 50 and
72 80 cm [15, 17, 22-24]. Total length at sexual maturity varied between localities and sex. In the Gulf
73 of Mexico, it ranges from 195 cm to 215 cm for males and from 204 cm to 226 cm for females [23,
74 25], while in South Africa, larger sizes were recorded, 235 cm and 240 cm for males and females,
75 respectively [24].

76 Female bull sharks exhibit reproductive philopatry and often use freshwater habitats as pupping
77 grounds [22, 26-29]. These freshwater habitats are also used by new-borns and juveniles as
78 nurseries for several years, providing protection from predators and resources [17, 22, 30-35]. This
79 philopatric behaviour may lead to a loss of genetic diversity, which could be compensated by male
80 dispersal [36-38], as it seems to occur in this species [28, 29]. It may also be linked to the
81 occurrence of multiple paternity in this species [21], which could compensate inbreeding
82 depression [39] due to female reproductive philopatry, or decrease the costs due to male harassment
83 during mating (i.e. convenience polyandry) [40, 41].

84 Data on reproductive cycles also remain scarce, and pupping and mating periods are still poorly
85 understood in many shark species, including the bull shark. A biennial reproductive cycle was
86 hypothesized by Brunnschweiler and Baensch [42], based on photographs and video footages taken

87 on a regular basis at a feeding site in Fiji. They identified mating scars from December to February
88 only, and pregnant females returning non-pregnant after being absent for several weeks at the end
89 of a calendar year. They thus assumed a gestation period of approximately one year followed by
90 an equivalent resting period [42]. Clark and von Schmidt [43] hypothesized a gestation period of
91 10-11 months in Florida, considered possible by Jenson [17], even though these latter did not
92 observe any seasonal reproductive pattern in a bull shark population from the Lake Nicaragua-Rio
93 San Juan system. In South Africa, Cliff and Dudley [24] caught six gravid females, and found
94 nearly full-term embryos both during the cold (June-August) and warm (November-February)
95 seasons, and thus assumed a prolonged mating season. In the south of Mozambique, Daly et al.
96 [44] showed migrations toward lower latitudes from July to October, and suggested that either
97 foraging or reproductive activity might be underlying mechanisms of such seasonal migration.

98 Here we further expand our understanding of bull shark reproductive biology using data from
99 Reunion Island control fishing programs, collected since 2012. To our knowledge, this study is
100 only the second investigating reproductive behaviours of the bull shark in the Western Indian Ocean
101 (see [24]), and the one with the highest number of gravid females, as data from 118 bull sharks,
102 including 16 gravid females, were examined. Combining morphometric data and molecular tools,
103 analyses of reproductive traits were used to estimate size at maturity and reproductive cycles while
104 specific microsatellite loci were used to investigate the frequency of multiple paternity in the
105 sampled litters.

106

107 **Material and Methods**

108 **Sampling and measurements**

109 Data analysed in this study were collected opportunistically from sharks caught during control
110 programs that were set up by the French government since 2012 for public safety following an
111 increase in attacks on humans along the West coast of Reunion Island [45]. Bottom longlines and
112 drumlines with a catch-a-live system (called smart-drumline) were used to catch bull sharks while
113 releasing by-catches alive. From 2012 to 2017, 118 bull sharks (58 females and 60 males) were
114 caught and dissected. Precaudal length (*PCL*), fork length (*FL*), total length (*TL*), the liver weight
115 (*W_l*) and the body mass (*W*) were recorded for each individual when possible. *PCL*, *FL* and *TL*

116 were measured as straight lines from the tip of the snout to the precaudal notch, the fork tail and
117 the tip of the upper caudal lobe, respectively. Unless specified, all lengths reported hereafter in this
118 study are *TL*. To convert lengths from other studies or for individuals for which the *TL* was not
119 measured, a linear regression was performed to estimate the *TL* from the *PCL* measure, using
120 animals for which both measures were taken. When it was not possible to weight the individuals,
121 the estimated weight (W_e) was calculated according to the following equations, with the weight in
122 kilograms and lengths in centimetres:

123 $W = 2.63 \times 10^{-5} PCL^{2.93}$ ($n = 266, R^2 = 0.9900$) for male bull sharks [24]

124 $W = 2.10 \times 10^{-5} PCL^{2.98}$ ($n = 306, R^2 = 0.9905$) for female bull sharks [24]

125 To determine the reproductive condition of males, the clasper lengths (measured from the point of
126 insertion at the anterior margin of the cloaca to the tip of the clasper), their degree of calcification
127 and their size relatively to the pelvic fins (smaller or larger) were noted [43]. Females were
128 considered mature based on i) the presence of embryos, ii) the presence of mature oocytes, iii) the
129 uterus width and iv) signs of recent parturition [43]. Gonads were also weighted (W_g) for both
130 sexes. Furthermore, when gravid females were caught (16 females, representing 164 embryos), for
131 each embryo, sex was determined based on the presence of claspers, and *PCL*, *FL*, *TL* and *W* were
132 measured. Finally a piece of muscle was also collected on all individuals (including embryos) for
133 microsatellite genotyping and stored in 90% ethanol until laboratory analyses.

134

135 **Microsatellite genotyping**

136 For each gravid female and its embryos, genomic DNA was extracted using Qiagen DNeasy Blood
137 & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

138 Genotyping was performed using the 21 loci already used in Pirog et al. [21] and laboratory
139 procedures were conducted as described in Pirog et al. [46]. The allelic sizes of the PCR products
140 were separated on an ABI 3730XL capillary sequencer at the Plateforme Gentyane (INRA,
141 Clermont-Ferrand, France) and scored with GENEMAPPER v.4.0 (Applied Biosystems) using the
142 Genescan LIZ-500 size standard (Applied Biosystems).

143

144 **Statistical analyses**

145 *TL* distribution of the studied individuals was plotted, and sex-related differences in length
146 distributions were tested using a Kolmogorov-Smirnov test [47].

147 Weight-size (W-PCL) relation was established for each sex, based on individuals for which both
148 weight and length were available, using the method of Pienaar and Thomson [48] for fitting a non-
149 linear regression model by least squares. Furthermore, whether these relations exhibited sex-related
150 differences, an analysis of covariance (ANCOVA) was performed on the logarithmic forms of these
151 equations ($\log(W) = \log(a) + b \times \log(PCL)$). Then, to check whether weight-size relations
152 established by Cliff and Dudley [24] also fitted our dataset, we tested whether significant
153 differences were retrieved between the observed (measured weights) and predicted (estimated
154 weight with equations from Cliff and Dudley [24]) using a Kolmogorov-Smirnov test [47]. This
155 was performed using R [49].

156 To assess size at maturity (L_{50}), clasper length and uterus width were plotted against *TL* for males
157 and females, respectively. L_{50} is defined as the length at which a randomly chosen individual has a
158 50% chance of being mature, and was estimated using the package *sizeMat* [50] for R [49]. First,
159 a Principal Component Analysis (PCA) was conducted using the *TL* and either the clasper length
160 or the uterus width in log base, to separate immature (juveniles) and mature (adults) individuals.
161 Individuals were assigned to each group using a hierarchical classification procedure. Using the
162 results of the classification, a linear discriminant analysis was performed to obtain a discriminating
163 function allowing to classify any individual as a juvenile or an adult based on the two variables
164 used (*TL* and either clasper length or uterus width). Then a logistic approach was used to estimate
165 L_{50} . In the regression analysis, *TL* was considered the explanatory variable and the classification
166 (juveniles: 0, adults: 1) was considered the response variable and these variables were fitted to a
167 logistic function with the form:

168
$$P = 1/[1 + e^{-(\beta_0 + \beta_1 * X)}]$$

169 where P is the probability of an individual of being mature at a determinate length X , β_0 (intercept)
170 and β_1 (slope) are the estimated parameters. The L_{50} is then calculated as:

171
$$L_{50} = -\beta_0/\beta_1.$$

172 Then, to describe the reproductive cycle, the frequency of potentially mature individuals (i.e.
173 $TL \geq L_{50}$) and mean embryo lengths for each litter were plotted against time (i.e. daily, all years
174 pooled). Furthermore, the gonadosomatic (GSI) and hepatosomatic (HSI) indices [51] were
175 calculated as $GSI = (W_g/W) \times 100$ and $HSI = (W_l/W) \times 100$. Their temporal distributions over time
176 were assessed to investigate seasonality in the reproduction (i.e. monthly, all years pooled). Finally
177 a Pearson correlation test was performed with R to infer the occurrence of correlations between the
178 number of embryos in a litter and the size of the mother.

179

180 **Analyses of multiple paternity**

181 The four litters already studied in Pirog et al. [21] were added to the present study in order to
182 identify males that may have sired several litters, leading to a total of 16 studied litters.

183 First the probability of detecting multiple paternity was calculated with PRDM [52]. This
184 probability is calculated given i) allelic frequencies of the microsatellite loci in the population, ii)
185 litter sizes, and iii) the number of sires of a litter. Populations were defined based on results of
186 genetic differentiation analyses performed in Pirog et al. [53], and allelic frequencies and
187 characteristics of the loci can be found in this latter study. A little differentiation was found with
188 microsatellite loci among localities from the Western Indian Ocean and the Western Pacific.
189 Genotypes from males collected in these regions were used to test whether they corresponded to
190 the inferred genotypes of the potential fathers.

191 For each different number of embryos observed in one litter ($n = 6 - 14$), six scenarios were defined
192 according to the number of fathers usually recorded in shark species ($n = 2 - 4$) [54] and different
193 paternal skews (number of embryos within the litter sired by each male): i) equal paternity (two
194 sires: 50% each; three sires: 33% each, four sires: 25% each), ii) a moderate skew (two sires: 66.7%,
195 33.3%; three sires: 57%, 28.5%, 14.5%) and iii) a high skew (two sires: 80%, 20%).

196 Then multiple paternity was considered when at least three non-maternal alleles were found in a
197 litter for at least two microsatellite loci. This was performed by manual counting of alleles. Second,
198 full and half-siblings within and among litters were assessed using the full pedigree likelihood
199 method implemented in COLONY v.2.0.4.5 [55]. A polygamous mating system was assumed for

200 both sexes to allow the assignment of half-siblings. A long-run with medium likelihood precision
201 and a genotyping error rate of 1% was performed.

202 Finally Pearson correlation tests were performed with R to infer the occurrence of correlations
203 between the number of embryos in the litter and the number of sires as well as between the size of
204 the mother and the number of fathers.

205

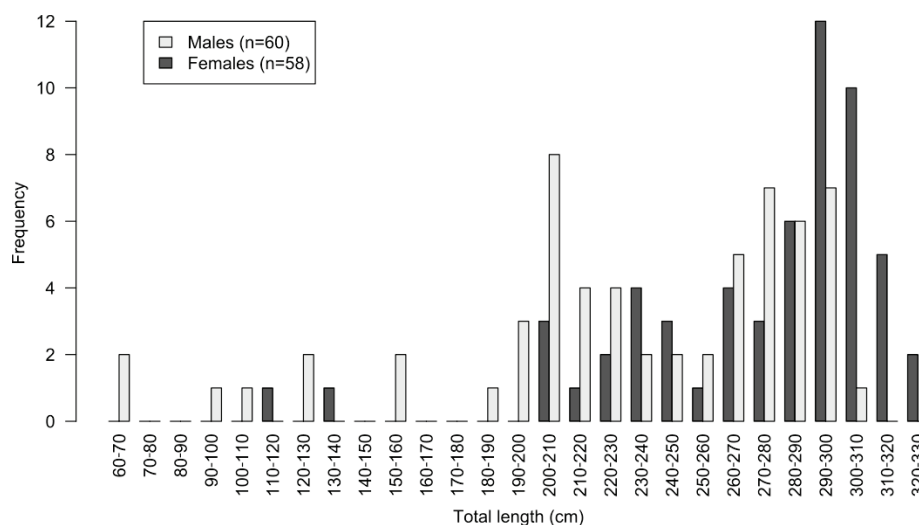
206 Results

207 Length-frequency, weight-length relationships and size at maturity

208 Length data were recorded for all 118 bull sharks captured (58 females and 60 males). Sex-ratio of
209 nearly 1:1 was thus observed. A significant and robust linear relationship exists between the
210 precaudal length (*PCL*) and the total length (*TL*):

$$211 TL = 1.279PCL + 6.916 \quad (n = 118, t_{116} = 111.08, R^2 = 0.99, P < 2.2 \times 10^{-16}).$$

212 Significantly different length distributions were found for males and females (two-samples
213 Kolmogorov-Smirnov test, $D = 0.39$, $P = 2.94 \times 10^{-4}$). Female lengths ranged from 111 cm to
214 325 cm (mean \pm SE = 273.90 ± 5.71 cm) and male lengths from 61 cm to 310 cm
215 (231.67 ± 7.69 cm). Females ranging from 280 to 320 cm were caught in greater abundance, while
216 males caught measured mostly around 200 – 230 cm and around 260 – 300 cm (Fig 1).



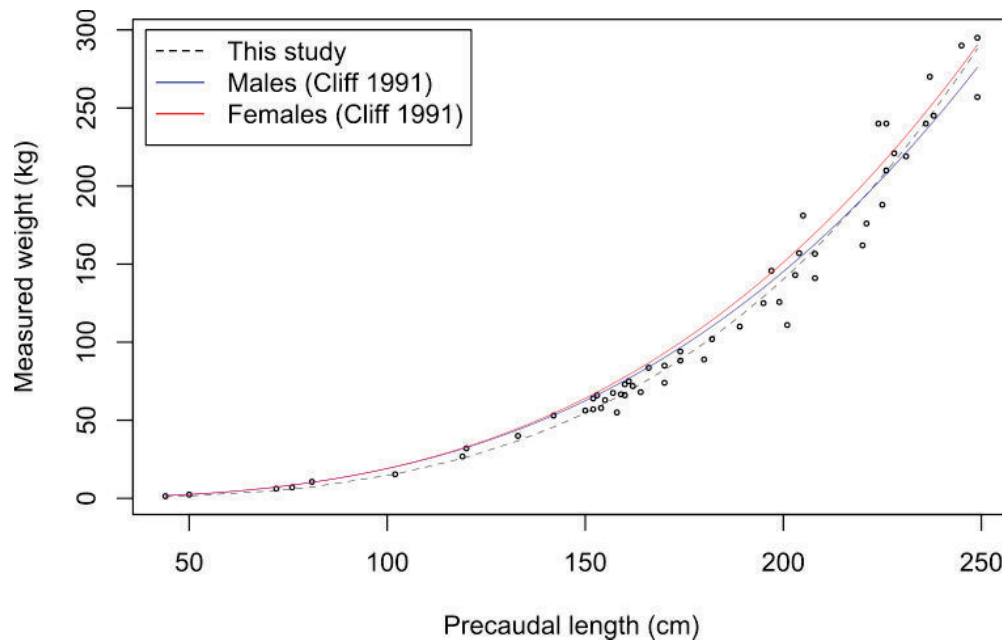
217

218 Fig 1. Length-frequency histogram for bull shark *Carcharhinus leucas*.

219 Weights were obtained from 32 males and 23 females and relationships were assessed with the
 220 precaudal length PCL , to compare the equations found with those from Cliff and Dudley [24]. The
 221 ANCOVA did not highlight a significant influence of the sex on the weight-size relation (intercept:
 222 $n = 55, F_{1,51} = 1.676, P = 0.20$; slope : $n = 55, F_{1,51} = 2.438, P = 0.12$). Thus a single length-weight
 223 equation was established for the bull sharks caught in Reunion Island:

224 $W = 4.00 \times 10^{-6} PCL^{3.28}$ ($n = 55, t_{53} = 32.72, R^2 = 0.987, P < 2.2 \times 10^{-16}$; Fig 2)

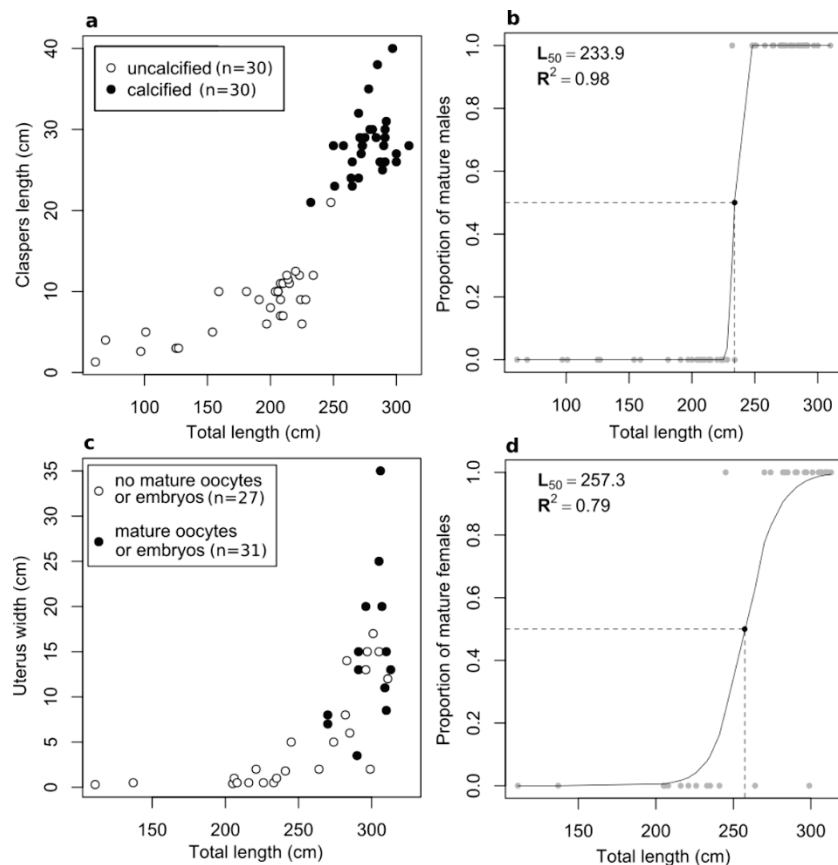
225 No significant differences were retrieved between the measured weights and those estimated with
 226 equations established in Cliff and Dudley [24] (two-samples Kolmogorov-Smirnov test, $D = 0.15$,
 227 $P = 0.55$)(Fig 2).



228
 229 **Fig 2. Relationship between precaudal length PCL and measured weight W for the bull shark**
 230 ***Carcharhinus leucas*.** This study (dotted black line): $W = 4.00 \times 10^{-6} PCL^{3.28}$ ($n = 55, R^2 = 0.987$);
 231 males [24] (blue line): $W = 2.63 \times 10^{-5} PCL^{2.93}$ ($n = 266, R^2 = 0.990$); females [24] (red line):
 232 $W = 2.10 \times 10^{-5} PCL^{2.98}$ ($n = 306, R^2 = 0.991$).

233
 234 Clasper length markedly increased in males larger than 230 cm, which was linked to claspers
 235 calcification (Fig 3a). The smallest male with calcified claspers measured 232 cm whereas the
 236 largest male with uncalcified claspers was 248 cm long. A clear break occurred between males
 237 with uncalcified claspers measuring less than 15 cm, and those with claspers measuring more than

238 20 cm, which were all calcified, except for one individual (Fig 3a). Male L_{50} was estimated to
 239 233.9 cm by the model (Fig 3b). Size at maturity for females was more difficult to detect, with
 240 various uterus widths for a same TL (Fig 3c). Nevertheless, an increase in uterus width seemed to
 241 occur for TL larger than 260 cm. The smallest female with mature oocytes or embryos measured
 242 260 cm while the largest non-gravid female nor presenting mature oocytes measured 311 cm
 243 (Fig 3c). Using the uterus width as a sign of maturity, female L_{50} was estimated to 257.3 cm
 244 (Fig 3d).



245
 246 **Fig 3. Maturity of bull sharks *Carcharhinus leucas* from Reunion Island.** (a) Length of
 247 calcified and uncalcified claspers versus male total length. (b) Proportion of mature males in 10 cm
 248 size intervals, dashed lines mark the size at which 50% of males reached maturity (L_{50}). (c) Uterus
 249 width versus female total length with occurrence of mature oocytes or embryos. (d) Proportion of
 250 mature females in 10 cm size intervals, dashed lines mark the size at which 50% of females reached
 251 maturity (L_{50}).

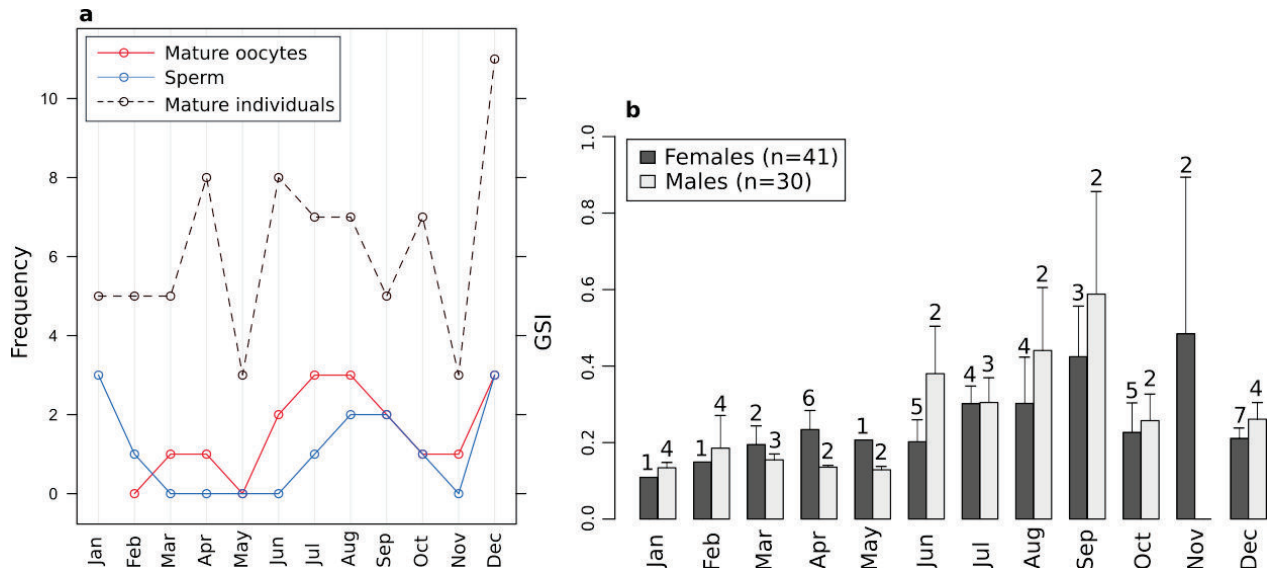
252

253

254 **Reproductive cycles**

255 To analyse the reproductive cycle, only individuals considered as mature were kept: 31 males with
256 $TL \geq 233.9$ cm and 43 females with $TL \geq 257.3$ cm. Females presenting mature oocytes ($n = 17$)
257 were mostly caught during the cold season, from June to September, but also in December during
258 the warm season (Fig 4a). Males with sperm ($n = 13$) were also mostly caught during these periods,
259 from July to October, and in December (Fig 4a). However, these data must be taken with caution
260 as they depend on the fishing effort, which was not constant over time, varying from month to
261 month and from year to year. In addition to the fact that they deal with small sample size implying
262 stochasticity, a decrease in the number of potentially mature individuals might thus just reflect a
263 period during which the fishing effort was weaker. One female presenting mating scars was caught
264 at the beginning of November 2016. Besides the monthly *GSI* varied greatly, but also between
265 individuals caught during similar months (Fig 4b), contrary to the *HSI*, which was constant for all
266 individuals (mean \pm SE: $HSI = 12.69 \pm 0.58\%$ for females and $HSI = 12.22 \pm 0.54\%$ for males).
267 For males, the *GSI* peaked in September and was significantly higher from June to December
268 compared to other months (Wilcoxon-test, $P = 6.5 \times 10^{-6}$). For females, *GSI* peaked in November
269 with higher values between July and November (mean $GSI = 0.32 \pm 0.05\%$) compared to the other
270 months (mean $GSI = 0.21 \pm 0.02\%$), though no significant variations were highlighted (Wilcoxon-
271 test, $P = 0.10$).

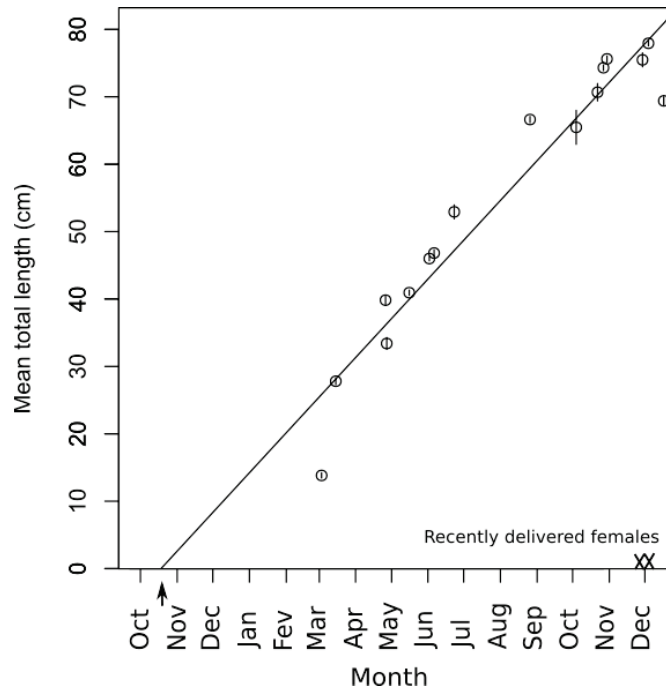
272



273
 274 **Fig 4. Analyses of reproductive cycles in the bull shark *Carcharhinus leucas*.** (a) Monthly
 275 number of males and females potentially mature (i.e., for which total length TL is greater than size
 276 at maturity L_{50} ; dashed line), and among them, the males presenting sperm (blue line) and the
 277 females presenting mature oocytes (red line). (b) Monthly variations of the gonado-somatic index
 278 (GSI) for potentially mature males and females ($TL \geq L_{50}$). Monthly number of sharks per sex are
 279 indicated above each bar.

280

281 Of the 16 litters sampled, none presented a sex-ratio significantly different from unity (χ^2 tests,
 282 $n = 5 - 14$, all $P > 0.05$). A total of 164 embryos were collected, with TL ranging from 12.2 to
 283 79 cm (mean = 56.51 ± 1.51 cm). The number of embryos per litter ranged from 5 to 14
 284 (mean = 10.31 ± 0.73). A significant correlation was found between the number of embryos per
 285 litter and the size of the mother, but this correlation was not strong (Pearson correlation test, $n = 16$,
 286 $t_{14} = 2.53$, $R^2 = 0.314$, $P = 0.02$). Litters with the smallest embryos were caught in March (two
 287 litters, mean embryos $TL = 13.82 \pm 0.39$ cm and 27.80 ± 0.50 cm) while litters with the largest
 288 embryos were caught toward the end of the year, in October and November (seven litters, mean
 289 embryos TL varying from 65.47 ± 0.46 cm to 77.94 ± 0.39 cm; Fig 5). The mean embryos lengths
 290 per litter were significantly correlated to the day of the year (Pearson test of correlation, $n = 16$,
 291 $t_{14} = 13.324$, $R^2 = 0.927$, $P = 2.41 \times 10^{-9}$). Furthermore, two recently delivered females were caught
 292 at the end of November and the beginning of December.



294

295 **Fig 5. Mean embryos length for each litter plotted against day of the year for the bull shark**
 296 ***Carcharhinus leucas* ($n = 16$ litters).** The line represents the linear regression linking the mean
 297 embryos length per litter (y) to the day of the year [x , coded as integers from 1 (1st January) to 365
 298 (31st December)], $y = 0.19x + 14.02$ ($n = 16$, $t_{14} = 13.324$, $R^2 = 0.927$, $P = 2.41 \times 10^{-9}$). Crosses
 299 indicate mature females with expanded, vacant uteri indicating they had delivered recently. The
 300 intersection between the regression line and the x -axis (indicated by the arrow) may correspond to
 301 the beginning of the gestation.

302

303 Multiple paternity

304 The probability of detecting multiple paternity in the bull shark litters sampled was high (ranging
 305 from 0.81 to 1 according to the number of sires and the reproductive skew) for all litter sizes, except
 306 for the two smallest ones (six and seven pups) assuming two sires and a high reproductive skew
 307 (probabilities of 0.67 and 0.72; Table 1). The true number of sires in these litters may thus be
 308 underestimated.

309

310 **Table 1. Probability of detecting multiple paternity for the bull shark *Carcharhinus leucas***
 311 **using 21 microsatellite loci.**

	Number of embryos							
	6	7	9	10	11	12	13	14
Paternal skews								
Two males (50:50)	0.90	0.94	0.97	0.98	0.98	0.99	0.99	0.99
Two males (66.7:33.3)	0.84	0.88	0.94	0.95	0.96	0.97	0.98	0.98
Two males (80:20)	0.67	0.72	0.81	0.84	0.87	0.89	0.91	0.92
Three males (33.3:33.3:33.4)	0.97	0.99	1.00	1.00	1.00	1.00	1.00	1.00
Three males (57:28.5:14.5)	0.92	0.95	0.98	0.99	0.99	0.99	1.00	1.00
Four males (25:25:25:25)	0.99	0.99	1.00	1.00	1.00	1.00	1.00	1.00

312 Six scenarios varying in number of sires and paternal skews are depicted.

313
 314 Among the four litters already studied with the same loci in Pirog et al. [21] (Litters 1 to 4), two
 315 exhibited evidence of multiple paternity. Among the 12 remaining litters (Litters 5 to 16), more
 316 than two paternal alleles for at least two loci were observed for seven litters, and in two other litters
 317 (Litters 10 and 12), one locus exhibited more than two paternal alleles. No litters exhibited more
 318 than four paternal alleles for a locus, which would have indicated the presence of three sires with
 319 certainty.

320 The analysis with COLONY identified 11 litters issued from two to five different males and the five
 321 remaining from only one male (Table 2). For Litter 12, for which one locus exhibited three paternal
 322 alleles, the analysis retrieved a possible genotyping error, and only assigned one sire to all embryos.
 323 Furthermore, while Litter 14 did not exhibit more than two paternal alleles for any loci, the
 324 maximum likelihood analysis performed by COLONY retrieved four sires. An important paternal
 325 skew was found for almost all litters with more than two sires, with a first male siring more than
 326 50% of the litter, and a second less than 30% of the litter (Table 2). Only Litter 8 (mean embryos
 327 $TL = 27.80 \pm 0.50$ cm), for which three sires were retrieved, presented two sires with almost equal
 328 contribution, with one siring six embryos and the other one, five (Table 2).

329

330 **Table 2. Results of the multiple paternity analysis performed with COLONY for 16 bull shark**
 331 ***Carcharhinus leucas* litters from Reunion Island.**

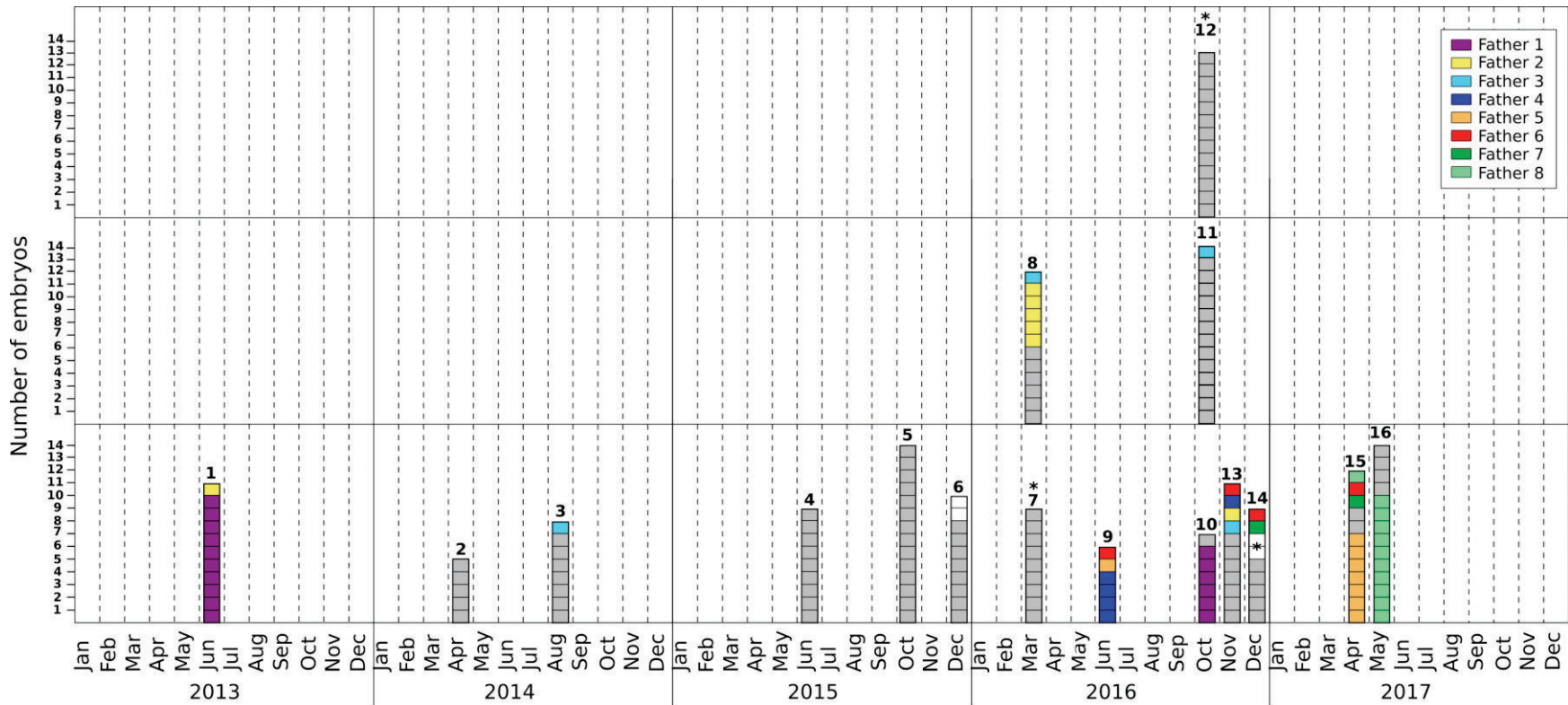
Litter ID	N_{embryo}	Mother TL	Mean embryos TL	Date of capture	N_{sires}	Paternal skew
Litter 1	11	285	46.82 (0.46)	06/06/2013	2	91:09:00
Litter 2	5	301	33.40 (0.87)	27/04/2014	1	-
Litter 3	8	295	66.63 (0.46)	26/08/2014	2	88:13:00
Litter 4	9	272	46.00 (0.33)	02/06/2015	1	-
Litter 5	14	322	70.68 (1.28)	22/10/2015	1	-
Litter 6	10	291	69.40 (0.60)	17/12/2015	2	80:20:00
Litter 7	9	274	13.82 (0.39)	03/03/2016	1	-
Litter 8	12	306	27.80 (0.50)	15/03/2016	3	50:42:08
Litter 9	6	267	52.93 (1.02)	23/06/2016	3	67:17:17
Litter 10	7	299	65.47 (2.51)	04/10/2016	2	86:14:00
Litter 11	14	300	74.31 (0.42)	27/10/2016	2	93:07:00
Litter 12	13	314	75.62 (0.43)	30/10/2016	1	-
Litter 13	11	294	75.48 (1.03)	29/11/2016	5	64:9:9:9
Litter 14	9	290	77.94 (0.39)	04/12/2016	4	56:22:11:11
Litter 15	12	317	39.83 (0.60)	26/04/2017	5	58:17:8:8
Litter 16	14	300	40.94 (0.38)	16/05/2017	2	71:29:00

332 N_{embryo} , number of embryos in the litter; N_{sires} , number of males that sired the litter according to
 333 COLONY results; Total lengths TL are in centimetres and in parentheses are indicated standard
 334 errors. In bold are indicated litters sired by several males.

335
 336 Furthermore, father genotypes were inferred and compared to genotypes of males collected in the
 337 Western Indian Ocean and in the Western Pacific during the study of Pirog et al. [53]. Two males
 338 collected around Reunion Island were identified as fathers of Litters 7 and 12 (both monoandrous,
 339 $P = 1.00$). One male tagged in Mozambique may have sired two embryos of Litter 14 ($P = 0.45$).
 340 The other fathers were not sampled during the study of Pirog et al. [53]. Overall 24 different fathers
 341 were identified for the 16 litters studied.

342 Interestingly, half-sibs were retrieved among different litters with probabilities higher than 0.80,
 343 indicating that a same male mated with several of the gravid females sampled (Fig 6). So, among
 344 the 24 fathers identified, eight males sired at least two litters (to a maximum of four litters for
 345 Fathers 3 and 6), either during the same mating season (Fathers 2, 3, 4, 6 and 8) or distinct mating
 346 seasons: one (e.g. Father 7 for Litters 14 and 15), two (Father 3 for Litter 3 vs Litters 8, 11 and 13)

347 or three (Father 1 for Litters 1 and 10 or Father 2 for Litter 1 vs Litters 8 and 13) (Fig 6). Concerning
348 reproductive skews in polyandrous litters, three cases were highlighted: i) fathers that always
349 contributed to only one embryo (Fathers 3, 6 and 7), ii) fathers that contributed to one embryo in
350 some litters and several in others (Fathers 2, 5, 4 and 8) and iii) fathers that always contributed to
351 most of the embryos from a litter (Father 1).



352

353 **Fig 6. Multiple paternity in 16 bull shark litters from females caught in Reunion Island.** Rectangles represent litters (numbers above
 354 correspond to litter ID as in Table 2), the height being proportional to the number of embryos in the litter. On the x-axis is indicated the
 355 date of capture. Colors represent fathers of the embryos. In grey and white are represented males that sired only one litter. Asterisks
 356 indicate the fathers already identified in Pirog et al. [53].

357 No correlations were found between the number of embryos per litter and the number of sires
358 (Pearson correlation test, $n = 16$, $t_{14} = 0.24$, $R^2 = 0.004$, $P = 0.81$), nor between the number of sires
359 and the size of the mother (Pearson correlation test, $n = 16$, $t_{14} = 0.30$, $R^2 = 0.006$, $P = 0.77$).

360

361 **Discussion**

362 From 118 bull sharks (*Carcharhinus leucas*) caught in Reunion Island (Western Indian Ocean),
363 biological and reproductive traits were studied here to improve our understanding of this still poorly
364 known species. Females were larger than males and size at maturity were larger than those reported
365 in the Atlantic for both males and females, but similar to those estimated in South Africa. The
366 analysis of the 16 litters collected from gravid females showed that the mean length of the embryos
367 was significantly correlated with time. Finally, multiple paternity was confirmed at least in 9 litters
368 over 16. Besides, some males were found to be fathers of different litters conceived during a same
369 or distinct mating seasons, with important paternal skew.

370

371 **Length-frequency, weight-length relationships and size at maturity**

372 Among the caught individuals, a clear discrepancy was observed between the size of males and
373 females, with a higher frequency of large ($TL > 270$ cm) females compared to males. Besides,
374 individuals of both sexes were larger in Reunion Island than in other localities, notably in the
375 Atlantic (Table 3). The largest animals caught in the present study measured 310 and 320 cm (male
376 and female, respectively) while, in the Gulf of Mexico, the largest animals measured from 245 to
377 254 cm for males and from 265 to 271 cm for females [23, 25, 43]. Comparatively, along the
378 eastern coast of South Africa, Bass et al. [56] reported maximal sizes of 299 and 300 cm, and Cliff
379 and Dudley [24] sizes of 276 and 284 cm, for males and females, respectively (Table 3). This study
380 thus reports some of the largest individuals to date, with females frequently measuring more than
381 290 cm. However, McCord and Lamberth [57] reported a 400 cm female in the Breede estuary on
382 the southern coast of South Africa, which seems to be the largest individual ever recorded.

383

384 **Table 3. Synthesis of the knowledge on the reproductive biology of the bull shark *Carcharhinus leucas*.**

Locality	Mating period	Parturition period	Size at birth	L_{50} Males	L_{50} Females	TL_{max} Males	TL_{max} Females	Study
Reunion Island	June-September	October-February	70-80	233.9	257.3	310	320	This study
South Africa east coast	-	probably November-December	60-70	225	225	299	300	[56]
South Africa east coast	prolonged mating season	-	80	235-250	235-250	276	284	[24]
South Africa east coast	-	-	60-70	247.2	250.9	279.2	285.3	[58]
Fiji	December-February	end of the calendar year	-	-	-	-	-	[42]
Gulf of Mexico-Florida	June-July	April-June	74-75	-	-	249	265	[43]
Gulf of Mexico-Florida	-	-	60-75	215	226	245	268	[23]
Gulf of Mexico-Florida	-	-	-	190-200	204	254	271	[25]
Lake Nicaragua-Rio San Juan system	no seasonal pattern	no seasonal pattern	50-75	160-165	160-170	-	235?	[17]
Brazil	-	November-February	70-82	-	-	-	275.5	[59]

385 L_{50} , size at maturity; TL_{max} , maximal total length. All sizes are in centimetres.

Weight-length relationships established for the bull shark in Reunion Island did not significantly differ for males and females, contrary to Cliff and Dudley [24] in South Africa. Nevertheless, their equations mostly differed for large individuals ($TL > 260$ cm), which have been less caught (especially females) in their study than in the present one. The relationship established here might thus be more accurate to estimate the weight of large individuals. Overall, equations established for South Africa and Reunion Island provided very similar results.

The size at maturity for both sexes was also larger than previously reported (Table 3 and references therein). Clasper sizes and calcification data indicated that males mature at ~ 234 cm, while uterus widths and presence of mature oocytes or embryos suggest a size at maturity of ~ 257 cm for females. Once again, sizes at maturity in Reunion Island are more similar to those estimated in South Africa [24, 58] than those of the Atlantic basin [17, 23, 25, 43, 59] (Table 3).

Overall, it seems that bull shark populations in the Western Indian Ocean are characterised by larger animals, with larger sizes at maturity, than in the Western Atlantic, which is consistent with the high genetic differentiation found between bull shark populations from the Atlantic and the Western Indian Ocean [53]. However the underlying mechanisms supporting this finding have to be identified.

Reproductive cycles

Among the 43 females that were caught during the period 2012 – 2017 in this study and that were potentially sexually mature (i.e. $TL \geq L_{50}$), 16 were gravid, representing 37% of the potentially mature females and 14% of the total sampling. This was well over what was reported in South Africa (8% of the mature females and less than 1% of the total sampling for the period 1978 – 1990, [24]) or in Florida (6.3% of the mature females and 3% of the total sampling for the period 1955 – 1964, [43]). The only other study reporting a high number of gravid females (65 over 200 sharks, representing 32.5% of the total sampling) was conducted in a known nursery area, the lake Nicaragua-Rio San Juan system [17]. Therefore, it is likely that female bull sharks use some areas along Reunion Island coasts as pupping and nursery grounds.

Litter sizes were variable (from 5 to 14 embryos), but fell in the range of previous studies [17-20]. Embryo length data presented here from 16 gravid females caught in eight different months of the

year provide the most complete picture of bull shark gestation to date. Indeed the only study with a higher number of litters included three collecting periods only, from June to August, a week in April, and four days in November [17].

Here, the largest mean length per litter was of 77.94 ± 0.39 cm. Nonetheless, two free-swimming juveniles measuring less than 70 cm were collected, suggesting a size at birth between 60 and 80 cm. Other studies reported sizes at birth varying from 50 – 75 cm in the lake Nicaragua-Rio San Juan system [17], to 80 cm in South Africa [24] and Brazil [59] (Table 3). In Reunion Island, litters sampled from October to December with mean embryos *TL* varying from 65.47 ± 0.46 cm to 77.94 ± 0.39 cm are thus likely to be near-parturition litters, indicating the end of the gestation at this period. According to anglers, new-borns are usually caught from the shore during the rainy season in Reunion Island, which extends from December to April. This reinforces the idea of a parturition period at the beginning of the austral summer, in October-November. Some studies also hypothesized a parturition time during the warm season, from November-December to February in the Southern Hemisphere [42, 56, 59], while around April-June in the Northern Hemisphere [43] (Table 3).

Assuming a linear growth of embryos in utero, gestation would begin in October of the previous year, suggesting thus a gestation period of 12 – 14 months. This period is extended compared to a gestation of 10 – 11 months proposed by Clark and von Schmidt [43] based on eight litters gathered from different studies and maybe underestimated. In addition, it was based on bull sharks from Florida and the Gulf of Mexico that seem to be smaller than individuals from Reunion Island. Therefore it is not excluded that gestation is longer in Reunion Island in relation with larger new-borns (Table 3). Such gestation duration remains in the range proposed for other shark species, such as the school shark *Galeorhinus galeus* (12-month gestation followed by a 24-month resting period, [60]) or the dusky shark *Carcharhinus obscurus* (22-month gestation and a one-year resting phase, [61]). It may remain in accordance with the biennial cycle proposed by Brunnschweiler and Baensch [42] and that is typical for carcharhinids [30, 43], but some further data would be needed to refine the description of the reproductive cycle.

Mating likely occurs during the cold season in Reunion Island from June to September. Indeed one male with oedematous claspers was caught in August 2015, correlated to higher *GSI* between July and September. Similarly, most females presenting mature oocytes were caught from June to

September. As gestation likely begins in October, sperm may be stored in the oviducal gland for some months between mating (i.e. insemination) and fertilization. Indeed, while not yet observed in the bull shark [17], sperm storage has been reported in many shark species [62], including several carcharhinid species [63]. The higher *GSI* observed for females in November (mean $GSI = 0.32 \pm 0.05\%$) may thus be linked to ovulation. Thus sperm storage would lead to an extended period of fertilization allowing females to store enough energy to invest in the production of offspring enhancing consequently the reproductive success [63], while limiting the mating period, which can be costly (injuries from males).

Some variability in the mean size of embryos during a same month (i.e. March and December) was observed. This could be due to i) sperm storage for a longer period leading to a delay of the gestation starting date (S1a Fig.), ii) an extended mating period as some individuals presenting sperm and mature oocytes were also recorded in December (with the reserves raised in the Results Section about the dependence on the fishing effort and the stochasticity due to very small sample size), or iii) that the *in utero* growth is not linear, but rather logarithmic with accelerated growth at the beginning of the gestation (S1b Fig.). This would imply a gestation starting date in January-February, i.e. a 10-11 months gestation in accordance with previous results. Unfortunately, the lack of litters with very small embryos in our sampling prevents us to test this last hypothesis. All in all, a natural variability may exist in the timing of reproduction and the development of embryos in relation to the experience and the physiological state of the females. The triggers of the reproduction are not yet documented, but one could imagine that it is related to the sea temperature or the food availability. These two parameters are both subjected to annual variability that is reflected by the variability observed in our results, these latter gathering data over six years.

Multiple paternity

Over 16 bull shark litters investigated, using allele manual counting, nine (56.25%) exhibited clear evidence of multiple paternity. According to the full pedigree likelihood method, two more litters may also be issued from several males, leading to a multiple paternity frequency of 68.75% (11 litters). The percentage of polyandrous litters in the bull shark is thus comparable to those of other sharks of the genus *Carcharhinus*, such as *Carcharhinus plumbeus* (85% of polyandrous litters, [64]) or *Carcharhinus amblyrhynchos* (66% of polyandrous litters, [65]). Multiple paternity is

thought to occur more frequently in philopatric species displaying low dispersal rates, as it may reduce the probability of breeding between relatives, thereby decreasing inbreeding depression [39, 66, 67]. Philopatry in the bull shark has already been documented [22, 26-29, 53], notably for females. In addition, males grasp females during copulation [16], inducing wounds to these latter [42]. So females may adopt a multiple mating strategy to reduce harassment cost, also called convenience polyandry [52, 68, 69]. No correlations were found between the number of sires and the number of embryos in the litter, which would mean that this multiple paternity does not lead to genetic benefits, confirming the hypothesis of convenience polyandry [21]. Besides, within a same litter, embryos sired by distinct fathers presented similar sizes, reinforcing the idea of the uncoupling between mating and fertilization, with sperm from all males being stored for some time before simultaneous fertilization of all eggs.

Furthermore we detected several males that each sired several litters, either conceived during the same mating season (five males; i.e. 21% of the sires of the 16 litters studied) or during distinct ones. This indicates that male bull sharks are polygynous. From our data, it does not seem that males select females based on their size, as no significant correlation was found between the size of the mother and the number of sires. Males that sired different litters did not always contribute to the litters in the same proportions (some siring always one embryo per litter and others, either one or several). Noteworthy, only one male contributed significantly to the two litters it sired. Besides, within all the polyandrous litters investigated here, an important paternal skew was highlighted, except for the one with the smallest embryos (Litter 8: mean embryos $TL = 27.80 \pm 0.50$ cm), for which two males participated almost equally to the litter. Mechanisms of paternal skews in bull sharks may thus be related to female choice, timing/order of males mating and sperm competition [39, 69-71]. Without excluding the mechanisms cited above, it may also point to post-zygotic processes, such as nutrients relocation between defective and viable embryos, as highlighted in the mouse *Mus musculus* [72].

The detection of polygyny indicates that male bull sharks may exhibit some mating site fidelity, coming back in a regular basis to specific places to mate. It also suggests that bull sharks may aggregate to mate, which would explain higher frequency of detection of tagged bull sharks recorded during the mating season (i.e. the cold season) close to the shore around Reunion Island [73]. Aggregation of individuals during a short and well-defined mating season may increase

encounter probability between partners and thereby promote polygamy. Polyandry, as well as polygyny, could also be an insurance against the possible infertility of some mates (either male or female) [74], explaining why some litters present only one sire (the other males that potentially mated with the female being infertile). Polyandry and paternal skew mechanisms in bull sharks, and sharks in general, are complex and require further investigations to be fully understood, as well as their implications for individual fitness and population dynamics.

Concluding remarks

The reproductive biology and behavior of the bull shark *Carcharhinus leucas* was described for the first time in Reunion Island (Western Indian Ocean). To our knowledge, this is also the most complete study to date on the biology of this species in general. Total length, size at maturity and size at birth are similar to those observed in South Africa and larger than those of individuals from the Atlantic, which is in accordance with the results of high genetic differentiation between these two regions [53].

Evidence of a seasonal reproductive cycle was highlighted, with parturition during the austral summer from October to December, and a gestation period of 12 – 14 months. Mating period most likely occurs during the austral winter from June to September, leading to possible aggregations of sharks near Reunion Island during this period [73]. Furthermore, the high number of gravid females caught compared to other studies tends to show that the bull shark presence close to the shores around Reunion Island is related to parturition. The time lag between the peak of *GSI* for males and females supports the idea that females store sperm for up to 4 months, which may ensure that females trigger fertilization when they are physiologically prepared. Finally, we provide evidence for frequent multiple paternity in the bull shark, as well as polygyny, both suggesting aggregation of bull sharks during the mating season, as well as some male fidelity to mating sites. These findings provide valuable information for the management of this species, both in Reunion Island and in the Indian Ocean.

Acknowledgments

We thank gratefully B. Rêche and D. Guyomard for support in providing samples. The study was financially supported by CHARC program and ECORECORUN program (DEAL). We acknowledge the Plateforme Gentyane of the Institut National de la Recherche Agronomique (INRA, Clermont-Ferrand, France) for guidance and technical support. Agathe Pirog was supported by a doctoral fellowship from Reunion regional council (European Social Fund).

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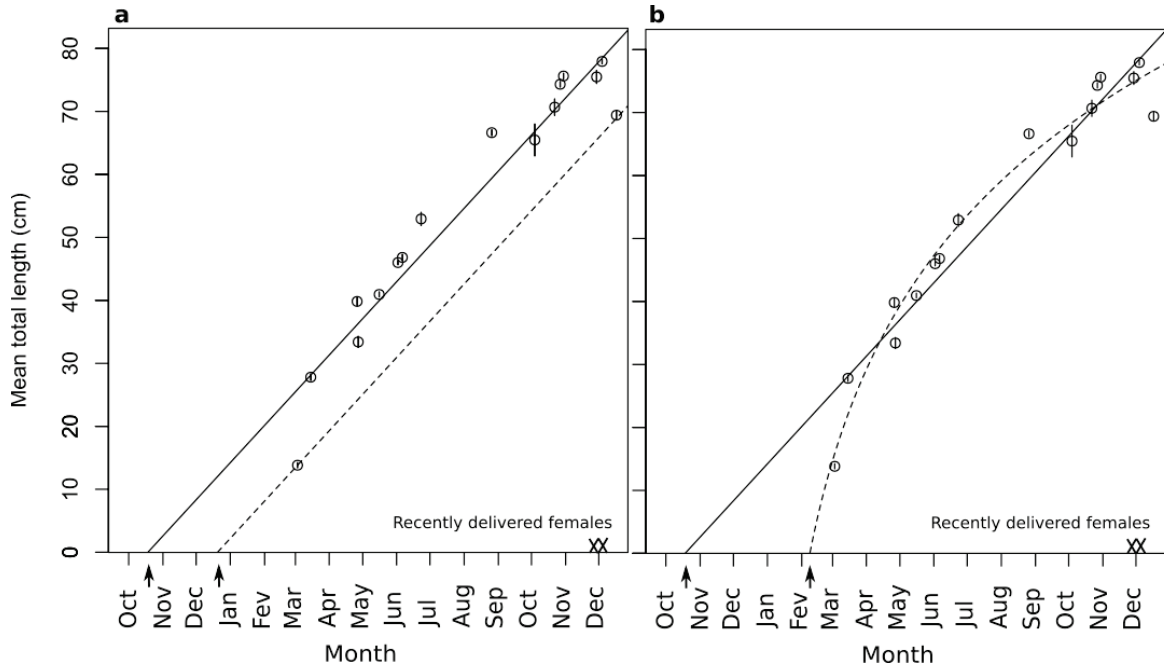
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Supporting information



S1 Fig. Mean embryos length for each litter plotted against day of the year in the bull shark *Carcharhinus leucas* ($n = 16$ litters). (a) Black line: linear regression linking the mean embryos length per litter (y) to the day of the year [x , coded as integers from 1 (1st January) to 365 (31st December)], $y = 0.19062x + 14.02362$ ($n = 16$, $t_{14} = 13.324$, $R^2 = 0.927$, $P = 2.41 \times 10^{-9}$); dotted line: translation of the first linear regression to fit the mean embryo sizes of the smallest litters caught in March and December ($y' = 0.19062x + 2$). (b) Black line: linear regression linking the mean embryos length per litter (y) to the day of the year [x , coded as integers from 1 (1st January) to 365 (31st December)], $y = 0.19062x + 14.02362$ ($n = 16$, $t_{14} = 13.324$, $R^2 = 0.927$, $P = 2.41 \times 10^{-9}$); dotted line: logarithmic regression linking the mean embryo length per litter (y) to the day of the year (x , coded as integers from 1 to 365), $y = 35.099 \times \log(x) - 129.009$ ($n = 16$, $t_{14} = 20.932$, $R^2 = 0.969$, $P = 5.796 \times 10^{-12}$).

Crosses indicate mature females with expanded, vacant uteri indicating they had delivered recently. The arrows indicate inferred beginning of gestation.

CHAPITRE 4. Diversité génétique et connectivité des populations du requin tigre *Galeocerdo cuvier*

Résumé

Chez le requin tigre *Galeocerdo cuvier*, les études de génétique des populations sont très récentes, datant de ces deux dernières années (2016 et 2017). Cette espèce, comme les autres requins, est difficile à étudier par des méthodes directes, d'autant qu'elle semble plus océanique que la plupart des Carcharhinidés. Par ailleurs, ce prédateur apical est soumis à diverses pressions d'origine anthropique ou naturelle au sein de son aire de répartition, ce qui peut impacter indirectement l'ensemble des communautés des écosystèmes qu'il occupe. Alors que la structure génétique mondiale des populations de requin tigre a déjà été étudiée, plusieurs questions restent irrésolues, notamment la structure génétique des populations des océans Indien et Pacifique, ainsi que l'histoire démographique de ces populations. Nous avons étudié ici ces paramètres, grâce à l'analyse de la diversité génétique de 286 individus échantillonnés dans l'Ouest de l'océan Indien et dans l'Ouest de l'océan Pacifique, en se fondant sur 27 marqueurs microsatellites et trois marqueurs mitochondriaux (*CR*, *COI*, *cytb*). Une faible (voire inexistante) différenciation génétique a été identifiée au sein de cette région, illustrant ainsi une importante connectivité génétique. Cela confirme les résultats des études précédentes, et est en accord avec les déplacements à grande échelle en milieu océanique réalisés par cette espèce. Par ailleurs, en utilisant le calcul bayésien approché afin de coupler l'information des deux types de marqueurs utilisés, un goulot d'étranglement récent a été détecté, ayant eu lieu pendant l'Holocène (il y a 2 000-3 000 ans). Ce goulot est probablement responsable de la faible diversité génétique trouvée chez le requin tigre, et une taille efficace très faible (111 individus) a été estimée lors de la réduction de la population. Ces résultats montrent que la population de requin tigre de l'Ouest de l'océan Indien et de l'Ouest du Pacifique est probablement très réduite et vulnérable aux pressions auxquelles elle est soumise. Des mesures de protection sont ainsi nécessaires pour la préserver.

Population connectivity and effective population size variations in the apex predator *Galeocerdo cuvier*

Agathe Pirog¹, Sébastien Jaquemet¹, Jeremy Cliff^{2,3}, Bonnie J. Holmes⁴, Nigel E. Hussey⁵, John Nevill⁶, Andrew J. Temple⁷, Laurent Vigliola^{8,9}, Hélène Magalon^{1,9}

¹ UMR ENTROPIE (Université de La Réunion/IRD/CNRS), Université de La Réunion, Saint Denis, La Réunion, France

² KwaZulu-Natal Sharks Board, Umhlanga Rocks, South Africa

³ Biomedical Resource Unit, University of KwaZulu-Natal, Durban, South Africa

⁴ School of Biomedical Sciences, University of Queensland, St Lucia, Brisbane, Qld., Australia

⁵ Great Lakes Institute for Environmental Research, University of Windsor, Windsor, ON, Canada

⁶ Environment Seychelles, Seychelles

⁷ School of Marine Science & Technology, Newcastle University, Newcastle-upon-Tyne, United Kingdom,

⁸ UMR ENTROPIE (Université de La Réunion/IRD/CNRS, Institut de Recherche pour le Développement, Nouméa, New Caledonia

⁹ Laboratoire d'Excellence CORAIL, Perpignan, France, www.labex-corail.fr

Keywords: approximation bayesian computation, bottleneck, effective population size, *Galeocerdo cuvier*, microsatellite DNA, mitochondrial DNA, population genetics, tiger shark

Corresponding Author:

Magalon Hélène

UMR ENTROPIE, Université de La Réunion, 15 Avenue René Cassin, CS 92003, 97744 Saint Denis Cedex 09, La Réunion, France

Email address: helene.magalon@univ-reunion.fr

Orcid number: 0000-0002-7061-955X

Running title: Population genetics of the tiger shark

Abstract

Population genetics have been increasingly applied to study large bodied sharks in the last 10 years. These elusive species are indeed difficult to study with direct methods, and yet knowledge is needed to assure both population management and preservation of these species especially sensitive to anthropogenic and climate pressures. It is notably the case for the tiger shark *Galeocerdo cuvier*, an apex predator likely playing important direct and indirect roles in marine ecosystems. While worldwide population genetic structure of this species has already been investigated, questions remain, notably on the population structure and demography history in the Western Indian Ocean and in the Western Pacific. We investigated these patterns by analysing the genetic diversity of 286 individuals using 27 microsatellite loci and three mitochondrial genes (*CR*, *COI*, *cytb*). A weak genetic differentiation was observed in this region, implying high genetic connectivity. This confirms previous studies, and the important oceanic use of this species. Using Approximate Bayesian Computation and coupling information from both marker types, a recent bottleneck occurring in the Holocene (2,000-3,000 years ago) was evidenced. It was probably responsible for the low genetic diversity observed, and an effective population size as low as 111 was estimated during the bottleneck. Altogether, these results point to a population extremely sensitive to pressures that may be depleted, and conservation measures are thus needed to preserve it.

Introduction

The tiger shark *Galeocerdo cuvier* is, with the great white shark *Carcharodon carcharias* and the whale shark *Rhincodon typus*, among the largest sharks that present specific challenges for researchers and managers. These species spend part of their lifetime in open and deep waters, rendering them difficult to observe and study, resulting in a paucity of data concerning basic aspects of their biology, such as migration patterns and population structure (Conrath, Musick, Carrier, & Heithaus, 2012; Musick, 2010). Their role in marine ecosystems remains also poorly understood, but, as marine predators, they are supposed to exercise an important function in marine food webs via top-down processes (Dudley & Simpfendorfer, 2006; Ferretti, Worm, Britten, Heithaus, & Lotze, 2010; Myers, Baum, Shepherd, Powers, & Peterson, 2007). A better understanding in the biology and the ecology of these sharks is thus needed to insure their conservation, especially because they present reproductive patterns specific of a K-strategy, with slow growth rate, late maturity and low fecundity (Musick, Burgess, Cailliet, Camhi, & Fordham, 2000), rendering them sensitive to over-exploitation and slow to rebound from depletion (Campana & F., 2016; Cortés, 2002; Dudley & Simpfendorfer, 2006; Ferretti et al., 2010; Myers & Worm, 2005; Worm et al., 2013). Furthermore, while their conservation remains a challenge, some of these species, e.g. the great white shark and the tiger shark, are also responsible of attacks on humans and a better knowledge of their biology is needed to set up adapted management plans while preserving their populations. These issues are maybe best exemplified by the tiger shark, which, being less emblematic than the great white shark, remains less studied, while it presents specific patterns of migration, reproduction and is exploited for its fins.

The tiger shark *G. cuvier* is a large carcharhinid measuring up to 5.5 m long (Meyer et al., 2014), present worldwide in tropical and subtropical waters (Compagno, 1984, 1990). It is described as a potentially keystone species in marine ecosystems by inflicting mortality on their preys or inducing behavioural modifications of these latter, thus modifying indirectly primary producer community structure, biomass and nutrient composition (Burkholder, Heithaus, Fourqurean, Wirsing, & Dill, 2013; Heithaus, Frid, Wirsing, & Worm, 2008; Wirsing, Heithaus, & Dill, 2007). While the tiger shark is listed as globally 'Near Threatened' by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Simpfendorfer, 2009), Clarke et al. (2006) estimated that approximately 400,000-500,000 tiger sharks are fished per year in the shark fin trade worldwide.

Furthermore, this species is a major catch of several shark control programs (Simpfendorfer, 2009), notably in the Indian and Pacific Oceans (further designated as Indian/Pacific Oceans), with long-term existing control programs in Australia (Holmes et al., 2012; Reid & Krogh, 1992; Simpfendorfer, 1992), South Africa (Cliff & Dudley, 1991; Dudley, 1997; Sumpton, Taylor, Gribble, McPherson, & Ham, 2011) and Hawaii (Wetherbee, Lowe, & Crow, 1994) and is also reported as bycatch in pelagic fisheries, notably in the Western Pacific (Polovina & Lau, 1993) and in the Southern (Afonso & Hazin, 2014) and North-Western (Baum et al., 2003) Atlantic. Trends in long-term catch and catch rates are difficult to obtain for sharks not specially targeted by fisheries. Nevertheless control programs established for decades as well as the use of logbook on longline fleets allow collecting data over prolonged temporal scales using standardised fishing methods, in order to assess the variation of population size through time. Using these data, tiger shark catch rates were shown increasing in KwaZulu-Natal, in South Africa (Dudley & Simpfendorfer, 2006), while declining notably in Queensland (Holmes et al., 2012) and New South Wales (Reid, Robbins, & Peddemors, 2011) in Australia and in the Northern Atlantic (Baum et al., 2003; Myers et al., 2007), likely related to population declines. Furthermore, these data are not useful only to assess population size trends, but also to understand habitat use patterns. Indeed, data of longline pelagic fisheries highlighted an important use of the oceanic realm by this species described as coastal (Domingo et al., 2016; Polovina & Lau, 1993), revealing a diversity of habitats used, which is confirmed by other data. Indeed, mark-recapture studies and satellite tagging have shown a wide heterogeneity of habitat use and foraging strategies in tiger shark populations across a range of spatial and temporal scales.

Some individuals move over very long distances (Ferreira et al., 2015; Hammerschlag, Gallagher, Wester, Luo, & Ault, 2012; Holmes et al., 2014; Lea et al., 2015; Werry et al., 2014), with records of two tiger sharks that crossed the Atlantic from South America and from North America to the East coast of Africa, travelling thousands of kilometres, respectively (Afonso, Garla, & Hazin, 2017; Kohler, Casey, & Turner, 1998; Kohler & Turner, 2001), while another one crossed the Indian Ocean from Australia to South Africa, travelling 8,000 km in 99 days (Heithaus, Wirsing, Dill, & Heithaus, 2007). On the other hand, tracking studies also revealed strong residency patterns, with some individuals maintaining large but defined home ranges and returning to specific places on a regular basis (Ferreira et al., 2015; Fitzpatrick et al., 2012; Heithaus, 2001; Holland, Wetherbee, Lowe, & Meyer, 1999; Lowe, Wetherbee, & Meyer, 2006). These patterns seem to be

linked to intrinsic states such as size and sex, but also to extrinsic drivers, notably prey abundance and water temperature. Indeed, smaller sharks do not use similar habitats than larger ones, probably to avoid predation, and use mainly coastal areas also richer in preys (Lowe, Wetherbee, Crow, & Tester, 1996). On the other hand, females use more shallow edge areas than males, and even if the reason of this difference remains unclear (Heithaus, Hamilton, Wirsing, & Dill, 2006), it may be linked to reproductive behaviours, with females avoiding male harassment (Sulikowski et al., 2016) or going to specific breeding places (Papastamatiou et al., 2013). Foraging strategies influence habitat use, and evolve as tiger sharks mature. Specific exploratory movements that enable sharks to discover new foraging grounds have been highlighted, which probably explains why foraging strategies are individual-specific (Heithaus et al., 2006; Holland et al., 1999; Meyer, Clark, Papastamatiou, Whitney, & Holland, 2009; Meyer, Papastamatiou, & Holland, 2010; Papastamatiou et al., 2011). Seasonal migrations related to prey abundance have been identified, notably in the French Frigate Atoll (Hawaii). Several tiger sharks visited the atoll in summer to forage on fledging albatrosses, before migrating over thousands of kilometres along the Hawaiian archipelago and coming back in subsequent years (Lowe et al., 2006; Meyer et al., 2010). These seasonal migrations may also be related to water temperature changes, as highlighted in Australia, with tiger sharks being less detected by acoustic receivers and lower catch rates during the cold season (June-August), when water temperatures drop below 19-20°C (Heithaus, 2001; Holmes et al., 2014; Wirsing, Heithaus, & Dill, 2006). All of these studies highlighted the complex migration patterns and habitat use of tiger sharks, but allowed to suggest that tiger sharks may occupy very large home ranges and demonstrate small-scale specific patterns of movement and habitat use due to a mix of intrinsic and extrinsic drivers. The consequences of these patterns on population connectivity and structure have only been recently studied using molecular markers.

The first study investigating population genetic dynamics of the tiger shark was primarily designed for species delimitation, and only examined 29 samples of tiger shark collected throughout the species distribution range with the mitochondrial NADH dehydrogenase subunit (ND2) gene (Naylor et al., 2012). They identified two monophyletic clades in the Atlantic and the Indian/Pacific Oceans, with no shared haplotype, and hypothesized the presence of two subspecies. This hypothesis was further refuted by Bernard et al. (2016), who used a wider sampling of 380 individuals from several sampling sites in the three oceanic basins, 10 microsatellite loci and two mitochondrial genes, the control region (*CR*) and the cytochrome oxidase c subunit I (*COI*). They

highlighted a long-term genetic isolation between tiger shark populations of the Atlantic and the Indian/Pacific Oceans, but with shared mitochondrial haplotypes, which is inconsistent with the two-subspecies hypothesis. Furthermore, samples from Hawaii seemed genetically differentiated from all other locations, which was hypothesized to be due to the more regionally migratory behaviour of these sharks highlighted by tagging studies (Meyer et al., 2010; Papastamatiou et al., 2013). Bernard et al. (2016) also identified a slightly stronger genetic differentiation with mitochondrial than with microsatellite data in the Indian/Pacific Oceans, even between Western and Eastern Australian coasts, perhaps linked to a stronger matrilineal structure due to sex-biased dispersal and female philopatry. Another study focused on the population structure of tiger sharks across the Eastern Indian Ocean and the Pacific, with 355 samples collected mainly around Australia (one location of the Western coast, one of the Northern coast, four along the Eastern coast) and from Hawaii, and eight samples from Brazil ($n = 8$) used as an outgroup (Holmes et al., 2017). Using nine microsatellite loci, Holmes et al. (2017) confirmed the genetic isolation of the Western Atlantic and the Indian/Pacific Oceans and the high connectivity around Australia, as found by Bernard et al. (2016) and with satellite tracking. Nevertheless they found contrasting results for samples from Hawaii, which were not genetically differentiated from other locations along Australian coasts, contrary to what was found by Bernard et al. (2016). The authors explain this discrepancy by the lower sample size from Eastern Australia (21 samples) used by Bernard et al. (2016) to assess F_{ST} and D_{est} estimates and to the STRUCTURE analysis, for which Bernard et al. (2016) chose to keep the results for three genetic clusters, while ΔK suggested two genetic clusters.

In the present study, we used a more intensive sampling in the Western Indian Ocean (244 samples from five locations) and samples from the Western Pacific (42 samples from three locations) with 27 microsatellite loci and three mitochondrial genes (*CR*, *COI* and *cytb*) to further investigate population structure and demographic patterns in the tiger shark *Galeocerdo cuvier*. As we used samples in common with Holmes et al. (2017) and the nine microsatellite loci they used, we were able to add their samples to ours to obtain a more precise picture of the population genetic dynamics of the tiger shark in the Indian and Pacific Oceans. We also added *CR* sequences obtained by Bernard et al. (2016) to ours, to further investigate the mitochondrial structure they highlighted in these oceans.

Material and Methods

Sampling

Samples were collected in five locations in the Western Indian Ocean (Zanzibar, ZAN: $n = 8$; South Africa, SAF: $n = 34$; the Seychelles, SEY: $n = 24$; Madagascar, MAD: $n = 11$; Reunion Island, RUN: $n = 167$), and in the Eastern Indian Ocean, along Australian Western coast (AUS1: $n = 9$). Samples were also collected in the Western Pacific on the North-East coast of Australia (Queensland, AUS2: $n = 10$) and in New Caledonia (NCA: $n = 23$; Figure 1). In Madagascar, samples were collected on teeth found in markets, and identified thanks to their morphology, specific of the species (Fulgosi & Mori, 1979). Species identity was also confirmed *a posteriori* thanks to the *CR* sequence. All other samples came from individuals caught by fishermen or fishing programs and from scientific projects (i.e. biopsies). All samples were preserved in 90% ethanol.

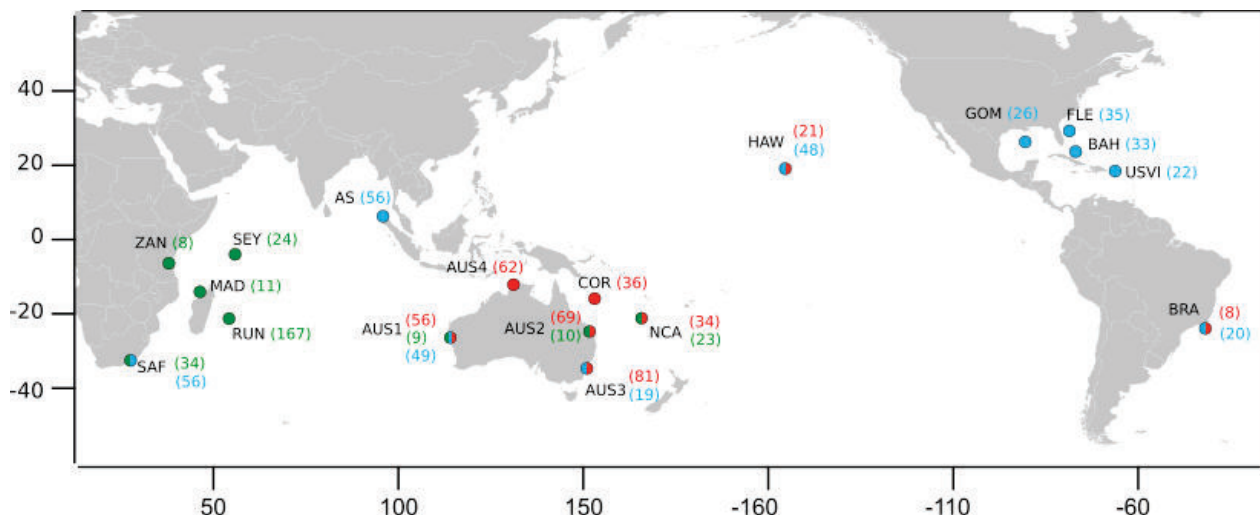


Figure 1. Map of tiger shark (*Galeocerdo cuvier*) sampling locations (ZAN, Zanzibar; SEY, Seychelles; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; AS, Andaman Sea; AUS1, Western Australian coast; AUS2, Queensland, Australia; AUS3, New South Wales, Australia; AUS4, Northern Territories, Australia; COR, Coral Sea; NCA, New Caledonia; HAW, Hawaii; GOM, Gulf of Mexico; FLE, Florida East Coast; BAH, Bahamas; USVI, US Virgin Islands; BRA, Brazil). In brackets are sample sizes. In green are indicated samples collected for this study and genotyped with 27 microsatellite loci and three mitochondrial genes. In red are indicated samples genotyped in Holmes et al. (2017) with the nine microsatellite loci developed by Bernard, Feldheim, and Shivji (2015). In blue are samples sequenced at the control region by Bernard et al. (2016).

Laboratory procedures

Genomic DNA was extracted using Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following manufacturer instructions, except for samples coming from Madagascar for which teeth were ground and then lysed overnight using 380 μ L of ATL and 20 μ L of Proteinase K.

The genotyping of all samples was performed at 30 microsatellite loci. Twenty-six of them were species-specific loci: Gc01 to Gc08 developed by Pirog, Jaquemet, Blaison, Soria, and Magalon (2016), the nine TGR-loci developed by Bernard et al. (2015), and the nine TIG-loci developed by Mendes et al. (2016). The remaining microsatellite loci were originally developed for the bull shark *Carcharhinus leucas* (Cl12, Cl14 and Cl17; Pirog, Blaison, Jaquemet, Soria, & Magalon, 2015) and the blacktip shark *Carcharhinus limbatus* (Cl100; Keeney & Heist, 2003), and successfully cross-amplified in the tiger shark. Loci Gc01 to Gc08 and Cl12, Cl14 and Cl17 were directly fluorochrome-labelled (using 6-FAM, PET, VIC or NED) and PCR reactions were performed following Pirog et al. (2016). Other loci were indirectly fluorochrome-labelled (using 6-FAM, PET, VIC or NED) and PCR reactions were performed following G elin, Postaire, Fauvelot, and Magalon (2017). All loci were multiplexed post-PCR in five panels (Supplementary Material S1). PCR products were genotyped using an ABI 3730XL capillary sequencer at the Plateforme Gentyane (INRA, Clermont-Ferrand, France). Allelic sizes were determined with GENEMAPPER v.4.0 (Applied Biosystems) using an internal size standard (Genescan LIZ-500, Applied Biosystems). From the nine loci developed by Bernard et al. (2015), we did not keep TGR233 as we found it difficult to read. From the loci developed by Mendes et al. (2016), we did not keep TIG05 that was difficult to read with a lot of failed amplifications, nor TIG25 that was monomorphic overall our samples. We thus kept 27 microsatellite loci for further analyses.

Additional genotyped and sequenced individuals from Holmes et al. (2017) and Bernard et al. (2016) were also added in the present study (Figure 1; Table 1). Individuals from Australia in this study are the same as in Holmes et al. (2017) in which they were genotyped with the nine microsatellite loci developed by Bernard et al. (2015). For these individuals and the eight microsatellites in common between the present study and Holmes et al. (2017), the genotypes were compared and allele lengths calibrated. To ensure the allele frequency bins were uniform between the studies at each locus, alleles frequencies were plotted and compared for each sampling location (Supplementary Material S2). We thus added genotypes from all the individuals from Holmes et

al. (2017) (Figure 1 and Table 1) enlarging the geographic coverage of our sampling: northern and south-eastern Australian coasts, Coral Sea, Hawaii and Brazil. We also expanded the number of individuals for some localities in common: western (AUS1) and north-eastern (AUS2) Australian coasts, and New Caledonia (Figure 1, Table 1).

Moreover, for some analyses (DAPC, see below), we also used the microsatellite genotypes from Bernard et al. (2016) keeping only the eight microsatellites in common with our study and Holmes et al. (2017). However we could not add them to our genotypes as we did not have individuals in common to calibrate the reading of the electrophoregrams.

In summary, four microsatellite datasets were used (Table 1): (1) 27-msat dataset (the 27 microsatellites on the individuals from this study only), (2) 8-msat dataset (the 8 microsatellites in common for all studies on the individuals from this study and those from Holmes et al. (2017) only), and (4) Bernard 8-msat dataset (the 8 microsatellites in common for all studies on the individuals from Bernard et al. (2016) only).

The mitochondrial DNA control region (*CR*) was amplified using the set of primers Gc-CR-F/Gc-CR-R (Pirog et al., 2016), the cytochrome oxidase c subunit I (*COI*) using the primer cocktails C_FishF1t1/C_FishR1t1 (Ivanova, Zemlak, Hanner, & Hebert, 2007; Ward & Myers, 2005) as described in Wong, Shivji, and Hanner (2009) and the cytochrome b (*cytb*) using GluDG/C61121H (Naylor, Ryburn, Fedrigo, & Lopez, 2005). This was performed for subsets of the whole dataset: 200 individuals for *CR*, 147 individuals for *COI* and 130 for *cytb*. Primers were used for both amplification and direct sequencing. PCR reactions were performed in a total volume of 25 μ L: 1X of MasterMix (Applied Biosystems), 0.3 μ M of forward and reverse primers/cocktails and 1.6 ng/ μ L of genomic DNA. The thermocycling program for *CR* is described in Pirog et al. (2016). For *COI* and *cytb*, the same program was used, except that the PCR annealing temperature was set to 53°C. Amplicons were sent for sequencing to Genoscreen (Lille, France) on a capillary sequencer ABI 3730XL (Applied Biosystems).

Moreover, to complement the mitochondrial analyses, *CR* sequences generated by Bernard et al. (2016) (GenBank accession numbers: KU847364-KU847386) were added to our dataset (named hereafter *CR* dataset), adding samples from 10 locations for *CR* analyses (Figure 1; Table 1): Gulf

of Mexico, Florida East Coast, Bahamas, US Virgin Islands, Brazil (referred as Western South Atlantic therein), South Africa (referred as Western South Indian Ocean therein), Andaman Sea, Western Australia (referred as Eastern South Indian Ocean therein), South-Eastern Australia (referred as Western South Pacific Ocean therein) and Hawaii (referred as Central Pacific Ocean therein).

Table 1. Summary of *Galeocerdo cuvier* sampling locations and number of individuals from this study, Holmes et al. (2017) and Bernard et al. (2016), as well as the molecular markers used in each study and the different datasets analysed in the present study. In bold are samples or molecular markers in common in several studies.

	This study	Holmes et al., 2017	Bernard et al., 2016
Sampling locations			
Western Indian Ocean			
ZAN	8		
SEY	24		55
SAF	34		
RUN	167		
Eastern Indian Ocean			
AS			31
AUS1	9	9+47	66
Western Pacific Ocean			
AUS4		62	
COR		36	
AUS2	10	10+59	
AUS3		81	21
NCA	23	22	
Central Pacific			
HAW		21	65
Northwestern Atlantic			
GOM			26
FLE			35
BAH			39
USVI			22
Southwestern Atlantic			
BRA		8	20
<i>microsatellite loci</i>	CI12; CI14; CI17 ¹ Gc01-Gc08 ²		
	Chi100 ³		Chi100
	TIG01; TIG07; TIG10; TIG12; TIG15; TIG17; TIG19 ⁴		
	TGR47; TGR212; TGR348; TGR891; TGR943; TGR1033; TGR1157; TGR1185⁵	TGR47; TGR212; TGR348; TGR891; TGR943; TGR1033; TGR1157; TGR1185	TGR47; TGR212; TGR348; TGR891; TGR943; TGR1033; TGR1157; TGR1185
		TGR233	TGR233
<i>mitochondrial loci</i>	CR; COI		CR; COI
	cytb		
<i>microsatellite datasets</i>	27-msat	Holmes 8-msat	Bernard 8-msat
		8-msat	
<i>mitochondrial datasets</i>	CR-COI-cytb		
	CR		CR

¹Pirog et al., 2015, ²Pirog et al., 2016, ³Keeney & Heist, 2003, ⁴Mendes et al., 2016, ⁵Bernard et al., 2015

Genetic diversity analyses

Null alleles and allelic drop-out occurrence and frequencies were assessed using MICROCHECKER v.2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004). Linkage disequilibrium (LD) between pairs of loci was tested using a likelihood-ratio test with 10,000 permutations in ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010). Diversity indices such as the number of alleles per locus N_a , observed and expected heterozygosities (H_O and H_E) and inbreeding coefficient F_{IS} (Weir & Cockerham, 1984) were assessed using FSTAT v.2.9.3.2 (Goudet, 1995). Departure from Hardy-Weinberg equilibrium (HWE) was tested using 5,000 permutations in FSTAT v.2.9.3.2 (Goudet, 1995). The mean allelic richness A_r and the mean private allelic richness A_{rp} were calculated using a rarefaction method, as implemented in HP-RARE v.1.0 (Kalinowski, 2005). This method accounts for differences in sample size by standardizing A_r and A_{rp} values across sampled locations by resampling the lowest number of genotypes available (i.e., 16 haploid gene copies or eight diploid genotypes in Zanzibar) in each location.

Mitochondrial sequences were quality checked and aligned using GENEIOUS v.8.1.2 (Kearse et al., 2012). Alignments were performed using the MAFFT method (Kato, Misawa, Kuma, & Miyata, 2002) for each gene separately first and then for the concatenated sequence *CR-COI-cytb*. Molecular diversity indices (i.e. number of haplotypes, number of segregating sites, haplotype (h) and nucleotide (π) diversities) were calculated for each region separately and for the concatenated dataset (called hereafter *CR-COI-cytb* dataset), using DNASP v.5.10.1 (Librado & Rozas, 2009).

Detection of partitioning schemes and substitution models within the concatenated sequence *CR-COI-cytb* was performed using PARTITIONFINDER v.2.1.1 (Guindon et al., 2010; Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017). BEAST v.1.8.4 (Drummond, Suchard, Xie, & Rambaut, 2012) was used to reconstruct phylogenetic relationships on the *CR* dataset, and on the mitochondrial concatenated *CR-COI-cytb* dataset. Bayesian Markov chain Monte Carlo (MCMC) analyses were performed assuming a HKY85 model of substitution as the latter was shown to best fit the data (see Results). Rate of variation among sites was modeled with a discrete gamma distribution with four rate categories. We assumed an uncorrelated lognormal relaxed clock to account for rate variation among lineages. To minimize prior assumptions about demographic history, we adopted an extended Bayesian skyline plot (EBSP) approach in order to integrate data over different

coalescent histories. Evolutionary model parameters were then estimated, with samples drawn from the posterior every 10^5 MCMC steps over a total of 10^8 steps from five independent runs. The first 10^7 steps were discarded as burn-in. Good mixing and convergence were assessed using TRACER v.1.6 (Rambaut, Suchard, Xie, & Drummond, 2014) and the best tree was selected using the maximum clade credibility option with TREEANNOTATOR v.1.8.4 (Drummond et al., 2012) and viewed with FIGTREE v.1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>). To further evaluate phylogenetic relations among haplotypes, TCS statistical parsimony networks (Clement, Posada, & Crandall, 2000) were constructed using POPART v.1.7 (Leigh & Bryant, 2015).

Population genetic structure

Two complementary clustering methods were used to investigate population structure in the tiger shark. First, Bayesian clustering analyses were performed using STRUCTURE v.2.3.4 (Falush, Stephens, & Pritchard, 2003; Pritchard, Stephens, & Donnelly, 2000). For any given number of clusters (K) between 1 and 10, individual assignment probabilities to each cluster were determined so as to minimize departures from HWE within clusters and maximize LD between clusters. Two analyses were performed, with and without the LOCPRIOR model, which uses prior sampling location information in the Bayesian clustering, to detect genetic population structure that might be weaker (Hubisz, Falush, Stephens, & Pritchard, 2009). Conditions were set to 10^6 chain length after a burn-in of 5×10^5 and 10 chains were run for each K , assuming correlated allele frequencies and the admixture model. For a given K , distinct modes were identified and, for each mode and each individual, the assignment probabilities to each cluster were averaged using CLUMPAK (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015). These analyses were performed on both 27-msat and 8-msat datasets. Secondly, a Discriminant Analysis of Principal Components (DAPC; Jombart, Devillard, & Balloux, 2010), that does not rely on HWE or LD to identify clusters contrary to STRUCTURE, was performed to check consistency between clustering methods based on different algorithms. This method transforms genotypes using PCA as a prior step to a discriminant analysis and defines clusters such as to minimize variations within them but maximize differentiation between them. DAPC was applied using the *adegenet* package (Jombart, 2008) for R (R Development Core Team 2017). This analysis was performed on the four microsatellite datasets (see Laboratory Procedures above). Methods traditionally used to detect the most likely

number of clusters within a dataset might provide different outputs. To cope with these inconsistencies, we chose to consider the highest number of clusters and the individual assignments that were retrieved by both analyses.

Assessing population differentiation between pairs of sampling locations, F_{ST} (Weir & Cockerham, 1984) and D_{est} (Jost, 2008) were estimated for the microsatellite data with ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010) and *DEMEtics* v.0.8-7 (Gerlach, Jueterbock, Kraemer, Deppermann, & Harmand, 2010), respectively, on both 27-msat and 8-msat datasets. Contrary to F_{ST} , which depends on within-population diversity and is affected by migration rates and effective population sizes, D_{est} , based on the effective number of alleles strictly reflects the genetic distance/differentiation between populations. For the mitochondrial *CR-COI-cytb* dataset as well as the *CR* dataset, the Φ_{ST} (Slatkin, 1995) was estimated using ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010). Significance of pairwise population differentiation indices was tested using 10,000 permutations.

Population demographics

To test for departures from a constant population size (Ramos-Onsins & Rozas, 2000), the summary statistics Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) were estimated from the concatenated mitochondrial dataset with ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010), with significance tested implementing 10^5 simulated samples.

Furthermore, to identify effective population size variations in the Western Indian and Western Pacific Oceans, as no population structure was highlighted between both regions (see Results), we used a coalescent framework through approximate Bayesian computation (ABC) using DIYABC v.2.1.0 (Cornuet et al., 2014) with both the 27-msat dataset and the *CR-COI-cytb* dataset. We defined N_0 the actual effective population size, N_I the ancestral effective population size, N_b the effective population size during a bottleneck, and N_e the effective population size during an expansion. Then seven scenarios were set up to test for: (Scenario 1) a recent (less than 500 generations) decrease ($N_0 < N_I$), (Scenario 2) a more ancient (between 10^3 and 5×10^5 generations in the past) decrease, (Scenario 3) a recent (less than 500 generations) expansion ($N_0 > N_I$), (Scenario 4) a more ancient (between 10^3 and 5×10^5 generations in the past) expansion,

(Scenario 5) an expansion followed by a decrease ($N_e > N_0, N_I$), (Scenario 6) a bottleneck ($N_b < N_0, N_I$) and (Scenario 7) a constant effective population size ($N_0 = N_I$) (Figure 2). For Scenarios 5 and 6, the end of the expansion/bottleneck was set at 5 generations in the past, which approximately corresponds to the interdiction of commercial exploitation of the tiger shark in Reunion Island (in 1999). Generation time was supposed to be around 7 -10 years (Branstetter, Musick, & Colvocoresses, 1987; Holmes et al., 2015; Kneebone, Natanson, Andrews, & Howell, 2008; Wintner & Dudley, 2000).

To run this analysis, we considered all the individuals from Reunion Island, as it is the location with the highest number of individuals and may be the most representative of the genetic diversity of the whole population. It has indeed been shown that pooling individuals from different sampling locations even with non-significant pairwise differentiation values may bias results (Lombaert et al., 2014). Nevertheless, the analysis was also run using only samples from New Caledonia ($n = 23$), to comfort the results.

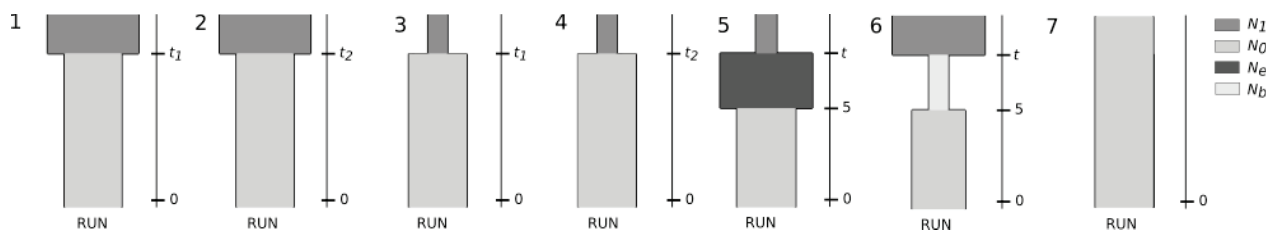


Figure 2. Graphical representations of the seven scenarios depicting possible variations of effective population size of *Galeocerdo cuvier* population, using individuals from Reunion Island (RUN). The time was measured backward in generations before present. N_0 , the actual effective population size; N_I , the ancestral effective population size; N_b , the effective population size during a bottleneck; N_e , the effective population size during an expansion; t_1 , beginning of decrease or expansion for Scenarios 1 and 3; t_2 , beginning of decrease or expansion for Scenarios 2 and 4; t , beginning of the expansion or bottleneck period for Scenarios 5 and 6.

For each scenario, 10^6 simulated datasets were run. To select the best-fit scenario, posterior probabilities were computed via logistic regression on the 1% of simulated datasets closest to the empirical data (Cornuet et al., 2008). Summary statistics were transformed by linear discrimination analysis prior to logistic regression to reduce correlation among explanatory variables and provide conservative estimates of scenario discrimination (Estoup et al., 2012). Posterior distributions of all parameters were then estimated from the selected model, based on the 1% of simulated datasets

closest to the empirical data. More details on the ABC analysis are provided in Supplementary Material S3.

Results

Genetic diversity analyses

Samples from Madagascar, all extracted from teeth, exhibited high amount of missing data (more than 50%) due to low-quality DNA and were not used for subsequent analyses.

27-msat dataset

Null alleles were detected for several loci in several sampling locations but were not constant among locations and were not correlated with significant deviations from HWE. All loci were thus kept for further analyses. Global significant LD was detected for 21 tests over 2,457 after FDR correction (0.85%, $P < 0.05$) only, and all loci were thus considered independent. The mean rarified allelic richness (\pm standard error SE) was rather constant among locations, varying from 2.86 ± 0.30 in AUS1 (Western Australia) to 3.05 ± 0.28 in New Caledonia while H_E varied from 0.52 ± 0.06 in Reunion Island to 0.60 ± 0.05 in Zanzibar and H_O from 0.49 ± 0.05 in South Africa to 0.60 ± 0.06 in Zanzibar (Table 2). No significant deviation from HWE was retrieved for any location. The mean rarified private allelic richness varied from 0.11 ± 0.04 in AUS1 (Western Australia) to 0.19 ± 0.05 in New Caledonia (Table 2) and was rather constant among locations.

8-msat dataset

The mean rarified allelic richness varied from 4.63 ± 0.73 in Brazil to 5.89 ± 1.11 in Coral Sea while H_E and H_O varied from 0.66 ± 0.04 in South Africa to 0.72 ± 0.07 in Brazil and from 0.59 ± 0.04 in South Africa to 0.73 ± 0.07 in Brazil, respectively (Table 2). No significant deviations from HWE were retrieved. The mean rarified private allelic richness was rather constant among locations but was higher for Brazil, varying from 0.07 ± 0.04 in New Caledonia to 0.49 ± 0.17 in Brazil (Table 2).

Table 2. Summary statistics for each sampling location (ZAN, Zanzibar; SEY, Seychelles; SAF, South Africa; RUN, Reunion Island; AUS1, Western Australian coast; AUS2, Queensland, Australia; AUS3, New South Wales, Australia; AUS4, Northern Territories, Australia; COR, Coral Sea; NCA, New Caledonia; HAW, Hawaii; BRA, Brazil) for the 27-msat and 8-msat datasets. Populations are ordered along a west to east and north to south gradient beginning at locations from the Eastern African coast.

27 loci	<i>N</i>	<i>A_r</i>	<i>A_{rp}</i>	<i>H_o</i>	<i>H_E</i>	<i>F_{IS}</i>
ZAN	8	3.01 (0.33)	0.14 (0.05)	0.60 (0.06)	0.60 (0.05)	0.01
SEY	24	2.96 (0.31)	0.14 (0.04)	0.57 (0.05)	0.57 (0.05)	0.01
RUN	167	3.02 (0.28)	0.19 (0.04)	0.50 (0.05)	0.52 (0.06)	0.05
SAF	34	3.02 (0.30)	0.16 (0.04)	0.49 (0.05)	0.55 (0.05)	0.12
AUS1	9	2.86 (0.30)	0.11 (0.04)	0.55 (0.05)	0.58 (0.05)	0.05
AUS2	10	2.99 (0.30)	0.18 (0.06)	0.53 (0.05)	0.59 (0.05)	0.1
NCA	23	3.05 (0.28)	0.18 (0.03)	0.53 (0.06)	0.58 (0.05)	0.09
8 loci	<i>N</i>	<i>A_r</i>	<i>A_{rp}</i>	<i>H_o</i>	<i>H_E</i>	<i>F_{IS}</i>
ZAN	8	5.75 (1.10)	0.25 (0.15)	0.61 (0.12)	0.67 (0.11)	0.08
SEY	24	5.66 (1.13)	0.17 (0.12)	0.65 (0.06)	0.69 (0.05)	0.06
SAF	34	5.43 (1.02)	0.11 (0.06)	0.59 (0.04)	0.66 (0.04)	0.07
RUN	167	5.79 (1.04)	0.11 (0.04)	0.65 (0.02)	0.69 (0.02)	0.04
AUS1	56	5.76 (1.02)	0.11 (0.04)	0.68 (0.03)	0.70 (0.03)	0.01
AUS4	62	5.81 (1.06)	0.18 (0.08)	0.71 (0.03)	0.70 (0.03)	-0.04
COR	37	5.89 (1.11)	0.08 (0.05)	0.64 (0.04)	0.68 (0.04)	0.04
AUS2	74	5.80 (1.03)	0.08 (0.03)	0.68 (0.03)	0.70 (0.03)	0.01
AUS3	81	5.80 (1.08)	0.10 (0.07)	0.70 (0.03)	0.69 (0.03)	-0.03
NCA	34	5.53 (0.97)	0.07 (0.04)	0.62 (0.04)	0.67 (0.04)	0.06
HAW	21	5.85 (1.12)	0.10 (0.07)	0.67 (0.06)	0.68 (0.06)	0.01
BRA	8	4.63 (0.73)	0.49 (0.17)	0.73 (0.07)	0.72 (0.07)	-0.16

Mitochondrial diversity indices for the three mitochondrial genes

We obtained sequences of 862 bp for *CR* (Pirog et al., 2016), 652 pb for *COI* and 931 bp for *cytb*. For *CR*, as haplotypes defined by Bernard et al. (2016) started with 7 bp of the tRNA-Pro segment, with a mutation occurring at the 4th bp, we read our sequences including this portion, resulting in a sequence of 869 bp.

Using only our samples, the three genes resolved 9, 4, 14 haplotypes with 7, 3 and 12 polymorphic sites, respectively. Total haplotype diversity (*h*) was lower for *COI* (0.09 ± 00) than for *cytb* (0.47 ± 0.00) and *CR* (0.48 ± 0.00). Similar results were observed for the total nucleotide diversity (π), varying from 0.00014 ± 0.00000 for *COI* to 0.00063 ± 0.00001 and 0.00068 ± 0.00000 for *cytb*

and *CR*, respectively. Overall, variations of haplotype and nucleotide diversities were not constant across locations and across genes, with lowest values estimated for Reunion Island ($h = 0.34 \pm 0.01$ and $\pi = 0.00047 \pm 0.00005$) and Zanzibar ($h = 0.00 \pm 0.00$ and $\pi = 0.00000 \pm 0.00000$) and highest values for AUS1 (Western Australia; $h = 0.78 \pm 0.44$ and $\pi = 0.00115 \pm 0.00032$) for *CR* and New Caledonia ($h = 0.61 \pm 0.03$ and $\pi = 0.00099 \pm 0.00018$) for *cytb*. For *COI*, nearly all locations showed null haplotype and nucleotide diversities, except for South Africa ($h = 0.07 \pm 0.01$ and $\pi = 0.00010 \pm 0.00004$), New Caledonia ($h = 0.09 \pm 0.02$ and $\pi = 0.00013 \pm 0.00006$) and Reunion Island ($h = 0.21 \pm 0.01$ and $\pi = 0.00032 \pm 0.00007$; Supplementary Material S4).

The concatenated *CR-COI-cytb* dataset (2,452 bp; $N_S = 127$) resolved 22 haplotypes and 20 polymorphic sites, with an overall haplotype diversity of 0.78 ± 0.00 and a nucleotide diversity of 0.00053 ± 0.00000 (Table 3a). No partitioning schemes were detected within the concatenated sequence, and the HKY85 model of substitution was selected. Bayesian analysis on the *CR-COI-cytb* dataset showed good convergence and mixing, with ESS above 200 (Supplementary Material S5). Nevertheless, no lineages were strongly supported as no internal nodes showed high support. This was retrieved on the TCS statistical parsimony network built from the *CR-COI-cytb* dataset, with all haplotypes separated by only one or two mutations, and no clear geographic structuring (Figure 3a). Three main haplotypes were identified, represented by 50, 25 and 9 individuals from distinct locations.

Mitochondrial diversity on the CR dataset

Within the *CR* dataset, we analysed 538 *CR* sequences and resolved 25 haplotypes and 16 polymorphic sites, with an overall haplotype diversity h of 0.74 ± 0.00 and a nucleotide diversity π of 0.00280 ± 0.00000 (Table 3b). We retrieved only two new haplotypes compared to the study of Bernard et al. (2016), represented by three individuals (from the Western Indian Ocean). From locations in common in both studies, South Africa and AUS1 (Western Australia), h and π for the *CR* did not vary when adding individuals from Bernard et al. (2016) (Table 3b and Supplementary Material S4). As shown by the TCS network built, samples collected for this study presented the

three main haplotypes identified by Bernard et al. (2016) in the Indian and Pacific Oceans and none of the haplotypes identified in the Western Atlantic (Figure 3b).

Table 3. Summary statistics for each sampling location of *Galeocerdo cuvier* for **(a)** the 2,452 bp concatenated *CR-COI-cytb* dataset and **(b)** the *CR* dataset: N_S , number of individuals sequenced in this study; $N_{Bernard}$, number of individuals sequenced in Bernard et al. (2016); $N_{S+Bernard}$, total number of individuals sequenced; H , number of haplotypes, h , haplotype diversity; S , number of polymorphic sites; π , nucleotide diversity. ZAN, Zanzibar; SEY, Seychelles; SAF, South Africa; RUN, Reunion Island; AS, Andaman Sea; AUS1, Western Australian coast; AUS2, Queensland, Australia; AUS3, New South Wales, Australia; NCA, New Caledonia; HAW, Hawaii; GOM, Gulf of Mexico; FLE, Florida East Coast; BAH, Bahamas; USVI, US Virgin Islands; BRA, Brazil.

(a) CR_COI_cytb		N_S		H	S	h	π
ZAN		8		2	1	0.43 (0.06)	0.00018 (0.00007)
SEY		20		8	8	0.76 (0.02)	0.00053 (0.00009)
SAF		23		6	5	0.62 (0.02)	0.00035 (0.00006)
RUN		39		9	8	0.77 (0.01)	0.00049 (0.00006)
AUS1		9		5	4	0.86 (0.03)	0.00050 (0.00013)
AUS2		10		4	3	0.78 (0.03)	0.00042 (0.00011)
NCA		18		8	9	0.82 (0.02)	0.00062 (0.00010)
Total		127		22	20	0.78 (0.00)	0.00053 (0.00000)
(b) CR	$N_{Bernard}$	N_S	$N_{S+Bernard}$	H	S	h	π
ZAN	-	8	8	2	1	0.43 (0.06)	0.00049 (0.00020)
SEY	-	20	20	4	3	0.57 (0.02)	0.00081 (0.00016)
SAF	56	24	80	4	3	0.39 (0.01)	0.00048 (0.00005)
RUN	-	103	103	6	5	0.34 (0.01)	0.00047 (0.00005)
AS	31	-	31	5	4	0.52 (0.02)	0.00084 (0.00013)
AUS1	49	9	58	9	6	0.78 (0.00)	0.00138 (0.00013)
AUS2	-	10	10	3	2	0.69 (0.03)	0.00095 (0.00026)
AUS3	19	-	19	4	3	0.68 (0.02)	0.00101 (0.00019)
NCA	-	25	25	3	2	0.56 (0.01)	0.00069 (0.00013)
HAW	48	-	48	2	1	0.48 (0.01)	0.00055 (0.00008)
GOM	26	-	26	2	1	0.21 (0.02)	0.00024 (0.00007)
FLE	35	-	35	4	7	0.49 (0.01)	0.00091 (0.00013)
BAH	33	-	33	5	7	0.60 (0.01)	0.00106 (0.00015)
USVI	22	-	22	7	9	0.80 (0.01)	0.00196 (0.00028)
BRA	20	-	20	9	11	0.87 (0.01)	0.00317 (0.00044)
Total	339	199	538	25	16	0.74 (0.00)	0.00280 (0.00000)

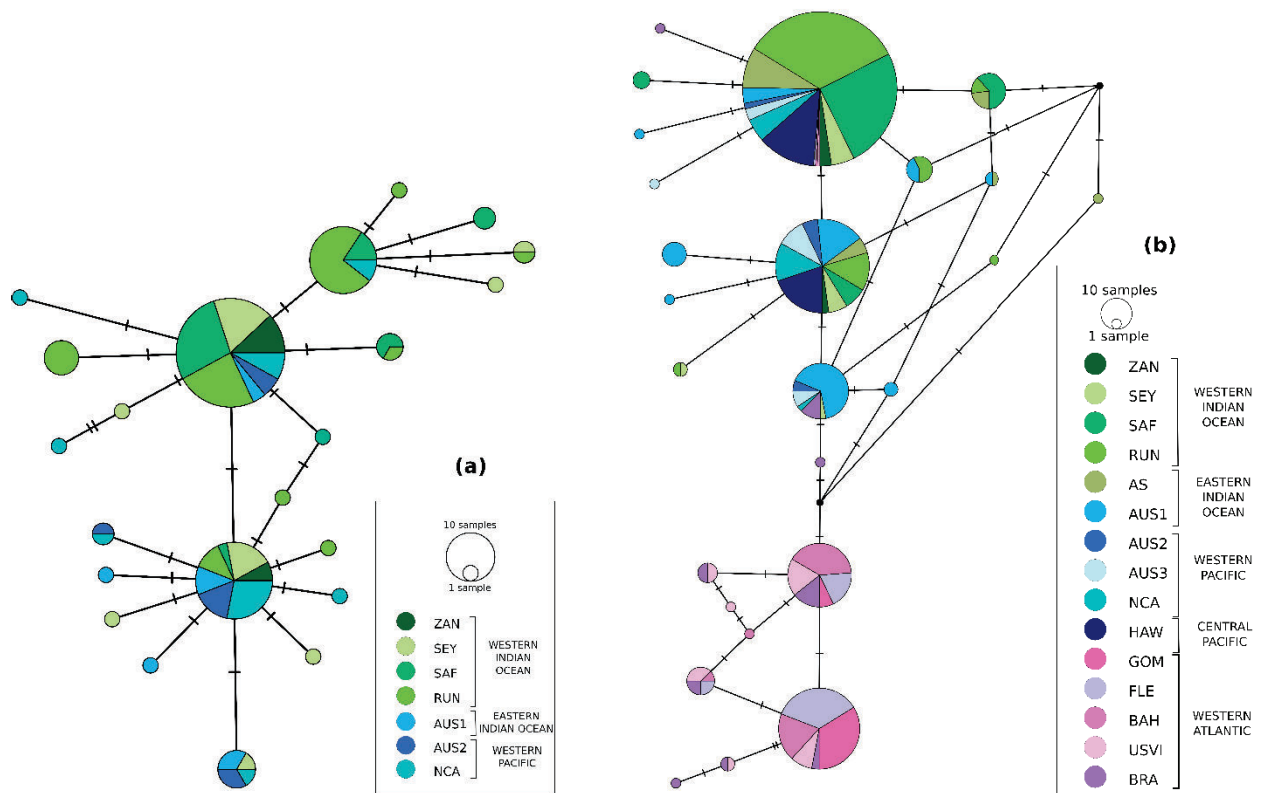


Figure 3. TCS statistical parsimony networks for the tiger shark *Galeocerdo cuvier* constructed with (a) the concatenated *CR-COI-cytb* dataset (22 haplotypes) and (b) the *CR* dataset (25 haplotypes). Each circle represents a haplotype and each trait, a mutation. Circle size is proportional to the number of individuals harbouring each haplotype and colours correspond to sampling locations (ZAN, Zanzibar; SEY, Seychelles; SAF, South Africa; RUN, Reunion Island; AS, Andaman Sea; AUS1, Western Australian coast; AUS2, Queensland, Australia; AUS3, New South Wales, Australia; NCA, New Caledonia; HAW, Hawaii; GOM, Gulf of Mexico; FLE, Florida East Coast; BAH, Bahamas; USVI, US Virgin Islands; BRA, Brazil).

Population genetic structure

Performing Bayesian clustering analyses without the LOCPRIOR model, no distinct genetic clusters were retrieved in both the 27-msat and the 8-msat datasets (Figure 4a and 4c).

Using the LOCPRIOR model on these both microsatellite datasets, a single mode regrouping the five runs was retrieved for each K . For the 8-msat dataset, the highest averaged log likelihood of observing the data (-15986.26 ± 11.59) was retrieved for $K = 3$. Individuals from the Western Indian Ocean (ZAN, SEY, SAF and RUN) belonged to one genetic cluster, those from the Eastern Indian Ocean (AUS1), the Western (AUS2, AUS3, AUS4, COR and NCA) and Central (HAW)

Pacific to a second one, and those from the Western Atlantic (BRA) to a third one (Figure 4d). Nevertheless, with the 27-msat dataset, the two clusters previously identified did not appear (Figure 4b). Indeed, while $K=4$ presented the highest likelihood of observing the data (-16235.32 ± 66.01), from $K=2$, each new cluster was mostly uninformative, with individual membership proportions in new clusters extremely low (respectively $3.43\% \pm 0.82\%$, $4.13\% \pm 0.74\%$ and $2.00\% \pm 0.45\%$ for $K=2$, $K=3$ and $K=4$).

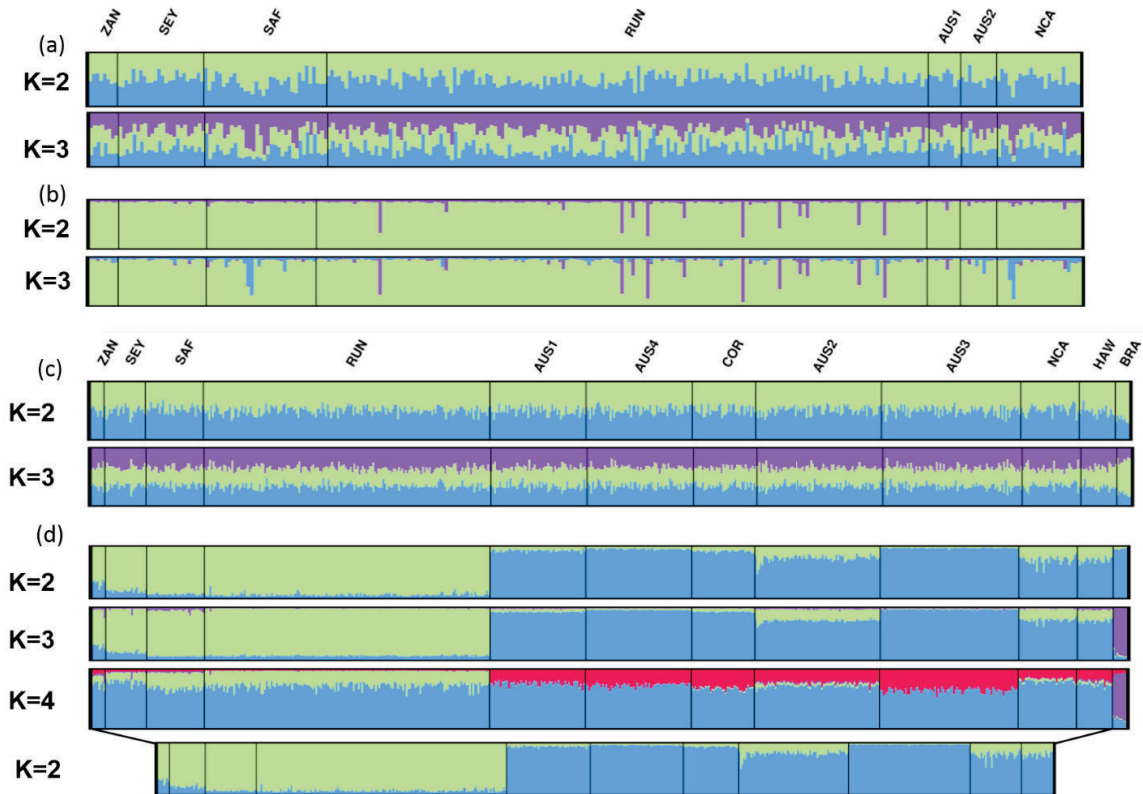


Figure 4. *Galeocerdo cuvier* assignment probabilities of individuals to putative clusters assuming correlated allele frequencies and admixture as performed by STRUCTURE. **(a)** Average probability of membership (y axis) of individuals ($N = 275$, x axis) for major modes of K varying from 2 to 3, with the 27-msat dataset and no *a priori* sampling location information. **(b)** Average probability of membership (y axis) of individuals ($N = 275$, x axis) for major modes of K varying from 2 to 3, with the 27-msat dataset and the LOCPRIOR model. **(c)** Average probability of membership (y axis) of individuals ($N = 606$, x axis) for major modes of K varying from 2 to 3, with the 8-msat dataset and no *a priori* sampling location information. **(d)** Average probability of membership (y axis) of individuals ($N = 606$, x axis) for major modes of K varying from 2 to 4, with the 8-msat dataset and the LOCPRIOR model. ZAN, Zanzibar; SEY, Seychelles; SAF, South Africa; RUN, Reunion Island; AUS1, Western Australian coast; AUS2, Queensland, Australia; AUS3, New South Wales, Australia; AUS4, Northern Territories, Australia; COR, Coral Sea; NCA, New Caledonia; HAW, Hawaii; BRA, Brazil.

Discriminant analyses of principal components performed on both 27-msat and 8-msat datasets did not retrieve distinct genetic clusters, with ellipses for each location overlapping (Figure 5a and 5b). For the analysis on the 27-msat dataset, the first axis explained 32.94% and the second 30.38% of total inertia (Figure 5b) and no clear groups were identified. For the 8-msat dataset, when assigning individuals to five clusters (i.e. defining $K = 5$), the first axis explaining 19.58% of total inertia and the second axis 18.01% and ellipses for each location overlapped, with no clear clusters identified (Figure 5b). Nevertheless, with the same dataset, for lower values of K (3 and 4), individuals seemed to cluster in three groups, not related to their geographical origins (Supplementary Material S6a). As DAPC analyses were not performed in the studies of Holmes et al. (2017) and Bernard et al. (2016), we performed them on both Holmes 8-msat and Bernard 8-msat datasets, to compare the results obtained (Figure 5c and 5d). Using Holmes 8-msat dataset, we observed the same patterns than with the 8-msat dataset (i.e. three clusters not related to the individuals geographical origin for K varying from 2 to 4, and for higher K , no clustering pattern). With the Bernard 8-msat dataset, individuals were clearly clustered in two groups, one gathering individuals from the Western Atlantic and one with individuals from the Indian and Pacific Oceans (Figure 5d). The three clusters identified with the 8-msat and Holmes 8-msat datasets were never observed with Bernard 8-msat dataset, for any K .

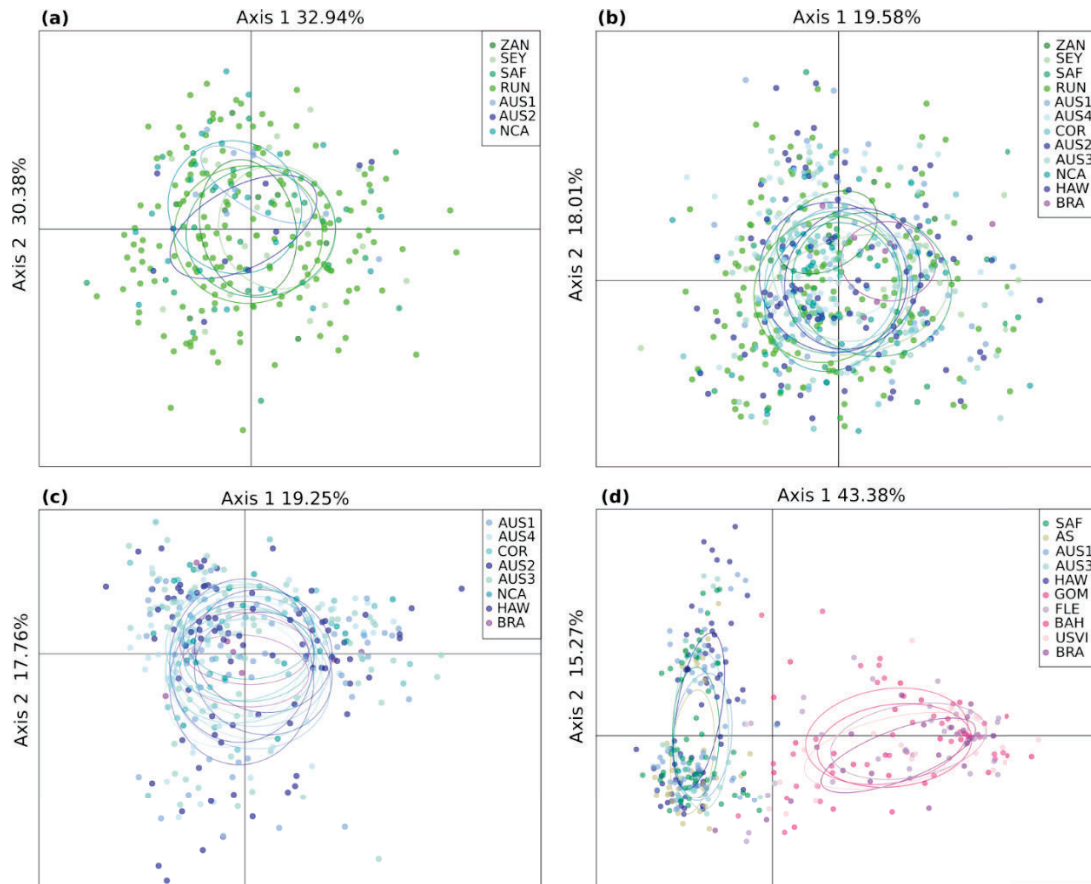


Figure 5. *Galeocerdo cuvier* scatterplot output from DAPC analyses performed using the four microsatellite datasets, and using the first and second components (a) 27-msat dataset, (b) 8-msat dataset, (c) Holmes 8-msat dataset, (d) Bernard 8-msat dataset. Dots represent individuals colored by their sampling location (ZAN, Zanzibar; SEY, Seychelles; SAF, South Africa; RUN, Reunion Island; AS, Andaman Sea; AUS1, Western Australian coast; AUS2, Queensland, Australia; AUS3, New South Wales, Australia; AUS4, Northern Territories, Australia; COR, Coral Sea; NCA, New Caledonia; HAW, Hawaii; GOM, Gulf of Mexico; FLE, Florida East Coast; BAH, Bahamas; USVI, US Virgin Islands; BRA, Brazil).

In conclusion, for both 27-msat and 8-msat datasets, neither the STRUCTURE analysis without the LOCPRIOR model, nor the DAPC highlighted clear genetic clusters, even between locations from the Indian/Pacific Oceans and the Western Atlantic. We chose not to take into account the genetic clusters identified by the STRUCTURE analysis with the LOCPRIOR model performed using the 8-msat dataset. They were not identified in other analyses, including the STRUCTURE analysis with the LOCPRIOR model performed with the 27-msat dataset, which, if this pattern clearly exists, should have attributed individuals from locations of the Western Indian Ocean (i.e. ZAN, SEY,

SAF and RUN) and those from around Australia and the Western Pacific (i.e. AUS1, AUS2 and NCA) in two different genetic clusters. This clustering may rather be related to a very weak differentiation, or to the fact that different persons read the electrophoregrams. Thus, as no clear geographic structuring was retrieved using these both microsatellite datasets, we did not perform analyses of molecular variances, and directly calculated pairwise differentiation estimates.

Concerning the 27-msat dataset, no pairwise F_{ST} values were found significantly different from 0 ($F_{ST} = [0.000, 0.012]$, all $P > 0.05$ after FDR correction; Table 4a). Nevertheless, weak but significant D_{est} values were retrieved between New Caledonia and all other locations ($D_{est} = [0.030, 0.052]$, all $P < 0.05$ after FDR correction), except Zanzibar ($D_{est} = 0.026^{NS}$). AUS2 (Queensland, Australia) was also significantly different from the Seychelles, South Africa and Reunion Island ($D_{est} = [0.034, 0.044]$, all $P < 0.05$ after FDR correction) and a low significant value was also retrieved between Reunion Island and South Africa ($D_{est} = 0.014$, $P < 0.05$ after FDR correction; Table 4a).

Considering the 8-msat dataset, significant pairwise F_{ST} values were only found between Brazil and all other locations ($F_{ST} = [0.030, 0.056]$, all $P < 0.05$ after FDR correction; Table 4b), except Zanzibar ($F_{ST} = 0.039^{NS}$) and between South Africa and Reunion Island, AUS1 (West Australia), AUS4 (North Australia) and AUS3 (East Australia) ($F_{ST} = [0.009, 0.011]$, all $P < 0.05$ after FDR correction). Significant D_{est} values were also retrieved between Brazil and all other locations ($D_{est} = [0.323, 0.406]$, all $P < 0.01$ after FDR correction), as well as between Reunion Island and South Africa, AUS1 (West Australia) and AUS3 (East Australia) ($D_{est} = [0.032, 0.056]$, all $P < 0.05$ after FDR correction), between Reunion Island and AUS1, AUS4 and AUS3 ($D_{est} = [0.017, 0.019]$, all $P < 0.05$ after FDR correction), and between AUS1 and AUS3 ($D_{est} = 0.021$, $P < 0.05$; Table 4b).

1 **Table 4.** Genetic differentiation between *Galeocerdo cuvier* sampling locations estimated with Weir and Cockerham's F_{ST} (lower-left
2 matrix) and Jost's D_{est} . (upper-right matrix) for **(a)** the 27-msat dataset, and **(b)** the 8-msat dataset. Tests significance were assessed after
3 FDR correction and values significantly different from zero are indicated in bold; *: $P < 0.05$, **: $P < 0.01$. In parentheses are indicated
4 the number of individuals used for analyses. ZAN, Zanzibar; SEY, Seychelles; SAF, South Africa; RUN, Reunion Island; AUS1,
5 Western Australian coast; AUS2, Queensland, Australia; AUS3, New South Wales, Australia; AUS4, Northern Territories, Australia;
6 COR, Coral Sea; NCA, New Caledonia; HAW, Hawaii; BRA, Brazil.

(a)	ZAN	SEY	SAF	RUN	AUS1	AUS2	NCA
ZAN (8)	-	0.000	0.000	0.000	0.000	0.019	0.026
SEY (24)	0.001	-	0.007	0.003	0.005	0.044*	0.047**
SAF (34)	0.000	0.002	-	0.014*	0.028	0.038*	0.052**
RUN (167)	0.000	0.000	0.006	-	0.014	0.034*	0.034**
AUS1 (9)	0.000	0.008	0.012	0.012	-	0.022	0.030*
AUS2 (10)	0.000	0.004	0.000	0.000	0.000	-	0.040*
NCA (23)	0.000	0.008	0.008	0.001	0.004	0.000	-

7

(b)	ZAN	SEY	SAF	RUN	AUS1	AUS4	COR	AUS2	AUS3	NCA	HAW	BRA
ZAN (8)	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.331**
SEY (24)	0.002	-	0.035	0.004	0.000	0.014	0.003	0.003	0.027	0.025	0.000	0.376**
SAF (34)	0.008	0.007	-	0.032*	0.056**	0.032	0.036	0.025	0.039*	0.033	0.033	0.342**
RUN (167)	0.000	0.003	0.010*	-	0.019*	0.017*	0.006	0.006	0.018**	0.017	0.005	0.377**
AUS1 (56)	0.002	0.000	0.011*	0.004	-	0.010	0.000	0.021	0.021*	0.019	0.000	0.366**
AUS4 (62)	0.000	0.007	0.009*	0.003	0.001	-	0	0.000	0.000	0.011	0.002	0.355**
COR (37)	0.001	0.007	0.011	0.000	0.000	0.001	-	0.000	0.000	0.008	0.019	0.406**
AUS2 (74)	0.000	0.004	0.008	0.000	0.003	0.000	0.000	-	0.000	0.013	0.017	0.355**
AUS3 (81)	0.000	0.007	0.009*	0.002	0.003	0.000	0.000	0.000	-	0.018	0.000	0.365**
NCA (34)	0.003	0.007	0.008	0.002	0.002	0.004	0.001	0.004	0.003	-	0.002	0.367**
HAW (21)	0.000	0.004	0.006	0.001	0.000	0.000	0.001	0.001	0.000	0.000	-	0.323**
BRA (8)	0.039	0.048*	0.033*	0.047**	0.037*	0.046**	0.051**	0.044**	0.038**	0.056**	0.030*	-

8

9 Pairwise Φ_{ST} values for the concatenated *CR-COI-cytb* dataset were highly significant between
10 Reunion Island and AUS1 (West Australia), AUS2 (East Australia) and New Caledonia as well as
11 between South Africa and AUS1 (West Australia), AUS2 (East Australia) and New Caledonia
12 ($\Phi_{ST} = [0.165, 0.387]$, all $P < 0.001$ after FDR correction; Table 5). Values were lower but also
13 significant between the Seychelles and South Africa and between the Seychelles and Reunion
14 Island ($\Phi_{ST} = 0.104$ and $\Phi_{ST} = 0.110$, all $P < 0.01$ after FDR correction; Table 5). When using the
15 *CR* dataset, high significant values were observed between locations of the North-Western Atlantic
16 Ocean (i.e. GOM, FLE, BAH and USVI) and locations from the Indian and Pacific Oceans
17 ($\Phi_{ST} = [0.697, 0.954]$, all $P < 0.001$ after FDR correction; Table 5), and also between Brazil and
18 locations from the Indian and Pacific Oceans ($\Phi_{ST} = [0.485, 0.793]$, all $P < 0.001$ after FDR
19 correction). Within the Indian/Pacific Oceans and the Western Atlantic, some locations were
20 significantly differentiated between each other, but it was not related to geographical distance or
21 coastline separating them (see Table 5).

Table 5. Genetic differentiation between *Galeocerdo cuvier* sampling locations estimated with Weir and Cockerham's Φ_{ST} for the concatenated *CR-COI-cytb* dataset (upper-right matrix; number of individuals indicated in parentheses on the first line) and for the *CR* dataset (lower-left matrix; number of individuals indicated in parentheses on the first column). ZAN, Zanzibar; SEY, Seychelles; SAF, South Africa; RUN, Reunion Island; AS, Andaman Sea; AUS1, Western Australian coast; AUS2, Queensland, Australia; AUS3, New South Wales, Australia; NCA, New Caledonia; HAW, Hawaii; GOM, Gulf of Mexico; FLE, Florida East Coast; BAH, Bahamas; USVI, US Virgin Islands; BRA, Brazil. Tests significance were assessed after FDR correction and values significantly different from zero are indicated in bold; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

	ZAN (8)	SEY (20)	SAF (23)	RUN (39)	AS	AUS1 (9)	AUS2 (10)	AUS3	NCA (18)	HAW	GOM	FLE	BAH	USVI	BRA
ZAN (8)	-	0.000	0.054	0.100		0.228	0.179		0.016						
SEY (20)	0.000	-	0.104*	0.110*		0.084	0.043		0.000						
SAF (80)	0.021	0.174***	-	0.033		0.387***	0.346***		0.183***						
RUN (103)	0.000	0.114*	0.020*	-		0.327***	0.290***		0.165**						
AS (31)	0.000	0.056	0.014	0.029	-										
AUS1 (58)	0.280**	0.215*	0.486***	0.463***	0.339***	-	0.000		0.010						
AUS2 (10)	0.207	0.074	0.467***	0.418***	0.258**	0.015	-		0.000						
AUS3 (19)	0.124	0.037	0.388***	0.340***	0.206**	0.073*	0.000	-							
NCA (25)	0.048	0.000	0.290***	0.236***	0.140**	0.182***	0.017	0.000	-						
HAW (48)	0.000	0.000	0.165***	0.111***	0.073*	0.300***	0.175*	0.109*	0.013	-					
GOM (26)	0.954***	0.922***	0.937***	0.936***	0.911***	0.818***	0.923***	0.904***	0.925***	0.931***	-				
FLE (35)	0.869***	0.857***	0.903***	0.907***	0.857***	0.774***	0.835***	0.835***	0.862***	0.885***	0.016	-			
BAH (33)	0.841***	0.832***	0.890***	0.895***	0.834***	0.745***	0.801***	0.806***	0.838***	0.867***	0.245***	0.082*	-		
USVI (22)	0.752***	0.764***	0.865***	0.874***	0.780***	0.697***	0.705***	0.731***	0.778***	0.829***	0.195***	0.067*	0.000	-	
BRA (20)	0.564***	0.598***	0.777***	0.793***	0.640***	0.518***	0.485***	0.542***	0.616***	0.707***	0.327***	0.222***	0.128***	0.059	-

Population demographics

Considering the concatenated mitochondrial sequence *CR-COI-cytb* and all locations separately, no evidence of historical population expansions or contractions were found when estimating Tajima's D (all $P > 0.05$; Supplementary Material S7). Nevertheless, pooling all locations, a significantly negative D value was found ($D = -1.83$, $P < 0.01$), highlighting a population expansion. Furthermore, all Fu's F_S estimates were significantly negative, either considering all locations separately (except for Zanzibar and Western Australia AUS2), or pooling all locations ($F_S = [-18.12, -1.99]$; all $P < 0.05$; Supplementary Material S7), also indicative of a population expansion.

Concerning the ABC analyses (Supplementary Material S8), observed data for all scenarios fell within simulated data, suggesting good model fit (Supplementary Material S8a). Scenario 6 (population decrease at time t from N_I to N_b , followed by an expansion from N_b to N_0 five generations ago; Figure 2) presented the highest posterior probability based both on the logistic regression-based estimates and the direct estimate of posterior probability (Supplementary Materials S8b and S8c). Other scenarios had no statistical support. Furthermore, posterior error rates were relatively low, with values of 0.342 and 0.294 using the direct and logistic approaches, respectively. We thus estimated parameter values using data simulated under Scenario 6 (Table 5). The population size decrease during the bottleneck event (N_b) was equal to 111 (95% Confidence Interval (CI) = [43, 369]) and Root Mean Square Error (RMSE) = 0.958, the ancestral effective population sizes (N_I) equal to 5,150 (95% CI = [1,120, 9,710], RMSE = 1.049) and the time the bottleneck begun (t) estimated to 319 (95% CI = [65, 913]) generations, corresponding to approximately 2,000 – 3,000 years (RMSE = 1.347). However, the actual effective population size N_0 was more difficult to infer (Supplementary Material S9 and Table 6).

Table 6. Characteristics of demographic parameter posterior distributions estimated using *Galeocerdo cuvier* individuals from Reunion Island with DIYABC under Scenario 6 (population decrease at time t from N_I to N_b , followed by an expansion from N_b to N_0 five generations ago). N_0 , the actual effective population size; N_I , the ancestral effective population size; N_b , the effective population size during a bottleneck; t , beginning of the bottleneck period; RMSE, Root Mean Square Error.

Parameter	median	2.5% quantile	97.5% quantile	RMSE
N_b	111	43	369	0.958
t	319	65	913	1.347
N_0	5760	639	9820	2.839
N_I	5150	1120	9710	1.049

Similar results were found when performing the analysis including only individuals from New Caledonia (Supplementary Materials S10, S11 and S12).

Discussion

This study investigates the population structure and demographics of the tiger shark *Galeocerdo cuvier* by adding new samples and new molecular markers to recent studies (Bernard et al., 2016; Holmes et al., 2017). Compared to these previous studies, we performed an intensive sampling in the Western Indian Ocean, area for which only one location was previously sampled, and used a higher number of microsatellite and mitochondrial loci, thus completing the picture of tiger shark population structuring. We confirmed here the genetic differentiation between the populations from the Indian/Pacific Oceans and the Western Atlantic, both with microsatellite and mitochondrial markers, while an important genetic connectivity was detected between the Indian and the Pacific Oceans, and within each ocean. Furthermore, we investigated for the first time variations of effective population size at the scale of the Indian/Pacific Oceans and highlighted the probable occurrence of a bottleneck 2,000-3,000 years ago.

Connectivity between the Western Atlantic and both the Western Indian Ocean and the Pacific

Genetic differentiation between tiger sharks from the Western Atlantic and Indian/Pacific Oceans could be investigated only with the 8-msat and *CR* datasets as we did not get individuals from the Western Atlantic. So adding new sampling sites from the Western Indian Ocean, the present study confirms a genetic differentiation between both regions. Indeed, STRUCTURE (using the LOCPRIOR model and at $K = 3$, i.e. not the first level of differentiation) and mitochondrial haplotype network clustered individuals from Brazil (and more largely the Atlantic when considering individuals from Bernard et al. (2016)) separately from the individuals sampled in the Indian and Pacific Oceans. Nevertheless, STRUCTURE without the LOCPRIOR model, nor DAPC retrieved this structuring between both regions. Meanwhile, the DAPC with the Bernard 8-msat dataset did identify the clear differentiation between the Indian/Pacific Oceans and the Western Atlantic. So the absence of clustering retrieved here with DAPC is thus unlikely to represent a biological pattern, but rather a mathematical artefact due to a low number of individuals from Brazil ($n = 8$). So we tend to support the conclusions of Bernard et al. (2016) and Holmes et al. (2017) concerning the global structuring pattern of the tiger shark populations, with populations from the Indian and Pacific Oceans genetically distinct to those from the Western Atlantic.

Population connectivity within the Western Indian Ocean and the Pacific

Patterns of genetic differentiation were more difficult to interpret in the Indian/Pacific Oceans. First, considering the 8-mast dataset, STRUCTURE with the LOCPRIOR model identified two genetic clusters in the Indian/Pacific Oceans, one gathering individuals from the Western Indian Ocean (i.e. ZAN, SEY, SAF and RUN) and the other, locations around Australia and from the Pacific (i.e. AUS1, AUS2, AUS3, AUS4, COR, NCA and HAW), i.e. from the Eastern Indian Ocean and the Pacific. However, STRUCTURE without the LOCPRIOR model and DAPC did not partition the individuals into distinct clusters. Considering the 27-msat dataset, none of the analyses performed identified different clusters in the Indian/Pacific Oceans. So increasing the number of markers has improved the resolution, highlighting the need of sufficient number of microsatellite loci for population genetics analyses (Meirmans, 2015; Putman & Carbone, 2014). Thus the clustering pattern identified by STRUCTURE (LOCPRIOR model) with the 8-msat dataset does not

reflect a biological pattern, but is rather due to the microsatellites characteristics when used in low number.

Microsatellite differentiation estimates between population pairs from this region were dependent on the differentiation index used (F_{ST} or D_{est}) and were not related to whether locations were connected by coastlines or separated by large oceanic expanses. If we only take into account differentiation values significantly different from zero for both indices (F_{ST} and D_{est}), genetic differentiation was only highlighted for the 8-msat dataset and not particularly between very distant locations, concerning South Africa and Reunion Island (not identified with the 27-msat dataset while similar number of individuals between both datasets), South Africa and Western Australia (AUS1) or South Africa and the North-Eastern Australia (AUS3). When using the 27-msat dataset, for none of the population pairs, were significantly different from 0, which would support the hypothesis of one genetic cluster in the Indian/Pacific Oceans. The same pattern was retrieved by Bernard et al. (2016) (except for Hawaii that was significantly differentiated from all other locations).

Furthermore the TCS haplotype networks constructed in this study and the one of Bernard et al. (2016), using either only the *CR* sequence or the *CR-COI-cytb* sequence, showed shared haplotypes among all locations sampled in the Indian and Pacific Oceans. Only three main haplotypes were identified in both studies, separated from one another by one mutation event. This confirms the absence of differentiation among locations from the Indian and Pacific Oceans. Some of the mitochondrial differentiation estimates calculated for both datasets (*CR* or *CR-COI-cytb*) were nevertheless significantly different from zero, which would point to some level of genetic differentiation. It concerned notably individuals from Reunion Island and South Africa, for which Φ_{ST} estimates were significantly different from individuals from the Seychelles, Australia, and New Caledonia, while no significant estimates were calculated between the individuals from Seychelles and those from Australia or New Caledonia, locations separated by almost equal distances than South Africa and Australia, which make these results difficult to interpret. One needs to remember that Φ_{ST} are based on haplotype frequencies and depend highly on the sampling.

Thus, altogether, a weak genetic differentiation was highlighted between locations in the Indian/Pacific Oceans, both with microsatellite and mitochondrial markers. It is known that tiger sharks present the ability to travel across large oceanic expanses (Ferreira et al., 2015;

Hammerschlag et al., 2012; Holmes et al., 2014; Werry et al., 2014), with records of transoceanic migrations in the Indian (Heithaus et al., 2007) or in the Atlantic (Afonso et al., 2017; Kohler et al., 1998; Kohler & Turner, 2001; Lea et al., 2015) Oceans. These observations are thus in accordance with the weak genetic differentiation highlighted in the Indian/Pacific Oceans in this study and previous ones.

To be or not to be philopatric?

Bernard et al. (2016) inferred from the mitochondrial differentiation estimates they retrieved, matrilineal population structure within the Indo-Pacific, which they linked to potential female site fidelity (i.e. philopatry) to reproductive areas, probably pupping sites. Female philopatry to nurseries has been hypothesized in many shark species, notably from discordances between mitochondrial and nuclear differentiation estimates (Karl, Castro, Lopez, Charvet, & Burgess, 2011; Pardini et al., 2001; Portnoy et al., 2014; Tillett, Meekan, Field, Thorburn, & Ovenden, 2012), but these discordances may also result from other processes (Prugnolle & de Meeus, 2002). Furthermore, the tiger shark seems to inhabit oceanic waters more frequently than other species such as the lemon shark *Negaprion brevirostris*, for which female philopatry to nurseries has been demonstrated with certainty (Feldheim et al., 2013). It may thus be less restrained in movements and females may deliver in various sites. It is notably interesting that no known nurseries of tiger sharks have been identified in the Western Indian Ocean nor in the Pacific, leaving open the fidelity of females to coastal waters to pup. The tiger shark is also one of the few species for which multiple paternity (polyandry) has not been identified, even if the number of litters investigated is low (four litters; Holmes et al., 2018). Yet this behavior is supposed to be linked to philopatry and more structured populations (Chapman, Prodohl, Gelsleichter, Manire, & Shivji, 2004), and the predominance of monoandry in the tiger shark may be another indication pointing to an absence of female philopatry to specific nurseries. All in all, further studies, notably identification of nurseries and genotyping of females and juveniles for several years, as well as satellite tracking are needed to fully resolve the occurrence or not of female fidelity to nurseries in the tiger shark.

Weak genetic diversity and bottleneck

Galeocerdo cuvier presents a weak genetic diversity with a very low number of mitochondrial haplotypes and haplotype diversity for the sequence studied compared to other shark species, as the bull shark *Carcharhinus leucas* (Pirog et al., submitted), the great white shark *Carcharodon carcharias* (Pardini et al., 2001), the blue shark *Prionace glauca* (Veríssimo et al., 2017), the blacktip shark *Carcharhinus melanopterus* (Vignaud et al., 2014) or the tope shark *Galeorhinus galeus* (Chabot, 2015; Chabot & Allen, 2009). Furthermore, using the same screening effort, we characterized 20 microsatellite loci for the bull shark (Pirog et al., 2015), and only eight for the tiger shark (Pirog et al., 2016), which also point to a weaker genetic diversity in the latter species. Weak genetic diversity has also been identified for another oceanic shark species, the basking shark *Cetorhinus maximus* (Hoelzel, Shivji, Magnussen, & Francis, 2006), for which only six haplotypes were identified using the *CR* marker, within its whole distribution range. This weak genetic diversity was thought to be due to the occurrence of a bottleneck during the Holocene (Hoelzel et al., 2006).

Here the Bayesian analysis performed here coupling both nuclear and mitochondrial information also evidenced the occurrence of a recent bottleneck experienced by the Indian/Pacific tiger shark population, 2,000-3,000 years ago (during the Holocene). Approximate Bayesian Computation has been proven to be one of the most accurate method to detect recent demographic changes, notably by studying a large marine mammal for which historic fisheries data were available, the Antarctic fur seal *Arctocephalus gazella* (Hoffman, Grant, Forcada, & Phillips, 2011). Estimates of effective population sizes for the tiger shark remained difficult to infer, but the ancestral size of this population was around 5,000 individuals (95% CI = [1,120, 9,710]) and the bottleneck resulted in an effective population size as low as 100 individuals (95% CI = [43, 369]). This bottleneck may well be responsible for the low genetic diversity presented by the species. Decreases of effective population sizes within the Holocene have also been identified for several large marine species, notably sea turtles (Molfetti et al., 2013), whales (Baker & Clapham, 2004) or elephant seals (de Bruyn et al., 2009), but also for terrestrial megafauna (Brook & Bowman, 2002). Notably, a similar signal to the one we retrieved here for the tiger shark was also identified for the scalloped hammerhead shark *Sphyrna lewini* in its Eastern Pacific range (Nance, Klimley, Galván-Magaña, Martínez-Ortíz, & Marko, 2011). Factors of population declines during the Holocene for such

species remain difficult to identify. This period is indeed characterised by a general warming that probably induced population expansions of many marine species (Marko et al., 2010; Uthicke & Benzie, 2003), and it is unclear how climate changes during this period may have led to population decreases of tropical and subtropical species. A widely spread hypothesis to extinctions during the Pleistocene-Holocene is the emergence of diseases (Koutavas, Lynch-Stieglitz, Marchitto, & Sachs, 2002), notably for corals or sea urchins (Aronson & Precht, 2001; Lessios, 1988). Nevertheless, little is known about diseases and their impacts in sharks, despite they seem to present a robust immune system (Luer, Walsh, & Bodine, 2004; Walsh et al., 2006). It is also possible that prehistoric fisheries are responsible for initiating this decrease, which was then intensified by modern fishing. Indeed, evidence of pelagic fishing was reported as soon as 42,000 years before present in the Western Pacific (O'Connor, Ono, & Clarkson, 2011). Other studies evidenced that prehistoric fishing may have impacted coastal ecosystems in many areas around the world (Cooke, 1992; Erlandson & Rick, 2010). Furthermore, presence of elasmobranchs (sharks, rays and skates) in prehistoric fisheries are difficult to prove as their cartilaginous skeleton were rarely preserved in archeological sites (Rick, Erlandson, Glassow, & Moss, 2002), and thus the impacts of prehistoric fishing on populations of these species may be underestimated. Regardless of the possible causes of tiger shark population decline in the Indian/Pacific Oceans, the extremely low effective population size estimated during the bottleneck ($N_b = 111$) is lower than the minimal estimated value of 500 thought to be required for a population to adapt to environmental changes or pressures (Frankham, Briscoe, & Ballou, 2010). While this tiger shark population seems to have been expanding since the bottleneck, we were not able to estimate the current effective population size, and this population may well be severely depleted.

Conclusion

We confirmed here the important genetic connectivity of tiger sharks within the Western Indian Ocean and the Western Pacific, both using microsatellites and mitochondrial markers. While mitochondrial differentiation estimates were slightly higher than microsatellite ones, further analyses are needed to confirm whether it reflects female philopatry to specific nurseries. Individuals from Indian and Pacific Oceans form a unique population. Management and preservation programs need thus to be set up on very large scales. Meanwhile, local fishing may

impact the whole population. Furthermore, the detection of a low genetic diversity as well as a recent bottleneck (in the Holocene), during which the effective population size of tiger sharks in this region may have dropped as low as 100, points to a possibly extremely depleted population. Further assessments of the health status of this population as well as conservation plans are thus particularly needed to preserve the tiger shark within the Western Indian Ocean and the Western Pacific.

Acknowledgments

We acknowledge the Plateforme Gentyane of the Institut National de la Recherche Agronomique (INRA, Clermont-Ferrand, France) for guidance and technical support. Agathe Pirog was supported by a doctoral fellowship from the region Reunion.

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Data accessibility

Microsatellite genotypes: Dryad doi:XXX.

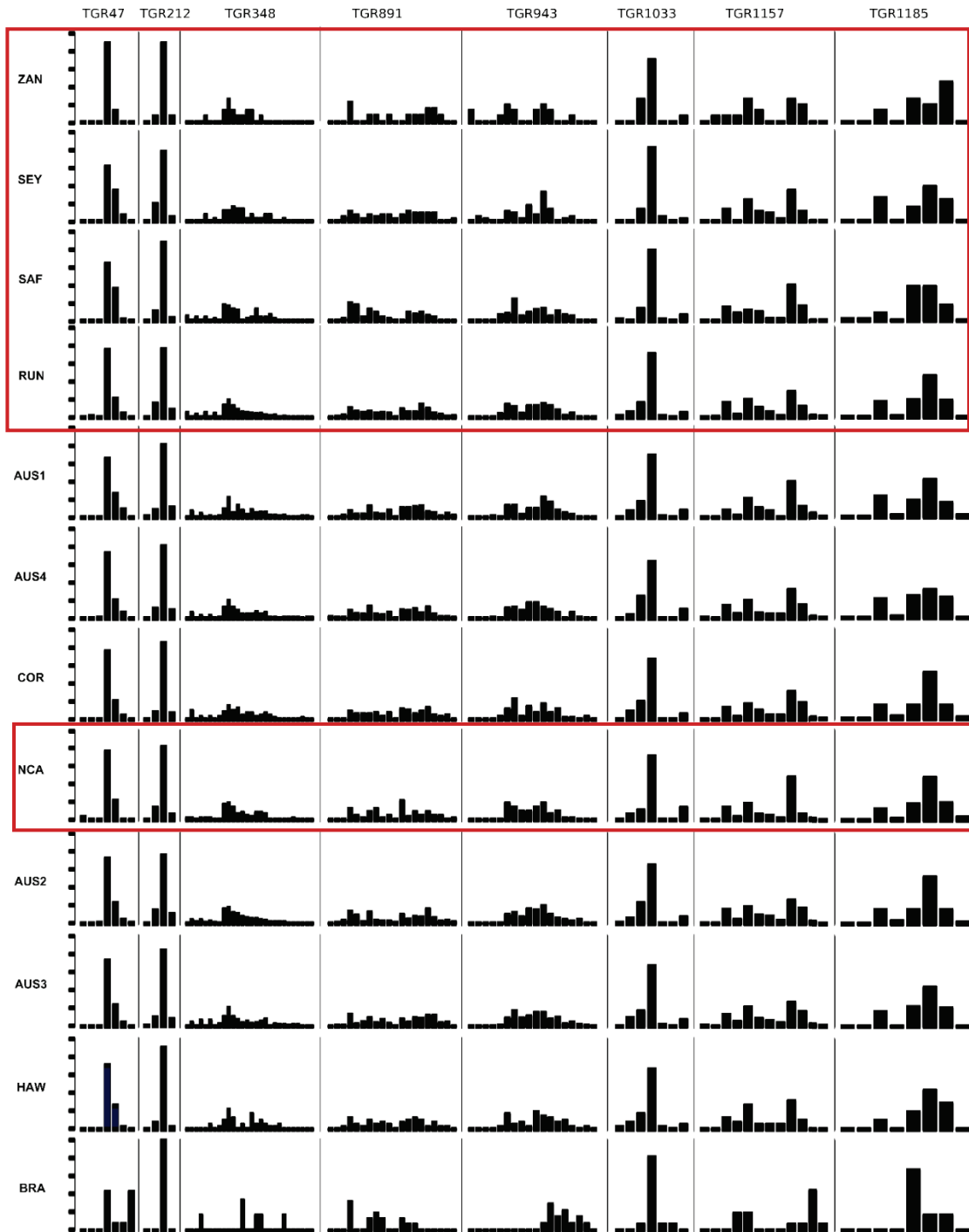
Mitochondrial sequences: GenBank Accession Numbers XX-XX

Supplementary Material

Supplementary Material S1. Panels used to multiplex loci post-PCR and allelic ranges (in base pairs, without M13-tail).

Locus Name	Panel	Fluorochrome	Allelic range	Reference
Gc02	1	VIC	93-105	Pirog et al. 2016
Gc03	1	PET	119-129	Pirog et al. 2016
TGR1185	1	6-FAM	145-161	Bernard et al.2015
ClI4	1	VIC	184-218	Pirog et al. 2015
TGR212	1	NED	194-200	Bernard et al.2015
ClI2	2	6-FAM	105-125	Pirog et al. 2015
TGR348	2	VIC	107-193	Bernard et al.2015
Gc04	2	6-FAM	195-199	Pirog et al. 2016
ClI100	2	NED	207-227	Keeney et al. 2003
Gc05	3	PET	105-123	Pirog et al. 2016
Gc01	3	VIC	135-147	Pirog et al. 2016
ClI7	3	6-FAM	167-181	Pirog et al. 2015
TGR943	3	NED	139-215	Bernard et al.2015
TIG15	3	PET	209-256	Mendes et al. 2016
TIG17	3	VIC	251-269	Mendes et al. 2016
Gc07	4	VIC	114-146	Pirog et al. 2016
TGR1157	4	PET	196-226	Bernard et al.2015
TGR891	4	NED	336-408	Bernard et al.2015
Gc06	5	6-FAM	116-122	Pirog et al. 2016
TGR47	5	PET	150-162	Bernard et al.2015
TGR1033	5	NED	164-180	Bernard et al.2015
Gc08	5	VIC	207-225	Pirog et al. 2016
TIG01	6	6-FAM	127-135	Mendes et al. 2016
TIG07	6	VIC	151-169	Mendes et al. 2016
TIG10	6	NED	248-258	Mendes et al. 2016
TIG19	6	6-FAM	321-335	Mendes et al. 2016
TIG12	6	VIC	342-366	Mendes et al. 2016

Supplementary Material S2. Allelic frequencies at the eight microsatellite studied in common in the present study (red square) and the study of Holmes *et al* (2017). (ZAN, Zanzibar; SEY, Seychelles; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; AS, Andaman Sea; AUS1, Western Australian coast; AUS2, Queensland, Australia; AUS3, New South Wales, Australia; AUS4, Northern Territories, Australia; COR, Coral Sea; NCA, New Caledonia; HAW, Hawaii; GOM, Gulf of Mexico; FLE, Florida East Coast; BAH, Bahamas; USVI, US Virgin Islands; BRA, Brazil)



Supplementary Material S3. Inference of demographic history using Approximate Bayesian Computation (ABC) and performed with DIYABC v.2.1.0

Scenarios

To identify effective population size variations in the Western Indian and Western Pacific Oceans, as no population structure was highlighted between both regions (see Results), we used a coalescent framework through approximate Bayesian computation (ABC) using DIYABC v.2.1.0 {Cornuet, 2014 #534} with both the 27-msat dataset and the *CR-COI-cytb* dataset. We defined N_0 the actual effective population size, N_I the ancestral effective population size, N_b the effective population size during a bottleneck, and N_e the effective population size during an expansion. Then seven scenarios were set up to test for: (Scenario 1) a recent (less than 500 generations) decrease ($N_0 < N_I$), (Scenario 2) a more ancient (between 10^3 and 5×10^5 generations in the past) decrease, (Scenario 3) a recent (less than 500 generations) expansion ($N_0 > N_I$), (Scenario 4) a more ancient (between 10^3 and 5×10^5 generations in the past) expansion, (Scenario 5) an expansion followed by a decrease ($N_e > N_0, N_I$), (Scenario 6) a bottleneck ($N_b < N_0, N_I$) and (Scenario 7) a constant effective population size ($N_0 = N_I$) (Figure 2). For Scenarios 5 and 6, the end of the expansion/bottleneck was set at 5 generations in the past, which approximately corresponds to the interdiction of commercial exploitation of the tiger shark in Reunion Island (in 1999). Generation time was supposed to be around 7 -10 years {Branstetter, 1987 #94;Holmes, 2015 #452;Wintner, 2000 #581;Kneebone, 2008 #93}.

Prior distribution

Parameters were drawn in the prior distributions described in the enclosed Table S1. Because of the lack of knowledge on effective sizes of populations of *G. cuvier*, ranges were chosen broad. For population sizes and times of effective population size variations, uniform distributions with large interval were chosen. Parameters for microsatellite mutation models were set by default following a generalized stepwise mutation model.

Table S1. Prior distributions of parameters used in the DIYABC analysis.

Parameters	Correspondence in scenarios	Priors
N_{high}	N_I for Scenarios 3 and 4	uniform [10^2 , 10^4]
N_{low}	N_I for Scenarios 1 and 2	uniform [10, 5×10^2]
N_{now}	N_0 for all scenarios	uniform [10, 10^4]
N_e	N_e for Scenario 5	uniform [10, 10^4]
N_b	N_b for Scenario 6	uniform [10, 5×10^3]
N_I	N_I for Scenarios 5 and 6	uniform [10, 10^4]
t_1 (in generations)	beginning of decrease or expansion for Scenarios 1 and 3	uniform [5, 5×10^2]
t_2 (in generations)	beginning of decrease or expansion for Scenarios 2 and 4	uniform [10^3 , 5×10^5]
t (in generations)	t	uniform [1, 10^3]
μ_{seq}	μ_{seq}	uniform [10^{-9} , 10^{-5}]
μ_{sat}	μ_{sat}	uniform [10^{-5} , 10^{-2}]
Constraint on parameters	$N_{high} > N_{now}$; $N_{low} < N_{now}$; $N_e > N_{now}$; $N_e > N_I$; $N_b < N_I$; $N_b < N_{now}$	

Prior choices were validated performing a principal component analysis (PCA) in the space of summary statistics for the first 100,000 datasets simulated with parameter values drawn from the prior parameters. The observed dataset was then projected as a supplementary individual to determine (visual check) whether it fell well within the variability of simulated data {Cornuet, 2010 #1471} (Figure S2).

Summary statistics

The genetic variation within the population was summarized using a set of 12 summary statistics, all the ones available in DIYABC v.2.1.0 {Cornuet, 2014 #534}. We thus used, for the 27 microsatellite loci, the mean number of alleles over loci K , the mean of Nei's genetic diversity H , the mean size variance and the mean Garza-Williamson index M . For the concatenated mitochondrial sequence, we used the number of haplotypes H , the number of segregating sites S , the mean number of pairwise differences P_i , the variance of the number of pairwise differences, Tajima's D , the mean of numbers of the rarest nucleotide at segregating sites and the variance of numbers of the rarest nucleotide at segregating sites.

Implementing ABC procedure

For each scenario, we performed 1×10^6 simulations using the same data, summary statistics and prior parameters, constructing, in whole, a reference table with 7×10^6 simulated datasets. To select the best-fit scenario, posterior probabilities were computed via logistic regression on the 1% of simulated data sets closest to the empirical data {Cornuet, 2008 #1088}. Summary statistics were transformed by linear discrimination analysis prior to logistic regression to reduce correlation among explanatory variables and provide conservative estimates of scenario discrimination {Estoup, 2012 #1089}. Posterior distributions of all parameters were then estimated from the selected model, based on the 1% of simulated data sets closest to the empirical data.

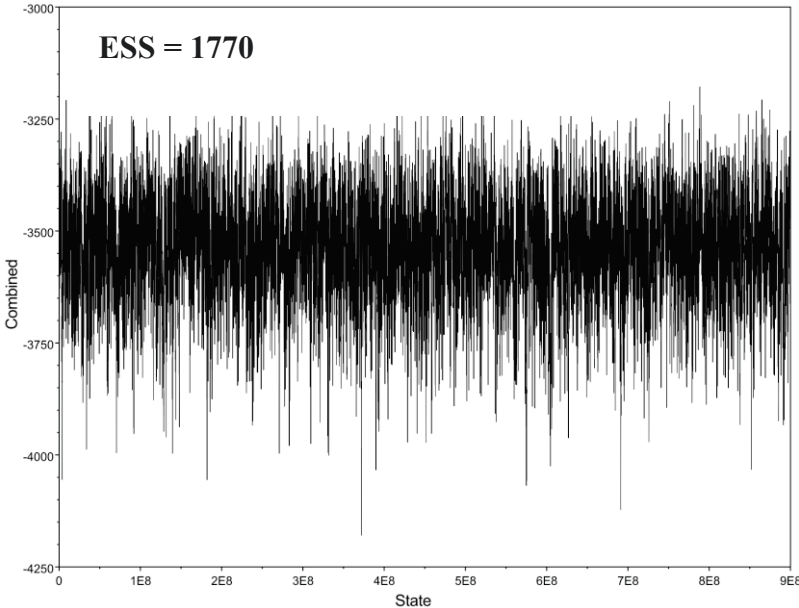
Then the confidence in scenario choice was performed by simulating pseudo-observed datasets drawing (with replacement) the scenario ID and parameter values from the 500 simulated datasets closest to the observed dataset (i.e. the 500 datasets of the reference table with the smallest Euclidian distance). For each of the pseudo observed dataset produced this way, we applied the same procedure than the one performed to select the best scenario, to estimate their respective posterior probabilities and measure the proportion of times the right scenario has the highest posterior probability. The posterior error rate is then given as a proportion of wrongly identified scenarios over the test datasets for both the direct and logistic approaches.

Finally, bias and mean square error of parameter estimations were computed by simulating 500 pseudo-observed datasets under the retained scenario (Scenario 6), and by comparing the real and estimated values of parameters. The parameters values of the pseudo-observed datasets were drawn from the posterior distributions estimated using a standard ABC procedure.

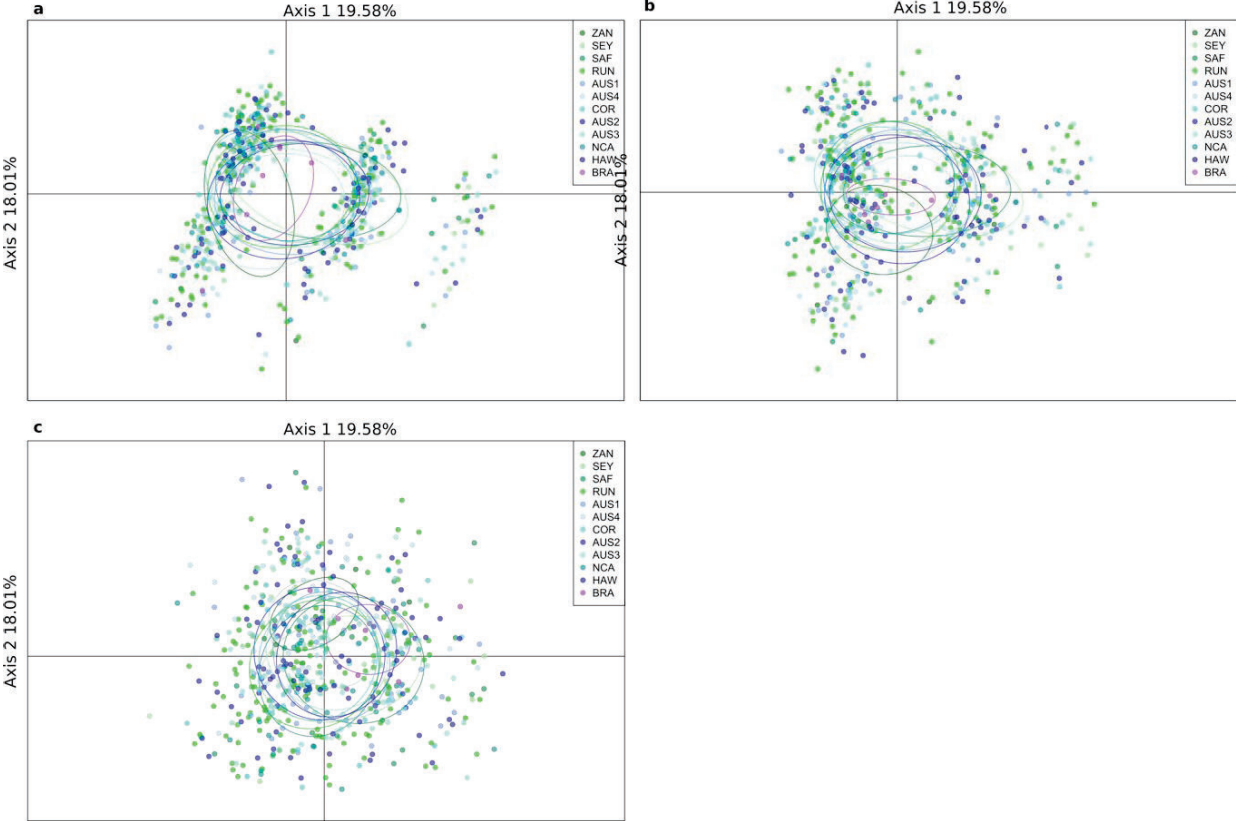
Supplementary Material S4. Summary statistics for each sampling locality (ZAN, Zanzibar; SEY, Seychelles; SAF, South Africa; MAD, Madagascar; RUN, Reunion Island; AUS1, Western Australian coast; AUS2, Queensland, Australia; NCA, New Caledonia) for the three mitochondrial markers used, the control region (*CR*, 862 bp), *COI* (652 pb) and *cytb* (931 bp): N_s , number of individuals sequenced; H , number of haplotypes, h , haplotype diversity; S , number of polymorphic sites; π , nucleotide diversity. In parentheses, are indicated standard errors.

<i>CR</i>	ZAN	SEY	SAF	RUN	AUS1	AUS2	NCA	TOT
N_s	8	20	24	103	9	10	25	199
H	2	4	4	6	4	3	3	9
S	1	3	3	5	3	2	2	7
h	0.43 (0.06)	0.57 (0.02)	0.37 (0.02)	0.34 (0.01)	0.78 (0.04)	0.69 (0.03)	0.56 (0.01)	0.48 (0.00)
π	0.00049 (0.00020)	0.00081 (0.00016)	0.00046 (0.00010)	0.00047 (0.00005)	0.00115 (0.00032)	0.00095 (0.00026)	0.00069 (0.00013)	0.00068 (0.00000)
<i>COI</i>	ZAN	SEY	SAF	RUN	AUS1	AUS2	NCA	TOT
N_s	8	24	30	43	9	10	23	147
H	1	1	2	2	1	1	2	4
S	0	0	1	1	0	0	1	3
h	0.00 (0.00)	0.00 (0.00)	0.07 (0.01)	0.21 (0.01)	0.00 (0.00)	0.00 (0.00)	0.09 (0.02)	0.09 (0.00)
π	0.00000 (0.00000)	0.00000 (0.00000)	0.00010 (0.00004)	0.00032 (0.00007)	0.00000 (0.00000)	0.00000 (0.00000)	0.00013 (0.00006)	0.00014 (0.00000)
<i>cytb</i>	ZAN	SEY	SAF	RUN	AUS1	AUS2	NCA	TOT
N_s	8	22	23	39	9	10	19	130
H	1	6	3	5	2	2	7	14
S	0	5	2	4	1	1	7	12
h	0.00 (0.00)	0.41 (0.03)	0.42 (0.02)	0.57 (0.01)	0.22 (0.06)	0.20 (0.05)	0.61 (0.03)	0.47 (0.00)
π	0.00000 (0.00000)	0.00066 (0.00013)	0.00048 (0.00010)	0.00070 (0.00010)	0.00024 (0.00012)	0.00022 (0.00010)	0.00099 (0.00018)	0.00063 (0.00001)

Supplementary Material S5. Posterior trace file of the BEAST analysis. Concatenated runs of the five analyses performed are shown, as well as the Effective Sample Sizes (ESS) of the posterior distribution.



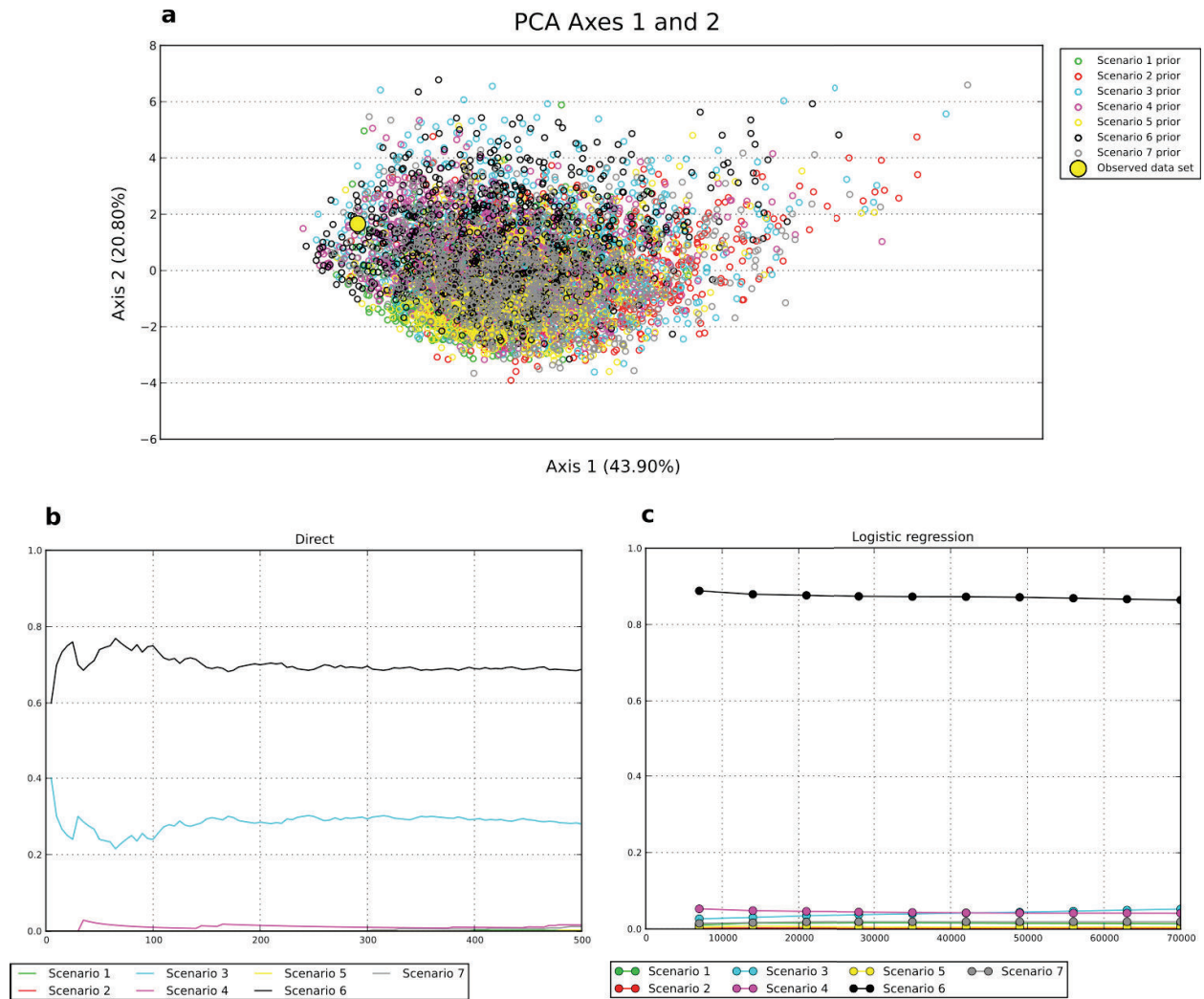
Supplementary Material S6. DAPC analyses performed for the 8-msat dataset, for (a) three clusters assumed, (b) four clusters assumed, (c) five clusters assumed.



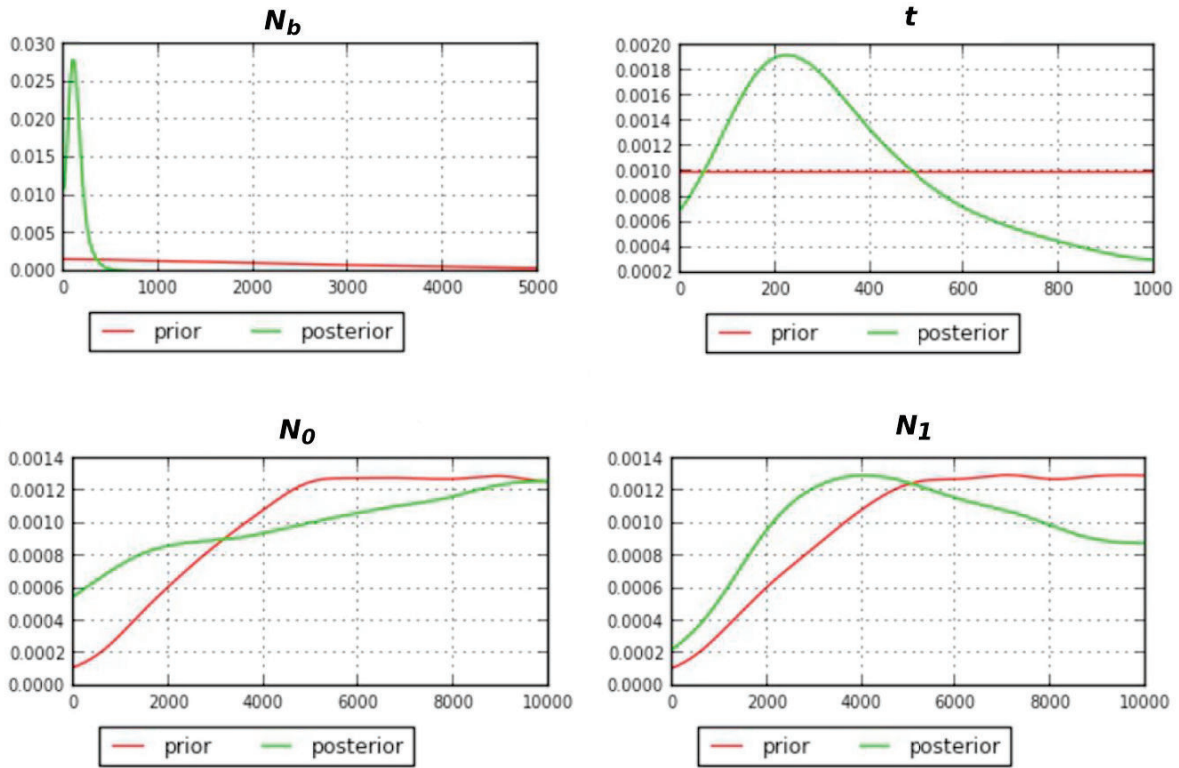
Supplementary Material S7. *Galeocerdo cuvier* demography statistics (Tajima's D and Fu's F_s) for the concatenated mitochondrial sequence *CR-COI-cytb* (Zanzibar, ZAN; Seychelles, SEY; South Africa, SAF; Reunion Island, RUN; Western Australian coast, AUS1; North-Eastern Australian coast, AUS2; New Caledonia, NCA); *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$

	D	F_s
<i>Western Indian Ocean</i>	-1.52	-11.34***
ZAN	0.33	0.54
SEY	-1.44	-3.88**
SAF	-1.07	-2.50*
RUN	-1.03	-3.74*
<i>Australia and Western Pacific</i>	-1.56	-4.74**
AUS1	-0.69	-1.99*
AUS2	-0.13	-0.83
NCA	-1.50	-3.56**
<i>All localities pooled</i>	-1.83**	-18.12***

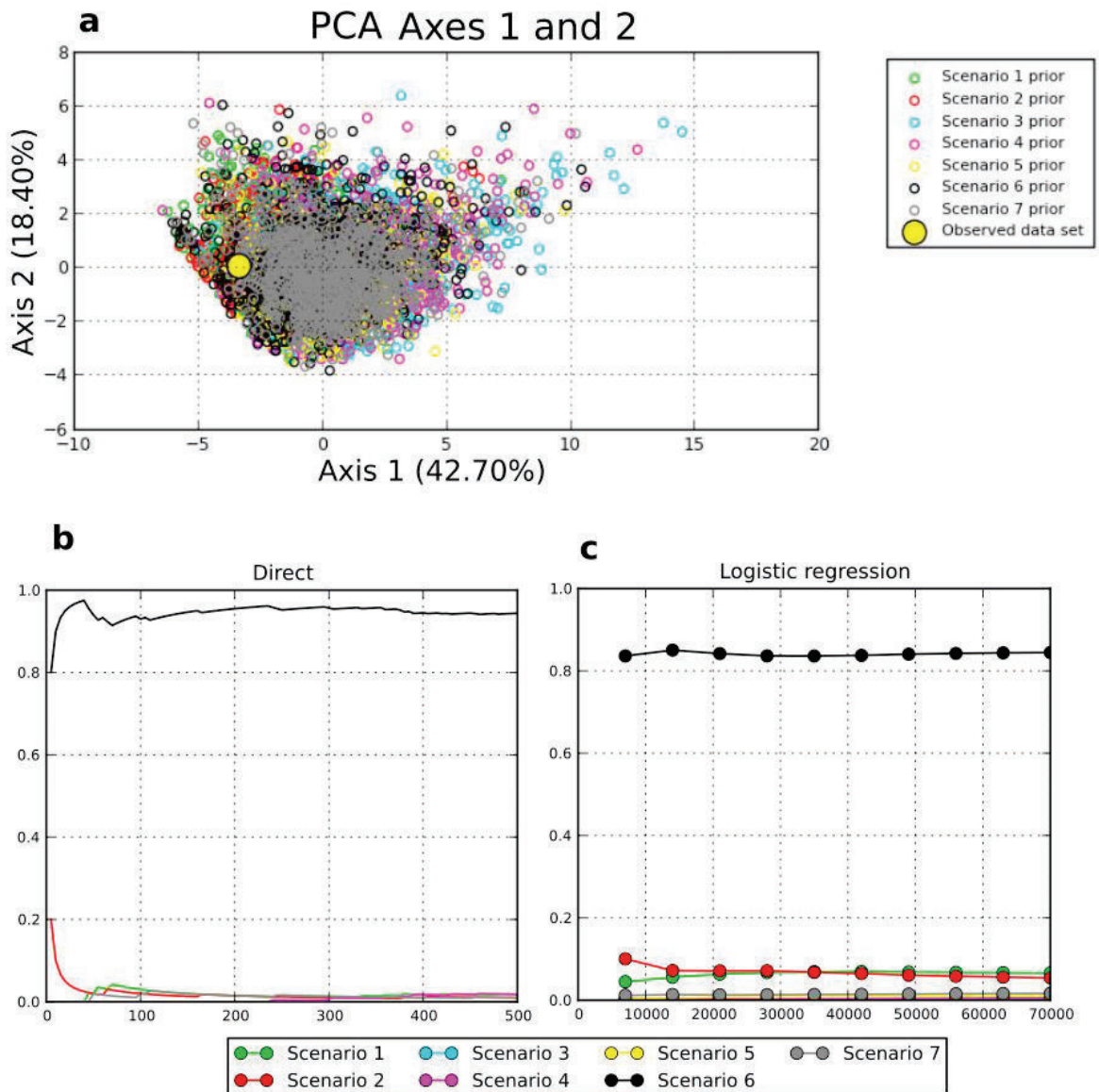
Supplementary Material S8. Fit of observed data to models selected using ABC analysis and scenario comparison using samples from Reunion Island. (a) Plane of the principle components analysis was generated from 70 summary statistics from simulations, using prior distributions of historical parameters: open circles are simulated data sets, generated using prior distributions of historical parameters; closed yellow circle is the observed data set. Results of model testing with (b) direct estimate of posterior probability (c) logistic regression estimate of posterior probability; ordinate is probability, abscissa is number of simulated data sets.



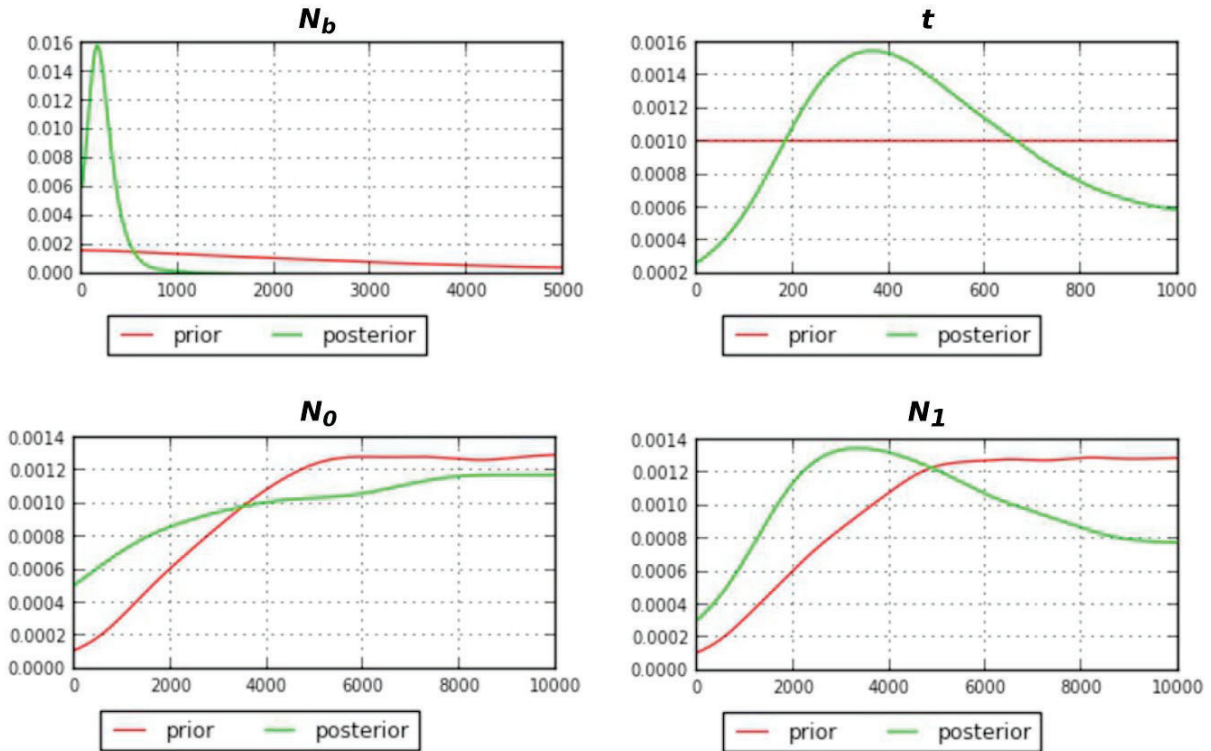
Supplementary Material S9. Posterior distributions of the demographic parameters drawn with Scenario 6 using samples from Reunion Island. N_0 , the actual effective population size; N_1 , the ancestral effective population size; N_b , the effective population size during a bottleneck; t , beginning of the bottleneck period.



Supplementary Material S10. Fit of observed data to models selected using ABC analysis and scenario comparison using samples from New Caledonia. (a) Plane of the principle components analysis was generated from 70 summary statistics from simulations, using prior distributions of historical parameters: open circles are simulated data sets, generated using prior distributions of historical parameters; closed yellow circle is the observed data set. Results of model testing with (b) direct estimate of posterior probability (c) logistic regression estimate of posterior probability; ordinate is probability, abscissa is number of simulated data sets.



Supplementary Material S11. Posterior distributions of the demographic parameters drawn with Scenario 6 using samples from New Caledonia. N_0 , the actual effective population size; N_1 , the ancestral effective population size; N_b , the effective population size during a bottleneck; t , beginning of the bottleneck period.



Supplementary Material S12. Characteristics of demographic parameter posterior distributions estimated using *Galeocerdo cuvier* samples from New Caledonia with DIYABC under Scenario 6. N_0 , the actual effective population size; N_I , the ancestral effective population size; N_b , the effective population size during a bottleneck; t , beginning of the bottleneck period; RMSE, Root Mean Square Error.

Parameter	median	2.5% quantile	97.5% quantile	RMSE
N_b	193	69	776	0.917
t	458	99	954	1.127
N_0	5660	694	9800	2.067
N_I	4750	989	9670	1.32

CHAPITRE 5. Modes de reproduction du requin tigre *Galeocerdo cuvier*

Résumé

Le requin tigre *Galeocerdo cuvier* est la seule espèce de la famille des Carcharhinidés se reproduisant par viviparité aplacentaire, et présente une utilisation de l'habitat océanique plus importante que plusieurs autres requins de cette famille. Ce grand prédateur, qui joue des rôles majeurs dans les écosystèmes marins, est ciblé par les pêcheries commerciales mais aussi par des programmes de régulation dans toute son aire de répartition. Comme mis en évidence dans le chapitre précédent, les populations de cette espèce pourraient être vulnérables et réduites, et l'amélioration des connaissances sur ses modes de reproduction et la dynamique de ces populations est nécessaire pour mettre en place des mesures de gestion efficaces. Dans ce chapitre, nous avons analysé les traits biologiques et reproducteurs de 150 requins tigre pêchés à La Réunion, comprenant cinq femelles gravides. Par ailleurs, 27 microsattellites spécifiques à cette espèce ont été utilisés afin d'étudier la présence de portées polyandres au sein de l'échantillonnage. Les longueurs totales des individus pêchés sont comprises entre 175 cm et 429 cm pour les femelles et entre 130 cm et 415 cm pour les mâles, et les tailles à maturité estimées sont de 278,5 cm pour les mâles et 336 cm pour les femelles. Alors que la distribution des longueurs totales ne dépend pas du sexe, les relations taille-poids établies sont significativement différentes pour les mâles et les femelles. La période d'accouplement semble avoir lieu en mai-juin, pendant la saison froide, et la parturition aurait lieu un an et demi plus tard, pendant la saison chaude (décembre-janvier). Néanmoins, le faible nombre de femelles potentiellement matures pêchées empêche d'étudier plus précisément le cycle de reproduction du requin tigre, et semble montrer que la présence de cette espèce autour de La Réunion est liée à une activité alimentaire plutôt que reproductrice. Enfin, les quatre portées pour lesquelles la paternité multiple a été testée sont chacune issues d'un seul mâle. Cette espèce semble donc monoandre. Ces résultats permettront d'aider à la mise en place de plans de gestion durable des populations du requin tigre.

In the vast oceanic world, no choice to be choosy: lessons from the tiger shark *Galeocerdo cuvier* reproductive biology

Agathe Pirog¹, H el ene Magalon^{1,2}, Thomas Poirout¹, S ebastien Jaquemet¹

¹ UMR ENTROPIE (Universit e de La R eunion/IRD/CNRS), Universit e de La R eunion, Saint Denis, La R eunion, France

² Laboratoire d'Excellence CORAIL, Perpignan, France, www.labex-corail.fr

* Corresponding author

Jaquemet S ebastien

UMR ENTROPIE, Universit e de La R eunion, 15 Avenue Ren e Cassin, CS 92003, 97744 Saint Denis Cedex 09, La R eunion, France

E-mail adress: sebastien.jaquemet@univ-reunion.fr

Short title: Reproductive biology of the tiger shark

Keywords: Chondrichthyes; Elasmobranch; Carcharhinidae; life history traits; reproductive cycle; polyandry

Abstract

The tiger shark *Galeocerdo cuvier* is the only Carcharhinidae reproducing by aplacental viviparity and seems more oceanic than several other species of this family. This large predator, playing important roles in marine ecosystems, is fished throughout its range, both in commercial fisheries and regulation programs, as it has been responsible of attacks on humans. Populations of this species may thus be depleted, and more knowledge on their reproductive behaviors and their population dynamics is needed. We investigated here the reproductive traits of 150 tiger sharks caught during a regulation program in Reunion Island (Western Indian Ocean), including five gravid females. Specific microsatellite loci were also used to investigate the occurrence of polyandry in the sampled litters. Total sizes ranged from 175 cm to 429 cm for females and from 130 cm to 415 cm for males and sizes at maturity estimated were of 278.5 cm for males and 336 cm for females. While the total length distribution was not dependent on sex, weight-size relations were not similar for males and females. A possible mating period in May-June, during the cold season, was observed, with a parturition probably occurring a year and a half later, during the warm season (December-January). Nevertheless, the low number of potentially mature females caught precluded further investigations on the reproductive cycle of the tiger shark, and may indicate that presence of this shark near Reunion Island shores may be more related to foraging than to reproductive activities. Finally, none of the four litters investigated was polyandrous, suggesting this species to be mainly (or even only) monoandrous. These results are valuable to improve preservation and management plans for this species.

Introduction

Large-bodied sharks are mostly elusive oceanic predators of the marine megafauna thought to influence their communities through direct predation and prey behavioural modification (Burkholder, Heithaus, Fourqurean, Wirsing, & Dill, 2013; Ferretti, Worm, Britten, Heithaus, & Lotze, 2010; Heithaus, Frid, Wirsing, & Worm, 2008; Heupel, Knip, Simpfendorfer, & Dulvy, 2014). Indeed, they typically consume large amounts of biomass, thereby potentially impacting trophic dynamics, through spatial redistribution of nutrients and energy. In addition their large-scale movements connect ocean ecosystems over large spatial scales and their large size often acts as a powerful way for physical reengineering of ecosystem structure (Estes, Heithaus, McCauley, Rasher, & Worm, 2016). Besides, they present life history characteristics specific of a *K*-strategy, with slow growth rates, low fecundities and late ages to sexual maturity (Cortés, 2000; Musick, Burgess, Cailliet, Camhi, & Fordham, 2000) while facing considerable exploitation (Campana & F., 2016; Worm et al., 2013). Particularly sensitive to fishing and environmental pressures (Myers & Worm, 2005), many populations have declined worldwide (Dulvy et al., 2014). The tiger shark *Galeocerdo cuvier* is caught in recreational, subsistence and targeted commercial fisheries (Afonso & Hazin, 2014; Baum et al., 2003; Clarke et al., 2006; Polovina & Lau, 1993), and is also subjected to lethal risk reduction programs due to attacks on humans throughout its range, notably in Australia (Holmes et al., 2012; Reid & Krogh, 1992; Simpfendorfer, 1992), South Africa (Cliff & Dudley, 1991; Dudley, 1997; Sumpton, Taylor, Gribble, McPherson, & Ham, 2011), and Hawaii (Wetherbee, Lowe, & Crow, 1994). This large predator inhabits tropical and warm temperate waters worldwide (Compagno, 1984), and as a megafauna species likely plays important roles in structuring marine communities (Burkholder et al., 2013; Heithaus et al., 2008; Wirsing, Heithaus, & Dill, 2007). Despite the charismatic nature of marine megafauna in general and large-bodied sharks in particular, much remains to be learned about these animals (Estes et al., 2016). The tiger shark does not escape the rule. Therefore more knowledge on their life history characteristics and reproductive behaviour are needed to adopt conservative management plans, including the management of potential human-shark interactions in coastal ecosystems.

The tiger shark is the only member of the Carcharhinidae reproducing by aplacental viviparity, embryos relying only on the yolk sac for nourishment, receiving no additional nutrients from the mother during gestation (Compagno, 1984; Parsons, Hoffmayer, Frank, & Bet-Sayad, 2008). Litter

sizes range from three (Whitney & Crow, 2007) to 82 (Bigelow & Schroeder, 1948) embryos, with mean number typically between 30 and 50 embryos per litter (Bigelow & Schroeder, 1948; Clark & von Schmidt, 1965; Simpfendorfer, 1992; Varghese, Unnikrishnan, Gulati, & Ayoob, 2017; Whitney & Crow, 2007). Important variations are recorded both for sizes at birth, ranging between 51 and 90 cm (Compagno, 1984; Randall, 1992; Simpfendorfer, 1992; Whitney & Crow, 2007), and for sizes at maturity, varying from 286 cm (eastern Arabian Sea; Varghese et al., 2017) to 318 cm (South Africa; Dicken, Cliff, & Winker, 2016) for males and from 300 cm (Gulf of Mexico; Clark & von Schmidt, 1965) to 359 cm (South Africa; Dicken et al., 2016) for females. To date, the most detailed study investigating the reproductive biology of this species was conducted in Hawaii, and proposed a triennial reproductive cycle, including a gestation lasting 15-16 months before which sperm is presumably stored in the oviducal gland for 4-5 months, and a resting period of one year after parturition (Whitney & Crow, 2007). Under this scenario, mating in this region was thought to take place in January and February and pupping in September and October of the following year. In Australia, Simpfendorfer (1992) suggested parturition to occur a little later in the year than in Hawaii, between October and January. In the Western Indian Ocean, Jaquemet, Smale, Blaison, Guyomard, and Soria (2013) suggested a pupping period in austral summer and a mating period in winter of the previous year, though these information have to be confirmed due to the paucity of data.

Furthermore, while multiple paternity (polyandry) seems frequent in many shark species (Byrne & Avise, 2012; Dibattista, Feldheim, Thibert-Plante, Gruber, & Hendry, 2008; Green, Appleyard, White, Tracey, & Ovenden, 2017; Pirog, Jaquemet, Soria, & Magalon, 2015; Portnoy, Piercy, Musick, Burgess, & Graves, 2007), the tiger shark seems mainly monoandrous (Holmes et al., 2018). This was assumed to be due to the oceanic nature of this species (Holmes et al., 2018), lowering probabilities of encounter between potential mates. Movements across the Indian and the Pacific Oceans (Heithaus, Wirsing, Dill, & Heithaus, 2007) and important genetic connectivity within the Indian and the Pacific Oceans (Bernard et al., 2016; Holmes et al., 2017; Pirog et al., submitted) have indeed been shown for tiger shark populations. Nevertheless, multiple paternity was investigated in four litters only, and further analyses are needed to confirm the absence of polyandry reported by Holmes et al. (2017).

Here we further expand knowledge on the biology of reproduction of the tiger shark using data collected in Reunion Island during control fishing programs since 2012. We examined data from 150 tiger sharks, including five gravid females. Analyses of reproductive traits were used to estimate size at maturity and reproductive cycles, while the use of specific microsatellite loci allowed assessing the occurrence of multiple paternity in the sampled litters.

Material and Methods

Sampling and measurements

Data analysed in this study were opportunistically collected within the frame of the Reunion Island shark control program that has been applied along the West coast of the island since 2012 and that was set up by the French government for public safety after several attacks on humans. From 2012 to 2017, 150 tiger sharks (87 females and 63 males) were caught and dissected. Precaudal length (*PCL*), fork length (*FL*), total length (*TL*), liver weight (*W_l*) and body mass (*W*, when possible) were measured for each individual. *PCL*, *FL* and *TL* were measured as straight lines from the tip of the snout to the precaudal notch, the fork tail and the tip of the upper caudal lobe, respectively. Unless specified, all lengths reported in this study are *TL*. To convert lengths from other studies or for individuals for which the *TL* was not measured, the *PCL* was used to calculate the *TL* from a linear regression calculated from other animals in the study. When it was not possible to weight animals, the estimated weight (*W_e*) was calculated according to the following equation, with the weight in kilograms and the fork length in centimetres:

$W = 2.5281 \times 10^{-6} FL^{3.2603}$ ($n = 187$, $R^2 = 0.9550$) for males and females (Kohler, Casey, & Turner, 1996).

To determine the reproductive condition of males, clasper length (measured from the point of insertion at the anterior margin of the cloaca to the tip of the clasper), their calcification, and their size relatively to the pelvic fins (smaller or larger) were noted (Clark & von Schmidt, 1965). Female sexual maturity was assessed based on (1) the presence of embryos, (2) the presence of mature oocytes, (3) the uterus width and (4) signs of recent parturition (Clark & von Schmidt, 1965). Gonads for both sexes were also weighted (*W_g*). When gravid females were caught (five, representing 135 embryos), for each embryo, sex was determined based on the presence of claspers

and *PCL*, *FL*, *TL* and *W* were measured. Finally a piece of muscle was also collected on all specimens (including embryos) for microsatellite genotyping, and stored in 90% ethanol until laboratory analyses.

Microsatellite genotyping

For each gravid female and its embryos, genomic DNA was extracted using Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Genotyping was performed using 27 microsatellite loci: 23 species-specific loci [Gc01 to Gc08 (Pirog, Jaquemet, Blaison, Soria, & Magalon, 2016), the nine TGR-loci (except TGR233; Bernard, Feldheim, & Shivji, 2015), the nine TIG-loci (except TIG05 and TIG25; Mendes et al., 2016)], as well as four loci developed for the bull shark *Carcharhinus leucas* (C112, C114 and C117; Pirog, Blaison, Jaquemet, Soria, & Magalon, 2015) and the blacktip shark *Carcharhinus limbatus* (Cli100; Keeney & Heist, 2003) that successfully cross-amplified in the tiger shark. Choices of loci and laboratory procedures are described in Pirog et al. (submitted). The allelic sizes of the PCR products were separated on an ABI 3730XL capillary sequencer at the Plateforme Gentyane (INRA, Clermont-Ferrand, France) and scored with GENEMAPPER v.4.0 (Applied Biosystems) using the Genescan LIZ-500 size standard (Applied Biosystems).

Statistical analyses

The length distribution of all the individuals was plotted, and significant different length distributions between sexes were tested using a Kolmogorov-Smirnov test (Chakravarti, Laha, & Roy, 1967).

Weight-size (*W-FL*) relations were established by calculating the length-weight equation (in the form $W = a \times FL^b$) for individuals weighted in this study, using the method of Pienaar and Thomson (1969) for fitting a non-linear regression model by least squares. This was performed for both sexes separately first, to test whether the relations established were significantly different using the logarithmic equation forms ($\log(W) = \log(a) + b \times \log(FL)$) and an analysis of covariance (ANCOVA). Then, to check whether weight-size relations established by Kohler et al. (1996) also fitted our dataset, we tested whether significant differences were retrieved between the measured weights and those estimated with Kohler et al. (1996) equations using a Kolmogorov-Smirnov test (Chakravarti et al., 1967). This was performed using R (R Development Core Team 2017).

To assess sizes at maturity, clasper length and uterus width were plotted against TL for males and females, respectively. Size at maturity (L_{50}) is defined as the length at which a randomly chosen individual has a 50% chance of being mature. It was estimated using the package *sizeMat* (Torrejon-Magallanes, 2017) for R (R Development Core Team 2017). First, a Principal Component Analysis (PCA) was conducted using the TL and either the clasper length or the uterus width in log base, to distinguish the two groups representing juveniles and adults. Individuals were assigned to each group using a hierarchical classification procedure. Using the results of the classification, a linear discriminant analysis was performed to obtain a discriminating function allowing to classify any individual as a juvenile or an adult based on the two variables used (TL and either clasper length or uterus width). Then a logistic approach was used to estimate L_{50} . In the regression analysis, TL was considered the explanatory variable, the classification (juveniles: 0; adults: 1) was considered the response variable and these variables were fitted to a logistic function with the form:

$$P = 1/[1 + e^{-(\beta_0 + \beta_1 * X)}]$$

where P is the probability of an individual of being mature at a determinate X length, β_0 (intercept) and β_1 (slope) are the estimated parameters. The L_{50} is then calculated as:

$$L_{50} = -\beta_0/\beta_1.$$

Then to analyse the duration of the gestation and reproductive cycles, presence of sperm and presence of mature oocytes for individuals considered as potentially mature (i.e. $TL > L_{50}$) were plotted monthly (all years pooled). Mean embryo lengths for each litter were also plotted against time (i.e. monthly, all years pooled). Furthermore, the gonadosomatic (GSI) and hepatosomatic (HSI) indices (Stevenson & Woods, 2006) were calculated as $GSI = (W_g/W) \times 100$ and $HSI = (W_l/W) \times 100$. These two indices were calculated monthly (all years pooled) for individuals considered as potentially mature (i.e. $TL > L_{50}$) to assess possible temporal variations.

Analyses of multiple paternity

As one litter only contained one embryo, it was not used to study multiple paternity. Thus, four tiger shark litters were used to study multiple paternity.

First the probability of detecting multiple paternity in the sampled litters was calculated with PRDM (Neff & Pitcher, 2002). This probability is calculated given (1) allelic frequencies of the microsatellite loci in the population, (2) litter sizes and (3) the number of sires of a litter. Populations were defined based on results of genetic differentiation analyses performed in Pirog et al. (submitted), and allelic frequencies and characteristics of the loci can be found in this previous study. Little differentiation was found with microsatellite loci among locations from the Indian and the Pacific Oceans. Thus, genotypes from males collected in these regions were used to test whether they corresponded to the inferred genotypes of the fathers of the litters.

As PRDM is limited to 25 loci, the analysis was performed removing the two less polymorphic loci (C112 and C117). For each litter size observed in this study ($n = [3, 45]$), six scenarios were defined, according to the number of fathers usually recorded in shark species (from 2 to 4) (Byrne & Avise, 2012) and different paternal skews (number of embryos within the litter sired by each male): (1) equal paternity (two sires: 50% each; three sires: 33% each; four sires: 25% each), (2) a moderate skew (two sires: 66.7%, 33.3%; three sires: 57%, 28.5%, 14.5%) and (3) a high skew (two sires: 80%, 20%).

Then multiple paternity was considered when at least three non-maternal alleles were found in a litter for at least two microsatellite loci, which was performed by manual counting of alleles. Second, full and half-siblings within and among litters were assessed using the full pedigree likelihood method implemented in COLONY v.2.0.4.5 (Jones & Wang, 2010). A polygamous mating system was assumed for both sexes to allow the assignment of half-siblings and a long-run with medium likelihood precision and a genotyping error rate of 1% was performed.

Results

Length-frequency, weight-length relations and size at maturity

From 2012 to 2017, 150 tiger sharks (87 females and 63 males) were caught, measured and analysed. A sex-ratio of 1:1.4 males to females was observed, not significantly different from unity (χ^2 test, $P > 0.05$, $n = 150$). A significant linear relationship between precaudal and total lengths exists:

$$TL = 1.169PCL + 35.697 \quad (n = 136, t_{134} = 78.249, R^2 = 0.979, P < 2.2 \times 10^{-16}).$$

No significant differences were found between length distributions of males and females (two-samples Kolmogorov-Smirnov test, $D = 0.12, P = 0.71$). Female TL ranged from 175 cm to 429 cm (mean \pm SE = 328.40 ± 5.81 cm), and male TL from 130 cm to 415 cm (mean \pm SE = 326.00 ± 6.31 cm; Figure 1). Most sharks sampled measured 270 – 410 cm.

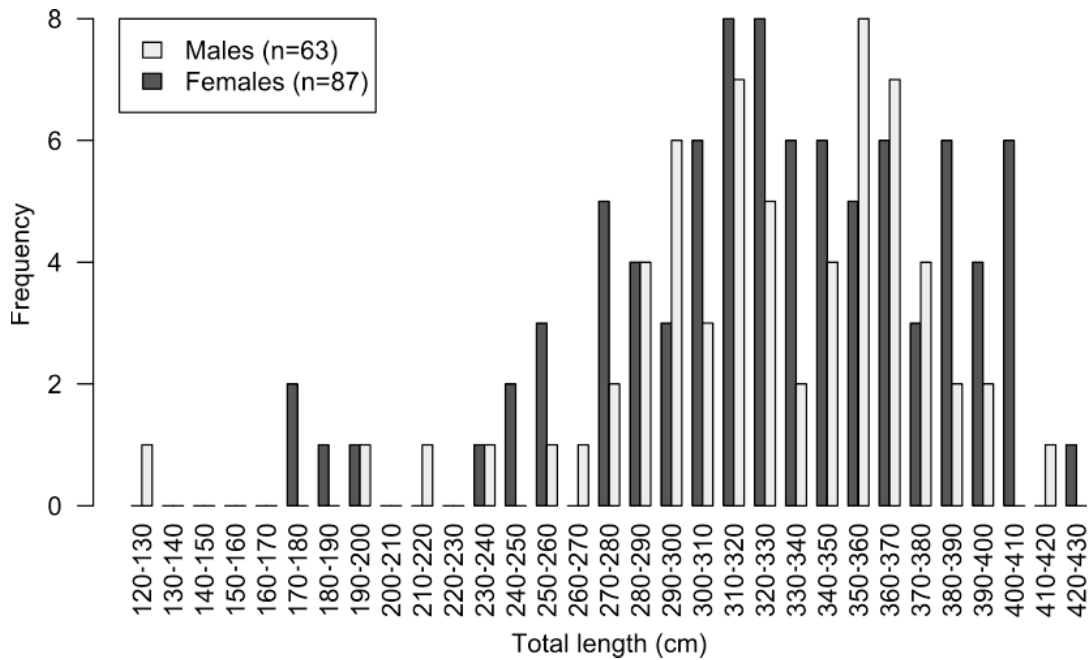


Figure 1. Length-frequency histogram for tiger sharks *Galeocerdo cuvier*.

Weights were obtained from 21 males and 28 females and allowed to establish the weight-length relationships (with the fork length FL) for the species in Reunion Island. As the ANCOVA performed showed no significantly different intercepts but significantly different slopes for males and females (intercept: $n = 49, F_{1,45} = 0.95, P = 0.34$; slope: $n = 49, F_{1,45} = 8.39, P = 0.01$), length-weight equations were calculated for each sex:

$$\text{Females: } W = 3.960 \times 10^{-5} FL^{2.753} \quad (n = 28, t_{26} = 29.896, R^2 = 0.986, P < 2.2 \times 10^{-16})$$

$$\text{Males: } W = 4.226 \times 10^{-8} FL^{3.989} \quad (n = 21, t_{19} = 26.416, R^2 = 0.987, P < 2.2 \times 10^{-16})$$

No significant differences were retrieved between the measured weights for each sex and those estimated with the equation established in Kohler et al. (1996) (two-samples Kolmogorov-Smirnov test; males, $D = 0.14, P = 0.98$; females, $D = 0.11, P = 0.99$)(Figure 2).

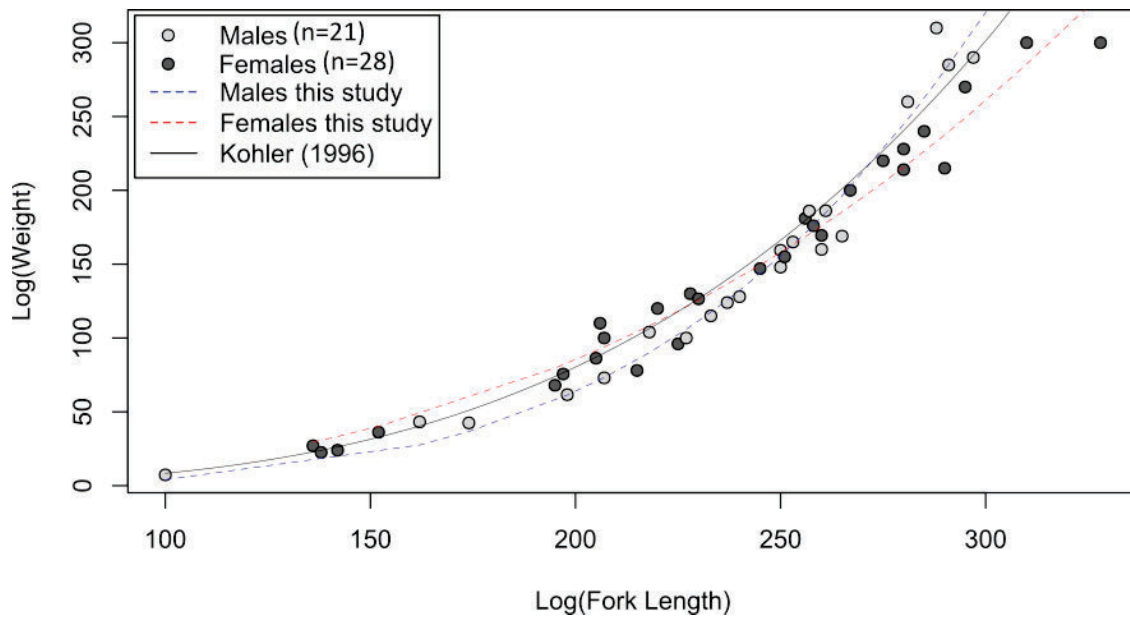


Figure 2. Relationship between fork length FL and measured weight W for the tiger shark *Galeocerdo cuvier*. This study, female (red line): $W = 3.960 \times 10^{-5} PCL^{2.753}$ ($n = 28$, $R^2 = 0.986$), male (blue line): $W = 4.226 \times 10^{-8} PCL^{3.989}$ ($n = 21$, $R^2 = 0.987$); Kohler et al. (1996) (black line): $W = 2.5281 \times 10^{-6} PCL^{3.2603}$ ($n = 187$, $R^2 = 0.955$).

Despite a low number of sharks measuring less than 260 cm ($n = 4$), clasper length seemed to increase in males larger than this size, which was linked to clasper calcification (Figure 3a). The smallest male with calcified claspers measured 280 cm whereas the largest male with uncalcified claspers was 323 cm TL . Male L_{50} was estimated to 278.5 cm by the model (Figure 3b). A low number of females presenting signs of maturity (mature oocytes or embryos, $n = 10$) and various uterus widths for the same TL rendered difficult to determine size at maturity (Figure 3c). Female L_{50} was estimated at 278.5 cm ($R^2 = 0.55$)(Figure 3d).

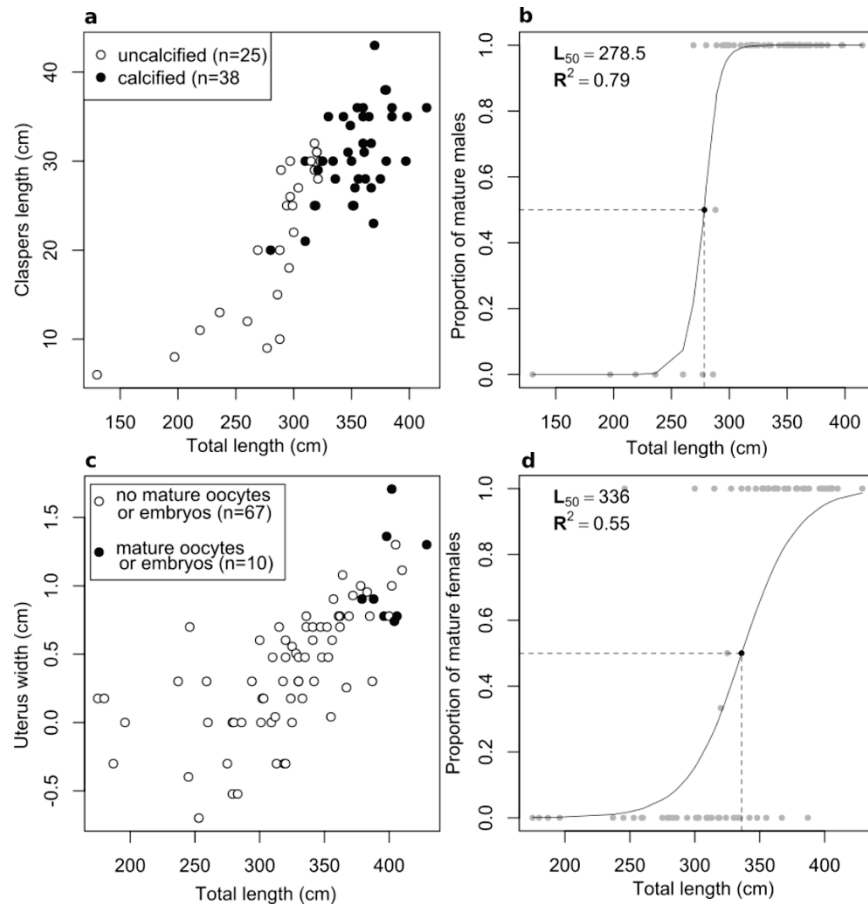


Figure 3. Maturity of tiger sharks *Galeocerdo cuvier* from Reunion Island. (a) Length of calcified and uncalcified claspers versus male total length. (b) Proportion of mature males in 10 cm size intervals, dashed lines mark the size at which 50% of males reached maturity (L_{50}). (c) Uterus width versus female total length with occurrence of mature oocytes or embryos. (d) Proportion of mature females in 10 cm size intervals, dashed lines mark the size at which 50% of females reached maturity (L_{50}).

Reproductive cycles

During the study, 56 males (89%) and 41 females (47%) measured more than L_{50} and were considered as potentially mature. Among these females, only three caught in April, September and October presented mature oocytes. Males with sperm ($n = 31$) were mostly caught in April, May and from September to December (Figure 4a). However, these data must be taken with caution as they depend on the fishing effort, which was not constant over time, varying from month to month and from year to year. In addition to the fact that it deals with small sample size implying stochasticity, a decrease in the number of potentially mature individuals might thus just reflect a period during which the fishing effort was weaker.

The *HSI* was rather constant among months or among individuals (mean \pm S.E.: *HSI* = $18.19 \pm 0.73\%$ and $17.17 \pm 0.51\%$ for females and males, respectively). The *GSI* did not show clear monthly variations, but seemed rather constant from one month to another, except a peak in June for males ($0.51 \pm 0.09\%$), and a peak in October for females ($0.39 \pm 0.20\%$; Figure 4b).

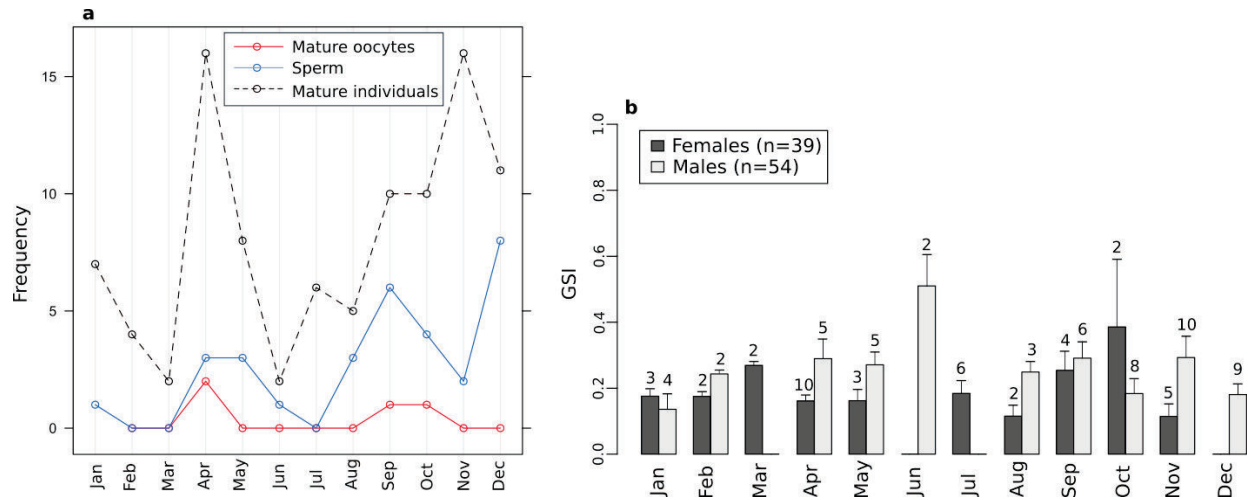


Figure 4. Analyses of reproductive cycles in the tiger shark *Galeocerdo cuvier*. (a) Monthly number of males and females potentially mature (i.e., for which total length *TL* is greater than size at maturity L_{50} ; dashed line), and among them, the males presenting sperm (blue line) and the females presenting mature oocytes (red line). (b) Monthly variations of the gonado-somatic index (*GSI*) for potentially mature males and females ($TL \geq L_{50}$). Monthly number of sharks per sex are indicated above each bar.

Five gravid females were caught during the studied period, representing 135 embryos collected. No litter presented a sex-ratio significantly different from unity (χ^2 tests, $n = 3 - 45$, all $P > 0.05$). One litter was not entirely sampled, as embryos were released by the mother during capture, explaining its low number of embryos ($n = 3$). Another female had one embryo only, measuring 77 cm, reported as stillborn. Other litter sizes ranged from 42 to 45 embryos (mean 43.67 ± 0.88). Embryo *TL* range was 29.2 – 87 cm with a mean of 64.79 ± 1.95 cm. The low number of litters prevents analysis of the timing of embryos development. However the two litters with the largest embryos were recorded in November and January (mean embryo *TL* of 80.81 ± 0.27 cm and 81.70 ± 0.31 cm; Figure 5), and the litter of the stillborn ($TL = 77$ cm) was sampled in March. A female with marks of parturition was also sampled in January 2016. Litters with the smallest embryos were caught in April and May (mean embryo *TL* of 42.0 ± 0.58 cm and 33.86 ± 0.32 cm; Figure 5).

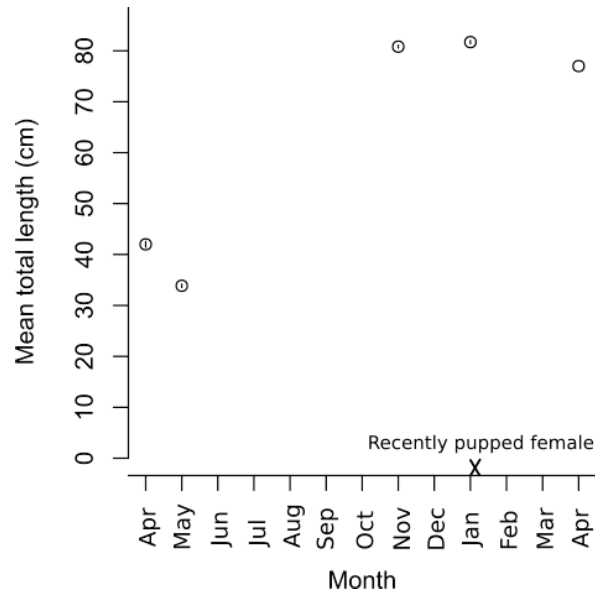


Figure 5. Mean embryo total length per litter plotted against month of the year in the tiger shark *Galeocerdo cuvier* ($n = 5$ litters). The cross indicates a mature female with expanded, vacant uterus indicating it had recently pupped.

Multiple paternity

For tiger shark litters of more than 4 embryos, the 25 most polymorphic loci allowed a strong probability to detect multiple paternity, for all numbers of sires and all reproductive skews tested ($P = 1.00$, Table 1). Detecting multiple paternity was only weak for the litter with three embryos, with a probability to be detected varying from 0.46 to 0.91, according to the number of sires and the reproductive skew (Table 1). The true number of sires for this litter may thus be underestimated.

Table 1 Probability of detecting multiple paternity for the tiger shark *Galeocerdo cuvier* using 25 microsatellite loci. Six scenarios varying in number of sires and paternal skews are depicted.

	Number of embryos			
	3	42	44	45
Paternal skews				
Two males (50:50)	0.72	1.00	1.00	1.00
Two males (66.7:33.3)	0.64	1.00	1.00	1.00
Two males (80:20)	0.46	1.00	1.00	1.00
Three males (33.3:33.3:33.4)	0.86	1.00	1.00	1.00
Three males (57:28.5:14.5)	0.76	1.00	1.00	1.00
Four males (25:25:25:25)	0.91	1.00	1.00	1.00

Nevertheless, manual allele counting indicated that only one sire contributed to each litter, with none of the four litters investigated exhibiting more than two paternal alleles for any of the 27 microsatellite loci used. This was confirmed by the analysis with COLONY, with all embryos of a same litter identified as full-sibs. Each litter was sired by a different male, not identified among the males sampled in the Western Indian Ocean and in the Western Pacific in the study of Pirog et al. (submitted).

Discussion

Biological and reproductive traits were studied here from 150 tiger sharks (*Galeocerdo cuvier*) captured in Reunion Island (Western Indian Ocean), to continue to improve knowledge on reproductive behaviors and population dynamics for this species. Females were not significantly larger than males, despite reaching larger sizes. Nevertheless, weight-size relations were related to the sex. Individual sizes as well as sizes at maturity were in the same range than previously observed. Finally, no polyandrous litters were detected.

Length-frequency, weight-length relations and size at maturity

While female tiger sharks reached larger sizes than males (429 cm vs 415 cm), no significant differences were found between sexes for the length distribution frequencies of caught individuals off Reunion Island. Most of the caught animals ranged from 270 to 400 cm. Similar size ranges were observed for tiger sharks caught in Hawaii (Whitney & Crow, 2007) and in South Africa (Dicken et al., 2016) while, in Australia, the range was 200 – 320 cm (Holmes et al., 2012). Branstetter, Musick, and Colvocoresses (1987) also observed a size discrepancy between tiger sharks caught in the Northwestern Atlantic, where individuals measuring more than 300 cm were frequently observed, and in the Gulf of Mexico, where the size ranged between 100 and 260 cm. Discrepancies may be due to the habitat, with more oceanic habitats preferred by larger individuals while smaller individuals would select more often coastal areas (Heithaus, Hamilton, Wirsing, & Dill, 2006; Lowe, Wetherbee, Crow, & Tester, 1996). As oceanic islands, Hawaii and Reunion Island constitute typical oceanic habitats even close to the shore, explaining consequently the

presence of large individuals. Fishing gears and hook size could also explain the differences in the size of the individuals.

Weight-length relationships established for Reunion Island were significantly different for males and females, with females weighting more than males for a same length for individuals measuring less than 316 cm (*TL*) (Varghese et al., 2017), and females weighted less than males when *TL* > 316 cm. This could suggest some sexual dimorphism and different growth strategies between sexes in the tiger shark. However this pattern was not identified by Kohler et al. (1996).

Sizes at maturity were estimated at 278.5 cm for males and 336 cm for females and they were in the same range than those estimated in other studies (Bass, D'Aubrey, & Kistnasamy, 1975; Branstetter et al., 1987; Dicken et al., 2016; Fourmanoir, 1961; Varghese et al., 2017; Whitney & Crow, 2007). In the present study, the accuracy of the size at maturity estimate was weak, especially for females. It was notably due to the low number of females captured presenting mature oocytes or carrying embryos ($n = 9$, i.e. 4 mature + 5 gravid females). These females represent only 11.5% of the females caught. Such a weak proportion of females presenting signs of maturity was also observed along the Eastern coast of South Africa (1.5%; Dicken et al., 2016) and in the Eastern Arabian Sea (10.6%; Varghese et al., 2017). So these locations along with Reunion Island might represent potential feeding sites rather than mating sites, as also suggested for Hawaii (Lowe et al., 1996; Meyer, Clark, Papastamatiou, Whitney, & Holland, 2009), South Africa (Dicken et al., 2016) and Australia (Heithaus, 2001; Werry et al., 2014). Mating in tiger sharks could thus occur in the open ocean rather than in coastal ecosystems, or, if occurring in coastal areas, these latter are not especially selected by females, but just haphazardly when meeting a mate. The lack of population genetic structure observed for this species in the Indian and Pacific Oceans (Bernard et al., 2016; Holmes et al., 2017; Pirog et al., submitted) may be explained by a lack of site fidelity to specific nurseries for parturition, which may occur in the open ocean as well as in coastal areas without distinction.

Reproductive cycles

The number of embryos recorded from the five litters examined ranged from one to 45 and fell in the range of embryos previously documented (e.g. Dicken et al., 2016; Randall, 1992; Simpfendorfer, 1992; Whitney, Robbins, Schultz, Bowen, & Holland, 2012). The capture of two litters in April with mean embryo lengths significantly different (42.00 ± 0.58 cm and

77.00 ± 0.00 cm) confirmed previous studies suggesting a gestation period longer than one year (Clark & von Schmidt, 1965; Whitney & Crow, 2007). Size at birth was estimated around 80 – 90 cm (Simpfendorfer, 1992; Varghese et al., 2017; Whitney & Crow, 2007). Thus litters collected in November, January and April in Reunion Island with embryos measuring nearly 80 cm were certainly near-term, suggesting a parturition period during the warm season (November-April), as suggested by Jaquemet et al. (2013). In South Africa, a near-term pregnant female was caught in February (Dicken et al., 2016) while the timing of captures of gravid sharks off the Eastern Cape Province of South Africa (Bass et al., 1975) and Madagascar (Fourmanoir, 1961) also suggested that pupping occurred during the austral summer. In the northern hemisphere, largest embryos collected in the eastern Arabian Sea as well as smallest free-swimming juveniles were caught in May (Varghese et al., 2017) while parturition seemed to occur in September-October in Hawaii (Whitney & Crow, 2007), corresponding to the warm season at these locations.

One male tiger shark presenting reproduction signs was caught in December 2014, while a female with scars probably due to mating was caught at the end of October 2015. Nevertheless, the higher *GSI* recorded for mature males in June ($0.51 \pm 0.09\%$) may also correspond to a higher reproductive activity during this period. Caution must nevertheless be taken when analysing this result, as no significant monthly variation was identified and this result may be due to the low number of samples per month. Nonetheless, this value is higher than those reported by Dicken et al. (2016) in South Africa (*GSI* ranging from 0.13 to 0.41% for males). Mature females also presented a slightly higher *GSI* in October ($0.39 \pm 0.20\%$), which may be linked to the ovulation period. Female tiger sharks are able to store sperm in the oviducal gland for 4 – 5 months until ovulation (Pratt, 1993; Whitney & Crow, 2007), and it is thus possible that the mating period occurs in May-June and that sperm is stored until fertilization in October. Assuming a gestation period of 15 – 16 months (Whitney & Crow, 2007), this cycle fits with a parturition period during the warm season. Nevertheless, more data would be needed to confirm seasonal cycles of tiger sharks in the South Western Indian Ocean.

Multiple paternity

No evidence of multiple paternity was highlighted studying the four litters with more than one embryo, representing 134 embryos investigated. This result is similar to a recent study in Australia comprising four tiger shark litters studied using nine microsatellite loci (Holmes et al., 2018). From

their litters, only one embryo in one litter was assigned to a second father, but the authors did not reject the possibility of genotyping errors. Overall, eight tiger shark litters were investigated using molecular tools, representing 246 embryos, all being monoandrous. Prevalence of genetic monogamy has also been reported in one other elasmobranch only, the bonnethead shark *Sphyrna tiburo* (Chapman, Prodohl, Gelsleichter, Manire, & Shivji, 2004): of the 22 litters collected in the Gulf of Mexico, 80% were issued from one sire. It has been hypothesized that species with large, highly dispersive populations would probably present higher levels of monogamous mating than species with smaller or more structured populations (Chapman et al., 2004). The tiger shark indeed presents weak population genetic differentiation across the Indian and the Pacific Oceans (Holmes et al., 2017; Pirog et al., submitted), and evidence of philopatry to nurseries remain scant (Bernard et al., 2016). As some studies suggested that multiple paternity may increase effective population size or maintain genetic diversity in populations (Sugg & Chesser, 1994; Zeh & Zeh, 2001), the prevalence of monoandry in the tiger shark may constrain effective population size and reduce the genetic diversity in this species (Pirog et al., submitted). The lower frequency of polyandry in oceanic shark species may also be explained by the more stable nature of the open ocean, while coastal ecosystems are known to be very variable according to environmental or anthropogenic changes (Cloern et al., 2016). The tiger shark seems indeed to display some characteristics of a species adapted to stable environments, with a low genetic diversity and a high investment in one reproductive event (high number of embryos, longer gestation period and reproductive cycle compared to other shark species).

Concluding remarks

This study explored the reproductive biology of the tiger shark *Galeocerdo cuvier* in Reunion Island and also contributes to increase the knowledge on this major predator of the tropical ocean, as it is still poorly documented. Evidence of a complex seasonal cycle was highlighted with parturition during the austral summer (December-January) and a gestation period longer than one year, confirming what was previously described in other locations. The potential time lag between the peak of *GSI* for males and females supports the idea that females store sperm for months, which was already suggested by Whitney and Crow (2007). Indeed, rate of encounters with mating partners for this oceanic species may be low, and sperm storage may allow females to benefit from each possible mating, by letting them the time to get physiologically prepared for gestation with

embryos developing on yolk sacs only (Pratt, 1993). The oceanic behavior of the tiger shark and the associated low probability to encounter a mate may also explain the high occurrence of monoandrous litters, an uncommon behavior in sharks, which may reduce genetic diversity and effective population size in this species. Interestingly, a low number of gravid females was sampled during the study, pointing that the presence of the tiger shark near Reunion Island coasts may not be related to parturition, but more likely to foraging. Indeed, this oceanic island may increase the feeding opportunities compared to the open ocean ecosystems. This observation was also made in Hawaii, South Africa and Australia, and parturition areas used by the tiger sharks remain unknown. The findings from this study nevertheless provide valuable information for the conservation of the iconic megafaunal species.

Acknowledgments

We thank gratefully B. Rêche and D. Guyomard for support in providing samples. The study was financially supported by CHARC program and Ecorecorun program (DEAL). We acknowledge the Plateforme Gentyane of the Institut National de la Recherche Agronomique (INRA, Clermont-Ferrand, France) for guidance and technical support. Agathe Pirog was supported by a doctoral fellowship from Reunion regional council (European Social Fund).

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SYNTHÈSE GÉNÉRALE ET PERSPECTIVES

Malgré le nombre toujours croissant de données et de connaissances acquises sur le monde qui nous entoure, bien des espèces restent méconnues, notamment dans l'environnement marin, et ce, même pour des espèces emblématiques suscitant depuis longtemps notre intérêt et notre curiosité. C'est notamment le cas des requins, longtemps perçus par le prisme des attaques sur l'Homme dont ils sont responsables. Ce groupe très diversifié joue pourtant des rôles variés dans les écosystèmes marins. En effet, les requins peuvent contrôler le fonctionnement des réseaux trophiques par leur consommation de biomasse et leur prédation, permettent la redistribution spatiale des nutriments et de l'énergie, peuvent connecter les écosystèmes marins grâce à leurs capacités de déplacement importantes (surtout pour les grands requins) et sont enfin capable de modifier la structure physique de ces écosystèmes lorsqu'ils bougent ou chassent (Estes et al., 2016). Par ailleurs, ils présentent une valeur économique non négligeable, suivant qu'ils soient pêchés ou que leur présence soit valorisée plus durablement par le tourisme, avec mise en place de plongées en cage ou sur sites de nourrissage (Gallagher & Hammerschlag, 2011; Gallagher et al., 2015). Pourtant, les populations de requins, surtout celles des grands requins côtiers, sont en déclin à travers le monde, du fait d'une surpêche et d'autres pressions anthropiques, auxquelles ils sont très sensibles de par leurs traits d'histoire de vie (Baum et al., 2003; Dulvy et al., 2008; Dulvy et al., 2014). Il apparait donc nécessaire d'acquérir des connaissances à la fois sur la structure de leurs populations, sur leur degré de connectivité et leur taille, et enfin sur leur dynamique.

Le travail de cette thèse a donc été d'étudier deux de ces espèces de grande taille mais discrètes, le requin bouledogue *Carcharhinus leucas* et le requin tigre *Galeocerdo cuvier*, méconnues et impliquées dans des attaques sur l'Homme avec une recrudescence inexplicée d'attaques autour de La Réunion depuis 2011, et d'apporter des éléments de réponse aux questions posées. Le requin bouledogue et le requin tigre appartiennent à la famille des Carcharinidés et présentent donc des caractéristiques communes. Ce sont des individus de grande taille à l'âge adulte, qui présentent des capacités de déplacement importantes. Pourtant, elles présentent chacune leurs spécificités. Elles utilisent des habitats légèrement différents : le requin bouledogue est euryhalin et davantage côtier que le requin tigre qui est considéré comme une espèce semi-océanique, c.-à-d. capable d'évoluer à la fois en milieu côtier et océanique. Ces différences d'utilisation d'habitats ont été mises en évidence à La Réunion (Trystram, 2016). Ils présentent également des modes de reproduction

différents : le requin tigre se reproduit par viviparité lécithotrophe (ou aplacentaire) alors que le bouledogue par viviparité placentaire. La structure de leurs populations et leurs modes de reproduction restaient largement méconnus au début de cette thèse, et il semblait important d'acquérir des connaissances pour évaluer si les spécificités mises en évidence pour ces deux espèces se reflétaient dans l'histoire démographique et la dynamique de leurs populations.

Au cours de cette synthèse, nous comparerons donc les résultats obtenus pour chaque espèce afin d'identifier les dissemblances et ressemblances observées, et ainsi tenter d'estimer les différents impacts que peuvent avoir les programmes de régulation ou les pêcheries selon les caractéristiques de l'espèce, en se concentrant plus particulièrement sur la situation réunionnaise. L'ensemble des résultats est également résumé dans le Tableau 2. Nous proposerons, par ailleurs, différentes perspectives afin de continuer à accroître nos connaissances sur ces espèces, toujours dans un but d'améliorer la gestion durable de leurs populations.

Tableau 2. Caractéristiques des populations du requin bouledogue *Carcharhinus leucas* et du requin tigre *Galeocerdo cuvier* mises en évidence au cours de ce travail de thèse.

	Requin bouledogue <i>Carcharhinus leucas</i>	Requin tigre <i>Galeocerdo cuvier</i>
Structure génétique		
Atlantique Ouest contre Ouest de l'océan Indien/Ouest du Pacifique	Différenciation complète	Différenciation mais quelques flux de gènes contemporains
Ouest de l'océan Indien contre Ouest du Pacifique	Différenciation avec soit absence de flux de gènes contemporains, soit migration des mâles mais pas des femelles	Aucune différenciation génétique
Ouest de l'océan Indien	Différenciation génétique faible voire inexistante. Philopatrie des femelles à des zones d'accouplement et/ou de mise bas ?	Aucune différenciation génétique
Utilisation de l'habitat	Espèce majoritairement côtière mais capable d'incursions dans le milieu océanique	Espèce majoritairement océanique
Diversité génétique et taille efficace		
Diversité génétique	Importante	Faible
Variations de taille efficace	Constante dans chaque bassin océanique	Goulot d'étranglement de la population des océans Indien et Pacifique il y a 2 000-3 000 ans
Taille efficace actuelle de chaque population	6 000-10 000 individus	?
Reproduction autour de La Réunion		
Durée de la gestation	11-12 mois	Environ 15 mois
Saison d'accouplement	Juin-septembre	Mai-juin
Saison de parturition	Décembre-janvier	Décembre-janvier
Stockage du sperme	2-4 mois	4-5 mois
Durée probable du cycle	Bisannuel	Trisannuel
Polygamie	60 % des portées étudiées polyandres et polygynie détectée	Absente (ou très rare)
Zones de mise bas	Zones côtières spécifiques, restant à identifier à La Réunion	Pleine mer ? à confirmer
Présence côtière autour de La Réunion	Habitat naturel avec présence renforcée pour la reproduction (accouplement et/ou mise bas)	Principalement liée à l'alimentation
Conséquences pour la gestion des populations	Populations ouvertes autour de La Réunion, efficacité probablement limitée des programmes de régulation Populations non sévèrement atteintes par les pressions naturelles et anthropiques mais la pêche dans les zones (et aux périodes) d'accouplement et/ou de mise bas pourrait avoir des conséquences sur le long terme sur la taille de ces populations, voire sur l'espèce	Populations ouvertes autour de La Réunion, efficacité probablement limitée des programmes de régulation Populations probablement réduites et vulnérables à la pêche. Révision du statut de conservation ?

1. Connectivité moindre pour les populations du requin bouledogue *Carcharhinus leucas*

Au cours de ce travail, nous avons analysé la variation génétique de 370 et 244 échantillons de requin bouledogue et de requin tigre, provenant de localités situées dans l'Ouest de l'océan Indien et l'Ouest de l'océan Pacifique, et même dans l'Ouest de l'océan Atlantique dans le cas du requin bouledogue. Ceci a été réalisé à l'aide d'un nombre important de marqueurs microsatellites (25 et 27 marqueurs pour le requin bouledogue et le requin tigre, respectivement) et de 3 marqueurs mitochondriaux. Cet échantillonnage conséquent, ainsi que l'utilisation d'un nombre important de marqueurs nucléaires et mitochondriaux présentant des modes d'évolution différents, a mis en évidence des patrons de structuration génétique des populations de requin bouledogue et de requin tigre similaires entre l'Ouest de l'Atlantique et les océans Indien et Pacifique, mais bien différents entre l'Ouest de l'océan Indien et l'Ouest de l'océan Pacifique.

En effet, même si cette étude n'inclut pas d'échantillons de requin tigre de l'Ouest de l'Atlantique, une différenciation génétique forte, estimée avec 10 marqueurs microsatellites et 2 marqueurs mitochondriaux, a été montrée entre les populations de requin tigre des océans Indien et Pacifique et celles de l'Ouest de l'Atlantique (Bernard et al., 2016), ce qui est semblable aux résultats trouvés ici pour le requin bouledogue (Chapitre 2 ; Pirog et al., soumis-a). Des résultats similaires ont été trouvés pour la majorité des grands requins, à la fois chez des espèces océaniques (par exemple, le requin baleine *Rhincodon typus* ; Castro et al., 2007; Vignaud et al., 2014a) et côtières (par exemple, le requin soyeux *Carcharhinus falciformis* ou le requin requiem des sables *Carcharhinus obscurus* ; Benavides et al., 2011; Clarke et al., 2015). Néanmoins, une absence complète de flux de gènes contemporains entre l'Ouest de l'Atlantique et l'Ouest de l'océan Indien a été mise en évidence ici chez le requin bouledogue (Chapitre 2 ; Pirog et al., soumis-a), contrairement au requin tigre, pour lequel des événements de dispersion efficace, certes probablement rares, auraient encore lieu entre ces deux régions, probablement par l'Atlantique Sud en passant au sud de la pointe de l'Afrique (Bernard et al., 2016). Par ailleurs, entre l'Ouest de l'océan Indien et l'Ouest de l'océan Pacifique, une absence de différenciation génétique a été retrouvée pour le requin tigre (Chapitre 4 ; Bernard et al., 2016; Holmes et al., 2017; Pirog et al., en préparation-a), alors que, pour le requin bouledogue, les flux de gènes contemporains entre ces deux régions sont soit inexistantes, soit assurés uniquement par les mâles (Chapitre 2 ; Pirog et al., soumis-a). En outre, la forte discordance retrouvée entre les estimations de différenciations nucléaire et mitochondriale entre ces deux

dernières régions pour le requin bouledogue n'a pas été identifiée chez le requin tigre. Enfin, au sein de l'Ouest de l'océan Indien et au sein de l'Ouest de l'océan Pacifique, une absence de différenciation a été retrouvée pour les deux espèces, suggérant des flux de gènes importants au sein de ces régions. Néanmoins, la possibilité qu'il existe de la philopatrie des femelles à des zones de nurseries ne peut pas être rejetée pour le requin bouledogue. Cette philopatrie pourrait expliquer les estimations de différenciation mitochondriale légèrement plus importantes entre les îles océaniques (c.-à-d. La Réunion et Rodrigues) et les autres localités échantillonnées pour le requin bouledogue (Chapitre 2 ; Pirog et al., soumis-a).

En conclusion, le requin tigre présente des populations connectées à une plus grande échelle géographique que celles du requin bouledogue, et effectuerait des déplacements réguliers en milieu océanique, sur de longues distances (plusieurs dizaines de milliers de kilomètres), ce qui est concordant avec des études de déplacement de l'espèce (Lea et al., 2015a). Le requin bouledogue présente cependant également des capacités de déplacement assez importantes, et se déplace probablement régulièrement le long des continents, sur des distances relativement grandes (plusieurs milliers de kilomètres ; Daly et al., 2014; Espinoza et al., 2016). Il semble également capable de traverser des eaux profondes (2 000 m de profondeur) sur des distances de quelques centaines de kilomètres, étant donné qu'aucune différenciation génétique n'a été mise en évidence entre le Mozambique et les Seychelles, par exemple. Ce résultat est en accord avec le suivi d'une femelle gravide qui a effectué un déplacement d'environ 2 000 km entre les Seychelles et Madagascar, très vraisemblablement pour aller mettre bas dans une rivière de la côte Est malgache (Lea et al., 2015a). Par ailleurs, deux requins bouledogue (un mâle et une femelle) marqués par marque satellite (miniPAT) à La Réunion ont effectué plusieurs incursions dans le milieu océanique de plusieurs centaines de kilomètres au cours des 6 mois de suivi (Soria et al., 2015).

Il est intéressant de noter que la connectivité plus importante retrouvée chez le requin tigre s'accompagne d'une plus faible diversité génétique.

2. Diversité génétique moins importante chez le requin tigre *Galeocerdo cuvier*

Ayant travaillé symétriquement sur le requin tigre et le requin bouledogue, nous avons utilisé les mêmes procédures pour développer les marqueurs moléculaires et réaliser les analyses génétiques, ce qui permet de comparer précisément les différences de diversité génétique observée chez ces

deux espèces. En effet, utilisant la même approche pour construire les banques microsatellites spécifiques à chaque espèce, nous avons identifié 20 marqueurs polymorphes chez le requin bouledogue, contre seulement 8 chez le requin tigre, avec un nombre moyen (\pm E.S.) d'allèles par locus de $4,55 \pm 0,54$ et de $3,38 \pm 0,71$, respectivement (sur 41 et 101 individus de La Réunion génotypés, respectivement) (Pirog et al., 2015a; Pirog et al., 2016). Par ailleurs, les trois marqueurs mitochondriaux utilisés pour chaque espèce, dont deux en commun (*CR* et *cytb*), ont permis l'analyse de séquences mitochondriales de longueurs relativement similaires (2 516 pb pour le requin bouledogue et 2 452 pb pour le requin tigre) (Chapitres 2 et 4 ; Pirog et al., en préparation-a; Pirog et al., soumis-a). Or, dans l'Ouest de l'océan Indien et l'Ouest du Pacifique, où 188 échantillons de requin bouledogue de 10 localités ont été analysés, ainsi que 127 échantillons de requin tigre de 7 localités, 27 haplotypes ont été identifiés pour le requin bouledogue, avec 36 sites polymorphes et une diversité haplotypique h de $0,91 \pm 0,00$, contre 22 haplotypes identifiés pour le requin tigre, avec 20 sites polymorphes et une diversité haplotypique h de $0,78 \pm 0,01$ (Chapitres 2 et 4 ; Pirog et al., en préparation-a; Pirog et al., soumis-a). Par ailleurs, seuls trois haplotypes majoritaires ont été identifiés chez le requin tigre, représentés par 66 % des individus séquencés (Chapitre 4 ; Pirog et al., en préparation-a). Une diversité génétique plus faible a donc été détectée pour le requin tigre, à la fois pour les marqueurs nucléaires et les marqueurs mitochondriaux.

La diversité génétique chez le requin bouledogue est assez similaire à celle trouvée pour d'autres espèces de grands requins, comme le requin blanc *Carcharodon carcharias* (Pardini et al., 2001), le requin peau bleue *Prionace glauca* (Veríssimo et al., 2017), le requin à pointes noires *Carcharhinus melanopterus* (Vignaud et al., 2014b) ou encore le requin milandre *Galeorhinus galeus* (Chabot & Allen, 2009; Chabot, 2015). Elle tend à montrer que les populations délimitées (c.-à-d. l'Ouest de l'océan Indien, l'Ouest du Pacifique et l'Ouest de l'Atlantique) ne sont pas encore extrêmement touchées par les pressions qu'elles subissent. Ceci est confirmé par les tailles efficaces estimées pour chacune de ces populations, variant de 6 000 à 10 000 individus (Chapitre 2 ; Pirog et al., soumis-a), et similaires à celles estimées pour d'autres requins considérés vulnérables, mais pas en danger immédiat (Chabot & Allen, 2009; Schultz et al., 2008). A l'opposé, la diversité génétique réduite mise en évidence chez le requin tigre pourrait indiquer une sensibilité plus importante des populations de cette espèce aux pressions anthropiques. La détection d'un goulot d'étranglement datant de moins de 3 000 ans dans les océans Indien et Pacifique pourrait bien confirmer que ces populations sont en effet sensibles à l'activité de l'Homme (Chapitre 4 ;

Pirog et al., en préparation-a). L'impossibilité de calculer la taille efficace actuelle limite néanmoins l'estimation de l'état de santé de ces populations et la prédiction de leur évolution.

Par ailleurs, il semble bien que ces différents patrons de diversité et de structuration génétique soient liés à des comportements de reproduction différents.

3. Patrons génétiques probablement liés à des comportements de reproduction différents

La plus grande structuration génétique identifiée chez le requin bouledogue, ainsi que son utilisation plus importante du milieu côtier, pourraient bien être liées à la philopatrie des individus à des zones de reproduction. Dans le terme « reproduction », on distinguera l'accouplement, la fécondation (des gamètes), la gestation et la mise-bas. En effet, il est depuis longtemps connu que ce requin utilise les estuaires ou grands fleuves pour mettre bas. Des femelles matures ont été suivies dans ces milieux, et des juvéniles sont fréquemment observés dans plusieurs de ces nurseries (Heupel et al., 2007; Heupel & Simpfendorfer, 2011; Jenson, 1976; Thorson et al., 1966; Thorson, 1976). Il est également supposé que les femelles seraient fidèles à des nurseries particulières pour mettre bas, peut-être celles où elles sont nées (philopatrie au site de naissance), comme démontré pour le requin citron *Negaprion brevirostris* (Feldheim et al., 2013). La discordance mito-nucléaire mise en évidence dans plusieurs études entre les indices de différenciation génétique calculés avec ces deux types de marqueurs (Karl et al., 2011; Pirog et al., soumis-a) suggère, en effet, de la philopatrie des femelles requin bouledogue à des nurseries spécifiques pour s'accoupler et/ou mettre bas, bien que d'autres études soient nécessaires pour confirmer ce comportement. Par ailleurs, nous avons mis en évidence ici pour la première fois de la philopatrie des mâles, qui reviendraient dans une même zone pour s'accoupler, et ce plusieurs années de suite (Chapitre 3 ; Pirog et al., soumis-b). Ce comportement n'a pas été identifié chez le requin tigre, pour lequel les zones de nurseries ou même d'accouplement (distinctes ou non des premières) sont encore très mal connues, voire inconnues. En effet, les différentes études ayant analysé la biologie de requins tigre pêchés lors de programmes de régulation n'ont observé qu'une faible proportion de femelles matures, que ce soit en Afrique du Sud (Dicken et al., 2016), dans l'Est de la mer d'Arabie (Varghese et al., 2017), ou à La Réunion (Pirog et al., en préparation-b). Cela peut suggérer que l'accouplement et la mise-bas ne se déroulent pas dans ces zones côtières mais soit (1) en pleine mer, soit (2) dans des zones côtières encore non identifiées et qui peuvent

être choisies de manière opportuniste par les femelles, rendant leur identification plus difficile (pas d'agrégations particulières de femelles matures ou de juvéniles). Ces deux hypothèses pourraient ainsi expliquer la connectivité génétique plus importante identifiée pour le requin tigre que pour le requin bouledogue.

En outre, le requin tigre semble majoritairement monoandre. À La Réunion (Pirog et al., soumis-b) et en Australie (Holmes et al., 2018), les portées étudiées grâce aux marqueurs microsatellites développés étaient issues chacune d'un seul père, contrairement au requin bouledogue (Pirog et al., 2015b) et à la majorité des espèces de requin pour lesquelles ce comportement a été étudié (Byrne & Avise, 2012; Lyons et al., 2017). En effet, chez le requin bouledogue, près de 60 % des portées étudiées ont été fécondées par plusieurs mâles, avec deux à cinq pères identifiés par portée (Pirog et al., soumis-b). Par ailleurs, plusieurs mâles ont été identifiés comme père d'embryons de portées différentes, conçues lors d'une même saison de reproduction ou au cours de saisons de reproduction différentes (successives ou une à deux saisons d'écart). Cela met en évidence, pour la première fois de manière formelle, de la polygynie chez le requin bouledogue (un mâle se reproduisant avec plusieurs femelles au cours d'une même saison de reproduction). Ces comportements, que ce soit la polyandrie ou la polygynie, pourraient ainsi être en lien avec des agrégations saisonnières de requins bouledogue pour s'accoupler (Pirog et al., soumis-b). Chez le requin tigre, l'absence de polyandrie pourrait être liée à l'absence de fidélité à des zones spécifiques d'accouplement, à l'utilisation prépondérante du milieu océanique et à des périodes de reproduction moins saisonnières (Withney & Crow 2007), limitant les rencontres entre partenaires potentiels. Ainsi la stratégie de reproduction du requin tigre serait fondée essentiellement sur des choix individuels qui dépendent moins des signaux environnementaux que d'un état physiologique favorable et de la rencontre d'individus matures. Cette stratégie pourrait refléter le fonctionnement des écosystèmes hauturiers tropicaux, dont la production de biomasse est moins saisonnière et prédictible que celle des écosystèmes côtiers. Elle pourrait également expliquer la plus faible diversité génétique trouvée, la polyandrie étant supposée augmenter la taille efficace des populations et maintenir la diversité génétique (Sugg & Chesser, 1994; Zeh & Zeh, 2001).

Les différents comportements de reproduction mis en évidence dans ce travail semblent donc bien être liés aux différents patrons de structuration génétique des populations identifiés chez le requin bouledogue et le requin tigre. Ces différences reflètent également très certainement des places et

rôles différents de ces deux espèces dans les écosystèmes marins en accord avec les caractéristiques des habitats dans lesquels ils évoluent.

4. Place des deux espèces dans les communautés marines

Les différences d'utilisation de l'habitat des requins bouledogue et tigre impliquent très certainement différents rôles dans les réseaux trophiques. En effet, le comportement semi-océanique du requin tigre semble congruent avec les résultats des analyses des contenus stomacaux et d'isotopes stables, montrant une exploitation plus importante des sources pélagiques chez cette espèce par rapport au requin bouledogue, autour de La Réunion (Trystram et al., 2017). Par ailleurs, la niche trophique du requin tigre semble plus large que celle du requin bouledogue et comporte des espèces différentes, impliquant qu'il exploite à la fois les ressources côtières et hauturières autour de La Réunion, contrairement au requin bouledogue qui exploite majoritairement des ressources côtières (Trystram et al., 2017). Le requin tigre aurait donc un rôle important dans le cycle des nutriments, permettant un flux entre les milieux côtiers et hauturiers (Heupel & Simpfendorfer, 2015; McCauley et al., 2012; Meyer et al., 2010; Schmitz et al., 2010; Williams et al., 2018). Le requin bouledogue, quant à lui, semble un acteur majeur des réseaux trophiques côtiers (Trystram et al., 2017). En effet, ces deux espèces identifiées comme des prédateurs apicaux par les analyses isotopiques, constituent très probablement des groupes clés des réseaux trophiques de surface autour de La Réunion (Trystram, 2016), mais aussi d'autres réseaux trophiques, notamment en Colombie et au Brésil (Bornatowski et al., 2014; Navia et al., 2010). En outre, les deux espèces présentent des patrons de spécialisation différents. Le requin bouledogue présente ainsi une variabilité interindividuelle des régimes alimentaires plus importante que le requin tigre, qui serait plus généraliste (Matich et al., 2011; Trystram et al., 2017) à l'échelle individuelle et populationnelle.

De manière générale, le requin bouledogue est donc une espèce plus adaptée aux milieux côtiers, connus pour leur variabilité spatiale et temporelle importante (Cloern et al., 2016). Le requin bouledogue présente, possiblement en relation avec cette variabilité, un régime alimentaire spécialisé aux écosystèmes qu'il occupe, avec notamment une variabilité régionale mise en évidence (Daly et al., 2013; Matich et al., 2011; Trystram et al., 2017). Cette spécialisation est aussi retrouvée au niveau génétique, avec des populations relativement structurées et une diversité

génétique importante. Par ailleurs, les comportements de reproduction identifiés, la polyandrie et la polygynie, pourraient également maintenir cette diversité génétique. Enfin, la fidélité potentielle des femelles à des nurseries connues pour fournir un abri et des ressources importantes aux juvéniles augmenterait le taux de survie de ce stade de vie particulièrement vulnérable. A l'opposé, le requin tigre présente des caractéristiques plus spécifiques aux habitats océaniques qui sont ouverts et plus stables que les habitats côtiers. Son régime alimentaire généraliste et opportuniste lui permet de tirer profit de tous types de proies rencontrées à travers son territoire, qui peut être très étendu géographiquement. Sa plus faible diversité génétique, de même que la prédominance de portées monoandres, pourrait également s'expliquer par l'adaptation de cette espèce à un milieu relativement stable dans le temps et l'espace. Cela semble également conforté par l'investissement important des femelles dans un unique événement de reproduction, avec un nombre élevé d'embryons par portée, une période de gestation longue et un cycle de reproduction trisannuel, moins fréquent chez les requins que les cycles de reproduction bisannuels (Whitney & Crow, 2007).

Ainsi, le requin bouledogue et le requin tigre sont biologiquement très différents, ce qui influe sur leurs places dans les communautés marines, mais aussi sur l'état de santé et la résistance de leurs populations aux pressions anthropiques. Cela implique donc des mesures de gestion adaptées à chaque espèce.

5. Implications pour la gestion des populations

Le requin tigre et le requin bouledogue sont soumis à des programmes de régulation à La Réunion, en Afrique du Sud et en Australie, où des requins sont prélevés du milieu pour limiter le risque d'attaques. Ces programmes de régulation sont généralement effectués dans une logique de supprimer les individus responsables des attaques. Ils doivent donc, pour être efficaces, être réalisés sur des populations restreintes géographiquement et fermées (Dudley, 1997; Reid et al., 2011). Chez le requin bouledogue, la différenciation génétique importante entre les populations de l'océan Indien et du Pacifique montre que les populations de ces deux régions évoluent de manière indépendante. Ainsi les pressions, telles la pêche commerciale ou de loisirs et/ou les programmes de régulation en place au sein de chacun de ces océans, impactent probablement les populations à l'échelle de chacun des océans. En revanche, la connectivité importante mise en évidence chez le

requin tigre à l'échelle des océans Indiens et Pacifique implique que la pêche ou des programmes de régulation auront un impact sur les populations à l'échelle des deux océans. Ainsi les programmes de régulation réalisés à La Réunion, en Afrique du Sud, ou en Australie seraient donc inefficaces à court terme, puisque les individus se déplacent de manière non négligeable dans toute la région Ouest de l'océan Indien et/ou dans tout l'Ouest du Pacifique. L'efficacité limitée des programmes de régulation sur la baisse des attaques des requins a déjà été montrée à Hawaii (Wetherbee et al., 1994), ainsi qu'en Australie et en Afrique du Sud (Cliff & Dudley, 2011; Gibbs & Warren, 2015; Sumpton et al., 2011). Ainsi, à Hawaii, entre 1959 et 1976 (17 ans), 300 000 dollars américains (USD) ont été investis dans des programmes de régulation, pendant lesquels 4 668 requins ont été pêchés, principalement des requins tigre *G. cuvier* et des requins gris *Carcharhinus plumbeus* (Wetherbee et al., 1994). D'après le registre comptabilisant au niveau mondial le nombre d'attaques de requins sur l'Homme (le « International Shark Attack File », ISAF), le taux d'attaques à Hawaii avant et après les programmes de régulation est resté constant (0,6 attaques par an en moyenne), et est depuis en augmentation (1,4 attaques par an en moyenne) (Wetherbee et al., 1994). Les auteurs concluent que le taux d'attaques de requins suit plus fidèlement l'augmentation de la population humaine des zones concernées que l'évolution des populations de requins (Wetherbee et al., 1994). Comparativement, à La Réunion, entre 2011 et 2014 (3 ans), environ 4 millions d'euros ont été directement engagés contre le risque requin (Fabing, 2014), pour financer le déploiement de dispositifs de protection et de prélèvements. En 2015, un nouveau programme de régulation a été financé, le programme Caprequins 2, dont le montant total s'élève à 1 million d'euros, pour une durée de deux ans. Entre juin 2015 et février 2017, 127 requins définis comme potentiellement dangereux ont ainsi été prélevés, 70 requin bouledogues et 57 requin tigres (CRA, 2017). Ces pêches touchent également d'autres espèces, capturées en tant que prises accessoires (notamment des requins marteau *Sphyrna lewini* et un grand requin blanc *Carcharodon carcharias*, deux espèces de statuts « en danger » et « vulnérable », respectivement, sur la liste rouge de l'UICN ; (Baum et al., 2007; Fergusson et al., 2009)). Le nouveau programme de pêche débuté en 2018 sous l'égide du Centre de Ressources et d'Appui (CRA) sur le risque requin a pour objectif de limiter au maximum l'impact de la pêche sur les espèces non ciblées. En parallèle, le CRA dirige également des études afin de développer d'autres moyens de protection non létaux. Le CRA dispose d'un budget annuel de ~650 000 euros.

Par ailleurs, nous avons identifié au cours de cette étude que les côtes réunionnaises pouvaient être une zone importante pour la reproduction du requin bouledogue, probablement utilisées à la fois comme nurseries pour les juvéniles, et également fréquentées lors des périodes d'accouplement. Ainsi, si la pêche effectuée touche principalement des individus matures en période de reproduction, elle pourrait entraîner à long terme un déclin de la population entière. En outre, la pêche du requin tigre depuis plusieurs décennies, et notamment à travers les océans Indien et Pacifique (Cliff & Dudley, 1991; Dudley, 1997; Holmes et al., 2012; Polovina & Lau, 1993; Reid & Krogh, 1992; Simpfendorfer, 1992, 2009; Sumpton et al., 2011; Wetherbee et al., 1994), pourrait avoir eu un impact non négligeable sur la santé des populations de cette espèce semi-océanique, comme montré par leur faible diversité génétique. Une révision de son statut, actuellement « quasi-menacé » sur la liste rouge des espèces menacées de l'Union Internationale pour la Conservation de la Nature (Simpfendorfer, 2009), pourrait être nécessaire afin de limiter la pêche et améliorer la protection de cette espèce. Ainsi, sur le long terme, les programmes de régulation mis en place dans plusieurs pays pourraient entraîner le déclin des populations de requin tigre et bouledogue, dont les conséquences sur l'équilibre des écosystèmes sont inconnues. En effet, la suppression d'un prédateur apical dans un réseau trophique peut entraîner des effets négatifs sur les niveaux inférieurs, d'autant plus si l'équilibre du réseau repose sur la présence de ce prédateur, comme cela semble être le cas à La Réunion (Trystram, 2016; Trystram et al., 2017). Ainsi, des données issues de 17 études de suivi de pêche de la Floride au Maine ont montré l'augmentation des populations de 12 espèces de petits Élasmobranches (requins, raies et chimères) liée à la diminution des populations de grands requins côtiers (Myers et al., 2007). En Afrique du Sud, la protection des plages depuis plus de 50 ans par la mise en place de filets permet d'accéder à des données sur le long terme. Ainsi, entre 1956 et 1976, dans ces filets, les prises par unité d'effort des grands requins ont diminué, alors que les compétitions sportives de pêche ont montré une prolifération des petits Élasmobranches. Cette prolifération a entraîné la diminution des populations de poissons osseux habituellement prisés par la pêche sportive (carangues, mérours, dorades, thonidés) (van der Elst, 1979). Enfin, en Australie, le déclin des populations de requin tigre semble impliquer un changement de comportement des tortues marines et des dugongs avec un effet en cascade jusqu'à diminuer l'étendue des herbiers (Heithaus et al., 2007a; Heithaus et al., 2008). En effet, la pression de prédation des requins tigre étant relâchée, les populations de tortues marines broutant les herbiers ont augmenté, diminuant la surface de ces derniers. Or ces écosystèmes servent d'habitat et de nurseries pour de nombreuses

espèces benthiques et jouent un rôle prépondérant dans le cycle des nutriments (Heithaus et al., 2007a; Heithaus et al., 2008).

L'écotourisme associé à la présence de requins est une solution de plus en plus souvent considérée pour assurer leur conservation tout en valorisant économiquement leur présence (Gallagher et al., 2015). Cet écotourisme concerne notamment la plongée sur des sites où les requins sont attirés afin d'être observés. Bien qu'encore controversées, ces activités, si elles sont mises en place dans un souci de protection des populations humaines et du respect de l'animal, constituent une source de bénéfices économiques qui s'avère non négligeable là où l'activité est développée (Clua et al., 2011; Dicken & Hosking, 2009; Vianna et al., 2012). Une méta-analyse mondiale conduite sur les bénéfices économiques de l'écotourisme lié aux requins a estimé qu'environ 590 000 personnes dépensent plus de 314 millions de dollars par an et soutiennent directement 10 000 emplois, afin d'observer les requins dans leur milieu naturel (Cisneros-Montemayor et al., 2013). D'après les évolutions observées, ce nombre pourrait doubler dans les 20 prochaines années, générant des revenus de plus de 780 millions de dollars. Comparativement, le bénéfice actuel des requins pêchés est estimé à 630 millions de dollars par an, et est en déclin depuis la dernière décennie (Worm et al., 2013). Les principales espèces de requin concernées par l'écotourisme sont le requin baleine *Rhincodon typus*, mais également le grand requin blanc *C. carcharias*, le requin tigre *G. cuvier*, les requins ange *Squatina* spp., les requins marteau *Sphyrna* spp. et les requins requiem *Carcharhinus* spp. (Cisneros-Montemayor et al., 2013). La présence de requins bouledogue et tigre est ainsi valorisée touristiquement à différents endroits dans le monde, notamment aux Fiji (Brunnschweiler & Earle, 2006), Hawaii (Meyer et al., 2009b) et en Afrique du Sud (Dicken & Hosking, 2009).

6. Vers une utilisation couplée de la génomique et du suivi satellite des populations

Ce travail a ainsi permis d'étudier précisément la structure génétique des populations de requin bouledogue et de requin tigre dans l'Ouest de l'océan Indien mais aussi à travers leur aire de répartition. Néanmoins, plusieurs questions restent encore sans réponses, ou du moins, pourraient être précisées. Il s'agit notamment :

1. de savoir si la discordance mito-nucléaire trouvée entre les indices de différenciation des populations de requin bouledogue de l'Ouest de l'océan Indien et de l'Ouest de l'océan Pacifique

reflète une absence complète de flux de gènes contemporains ou des flux assurés uniquement par les mâles ;

2. d'estimer plus précisément la taille et l'état de santé des populations de ces requins, et plus particulièrement du requin tigre, dans l'océan Indien et l'océan Pacifique. En effet, bien qu'au cours de cette étude nous avons mis en évidence une sensibilité particulière de cette espèce et une diversité génétique faible, nous n'avons pas encore d'idée précise de la taille du stock dans cette région ;

3. d'étudier plus précisément les comportements philopatrics du requin bouledogue dans l'Ouest de l'océan Indien, et plus particulièrement à La Réunion. Quelles sont les zones de nurseries ? À quelle fréquence reviennent les mâles et/ou les femelles sur ces zones ?

4. d'identifier les zones de mise bas du requin tigre. Se situent-elles en plein océan ? Ou dans des sites côtiers encore inconnus ?

Ces questions pourraient notamment être résolues en utilisant à la fois des méthodes indirectes moléculaires et des méthodes de suivi directes. Les technologies de séquençage nouvelle génération (Davey et al., 2011), en plein essor actuellement, peuvent permettre de pallier le manque de précision des marqueurs utilisés ici pour répondre à ces questions. En effet, le génotypage de type RAD (Restriction-site Associated DNA) permet de séquencer tout le génome et d'augmenter par un facteur 500 le nombre de marqueurs qui permettent de discriminer les individus (Baird et al., 2008), et ce pour un coût et un temps bien moindres qu'il n'y a, ne serait-ce, que trois ans. Ainsi, on passerait de 20 marqueurs à 10 000, permettant d'affiner les analyses et d'augmenter la puissance des tests statistiques. Ces marqueurs seraient ainsi très utiles pour affiner les estimations de taille efficace réalisées au cours de cette étude, mais pourraient aussi potentiellement permettre de résoudre la discordance mito-nucléaire trouvée pour le requin bouledogue.

Par ailleurs, les méthodes de suivi satellites deviennent également de plus en plus accessibles, et pourraient être utilisées pour étudier les migrations actuelles des individus au sein de l'océan Indien (Hays et al., 2016), mais aussi d'étudier la connectivité potentielle des populations de requin bouledogue de l'océan Indien et de l'océan Pacifique. Il faut néanmoins garder à l'esprit qu'elles ne permettent que de voir les déplacements des individus, et non les déplacements efficaces, c.-à-d. avec reproduction. Elles présentent toutefois l'avantage, comparativement au suivi acoustique, de

ne pas limiter spatialement le suivi des animaux. En effet, ce suivi satellite ne nécessite pas l'installation d'un réseau de récepteurs acoustiques qui quadrillerait ces régions et/ou la coordination des scientifiques de tous les pays de ces régions pour s'accorder sur le type de marquage et de balises utilisés. Enfin, cela permettrait d'identifier plus précisément les zones de nurseries. Leur suivi temporel permettrait d'étudier plus finement la fidélité des individus à ces zones. Cette approche pourrait par ailleurs être couplée à l'étude des génotypes des juvéniles sur plusieurs générations, afin de déterminer les lignées parentales au sein de ces zones. Tout comme Hawaii, la Floride ou l'Afrique du Sud, La Réunion pourrait devenir un haut lieu de la recherche internationale sur les requins et voir ainsi son rayonnement s'accroître à l'échelle régionale, voire au-delà, en matière de recherche scientifique.

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ANNEXES

En annexes sont présentés les articles en premier auteur ou co-auteur écrits en parallèle des travaux de cette thèse.

1. Clonal structure through space and time: High stability in the holothurian *Stichopus chloronotus* (Echinodermata)

Pirog A, Gélin P, Bédier A, Bianchetti G, Georget S, Frouin P, Magalon H. (2017). *Ecology and Evolution*.

L'annexe 1 est un article étudiant la stabilité de la structure clonale spatiale et temporelle des populations de l'holothurie verte *Stichopus chloronotus* à La Réunion.

2. Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean

Diogene J, Reverté L, Rambla-Alegre M, del Río V, De la Iglesia P, Campàs M, Palacios O, Flores C, Caixach J, Ralijaona C, Razanajatovo I, **Pirog A**, Magalon H, Arnich N, Turquet J. (2017). *Scientific Reports*.

L'annexe 2 est un article reportant la présence de ciguatoxines lors d'un épisode d'empoisonnement après ingestion de viande de requin à Madagascar (Ouest de l'océan Indien).

3. Artificial daily fluctuations of river discharge affect the larval drift and survival of a tropical amphidromous goby

Lagarde R, Teichert N, Faivre L, Grondin H, Magalon H, **Pirog A**, Valade P, Ponton D. (2017). *Ecology of Freshwater Fish*.

L'annexe 3 est un article traitant de l'influence des variations journalières du courant d'une rivière comportant un barrage sur la dévalaison des larves du Gobiidé *Sicyopterus lagocephalus*.

4. Temporal variability of larval drift of tropical amphidromous gobies along a watershed in Réunion Island

Lagarde R, Teichert N, Grondin H, Magalon H, **Pirog A**, Ponton D. (2016). *Canadian Journal of Fisheries and Aquatic Sciences*.

L'annexe 4 est un article traitant de la dévalaison des larves de deux espèces de Gobiidés indigènes de La Réunion, *Sicyopterus lagocephalus* et *Cotylopus acutipinnis*.

Annexe 1


Clonal structure through space and time: High stability in the holothurian *Stichopus chloronotus* (Echinodermata)

Pirog A, G lin P, B dier A, Bianchetti G, Georget S, Frouin P, Magalon H. (2017). *Ecology and Evolution*.

L'annexe 1 est un article  tudiant la stabilit  de la structure clonale spatiale et temporelle des populations de l'holothurie verte *Stichopus chloronotus*   La R union.

ORIGINAL RESEARCH

Clonal structure through space and time: High stability in the holothurian *Stichopus chloronotus* (Echinodermata)

Agathe Pirog¹  | Pauline G  lin^{1,2} | Alexandre B  dier¹ | Gr  goire Bianchetti¹ |
St  phane Georget¹ | Patrick Frouin^{1,2} | H  l  ne Magalon^{1,2}

¹UMR ENTROPIE (Universit   de La R  union, IRD, CNRS), Saint Denis, La R  union, France

²Laboratory of Excellence CORAIL, Perpignan, France

Correspondence

H  l  ne Magalon, UMR ENTROPIE, Universit   de La R  union, Saint Denis, La R  union, France.

Email: helene.magalon@univ-reunion.fr

Abstract

Sea cucumbers are increasingly exploited for human consumption and for their curative properties, and many wild populations are now depleted or in danger of extinction. While aquaculture is seen as an alternative to fisheries and as a mean to restore wild populations, more knowledge is needed on their reproductive strategies to render this practice efficient, notably for fissiparous holothurians, which are some of the mobile animals able of asexual reproduction by transverse fission. Little information is available on their population genetic diversity and structure. Here, the clonal structure of populations of the fissiparous sea cucumber *Stichopus chloronotus* has been investigated using nine microsatellite loci and a random sampling, at different spatial (intra-reef and inter-reef) and temporal (inter-season and inter-year) scales. Our findings highlight the importance of asexual reproduction in maintaining these populations, and the prevalence of the "initial seedling recruitment" strategy (ISR), leading to a high stability of clonal composition over seasons and years. It also seemed that clonal propagation was limited to the reef scale (<10 km) while reefs were connected by sexual dispersal. This is the first time that clonal structure in sea cucumbers has been studied at such a fine scale, with a specific sampling strategy. It provides key findings on the genetic diversity and structure of fissiparous sea cucumbers, which will be useful for the management of wild populations and aquaculture.

KEYWORDS

microsatellite, multilocus genotype, network, sea cucumber, *Stichopus chloronotus*

1 | INTRODUCTION

Studying and understanding the way species reproduce (sexually, asexually, or both) are not only an academic matter, but are of practical importance for species management because of the consequences on population genetic diversity, connectivity, and dynamics. While sexual reproduction allows to maintain genetic diversity, asexual reproduction leads to the rapid propagation of the fittest genotypes and their predominance in the populations (Hamilton, 1980; Williams, 1975).

Assessment of sexual and asexual reproduction in the wild thus allows to predict population ability (1) to adapt to disturbances (thanks to a sufficient genetic diversity) (Felsenstein, 1974; Waxman & Peck, 1999) and (2) to colonize rapidly new environments and maintain locally (thanks to asexual reproduction). Thus, species reproducing both sexually and asexually may have a considerable potential to evolve because they receive benefits from both modes. Nevertheless, these organisms generally favor one mode over the other, leading to strong implications for the evolution of populations and species: Different

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recruitment strategies (sexual or asexual) will affect dispersal (not performed by the same entities) and spatial occupation and will determine which entities (the alleles or the clonal lineages) will persist and evolve under natural selection (see Ayala, 1998). Besides the theoretical aspect, determining the modes of reproduction and their relative parts is of particular interest for organisms of economic interests. For example, in agronomy, asexual reproduction may lead to the rapid fixation of any desired genotype, while sexual reproduction will maintain a sufficient genetic diversity to sustain genetic improvement over the long term and to limit vulnerability to disease and pest epidemics (Hamilton, 1980; McKey, Elias, Pujol, & Duputié, 2010). Influence of the mode of reproduction has been poorly studied in animal breeding, as few of them reproduce asexually. Nevertheless, among exploited marine species, sea cucumbers (holothurians, Echinodermata) are good candidates.

Performing sediment bioturbation (Franklin, 1980; Purcell, 2010; Uthicke, 1999), holothurians provide a vital ecosystem service, notably for coral reefs, several species being described as "keystone species" (Paine, 1969; Power et al., 1996; Uthicke, Schaffelke, & Byrne, 2009). Meanwhile, presenting a high commercial value, approximately 66 species, mainly with a tropical distribution, are currently exploited by fisheries over the world (Conand, 2008; Purcell, 2010; Purcell, Hair, & Mills, 2012; Purcell, Samyn, & Conand, 2012). For the last thirty years, exploitation of wild populations has increased sevenfold and populations of the most exploited species (e.g., *Apostichopus japonicus*, *Holothuria scabra*, *Isostichopus fuscus*, *H. lessoni*, *H. nobilis*, *H. whitmaei*, and *Thelenota ananas*) become depleted and are now subjected to an extinction risk (Purcell, Polidoro, Hamel, Gamboa, & Mercier, 2014). Unsurprisingly, exploitation now turns toward less valuable species, such as *Stichopus chloronotus*, *S. hermani*, or *H. edulis* (Conand, 2008). Thus, to limit overfishing, aquaculture has become more and more developed, but needs more knowledge about (1) species modes of reproduction, (2) population genetic diversity to avoid genetic risk (i.e., introduction of new animals compromising the genetic integrity of local populations by large scale translocations and inbreeding), and (3) pathology.

Until now, only sexual holothurian species have been studied for aquaculture production, but their breeding remains difficult as they are broadcast spawners, leading to restricted successful fertilization and low larval survival rates (Eriksson, Robinson, Slater, & Troell, 2012). Besides, 16 holothurian species, which are exploited and could be bred in aquaculture, are able of asexual reproduction by transverse fission (reviewed in Dolmatov, 2014). Fission results into two new sections, and each regenerates the respective missing organs and tissues to form a complete animal, thus leading to clonal propagation. For most tropical fissiparous holothurians, sexual and asexual reproductions are asynchronous in time, occurring during the warm and the cold seasons, respectively (Conand, Jerome, Dijoux, & Garryer, 1998; Conand, Uthicke, & Hoareau, 2002; Uthicke, 1997), with some variations among species (Lee, Uthicke, & Byrne, 2009; Purwati, 2004). It has thus been hypothesized that fission in holothurians would maintain population stability, even when recruitment is low (Chao, Chen, & Alexander, 1994; Conand, 1996; Emson & Mladenov, 1987;

Mladenov, 1996; Uthicke, 1997; Uthicke, Benzie, & Ballment, 1998). However, why these distinct reproductive strategies would occur at different rates remains unclear.

Asexual reproduction in holothurians has been particularly studied in *S. chloronotus*, the greenfish sea cucumber (Conand et al., 1998; Reichenbach & Holloway, 1995; Uthicke, Benzie, & Ballment, 1999; Uthicke & Conand, 2004; Uthicke, Conand, & Benzie, 2001). This species is found in the Indian and Pacific Oceans, inhabiting reef flats and back-reef areas that are less than 20 m deep (Franklin, 1980). While presenting a moderate commercial value (Conand, 2008) but easily accessible for harvesting by hand or by free diving, it is exploited throughout its distribution range in artisanal and semi-industrial fisheries, especially in Indonesia (Purcell, Hair, et al., 2012; Purcell, Samyn, et al., 2012). In the Western Indian Ocean, it is notably fished in Mauritius, Madagascar, and Kenya, for exportation to the Asian market (Conand et al., 2007; Muthiga & Conand, 2014; Purcell, Hair, et al., 2012; Purcell, Samyn, et al., 2012). Aquaculture production of this species is thus under consideration, but requires a better understanding of the relative contribution of sexual and asexual reproductions in the maintenance and the genetic diversity of its populations to be efficient. Sexual and asexual reproductions in this species occur generally during the warm and the cold seasons, respectively (Conand et al., 1998, 2002; Uthicke, 1997). High fission rates were observed in the Great Barrier Reef and Reunion Island (from 19% to 60% according to the population; Conand et al., 2002; Uthicke, 1997). The high prevalence of asexual reproduction in these populations has been confirmed using molecular markers (allozymes and then AFLP) by clone identification (Uthicke & Conand, 2004; Uthicke et al., 1999, 2001). Nevertheless, the contribution of asexual reproduction to the replenishment of local populations has only been assessed over short time frames and may vary seasonally and over time, as well as the occurrence of new genotypes through sexual reproduction.

Using nine microsatellite loci, we investigate the clonal diversity and composition in populations of the fissiparous holothurian *S. chloronotus* from Reunion Island (Western Indian Ocean), where fishing is forbidden and thus does not represent a selection pressure. This was done through space and time and at a finer scale than previously achieved, to assess the relative roles of asexual and sexual reproductions in the maintenance of these populations and their genetic diversity. Indeed, performing a random sampling scheme, we studied the genetic structure (1) at different spatial scales, from the intra-reef to the inter-reef, to estimate clonal dispersal distances, and (2) at different temporal scales, from the inter-season (cold and warm seasons) to the inter-year (two years apart), to study clonal persistence through time.

2 | MATERIALS AND METHODS

2.1 | Sampling

Sampling of the greenfish sea cucumber *S. chloronotus* was conducted at four reefs from the west coast of Reunion Island (21°06'S, 55°36'E), South Western Indian Ocean, 700 km east from Madagascar. Individuals were sampled one by one *in situ* in the back-reef area

(1–1.5 m depth), while snorkeling. For each individual, one or two papillae were instantaneously collected using cutting pliers (not destructive for the animal), and the longitudinal size was measured before releasing the animal where it was found. Samples were preserved in 95% ethanol and stored at room temperature.

2.1.1 | Spatial sampling

Six study sites were defined according to the density of *S. chloronotus*, in the back-reef area of each reef. This species shows an aggregative distribution with a succession of high-density patches (between 1 ind. m⁻² and 3.15 ind.m⁻²; Conand et al., 1998, 2002) and zones of low densities (around 0.37 ind.m⁻²; Frouin P. personal communication), along the west coast of Reunion Island.

High-density sites

Three sites were chosen because of their high densities (named hereafter HIGH1, HIGH2, and HIGH3; Figure 1, Table 1), to study clonal structure at a fine scale. For each of these sites, we sampled two replicates, called stations (named S1 and S2), to assess clonal variations

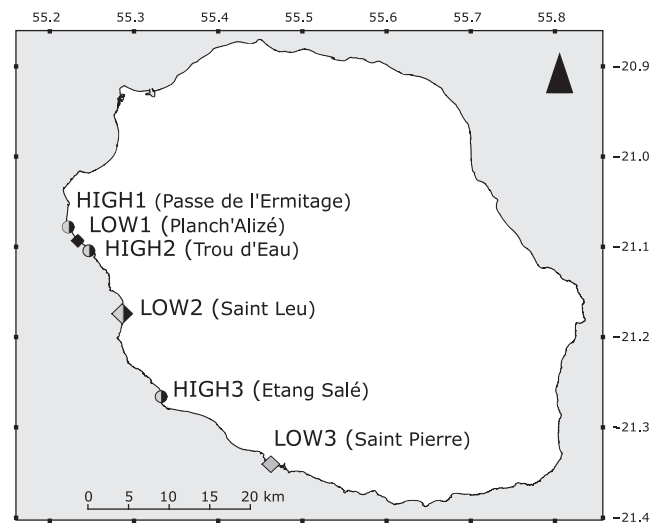


FIGURE 1 Map of the sampling sites of *Stichopus chloronotus* in Reunion Island. Circles represent high-density sites while diamonds low-density sites. In black, sites sampled during T0; in gray, sites sampled during T2

at the site scale. They were chosen near the center of the aggregation patch, approximately 150 m apart. For each of these stations, a random sampling procedure was performed in nested circular plots (radii of 5 m, 10 m, 20 m and 40 m) according to Baums, Miller, and Hellberg (2006). Within each circular strip, 16 individuals were randomly chosen. Thus, for each station, 64 individuals were sampled. Practically, GPS coordinates of the centers of each station were recorded and coordinates in each strip were generated randomly with increments set to 10 degrees and 10 cm, to avoid resampling of the same individual. For samples collected in circles of radii 5 m and 10 m, coordinates were located using a compass and a measuring tape attached to the center of the station. For samples collected in circles of radii 20 m and 40 m, coordinates were located using a GPS device. The nearest individual to each coordinate was sampled.

Low-density sites

To study clonal propagation among *S. chloronotus* patches along the west coast of Reunion Island and to test the influence of population density on clonal structure, three other sites presenting low densities of *S. chloronotus* were added (named hereafter LOW1, LOW2, LOW3; Figure 1, Table 1). Individuals were haphazardly sampled over hundreds of meters while snorkeling parallel to the coast. For each site, 32 individuals were sampled.

Location of sampling sites in Reunion Island reefs

Among these sites, three (HIGH1, LOW1, and HIGH2) were located in the reef complex of La Saline-Ermitage, approximately 7.5 km long. The three other sites (LOW2, HIGH3, and LOW3) were located in three distinct reefs: Saint Leu (2 km long), Étang Salé (1 km long), and Saint Pierre (2 km long), respectively (Figure 1, Table 1). HIGH1/LOW1/HIGH2 group in La Saline-Ermitage complex and LOW2 were 9 km apart while LOW2 and HIGH3 were distant by 15 km and HIGH3 and LOW3 by 16 km (Figure 1).

2.1.2 | Temporal sampling

As the relative importance of each mode of reproduction may vary according to the season, sampling was performed in the cold season (October) and the successive warm season (February–March), and this two years apart, in 2013/2014 (T0) and 2015/2016 (T2).

TABLE 1 Sampling of *Stichopus chloronotus* populations in Reunion Island for both sampling periods and for both seasons (cold + warm)

Reef	Locality	Sites	N T0 (2 stations)	N T2 (1 station)	Latitude (S)	Longitude (E)
La Saline-Ermitage reef	Passe de l'Ermitage	HIGH1	128 + 128	64 + 64	21°05'5.58"	55°13'32.39"
La Saline-Ermitage reef	Trou d'Eau	HIGH2	128 + 128	64 + 64	21°06'11.52"	55°14'44.61"
Étang Salé	Étang Salé	HIGH3	128 + 128	64 + 64	21°16'5.89"	55°19'57.29"
La Saline-Ermitage reef	Planch'Alizé	LOW1	32 + 32		21°05'41.70"	55°14'1.65"
Saint Leu	Saint Leu	LOW2	32 + 32	48 + 48	21°10'41.76"	55°17'10.58"
Saint Pierre	Saint Pierre	LOW3		48 + 48	21°20'32.85"	55°27'41.44"
Total			896	576		

T0

For T0, the exact same sampling scheme was performed for both seasons following the methodology of spatial sampling described above, but LOW3 was not sampled. A total of 896 individuals were thus sampled.

T2

For all high-density sites, preliminary analyses performed before T2 did not highlight variations in the clonal structure between both stations within site (see Section 3). Thus, we sampled only one station for each site during T2. Furthermore, we decided not to sample once again at LOW1 as it did not show any differentiation with HIGH1 during T0, and we added another reef not sampled at T0, at the southern limit of the west coast of Reunion Island, showing low densities of *S. chloronotus* (LOW3) (Figure 1). Moreover, during T2, we sampled 48 individuals per low-density site. In sum, during T0 and T2, two low-density sites were sampled: LOW1/LOW2 (32 individuals per site) and LOW2/LOW3 (48 individuals per site), respectively. Thus, 576 individuals were sampled during T2.

2.1.3 | Density

During T2, for each high-density site, population densities were measured using a 1 x 1 m quadrat, randomly thrown at each sampling point and all *S. chloronotus* individuals within the quadrat were counted. We thus performed 64 density measures for each high-density site, for each season, during T2.

In total, 1,472 individuals were sampled during this study (Table 1).

2.2 | Laboratory procedures

Total genomic DNA was extracted using Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) from one papilla.

We tested microsatellite loci previously developed and characterized for *S. chloronotus* (10 loci; Taquet, Nagai, Yasuda, & Nadaoka, 2011), *S. horrens* (16 loci; Yuan, Xia, Zhang, & Hu, 2012), and *S. monotuberculatus* (15 loci; Xia, Hu, Fan, Luo, & Zhang, 2010). These 41 microsatellite loci were tested on eight individuals (six from Reunion Island and two from New Caledonia).

From the 41 microsatellite loci tested, 13 amplified and were easily readable and thus were selected to genotype all the samples: the 10 microsatellite loci developed for *S. chloronotus* (Taquet et al., 2011), as well as three developed for *S. monotuberculatus* (Sm007, Sm010, and Sm014; Xia et al., 2010). Among these 13 microsatellite loci, four were monomorphic overall our samples (Sc08, Sc20, Sc41, and Sm010) and we thus kept nine polymorphic loci to perform analyses on the 1,472 sampled individuals.

Genotyping was performed similarly for polymorphic tests and total genotyping. Forward primers were indirectly fluorochrome labeled (6-FAM, VIC, NED) and were multiplexed post-PCR in two panels (Appendix S1). Amplification reactions as well as the thermocycling program was similar as previously described in Postaire, Aurelle, Bourmaud, Bruggemann, and Magalon (2015). PCR products were

genotyped using an ABI 3730 genetic analyzer (Applied Biosystems, Foster city, CA), and allelic sizes were determined using GeneMapper v 4.0 (Applied Biosystems, Foster city, CA).

2.3 | Data analyses

2.3.1 | Analysis of clonal structure

Clonal diversity

To analyze the clonal structure, we only kept individuals whose multi-locus genotypes (MLG) presented no missing data. First, the number of distinct MLGs (N_{MLG}) was assessed, and then, the clones were identified, a clone being defined as a set of individuals that present the same MLG, using the package RClone (Arnaud-Haond, Duarte, Alberto, & Serrao, 2007; Bailleul, Stoeckel, & Arnaud-Haond, 2016). To assess the relevance of the loci used (number and variability), we estimated the probability that two individuals randomly sampled share exactly the same alleles over all loci (i.e., the same MLG) just by chance rather than the result of asexual reproduction (probability of identity P_{ID} ; Waits, Luikart, & Taberlet, 2001) with GIMLET v. 1.3.3 (Valière, 2002). Then, for identical MLGs, the probability $P_{SEX}(F_{IS})$ of detecting more than once the same MLG issued from distinct reproductive events was estimated using RClone. This probability takes into account possible departure from Hardy-Weinberg equilibrium (HWE) (Arnaud-Haond & Belkhir, 2007).

To assess the relative part of asexual reproduction, the clonal richness R was calculated for each population (Dorken & Eckert, 2001), a population being defined by all the individuals sampled at the same place (station or site) and the same date. To discriminate identical MLGs due to asexual reproduction from those due to the potential weak variability of the loci used, clonal richness was assessed from simulated populations obtained without fission. These populations were composed of the individuals obtained at the next generation for each observed population, considering (1) only sexual reproduction including selfing (i.e., no fission), (2) panmixia, (3) unlinked loci, and (4) constant population size. Only T0 cold season (TO_{cold}) was considered, as no significant clonal composition changes between seasons and sampling periods were observed (see Section 3). The simulated multilocus genotypes were created by randomly sampling two alleles for each of the studied loci in the gamete pool (corresponding to the allele frequencies in each observed population). For each observed population, 10^3 datasets were simulated, and a mean clonal richness R_{sim} was then assessed, as well as the 95% confidence interval and the probability to observe a clonal richness weaker than the one observed (P -value).

Furthermore, slightly different MLGs may be due to somatic mutations or scoring errors, corresponding to the same multi-locus lineage (MLL) (Arnaud-Haond et al., 2007), possibly overestimating the number of clones. Pairwise genetic distances between MLGs were computed, based on Rozenfeld's genetic distance (Rozenfeld et al., 2007) using RClone. From this distribution, a threshold was determined, under which genetic distances were considered to be due to somatic mutations or scoring errors, and characterized distinct MLGs belonging to the same MLL.

To assess clonal heterogeneity in the studied sites, the parameter β of the Pareto distribution and Pielou's evenness (J') were estimated to describe whether the repeated MLGs were uniformly distributed rather than one predominant in each station.

The sampling design performed in the three high-density sites allowed estimating the spatial arrangement of the clonemates. It was estimated using aggregation (A_c) and edge effect (E_e) indices, and their significance was tested with 10^3 permutations using RClone.

Network analysis

A network was constructed using all distinct MLGs identified, to assess the genetic structure due to sexual reproduction at the island scale, whatever the season or the sampling period.

Then, to be able to compare the network indices between both sampling periods (T0 and T2) to assess the temporal variation in genetic structure, a network was also built for each period including all the distinct MLGs found at the common sites between both periods (HIGH1, HIGH2, LOW2, HIGH3). Finally, to assess the clonal structure at the site scale, a network was built for each station and for each season.

All networks were built using Rozenfeld's distance (Rozenfeld et al., 2007) with EDENetworks v. 2.18 (Kivelä, Arnaud-Haond, & Saramäki, 2015). Nodes represent MLGs and links present the genetic distance between two MLGs. For each analysis, a "fully connected" network was built, including all links between all MLGs, and we then searched for the percolation threshold (Dpe) (Becheler, Benkara, Moalic, Hily, & Arnaud-Haond, 2014; Moalic, Arnaud-Haond, Perrin, Pearson, & Serrao, 2011; Rozenfeld et al., 2007), allowing to identify the first significant level of limitation of gene flow within the system, and thus different clusters (connected components). The clustering coefficient of the whole network $\langle CC \rangle$ was then assessed (Rozenfeld et al., 2007).

2.3.2 | Genetic diversity and differentiation

Asexual reproduction in *S. chloronotus* leads to two distinct individuals with the same MLG, and each can be considered to participate equally to the reproduction, either sexual (gamete broadcasting) or asexual. So, to perform genetic diversity and population structure analyses at the island scale, it seems relevant to keep all individuals, regardless of their MLG, to take into account the fact that a predominant MLG may have a better fitness than a rare one, even if it has been reported that considering all the members of a clone to assess differentiation indices may bias results. Noteworthy, when keeping one representative per MLG for each population (i.e., keeping only distinct MLGs), a very weak number of individuals remained to perform these analyses (Table 2; N_{MLG} ranging from 2 to 14 with a mean number of distinct MLGs per population of 6.03) and would induce too much bias.

Indices were assessed for each population. The mean number of alleles (N_a) was assessed using FSTAT v. 2.9.3.2 (Goudet, 1995) and the mean number of private alleles per site (N_{ap}) using the R-package PopGenKit v. 1.0 (Paquette, 2012). We also assessed the expected and observed heterozygosities, respectively, H_E and H_O , and the

inbreeding coefficient F_{IS} to test for departures from HWE using Arlequin v. 3.5.1.3 (Excoffier & Lischer, 2010).

Population differentiation indices, F_{ST} (Weir & Cockerham, 1984) and D_{est} (Jost, 2008), were estimated between stations of a same site and among sites for a given season and sampling period (spatial differentiation). Additionally, they were assessed among seasons and sampling periods for a same site (temporal differentiation). They were also assessed between pairs of high-density sites (all sampling dates pooled), keeping either all individuals or only one representative per MLG, to study connectivity among sites due to sexual reproduction. Indeed, the number of distinct MLGs in high-density sites (from 16 to 22) was greater than in low-density sites, although remaining weak for these calculations even if all sampling dates were pooled.

2.3.3 | Statistical analyses related to population density and individual size

Among high-density sites, we tested whether the variable "density" during T2 was significantly dependent on factors "site" (three levels: HIGH1/HIGH2/HIGH3) and "season" (two levels: warm/cold) using a two-way ANOVA.

We tested the effects of the factors "density" (two levels: low/high) and "season" (two levels: cold/warm) on the variable "individual size," performing a two-way ANOVA.

When necessary, pairwise Student's *t*-tests were performed. All these analyses were performed using the statistical software R (R Development Core Team 2004).

3 | RESULTS

3.1 | MLG identification

Over the 1,472 individuals genotyped, 1,456 (98.9%) presented MLGs without missing data. The variability of the microsatellite loci used here resulted in a probability of identity P_{ID} of 2.356×10^{-3} .

Over the 1,456 individuals with MLGs without missing data, a total of 74 distinct MLGs were detected, and among them, 31 were shared by several individuals. Over these 31 MLGs, seven appeared to be the result of different reproductive events ($P_{SEX}(F_{IS}) > 0.05$). Six of these seven MLGs were rare and were identified in 21 individuals (1.4% of the whole dataset). The last one (MLG27) was identified in LOW3 and was the most represented at this site (92/94 individuals). Furthermore, when departure from HWE was not taken into account, individuals presenting this MLG likely originated from a same reproductive event ($P_{SEX} = 1.46 \times 10^{-37}$).

For each site (only TO_{cold} considered), the observed clonal richness was significantly lower than the ones calculated from the simulated populations without fission (Appendix S2), even for HIGH2, which represented very few distinct MLGs and very weak clonal richness.

To take into account somatic mutations and eventual scoring errors, the threshold in the genetic distance distribution was set at one mutation step, as the first gap in the distribution of pairwise allelic differences was found between 0 and two mutation steps, as in Schnittler

TABLE 2 Indices of genetic diversity and clonal structure for *Stichopus chloronotus* populations from Reunion Island, at six different sites, at two seasons per year and two years apart: (a) T0: 2013/2014 and (b) T2: 2015/2016

(a)																
T0																
Season	Reef	Site	Station	N	N_{MLG}	R	J'	β	A_c	E_e	N_d	N_{ap}	H_o	H_E	F_{IS}	
Cold	La Saline-Ermitage	HIGH1	S1	63	7	0.1	0.68	0.19	0.01	-0.17	2.00 (0.15)	0.00 (0.00)	0.49 (0.03)	0.28 (0.03)	-0.18***	
		HIGH2	S2	64	7	0.1	0.61	0.14	-0.2	0.64	2.22 (0.20)	0.11 (0.04)	0.65 (0.02)	0.33 (0.03)	-0.32***	
	La Saline-Ermitage	HIGH2	S1	63	3	0.03	0.15	0.01	-0.01	0.39	1.56 (0.09)	0.00 (0.00)	0.75 (0.06)	0.17 (0.03)	-0.97***	
		HIGH2	S2	64	6	0.08	0.22	0.02	0.17	0.22	2.11 (0.10)	0.44 (0.09)	0.44 (0.06)	0.18 (0.03)	-0.89***	
	Etang Salé	HIGH3	S1	64	7	0.1	0.56	0.12	0.13	0.19	2.22 (0.10)	0.00 (0.00)	0.43 (0.05)	0.30 (0.03)	-0.29***	
		HIGH3	S2	64	14	0.21	0.65	0.18	-0.13	0.1	2.33 (0.14)	0.00 (0.00)	0.35 (0.05)	0.27 (0.03)	-0.17***	
La Saline-Ermitage	LOW1			31	5	0.13	0.78	0.24	-	-	1.89 (0.17)	0.00 (0.00)	0.61 (0.02)	0.31 (0.05)	-0.31***	
	LOW2			32	3	0.06	0.6	0.08	-	-	2.00 (0.18)	0.00 (0.00)	0.57 (0.07)	0.25 (0.04)	-0.54***	
Warm	La Saline-Ermitage	HIGH1	S1	62	8	0.11	0.62	0.16	-0.17	-0.16	2.00 (0.16)	0.00 (0.00)	0.43 (0.03)	0.25 (0.03)	-0.15***	
		HIGH2	S2	64	7	0.1	0.72	0.2	0.09	0.24	2.00 (0.15)	0.00 (0.00)	0.48 (0.03)	0.26 (0.03)	-0.21***	
	La Saline-Ermitage	HIGH2	S1	64	4	0.05	0.17	0.01	0.15	-0.23	1.89 (0.12)	0.00 (0.00)	0.49 (0.07)	0.18 (0.03)	-0.87***	
		HIGH2	S2	63	6	0.08	0.26	0.02	-0.22	0.38	1.89 (0.10)	0.00 (0.00)	0.51 (0.07)	0.18 (0.03)	-0.88***	
	Etang Salé	HIGH3	S1	64	6	0.08	0.74	0.2	0.15	-0.78	1.89 (0.08)	0.00 (0.00)	0.44 (0.05)	0.26 (0.03)	-0.30***	
		HIGH3	S2	64	7	0.1	0.57	0.11	-0.1	0.14	2.11 (0.10)	0.00 (0.00)	0.41 (0.05)	0.28 (0.03)	-0.29***	
La Saline-Ermitage	LOW1			32	6	0.16	0.76	0.24	-	-	1.89 (0.16)	0.00 (0.00)	0.64 (0.02)	0.32 (0.05)	-0.33***	
	LOW2			31	5	0.13	0.62	0.13	-	-	1.78 (0.12)	0.00 (0.00)	0.56 (0.07)	0.26 (0.04)	-0.47***	
(b)																
T2																
Season	Reef	Site	Station	N	N_{MLG}	R	J'	β	A_c	E_e	N_d	N_{ap}	H_o	H_E	F_{IS}	
Cold	La Saline-Ermitage	HIGH1	S2	64	11	0.16	0.71	0.24	0.05	0.18	2.33 (0.20)	0.00 (0.00)	0.50 (0.02)	0.29 (0.03)	-0.14***	
		HIGH2	S2	64	6	0.08	0.29	0.03	0.25*	0.39	2.11 (0.16)	0.11 (0.04)	0.48 (0.06)	0.19 (0.03)	-0.73***	
	Etang Salé	HIGH3	S1	61	8	0.12	0.63	0.17	-0.02	0.44	2.33 (0.19)	0.11 (0.04)	0.52 (0.05)	0.31 (0.03)	-0.29***	
		LOW2			45	6	0.11	0.62	0.14	-	-	1.78 (0.10)	0.00 (0.00)	0.59 (0.05)	0.28 (0.03)	-0.43***
	Saint Pierre	LOW3			46	2	0.02	0.15	0.01	-	-	1.33 (0.07)	0.00 (0.00)	0.99 (0.00)	0.17 (0.04)	-0.99***
		HIGH1	S2	63	7	0.1	0.64	0.15	0.07	-0.24	2.00 (0.15)	0.00 (0.00)	0.46 (0.03)	0.26 (0.03)	-0.20***	
Warm	La Saline-Ermitage	HIGH2	S2	64	6	0.08	0.33	0.04	0.03	-0.18	1.78 (0.12)	0.00 (0.00)	0.58 (0.06)	0.20 (0.03)	-0.60***	
		HIGH3	S1	64	5	0.06	0.55	0.09	0.12	0.19	2.11 (0.15)	0.00 (0.00)	0.54 (0.05)	0.30 (0.03)	-0.42***	
	Saint Leu	LOW2			48	3	0.04	0.84	0.26	-	-	1.78 (0.10)	0.00 (0.00)	0.64 (0.04)	0.29 (0.03)	-0.46***
		LOW3			48	2	0.02	0.15	0.01	-	-	1.44 (0.08)	0.00 (0.00)	0.76 (0.07)	0.17 (0.04)	-0.99***

N: number of individuals presenting no missing data; N_{MLG} : number of distinct multilocus genotypes; R: clonal richness; J' : Pielou's index; β : parameter of the Pareto distribution (-1 * regression slope); A_c : aggregation index; E_e : edge effect. For A_c and E_e indices, none value was significantly different from 0, except one (* $p < 0.05$). N_d : mean allelic number per site, N_{ap} : mean number of private alleles per site, H_o and H_E : observed and expected heterozygosities, respectively. In parentheses: standard errors. F_{IS} : inbreeding coefficient and significant deviations from Hardy-Weinberg equilibrium are indicated as follows: * $P < .05$; ** $P < .01$; *** $P < .001$.

and Eusemann (2010) and Gitzendanner, Weekley, Germain-Aubrey, Soltis, and Soltis (2012). As pairwise MLGs differed by at least two mutation steps, all distinct MLGs were considered as distinct MLLs. To avoid redundancy, we further kept the term MLG rather than MLL for the 74 distinct MLGs found among our sampled individuals.

3.2 | Genetic diversity

The genetic diversity of the loci used was low, with a number of alleles per locus varying from 2 (Sc01, Sc10, and Sm007) to 9 (Sc33) and the mean allele number per site (N_a) from 1.33 to 2.33 (Table 2a,b). Private alleles [mean \pm standard error (SE)] were identified at (T0^{cold}) in HIGH1 and HIGH2 ($N_{ap} = 0.11 \pm 0.04$ and 0.44 ± 0.09 , respectively) and at T2^{cold} in HIGH2 and HIGH3 ($N_{ap} = 0.11 \pm 0.04$).

H_o varied from 0.35 to 0.99 while H_E varied from 0.17 to 0.33 (Table 2a,b). Significant deviations from HWE were found for all populations, highlighting high heterozygote excesses ($F_{IS} = [-0.99, -0.14]$; $P < .001$; Table 2a,b).

3.3 | Spatial distribution of clones

3.3.1 | Between stations within site

The sampling of two stations for each high-density site (HIGH1, HIGH2, and HIGH3) during T0 allowed studying the clonal distribution at the intra-reef scale. During T0^{cold}, over the 384 individuals sampled in the

three high-density sites, 381 individuals presented MLGs without missing data and 32 distinct MLGs were identified. Among these MLGs, 15 were represented by several individuals and 17 were found only once.

For a given high-density site, both stations presented similar values for all the clonal parameters investigated here (R , J' , and β ; Table 2a). All these values were weak indicating that each station was composed by one to three MLGs that were represented by most individuals, along with some rare ones (represented by one or two individuals). Above all, the most frequent MLGs were the same between stations: MLG01, MLG02, and MLG03 for HIGH1; MLG03 for HIGH2; MLG04, MLG05, and MLG06 for HIGH3. Some rare MLGs were also shared between both stations of each site, for instance MLG12 for HIGH1 (Figure 2). Whichever the station, no significant spatial aggregation of clonemates (A_c , all $P > .05$) or significant edge effects (E_e , all $P > .05$) were detected (Table 2a).

Finally, for all high-density sites, even if some were significantly different from 0 due to repeated MLGs, low values of differentiation indices were found between stations from a same site ($F_{ST} = [0.00, 0.04]$, $D_{est} = [0.00, 0.02]$; Table 3). Thus, within an aggregation patch (site), clonemates and individuals with unique MLGs were randomly distributed. Therefore, sampling at one station should allow characterizing asexual reproduction within the site.

3.3.2 | Among sites

At T0^{cold}, considering all the sites (i.e., 444 individuals with MLGs without missing data), 91.9% of the individuals belonged

FIGURE 2 Clonal distribution within each station and each site for each sampling year and season. Each sector represents a multi-locus genotype (MLG) and is proportional to the number of individuals presenting this MLG. A specific color was assigned to each MLG shared by at least two individuals, while MLGs presented by only one individual were left blank. Numbers in each cell indicate the number of individuals whose MLGs present no missing data. Numbers in parentheses in the legend indicate the number of individuals presenting the MLG

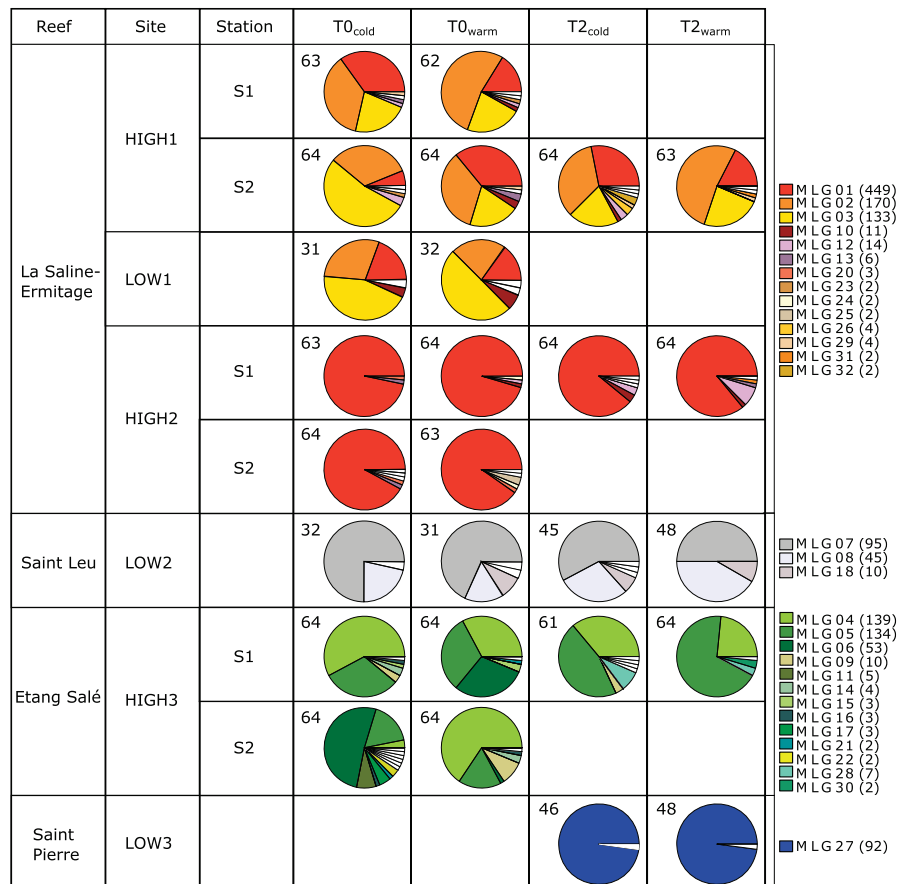


TABLE 3 Genetic differentiation between stations sampled at T_{0_cold} estimated with Weir and Cockerham's F_{ST} (lower-left matrix) and Jost's D_{est} (upper-right matrix) estimates

	HIGH1 S1	HIGH1 S2	HIGH2 S1	HIGH2 S2	HIGH3 S1	HIGH3 S2	LOW1	LOW2
HIGH1 S1	-	0.02	0.07	0.06	0.06	0.07	0.00	0.13
HIGH1 S2	0.03	-	0.13	0.12	0.10	0.11	0.00	0.14
HIGH2 S1	0.13	0.22	-	0	0.03	0.02	0.09	0.20
HIGH2 S2	0.13	0.21	0.00	-	0.03	0.02	0.09	0.20
HIGH3 S1	0.09	0.13	0.08	0.08	-	0.01	0.08	0.21
HIGH3 S2	0.11	0.16	0.05	0.05	0.04	-	0.09	0.19
LOW1	0.00	0.00	0.19	0.18	0.10	0.13	-	0.13
LOW2	0.19	0.19	0.36	0.35	0.29	0.27	0.18	-

white: $P > .05$; light gray: $P < .01$; gray: $P < .001$.

to one of the ten most represented MLGs and one MLG was over-represented (MLG01: 34.2%). Interestingly, some of these MLGs were shared between HIGH1 and LOW1 (MLG01, MLG02, and MLG03) and among HIGH1, LOW1, and HIGH2 (MLG01), sites that belong to the same reef (3 km apart). Conversely, no MLGs were shared among sites located in different reefs, that is, among the complex HIGH1/LOW1/HIGH2, LOW2, and HIGH3 (Figure 2).

Indices of clonal richness R were very low for all sites, from 0.03 to 0.21 (Table 2a). All the sites presented similar values of Pielou's indices J' (0.56–0.78), except HIGH2 that presented the lowest ones (0.15–0.22) (Table 2a). The parameter β of the Pareto distribution showed similar results: a very low value for HIGH2 (from 0.01 to 0.02), and higher values for the other sites, from 0.12 to 0.24 (Table 2a). Indeed, MLG01 was highly represented in HIGH2, from 59 to 61 individuals according to the station, representing 92%–97% of the individuals (Figure 2). None of these indices were significantly different between high-density and low-density sites (R : pairwise Wilcoxon test, $W = 4$, $P = 1.00$; J' : pairwise Wilcoxon test, $W = 3$, $P = .43$; β : pairwise Wilcoxon test, $W = 4$, $P = 0.64$).

Furthermore, differentiation indices were calculated among sites, pooling both stations for each site. They were highly significantly positive among all sites ($F_{ST} = [0.06, 0.37]$ and $D_{est} = [0.02, 0.20]$; all $P < 0.001$) except between HIGH1 and LOW1 that belong to the same reef complex (Appendix S3a). Thus, as similar MLGs and no differentiation were found between these sites, LOW1 was not sampled at T2, but replaced by another low-density site (LOW3), where two new MLGs were identified with one over-represented (47/48 individuals).

Finally, the network constructed using all MLGs identified in the study ($N_{MLG} = 74$) did not highlight a clear geographic structuring, with strong links among MLGs sampled in different reefs (Figure 3). Furthermore, F_{ST} and D_{est} estimates calculated among high-density sites using one representative per MLG (all sampling dates pooled) were lower ($F_{ST} = [0.03, 0.04]$, all $P < .01$; $D_{est} = [0.01, 0.03]$, all $P < .05$ except one) than those calculated keeping all individuals ($F_{ST} = [0.06, 0.17]$ and $D_{est} = [0.02, 0.09]$; all $P < .001$) (Appendix S3b,c).

3.4 | Temporal variation of clonal structure

3.4.1 | Interseasonal variability

For both sampling periods, similar dominant MLGs were found at each site for both seasons, but some rare MLGs appeared or disappeared between seasons (Figure 2). Mean clonal richnesses (R) were not significantly different between seasons for both sampling periods

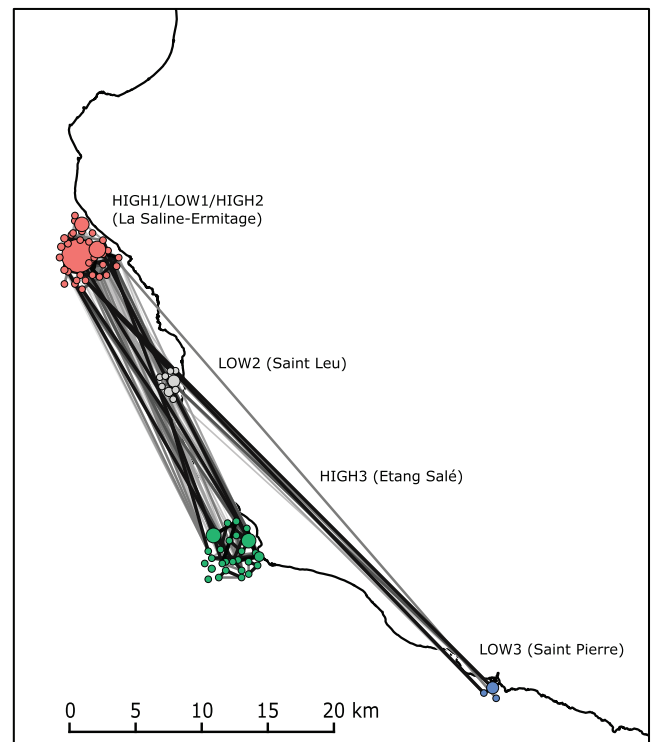


FIGURE 3 Network topology of multi-locus genotypes (MLGs) identified in *Stichopus chloronotus* whole sampling based on the Rozenfeld's distance. Only links with distances smaller or equal to the percolation threshold ($D_{pe} = 0.89$) are presented. Nodes, representing MLGs, are arranged according to their geographic coordinates. Node size is proportional to the number of individuals harboring each MLG. Node colors correspond to reefs

(T0: pairwise Wilcoxon test, $W = 8$, $P = .42$; T2: pairwise Wilcoxon test, $W = 19$, $P = .22$). Similar results were found concerning mean Pielou's indices J' (T0: pairwise Wilcoxon test, $W = 8$, $P = .42$; T2: pairwise Wilcoxon test, $W = 12$, $P = 1$) and the parameter β of the Pareto distribution (T0: pairwise Wilcoxon test, $W = 8$, $P = .42$; T2: pairwise Wilcoxon test, $W = 13$, $P = 1$).

Differentiation indices were also estimated between both seasons at a given site, to test for temporal differentiation within sites. Low differentiation values were found for all sites between both seasons whatever the sampling period ($F_{ST} = [0.000, 0.020]$; $D_{est} = [0.000, 0.011]$; Table 4).

3.4.2 | Interannual variability

Among the 37 distinct MLGs identified during T2, 23 were new. Among these 23 MLGs, three were identified in LOW3 representing all the individuals of this site and the 20 others were rare, presented at most by seven individuals and representing 5.1% of the individuals sampled during T2 (Figure 2). These 20 new rare MLGs were not exclusively represented by small individuals (only 9.7% measuring less than 7 cm), which would have indicated that they were originated from the previous event of sexual reproduction. The 14 MLGs already found during T0 were the most frequent ones, representing 78% of the individuals sampled during T2. For each site, the most frequent MLGs at T2 were the same as in T0. Furthermore, in LOW2 and HIGH3, it seems that between the two sampling periods (T0_{cold} and T2_{warm}), the frequency of the most represented MLG identified in each site in T0 decreased in favor of another one (MLG07 and MLG08 in LOW2; MLG04 and MLG05 in HIGH3; Figure 2). At a lesser extent, this pattern was also observed in HIGH2, with MLG12, that was not present at T0_{cold} but with a frequency of 7.8% at T2_{warm} (Figure 2).

Similar to T0, the clonal parameters were not significantly different between high-density and low-density sites (R : pairwise Wilcoxon test,

$W = 20$, $P = .11$; J' : pairwise Wilcoxon test, $W = 15$, $P = 0.61$; β : pairwise Wilcoxon test, $W = 15$, $P = 0.61$). Likewise, differentiation indices were highly significantly positive among all sites ($F_{ST} = [0.06, 0.37]$ and $D_{est} = [0.02, 0.19]$; all $P < .001$) (Appendix S3a). In order to compare clonal parameters between T0 and T2, LOW1 and LOW3 were not included in further analyses, as these sites were only sampled during one of the two periods and, for each high-density site, only the station sampled at both periods was kept. We found no significant difference of clonal richness between both periods (pairwise Wilcoxon test, $W = 31$, $P = 0.96$), and similar results were obtained when studying the Pielou's index J' (pairwise Wilcoxon test, $W = 27$, $P = .65$) and the parameter β of the Pareto distribution (pairwise Wilcoxon test, $W = 23$, $P = .37$).

Differentiation indices calculated for all sites between T0 and T2 were low, with F_{ST} values varying from 0.000 to 0.047 and D_{est} values varying from 0.000 to 0.027 (Table 4). Highest values were found in HIGH3 ($F_{ST} = 0.047$ and $D_{est} = 0.027$; $P < .001$) and may be related to the change in dominant MLGs frequencies.

The global network built on the basis of MLGs in T0 ($N_{MLG_{T0}} = 29$) was rather similar to the one obtained in T2 ($N_{MLG_{T2}} = 34$) with identical percolation thresholds ($D_{pe} = 1.11$). The clustering coefficient $\langle CC \rangle$ was higher in T2 than in T0 (0.52 and 0.45, respectively), suggesting an increase in the hierarchical differentiation among clusters (Appendix S4).

In order to see whether this result was due to an increase in differentiation at the island or site scale, networks were also constructed for each station. Clustering coefficients were variable, with no clear pattern of variations (Appendix S5). Indeed, for one station, for example, HIGH1S2, the clustering coefficient increased between T0_{cold} and T0_{warm} (from 0.74 to 0.83) and decreased between T2_{cold} and T2_{warm} (from 0.45 to 0.31). It thus seems that the increase in the hierarchical differentiation among clusters highlighted at the island scale is not related to an increase at the site scale.

TABLE 4 Genetic differentiation between both seasons of a given sampling period (T0 or T2), and between sampling periods, estimated with Weir and Cockerham's F_{ST} (upper value) and Jost's D_{est} (lower value)

	T0 cold/warm	T2 cold/warm	T0/T2
HIGH1	0.012**	0.009	0.000
	0.007**	0.006	0.000
HIGH2	0.000	0.000	0.001**
	0.000	0.000	0.000
HIGH3	0.004	0.020*	0.047***
	0.002**	0.011**	0.027***
LOW1	0.000	-	-
	0.000	-	-
LOW2	0.000	0.000	0.013*
	0.000	0.000	0.006**
LOW3	-	0.000	-
	-	0.000	-

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.5 | Variation in population density and individual size

3.5.1 | Population density

At T2, considering the high-density sites only, the two-way ANOVA showed that the population density was significantly dependent on the site ($F(2,378) = 3.02$; $P < 10^{-16}$), but not on the season ($F(1,378) = 3.87$; $P = .44$) with no interaction between season and site ($F(2,378) = 3.02$; $P = .17$) (see Appendix S6b). Density (mean \pm SE) was higher in HIGH2 ($4.3 \pm 0.3 \text{ ind.m}^{-2}$), than in HIGH3 ($2.3 \pm 0.2 \text{ ind.m}^{-2}$) and in HIGH1 ($1.2 \pm 0.1 \text{ ind.m}^{-2}$) (pairwise Student's t -tests; Appendix S6a,c).

3.5.2 | Individual size

The two-way ANOVA showed that the size was significantly dependent on the season ($F(1,1466) = 3.85$; $P < 2.2 \times 10^{-16}$) and the density ($F(1,1466) = 3.85$; $P = 2.56 \times 10^{-11}$) with an interaction between both

factors ($F(1,1466) = 3.85$; $P = 3.12 \times 10^{-13}$) (Appendix S6e). The size variation among high- and low-density sites was more important in the warm season (mean \pm SE = 12.3 ± 0.1 cm and 17.2 ± 0.4 cm, respectively) than in the cold season (mean \pm SE = 11.7 ± 0.2 cm and 13.1 ± 0.4 cm, respectively) (Appendix S6d,f).

4 | DISCUSSION

Our results revealed a high proportion of asexual reproduction in the greenfish sea cucumber *S. chloronotus* on the west coast of Reunion Island. Each population was composed of few clones, each with a lot of clonemates. At the island scale (six sites), *S. chloronotus* showed a weak clonal propagation among reefs while sexual dispersal seemed to be over longer distances. Additionally, sampling through time allowed detecting a high temporal stability in clonal composition, between seasons and years. Moreover, while the size of the individuals varied following the season and the density, this latter had no effect on clonal structure.

4.1 | Importance of asexual reproduction in population maintenance

Using nine microsatellite loci, we detected a very low clonal richness at all sites, highlighting the importance of asexual reproduction in propagation and maintenance of *S. chloronotus* in Reunion Island. Nevertheless, we found a moderate value of P_{ID} (2×10^{-3}) compared to other studies (10^{-4} – 10^{-5} ; Waits et al., 2001) and seven $P_{SEX}(F_{IS})$ that did not differ significantly from zero. This may indicate that a small proportion of the genotypic diversity may remain undetected, due to the lack of statistical power induced by datasets with a very low clonal diversity (Arnaud-Haond et al., 2007). However, for the six $P_{SEX}(F_{IS}) > 0.05$ found for rare MLGs, even if these MLGs were the result of different reproductive events, the overestimation of asexual reproduction that it entailed might be very slight, because it implied a weak number of individuals compared to the whole dataset (1.4%). As for MLG27 (found in LOW3 and representing almost all the individuals sampled at this site), it is highly unlikely that most of the individuals presenting this MLG were not the result of fission. Nevertheless, we cannot rule out that some of these individuals might have been produced by sexual reproduction (notably between two individuals presenting this MLG). Furthermore, the simulated clonal richnesses considering sexual reproduction only were significantly greater than the ones observed, even for HIGH2 presenting very few MLGs and a very weak clonal richness. So the loci used are discriminant enough to retrieve most of the genetic diversity of the studied populations and to detect asexual reproduction.

The clonal richness was very weak for all the populations (0.02–0.21), indicating that asexual reproduction is preponderant in these populations. The importance of asexual reproduction in *S. chloronotus* has already been demonstrated studying fission rates. In the Great Barrier Reef, they varied from 19% to 43% according to the population (Uthicke, 1997) and, in Reunion Island, from 50% to 60% at Trou d'Eau

(HIGH2 herein) and Etang Salé (HIGH3 herein), respectively (Conand et al., 2002). Then, it has been confirmed using allozymes (Uthicke et al., 1999) and AFLP (Uthicke & Conand, 2004). In *S. chloronotus* populations from Reunion Island, using allozymes, only one or two genotypes were retrieved per population (approximately 50 individuals) (Uthicke et al., 2001). Furthermore, using AFLP loci to study populations from the Western Indian Ocean (Reunion Island, Madagascar) and from the Great Barrier Reef, Uthicke and Conand (2004) detected 51 genotypes in the whole sample ($n = 149$), with up to 20 individuals (nearly all the individuals of a population) presenting the same genotype. Besides, the importance of asexual reproduction varied according to the population. One population in the Great Barrier Reef (Great Palm Island), one in Reunion Island (Trou d'Eau/HIGH2), and at a lesser extent in Saint Leu (LOW2 herein), exhibited higher levels of asexuality, with at least 79% of the sampled individuals belonging to the same clone (Uthicke et al., 1999). This pattern was confirmed here, with more than 90% of the individuals sampled in Trou d'Eau/HIGH2 presenting the same MLG. We also highlighted this pattern in Saint Pierre (LOW3 herein), site not studied previously and at the southern end of the island.

Five possible factors may influence fission rates: habitat instability (related to environmental stress), optimum individual size, food availability, juvenile mortality, and larval supply (Uthicke, 2001). Here, asexual reproduction does not seem to be related to individual size. Indeed, individuals were smaller in high-density sites than in low-density sites, especially at the warm season. However, no indices of clonal structure were significantly different between high- and low-density sites, nor between seasons. Two sites showed an extreme clonal structure, HIGH2 and LOW3, with one MLG highly predominant (more than 90% of the individuals sampled). The importance of fission highlighted in HIGH2 (Trou d'Eau) may be related to higher food availability, as this site is subject to stream inputs enriched in nutrients (Cuet, Naim, Faure, & Conan, 1988; Naim, Cuet, & Mangar, 2000). This higher food availability may also explain the higher population density observed at this site, as already suggested (Conand et al., 1998, 2002; Uthicke et al., 2001). This high fission rate may be also due to a toxic stress induced by the input of pesticides and organic contaminants that are carried downstream into the reef. Kolasinski, Taddei, Cuet, and Frouin (2010) showed that *Holothuria leucospilota* individuals also sampled in HIGH2 (Trou d'Eau) presented either lower acetylcholinesterase (AChE) activity levels or faster Cuvierian tubules expulsion than in other sites of the La Saline-Ermitage reef complex, which are two stress indicators. Nevertheless, this stress increase was not highlighted in sampled individuals of *H. atra* (Kolasinski et al., 2010) and may be species related. Likewise, in Saint Pierre (LOW3), freshwater inputs may explain the high fission rate observed. This reef is also submitted to currents and strong austral oceanic swell (Lecacheux et al., 2012). Thus, the high levels of asexual reproduction highlighted there may be related to high environmental instability. It would be interesting to test for stress indicators in *S. chloronotus* at all the study sites.

The predominance of one MLG in Saint Pierre (LOW3), and at a lesser extent in Saint Leu (LOW2), may also be explained by an important larval mortality due to specific environmental conditions. These

sites also exhibited low densities of *S. chloronotus*, maybe due to a high post-settlement mortality of larvae; the most frequent MLG found in each of these sites would then be the fittest to local environmental conditions. On the other hand, selection may not play any role, but just chance, as in gene surfing (Hallatschek, Hersen, Ramanathan, & Nelson, 2007) or equal chance hypothesis in ecology (Sale, 1979): the first to arrive, the first to propagate.

4.2 | Clonal propagation in space

While echinoderms present various patterns of asexual reproduction, it only occurs by fission in holothurians (Dolmatov, 2014). One species only, *Parastichopus californicus*, has been found to reproduce asexually during the larval stage, with fission occurring at the doliolaria stage (Eaves & Palmer, 2003). For all other species investigated, fission only occurred at the adult stage. Thus, clonal dispersal in *S. chloronotus* may only be achieved by adults.

Movements of holothurians are difficult to assess, as tagging methods are not always well-adapted, tags being rejected by the tegument according to the species (Conand, 1991, 1993). However, some studies managed to estimate the motion speed of *H. atra* and *H. leucospilota*, which was *ca.* 52 m.day⁻¹ (Bakus, 1973) and 41 m.day⁻¹ (Taddei, 2006), respectively. Thus, *S. chloronotus* may present similar motion speed. Here, shared MLGs were commonly found between both stations within site, which were separated by about 150 m. This pattern seems congruent with motion speeds highlighted in other sea cucumber species. Thus, *S. chloronotus* individuals may be highly mobile within an aggregation patch, at the scale of several hundred meters.

Furthermore, even if shared MLGs were found in sites located in the same reef, no common MLGs were identified among reefs, indicating that clonal propagation may be restricted to the reef scale. Using AFLP, Uthicke and Conand (2004) found two individuals in Trou d'Eau (HIGH2) presenting a genotype commonly identified in Saint Leu (LOW2), and one individual in the latter site that presented the genotype mainly identified in HIGH2. The fact that we did not identify shared MLGs among reefs may be related to the higher discriminant power of microsatellites. It may also be possible that the propagation of a MLG from one reef to another and its maintenance in the new reef is extremely rare.

Meanwhile, the lack of geographic structure in the networks built keeping one representative per MLG may represent gene flow among reefs due to sexual reproduction. This is confirmed by the lower F_{ST} and D_{est} estimates assessed among high-density sites keeping one representative per MLG as compared to those assessed keeping all individuals, as well as the low or absent number of private alleles within each site. During sexual reproduction, gametes are released directly into the water column and fertilized eggs rapidly develop into free-swimming planktotrophic larvae, the main dispersal stage of sea cucumbers. Little information on the larval life span is available, but larvae seem to spend a couple to several weeks in the water column before settling (reviewed in Smiley, McEuen, Chaffee, & Krishnan, 1991), possibly allowing dispersal among reefs. Thus, sexual reproduction may maintain

connectivity among *S. chloronotus* patches of Reunion Island whereas clonal propagation may take place at the reef scale only.

4.3 | Clone persistence through time

Sampling at both seasons within a year was performed because of higher fission rates in the cold season and a predominance of sexual reproduction in the warm season. Gamete spawning in this species occurs between November and February (Hoareau & Conand, 2002). Conand (1988) assessed the parameters of von Bertalanffy equation: $L_t = L_\infty (1 - e^{-k(t-t_0)})$, with $L_\infty = 342.24$, $k = 0.45$, and $t_0 = -0.25$, allowing to estimate the growth rate of *S. chloronotus*. Thus, when the warm season sampling occurred in March, the new individuals issued from sexual reproduction could be aged from 1 to 4 months, that is, measuring approximately from 4.7 to 7.9 cm long. Even if the mean size of the individuals sampled was higher (12.6 cm), 5%–20% of small individuals (< 8 cm) without fission evidence were sampled at each season for each period (90 individuals < 8 cm at $T_{0_{cold}}$, 70 at $T_{0_{warm}}$, 34 at $T_{2_{cold}}$ and 16 at $T_{2_{warm}}$). These small individuals should represent the genetic contribution of the sexual reproduction event from the previous season, remaining weak compared to the part of asexual reproduction. Furthermore, for each site studied two years apart, *S. chloronotus* patches were dominated by one to three MLGs, which were found in similar proportions, showing a high stability through time of their clonal composition, over several years. The networks built for each sampling period also presented a rather similar structure, confirming the stability of the populations' genetic composition. Conversely, the majority of the rare MLGs (presented by few individuals, i.e., <5% for each population) were not retrieved from one sampling period to the other, highlighting the variability of these MLGs. This variability was also found in the frequency of certain MLGs, which decreased (MLG07 and MLG04) or increased (MLG08 and MLG05) between both sampling periods. Thus *S. chloronotus* populations from Reunion Island seem constituted of a core of large and stable clones, with some rare transient MLGs issued from sexual reproduction. The genotypic composition of these populations may be an indicator of the recruitment strategy in this species. Two opposed recruitment strategies have been described for partially clonal species, especially in terrestrial plants and in marine sessile organisms (seagrass and corals), the "initial seedling recruitment" (ISR) and the "repeated seedling recruitment" (RSR) (Eriksson, 1993). In ISR species, populations originate from a single event of colonization, and no local sexual recruitment occurs after the establishment of the initial cohort. On the opposite, in RSR species, new MLGs appear continuously in local populations, resulting from sexual recruitment. Partially clonal species are likely to present recruitment strategies mixing both extremes in different proportions. Thus, the ISR strategy seems to be privileged in meadows of seagrass species such as *Zostera marina* (Thorsten, Christoffer, Wytze, & Jeanine, 1999), or in some coral populations of *Pocillopora damicornis sensu lato* (Gorospe & Karl, 2013), *Pocillopora damicornis* β (Gélin et al., 2017), *Acropora palmata*, and *Acropora cervicornis* (Japaud, Bouchon, Manceau, & Fauvelot, 2015) whereas the RSR strategy seems predominant in populations of the

seagrass *Cymodocea nodosa* (Ruggiero, Capone, Pirozzi, Reusch, & Procaccini, 2005).

The prevalence of few dominant MLGs that seem to be present for several years highlights the predominance of the ISR strategy (Eriksson, 1993) in *S. chloronotus* populations at Reunion Island. ISR strategy is dominant in populations with important intraspecific competition or with a low level of environmental and demographic variations, as fitter MLGs settle at the expense of others (Eriksson, 1993; Hartnett & Bazzaz, 1985). Nevertheless, the presence of rare MLGs not found two years apart shows the settlement of new MLGs, allowing the maintenance of some genetic diversity in populations under highly variable environments. This pattern showing some dominant clones with transient rare ones has already been highlighted in *Z. marina* meadows using a temporal genetic monitoring (Becheler et al., 2014). If the predominance of the ISR strategy seems congruent with the high densities and thus the potentially high intraspecific competition of *S. chloronotus* in HIGH2, it is more difficult to explain the predominance of one clone in LOW3 (Saint Pierre). At this site, *S. chloronotus* densities are very low and individuals are frequently disturbed by ocean swells and currents, freeing microsites for new recruits, more in adequacy with a RSR strategy. Some further work is required to fully understand patterns triggering asexual propagation in this population.

Our findings on the importance of clonal reproduction in the Reunion Island populations may benefit aquaculture production of *S. chloronotus*. Aquaculture of sea cucumbers is realized in different ways, in land-based aquaculture ponds or by sea ranching: Individuals are maintained *in situ* in more or less closed areas with no additional food, the juveniles either coming from hatchery process or from the wild (for a review, see Purcell, Hair, et al., 2012; Purcell, Samyn, et al., 2012). The weak clonal propagation of *S. chloronotus* and the high fission rate in its populations could allow sea ranching with juveniles present in the wild, as soon as local conditions are optimal, especially regarding the feeding ground. Its short-range propagation also spares the use of sea pens to close the ranching areas, diminishing the costs of pen construction, cleaning, and repairing (Robinson & Pascal, 2012). Second, we highlighted here a specific clonal structure within Reunion Island reefs, each with a specific assemblage of few clones that seem locally adapted. So it might be wise to farm the animals where they are aggregated and not to translocate them too far from their source environment. Understanding which local environmental factors drive these high rates of asexual reproduction and these high-density populations, especially in Reunion Island, has to be addressed by future research.

5 | CONCLUSION

To our knowledge, this study is the first to study genetic structure of fissiparous sea cucumbers at different spatial scales, adding a temporal monitoring. It reveals as follows:

- The importance of asexual reproduction to maintain populations of *S. chloronotus* and their weak clonal richness: While evidently

well-adapted to local conditions, this lack of genetic diversity may reduce their abilities to adapt to new environmental conditions.

- The high stability of clonal composition through time: The maintenance of the same predominant clones over seasons and years with weak inputs through sexual reproduction indicates the prevalence of the Initial Seedling Recruitment strategy in *S. chloronotus*. It is probably due to the stability of environmental conditions.
- A limited clonal propagation at the reef scale even if reefs seemed connected through larval dispersal, which could potentially influence the gene flow among *S. chloronotus* populations.

During this study, no exceptional events, such as cyclones, pollution, or fisheries, occurred that could have completely changed the clonal structure of these populations. In perspective, it would be interesting to pursue the temporal monitoring of these populations to assess the impact of exceptional events on their evolution in terms of clonal composition and genetic diversity.

ACKNOWLEDGMENTS

We thank Sophie Bureau for field assistance and Chantal Conand for her comments on a first draft. We acknowledge the Plateforme Gentyane of the Institut National de la Recherche Agronomique (INRA, Clermont-Ferrand, France) for guidance and technical support. Alexandre Bédier and Agathe Pirog were supported by a doctoral fellowship from the Conseil régional de La Réunion.

CONFLICT OF INTEREST

None declared.

DATA ACCESSIBILITY

Microsatellite genotypes: Dryad doi: 10.5061/dryad.n5140.

AUTHOR CONTRIBUTIONS

AP, PG, AB, GB, SG, PF, and HM performed sampling. AP, PG, AB, SG, GB, and HM analyzed data. PF and HM designed research. AP, PG, PF, and HM wrote the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Pirog A, Gélín P, Bédier A, et al. Clonal structure through space and time: High stability in the holothurian *Stichopus chloronotus* (Echinodermata). *Ecol Evol*. 2017;00:1–14. <https://doi.org/10.1002/ece3.3285>


Annexe 2

Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean

Diogene J, Reverté L, Rambla-Alegre M, del Río V, De la Iglesia P, Campàs M, Palacios O, Flores C, Caixach J, Ralijaona C, Razanajatovo I, **Pirog A**, Magalon H, Arnich N, Turquet J. (2017). *Scientific Reports*.

L'annexe 2 est un article reportant la présence de ciguatoxines lors d'un épisode d'empoisonnement après ingestion de viande de requin à Madagascar (Ouest de l'océan Indien).

SCIENTIFIC REPORTS



OPEN

Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean

Jorge Diogène¹, Laia Reverté¹, Maria Rambla-Alegre¹, Vanessa del Río¹, Pablo de la Iglesia¹, Mònica Campàs¹, Oscar Palacios², Cintia Flores², Josep Caixach², Christian Ralijaona³, Iony Razanajatovo⁴, Agathe Pirog⁵, Hélène Magalon⁵, Nathalie Arnich⁶ & Jean Turquet⁷

Severe food poisoning events after the consumption of sharks have been reported since the 1940s; however, there has been no clear understanding of their cause. Herein, we report for the first time the presence of ciguatoxins (CTXs) in sharks. The identification by mass spectrometry of CTXs, including two new analogues, in a bull shark (*Carcharhinus leucas*) that was consumed by humans, causing the poisoning and death of 11 people in Madagascar in 2013 is described. Typical neurotoxic ciguatera symptoms were recorded in patients, and toxicological assays on extracts of the shark demonstrated CTX-like activity. These results confirm this episode as a ciguatera poisoning event and expand the range of pelagic fish species that are involved in ciguatera in the Indian Ocean. Additionally, gambieric acid D, a molecule originally described in CTX-producing microalgae, was identified for the first time in fish. This finding can contribute to a better understanding of trophic relations within food webs. The present work confirms that consumption of sharks from the Indian Ocean should be considered a ciguatera risk, and actions should be taken to evaluate its magnitude and risk in order to manage shark fisheries.

Ciguatera is a well-known food poisoning that occurs when fish containing ciguatoxins (CTXs) are consumed. These potent neurotoxins are produced by microalgae of the genus *Gambierdiscus*^{1–3} and *Fukuyoa*^{4,5}. Ciguatoxins produced by these microalgae may be transferred along the food web, eventually reaching carnivorous fish like barracuda or amberjack. Ciguatera is the main cause of seafood poisoning due to the consumption of fish, and estimations point out around 50,000–500,000 people are affected by ciguatera each year⁶, although these should be re-evaluated for a better assessment of the present impact of ciguatera.

Numerous incidences of human poisoning after the consumption of several species of shark have been reported since the 1940s. These cases have been proposed to be ciguatera events according to the toxicity in animal assays or due to the symptoms in patients⁷. However, the presence of CTXs has never been confirmed in sharks. In Madagascar, a first possible event of ciguatera was described in 1993, after the consumption of shark in Manakara (south-east coast) and was noted for its unprecedented severity. Several hundred people (between 200 and 500 depending on the different authors) were poisoned due to the consumption of a shark, either a bull shark (*Carcharhinus leucas*) or a pigeye shark (*C. amboinensis*), two species that are difficult to distinguish. This event resulted in the death of between 60 and 98 people, depending on the different authors, a fatality rate of 20 to 30%^{8,9}. In this particular event, patients presented almost exclusively neurological symptoms. Boisier *et al.* identified two toxic extracts from the liver of the shark, which were proposed to be the causative agent of the poisoning, and tentatively named the new toxins as carchatoxin-A and carchatoxin-B⁹. However, the toxicity levels of the shark flesh did not match that of the liver extracts, and thus, toxicity remained unexplained. No further information

¹Marine Environmental Monitoring, IRTA, Ctra. Poble Nou, km 5.5, 43540, Sant Carles de la Ràpita, Spain. ²Mass Spectrometry Laboratory/Organic Pollutants, IDAEA-CSIC, Jordi Girona 18, 08034, Barcelona, Spain. ³IHSM, Institut Halieutique des Sciences Marines de Tuléar, Université de Toliara, Toliara, Madagascar. ⁴IPM Institut Pasteur Madagascar, Laboratoire d'Epidémiologie-Surveillance, BP 1274 - Avaradoha, 101, Antananarivo, Madagascar. ⁵UMR ENTROPIE Univ. Réunion/IRD/CNRS, Faculté des Sciences et Technologies, Université de La Réunion, 15 Bd René Cassin, CS 92003, 97744 St Denis Cedex 09, La Réunion, France. ⁶ANSES French Agency for Food, Environmental and Occupational Health & Safety, Unit on Food Risk Assessment, Risk Assessment Department, 14 rue Pierre et Marie Curie - 94701, Maisons-Alfort Cedex, France. ⁷HYDROREUNION, CBEM, C/O CYROI, 2, Rue Maxime Rivière, 97490, Sainte Clotilde, La Réunion, France. Correspondence and requests for materials should be addressed to J.D. (email: jorge.diogene@irta.cat)

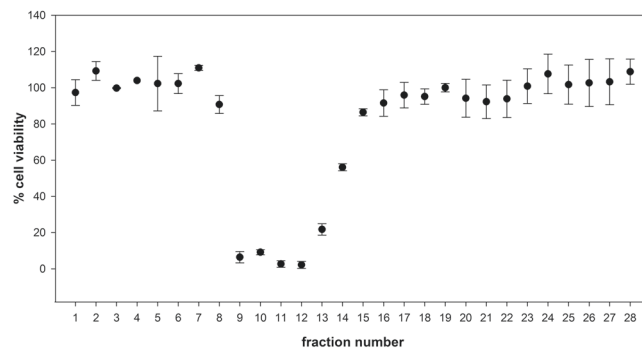


Figure 1. Toxicity of stomach fractions by cell-based assay (CBA). Toxicity was estimated according to the cell viability obtained after exposing cells to 0.27 μL of each fraction/mL. Error bars represent standard deviation (SD) values for 3 replicates ($n = 3$).

regarding the chemical behaviour, structure, toxicity or mechanism of action of carchatoxins has been published since then. Another event, occurring between November 14 and 19, 2013 at Fenerive-Est in Madagascar, caused the poisoning of 97 people that presented ciguatera symptoms after eating the flesh, the liver or the head of a bull shark, 11 of whom died. Our preliminary laboratory results of this particular event were communicated to the French “Agence nationale de sécurité sanitaire de l’alimentation, de l’environnement et du travail” (ANSES) in order to quickly manage the potential risk of food poisoning by shark consumption in the Madagascar area¹⁰. Further data regarding the epidemiology of this event, described that the major symptoms were neurological and digestive¹¹.

We report herein the confirmation of ciguatera, caused by consumption of this bull shark (*C. leucas*) in Madagascar in November 2013. This was based on the evidence of symptoms in patients and in mice, cellular toxicity, and unequivocal identification of CTXs by liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS). To the best of our knowledge, this is the first identification of CTXs in sharks.

Results

Poisoning event in Madagascar. In November 2013, an outbreak of fish poisoning following the consumption of shark was reported in the district of Fenoarivo Atsinanana (Fenerive-Est, Madagascar). According to the information transmitted to the ANSES by the “French Institute for Public Health Surveillance,” dated 22 April 2014, 124 people, 11 of whom died, were poisoned after consuming the flesh, liver, head and part of the viscera of a shark¹¹. The patients developed symptoms between 2 and 12 h following ingestion of their meal, and the predominant neurological signs were paraesthesia of the extremities, dysesthesia, and reversing sensitivity of hot and cold. These symptoms were accompanied by headache, dizziness, and arthralgia. The digestive symptoms were moderate and inconsistent. The clinical profile was similar to that of patients that had previously been poisoned after consumption of shark in Madagascar⁹. A detailed epidemiological report is presented by Rabenarison *et al.*¹¹, and additional investigations conducted by agents of the “Institut Halieutique et des Sciences Marines” (Tulear, Madagascar) concluded that the shark in question was a female of about 1.5 m in length. Samples of the fish implicated in the episode, and used in our study, consisted of salted stomach, three dried fins, and partially cooked flesh. The genetic analyses that we performed using 22 microsatellite loci on the five samples demonstrated that they all belong to the species *C. leucas* and surely to the same individual (identical multilocus genotypes; Supplementary Table S1 and Fig. 1).

Toxicity evaluation of shark samples by mouse bioassay (MBA). The MBA performed on the flesh samples showed toxicity, although quantification was not possible due to the limited amount of samples. The single dose injected corresponds to an amount of 50 g equiv. flesh/mouse. The symptoms observed in the mice included: paralysis of limbs, dyspnoea, convulsions, mild diarrhea, and mortality from respiratory failure between 3 and 4 hours after injection of the extract. Toxicity and symptoms in mice were similar to those previously described in the study on shark toxins in Madagascar⁹. The MBA performed on the stomach sample showed a very high toxicity. Even at low doses, when mortalities were observed these were always rapid (survival time less than 1.5 h) and otherwise the mice recovered. The symptoms observed were dominated by neurological problems, including difficulty with breathing, followed by a severe respiratory arrest. In some mice, hyper-salivation was also observed. The lowest dose tested that resulted in the death of mice was estimated at 72 mg equiv. stomach per mouse of 20–22 g. Fin samples were not tested by MBA.

Evaluation of ciguatoxin-like activity by Neuro-2a cell-based assay (CBA). Neuro-2a cells exposed to Pacific ciguatoxin P-CTX-1 (CTX1B) standard presented the expected dose-response curve with a regression factor (R^2) of 0.996. The concentration of P-CTX-1 that caused a 50% cell mortality (IC_{50}) was 19.9 $\mu\text{g}/\text{mL}$. The limit of detection (LOD), defined as the concentration of P-CTX-1 that causes a 20% of cell mortality (IC_{20}), was 8.4 $\mu\text{g}/\text{mL}$ (Supplementary Figure S2). Considering a maximum exposure concentration of 200 mg/mL for flesh, 100 mg/mL for stomach and fins 1 and 3, and 50 mg/mL for fin 2 (Supplementary Figure S3), the effective LODs (eLODs) for P-CTX-1 in shark samples were 0.04 μg P-CTX-1 equiv./kg for flesh, 0.08 μg P-CTX-1 equiv./kg for

Crude extract	MBA (μg P-CTX-1 equiv./kg tissue)	CBA (μg P-CTX-1 equiv./kg tissue)	LC-ESI-HRMS (μg P-CTX-1 equiv./kg tissue)		
			I-CTX-1&2	I-CTX-3&4	Σ I-CTXs
flesh	n.q.	0.06	n.d.	n.d.	n.d.
stomach	83	92.78	6.54	9.74	16.28
fin 1	—	0.12	—	—	—
fin 2	—	0.79	n.d.	n.d.	n.d.
fin 3	—	0.17	—	—	—

Table 1. Concentration of P-CTX-1 equiv./kg tissue in crude stomach, flesh and fin extracts as determined by mouse bio-assay (MBA), Neuro-2a cell-based assay (CBA) and liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS).

Fractions	CBA (% P-CTX-1 equiv.)	LC-ESI-HRMS (% P-CTX-1 equiv.)		
		I-CTX-1&2	I-CTX-3&4	Σ I-CTXs
F8	0.23	n.d.	n.d.	n.d.
F9	5.15	n.d.	5.67	5.67
F10	5.80	n.d.	29.66	29.66
F11	9.37	13.87	15.75	29.62
F12	8.05	16.12	3.86	19.98
F13	1.79	n.d.	n.d.	n.d.
F14	1.44	n.d.	n.d.	n.d.
F15	2.30	n.d.	n.d.	n.d.
F16	0.62	n.d.	n.d.	n.d.
F17	0.29	n.d.	n.d.	n.d.
F18	0.27	n.d.	n.d.	n.d.
F19	0.33	n.d.	n.d.	n.d.
F20	0.23	n.d.	n.d.	n.d.
F21	0.19	n.d.	n.d.	n.d.
F22	0.12	n.d.	n.d.	n.d.

Table 2. Distribution of ciguatoxins (CTXs) after fractionation of the stomach crude extract. Percentages of P-CTX-1 equiv. recovered in each fraction in relation to the P-CTX-1 equiv. injected, estimated by the Neuro-2a cell-based assay (CBA) and liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS).

stomach and fins 1 and 3, and 0.17 μg P-CTX-1 equiv./kg for fin 2. Flesh, stomach and fins 1 to 3 crude extracts of *C. leucas* contained 0.06, 92.78, 0.12, 0.79 and 0.17 μg P-CTX-1 equiv./kg matrix, respectively (Table 1).

With the aim of separating the different compounds to better identify the toxin profile of the most toxic sample, the stomach extract was fractionated by high-performance liquid chromatography (HPLC). In order to identify the distribution of the toxin within the 28 fractions recovered, cells were first exposed to 2.17 μL of each fraction/mL. CTX-like activity was observed in fractions F8 to F22. Fractions F8 and F17 to F22 fell within the working range (IC_{20} – IC_{80}), and CTX-like content was able to be quantified. The use of lower fraction volume (0.27 μL of each fraction/mL) was required to quantify fractions F13 and F14 (Fig. 1). Further dilution was required to quantify fractions F9 to F12. Distribution of ciguatoxins after fractionation of the stomach crude extract is shown in Table 2.

Confirmation of ciguatoxins (CTXs) by liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS).

A liquid chromatography electrospray ionization high-resolution mass spectrometry (LC-ESI-HRMS) method was developed for the analysis of CTXs in extracts of *C. leucas*, based on previous LC-MS/MS methods^{3,12,13}. The spectra of I-CTXs were dominated by $[\text{M}+\text{H}]^+$, $[\text{M}+\text{NH}_4]^+$, $[\text{M}+\text{Na}]^+$, $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$, $[\text{M}+\text{H}-2\text{H}_2\text{O}]^+$ in accordance with Hamilton *et al.*¹³. The adduct ions giving higher signals, specifically $[\text{M}+\text{NH}_4]^+$ and $[\text{M}+\text{Na}]^+$, were chosen for confirmation and quantification purposes.

Crude extracts of flesh, stomach and fin 2 were analyzed by LC-ESI-HRMS. The presence of CTX analogues was not observed in the flesh nor in the fin 2 crude extracts. A possible explanation is that the LOD attained by CBA (0.04 μg P-CTX-1 equiv./kg flesh tissue and 0.17 μg P-CTX-1 equiv./kg fin 2 tissue) is lower than the LOD attained by LC-ESI-HRMS (0.5 μg P-CTX-1 equiv./kg tissue). In the stomach crude extract, the CTX analogues I-CTX-1&2 and I-CTX-3&4 were detected and quantified (Table 1).

All analogues were confirmed using their theoretical accurate mass (m/z), measured m/z , and mass accuracy (ppm): i) I-CTX-1&2 ($[\text{C}_{62}\text{H}_{92}\text{O}_{19}\text{NH}_4]^+$ and $[\text{C}_{62}\text{H}_{92}\text{O}_{19}\text{Na}]^+$): 1158.6571, 1158.6606, <5.49 ppm and 1163.6125, 1163.6148, <4.93 ppm, respectively; and ii) I-CTX-3&4 ($[\text{C}_{62}\text{H}_{92}\text{O}_{20}\text{NH}_4]^+$ and $[\text{C}_{62}\text{H}_{92}\text{O}_{20}\text{Na}]^+$): 1174.6540,

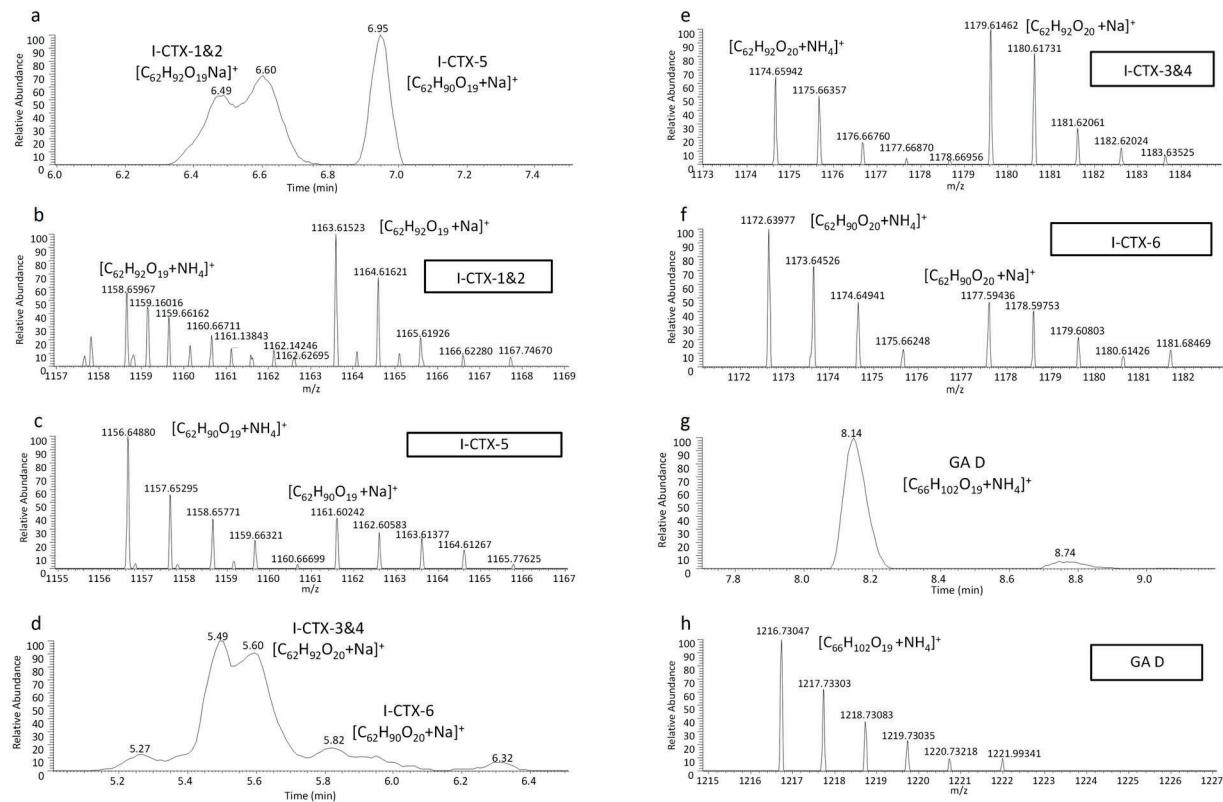


Figure 2. Evidence for the presence of ciguatoxins (CTXs) and gambieric acid D (GA D) in shark tissues. (a) Extracted ion chromatogram of I-CTX-1&2 $[M+Na]^+$ at 1163.6125 and HRMS exact mass spectra of (b) I-CTX-1&2 $[M+Na]^+$ at 1163.6125 and (c) I-CTX-5 ($C_{62}H_{90}O_{19}$) $[M+Na]^+$ at 1161.6070, in fraction F12 from stomach; (d) extracted ion chromatogram of I-CTX-3&4 $[M+Na]^+$ at 1179.6084 and HRMS exact mass spectra of (e) I-CTX-3&4 $[M+Na]^+$ at 1179.6084 and (f) I-CTX-6 ($C_{62}H_{90}O_{20}$) $[M+Na]^+$ at 1177.5910, in stomach crude; (g) extracted ion chromatogram of GA D $[M+NH_4]^+$ at 1216.7354 and (h) HRMS exact mass spectra of GA D $[M+NH_4]^+$ at 1216.7354, in flesh crude extract, respectively.

1174.6565, <6.31 ppm and 1179.6084, 1179.6146, <5.89 ppm, respectively. From a qualitative point of view, both LC-ESI-HRMS and CBA showed the presence of P-CTX-1 equiv. in the stomach crude extract. However, lower contents of P-CTX-1 equiv. were estimated by LC-ESI-HRMS in relation to the CBA. This difference in the quantification could be attributed to the different principles of the techniques: while LC-ESI-HRMS is based on structural identification of specific CTX analogues, and may neglect some non-described CTX analogues, CBA measures a composite toxicity, which is a global response indicative of the toxic effect of several CTX analogues on cells.

Having identified the toxic stomach fractions using the CBA, fractions F8 to F22 were analyzed by LC-ESI-HRMS for toxin identification. Fractionation of the stomach crude extract reduced matrix interferences and confirmed the presence of I-CTX-1&2 and/or I-CTX-3&4 in fractions F9 to F12, the most toxic ones by CBA (Table 2). Extracted ion chromatograms for I-CTX-1&2 and I-CTX-3&4 found in fraction F12 from stomach are shown in Fig. 2a and d, respectively. Full HRMS exact mass spectra of I-CTX-1&2 and I-CTX-3&4 (Fig. 2b and e, respectively) confirmed the presence of these toxins. The isotopic pattern of each signal was taken into consideration in assigning their molecular formula. In addition, these toxins showed a profile similar to P-CTX-1 according to $[M+NH_4]^+$ and $[M+Na]^+$. As for the analysis of crude extracts, the percentages of P-CTX-1 equiv. in fractions F9 to F12 determined by LC-ESI-HRMS were higher than those obtained by CBA. Nevertheless, both techniques concluded that fractions F9 to F12 contained the highest CTX content among all fractions.

Two new CTX analogues (I-CTX-5 and I-CTX-6) were detected by LC-ESI-HRMS (Fig. 2c and f). The identification of these new CTX analogues related to I-CTXs in the stomach crude extract and fractions was given using the restrictive criteria to propose elemental formulae. Ion assignment indicated that the new CTX analogues had nearly the same molecular formula as I-CTX-1&2 and I-CTX-3&4 with only 2H less, which corresponds to the formation of a double bond. The ring double bond equivalents (RDBEs) for both unknown compounds was 16.5, which corresponds to the 15.5 RDBE value from CTX analogues (I-CTX-1&2 and I-CTX3&4). Analogues were confirmed according to their theoretical accurate m/z , measured m/z , and mass accuracy (ppm): i) I-CTX-5 ($[C_{62}H_{90}O_{19}NH_4]^+$ and $[C_{62}H_{90}O_{19}Na]^+$): 1156.6414, 1156.6479, 5.57 ppm and 1161.5968, 1161.6030, 5.29 ppm, respectively; and ii) I-CTX-6 ($[C_{62}H_{90}O_{20}NH_4]^+$ and $[C_{62}H_{90}O_{20}Na]^+$): 1172.6496, 1172.6381, -1.44 ppm and 1177.5918, 1177.5953, 3.03 ppm in fraction F12 from stomach, respectively. Full HRMS exact mass spectra of both new I-CTXs in Fig. 2c and f confirm the presence of these toxins.

Identification of gambieric acid D (GA D) by liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS). Gambieric acid D ($C_{66}H_{102}O_{19}$) (GA D), which is also produced by *Gambierdiscus* spp., was identified in the flesh crude extract of the shark using the theoretical accurate m/z , measured m/z , and mass accuracy (ppm) of $[C_{66}H_{102}O_{19}NH_4]^+$: 1216.7354, 1216.7304, -4.02 ppm at 8.14 min, respectively. Quantification by LC-ESI-HRMS was not carried out due to the lack of GA D standard solutions. Extracted ion chromatograms (Fig. 2g) and HRMS exact mass spectra (Fig. 2h) confirm the presence of GA D by the m/z of most abundant ion peaks $[M+NH_4]^+$. No GA D was identified in the stomach or in the fins.

Discussion

The methodology presented in this paper, which combines a multi-disciplinary approach focused on epidemiology, toxicology and instrumental analysis, has proved to be effective in the identification of CTXs in seafood and contributes to a better characterization of the present incidence of ciguatera.

This is the first evidence of the presence of CTXs in sharks. The identification of CTXs in the shark responsible for a food poisoning event in Madagascar presented herein, along with additional observations, confirm the episode as a ciguatera event and the suspected implication of sharks in ciguatera⁷. The evidences that support this confirmation are: i) the symptoms observed in patients matched those of ciguatera, ii) injection of flesh and stomach crude extracts to mice resulted in symptoms characteristic of CTXs, which were comparable to those reported for I-CTX in the bony fish *Lutjanus sebae* from the Indian Ocean¹³, iii) neuroblastoma cells exposed to flesh, stomach and fin crude extracts showed the characteristic toxicity of CTXs through activation of voltage-gated sodium channels, and iv) LC-ESI-HRMS provided the identification of I-CTX-1&2 and I-CTX-3&4 in the stomach extracts. The stomach was extremely toxic with an estimation of 92.78 μ g P-CTX-1 equiv./kg by cell-based assay (CBA), a concentration approximately 10,000 times the guidance level concentration of P-CTX-1 causing ciguatera in humans, established at 0.01 μ g P-CTX-1/kg by the FDA¹⁴, and considered by EFSA¹⁵ as the level expected not to exert effects in sensitive individuals. The estimation of the stomach levels by LC-ESI-HRMS was lower than determined by CBA, 16.28 P-CTX-1 equiv./kg; however, this level is well above the FDA guidance level. Flesh and fins presented toxicity with an estimation by CBA of 0.06 μ g P-CTX-1 equiv./kg in the flesh and 0.12, 0.79 and 0.17 μ g P-CTX-1 equiv./kg in fins 1 to 3, respectively, which are also above the FDA guidance level. Identifying CTXs in viscera is significant, since local food habits from the Indian Ocean include eating liver and viscera¹¹, some of which are dried and salted. Since the liver of the shark was not available, the possible presence of carchatoxins previously described in other shark poisoning events¹⁶ could not be studied.

Currently, only four I-CTX analogues (I-CTX-1&2 and I-CTX-3&4) have been described in the literature¹³. As for the already known CTXs, I-CTX-1&2 and I-CTX-3&4, our results obtained in the stomach of shark revealed higher amount of I-CTX-3&4 in relation to I-CTX-1&2 (60% vs 40% of the total amount of I-CTXs estimated by LC-ESI-HRMS). Contrarily, in that previous study on Indian CTXs in one fish (*Lutjanus sebae*), a lower amount of I-CTX-3&4 in relation to I-CTX-1&2 was described¹³. This difference may be explained by the tissue evaluated, since in their work the whole fish was analysed¹³. Additionally, Hamilton and collaborators postulated that I-CTXs-1&2 might originate from dinoflagellates and that I-CTX-3&4 would be metabolites produced in fish¹³. Since sharks are higher in the trophic webs than *L. sebae*, this may explain the higher amounts of I-CTX3&4 in relation to I-CTX-1&2 obtained in shark stomach. Herein, two new I-CTX analogues have been identified by LC-ESI-HRMS in the stomach extract and fractions, I-CTX-5 showing 2H less than I-CTX-1&2 and I-CTX-6 showing 2H less than I-CTX-3&4, which corresponds to the formation of a double bond. This result widens the number of CTXs analogues possibly present in nature^{3,12,13} and this will impact our understanding of ciguatera. First, the identification of new CTX analogues may indicate that the metabolism of CTXs may be more complex than previously foreseen. Second, these new CTX analogues possibly present in seafood will need to be taken into account for a better evaluation of ciguatera risks.

Gambieric acid D (GA D)¹⁷ was identified in shark flesh. To the best of our knowledge, this work is the first report of GA in any organism other than *Gambierdiscus* spp.¹⁶. The identification of GA D in the flesh of the shark evidences, additionally to the presence of CTXs, the link between *Gambierdiscus* spp. and this particular food poisoning event. Identifying GA D in sharks that, as carnivorous pelagic fish are situated at the highest levels of the marine food webs, demonstrates how stable these molecules may be throughout their transfer and metabolic transformations along the food webs. The identification of molecules produced by microorganisms in animals situated at higher trophic levels, such as GA D would constitute, as for the analysis of fatty acids or stable isotopes, a good strategy to understand trophic relations in the ecosystems. Gambieric acid A has demonstrated no toxicity in mice, while a mixture of GA C and GA D was moderately toxic to mouse lymphoma cells L5178Y^{18,19}. Additionally, GA A has been demonstrated to bind to the voltage gated sodium channels in synaptosomes isolated from rat brains²⁰ in the same manner as CTXs, but with much less affinity to these than CTXs, possibly explaining its low toxicity. To better understand the potential harmful effects of GAs, and more specifically of GA D, their toxicity should be further characterized; however, at this moment reference material for GAs is not commercially available. Urgent need exists for the availability of certified standards for CTXs and GAs.

About 100 million sharks are caught each year world-wide²¹, and the global shark fin trade is estimated to be worth US \$ 400–500 million a year²². The identification of CTXs in shark from the Indian Ocean may favour the re-consideration of local food safety measures that could affect the shark fisheries industry, which is of special relevance in areas such Madagascar²². The present work confirms that shark consumption, in this example, a bull shark (*C. leucas*), from the Indian Ocean should be considered a ciguatera risk, and actions should be taken to evaluate its magnitude and risk in order to manage shark fisheries. As for the numerous suspicious cases of ciguatera involving sharks⁷, it may be postulated that sharks with CTXs will not be restricted to the species *C. leucas* and to the Indian Ocean. Consequently, other species of shark and other oceans should also be considered for ciguatera evaluation, especially to account for the migration of sharks and current changes in the geographical distribution of sharks due to fishing pressure and global warming^{23,24}.

Methods

Samples. Shark samples were collected and analyzed in the framework of a research and development agreement funded by ANSES. Five samples were recovered by the health authorities of Madagascar “Agence de Contrôle Sanitaire et de la Qualité des Denrées Alimentaires de Madagascar” (ACSQDA) and were transferred to the laboratory by the WHO and the “Pasteur Institute of Madagascar”. The salted stomach, three dried fins and partially cooked flesh samples were stored at -25°C until extraction. Samples of flesh and stomach were crushed and homogenized before starting the analyses. Until extraction, they were stored at -25°C .

Shark species identification through microsatellite genotyping and Dloop sequencing. The five samples (flesh, stomach and fins 1 to 3) from elasmobranch tissues, suspected to be from bull shark (*C. leucas*), were used for identification. Total genomic DNA was extracted using Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). The mitochondrial control region (D-loop; 832 bp) was also sequenced using primers designed previously²⁵, but failed to amplify due to DNA degradation before collection. Therefore, genotyping was performed using a total of 22 microsatellite loci [20 loci developed for *C. leucas*²⁶, one locus (Cli106) for *C. limbatus*²⁷ and one locus (Gc01) for *Galeocerdo cuvier*²⁸]. All amplifications were performed as described in the literature²⁶. Then, identical multi-locus genotypes (MLGs) were identified using the software GenClone v. 2.0²⁹ and, using the software STRUCTURE v. 2.3.1³⁰, assignment tests were performed together with MLGs obtained from other carcharhinid shark species (*C. leucas*, *C. obscurus* and *C. plumbeus*) that are known to amplify with the same loci.

Toxin standards. Pacific type 1 CTX (P-CTX-1 or P-CTX-1), P-CTX-2 and P-CTX-3 standard solutions were provided by Pr. Richard J. Lewis (The Queensland University, Australia). P-CTX-1 standard was used for CBA and LC-ESI-HRMS analysis. P-CTX-2, P-CTX-3 standards were used only for LC-ESI-HRMS analysis.

Flesh, stomach and fins extraction. Samples were extracted and purified according to the protocol described in the literature³¹ with minor modifications provided by ANSES. In brief, $10\text{ g} \pm 0.1\text{ g}$ of flesh, stomach or fins homogenates were placed in 50-mL tubes. Samples were extracted in 20 mL of acetone and homogenized with an Ultraturax blender. Samples were heated in the sealed tube at 70°C for 10 min in a water bath. The supernatant was recovered by centrifugation at 3,000 g for 10 min at 4°C and filtered using 0.45- μm nylon filters. The sample pellets were re-extracted with acetone and supernatants were pooled and evaporated until dry. Liquid/liquid partition was then performed twice in the tubes with 20 mL of water/diethyl ether (DEE) (1:4, v-v). The DEE upper phase was recovered and pooled with the second DEE partition. Both DEE phases were evaporated until dry. The dried extracts were then dissolved in 4 mL of *n*-hexane and 2 mL of methanol/water (4:1, v-v). The hexane upper phase was removed. This liquid/liquid partition was repeated three times and the methanol phases were pooled and evaporated until dry. Finally, the resulting residues were re-dissolved in 4 mL of HPLC-grade methanol and preserved at -20°C until analyzed.

Stomach extract fractionation. A total of 2,750 μL of stomach extract were evaporated until dry using $\text{N}_{2(\text{g})}$, and re-dissolved in 1,000 μL of HPLC-grade methanol. The analytical fractionation of this extract was performed as described before for the chromatographic separation of CTXs³. Once the chromatographic run started, fractions were collected every 30 seconds ($n = 28$). After fractionation, the volume of each tube was evaporated to dryness, re-dissolved in 500 μL of HPLC-grade methanol and preserved at -20°C until analyzed. Stomach fractions were analyzed by LC-ESI-HRMS and CBA, but not by MBA.

Mouse bioassay. The protocol used at HYDROREUNION was validated beforehand by the ethics committee (Protocol agreement n° EU0450 - GIP CYROI - APAFiS - Autor. APAFiS #2641-2015110916009490) and was in accordance with the regulations in force. This protocol is based on a standard method developed by ANSES (CATNAT-10). The extracts of shark were solubilized in Tween-60 1–5% saline solution, and then injected into three mice (male, OF1; $20 \pm 2\text{ g}$) by intraperitoneal (i.p.) route. The mice were observed continuously during the first 2 h, and then monitored regularly up to 24 h after injection. The interpretation of the results was based on the symptoms observed and the time-to-death of the mice. The typical symptoms of the presence of CTXs include profuse diarrhea, piloerection, respiratory disorders, dyspnoea and, when using male mice, transient pre-erectile cyanosis of the penis (which can become priapism). With the Indian Ocean toxins, this last symptom is observed only very rarely. It therefore does not appear in the classical description for this region. The death of 1 or 2 mice within 24 h was deemed a positive result indicating the presence of CTXs (sample therefore non-edible).

Assessment of ciguatoxin-like activity by Neuro-2a cell-based assay. Neuroblastoma mice (Neuro-2a cell line: CCL-131) were purchased from the American Type Culture Collection (ATCC) (LGC standards S.L.U., Barcelona, Spain). The presence of CTX-like activity in shark tissues extracts was evaluated on Neuro-2a cells according to the method based on the use of ouabain and veratridine published by Caillaud and co-workers³². Briefly, cells were exposed to the P-CTX-1 standard and to shark extracts for 24 h, and the CTX-like activity was measured in the presence of ouabain and veratridine with the MTT colorimetric assay³³. Previous to the analysis of flesh, stomach and fins crude extracts or stomach fractions by CBA, methanol was removed from the extracts/fractions and P-CTX-1 standard solution by evaporation under $\text{N}_{2(\text{g})}$ and re-dissolved in RPMI medium. CTX-like activity was estimated with respect to P-CTX-1.

Liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS). An Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer equipped with heated

electrospray source (H-ESI II), a Surveyor MS Plus pump and an Accela Open AS auto-sampler kept isothermal at 15 °C (Thermo Fisher Scientific, San Jose, California) were used for the analysis by LC-ESI-HRMS.

The chromatographic separation was performed on a reversed-phase Hypersil Gold C₁₈ (50 mm × 2.1 mm, 1.9 μm) (Thermo Fisher, Scientific, Bremen, Germany) at a flow rate of 250 μL/min. Mobile phase A was water and B was acetonitrile/water (95:5), both containing 2 mM ammonium formate and 0.1% formic acid. The gradient elution program for the analysis was: 30% B 1 min, 30–40% B 2 min, 40–50% B 1 min, 50–90% B 5 min, 90% B 3 min and return to initial conditions for re-equilibrate (11 min 30% B). A 5-μL injection volume was used. The total duration of the method was 25 min.

The analyses were carried out in positive electrospray ionization (ESI+) mode, and the instrument was calibrated daily. P-CTX-1 was used to optimize the source, transmission and HRMS conditions in positive mode. The final parameters were: spray voltage of 4.0 kV, capillary temperature of 275 °C, heater temperature of 300 °C, sheath gas flow rate of 35 psi and auxiliary gas flow rate of 10 (arbitrary units). In addition, capillary voltage of 47.5 V, tube lens voltage of 186 V and skimmer voltage of 18 V were used. Nitrogen (purity > 99.999%) was employed as sheath gas, auxiliary gas and collision gas. The mass range was *m/z* 400–1,500 in full scan acquisition mode. The resolution was 50,000 (*m/z* 200, FWHM) at a scan rate 2 Hz, the automatic gain control (AGC) was set as “balanced” (1e⁶) with a maximum injection time of 250 ms. The data was processed with Xcalibur 2.2 SP1 software (Thermo Fisher Scientific, Bremen, Germany).

Automatic identification/quantification were performed. The peaks were extracted from the chromatogram using the exact mass of both [M+NH₄]⁺ and [M+Na]⁺ diagnostic ions, the mass accuracy (±10 ppm extraction window), and the retention time window. In addition to HRMS and accuracy parameters for identification, in the present study, to be confident of the identification and the proposed elemental formulae, the following restrictive criteria were applied: elements considered were restricted in accordance with CTXs molecular formulae and adduct signals [C 55 to 70, H 64 to 110, O 11 to 25, N 0 to 1, and cations (Na) 0 to 1]; the isotopic pattern was matched and the charge, the ring double bond equivalents (RDBEs) and nitrogen rule were taken into account. Additionally, the monoisotopic pattern (M+1 ion) of these signals was used to assist in the further confirmation of the toxin's identity. Therefore, in total four diagnostic signals were used for toxin identification. The relative ion intensities between [M+NH₄]⁺, [M+Na]⁺ and their M+1 ions were calculated and matched taking into account a tolerance according to the EU Decision 2002/657/EC. The toxins in study are characterised by a vast amount of carbon atoms, leading to relative abundances of the ¹³C isotopic ion higher than 65%. This high sensitivity of M+1 ion render these signals a high identification potential. The combination of high resolution, AMM and restrictive criteria was crucial for identification of both targeted and unknown compounds, as well as precise quantification of analytes. An external standard calibration was carried out from 12.5 to 100 ng/mL using P-CTX-1 and showed good linearity (R² = 0.996), with the LOD being 1.25 ng/mL. Due to the lack of proper analytical standards for all CTX congeners, in order to calculate concentrations of CTX analogues it was assumed that related analogues would give a similar response to that obtained with the P-CTX-1 standard.

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Acknowledgements

We kindly acknowledge Dr. Bernard Reche, the veterinarian in charge of the shark autopsies in La Réunion and who confirmed the viscera sample was stomach, and also Erwan Lagadec (CRVOI) for performing the DNA extraction. We also thank the World Health Organization (WHO) for facilitating the transfer of samples from Madagascar to Reunion Island. The authors acknowledge Dr. Chris Rodgers for his critical review of the manuscript and Dr. Melissa Dunkle for assistance with the English. The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007–2013) under the ECsafeSEAFOOD project (grant agreement no. 311820) and from CERCA/Generalitat de Catalunya program.

Author Contributions

J.D., J.T., M.C. and P.D.I. designed the study. J.T., I.R. and C.R. contributed to obtaining and transferring the samples. N.A. contributed to gathering epidemiological records. H.M. and A.P. contributed to genetic analyses. L.R. and V.d.R. performed the sample preparation and fractionation. J.T. performed the mouse bioassay quantification. L.R. performed the cell based assay quantification. M.R.-A., O.P. and C.F. performed LC-HRMS quantification and interpretation of CTXs in the shark extracts. J.D., J.T., L.R., M.C., M.R.-A., P.D.I., H.M., A.P. and J.C. discussed the results and participated in the writing.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-08682-8

Competing Interests: The authors declare that they have no competing interests.

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Annexe 3

Artificial daily fluctuations of river discharge affect the larval drift and survival of a tropical amphidromous goby

Lagarde R, Teichert N, Faivre L, Grondin H, Magalon H, Pirog A, Valade P, Ponton D. (2017). *Ecology of Freshwater Fish*.

L'annexe 3 est un article traitant de l'influence des variations journalières du courant d'une rivière comportant un barrage sur la dévalaison des larves du Gobiidé *Sicyopterus lagocephalus*.

Artificial daily fluctuations of river discharge affect the larval drift and survival of a tropical amphidromous goby

Raphaël Lagarde^{1,2,3}  | Nils Teichert^{1,4} | Laëticia Faivre¹ | Henri Grondin¹ |
Hélène Magalon² | Agathe Pirog² | Pierre Valade⁵ | Dominique Ponton³

¹Hydrô Réunion, Z.I. Les Sables, Etang Salé, France

²ENTROPIE, Université de La Réunion, IRD, CNRS, Laboratoire d'Excellence CORAIL, Saint Denis, France

³ENTROPIE, IRD, Université de La Réunion, CNRS, Laboratoire d'Excellence CORAIL, Le Port, France

⁴Irstea, UR EABX, Cestas, France

⁵OCEA Consult, Ravine des Cabris, France

Correspondence

Raphaël Lagarde, Hydrô Réunion, Z.I. Les Sables, Etang Salé, France.
Email: lagarde.arda@orange.fr

Funding information

European Regional Development Fund; Région Réunion; Office National de l'Eau et de Milieux Aquatiques

Abstract

Amphidromous fish species reproduce in rivers, and their larvae immediately drift to the sea after hatching. Most of these larvae survive in freshwater for a few days only, rapidly reaching sea water is thus essential. Being of small size, especially among species of the Sicydiinae subfamily, the larvae possess poor swimming abilities; their drift dynamics is considered to be mainly passive and influenced by flow conditions. The influence of daily fluctuations in flow regime on *Sicyopterus lagocephalus* (Sicydiinae) larval drift patterns was studied using drift nets at three sites along the dammed Langevin River in Reunion Island. The river flow was stable at the upstream site when frequent anthropogenic river flow fluctuations were observed at the two sites downstream to the dam. Weak diel larval drift dynamics were detected at the upstream site and the first site under anthropogenic river flow conditions. In contrast, larval drift dynamics at the most downstream site was strongly influenced by anthropogenic daily fluctuations of discharge: the abundance of drifting larvae increased with peaks of discharge, regardless of the time. As higher mortality rates of drifting larvae were observed during discharge peaks, the benefit of a more rapid travel to the ocean associated with these peaks should be lower than expected. It is concluded that peaks of discharge increase the number of larvae drifting to the sea probably due to higher physical constraints on the egg clutches, but also decrease the chance of survival for larvae that may be nonfully developed.

KEYWORDS

diadromy, downstream migration, hydrology, mortality, Sicydiinae, *Sicyopterus lagocephalus*

1 | INTRODUCTION

Flow regime is a major environmental driver of riverine fish ecology (Humphries, Serafini, & King, 2002; Winemiller, 1989). It notably influences the downstream movement, or drift, of fish larvae between their hatching and nursery habitats (Barthem, da Costa, Cassemiro, Leite, & Silva, 2014; Pavlov, Mikheev, Lupandin, & Skorobogatov, 2008). For example, high abundances of drifting larvae are frequently observed during periods of elevated river discharge (Charteris, Allibone, & Death, 2003; Næsje, Jonssons, & Skurdal, 1995). In addition to flow regime, larval behaviours may also, to some extent, influence larval

drift (Lechner, Keckeis, & Humphries, 2016). Larvae of large size in low, steady flow conditions are either able to increase their downstream movements by swimming in the flow direction, or decrease it by swimming against the flow direction, or towards areas of low flow velocity (Lechner et al., 2014; Pavlov et al., 2008). In contrast, small larvae mostly drift passively as their swimming capacities are limited (Braaten et al., 2012; Lechner et al., 2016).

Amphidromous fish species spawn in rivers. Immediately after hatching, their larvae drift down to the sea and develop for months before returning to freshwater (Radtko, Kinzie, & Folsom, 1988; Teichert, Valade, Grondin, et al., 2016). The different hatching (i.e. river) and

nursery (i.e. sea) habitats highlight the importance of the larval drift in the amphidromous life cycle. Although some amphidromous species can spend their entire larval stage in freshwater (Closs, Smith, Barry, & Markwitz, 2003; Hogan, Blum, Gilliam, Bickford, & McIntyre, 2014), for many other species their survival time in freshwater varies from 1 to 7 days only (Iguchi & Mizuno, 1999; Valade et al., 2009). As these larvae must reach the sea within a constrained time window to successfully develop, reducing the period of drifting between their hatching habitats and the sea thus appears to be essential for limiting their mortality.

Among amphidromous species, the gobies of the Sicydiinae subfamily are a species-rich group of more than 100 described species (Taillebois et al., 2014). These species mainly occur in small tropical islands. The swimming abilities of their larvae are limited by their small size, which never exceeds 2 mm notochord length at hatching (Iida, Watanabe, & Tsukamoto, 2013; Kinzie, 1993; Valade et al., 2009; Yamasaki & Tachihara, 2006). Once they hatch, the larvae present a single type of behaviour: "swim up-sink down" (Bell & Brown, 1995). The drifting mode of Sicydiinae larvae thus could be considered as mainly passive, and therefore, the flow regime and the eventual spawning strategies of adults are probably the only two factors affecting the time of larval freshwater drift.

For Sicydiinae species, a downstream spawning migration of adults has been hypothesised as one of the strategies aimed to reduce the time that larvae drift in freshwater environment. Indeed, the release of eggs in proximity to the estuary would reduce the distance between spawning grounds and sea. This hypothesis has been developed by Lord, Tabouret, Claverie, Pécheyrans, and Keith (2011) based on the variations in otolith elemental composition in adults of *Sicyopterus lagocephalus* and *S. aiensis* in Vanuatu rivers. However, a recent study focused on the migration of adults and juveniles of two Sicydiinae species, including *S. lagocephalus*, in tropical rivers (Lagarde, Teichert, Boussarie, Grondin, & Valade, 2015), suggested that the downstream displacements of adults depend solely on hydrological disturbances during cyclonic floods. If spawning location is not intended to reduce the time spent by larvae in freshwater, the only strategy for reaching the ocean more rapidly would be that larvae drift faster, mainly during periods of elevated river discharge. Indeed, the increase in flow velocity associated with elevated discharge could significantly reduce the time larvae spend in rivers. This hypothesis of synchrony between larval drift peak and elevated discharge is supported by observations made by Way, Burky, Harding, Hau, and Puleloa (1998) and Lagarde et al. (2017). In both studies, a synchronicity between peaks of Sicydiinae larval drift in rivers and elevated discharge was demonstrated on a seasonal scale. However, the question of how daily fluctuations of rivers discharge influence the drift dynamics of Sicydiinae larvae in freshwater remains.

The first objective of this study was to confirm the previously observed diel variations of Sicydiinae larvae abundances in natural and stable flow conditions (Lagarde et al., 2017). The second objective was to test the hypothesis that daily anthropogenic fluctuations of river discharge strongly affect the larval drift dynamics of Sicydiinae (i.e. larval abundance, transport duration and larval survivorship). In other

terms, the objective was to verify whether a benefit in terms of survival really exists when larval drifting time is reduced during periods of elevated discharge.

2 | MATERIAL AND METHODS

2.1 | Fish species studied

In Reunion Island, a small volcanic island in the south-western Indian Ocean (Figure 1a), two Sicydiinae gobies, *Sicyopterus lagocephalus* and *Cotylopus acutipinnis*, dominate the freshwater fish assemblages. These two species present an important cultural value as traditional fishermen mainly target them (Aboussouan, 1969; Bell, 1999). They are present from the estuaries to upstream reaches (Teichert, Valade, Lim, et al., 2014) as they can climb waterfalls tens of metres high due to their efficient climbing abilities (Blob, Rai, Julius, & Schoenfuss, 2006; Schoenfuss & Blob, 2007; Voegtli, Larinier, & Bosc, 2002). Reproduction of both species occurs in middle and upper reaches of rivers during the warmer months, that is December to May (Teichert, Valade, Fostier, Grondin, & Gaudin, 2016; Teichert, Valade, Fostier, Lagarde, & Gaudin, 2014). Females lay eggs under pebbles and rocks in runs and riffles (Teichert, Valade, Bosc, Richardson, & Gaudin, 2013). After egg fertilisation, the male guards the eggs until the larvae hatch (Teichert, Keith, et al., 2013).

2.2 | Sampling sites

The study was conducted at three sites along the watershed of Langevin River (Figure 1b). This torrential and very turbulent river flows southward from its source located on the south flank of the Piton de la Fournaise volcano. Approximately 500 m upstream to the estuary, a waterfall tens of metres high impedes the upstream migration of most amphidromous fish species. Due to their climbing capacities, only *S. lagocephalus* and *C. acutipinnis* can pass this waterfall and reach upstream river sections. Three intermittent sectors are present along the river due to the porosity of the bedrock (Figure 1c). Water flows through these sectors only a few days per year during flood conditions. In addition to natural flow variability within the Langevin River watershed, hydroelectric management of a dam, located at 6.5 km upstream to the estuary and 215 m above sea level (a.s.l.), results in diel fluctuations of river discharge.

The most upstream sampling site (site A, Figure 1c) was located immediately upstream of the dam at 215 m a.s.l. The river width at this site was approximately 20 m. The riverine habitats upstream to site A consisted of riffles and pools, several lotic areas being suitable for *S. lagocephalus* and *C. acutipinnis* to reproduce. A gauging station was located approximately 100 m upstream to site A.

Site B (Figure 1c) was located immediately downstream to the outlet channel of the hydropower plant. The river width at site B was approximately 5 m. The concrete-built channel presented no substratum suitable for *S. lagocephalus* and *C. acutipinnis* to reproduce, so it was assumed that no reproductive activity occurred in this channel during the study. On 17 February 2016, the dam was not operative and no

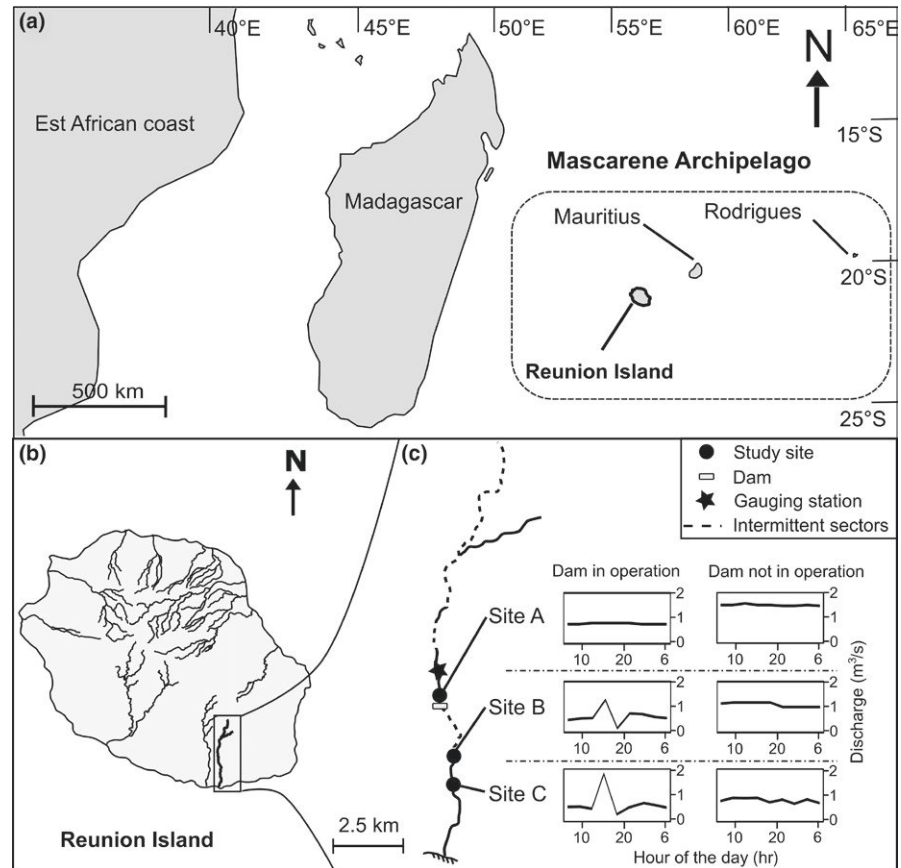


FIGURE 1 Sampling sites along the Langevin River watershed (c), Reunion Island (b), Southwestern Indian Ocean (a). Discharge fluctuations on 22 January 2016, when the dam was in operation, and on 17 February 2016, when the dam was not in operation are presented as examples for each site. Water loss can be observed between sites A and B on 17 February 2016 due to the porosity of the bed rock

water was flowing in the channel upstream to the usual location of site B. The sampling site was thus moved to the river bed, approximately 50 m upstream to its usual location. At this occasion, the river width at site B was approximately 10 m.

The most downstream sampling site (site C, Figure 1c) was located at 50 m a.s.l., 2 km downstream to site B and 2.5 km upstream to the estuary. The river width at this site was approximately 18 m. The riverine habitat between sites B and C consisted of riffles and pools, several lotic areas being suitable for *S. lagocephalus* and *C. acutipinnis* to reproduce. Egg clutches of Sicydiinae gobies had been observed in the river between both sites during a previous study (Teichert, Valade, et al., 2013).

During the study, data from site A (Figure 1c) were used to describe the daily variations of larval abundances in natural flow conditions. On 17 February 2016, when the dam was not operative, the river flowed between sites A, B and C without anthropogenic disturbances or natural input from tributaries (Figure 1c). During this sampling session only, data from sites B and C were also used to describe the variations of larval abundances in natural flow conditions. For all the other sampling sessions, the dam was operative and the whole river discharge was diverted into the hydropower plant. Consequently, the river section between the dam and site B was dried up. Larvae originating from upstream reaches situated nearby site A thus had to survive the passage through hydroelectric structures to reach site B. The diel fluctuations of discharge observed at sites B and C (Figure 1c) were due to hydro-power management. When the dam was operative, data from site C

were used to describe the effect of daily anthropogenic fluctuations of river discharge on larval drift dynamics. Data from site B were used for controlling that larvae coming from upstream to the dam, and observed at site A, did not significantly influence the larval drift dynamics observed at site C.

2.3 | Sampling procedure

A total of six sampling sessions were performed simultaneously at the three sites every 2 weeks from January 2016 to March 2016. This period was selected as it corresponds to the main season of reproduction of Sicydiinae gobies in Reunion Island, especially in upstream sites (Teichert, Valade, Fostier, et al., 2014).

Each sampling covered an entire 24-hr cycle and consisted in immersing simultaneously two conical nets nine to ten times at each site. The first nine two-net samples were scheduled every 3 hr. The additional, tenth, two-net sample was performed during the first peak of discharge of the day if this peak did not occur during the scheduled samples. Peaks of discharge were detected based on constant visual observations of water height at site C. Synchronicity of sampling schedule among sites was maintained by regular communications among the teams working at each site using mobile phones. At each station, the two nets were immersed for 5–30 min (D_{net}) to filter a volume of 3–6 m³. This volume captures tens of larvae (Lagarde et al., 2017) and limits the quantity of organic and mineral debris in the net. The nets were similar to those used for quantitative

drift sampling in small streams by Bell (1994, 2007). Their mouth diameter was 0.12 m, cod end diameter 0.06 m, length 1 m, and mesh size 80 μm . One of the two nets was fixed in the centre of the stream flow, whereas the other one was randomly positioned along the lotic areas of the river cross-section. The distances between the two nets ranged between 1.2 and 8.7 m (mean \pm standard deviation [SD] = 3.9 m \pm 2.1) at site A, between 0.2 and 2.4 m (0.7 m \pm 0.5) at site B and between 0.2 and 2.7 m (1.3 m \pm 0.7) at site C. Each net was set separately, the second net being set within 10 min after the first net. Using two nets quasi simultaneously, homogeneity of larvae distribution across the river was quantified (Lagarde et al., 2017). Each net was placed facing upstream at about 50% of the water depth. It was attached to an iron rod hammered into the substratum. Two measures of water velocity (v_1 and v_2) were performed in front of the mouth of each net, immediately after being set and before being removed. Measures were performed with a FLO-MATE 2000 flow meter (Cometec) or a Z4000 current meter (OTT) at site A, a M1 current meter (SEBA Hydrometry) at site B, and a MF PRO flow meter (OTT) at site C. All these apparatuses gave values with the same precision.

The sampled volume (V_{net}) was estimated with Equation (1):

$$V_{\text{net}} = \frac{v_1 + v_2}{2} \times \pi \times r^2 \times D_{\text{net}} \quad (1)$$

where v_1 and v_2 are the instantaneous water velocities measured in m/s before and at the end of the sampling respectively; r , the radius of the net mouth (i.e. 0.06 m); and D_{net} , the sampling duration (s).

2.4 | Larvae sorting and counting

After recovering each net, the content of each cod end was immediately transferred into a 2-L bottle filled with water from the river. The sample was kept still for a minimum of 1 hr and a maximum of 11 hr in the bottle to let organic and mineral debris settle down. This delay was assumed not to affect the number of alive larvae as the first mortality of Sicydiinae larvae usually occurs after approximately 24 hr spent in freshwater (Iida et al., 2010; Valade et al., 2009). This assumption was confirmed by visual observations of active larvae 11 hr after sampling. Larvae remained in the water column thanks to their "swim up-sink down" behaviour (Bell & Brown, 1995; Valade et al., 2009). Larvae were then collected by carefully pouring the contents of the bottle on an 80- μm -mesh sieve. The pouring was stopped before the overflow of vegetal and mineral debris. The water was returned to the bottle in order to repeat the decanting procedure twice. All larvae were transferred to a counting dish and counted by removing them one by one using a 15- μl micropipette. When the number of larvae appeared to be too high for direct count, the larvae were fixed with 95% alcohol solution, and their number was estimated later in the laboratory using a Dolfus tray. The total number of larvae (N_{larvae}) corresponded to the sum of all the larvae removed at the end of the three decant process. For each sample, the larvae were placed in a labelled 1-ml Eppendorf[®] and fixed with 95% alcohol solution.

2.5 | Estimation of river discharge and fluctuations of water height

Data from the gauging station located upstream to site A were obtained online (www.eaureunion.fr, accessed in July 2016). The instantaneous river discharge (Q_{river} , m^3/s) was recorded every 12 min.

At site B, the discharge at the outlet of the dam was estimated every hour by the hydroelectric company (Electricité de France) based on the mean hourly hydroelectric production. These data were used for estimating the river discharge during each two-net sample at this site, except on 17 February 2016 when the dam was not operative.

The river discharge at site C was estimated during each two-net sample using the velocity-area method. The river cross-section was divided into a minimum of ten vertical subsections. In each subsection, the water velocity was measured using a flow meter (type MF PRO, OTT) at up to three different water depths. The discharge of each subsection was estimated by multiplying the subsection area by its mean water velocity. The river discharge corresponded to the sum of the discharges of all subsections. The methods used for the measurement of flow velocities and for the estimation of discharge were based on the recommendation of the standard International Organization for Standardization (ISO, 2007).

The duration of the water height rising from base flow to the peak of discharge was identified based on measurements of water height at site C made every minute using a pressure sensor (Mini Diver SWS Technology) with an accuracy of ± 1 cm. The sensor was attached to an iron rod hammered into the substratum. As the position of the sensor varied between sampling sessions, only the relative variation in water height (i.e. difference between the water height at a given time and the minimum water height observed during the sampling session) was used.

2.6 | Variations of larval abundance

Instantaneous larval abundance was approximated with larval density (D_{larvae} , ind/m^3) calculated for each net with Equation (2):

$$D_{\text{larvae}} = \frac{N_{\text{larvae}}}{V_{\text{net}}} \quad (2)$$

where N_{larvae} is the number of larvae caught in the net and V_{net} is the sampled volume (m^3). Differences in the larval density between each net of the two-net samples were tested for the whole data set using a paired Student's t test. The homogeneity of the spatial distribution of larval density along the cross-section of the river was checked using orthogonal regressions between the larval densities estimated for each net of the two-net samples.

The daily variations in larval densities were analysed at each study site using periodic linear regressions as proposed by Bell (2007). Periodic linear regressions are frequently used for modelling predictable cyclical events, and allow the use of time as a continuous variable rather than a series of arbitrary categories (Bell, 2008; Bliss, 1958). This is particularly appropriate for this study as the time interval between two successive two-net samples (from 1 to 3 hr), and the

number of two-net samples per sampling session (from 9 to 10), varied between sampling sessions.

A mixed periodic model was adjusted taking into account each sampling session in order to integrate the variability of larval density among dates (3):

$$D_{\text{larvae}} = \beta_0 + \beta_1 \sin(R_h) + \beta_2 \cos(R_h) + r(1 + \sin(R_h) + \cos(R_h)) | \text{Session} \quad (3)$$

where D_{larvae} represents the instantaneous larval density; R_h , the radian transformed hour of the day (hour $\times 2\pi/24$); β_0 , the mesor (i.e. mean response of the biological trait over cycles); β_1 and β_2 , the regression coefficients; and r , the matrix of random individual effects for the sampling sessions. Residuals of regressions were plotted as a function of a theoretical normal distribution (quantile–quantile plot) to verify the assumption of normality. The significance of parameters was calculated based on the F -statistic using the Satterthwaite's approximation method for fixed part, and on likelihood ratio tests for random part (chi-square tests). The peak position (P , hour of the day) was determined for the mixed periodic linear model from Equation (4):

$$P = 24 \times \frac{\arctan\left(\frac{\beta_1}{\beta_2}\right) + \text{QC}}{2\pi} \quad (4)$$

where QC is a quadrant correction (QC = 0 if β_1 and β_2 are both positive, QC = π if β_1 is positive and β_2 negative or if both are negative and QC = 2π if β_1 is negative and β_2 positive). This peak was considered significant even if one part of the periodic model (i.e. β_1 or β_2) was not significant because cycles cannot be expressed in less than two dimensions (Bell, 2007). At site C, one outlier was excluded from this analysis to verify the assumption of normally distributed residuals.

2.7 | Estimation of larval drifting time and survival probability

The mean flow velocity of the river reaches downstream to site C was used to approximate the time taken by larvae drifting from site C to the sea. The estimation of mean flow velocity in this reach was performed twice: on a date when river discharge was low, and on a date when it was elevated. On each date, water velocity was measured at 40% of the water depth using a flow meter (type MF PRO, OTT) in several points along 12–14 transects following the protocol described by Girard, Lamouroux, and Mons (2014).

Estimations of survival probability were performed on larvae collected at sites A and C during three different sampling sessions when variations of river discharge were observed. These three dates were selected based on staff availability. For each of the three sampling sessions, survival probability was studied for larvae captured during the first two-net sample of the session, and for larvae captured during the additional two-net sample performed immediately after the first peak of discharge observed at site C. Larvae captured at site C were used to study the effect of discharge conditions on larval survival when those captured at site A were used for controlling the effect of the hour of the day on larval survival. A maximum of 60 larvae were subsampled per two-net sample. If the number of

larvae captured in the two-net sample was lower than 60, all larvae were sampled. At site A, an additional third net was set immediately after the two-net sample in order to increase the number of larvae for this experiment.

Immediately after being captured, the larvae were transferred to the laboratory where they were sorted and counted following the protocol described above. They were placed in 500-ml beakers at $23.5 \pm 0.5^\circ\text{C}$, a temperature corresponding to the mean daily temperature observed in downstream reaches of the Langevin River during summer (Teichert, Valade, Fostier, et al., 2014). Every 6–8 hr, the dissolved oxygen (mg/L) in the beakers was measured using an oximeter (HQ30D; Hatch), and the dead larvae were removed using a 15- μl micropipette, and preserved in 95% alcohol solution. Larvae were considered dead when lying on the bottom of the beaker and not responding to a gentle stimulation using a soft paintbrush.

Estimations of larval survival probability with time were performed based on the Kaplan–Meier method. Due to the low number of larvae captured during some two-net samples, results of survival time from the three different sampling sessions were summed. Results were expressed for low and peak discharge at site C, and the corresponding time of sampling at site A, the station where hydrological conditions were not modified. Finally, the difference of survival probability with time among the four different groups was tested using a log-rank test.

All statistical analyses were performed with the open source R v. 3.3.1 software (R Development Core Team, 2016). The packages *lme4* (Bates, Maechler, Bolker, & Walker, 2015) and *lmerTest* (Kuznetsova, Brockhoff, & Christensen, 2015) were used for the mixed periodic model analyses, and the package *survival* (Therneau, 2015) for the estimations of larval survival time.

2.8 | Identification of the larvae to the species level

No morphological criteria allow to discriminate the larvae of *S. lagocephalus* and *C. acutipinnis* (Teichert, 2012). The larvae of both species present similar size, shape, colour and repartition of their chromatophores. Criteria commonly used for morphological identification of amphidromous fish larvae (Lindstrom, 1999) are thus not useful for identifying these two species.

Identification at the species level was thus based on DNA analyses. A sample of 480 larvae was selected, 288 being randomly subsampled among the 407 larvae used for survival experiments, and 78 and 114 being randomly subsampled among the two-net samples from sites A and C respectively. As no peak of discharge was observed on 17 February 2016, no larvae from this sampling session were sampled for DNA analysis.

Total genomic DNA was extracted from each whole larva using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Following the same protocol as in Lagarde et al. (2017), genotyping was performed using the microsatellite locus SIC122, which expresses specific alleles for each species (at 166 bp for *C. acutipinnis*, and from 194 to 242 bp for *S. lagocephalus*; Hoareau, Barbisan, Dubois, Zane, & Berrebi, 2009). Amplicons were run on an ABI

TABLE 1 Sampling dates at each site and corresponding number of two-net samples, number of samples (numbers in parentheses correspond to the use of a third net in parallel to some two-net samples in sites A and B for increasing the number of larvae caught, see text for details), filtrated volume (m³), number of Sicydiinae larvae captured, number of larvae used for DNA analyses and number of individuals successfully identified for each species. On 15 March 2016, peak of discharge occurred immediately after the second two-net sample of the day; therefore, no supplementary two-net sample was performed at site A. On the same date, one of the nets was still immersed at site B during the increase of discharge and the sample was thus lost

Site	Date	Number of two-net samples	Number of samples	Filtrated volume (m ³)	Number of Sicydiinae larvae	Number of larvae used for DNA analyses	Number of <i>Sicyopterus lagocephalus</i>	Number of <i>Cotylopus acutipinnis</i>
A	22/01/2016	9	18	95	281	10	10	0
	03/02/2016	10	20 (+2)	129	809	59	58	1
	17/02/2016	9	18 (+1)	106	448	16	11	0
	02/03/2016	10	20 (+2)	119	225	19	19	0
	15/03/2016	9	18 (+1)	110	243	64	49	1
	30/03/2016	9	18	90	521	18	18	0
B	22/01/2016	9	18	79	42	—	—	—
	03/02/2016	10	20 (+2)	126	95	—	—	—
	17/02/2016	9	18	70	205	—	—	—
	02/03/2016	10	20 (+2)	96	189	—	—	—
	15/03/2016	10	18 (+1)	100	172	—	—	—
	30/03/2016	9	18	82	216	—	—	—
C	22/01/2016	9	18	53	8,829	40	22	0
	03/02/2016	10	20	74	1,062	46	43	0
	17/02/2016	9	18	82	1,061	—	—	—
	02/03/2016	10	20	98	2,319	85	73	0
	15/03/2016	10	20	87	2,141	83	64	0
	30/03/2016	9	18	75	788	40	35	0
Total	—	171	349	1,671	19,646	480	402	2

3730 XL sequencer (Applied Biosystems, Foster City, CA, USA), and allele size was determined using Genemapper v 4.0 (Applied Biosystems).

3 | RESULTS

3.1 | Sampling effort and relative proportion of species

Drifting larvae of Sicydiinae were present at all sampling sessions and each site (Table 1). At site A, a total of 2,527 larvae were collected in the 118 samples. Among the 186 larvae sampled for DNA analysis, 167 were successfully identified with 165 (99%) presenting the specific alleles of *S. lagocephalus* and two (1%) presenting the specific alleles of *C. acutipinnis*. At sites B and C, a total of 919 and 16,200 larvae were collected in the 118 and 114 samples respectively. Among the 294 larvae from site C sampled for DNA analysis, 237 were successfully identified as being *S. lagocephalus*. The unsuccessful identification of species was always due to a lack of DNA amplification that may be related to the low quantity of DNA per larva, or poor preservation of some samples. The total proportion of larvae at sites A and C presenting the specific alleles of *C. acutipinnis* was less than 0.5%. *Sicyopterus lagocephalus* and *C. acutipinnis* are the only two amphidromous fish species present in sampling sites, and the proportion of

C. acutipinnis in samples appeared very low. The following results can thus be considered to mainly concern *S. lagocephalus* individuals.

All sites considered together, larval densities estimated for each sample of the two-net samples ranged from 0 to 1,346 ind/m³. They did not significantly differ between the fixed and the randomly positioned nets of the two-net samples (paired Student's *t* test, $n = 169$, $t_{168} = 1.0$, $p = .31$). The orthogonal relationship of the log-transformed larval density estimated from the fixed and the randomly positioned nets indicated that the larval density was similar across the river cross-section ($n = 169$; slope \pm standard error [SE] = 0.99 ± 0.04 ; intercept \pm SE = 0.01 ± 0.07 ; $r^2 = .76$). As a consequence, the mean value (\bar{D}_{larvae}) of all nets for each two-net sample was used for the following analyses. When, for technical reasons, the estimation of the larval density was available for only one net of the two-net sample ($N = 2$, i.e. 1.2% of the two-net samples), this unique value was used.

3.2 | Diel variations in larval density of *S. lagocephalus*

The larval density (\bar{D}_{larvae}) of *S. lagocephalus* varied between 0.19 and 22 ind/m³ (mean \pm SD = 4.2 ± 4.2 ind/m³) at site A, between 0 and 5.8 ind/m³ (1.8 ± 1.5 ind/m³) at site B and between 2.9 and 1,120 ind/

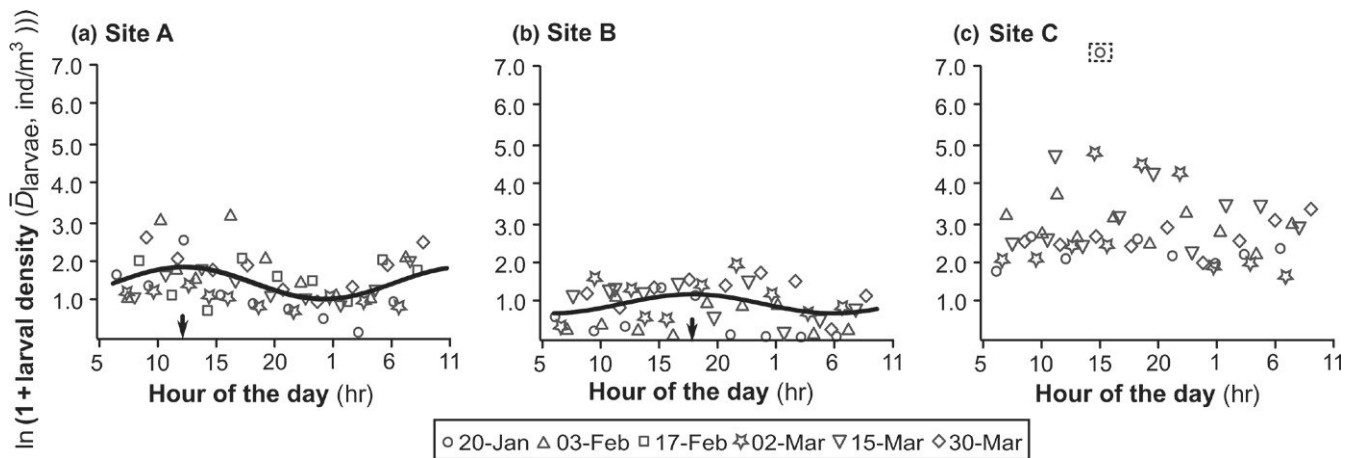


FIGURE 2 Log-transformed larval densities of *Sicyopterus lagocephalus* according to the hour of the day at sites A (a), B (b) and C (c). Open symbols differ for each date, the solid black line corresponds to the predictions for the fixed part of the periodic linear mixed models, and the black arrows correspond to the daily peak predicted for the fixed part of the periodic linear mixed model. The circled value at site C is an outlier which was excluded from the analysis to verify the assumption of normally distributed residuals. As no significant variation of discharge was observed on 17 February 2016, data from this sampling session were not included in the analysis at sites B and C

TABLE 2 Results of the periodic linear mixed models for sites A, B and C with the sampling session considered as a random effect. At site C, an outlier was excluded from the analysis to verify the assumption of normally distributed residuals (see Figure 2 for details). With β_0 : mesor, that is mean response of the biological trait over cycles, β_1 and β_2 : regression coefficients, *df*: degree of freedom estimated using the Satterthwaite's approximation method, *F*: *F*-statistic, and *p*: associated probability. As no significant variation of discharge was observed on 17 February 2016, data from this sampling session were not included in the analysis for sites B and C

	Coefficient	Estimate	<i>df</i>	<i>F</i>	<i>p</i>
Fixed effects					
Site A	β_0	1.42	—	—	—
	β_1	-0.01	4.82	0.00	.96
	β_2	-0.41	4.84	11.10	.02
Site B	β_0	0.86	—	—	—
	β_1	-0.26	23.99	9.26	< .01
	β_2	-0.01	3.98	0.01	.92
Site C	β_0	2.38	—	—	—
	β_1	-0.33	13.71	1.60	.27
	β_2	-0.11	13.71	0.50	.49
	Coefficient	χ^2	<i>df</i>	<i>p</i>	
Random effects					
Site A	β_1	0.66	3	.90	
	β_2	1.44	3	.70	
Site B	β_1	0.12	3	1.00	
	β_2	1.89	3	.60	
Site C	β_1	4.86	3	.90	
	β_2	0.46	3	.20	

m^3 ($34.7 \pm 146.2 \text{ ind}/m^3$) at site C (Figure 2). The estimated fixed parameters, β_1 and β_2 , of the periodic models explained a significant part of larval densities variability at sites A and B, but not at site C (Table 2, Figure 2). As no significant variation of discharge was observed on 17 February 2016, data from this sampling session were excluded from the analysis at sites B and C. At sites A and B, the predicted peaks of

larval densities were at 12:03 and 17:48 respectively. The variance explained by the random periodic parameters included in each model was never significant for the three sites (chi-square test, $df = 3$, $p \geq .2$ in all sites). It means that the larval density dynamics considered separately for each sampling session were not different from the larval density dynamics described for all the sampling sessions considered altogether.

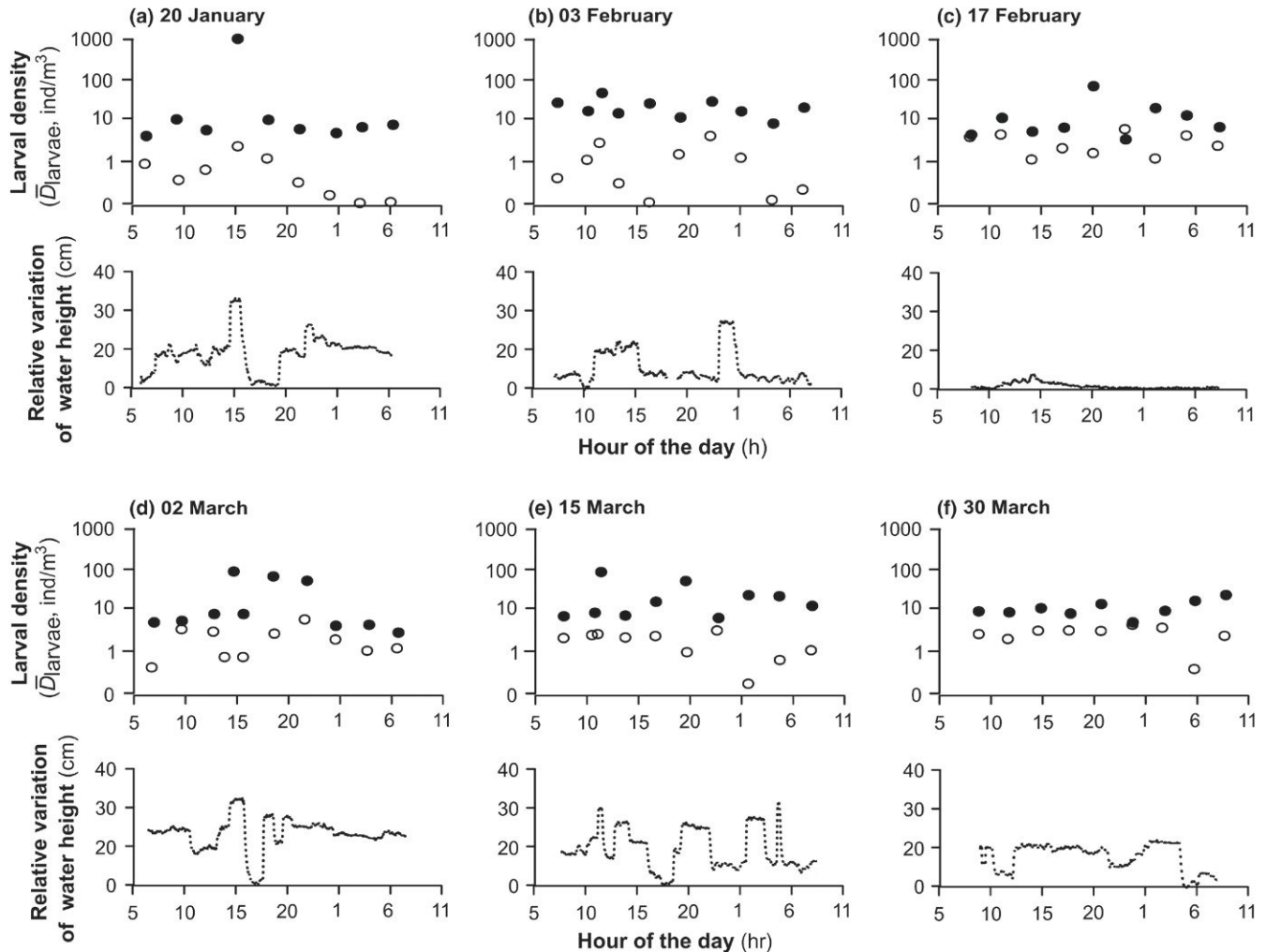


FIGURE 3 Log-transformed larval densities of *Sicyopterus lagocephalus* at site B (open symbols) and site C (filled symbols), and relative fluctuations of water height at site C (dashed line) according to the hour of the day for each sampling session (from a to f)

3.3 | Effects of anthropogenic fluctuation of discharge on larval densities of *S. lagocephalus* at sites B and C

The larval densities observed at site C were always higher than those observed at site B with the exception of a single event at 23:00 on 17 February 2016 when the dam was not operating (Figure 3c). In addition, the maximum larval density at site C was at 20:00 on this day when the dam was not operating (Figure 3c). During the whole study, the relative fluctuations of water height at site C varied between 4 and 33 cm and the duration of the water height increase from base flow to the peak of discharge lasted from 12 to 17 min.

At site B, the discharge difference between two successive two-net samples ranged from -1.9 to $+1.6$ m³/s when the larval densities of *S. lagocephalus* difference ranged from -3.6 to $+3.5$ ind/m³ (Figure 4a). At site C, the discharge difference between two successive two-net samples ranged from -2.1 to $+1.9$ m³/s when the difference in larval densities ranged from $-1,110.9$ to $+1,114.9$ ind/m³ (Figure 4b). The log-transformed difference of larval density between

two successive two-net samples was correlated to the difference of discharge between the same two-net samples at site C ($n = 42$; $r^2 = .36$; $p < .001$, Figure 4), but not at site B ($n = 41$; $r^2 = .01$; $p = .6$, Figure 4). As no significant variation of discharge was observed on 17 February 2016, data from this sampling session were excluded from the analysis.

3.4 | Estimation of larval drifting time and survival probability

The estimation of the mean flow velocity downstream to site C was performed on 7 April 2016 (118 measurements) and on 23 May 2016 (82 measurements). On 7 April 2016, the estimated discharge was 1.0 m³/s for a mean flow velocity of 0.29 m/s while on 23 May 2016, discharge reached 1.6 m³/s and flow velocity 0.35 m/s. As a result, it would take 144 min for passive larvae to travel from site C to the estuary (distance = 2.5 km) on 7 April 2016, and 119 min on 23 May 2016. Thus, an increase in mean flow velocity from 0.29 to 0.35 m/s downstream to site C would result in an approximately 25-min reduction of larval drifting time.

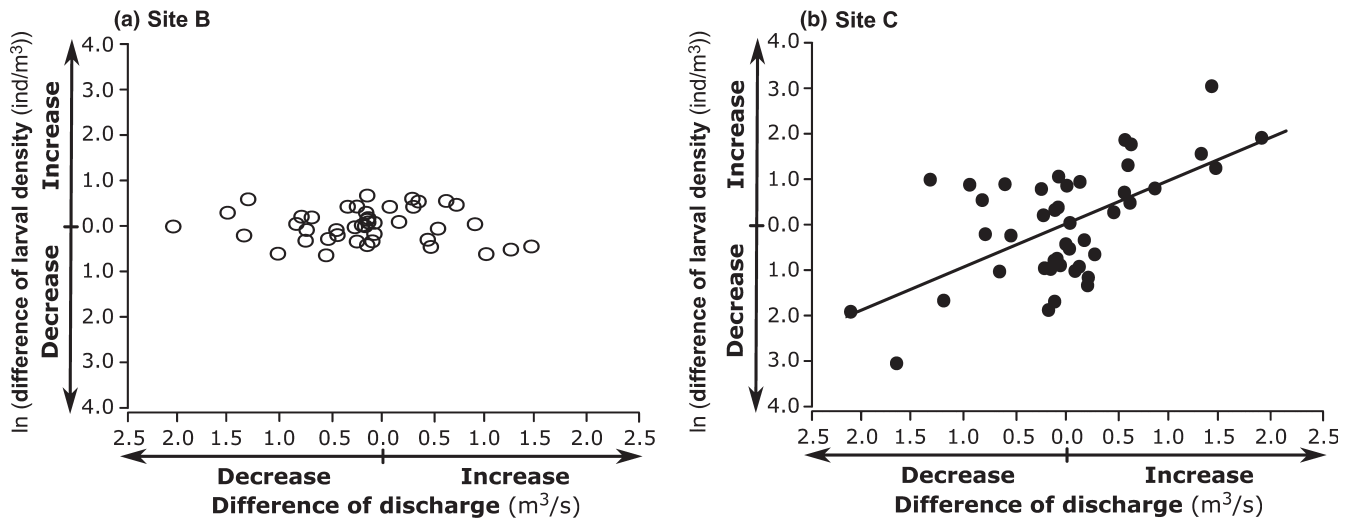


FIGURE 4 Linear regression relating the difference of discharge to the difference of the log-transformed larval density of *Sicyopterus lagocephalus* between two successive two-net samples (a) at site B (open symbols) and (b) at site C (filled symbols). As no significant variation of discharge was observed on 17 February 2016, data from this sampling session were not included in the analysis

TABLE 3 Sampling conditions at sites A and C, and number of larvae used for the survival experiment. The sample obtained from site A on 2 March 2016 at 07:00 was lost during transportation to the laboratory. The number of larvae from these experiments used for DNA analyses is also indicated

Site	Date	Sampling time	River discharge m ³ /s	Survival experiment treatment groups	Number of larvae used in survival experiment	Number of larvae subsamped for DNA analyses
A	03/02/2016	07:00	0.95	Control_low	25	20
	03/02/2016	11:15	0.95	Control_peak	26	17
	02/03/2016	07:00	1.68	Control_low	—	—
	02/03/2016	14:30	1.68	Control_peak	23	15
	15/03/2016	07:30	1.5	Control_low	26	18
	15/03/2016	10:30	1.5	Control_peak	42	38
C	03/02/2016	07:00	0.58	Low	10	8
	03/02/2016	11:15	1.14	Peak	32	28
	02/03/2016	07:00	1.78	Low	43	24
	02/03/2016	14:30	2.92	Peak	65	50
	15/03/2016	07:30	1.05	Low	51	32
	15/03/2016	11:00	1.52	Peak	63	38

Estimations of *S. lagocephalus* larval survival probability with time were performed on a total of 406 larvae caught on 3 February, 2 March and 15 March 2016 (Table 3). Among these larvae, 105 were captured during low discharge conditions, later identified as “low,” and 161 during peak discharge conditions, identified as “peak,” in site C. This corresponded to 51 and 90 larvae captured at the same time at site A where stable conditions of discharge prevailed. Larvae captured at site A between 07:00 and 07:30 were identified as “control_low” and larvae captured at the moment of the second sampling as “control_peak”. The mean dissolved oxygen concentrations in the beakers did not differ among the four groups of larvae “low,” “peak,” “control_low” and “control_peak” (ANOVA, $n = 206$, $F_{3,202} = 1.76$; $p = .16$). The maximum survival times for the four groups of larvae were similar and

ranged between 130 and 140 hr (Figure 5). However, survival probabilities over time significantly differed among groups (log-rank test, $df = 3$, $\chi^2 = 32$, $p < .001$), and the larvae captured during the peaks of discharge at site C had a lower survival probability, especially during the first 24 hr (Figure 5).

4 | DISCUSSION

4.1 | Diel variations of larval abundances of *Sicyopterus lagocephalus* in natural flow conditions

Among the 404 larvae successfully identified by DNA analyses, 402 (99.5%) were identified as *S. lagocephalus*. The proportion of

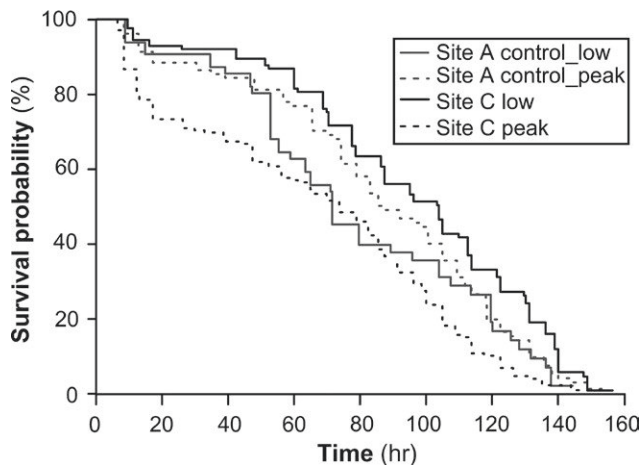


FIGURE 5 Mean larval survival probability over time based on the Kaplan–Meier method for the four groups of *Sicyopterus lagocephalus* larvae sampled on 3 February, 2 March and 15 March 2016. Larvae from the groups “low” and “control_low” were captured between 07:00 and 07:30 when no peak of discharge was observed at site C. Larvae from the groups “peak” and “control_peak” were captured during the peaks of discharge observed at site C, see Table 3 for details on sampling conditions. The origin of the x-axis is the time of capture of the larvae

C. acutipinnis larvae in this study can thus be considered as negligible, and the observed dynamics can be primarily attributed to *S. lagocephalus*. The very low proportion of *C. acutipinnis* larvae in Langevin River is not surprising as Teichert, Keith, et al. (2013) estimated that 99% of the mature adults present on the spawning grounds in this river were *S. lagocephalus* and that the number of *C. acutipinnis* eggs clutches was negligible. DNA-based identification of drifting larvae confirmed the observations made several years before, and demonstrated that larvae present in a river could be a good indicator of the adults present in upstream reaches.

Diel variations of abundances of *S. lagocephalus* larvae in natural and stable flow conditions were confirmed. The diel dynamics of abundances of *S. lagocephalus* larvae (i.e. larval density) captured in upstream site (site A) during all sampling sessions presented a weak but significant peak at around midday (precisely 12:03). The weak diel dynamics of larval drift in upstream reaches has already been documented for *S. lagocephalus* and *C. acutipinnis* in another watershed of Reunion Island (Lagarde et al., 2017). This observation has also been made for *Rhinogobius brunneus*, an amphidromous goby, in Japan (Iguchi & Mizuno, 1990), and for amphidromous shrimps in Puerto Rico (March, Benstead, Pringle, & Scatena, 1998). On 17 February 2016, the dam was not in operation, and the discharge remained stable in downstream reaches. At the most downstream site (site C), the peak of larval drift was observed few hours after night fall. This observation is consistent with the observations of larval drift dynamics made in downstream reaches of another watershed in Reunion Island for *S. lagocephalus* and *C. acutipinnis* (Lagarde et al., 2017). It is also consistent with observations made for other Sicydiinae species in Hawaii (Lindstrom, 1998; Luton, Brasher, Durkin, & Little, 2005).

4.2 | Effect of anthropogenic fluctuations of discharge on larval abundances of *Sicyopterus lagocephalus*

Larval abundances of *S. lagocephalus* were strongly affected by daily anthropogenic fluctuations of river discharge. The most elevated larval abundances observed at site C were clearly related to the rapid increases of discharge at this site. The number of larvae captured in site B was low compared to site C (919 larvae captured for a total filtered volume of 553 m³ at site B vs. 16,200 larvae captured for a total filtered volume of 469 m³ at site C). Larvae coming from upstream to the dam thus represented a negligible proportion of larvae caught at site C. Larval abundances at site C can be attributed to the spawning grounds present within the 2 km of the river between sites B and C.

Interestingly, when the dam was operative, a peak of drifting larvae was observed at the end of the afternoon (17:45) at site B. This peak was approximately 6 hr after the peak observed at site A. As no habitat appeared suitable for *S. lagocephalus* to reproduce between sites A and B, the larvae caught at site B probably originated from upstream reaches situated nearby site A. Thus, the 6 hr spent by larvae travelling between sites A and B reflected the time larvae took passing through the different hydroelectric structures between the water diversion at the dam and the outlet channel.

The variation of larval abundances at site C can be explained by the dynamics of hatching (Bradbury, Campana, Bentzen, & Snelgrove, 2004), or a coordinated and active swimming behaviour of larvae (Iguchi & Mizuno, 1991; Jarvis & Closs, 2015). This latter hypothesis is very unlikely as Sicydiinae gobies are known to have very poor swimming abilities (Bell & Brown, 1995; Valade et al., 2009). Moreover, the torrential and very turbulent flow conditions in the Langevin River would impede any active displacement of tiny larvae. In addition, the mean flow velocity in the short length of the river section upstream to site C was elevated (approximately 30 cm/s). Larvae coming from upstream spawning grounds were thus rapidly transported to site C. So, the most probable explanation for the variation of larval abundances observed at site C is the dynamics of hatching.

4.3 | Effect of anthropogenic fluctuations of discharge on larval drifting time and survival probability of *Sicyopterus lagocephalus*

The larval transport duration of *S. lagocephalus* was slightly reduced during anthropogenic peaks of discharge. Oppositely, larval survival probability dropped during these peaks. The influence of river discharge on larval drift dynamics of Sicydiinae gobies has been described at the seasonal scale for *Lentipes concolor* in Hawaii (Way et al., 1998), and for *S. lagocephalus* and *C. acutipinnis* in another watershed in Reunion Island (Lagarde et al., 2017). At the seasonal scales, these authors hypothesised that elevated flow velocity induced by increase of discharge during rainy events reduced the drifting duration of Sicydiinae larvae, and could be a strategy to reduce the risk of mortality in freshwater. The present study demonstrates that, on a daily scale, the mortality of larvae drifting during

the short periods of elevated discharge may not be reduced but increased. Indeed, larvae survival probability was lower during peaks of discharge compared to low flow conditions. Consequently, the artificial increases in flow velocity probably do not counterbalance the increase in larval mortality as the time spent by larvae drifting to the estuary is not drastically reduced. The gain in time is only few minutes when discharge artificially increases from 1.0 to 1.6 m³/s. Interestingly, the anthropogenic increases in discharge observed during this study were much less important than those associated with flash floods, which are frequent during the rainy season. The reduction of drifting time during natural flash floods is probably much greater than during artificial increases in flow velocity. For example, during a flood of low intensity recorded upstream to site A on 23 January 2016, the discharge increased of ca. 6 m³/s in less than 60 min. This value was much higher than the maximum increase of artificial discharge observed during this study (1.9 m³/s in 15 min). During natural flash floods, the reduction of drifting time, which may increase larvae survival by decreasing the time spent in freshwater, probably offsets the elevated probability of mortality. The positive effect of flash floods would be more important when the distance between spawning grounds and estuary increases.

4.4 | Influence of biological and environmental factors on larval drift dynamics of *Sicyopterus lagocephalus*

This study highlighted the direct relationship between increasing discharge and the quantity of drifting larvae of *S. lagocephalus* as well as their drifting time and survival probability in freshwater. These larval drift dynamics were related to hatching dynamics associated with peak of discharges in the downstream site (i.e. site C). Three factors can influence the dynamics of hatching: (i) the spawning strategy of adults, (ii) the behaviour of guarding males and/or (iii) the environmental factors (i.e. the river flow). Adults can influence the dynamics of hatching by spawning at a particular time. In unpredictable flow environment, some fish species can use the increase of discharge as a cue for spawning (Lytle & Poff, 2004). The guarding males of amphidromous gobies have also been suspected to actively provoke hatching. Indeed, Maeda and Tachihara (2010) suggested that the guarding males are poking and fanning the eggs more intensively to provoke hatching at a particular time of the day (i.e. at night). Similarly, males could also provoke hatching when sensing an increase of discharge. Finally, environmental factors such as the increase of shear stress on eggs clutches with river discharge (Lamouroux, Statzner, Fuchs, Kohmann, & Schmedtje, 1992; Statzner & Müller, 1989) can also promote hatching. Næsje et al. (1995) hypothesised that the increase of drifting larvae of two *Coregonus* species with increasing river discharge was due to the flow turbulence making the larvae hatch.

That the dynamics of hatching during this study was influenced by the spawning strategy of adults is very unlikely. Discharge peaks occurred unpredictably between 10:00 and 01:00 at site C, and no clear recurrent pattern was observed among sampling sessions. Moreover, Sicydiinae embryos develop in 3 days at temperatures ranging

between 19 and 21°C (Lindstrom, 1998). Adults are mostly likely unable to predict 3 days in advance when discharge peaks will occur.

Although a quantification of the influence of the guarding males' behaviour, or increase of shear stress on eggs clutches, was not performed during this study, it is likely that hatching was provoked by an increase of physical constraints. This increase of physical constraints on eggs clutches could force larvae that had not completed their embryonic development to hatch. The high proportion of incompletely developed larvae during the peaks of discharge may explain why their survival was lower.

This study demonstrated that larval abundance, larval transport duration and larval survivorship of Sicydiinae are strongly affected by daily anthropogenic fluctuations of river discharge. This strong relationship between environmental factors and larval drift dynamics of Sicydiinae in freshwater was largely expected for these small larvae released in highly turbulent flow conditions. However, more research remains needed to describe how larval drift dynamics varies with environmental factors regarding river morphology and flow conditions. As *S. lagocephalus* inhabits many tropical islands with varying river morphologies (Keith, Galewski, Cattaneo-Berberi, Hoareau, & Berberi, 2005), it will be interesting to study how the larval drift dynamics of this species adapts to different flow regimes.

4.5 | Implication for management and conservation

This study demonstrated that anthropogenic fluctuations of river discharge can have harmful effects on larval drift dynamics when the associated decrease of larval transport duration is low compared to the increase of larval mortality. The harmful effect of erratic diurnal flow pattern due to hydropower operations is well recognised for several aquatic organisms (Bunn & Arthington, 2002) and especially for fish larvae (Weyers, Jennings, & Freeman, 2003). However, most of the management and conservation recommendations concerning the larvae of amphidromous species have been focused on the reduction of direct mortality associated with entrainment of larvae into the water intakes (Benstead, March, Pringle, & Scatena, 1999; Jarvis & Closs, 2015). The much lower larval survivorship of *S. lagocephalus* observed during the peaks of discharge in Langevin River suggests that the impact of daily anthropogenic fluctuations of discharge should be further considered when building management and conservation plans for amphidromous species. The strong correlation between the abundances of drifting larvae and the amplitude of the discharge peaks suggests that reducing the amplitude of these peaks would efficiently mitigate their harmful effect as already suggested by Nagrodski, Raby, Hasler, Taylor, and Cooke (2012) and Bruder et al. (2016). Such operational measures and their monitoring would bring crucial data to address the knowledge gaps concerning the impacts of hydropower operations on amphidromous species.

ACKNOWLEDGEMENTS

This study was supported by the European Regional Development Fund, Région Réunion (Avenue René Cassin, BP 7190, 97719 Saint

Denis Cedex 9) and Office National de l'Eau et de Milieux Aquatiques (5 square Félix Nadar 94300 Vincennes). We are very thankful to the Anglers Federation of Réunion Island for providing a room to install our field laboratory, to Frédéric Cellier from Electricité De France for supplying the discharge data at site B and to all the persons who assisted us on the field especially Audrey Philippe, Guillaume Borie, Ananda Raichon, Clarisse Laroque and Dominique Hoarau. We acknowledge the Plateforme Gentyane of the Institut National de la Recherche Agronomique (INRA, Clermont-Ferrand, France) for genotyping support. We are also very grateful to Evgeny Romanov and Christophe LeMoine for their constructive comments and suggestions concerning this manuscript. We thank three anonymous referees for useful comments on previous versions of this paper.

ORCID

Raphaël Lagarde  <http://orcid.org/0000-0001-9809-1673>

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How to cite this article: Lagarde R, Teichert N, Faivre L, et al. Artificial daily fluctuations of river discharge affect the larval drift and survival of a tropical amphidromous goby. *Ecol Freshw Fish*. 2017;00:1–14. <https://doi.org/10.1111/eff.12381>

Annexe 4

Temporal variability of larval drift of tropical amphidromous gobies along a watershed in Réunion Island

Lagarde R, Teichert N, Grondin H, Magalon H, Pirog A, Ponton D. (2016). *Canadian Journal of Fisheries and Aquatic Sciences*.

L'annexe 4 est un article traitant de la dévalaison des larves de deux espèces de Gobiidés indigènes de La Réunion, *Sicyopterus lagocephalus* et *Cotylopus acutipinnis*.

Temporal variability of larval drift of tropical amphidromous gobies along a watershed in Réunion Island¹

Raphaël Lagarde, Nils Teichert, Henri Grondin, Hélène Magalon, Agathe Pirog, and Dominique Ponton

Abstract: Freshwater gobies of tropical islands are amphidromous: adults reproduce in rivers and larvae passively drift down to the sea immediately after hatching. Describing the phenology of this larval drift is essential to understanding the population dynamics of amphidromous gobies and to developing ecologically based recommendations for managing the watersheds. The larval drift patterns of two amphidromous gobies of Réunion Island, *Sicyopterus lagocephalus* and *Cotylopus acutipinnis*, were studied on a monthly basis for 1 year using plankton nets at two sites on the Mât River, located 20 km and 7 km from the river mouth. Genetic analyses showed that larval assemblages were dominated at 90% by *S. lagocephalus*, although the proportion of *C. acutipinnis* increased during early summer. Our findings highlighted a marked larval drift peak in full austral summer. The diel larval drift dynamic was described using periodic linear models. A diel pattern was only observed downstream, peaking a few hours after sunset. Finally, this study presents a new approach that improves standard methods for sampling and analyzing larval drift of amphidromous species.

Résumé : Les gobies dulçaquicoles des îles tropicales sont amphidromes : les adultes se reproduisent dans les rivières et, immédiatement après l'éclosion, les larves dérivent passivement jusqu'à la mer. La description de la phénologie de la dérive des larves en eau douce est indispensable à la compréhension de la dynamique des populations de ces gobies amphidromes et pour préconiser des mesures de gestion des bassins versants adaptées à leur écologie. Les patrons de dérive des larves de deux espèces de gobies amphidromes de l'île de La Réunion, *Sicyopterus lagocephalus* et *Cotylopus acutipinnis*, ont été décrits mensuellement pendant une année à l'aide de filets planctoniques au niveau de deux sites de la rivière du Mât, situés respectivement à 20 km et 7 km de l'embouchure. Les analyses génétiques ont montré que les assemblages larvaires étaient dominés à 90 % par *S. lagocephalus*. Cependant, la proportion de *C. acutipinnis* était plus importante en début d'été. Nos résultats ont mis en évidence que la dérive des larves avait lieu principalement pendant l'été austral. La dynamique journalière de dérive des larves a été décrite à l'aide de modèles périodiques linéaires. Un patron journalier n'a été observé qu'en aval avec un pic quelques heures après la tombée de la nuit. Enfin, cette étude présente une approche originale, basée sur l'amélioration des méthodes standards d'échantillonnage et d'analyse des données de dérive des larves des espèces amphidromes.

Introduction

Diadromy is a life history strategy involving migrations between freshwater and marine biomes (Myers 1949). Within diadromy, amphidromy is a subcategory in which the longest part of the individual's growing period, as well as its reproduction, occurs in freshwater habitats (Manacop 1953). Newly hatched larvae of amphidromous species immediately drift down to the sea where they grow for months (Radtke et al. 1988; Bell et al. 1995). At the end of their larval stage, they return to estuaries and settle in rivers to complete their life cycle as juveniles and adults (McDowall 2007). Amphidromy is widespread among fish species (McDowall 1988), especially in tropical islands where most indigenous fishes are amphidromous (Keith 2003). The highly dispersive capacities of amphidromous fishes have been demonstrated for several species (Chubb et al. 1998; Berrebi et al. 2005). The drift of their larvae

in rivers corresponds to the first part of this dispersal phase. Benefits of dispersal are numerous: avoiding the intergenerational competition, reducing the risks of inbreeding, and spreading larvae away from temporally unsuitable habitats (Burgess et al. 2015). The fact that rivers can become temporally unsuitable for fish species has been discussed particularly in the tropical context where cyclonic floods, droughts during the dry season, or even volcanic eruptions can occur and lead to temporary extirpation of fish populations (McDowall 2010). In the context of such unpredictable conditions, the dispersal stages of amphidromous species contribute to explaining the success of this life strategy in tropical islands (McDowall 2010). On the other hand, the risk of mortality during the larval stage is supposed to be huge. For example, Artzrouni et al. (2014) evaluated that the survival probability throughout the whole larval stage of *Sicyopterus lagocephalus* may

Received 1 March 2016. Accepted 12 May 2016.

R. Lagarde. Hydrô Réunion, Z.I. Les Sables, 97427 Etang Salé, La Réunion, France; UMR ENTROPIE (IRD, Université de La Réunion, CNRS), Laboratoire d'Excellence CORAIL, 15 Avenue René Cassin, CS 92003, F-97744 Saint Denis, Cedex 09, La Réunion, France; UMR ENTROPIE (IRD, Université de La Réunion, CNRS), Laboratoire d'Excellence CORAIL, c/o Hydrô Réunion Station Marine, Port ouest, Magasin 10, 97420 Le Port, La Réunion, France.
N. Teichert. Hydrô Réunion, Z.I. Les Sables, 97427 Etang Salé, La Réunion, France; Irstea, UR EABX, 50 Avenue de Verdun, F 33612 Cestas, Cedex, France.
H. Grondin. Hydrô Réunion, Z.I. Les Sables, 97427 Etang Salé, La Réunion, France.
H. Magalon and A. Pirog. UMR ENTROPIE (IRD, Université de La Réunion, CNRS), Laboratoire d'Excellence CORAIL, 15 Avenue René Cassin, CS 92003, F-97744 Saint Denis, Cedex 09, La Réunion, France.
D. Ponton. UMR ENTROPIE (IRD, Université de La Réunion, CNRS), Laboratoire d'Excellence CORAIL, c/o Hydrô Réunion Station Marine, Port ouest, Magasin 10, 97420 Le Port, La Réunion, France.

Corresponding author: Raphaël Lagarde (email: lagarde.arda@orange.fr).

¹This article is part of the special issue "Dispersal during early life history of fish", a product of the 39th Annual Larval Fish Conference, Vienna, Austria, 12–17 July 2015.

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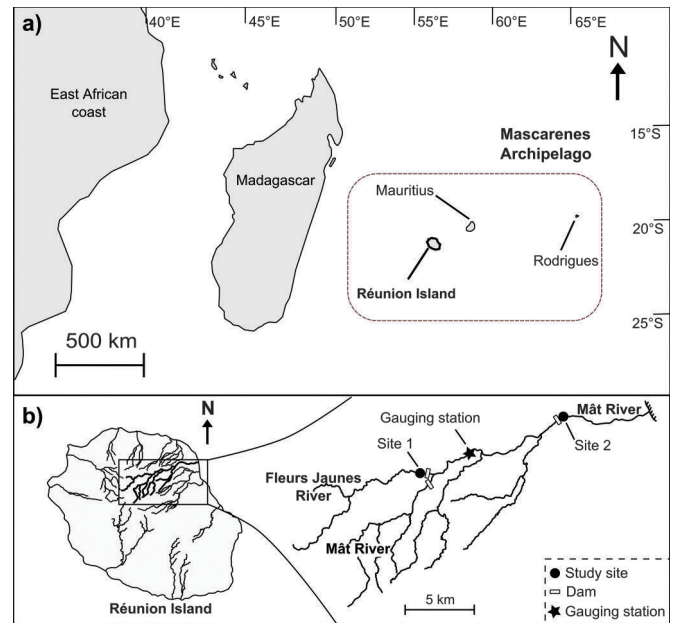
range from 10^{-4} to 10^{-5} . Even during the short period of freshwater larval drift, Bell (2009) estimated that mortality could be as high as 50% per hour for *Sicydium* species. In this context, it is likely that amphidromous species have developed different strategies for reducing the mortality of their larvae when drifting in freshwater streams and rivers (McDowall 2009).

Superimposed on these natural risks of mortality, drifting larvae of amphidromous fishes also face numerous anthropogenic threats. Alteration of the river continuity and the hydrological regimes due to dams can reduce the access to growing-spawning habitats and (or) their availability (Brasher 2003; Cooney and Kwak 2013). Larval survival can also be severely impaired by dams because an important part of the drifting larvae can be entrained within the diverted waters (Benstead et al. 1999). Moreover, alteration of downstream flow regime can increase the risk of river droughts and the duration of these droughts and can also result in the closure of the river mouth (Lill et al. 2013). The consequences of these flow alterations can lead to a total loss of the larvae drifting downstream (McDowall 1995). Despite the fact that harmful effects of dams on drifting larvae have been documented for years, this critical period of the amphidromous fish life cycle has not received much attention from managers (Benstead et al. 1999; Lagarde et al. 2015). This may be due to the inefficiency of existing methods (i.e., intake screens, behavioral barriers, etc.) to avoid migrating small-sized larvae being entrained within diverted waters (Larinier et al. 1994).

In Réunion Island, a 2500 km² volcanic island in the southwestern Indian Ocean, the freshwater fish assemblages are dominated by two amphidromous gobies, *Sicyopterus lagocephalus* and *Cotylopus acutipinnis*. These two species are culturally important for local populations because the individuals entering estuaries at the end of their marine larval life are traditionally fished (Aboussouan 1969; Bell 1999). Once in freshwater, postlarvae transform into juveniles and colonize the watersheds up to the headwaters thanks to their efficient climbing abilities (Blob et al. 2006; Schoenfuss and Blob 2007). The abundance of *S. lagocephalus* adults gradually decreases from estuaries to upper habitats located approximately 30 km upstream and at more than 600 m above sea level (a.s.l.), whereas the abundance of *C. acutipinnis* adults is equally distributed throughout the rivers (Teichert et al. 2014a). Reproduction of both species occurs during the warmer months in the middle and upper reaches, but *C. acutipinnis* is capable of spawning at lower temperatures than *S. lagocephalus* (Teichert et al. 2014b, 2016). Thus, reproduction in upstream reaches extends from December to May when the temperature is higher than 19 °C, whereas reproduction can occur during the whole year in downstream reaches (Teichert et al. 2014b). Both species present similar fecundities, ranging from 13 500 to 14 900 oocytes per gram (wet mass) of female and can spawn several times during the reproductive season, with an interspawning interval of approximately 1 month (Teichert et al. 2014b, 2016). Females lay egg clutches under pebbles and rocks in runs and riffles (Teichert et al. 2013a). After fertilizing the eggs, the male guards the nest until the larvae hatch (Teichert et al. 2013b). Immediately after hatching, larvae, which are at larval stage 1 according to Valade et al. (2009) and Ellien et al. (2016), passively drift down to the sea. Their only activity is a swim up – sink down behavior and they die if they do not reach the sea within few days after hatching (Valade et al. 2009).

The main objective of this study was to establish the spatiotemporal patterns in drifting larvae of *S. lagocephalus* and *C. acutipinnis*. As the number of drifting larvae should reflect both direct (i.e., on the larvae) and indirect (i.e., on the spawning population) disturbances, the ultimate aim was to evaluate the advantages of surveying drifting larvae for assessing the biotic integrity of tropical rivers.

Fig. 1. Location of (a) Réunion Island in the southwestern Indian Ocean and (b) studied sites in the Mât River watershed, Réunion Island.



Material and methods

Sampling sites

This study was conducted in two sites along the watershed of the Mât River on the east side of Réunion Island (Fig. 1). This torrential river flows over 36.3 km from its spring at 2850 m above sea level (a.s.l.). Important hydrological variations are recorded between the dry and the rainy seasons, with the rainy season spanning from November to April, during which time short and intensive cyclonic floods with peak discharge up to 300 times the mean annual discharge can occur (Robert and Davy 1988). One sampling site (site 1; Fig. 1b) was located at 385 m a.s.l. and 20.1 km from the river mouth. The river width at this site was approximately 14 m. A dam is present immediately downstream, but the diversion of water was not operating during the sampling period. The other site (site 2; Fig. 1b) was located downstream to the main tributary (Fleurs Jaunes River), at 120 m a.s.l. and 6.7 km from the river mouth. The river width at this site was approximately 19 m. A dam is located 150 m upstream of site 2. Water diversion due to this dam has not been operative for years; however, the dam disturbs the upstream migration of most diadromous fish and crustacean species and only *S. lagocephalus*, *C. acutipinnis*, freshwater eels (*Anguilla* spp.), and “chevaquines” (shrimps) (*Atyoida serrata*) can usually reach upstream habitats thanks to their climbing capacities. The riverine habitat was very similar in both sites, with no canopy cover and running water over a substratum composed mainly of small boulders and cobbles. Such substratum composition is suitable for *S. lagocephalus* and *C. acutipinnis* to build their spawning nests (Teichert et al. 2013a), and egg clutches have been observed under boulders and cobbles in both sites (R. Lagarde, personal observations). This observation is consistent with a previous study in which egg clutches were observed in different places on the Mât River from 7 to 20 km to the sea (Teichert et al. 2013a). It is thus likely that spawning grounds of Sicydiinae are present from site 2 to the upper limit of presence of those species, which is several kilometres upstream to site 1.

Sampling plan

In total, 14 sampling sessions were performed from May 2013 to June 2014 at site 1 and 13 sampling sessions were performed from

July 2013 to July 2014 at site 2. The frequency of the sampling sessions was approximately monthly, depending on meteorological conditions and staff availability. During the study, sunrise ranged from 0530 to 0655 and sunset ranged from 1745 to 1905. At the beginning of each sampling session, an estimate of the river discharge (Q_{river} , $\text{m}^3 \cdot \text{s}^{-1}$) was performed. The river cross section was divided into a minimum of 10 vertical subsections. In each subsection, the water velocity was measured using a current meter (Mini Current Meter type M1, SEBA Hydrometrie GmbH, Kaufbeuren, Germany, at one to seven locations, depending on the water depth. The discharge of each subsection was estimated by multiplying the subsection area by its mean water velocity. The river discharge corresponded to the sum of the discharges of all subsections. The number of subsections, the position of each measurement, and the method used to calculate the mean velocity for each subsection were based on the recommendation of the standard NF EN ISO 748:2007 (International Organization for Standardization (ISO) 2007).

A gauging station located 3.8 km downstream of the junction of the Mât and the Fleurs Jaunes rivers (Fig. 1b) recorded the instantaneous discharge every 6 min during the entire study except during a cyclonic flood event on 2 January 2014 and for 45 days following the event. This flood presented a peak discharge of approximately 30 times the mean annual discharge (Julien Bonnier, Office de l'eau Réunion, personal communication). Data of this gauging station are available online (www.eaureunion.fr, accessed in October 2015). Due to this flood event, data of the gauging station were only available from May to December 2013 and from February to July 2014. For all of the sampling sessions during which the gauging station recorded data, the instantaneous flow estimated every 6 min at this station remained within 4% (SD = 1%) of the mean diel flow recorded. The only exception was on 27 November 2013, when a small flooding event started at approximately 1830. Sampling was stopped for safety reasons, and only the first three two-net samples obtained from 0922 to 1546 were kept for analyses. As a consequence, for all of the sampling sessions except one, the river discharge estimated manually at each sampling site was considered to be constant over the whole duration of the sampling session.

Sampling procedure

At each site, one sampling session consisted of two conical nets set every 2–4 h over a minimal period of 24 h. The nets were similar to those developed by Bell (1994, 2007) for quantitative drift sampling in small streams. Their mouth diameter was 0.12 m, cod end diameter was 0.06 m, length was 1 m, and mesh size was 80 μm . One of the two nets was fixed in the center of the stream flow, while the other one was randomly positioned along the river cross section. The distances between the two nets ranged between 0.3 m and 6.3 m (mean \pm SD = 2.1 m \pm 1.3) in site 1 and between 0.2 m and 16.8 m (5.0 m \pm 4.1) in site 2. Each net was set separately, but the second net was always set within a period never exceeding 10 min after setting of the first net. Using two nets quasi simultaneously, with one of the nets having a random position in the river, allowed us to take into account the eventual heterogeneity in the spatial distribution of larval flow along the cross section of the river. To save time, if no larvae were observed in the first two two-net samples, only two to five two-net samples were performed.

Each net was set facing upstream at about 50% of the water depth, held in place by a removable fitting attached to an iron rod hammered into the substratum. Each net was immersed between 1 and 20 min (D_{net}), depending on the water velocity, to filter a volume of about 2 m^3 (mean \pm SD = 2.4 \pm 0.8). As the nets were not equipped with a current meter, two measures of water velocity (v_1 and v_2) were performed in front of the mouth of each net, immediately after being set and before being removed, with a current

meter (Mini Current Meter type M1, SEBA Hydrometrie GmbH). The sampled volume (V_{net}) was estimated according to eq. 1:

$$(1) \quad V_{\text{net}} = \frac{v_1 + v_2}{2} \times \pi \times r^2 \times D_{\text{net}}$$

where v_1 and v_2 are the instantaneous measures of water velocities ($\text{m} \cdot \text{s}^{-1}$) before and after the sampling, r is the radius of the net (i.e., 0.06 m), and D_{net} is the sampling duration (s).

After removing the net, the solid elements were immediately transferred from the cod end into a 2 L bottle filled with water from the river to keep the larvae alive. The larvae were counted alive as their movements facilitate their detection, sorting, and identification (Bell 2007). First, the sample was kept still for at least 2 h in the bottle to let vegetal and mineral debris to settle. Larvae remained in the water column thanks to their behavior of alternately actively swimming upward and passively sinking (Bell and Brown 1995; Valade et al. 2009). Larvae were then collected by carefully pouring the contents of the bottle on an 80 μm mesh sieve. The pouring was stopped before the overflow of vegetal and mineral debris. The water was returned to the bottle to repeat the decanting procedure twice. Larvae were transferred to a counting dish. All of the larvae in the counting dish were counted by removing each individual using a 15 μL micropipette (Bell 2007). The total number of larvae (N_{larvae}) was the sum of all of the larvae captured at the end of the three-decant process. For each sample, the larvae were placed in a labeled 1 mL Eppendorf tube and fixed with 95% alcohol solution. This live counting procedure was performed within a period never exceeding 15 h after sampling. This delay was assumed to not affect the number of living larvae as the first mortality of Sicydiinae larvae usually occurs after approximately 24 h spent in freshwater (Valade et al. 2009; Iida et al. 2010). This assumption was confirmed by visual observations of active larvae 15 h after sampling.

The instantaneous larval flow (q_{larvae} , individuals (ind) $\cdot \text{s}^{-1}$), corresponding to the number of larvae per second drifting through the river cross section, was calculated. The use of larval flow instead of larval density (i.e., number of larvae per filtrated volume) was preferred because values of larval flow are not affected by dilution effect when the discharge increases (Barthem et al. 2014). Comparison of larval drift intensities between sites and seasons can thus be performed even if discharge varies. The instantaneous larval flow was calculated for each net according to eq. 2:

$$(2) \quad q_{\text{larvae}} = \frac{N_{\text{larvae}}}{V_{\text{net}}} \times Q_{\text{river}}$$

where N_{larvae} is the number of larvae caught in the net, V_{net} is the sampled volume (m^3), and Q_{river} is the river discharge ($\text{m}^3 \cdot \text{s}^{-1}$).

Differences in larval flow between each net of the two-net samples were tested for the whole data set using a paired Student's t test. When no significant difference was detected, the mean value of both nets was used as an estimate of the instantaneous larval flow.

Genetic identification of species

No morphological criteria discriminate the larvae of *S. lagocephalus* and *C. acutipinnis* (Teichert 2012). The larvae of both species present the same size, shape, color, and a similar repartition of their chromatophores. Criteria commonly used for morphological identification of amphidromous fish larvae (Lindstrom 1999; Bell 2007; Maeda and Tachihara 2010; Jarvis and Closs 2015) are thus not useful for *S. lagocephalus* and *C. acutipinnis*. Identification at the species level can only be based on DNA analysis.

A subsample of 768 larvae was randomly selected among the dates covering the whole period during which the number of

larvae caught was greater than 30. As a result, the number of larvae selected per date ranged from 33 to 57. Total genomic DNA was extracted from each whole larva using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Genotyping was performed using the microsatellite locus SIC122, which expresses specific alleles for each species (at 166 base pairs (bp) for *C. acutipinnis* and from 194 to 242 bp for *S. lagocephalus*; Hoareau et al. 2009). The forward primer was indirectly fluorochrome labeled (6-FAM) as described in Schuelke (2000), and polymerase chain reactions (PCR) were performed in a total volume of 10 μL : 5 μL of 2 \times MasterMix (Applied Biosystems, Foster City, California, USA), 2.25 μL of demineralized water, 0.25 μL (1 $\mu\text{mol}\cdot\text{L}^{-1}$) of forward primer to which a M13 tail was added at the 5' end, 0.25 μL (10 $\mu\text{mol}\cdot\text{L}^{-1}$) of reverse primer, 0.25 μL (10 $\mu\text{mol}\cdot\text{L}^{-1}$) of a fluorophore-labeled universal M13 primer, and 2 μL of genomic DNA. The thermocycling program was as follow: 1 \times 5 min at 94 $^{\circ}\text{C}$; 7 \times [30 s at 94 $^{\circ}\text{C}$, 30 s at 62 $^{\circ}\text{C}$ (-1 $^{\circ}\text{C}$ each cycle), 30 s at 72 $^{\circ}\text{C}$]; 40 \times [30 s at 94 $^{\circ}\text{C}$, 30 s at 55 $^{\circ}\text{C}$, 30 s at 72 $^{\circ}\text{C}$]; 8 \times [30 s at 95 $^{\circ}\text{C}$, 30 s at 56 $^{\circ}\text{C}$, 30 s at 72 $^{\circ}\text{C}$]; and 1 \times 5 min at 72 $^{\circ}\text{C}$. Amplicons were run on an ABI 3730 XL sequencer (Applied Biosystems), and allele size was determined using Genemapper ver. 4.0 (Applied Biosystems).

Diel variations of larval flow

In each site, the diel variations of instantaneous larval flow were analyzed using periodic linear regressions as proposed by Bell (2007). Only the sampling sessions during which more than 100 larvae were caught and spanning over a minimum of 24 h were used to fit the regressions (four dates for site 1 and seven dates for site 2). With regard to the very low proportion of *C. acutipinnis* larvae captured during most of these dates (see Results), it appeared impossible to consider this species separately in the analyses. Indeed, the estimated number of *C. acutipinnis* larvae per sampling session was always less than 100 for all of the dates. Periodic linear regressions are frequently used to model predictable cyclical events and allow the use of time as a continuous variable rather than a series of arbitrary categories (Bliss 1958; Bell 2008). This is particularly appropriate for our study as the time interval between two successive two-net samples (from 2 to 4 h) and the number of two-net samples per sampling session (from nine to 12) were not constant between sampling sessions.

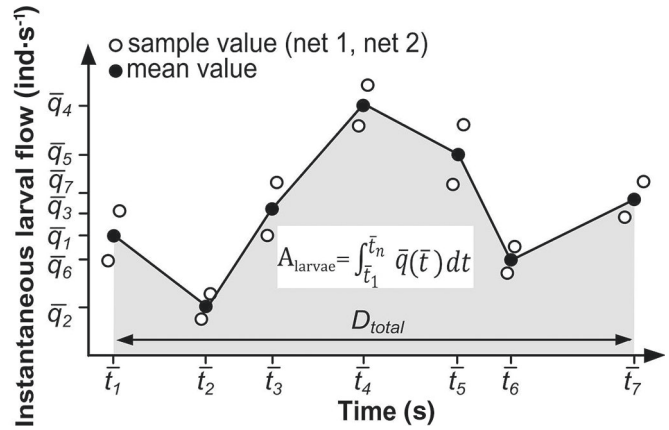
For each site, a mixed periodic model was adjusted with varying mesor and regression coefficients for each sampling session to integrate the variability of larval flow between dates (eq. 3):

$$(3) \quad Y = \beta_0 + \beta_1 \sin(R_{it}) + \beta_2 \cos(R_{it}) + r(1 + \sin(R_{it}) + \cos(R_{it}) | \text{Session})$$

where Y represents the instantaneous larval flow, R_{it} is the radian-transformed hour of the day (hour $\times 2\pi/24$), β_0 is the mesor (i.e., mean response of the biological trait over cycles), β_1 and β_2 are the regression coefficients, and r is the matrix of individual effects for the sampling sessions. Residuals of regressions were plotted as a function of a theoretical normal distribution (quantile–quantile plot) to verify the assumption of normality. The significance of parameters was calculated based on the F statistic using Satterthwaite's approximation method for the fixed part and on likelihood ratio tests for the random part (χ^2 tests). The peak position (P , hour of the day) was determined for each periodic linear model according to eq. 4:

$$(4) \quad P = 365 \times \frac{\arctan\left(\frac{\beta_1}{\beta_2}\right) + \text{QC}}{2\pi}$$

Fig. 2. Schematic representation of the method used for estimating the daily abundance of larvae (A_{larvae}) from instantaneous larval flow (q_{larvae}); \bar{t}_n and \bar{q}_n represent the mean time and mean instantaneous larval flow of the two-net samples, respectively, and D_{total} represents the total duration of sampling (i.e., time between the first and the last two-net samples of the sampling session).



where QC is a quadrant correction (QC = 0 if β_1 and β_2 are both positive, QC = π if β_1 is positive and β_2 is negative or if both are negative, and QC = 2π if β_1 is negative and β_2 positive). This peak was considered significant even if one part of the periodic model (i.e., β_1 or β_2) was not significant because cycles cannot be expressed in less than two dimensions (Bell 2007).

As the random part appeared significant for each site (see Results), periodic linear models were adjusted for each of the 11 sampling sessions (four for site 1 and seven for site 2) according to eq. 5:

$$(5) \quad Y = \beta_{n,0} + \beta_{n,1} \sin(R_{it}) + \beta_{n,2} \cos(R_{it})$$

where Y represents the instantaneous larval flow, R_{it} is the radian-transformed hour of the day (hour $\times 2\pi/24$), and $\beta_{n,0}$, $\beta_{n,1}$, and $\beta_{n,2}$ are the mesor and the regression coefficients for sampling session n , respectively. The significance of each of the parameters $\beta_{n,1}$ and $\beta_{n,2}$ was calculated based on the F statistic.

Diel abundance of larvae

The diel abundance of larvae corresponds to the number of larvae drifting through the river cross section during 24 h. Because the intervals between two-net samples within a sampling session were not constant and the larval flow varied with time during a sampling session, a simple arithmetic mean of larval instantaneous flow (q_{larvae}) did not appear adequate to estimate the diel abundance of larvae. Diel abundances of larvae (A_{larvae} , ind·day $^{-1}$) were thus obtained by integrating the instantaneous larval flow values obtained over 24 h (Fig. 2) according to eq. 6:

$$(6) \quad A_{\text{larvae}} = \frac{\int_{\bar{t}_1}^{\bar{t}_n} \bar{q}_{\text{larvae}}(\bar{t}) dt}{D_{\text{total}}} \times 24 \times 3600$$

where n is the number of two-net samples obtained during a sampling session, \bar{t} is the mean time of sampling (s) of the two-net samples ($(t_{\text{net1}} + t_{\text{net2}})/2$, with t_{net1} and t_{net2} corresponding to the median times of sampling for net 1 and net 2, respectively) ranging from $\bar{t}_1 = 0$ to $\bar{t}_n = D_{\text{total}}$, D_{total} corresponds to the total sampling duration (s) of the sampling session, and \bar{q}_{larvae} is the mean value of instantaneous larval flow (ind·s $^{-1}$) of the two-net sample ($(q_{\text{larvae_net1}} + q_{\text{larvae_net2}})/2$).

Table 1. Summary of the sampling effort and the number of successfully identified individuals of each species for site 1 and site 2.

Date (day/month/year)	Time of the first two-net sample (hours) ^a	No. of nets	Total filtrated volume (m ³)	Abundance of captured larvae	Mean river discharge (m ³ ·s ⁻¹)	No. of genetically identified	No. of <i>S. lagocephalus</i>	No. of <i>C. acutipinnis</i>
Site 1								
29/05/2013	0750	10	31.9	41	1.6	—	—	—
20/06/2013	1020	4	14.4	1	1.2	—	—	—
30/07/2013	1032	4	10.5	0	1.0	—	—	—
22/08/2013	1256	4	8.0	0	0.8	—	—	—
26/09/2013	1019	4	11.2	0	0.7	—	—	—
24/10/2013	0735	14	37.6	0	0.6	—	—	—
21/11/2013	0645	16	43.1	0	0.6	—	—	—
12/12/2013	0710	20	42.9	75	1.1	42	33	9
28/01/2014	0748	18	39.9	1842	3.6	50	48	2
20/02/2014	0745	18	14.6	436	3.1	47	43	4
27/03/2014	0708	20	49.7	371	2.0	47	43	4
23/04/2014	0735	18	43.5	420	1.9	56	54	2
19/05/2014	0700	18	50.1	36	1.7	33	33	0
18/06/2014	0737	14	43.1	0	1.1	—	—	—
Site 2								
01/07/2013	1204	26	83.2	65	4.8	—	—	—
22/07/2013	1153	16	45.6	2	3.9	—	—	—
19/08/2013	1023	16	44.7	0	3.6	—	—	—
16/09/2013	1058	18	55.1	1	3.2	—	—	—
28/10/2013	0907	24	58.8	139	2.7	53	46	7
27/11/2013	0922	6 ^b	17.1	77	5.9	51	29	22
16/12/2013	0725	20	49.3	486	3.5	54	54	0
04/02/2014	0808	17 ^c	16.4	596	11.6	51	47	4
23/02/2014	0732	18	17.6	736	8.7	50	49	1
20/03/2014	0723	20	42.3	736	6.6	54	53	1
05/05/2014	0723	18	43.0	598	6.0	54	46	8
02/06/2014	0703	22	71.4	280	6.7	54	42	12
02/07/2014	0713	18	55.5	80	4.1	50	48	2

^aTime is expressed in the 24-h system.

^bSampling stopped for safety reasons (see Material and methods).

^cTechnical issue with one net of the first two-net samples of the sampling session.

All statistical analyses were performed with the open-source R software (ver. 3.1.2; R Core Team 2013), implemented with the lme4 (Bates et al. 2014) and lmerTest (Kuznetsova et al. 2015) packages for the mixed-model analyses and the tis package (Hallman 2014) for diel abundance estimation.

Results

Sampling effort and relative proportion of species

Drifting larvae were caught between 29 May and 20 June 2013 and between 12 December 2013 and 19 May 2014 in site 1 (Table 1). In site 2, drifting larvae were caught during the whole sampling period except on 19 August 2013. The nets set in site 1 ($n = 182$) and site 2 ($n = 239$) allowed us to collect a total of 3222 and 3796 larvae, respectively. The number of larvae captured per sampling session ranged from 0 to 1842 in site 1 and from 0 to 736 in site 2.

The measured river discharge ranged from 0.6 to 3.6 m³·s⁻¹ in site 1 and from 2.7 to 11.6 m³·s⁻¹ in site 2 (Table 1). Maximum values of river discharges were observed during the first sampling sessions after the cyclonic flood and then decreased in the following months.

The genetic identification of larvae at the species level was successfully achieved for 746 individuals (between 33 and 56 larvae per sampling session) among the 768 larvae randomly selected. A total of 668 larvae (90%) presented the alleles of *S. lagocephalus*, whereas 78 (10%) presented the allele 166 of *C. acutipinnis* (Table 1). In site 1, the mean proportion of *S. lagocephalus*, weighted by the diel abundance of larvae, was 94%. The lowest proportion of *S. lagocephalus* (79%) was observed on 12 December 2013. In site 2, the mean proportion of *S. lagocephalus*, weighted by the diel abundance of larvae, was 92%. The lowest proportion of *S. lagocephalus* (57%) was observed on 27 November 2013, and proportions of 85%

and 78% were observed on 5 May 2014 and 2 June 2014, respectively.

Diel variations of larval flow

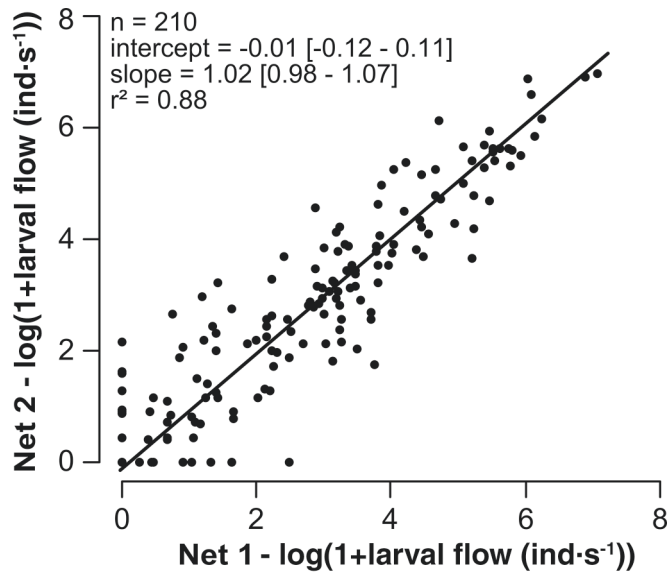
For the two sites considered together, instantaneous larval flows ranged from 0 to 2 275 ind·s⁻¹ and did not significantly differ between each net of the two-net samples (paired Student's *t* test, $n = 210$, $t_{208} = -1.40$, $P = 0.16$). The orthogonal relationship of the log-transformed larval flow estimated from the fixed net (net 1) and from the randomly positioned net (net 2) illustrated that larval flow was similar across the river's section (Fig. 3). As a consequence, the mean value (\bar{q}_{larvae}) of both nets was used for the following analyses.

The instantaneous larval flow (\bar{q}_{larvae}) varied through the day, with diel coefficients of variation varying from 17.2% to 141.4% for site 1 and from 41.5% to 300.0% for site 2. The diel pattern of larval flow generally showed a unique peak, sometimes two peaks (Fig. 4).

In site 1, the part of variance explained by fixed periodic parameters β_1 and β_2 was not significant, reflecting the absence of consensual diel periodic effect (Table 2; Fig. 4a). In contrast, the fixed periodic parameters estimated for site 2 explained a significant part of larval flow variability and predicted a consensual peak of larval flow at 2230 (Table 2; Fig. 4b). The random periodic parameters included in each model explained a significant part of variance (χ^2 test, $df = 3$, $P < 0.05$ in both sites), reflecting that the hour of the day had an effect on the larval flow for some sampling sessions.

The periodic linear models adjusted separately for each sampling session were consistent with the observed values of larval flow (Fig. 4). For site 1, the part of variance explained by the parameters $\beta_{n,1}$ and $\beta_{n,2}$, used to estimate the location of the peaks,

Fig. 3. Orthogonal regression of the estimated instantaneous larval flows ($\log + 1$) between the fixed net (net 1) and the randomly positioned net (net 2). Data from both sampling sites and all sampling sessions are pooled ($n = 210$). The values of the intercept and slope are given with 95% confidence intervals, and r^2 is the Pearson's correlation coefficient.



was only significant for two sampling sessions over the four. The two significant predicted peaks on 21 February and 28 March 2014 occurred during the afternoon (i.e., at 1230 and 1335; F test, $P < 0.05$; Fig. 4a). For site 2, predicted peaks occurred between 1955 and 0010, except on 16 December 2013 when the predicted peak occurred at 1230. The part of variance explained by the parameters $\beta_{n,1}$ and $\beta_{n,2}$, used to estimate the location of the peaks, was significant (F test, $p < 0.05$; Fig. 4b), except on 28 October 2013.

Diel abundance of larvae

The diel abundance of larvae ranged between 0 and 17×10^6 larvae·day⁻¹ for site 1 (Fig. 5a) and between 0 and 44×10^6 larvae·day⁻¹ for site 2 (Fig. 5b). In both sites, a marked maximum was observed in January–February. A good concordance was observed in both sites between the highest diel abundances of larvae and the highest values of river discharge.

Discussion

Temporal and spatial dynamics of larval flow

A weak but significant diel pattern of larval drift was observed for both species of Sicydiinae in the downstream site, showing a consensual peak of larvae a few hours after sunset. The same pattern has been reported in similar tropical contexts for several amphidromous gobies (Lindstrom 1998), among them Sicydiinae species (Bell 2007), or for crustaceans (March et al. 1998; Benstead et al. 1999). Nocturnal drift may correspond to a strategy for reducing predation (Lucas and Baras 2001). The poor swimming abilities of Sicydiinae larvae (Bell and Brown 1995) suggest that drifting a few hours after sunset is more probably due to a synchronism of hatching instead of an active behavior of the larvae. Two hypotheses could explain this synchronism in hatching time. First, it could be the result of a preferred spawning time of adults during the day. The short incubation duration of Sicydiinae eggs, approximately 3 days according to Lindstrom (1998), makes plausible the hypothesis that spawning events over a short period of time can lead to the larvae hatching at a given time. For example, if adults spawn mainly at sunset, larvae could hatch approximately at sunset 3 days later. Synchronicity between diel spawn-

ing behavior of adults and diel larval drift has been observed in other species such as *Osmerus mordax* (Bradbury et al. 2004). These authors correlated hatching of eggs with the decrease of luminosity at sunset. However, the egg clutches of Sicydiinae are buried under cobbles, so that luminosity in the nest is probably constantly low and makes this explanation less likely. The second hypothesis is that hatching could be provoked by the guarding male poking and fanning the eggs, as suggested by Maeda and Tachihara (2010).

Conversely, no clear diel pattern of larval drift was observed upstream. However, two significant peaks of larval drift, calculated for two different dates, were found to occur in the afternoon. The absence of clear diel larval drift pattern in upstream areas has already been observed for several amphidromous species, e.g., *Rhinogobius brunneus* by Iguchi and Mizuno (1990) or Palaemonidae and Atyidae shrimps by March et al. (1998). Two types of explanations were given for the lack of diel pattern of drifting larvae in upper reaches. First, Iguchi and Mizuno (1990) suggested that the higher complexity of hydrodynamics in upstream reaches could delay the drift of larvae due to their retention in eddies. Secondly, March et al. (1998) made the hypothesis that the absence of predators in upstream reaches could offset the advantage of nocturnal drift. The river sampled in Réunion Island presents a highly turbulent flow from head waters to the estuary; thus, it is unlikely that the different patterns of larval drift could be related to differences in hydraulic conditions between upstream and downstream sites. The hypothesis of March et al. (1998) seems more consistent regarding the structure of fish populations in the rivers of Réunion Island. Indeed, most indigenous predators such as *Eleotris* spp., *Kuhlia* spp., and *Agonostomus* sp. are present in downstream reaches only (Keith et al. 2006). *Atyoida serrata*, a common crustacean species of upstream reaches, is a detritivore species feeding mainly on organic matter and algae (Keith et al. 2006). So, the only predatory species commonly observed upstream are *Anguilla* spp., which are probably not predators of newly hatched larvae due to their very small size. Assuming that the mean flow velocity is $50 \text{ cm}\cdot\text{s}^{-1}$ between the two studied sites, which are approximately 13 km apart, the time that larvae would take for passively drifting from site 1 to site 2 would be approximately 7 h. Thus, larvae caught in higher abundances during the afternoon at the upstream site would reach the downstream reaches of the river, where most of their predators are present, at night. However, estimation of the contribution of larvae from site 1 to the observed dynamics in site 2 is much more complicated due to the continuous presence of spawning grounds between both sites.

A very strong seasonal peak of larval drift, matching the period of highest flow, was observed in both studied sites. The period during which drifting larvae were observed corresponded to the reproductive season that was expected based on ovary maturation of *S. lagocephalus* (Teichert et al. 2014b) and *C. acutipinnis* (Teichert et al. 2016). However, the larval drift peaks were more intense than what could be speculated from these previous studies. High flow conditions thus appeared to strongly influence the larval drift of Sicydiinae in Réunion Island rivers, as observed for amphidromous gobies in Hawaiian rivers (Lindstrom 1998; Way et al. 1998). This synchronism between high flow and high larval drift could be a strategy to reduce the drifting time and thus the risk of mortality at this stage (Bell 2009) due to starvation (Moriyama et al. 1998; Iguchi and Mizuno 1999), developmental constraints (Bell and Brown 1995), or predation. Predation is probably also lower during high flow due to the increased turbidity limiting the detection of larvae by predators (Araujo-Lima et al. 2001).

Relative proportion of species

The relative proportion of larvae observed was only partially in agreement with the spatial repartition of adults of *S. lagocephalus* and *C. acutipinnis* and what is known about their reproductive biology. However, the overall proportion of *S. lagocephalus* larvae caught during this study (90%) was consistent with the common

Fig. 4. Instantaneous larval flow (log + 1) and predictions of the periodic linear mixed models (solid black line) according to the hour of the day for (a) site 1 and (b) site 2. The vertical dashed line represents the location of the consensual daily peak predicted by the periodic mixed model. The grey dashed lines and associated symbols represent the larval flow (log + 1) and the periodic linear model adjusted separately for each sampling session from (a) 28 January 2014 to 24 April 2014 and (b) 28 October 2013 to 2 June 2014. The significance of each model is indicated as follows: ns, nonsignificant; *, $P < 0.05$; and **, $P < 0.01$. The black arrows represent the locations of the predicted individual daily peaks.

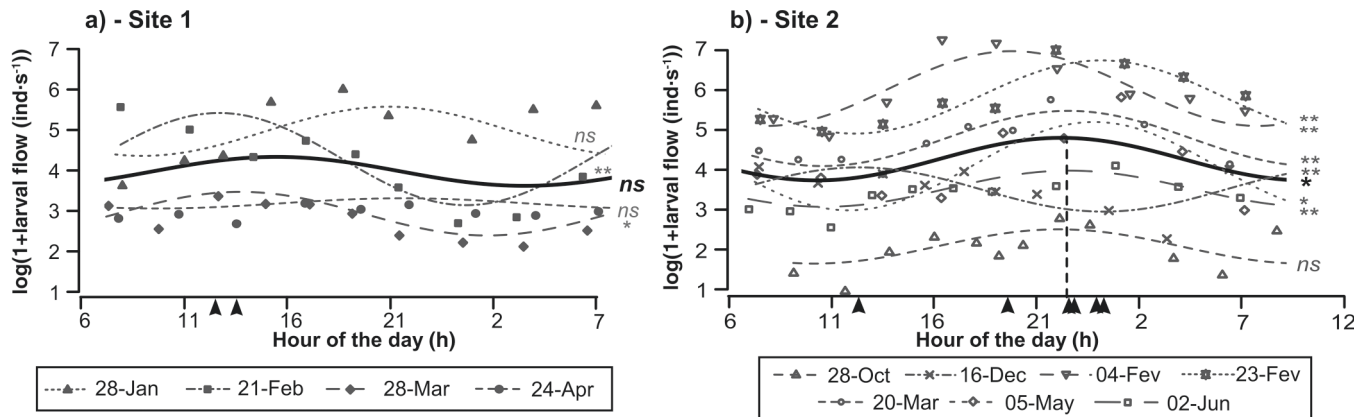


Table 2. Summary of the mixed periodic models for site 1 and site 2 with the sampling session as random effect.

Coefficient	Estimate	df	F	P
Site 1				
β_0	3.69	—	—	—
β_1	-0.23	5.97	3.66	0.47
β_2	-0.27	2.99	0.67	0.11
Site 2				
β_0	4.14	—	—	—
β_1	-0.21	5.47	3.94	0.09
β_2	0.49	5.91	6.14	0.05

Note: The significance of fixed effects is based on Satterthwaite's method.

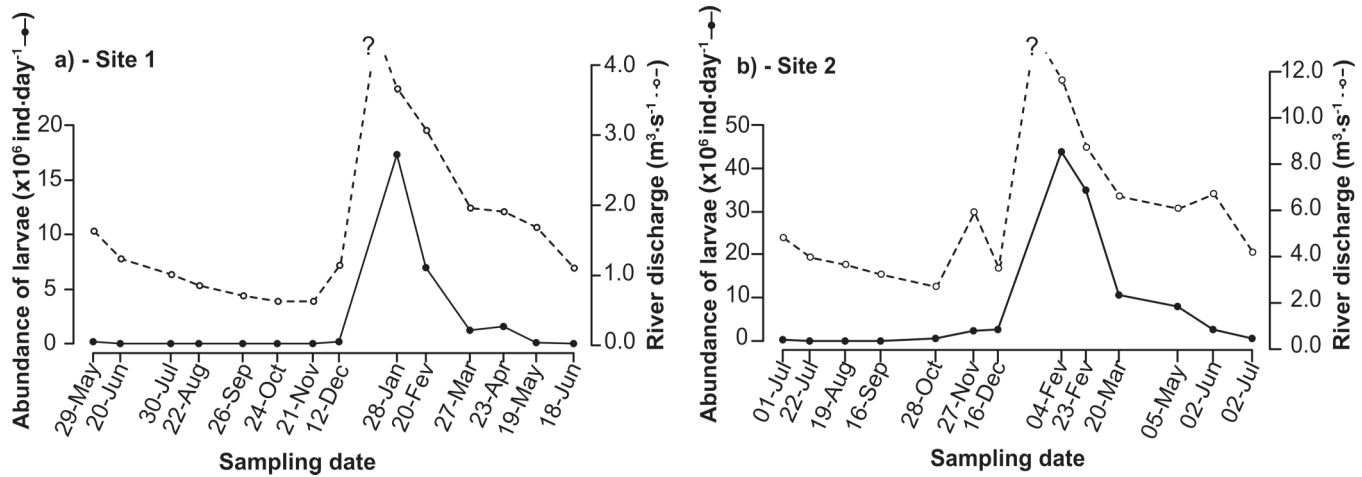
proportion of adults of this species in rivers of Réunion Island. Indeed, fish monitoring programs have demonstrated that this species generally represents more than 80% of the total abundance of Sicydiinae, whereas *C. acutipinnis* represents only 20% (Mérigoux et al. 2012). However, the proportion of *S. lagocephalus* adults is known to decrease from downstream to upstream reaches (Teichert et al. 2014a). Moreover, although the fecundity and interspawning intervals of both species are similar, *C. acutipinnis* is able to spawn at lower temperatures than *S. lagocephalus* (Teichert et al. 2014b, 2016). As a consequence, a lower proportion of *S. lagocephalus* larvae was expected upstream, as well as at the beginning and end of the reproductive season, when the mean diel water temperatures are below 19 °C. However, in this study, the proportions of *S. lagocephalus* larvae were approximately the same at both sites and were lower only at the beginning of the reproductive season (October–November). The only plausible explanation for this repartition of larvae would be a lower resilience of *C. acutipinnis* spawning population compared with *S. lagocephalus* after cyclonic flood events. A recent study in the same watershed highlighted important movements of adults of both species, probably due to the recolonization of upstream habitats after a cyclonic flood by washed-down individuals (Lagarde et al. 2015). As *C. acutipinnis* individuals are known to present slightly lesser climbing ability than *S. lagocephalus* (Voegtlé et al. 2002), they would probably take more time to return to their upstream spawning grounds after a cyclonic flood event. The lesser abilities of *C. acutipinnis* to recolonize upstream habitats could explain why the proportion of their larvae was lower than expected at the upstream site, especially after the cyclonic flood occurred.

Is it possible to use larval drift for assessing the biotic integrity of tropical rivers?

Fishes have been used as indicators of the biotic integrity of riverine habitats for years in both temperate (Karr 1981) and, more recently, tropical areas (Mérigoux et al. 2012; Kido 2013), as they integrate the impact of natural and anthropogenic disturbances on riverine communities. In temperate streams, those indicators are generally based on estimations of the abundances of adult and juvenile fishes, as the numbers of adults and juveniles largely depend on the biotic integrity of growing and spawning habitats present in the watersheds. In rivers of tropical islands, the abundances of amphidromous fishes are strongly influenced by the number of juveniles returning in the watershed and by the biotic integrity of the habitats in which they will grow (McDowall and Taylor 2000). Thus, monitoring adults and juveniles of tropical amphidromous fishes does not directly reflect the biotic integrity of spawning habitat within a watershed because of the marine dispersal phase at the end of which no homing exists (Berrebi et al. 2005). In this context, the abundance of drifting larvae could be a good proxy of the biotic integrity of both growing and spawning habitats because it depends on the biomass of adults and their achievement of reproduction within the watershed. Monitoring larval drift could also represent an interesting complementary approach to classical fish monitoring methods based on electrofishing or visual counts and to adult habitat surveys. Indeed, it integrates the biotic integrity not only of habitats located in the sampling site, but also of all habitats up to several kilometres upstream to the sampling site. Monitoring larval drift could also allow evaluation of species richness (Luton et al. 2005) of rivers, especially as progress in genetic techniques will soon allow efficient larval identification and quantification at the species level (Loh et al. 2014). However, evaluation of species richness would be easier and more efficient using environmental DNA methods (Valentini et al. 2016). Anyway, the challenge will be to obtain unbiased estimation of numbers of larvae for several species to use monitoring larval data as complementary indicators of the biotic integrity of riverine habitats.

Before using larval drift for assessing the biotic integrity of riverine habitats, numerous gaps in the knowledge about the biology and ecology of amphidromous species have to be filled. Firstly, knowing how far each species is present upstream to a monitoring site is mandatory. Indeed, the absence of drifting larvae of one species in the samples could simply come from the fact that adults are absent upstream. Secondly, the localization of the

Fig. 5. Seasonal fluctuation in total daily abundance of larvae (solid line and solid circles) and river discharge measured during the sampling session (dashed line and open circles) for (a) site 1 and (b) site 2. The discontinuity in the discharge curve (dashed line) gives the approximate date of the cyclonic flood.



growing and spawning habitats of each species (Teichert et al. 2013a, 2014a; Girard et al. 2014) appears essential. For example, Koster et al. (2013) observed that *Prototroctes maraena*, an amphidromous Osmeriformes from Australia, migrated downstream to their spawning grounds located 5 km up from the estuary, whereas adults were present up to 30 km upstream during the rest of year. In the case of this species, looking for drifting larvae in upstream reaches would be unsuccessful as growing and spawning habitats of adults were spatially separated. Finally, an estimate of the mortality of drifting larvae (Bell 2009) would be necessary to evaluate the spatial representativeness of the information obtained at each monitoring site. Indeed, Bell (2009) estimated that more than 99% of the drifting larvae that he caught had hatched in a 5 km section of river upstream from his sampling sites. In this situation, drift samples would be representative of the biotic integrity of riverine habitats located from the sampling site to less than 5 km upstream only. However, Bell's results varied over several orders of magnitude at a given sampling site and between sampling sites. It thus appears important to reevaluate how far upstream the larvae sampled at a given site are coming from in rivers presenting different biotic and abiotic conditions. Once this knowledge is obtained, determining relationships between larval drift and anthropogenic pressures would be possible and indicators of the biotic integrity of riverine habitats could be developed (Delpech et al. 2010; Kido 2013). In summary, before using larval drift for assessing the biotic integrity of riverine tropical habitats, future research programs should focus on the spatial distribution of adults of amphidromous fishes, localization of their growing and spawning habitats, and estimates of the mortality of their drifting larvae.

Acknowledgements

This study was supported by the European Regional Development Fund and the European Social Fund, Région Réunion (Avenue René Cassin, BP 7190, 97719 Saint Denis, Cedex 9), Electricité De France (22–30 Avenue de Wagram, 75382 Paris, Cedex 8), and Office National de l'Eau et de Milieux Aquatiques (5 square Félix Nadar 94300 Vincennes). We are very thankful to everyone who assisted us in the field, especially Germain Boussarie and Dominique Hoarau. We also thank the two anonymous reviewers for their detailed and constructive comments on a previous version of this manuscript. This is publication No. 134 of UMR ENTROPIE.

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Abstract

As apex predators, large sharks play key roles in the structuring and functioning of marine communities. Knowledge on the structure and dynamics of their populations remains limited, while they are particularly sensitive to anthropogenic pressures. Furthermore they may interact with human activities in coastal areas, sometimes leading to attacks, as it is the case in Reunion Island notably since 2011. Improving the knowledge on these species is thus essential to define efficient management plans.

This PhD thesis focuses on two large shark species belonging to the Carcharhinidae family, the bull shark *Carcharhinus leucas* and the tiger shark *Galeocerdo cuvier*. The aims are to study (1) the genetic structuring and connectivity of their populations, (2) the genetic diversity and effective population size of the delimited populations and (3) the reproductive modes of both species. The two first objectives were addressed thanks to the theoretical framework of population genetics (assignment tests, genetic differentiation indices, mitochondrial haplotype networks, approximate Bayesian computation) coupled with samples collected within two regions: the Western Indian Ocean and the Western Pacific. For the last objective, biological traits of sharks fished in Reunion Island were analysed to describe some of the reproductive parameters for each species.

A strong genetic differentiation was thus highlighted between bull shark populations from both regions, due to either an absence of contemporary gene flow or to an absence of female gene flow only. On the opposite, tiger shark populations seem genetically homogenous, with important genetic connectivity between both regions. Within each region, no genetic differentiation among localities was highlighted for both species. Furthermore a weaker genetic diversity was identified for the tiger shark, probably linked to the occurrence of a recent bottleneck occurring less than 3,000 years ago. Concerning the bull shark, effective population sizes estimated for each delimited population (6,000 to 10,000 individuals) were in the same range than those estimated for other shark species. Finally, around Reunion Island, bull and tiger shark populations present different dynamics, linked to their reproductive modes. Bull shark individuals from both sexes seem to exhibit some fidelity to specific coastal sites (philopatry) to mate and/or deliver embryos, and litters are frequently issued from several fathers (polyandry). On the opposite, mating and pupping areas of the tiger shark remain poorly known, and this species seems exclusively monoandrous, probably linked to its semi-oceanic nature, lowering encounter probabilities of the mates.

This work thus highlights the high dispersal abilities of both species. Besides their populations present different dynamics, leading to different sensitivities to anthropogenic pressures. These results point out the need to adopt management plans specific to each species.

Keywords: population structure, effective population size, bull shark, tiger shark, *Carcharhinus leucas*, *Galeocerdo cuvier*, reproductive cycles, polyandry, philopatry

Résumé

Les grands requins jouent, en tant que prédateurs apicaux, un rôle important dans la structuration et le fonctionnement des communautés marines. La compréhension de la structure et de la dynamique de leurs populations reste limitée alors qu'ils sont particulièrement vulnérables aux pressions anthropiques. Ils peuvent par ailleurs interagir avec les activités humaines en milieu littoral, conduisant parfois à des attaques, ce qui est notamment le cas à La Réunion, particulièrement depuis 2011. Il est donc primordial d'améliorer nos connaissances sur ces espèces afin de définir des plans de gestion durable.

Cette thèse porte ainsi sur deux espèces de grands requins de la famille des Carcharhinidés, le requin bouledogue *Carcharhinus leucas* et le requin tigre *Galeocerdo cuvier*. Ainsi, les objectifs de cette thèse sont d'étudier (1) la structure et la connectivité génétique de leurs populations, (2) la diversité génétique et la taille efficace des populations ainsi identifiées et (3) les modes de reproduction de ces deux espèces. Les deux premiers objectifs ont été abordés grâce au cadre théorique de la génétique des populations (tests d'assignement, indices de différenciation génétique, réseaux d'haplotypes mitochondriaux, calcul bayésien approché) couplé à un échantillonnage dans deux régions : l'Ouest de l'océan Indien et l'Ouest du Pacifique. Le dernier objectif a consisté en l'analyse des traits biologiques de requins pêchés à La Réunion, afin de décrire certains paramètres de la reproduction pour chaque espèce.

Une différenciation génétique importante a ainsi été identifiée entre les populations de requin bouledogue des deux régions étudiées, reflétant soit une absence totale de flux de gènes contemporains, soit des flux de gènes uniquement assurés par les mâles. À l'inverse, les populations de requin tigre de ces deux régions sont homogènes génétiquement, avec des flux de gènes importants. Par ailleurs, au sein de chacune de ces régions, aucune différenciation génétique n'a été identifiée pour ces deux espèces. En outre, une plus faible diversité génétique a été identifiée chez le requin tigre que chez le requin bouledogue, qui pourrait être liée à une diminution forte des effectifs datant de moins de 3 000 ans. Pour le requin bouledogue, la taille efficace estimée des populations délimitées (6 000 à 10 000 individus) est similaire à celles trouvées pour d'autres espèces de requin. Autour de La Réunion, les populations des requins bouledogue et tigre suivent des dynamiques bien différentes, liées à leurs modes de reproduction. Chez le requin bouledogue, les individus des deux sexes semblent fidèles à des zones côtières particulières (philopatrie) pour s'accoupler et/ou mettre bas, et les portées sont fréquemment issues de plusieurs pères (polyandrie). À l'inverse, les zones d'accouplement et de mise bas du requin tigre restent mal connues, et cette espèce semble exclusivement monoandre, caractéristiques liées à sa nature semi-océanique.

Les travaux de cette thèse montrent les capacités de dispersion importantes de ces deux espèces. En outre, leurs populations présentent des dynamiques différentes qui induisent une vulnérabilité différente aux pressions anthropiques. Ces résultats pointent la nécessité d'adopter des mesures de gestion spécifiques à chaque espèce.

Mots clés : structure des populations, taille efficace, requin bouledogue, requin tigre, *Carcharhinus leucas*, *Galeocerdo cuvier*, cycles de reproduction, polyandrie, philopatrie

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