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Les communautés microbiennes au sein des populations de tiques : origine, diversité et structure

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Écologie de la santé

École doctorale GAIA

Unité de recherche MIVEGEC

Les communautés microbiennes au sein des populations de tiques : origine, diversité et structure

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Le 26 novembre 2019

Sous la direction de Olivier DURON

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UNIVERSITÉ
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Résumé : Le microbiome désigne les communautés écologiques de micro-organismes hébergés par un hôte. De nos jours, les microbiomes sont perçus comme de véritables génomes accessoires déterminant le phénotype étendu de leurs hôtes. Les microbiomes, à l'image de leurs hôtes, sont extrêmement divers et structurés par tout un panel de facteurs biotiques, abiotiques et stochastiques. Ces facteurs varient en fonction de l'échelle résolutive concernée : la structure des microbiomes ne sera pas impactée par les mêmes facteurs au niveau de l'individu hôte ou de la métacommunauté d'hôtes. Dans ce contexte fondamental de l'écologie des communautés, ma thèse a notamment essayé de déterminer **quels sont les facteurs structurants d'un microbiome à différentes échelles résolutives**. Pour répondre à cette question, nous avons étudié les cortèges microbiens abrités par les tiques de Guyane. Ce modèle est particulièrement pertinent pour deux raisons : 1/ Il est admis que les tiques hébergent des communautés microbiennes complexes potentiellement assujetties à des facteurs structurants complexes. 2/ Il n'existe aucune donnée récente sur la diversité, la biologie ou le risque infectieux associés aux tiques de Guyane. Par conséquent, ces travaux de thèses ont, premièrement permis, de décrire des espèces de tiques jamais observées en Guyane et de caractériser des souches inconnues de bactéries potentiellement pathogènes, dont une nouvelle borrelie proche de celles responsables de la maladie de Lyme et de fièvres récurrentes. Deuxièmement, nous avons démontré qu'il existait de nombreux biais dans les études de microbiomes des tiques et que cela impactait l'interprétation des données de structuration microbienne. Cela se traduit notamment par l'observation artéfactuelle d'une forte diversité microbienne liée essentiellement au microbiome cuticulaire. En réalité, les microbiomes des tiques apparaissent majoritairement structurés par l'identité, la présence/absence et les interactions entre des bactéries intracellulaires mutualistes des tiques, que ce soit à une échelle intra-spécifique ou inter-spécifique. Enfin, nous avons mis en évidence que l'association entre les tiques et leur microbiome était également soumise à des patrons coévolutifs typiques des symbiotes intracellulaires d'arthropodes, ainsi qu'à des facteurs phylogéographique et bioécologique dépendant de l'hôte. Toutefois, il apparaît que la structuration des microbiomes chez les tiques est encore loin d'être totalement comprise, et que de futures études seront encore nécessaires pour démêler les différentes controverses sur la réalité biologique des microbiomes hébergés par les tiques.

Mots-clés : microbiome, tiques, Guyane, structuration microbienne, coévolution, endosymbiose, agent pathogène transmis par les tiques

Abstract: Microbiome refers to the ecological communities of microorganisms hosted by a host. Microbiomes are now perceived as true accessory genomes that determine the extended phenotype of their hosts. They are, like their hosts, extremely diverse and structured by a wide range of biotic, abiotic and stochastic drivers. These factors vary according to the resolution scale: the structure of microbiomes will not be impacted by the same factors at an individual scale or an interspecific scale. In this fundamental context of community ecology, my thesis tried to determine **what the microbiome drivers at different resolution scales are**. To answer this question, we studied the microbial community sheltered by ticks in French Guiana. This model is particularly relevant for two reasons: 1/ It is recognized that ticks harbour complex microbial communities that appear to be subject to a diversity of potential drivers. 2/ There are no recent data on the diversity, biology or infectious risks associated with ticks in French Guiana. As a result, in this thesis we described tick species which were never observed in French Guiana and characterized unknown strains of potentially pathogenic bacteria, including a new borrelia related to Lyme disease and to relapsing fever ones. Then, we demonstrated the ubiquity of biases in tick microbiome studies and their impact on the interpretation of microbiome data by confusing cuticular and internal microbes. Tick microbiomes actually appear mainly structured by the identity, the presence/absence and the interactions between mutualistic intracellular bacteria of ticks, whether on an intra- or interspecific scale. Finally, we have shown that the association between ticks and their microbiome is also subject to co-evolutionary patterns, typical of intracellular symbionts of arthropods, as well as phylogeographic and bio-ecological factors depending of the tick hosts. However, the microbiome structure in ticks, is still far from being fully understood and future studies are necessary to resolve the various controversies on the biological reality of microbiomes hosted by ticks.

Key words: microbiome, ticks, French Guiana, microbial structuring, coevolution, endosymbiosis, tick-borne pathogen

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Contexte général et objectifs principaux de la thèse

Les premières observations de micro-organismes remontent au XVII^{ème} siècle et sont l'œuvre d'Antoni van Leeuwenhoek (1632-1723). Il est aussi le premier à décrire ce que, quatre siècles plus tard, Joshua Lederberg appellera microbiome (Dobell, 1932 ; Hooper and Gordon, 2001 ; Lederberg, 2001). Ce terme désigne une communauté écologique de micro-organismes occupant un même environnement. L'exemple le plus emblématique est sans doute représenté par les micro-organismes commensaux, mutualistes et pathogènes qui occupent différentes niches du corps humain (tube digestif, épiderme, muqueuses, etc). Le microbiome humain se compose d'une grande diversité d'organismes : bactéries, Archaea, virus, protozoaires, nématodes ou encore champignons qui se partagent les écosystèmes délimités par l'hôte. Au gré des quinze dernières années, grâce aux avancées en génomique et en microbiologie, notre compréhension du microbiome a grandement évolué. Chez l'humain, le microbiome est considéré aujourd'hui comme un ensemble de génomes accessoires qui détermine le phénotype étendu de leurs hôtes. Par exemple, le microbiome intestinal, ou tout du moins ses membres bactériens (bactériome), assurent de nombreuses fonctions biochimiques importantes pour leur hôte (*e.g.* dégradation de composés complexes ou encore synthèse de vitamines) ; de même, de multiples et diverses pathologies humaines seraient associées avec des troubles du bactériome intestinal (Hanning and Diaz-Sanchez, 2015 ; Kinross *et al.*, 2011). En plus de souligner le caractère déterminant de ces génomes accessoires, ces exemples suggèrent l'existence d'interactions complexes microbiomehôte résultant de processus coévolutifs (Ley *et al.*, 2008a, 2008b). Le microbiome et son hôte seraient tellement intrinsèquement liés que ces derniers peuvent désormais être considérés comme un seul supra-organisme appelé holobionte (Gilbert *et*

al., 2012 ; Gordon *et al.*, 2013 ; Zilber-Rosenberg and Rosenberg, 2008). Les microbiomes ne sont bien sûr pas l'apanage des humains : ils se retrouvent effectivement chez une multitude d'organismes (si ce n'est la totalité) au sein de trois règnes du vivant que sont les plantes (Turner *et al.*, 2013), les animaux (Hanning and Diaz-Sanchez, 2015) et les protistes (*e.g.* Delafont *et al.*, 2013), suggérant donc un rôle déterminant dans l'évolution et la diversification du vivant (Woese, 2002).

Au même titre que les communautés microbiennes libres, la structuration des microbiomes intra-hôte est assujettie à un déterminisme complexe. En fonction de l'échelle de résolution considérée, les facteurs structurants des microbiomes semblent différer en partie. Par exemple, à l'échelle interindividuelle, comme chez l'humain, il existerait une corrélation entre la présence de certains taxons microbiens et des facteurs comme l'âge, la génétique, l'environnement local ou l'alimentation (The Human Microbiome Project Consortium *et al.*, 2012). Alors qu'à une échelle plus élevée, comme la classe des mammifères, le régime alimentaire et la physiologie de l'intestin en corrélation avec la phylogénie de l'hôte structureraient les variations majeures de composition du bactériome intestinal (Groussin *et al.*, 2017 ; Ley *et al.*, 2008a ; Muegge *et al.*, 2011). La corrélation entre la phylogénie de l'hôte et la structure de son microbiome est par ailleurs appelée « phylosymbiose » (Brooks *et al.*, 2016 ; Mazel *et al.*, 2018). De manière intéressante, ce patron de phylosymbiose semble généralisable à de nombreuses classes ou embranchements d'animaux, comme les insectes ou les éponges (Brooks *et al.*, 2016 ; Easson and Thacker, 2014). Cependant, les principaux facteurs structurants des microbiomes au sein de leurs hôtes restent encore peu compris tout en étant un concept fondamental de l'écologie des communautés. **C'est dans ce contexte que ma thèse s'est déroulée, durant laquelle nous avons essayé de répondre à la question suivante : quels sont les facteurs structurants d'un microbiome au niveau de différentes**

échelles taxonomiques ou individuelles de l'hôte ? Pour répondre à cette question, nous avons étudié les cortèges microbiens abrités par les tiques en Guyane. A ce titre, les expériences relatives à ce projet de thèse (2016-2019) ont été financées par le Labex CEBA (Centre d'Etude de la Biodiversité Amazonienne).

Les tiques sont des arthropodes hématophages stricts surtout connues pour leur rôle dans la transmission de maladies : elles sont effectivement le second vecteur d'agents pathogènes (après les moustiques) le plus important chez l'humain et chez les animaux (de la Fuente *et al.*, 2008). Alors que les tiques du continent Européen sont relativement bien connues et étudiées dans ce contexte, les tiques de Guyane ne font l'objet de pratiquement aucune étude depuis une cinquantaine d'années. Cependant, d'après le dernier inventaire acarologique publié en 1958 (Floch and Fauran, 1958), 30 espèces de tiques dont 22 du genre *Amblyomma* seraient présentes en Guyane : un patron de diversité relativement similaire à celui de la France métropolitaine qui compte 41 espèces de tiques dont 18 du genre *Ixodes* (Guiguen and Degeilh, 2001). Cette absence totale de données récentes sur la diversité, la répartition et la biologie-écologie des tiques entraînent une très grande méconnaissance du risque acarologique en Guyane. Il existe donc un réel besoin d'étudier les communautés de parasites potentiellement hébergées par les tiques de Guyane. De plus, comme nous allons le voir en introduction, les tiques n'hébergent pas seulement des agents pathogènes mais également un microbiome extrêmement divers qui semble assujéti à un très grand nombre de facteurs structurants, allant par exemple de la phylogénie de l'hôte à la présence d'agents pathogènes (Abraham *et al.*, 2017 ; Díaz-Sánchez *et al.*, 2019 ; Narasimhan and Fikrig, 2015).

Ainsi, le nombre de facteurs structurants potentiels des microbiomes de tiques et l'ignorance du risque acarologique et des tiques présentes en Guyane, font de ces dernières un modèle particulièrement pertinent pour le déroulement de cette thèse. Plus

précisément nous avons appréhendé, par des outils bio-informatiques, phylogéniques et d'analyses de données, comment ces communautés microbiennes sont structurées par les différentes échelles taxonomiques ou individuelles de l'hôte. Pour étudier ces processus, trois écueils techniques ont été surmontés : 1) inventorier les tiques de Guyane en couplant des analyses morphologiques et de biologie moléculaire 2) caractériser le microbiome de ces tiques par séquençage haut débit (NGS), barcoding moléculaire et typage multi-locus (MLST) 3) identifier quels facteurs structurent ces communautés microbiennes. **Suivant ces contraintes scientifiques et techniques, cette thèse se divise en six parties dont quatre chapitres.** La première partie est une introduction générale présentant les différents concepts abordés et aspects du modèle. Le chapitre 1 correspond à l'étude de la diversité des tiques de Guyane. Puis, dans le chapitre 2 nous présentons les biais méthodologiques existants pour la caractérisation des microbiomes de tiques tout en discutant la diversité bactérienne retrouvée chez les tiques de Guyane. Nous nous servons des résultats du chapitre 2 pour délimiter certains facteurs structurants des communautés microbiennes dans le chapitre 3. Afin d'investiguer de possibles effets structurants de la phylogéographie de l'hôte ou du spectre d'hôte sur le microbiome des tiques, nous nous sommes principalement concentrés sur un seul genre de tique : *Amblyomma*. Le chapitre 4 est la partie s'intéressant à la diversité des potentiels agents pathogènes retrouvés au sein des tiques de Guyane avec en point d'orgue, la caractérisation moléculaire et phylogénique de souches et d'espèces inconnues de bactéries potentiellement pathogènes pour les vertébrés. Enfin, dans la dernière partie de ce manuscrit, nous mettons en perspective les résultats obtenus au cours de cette thèse et proposons des pistes de recherche pour l'avenir.

Liste des articles écrits durant cette thèse :

En préparation / phase de soumission / review :

- . **Binetruy F**, Buysse M, Barosi R & Duron O. Microbial community structure reveals instability of nutritional symbiosis during evolutionary radiation of Amblyomma ticks. En préparation (À partir de la page 115 de ce manuscrit, chapitre III).
- . **Binetruy F**, Buysse M, Barosi R & Duron O. Novel *Rickettsia* genotypes, species and group in ticks of French Guiana, South America. Soumis à *Scientific Reports*. (À partir de la page 174, chapitre IV).
- . **Binetruy F**, Garnier S, Boulanger N, Loire E, Buysse M, Noël V, Barosi R & Duron O. A novel *Borrelia* species, divergent to Lyme disease and relapsing fevers groups, in passerine-transported ticks of French Guiana, South America. En préparation. (À partir de la page 216 de ce manuscrit, chapitre IV).

Acceptés :

- . **Binetruy F**, Chevillon C, de Thoisy B, Garnier S & Olivier Duron. Survey of ticks in French Guiana. *Ticks and Tick-borne Diseases*, 2019. (À partir de la page 79 de ce manuscrit, chapitre I).
- . **Binetruy F**, Dupraz M, Buysse M & Duron O. Surface sterilization methods impact measures of internal microbial diversity in ticks. *Parasites & Vectors*, 2019. (À partir de la page 97 de ce manuscrit, chapitre II).
- . **Binetruy F**, Bailly X, Chevillon C, Martin OY, Bernasconi MV & Duron O. Phylogenetics of the *Spiroplasma ixodetis* endosymbiont reveals past transfers between ticks and other arthropods. *Ticks and Tick-borne Diseases*, 2019. (Annexe A de ce manuscrit).
- . Bonnet S, **Binetruy F**, Hernandez A, Duron O. The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. *Frontiers in Cellular and Infection Microbiology*, 2017. (À partir de la page 41 de ce manuscrit, introduction).

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Introduction générale

1. Microbiome et Symbiose

1.1 Vue d'ensemble

En partant du postulat que le microbiome et son hôte ne forment qu'un seul supra-organisme ou holobionte régi par un réseau complexe d'interactions (Figure 1) (Gilbert *et al.*, 2012 ; Gordon *et al.*, 2013 ; Theis *et al.*, 2016 ; Zilber-Rosenberg and Rosenberg, 2008), la notion d'individu à proprement parler devient obsolète. À la place, il convient de nous décrire comme des organismes symbiotiques, au même titre que les célèbres lichens composés d'une association tripartite : champignon-algue-microbiome (*e.g.* Cernava *et al.*, 2017). En effet, comme l'a judicieusement proposé Lynn Margulis en 1991, toute association physique entre individus d'espèces différentes (appelés « bionts »), impactant d'une quelconque manière leur cycle biologique, forme une symbiose et donne naissance à un holobionte (Figure 1) (Margulis and Fester, 1991). Le microbiome est ainsi un membre à part entière de la symbiose l'associant avec son hôte. Dans sa définition la plus pure, la symbiose signifie « des organismes dissemblables qui vivent ensemble durablement » (Perru, 2006), les plus grands étant définis comme hôtes et les plus petits comme symbiontes. Les exemples les plus emblématiques de symbioses sont ce que l'on appelle des « endosymbioses », où un hôte va héberger en son sein un endosymbionte. Ce type d'organisme a profondément influencé l'évolution et l'écologie de nombreuses espèces (Margulis, 1993 ; Wernegreen, 2012).

Les endosymbiontes vivent typiquement à l'intérieur de leurs hôtes, parfois exclusivement dans leurs cellules, et ont évolué en / par des interactions complexes avec ces derniers. Les mitochondries et les chloroplastes, tous deux issus de bactéries intracellulaires, sont les exemples les plus connus d'endosymbiose. Dans le cas des mitochondries, l'internalisation d'une alphaprotéobactérie par une archéobactérie il y a

plus de 2 milliards d'années a conduit à une complexification de nombreuses fonctions cellulaires et à l'apparition des eucaryotes (Gray *et al.*, 1999 ; Margulis, 1970). Dans le cas des chloroplastes, une endosymbiose secondaire avec une cyanobactérie photosynthétique il y a 1,5 milliard d'années a permis l'acquisition de l'autotrophie par la cellule eucaryote et, *de facto*, l'apparition des plantes (Keeling, 2010). De nombreux autres cas d'endosymbiose sont aujourd'hui connus et, quoique plus récents que les mitochondries et les chloroplastes, ils concernent une multitude d'organismes. Par exemple, tout comme les plantes, l'acquisition d'un endosymbionte phototrophe a permis l'utilisation de la lumière comme source d'énergie exclusive par une amibe originellement hétérotrophe, *Paulinella chromatophora* (Nakayama and Archibald, 2012 ; Yoon *et al.*, 2006). De manière similaire, l'utilisation d'endosymbiontes chimiotrophes permet la conversion de molécules inorganiques en énergie, permettant à de nombreux invertébrés la possibilité de coloniser des milieux extrêmes tels que les sources hydrothermales (Dubilier *et al.*, 2008 ; Wernegreen, 2012). Ainsi, en associant durablement des lignées évolutives issues de différents domaines du vivant, l'endosymbiose crée une combinaison inédite de fonctions biochimiques qui permet l'exploitation d'environnements *a priori* hostiles (Gray *et al.*, 1999 ; Keeling, 2010). Dès lors, ces exemples illustrent combien l'endosymbiose, et *a fortiori* les microbiomes, ont fortement structuré l'évolution du vivant et sa diversification écologique (Wernegreen, 2012).

Dans cette thèse, nous allons nous concentrer sur l'endosymbiose et donc nous intéresser essentiellement aux bactériomes internes. Néanmoins, il faut garder à l'esprit que la symbiose entre son hôte et son microbiome externe, appelée ectosymbiose, est tout aussi impactante que l'endosymbiose. Par exemple, ce type de symbiose est impliqué dans la protection contre des agents pathogènes (Currie *et al.*, 1999 ; Schommer and Gallo, 2013), la reconnaissance chez les insectes sociaux (Davis *et al.*, 2013 ; Dosmann *et al.*,

2016) ou de manière plus originale, dans la magnétoréception chez des protistes marins (Monteil *et al.*, 2019).

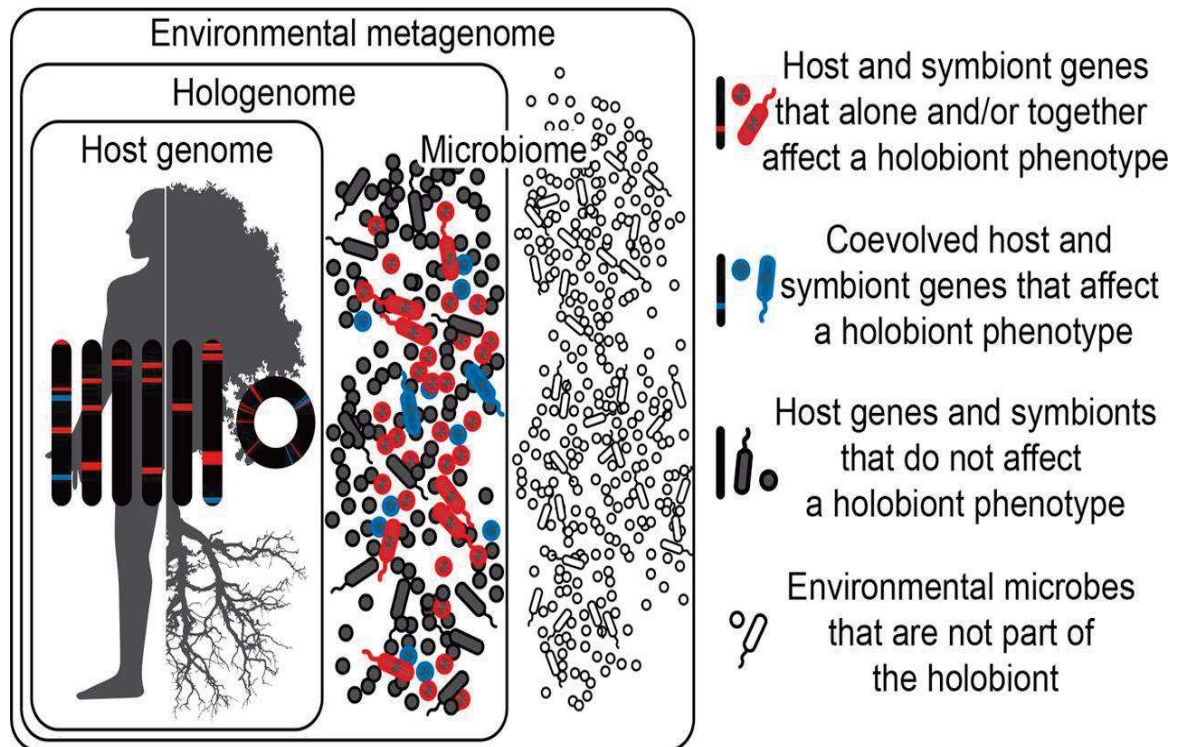


Figure 1 : Les différents composants d'un holobionte.

Les différents types de symbiontes qui vont être retrouvés dans un hôte forment un holobionte. Certains affectent le phénotype de l'holobionte et ont évolué conjointement avec l'hôte (bleu), d'autres affectent le phénotype de l'holobionte mais n'ont pas évolué conjointement avec l'hôte (rouge). Enfin certains n'impactent en rien le phénotype de l'holobionte (gris). Les différents micro-organismes peuvent être acquis de différentes façons et peuvent être constants ou inconstants chez l'hôte. Par conséquent, les phénotypes des holobiontes peuvent changer dans le temps et l'espace à mesure que les symbiontes entrent et sortent de ces derniers. Les microbes présents dans l'environnement ne font pas partie de l'holobionte (blanc). On appelle hologénome le regroupement de tous les génomes d'un holobionte à un instant donné : hôte + microbiome. Les hologénomes impactent donc ce que l'on appelle le phénotype étendu d'un hôte. D'après Theis *et al.* (2016).

1.2 Stratégies évolutives

Les exemples cités ci-dessus mettent en avant des relations dites mutualistes, où un symbionte et un hôte obtiennent un bénéfice mutuel de leur association. Pourtant, il est important de rappeler que d'après sa définition originelle, la symbiose comprend également toutes les interactions existantes au sein d'un holobionte (Perru, 2006). Ainsi, comme nous l'avons vu dans la partie contexte général, les microbiomes comprennent tous les types de symbiontes le long d'un continuum parasites-mutualistes (l'association profite au symbionte mais est délétère pour l'hôte) en passant par des commensaux (le symbionte bénéficie de l'hôte sans impacter ce dernier) (Ewald, 1987). En réalité, il est très souvent problématique d'assigner des phénotypes mutualistes/commensaux/parasites aux associations symbiotiques. En effet, l'hôte est généralement bénéfique pour ses symbiontes, mais ces derniers ont, quoi qu'il arrive, un coût physiologique sur leur hôte (*e.g.* Fleury *et al.*, 2000 ; Shigenobu *et al.*, 2000). Dans une association mutualiste, ces coûts sont contrebalancés par des bénéfices apportés à l'hôte qui sont supérieurs aux coûts (*e.g.* Fleury *et al.*, 2000 ; Shigenobu *et al.*, 2000) : c'est donc cette balance coûts/bénéfices qui va définir la nature de l'association symbiotique. Toutefois, la nature des associations peut se déplacer le long du continuum et passer d'un état à l'autre, notamment en fonction de la phénologie de l'hôte, de la présence d'autres micro-organismes ou d'autres conditions environnementales variables en fonction du phénotype de l'hôte (Clay, 1988 ; Saffo, 1992). De plus, un même symbionte peut être en association mutualiste avec un hôte mais également agir comme parasite d'un autre. C'est par exemple le cas de la bactérie *Photorhabdus* qui est un mutualiste ou un agent pathogène selon qu'elle habite l'intestin des nématodes ou l'hémolymphe des insectes (Clarke, 2008 ; French-Constant *et al.*, 2003). Globalement, un holobionte va donc être

composé à un instant T d'une multitude de symbiontes avec des stratégies évolutives différentes, parfois antagonistes, parfois complémentaires, pouvant se déplacer et évoluer le long du continuum mutualiste-parasite et modifiant le phénotype de l'hôte (Figure 1).

1.2.1 Mode de transmission

L'acquisition d'un symbionte par un hôte va dépendre de plusieurs facteurs écologiques, mais elle est également fortement assujettie à la stratégie évolutive suivie par ledit symbionte. Effectivement, un parasite aura plutôt tendance à se transmettre de manière horizontale (TH) (Anderson and May, 1982), alors qu'un symbionte mutualiste suivra typiquement une transmission verticale (TV) (Moran *et al.*, 2008). Logiquement ces deux stratégies évolutives apparaissent antagonistes : un parasite peut se transmettre même si ce dernier impacte fortement de manière négative la valeur sélective (ou *fitness*, capacité d'un individu d'un certain génotype à se reproduire) de son hôte en « consommant » les ressources de l'hôte ; inversement, un symbionte à TV a tout intérêt à favoriser le succès reproducteur de son hôte en lui apportant divers bénéfices, puisque sa survie et sa transmission dépendent de ce dernier. Ainsi, une TV favorisera l'apparition d'associations mutualistes et coévolutivement stables entre symbiontes et hôtes (Ewald, 1987 ; Lipsitch *et al.*, 1995), bien que des stratégies alternatives, comme le parasitisme de la reproduction, soient également communes (Engelstädter and Hurst, 2009 ; Moran *et al.*, 2008).

De manière intéressante, un symbionte peut utiliser et mélanger les modes de transmissions en fonction de différents facteurs (Ebert, 2013 ; Ewald, 1987 ; Russell, 2019). En effet, de nombreux agents pathogènes sont capables de se transmettre *via* les

deux modes de transmissions, c'est par exemple le cas du VIH qui se transmet essentiellement *via* TH mais qui peut également se transmettre verticalement de la mère à l'enfant lors de la grossesse, l'accouchement ou l'allaitement (Maartens *et al.*, 2014). Globalement, les infections à mode de transmissions mixtes semblent se situer le long d'un continuum, où les parasites transmis principalement horizontalement tendent vers une virulence (pouvoir pathogène d'un micro-organisme chez un hôte, caractérisé par son aptitude à se multiplier chez ce dernier) élevée, et les agents transmis principalement verticalement vers une virulence plus faible (Ewald, 1987 ; Lipsitch *et al.*, 1996).

De manière générale Russell (2019) a montré que le mode de transmission mixte était fréquent puisque sur un total de 528 symbioses analysées, 21% des symbiontes se transmettaient strictement horizontalement, 36% *via* une forme quelconque de transmission mixte, et 43% se transmettaient strictement verticalement. Toutefois, les taxons catégorisés comme à TV exclusives apparaissent rares et possiblement limités à certains endosymbiontes mutualistes d'invertébrés (Moran *et al.*, 2008). De plus, certains endosymbiontes mutualistes à TV strictes peuvent occasionnellement être transmis horizontalement, des TH entre individus d'une même espèce hôte, voire d'espèces différentes, pouvant effectivement se produire (Duron and Hurst, 2013 ; Ferrari and Vavre, 2011 ; Moran *et al.*, 2008). Ce phénomène a été observé par exemple chez certaines espèces de pucerons, ou encore de parasitoïdes, où des TH de symbionte mutualiste semblent relativement fréquentes (Duron *et al.*, 2010 ; Henry *et al.*, 2013 ; Meseguer *et al.*, 2017). La réalité est plus complexe qu'il n'y paraît : la majorité des symbiontes à TV, comme par exemple le célèbre parasite de la reproduction d'arthropode *Wolbachia*, se transmet effectivement verticalement d'une génération à l'autre de façon remarquablement stable ; pourtant, les nombreuses incongruences phylogénétiques entre les histoires évolutives des hôtes et de leurs symbiontes nous montrent que les TH

se produisent fréquemment à une échelle évolutive (Moran *et al.*, 2008 ; Werren *et al.*, 2008).

Dans le contexte d'un holobionte, les microbiomes sont eux aussi maintenant considérés comme à transmission mixte (Ebert, 2013). En effet, de nombreux exemples montrent qu'une partie du microbiome de la mère (voire du père dans certains cas) est transmise verticalement à la descendance, mais que cette dernière va également acquérir une partie de son microbiome *via* ses interactions avec son environnement et d'autres holobiontes. Par exemple, la transmission maternelle du microbiome des souris est si stable au fil des générations que les relations de parenté se reflètent dans la composition des communautés microbiennes (Ley *et al.*, 2006). Cependant, la composition des deux microbiomes n'est pas complètement identique, appuyant l'idée d'une transmission mixte. De manière surprenante, cette stratégie de transmission des microbiomes est également retrouvée chez les non-animaux. Effectivement, chez les plantes l'acquisition de leur microbiome se produit *via* TH depuis l'environnement, incluant par exemple les phytophages, et également grâce à la TV à partir des graines (Compant *et al.*, 2019). Néanmoins, il a été montré que certaines bactéries endophytes pourraient se transmettre de manière mixte, en infectant une graine depuis l'environnement puis en se transmettant verticalement *via* les graines de la plante résultante (Ferreira *et al.*, 2008).

1.2.2 Phénotype étendu de l'hôte

Bien que la stratégie évolutive d'un symbionte soit en partie définie par son mode de transmission, il est important de caractériser l'impact d'un symbionte sur le phénotype étendu de l'hôte pour finement évaluer cette stratégie, puisqu'un même phénotype de symbionte peut se transmettre par différentes voies. Nous avons vu précédemment que

les endosymbiontes pouvaient complètement changer le phénotype de leur hôte en leur fournissant de nouvelles capacités biochimiques. Les symbiontes sont donc des "acteurs cachés" dans de nombreux processus écologiques et évolutifs (Frago *et al.*, 2012). Ces microbiomes contiennent des gènes dix fois plus nombreux que l'information génétique de l'hôte (Colston and Jackson, 2016) ; ainsi, par exemple, au moins 20 % des petites molécules du sang humain sont des produits de nos symbiontes (Rook *et al.*, 2017).

Au sein de nombreux holobiontes, les effets du microbiome intestinal sur le phénotype de leur hôte commencent à être en partie connus. Les exemples les plus pertinents concernent très certainement l'aspect nutritionnel de l'hôte, avec en tête de liste, les symbiontes intestinaux des ruminants ayant permis l'apparition de l'herbivorie en leur permettant, entre autres, de digérer et d'assimiler la cellulose (Groussin *et al.*, 2017 ; Hobson and Stewart, 1997). De même, chez les termites le microbiome intestinal permet la digestion des composants alimentaires complexes (Brune, 2014) ; chez l'humain ou la drosophile, ce dernier peut subvenir à des régimes pauvres en nutriments (Flint *et al.*, 2012 ; Wong *et al.*, 2014). Au-delà de l'impact sur la nutrition de leur hôte, les symbiontes intestinaux influenceraient tout un ensemble de fonctionnalités telles que la régulation circadienne (Leone *et al.*, 2015), le développement tissulaire (Shin *et al.*, 2011) ou encore la protection contre des agents pathogènes (Dillon *et al.*, 2005). Plus récemment, des études ont montré que le microbiome intestinal pourrait également contribuer au phénomène de spéciation (Brucker and Bordenstein, 2013), influencer les systèmes d'accouplement et de reproduction et, de façon surprenante, impacter les interactions sociales d'un individu en modifiant la communication interspécifique et intraspécifique (Archie and Tung, 2015 ; Morimoto *et al.*, 2017 ; Walsh *et al.*, 2017). Enfin, il a été montré que des changements dans la composition microbienne de l'intestin pouvaient entraîner des changements phénotypiques chez l'hôte, jusqu'à provoquer des

pathologies et donc impacter négativement la valeur sélective de l'holobionte (*e.g.* Kinross et al., 2011). Tous ces exemples supportent concrètement le concept d'holobionte et démontrent à quel point un hôte et son microbiome interagissent de manière très intime. Toutefois, ce concept est encore plus flagrant chez certains arthropodes où cette interaction est poussée à son extrême.

En effet, de nombreux arthropodes hébergent des endosymbiontes se transmettant quasi-exclusivement de manière verticale *via* le cytoplasme des œufs, tout comme les mitochondries (Moran *et al.*, 2008), et plus rarement par le nucleus des spermatozoïde (De Vooght *et al.*, 2015 ; Watanabe *et al.*, 2014), constituant ainsi de véritables génomes accessoires de leurs hôtes (Dale and Moran, 2006 ; Duron and Hurst, 2013 ; Ferrari and Vavre, 2011 ; Moran *et al.*, 2008). Deux catégories sont classiquement distinguées en fonction de leurs effets sur le phénotype de l'hôte (Baumann, 2005 ; Buchner, 1965 ; Hansen and Moran, 2014 ; Moran *et al.*, 2008) :

1- Les symbiontes primaires (P-symbiontes) sont des mutualistes obligatoires, nécessaires à la survie ou à la reproduction de leurs hôtes, qui ont d'ailleurs généralement un organe spécifiquement alloué au P-symbiontes : le bactériome, composé de cellules spécialisées appelées bactériocytes. De par leur nature obligatoire, les P-symbiontes sont présents chez tous les individus au sein des espèces hôtes. Ils ont souvent un rôle nutritionnel en permettant à leur hôte d'utiliser des niches trophiques spécialisées. Les P-symbiontes se rencontrent donc communément chez les arthropodes dont le régime alimentaire se réduit à une ressource exclusive comme le phloème ou le sang (Wernegreen, 2012). Ainsi, si le phloème est riche en sucres, il est en revanche pauvre en d'autres nutriments comme les protéines et constitue donc un régime biaisé (Douglas, 1998). De ce fait, seuls certains insectes de l'ordre des Hémiptères sont capables de se nourrir uniquement de phloème sans présenter de carences, et ce grâce à des P-

symbiontes (Baumann and Baumann, 1994 ; Baumann, 2005 ; Moran *et al.*, 2008). Par exemple, la bactérie *Buchnera aphidicola* synthétise certaines vitamines, stéroïdes et acides aminés essentiels qui sont absents du phloème consommé par ses hôtes pucerons (Baumann and Baumann, 1994 ; Baumann, 2005 ; Douglas, 1998 ; Shigenobu *et al.*, 2000). De la même manière, l'hématophagie stricte représente également un régime alimentaire biaisé. Le sang des vertébrés, bien que riche en protéines, est en effet pauvre en certains métabolites essentiels à l'homéostasie, comme les vitamines B. Ainsi, par exemple, la bactérie *Wigglesworthia* synthétise des vitamines essentielles à la reproduction de son hôte, la glossine (Michalkova *et al.*, 2014). A l'instar de la glossine, la quasi-totalité des hématophages stricts (poux, punaises de lit, triatomés, hippoboscques...) hébergent des P-symbiotes qui auraient tous la capacité de synthétiser, entre autres, certaines vitamines B (Rio *et al.*, 2016). Plus généralement, une spécialisation trophique extrême ne semble possible chez les arthropodes que par endosymbiose (Moran & *al.*, 2008 ; Wernegreen, 2012).

2- Les symbiotes facultatifs ou secondaires (S-symbiotes) ne sont nécessaires ni à la survie ni à la reproduction de leurs hôtes. Ils présentent donc des prévalences variables entre populations hôtes. Deux sous-catégories sont classiquement distinguées en dépit de nombreux cas intermédiaires, en fonction de leur phénotype : les mutualistes facultatifs et les manipulateurs de la reproduction (Moran *et al.*, 2008). Les premiers confèrent une résistance aux stress biotiques (*e.g.* parasitoïdes, parasites) ou abiotiques (*e.g.* chocs thermiques), augmentant ainsi la valeur sélective de leurs hôtes sous certaines conditions environnementales (*e.g.* Montllor *et al.*, 2002 ; Scarborough *et al.*, 2005 ; Vorburger *et al.*, 2010). Le plus connu, la bactérie *Hamiltonella defensa* procure aux pucerons une résistance aux guêpes parasitoïdes, ce qui lui permet d'atteindre de fortes fréquences dans certaines populations hôtes (Oliver *et al.*, 2003). Les manipulateurs de la

reproduction quant à eux (*e.g.* les bactéries *Wolbachia* ou *Cardinium*) diffusent dans les populations hôtes *via* quatre grands types de manipulations : la féminisation de mâles génétiques en femelles fonctionnelles, l'induction de parthénogenèse, le *male-killing* (défini par la mort exclusive des mâles) et/ou une forme de stérilité conditionnelle appelée incompatibilité cytoplasmique (Engelstadter & Hurst, 2009 ; Stouthamer & *al.*, 1999 ; Werren & *al.*, 2008). La finalité de ces manipulations est toujours la même : favoriser chez la femelle infectée la production ou la valeur sélective de filles, c'est-à-dire du seul sexe transmetteur de l'infection.

Les bactéries héritables, P-symbiotes comme S-symbiotes, sont ainsi souvent à l'origine d'acquisition de traits évolutifs majeurs qui influencent l'adaptation des arthropodes à de nouveaux habitats ou interfèrent dans l'évolution de leurs systèmes de reproduction (Dale and Moran, 2006 ; Duron and Hurst, 2013 ; Ferrari and Vavre, 2011 ; Moran *et al.*, 2008).

1.2.3 Coévolution Symbionte-Hôte

Du fait de cette complexité, il est difficile à l'heure actuelle de comprendre, dans sa globalité, la coévolution entre un microbiome et son hôte (Davenport *et al.*, 2017). Il est donc plus facile d'appréhender ce phénomène de coévolution grâce à des modèles « simples » de symbioses. A la vue des différents exemples de symbioses présentés jusqu'ici, avec des effets phénotypiques divers (certains bénéfiques, d'autres nuisibles pour l'hôte), il est clair que la symbiose joue un rôle important sur la valeur sélective de l'hôte et la transmission des symbiotes. L'acquisition de symbiotes capables de TV peut être perçue comme une source de variations génétiques héréditaires comparable aux mutations génétiques, à la différence qu'aucun des gènes fonctionnels de l'hôte n'est

altéré dans le processus (Ferrari and Vavre, 2011). La symbiose apparaît donc comme un mécanisme évolutif majeur et pourrait en effet être l'un des mécanismes clés dans l'innovation biologique et la spéciation (Margulis and Fester, 1991 ; Maynard Smith, 1989).

Ce mécanisme est ineffablement caractérisé par l'histoire évolutive des cellules eucaryotes ou par celles liant les P-symbiontes et leurs hôtes arthropodes. En effet, ces endosymbiontes ont en commun d'être si étroitement liés à leurs hôtes qu'ils en sont devenus indissociables, constituant de quasi-organelles comparables aux mitochondries et chloroplastes. Toutefois, en fonction de la stratégie évolutive d'un symbionte et donc de son impact sur le phénotype étendu de l'hôte, la symbiose aboutira à des histoires coévolutives différentes. Les endosymbiontes d'arthropodes, quant à eux, illustrent parfaitement ces différences évolutives.

Effectivement, le caractère obligatoire de l'association entre P-symbionte et son hôte assure la persistance et la stabilité de l'infection chez ce dernier sur de longues périodes évolutives. Par conséquent, certaines symbioses existent depuis des millions d'années, c'est notamment le cas de l'association entre la bactérie *Sulcia* et son hôte cicadelle qui durerait depuis plus de 270 millions d'années (Moran *et al.*, 2005). D'un point de vue évolutif, le maintien de ces associations *via* TV sur de telles périodes se traduit par une co-diversification entre les deux partenaires. Cette évolution est typiquement observée par une congruence entre la phylogénie des P-symbiontes et celle de leur hôte, appelée co-cladogénèse (Figure 2) (Lo *et al.*, 2003 ; Moran *et al.*, 2008 ; Munson *et al.*, 1991). Ainsi, par exemple, la relation symbiotique entre les bactéries *Buchnera* et les pucerons se manifeste par une stricte co-spéciation depuis son établissement il y a approximativement 140 millions d'années (Moran *et al.*, 2008 ; Pérez-Brocal *et al.*, 2011). De nombreux cas similaires sont connus parmi lesquels les systèmes *Wigglesworthia* et

glossines (> 40 MA) ou *Blattabacterium* et cafards (150 MA) (Chen *et al.*, 1999 ; Lo *et al.*, 2003).

Les S-symbiontes suivent quant à eux une évolution bien différente marquée par la rareté des cas de co-diversification : des S-symbiontes phylogénétiquement proches sont souvent présents dans des espèces d'hôtes peu apparentées. Ces incongruences reflètent les fréquents TH de ces symbiontes au sein des arthropodes (Figure 2). Ceci est typiquement reporté pour des manipulateurs de la reproduction tels que *Wolbachia*, *Arsenophonus* ou *Cardinium* (Jousselin *et al.*, 2013 ; O'Neill *et al.*, 1992 ; Rousset *et al.*, 1992 ; Russell *et al.*, 2009 ; Vavre *et al.*, 1999). Cependant les événements de transmission horizontale de S-symbiontes restent considérés comme rares, donc comme se produisant sur une échelle évolutive plutôt qu'écologique. Ce mode de transmission mixte permet ainsi l'infection de nouveaux hôtes et de contrer une extinction du symbionte en cas de conditions bio-écologiques désavantageuses chez un hôte (*e.g.* compétition intersymbiotique pour la TV). Il en résulte une dynamique évolutive d'acquisition et de perte de ces symbiontes (Bailly-Bechet *et al.*, 2017). Pour aller plus loin, il a été proposé que malgré sa complexité intrinsèque, un S-symbionte se comporte fondamentalement comme un locus diallélique dans une population hôte haploïde : le premier allèle correspondant à la présence du symbiote chez l'hôte et le second à son absence (Olivieri, 2010). Par conséquent, les fréquences de ces symbiontes seront par la suite modulées par les forces évolutives classiques régulant les fréquences alléliques (Olivieri, 2010), c'est-à-dire la sélection naturelle, la dérive génétique, la migration génétique et les mutations.

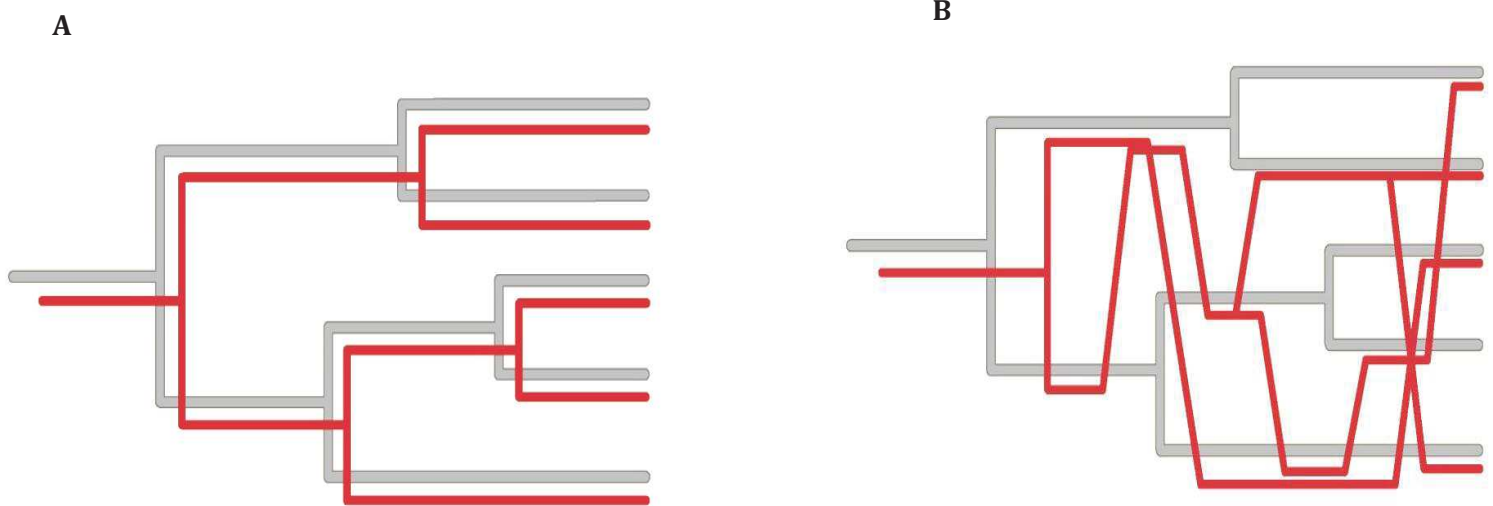


Figure 2 : Les différents patrons de coévolution entre les symbiontes et les arthropodes.

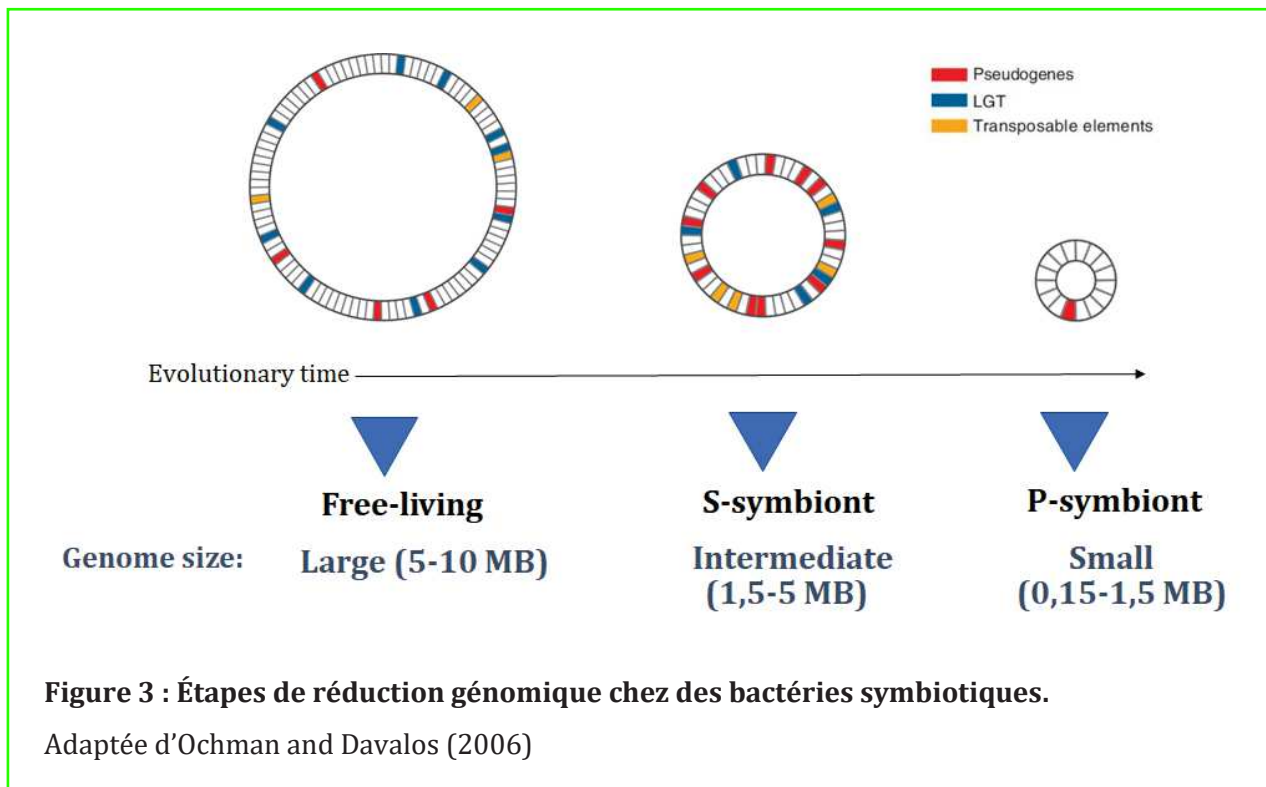
La figure 2A représente un patron de co-cladogénèse parfaite typiquement formé entre la phylogénie d'un P-symbionte (en rouge) et celle de son hôte arthropode (en gris), ce patron est par exemple retrouvé chez le modèle symbiotique *Hamiltonella*/pucerons. La figure 2B, quant à elle, illustre un patron de transmission mixte généralement emprunté par les S-symbiontes chez leur hôte. Cela se traduit par une phylogénie du symbionte (en rouge) présentant peu de congruence avec celle de son hôte (en gris). Adaptée de Dale and Moran (2006).

1.2.4 Impacts de la coévolution sur l'hologéome : exemple des holobiontes arthropodes

En plus de favoriser les phénomènes de spéciations, la coévolution qui se joue au sein d'une symbiose peut avoir comme conséquence des modifications de l'hologéome. En effet, au cours de leur association avec leurs hôtes arthropodes, les P-symbiontes subiront une réduction drastique de la taille de leur génome tout en subissant le « cliquet de Müller », entraînant une dérive génétique et la fixation de mutations délétères (Figure 3) (McCutcheon *et al.*, 2019 ; McCutcheon and Moran, 2012 ; Moran, 1996 ; Ochman and Davalos, 2006 ; Rispé and Moran, 2000). Ce processus s'explique notamment par le mode

de vie intracellulaire de ces symbiontes, leur confinement dans des bactériomes et les importants goulots d'étranglement (*bottleneck*) lors de leur transmission intergénérationnelle. Tout en limitant les TH de gènes entre bactéries, les bactériomes représentent un environnement « sur mesure » pour ces symbiontes. Ainsi, de nombreux gènes correspondant à une stratégie évolutive orientée vers le parasitisme ou vers le mode de vie libre deviennent obsolètes, évoluent en pseudogènes (gènes inactifs) faute de pression de sélection, et sont donc éliminés au fil du temps. Seuls les gènes apportant un avantage sélectif à l'holobionte ou indispensables au fonctionnement cellulaire basique sont conservés (McCutcheon *et al.*, 2019 ; McCutcheon and Moran, 2012 ; Moran, 1996 ; Ochman and Davalos, 2006 ; Rispé and Moran, 2000). De plus, ce phénomène de réduction génomique semble intervenir peu après l'établissement de la symbiose, se produisant à un taux d'érosion relativement rapide d'un point de vue évolutif (Gil *et al.*, 2003 ; McCutcheon *et al.*, 2019). Par conséquent, les génomes des P-symbiontes présentent des tailles extrêmement faibles tout en étant enrichis en bases AT puisque leur composition en bases nucléotidiques est biaisée par l'accumulation de mutations. Avec des tailles de génomes parfois en dessous de 200 kb, comme le symbionte *Tremblaya* en symbiose avec une cochenille (138 kb) (López-Madrugal *et al.*, 2011) ou *Carsonella* en symbiose avec une psylle (160 kb) (Nakabachi *et al.*, 2006), ces micro-organismes exhibent les plus petits génomes bactériens (non synthétiques) connus. De manière intéressante, le génome de ces deux endosymbiontes a atteint un tel degré de réduction que ces organismes ne peuvent plus « vivre » de manière autonome et ne sont donc plus considérés comme bactéries mais comme des types d'organelles à part entière (López-Madrugal *et al.*, 2011 ; Nakabachi *et al.*, 2006). Globalement, un long processus co-évolutif est nécessaire pour qu'un symbionte devienne une quasi-organelle, passant typiquement d'un mode de vie libre ou parasitaire à une stratégie évolutive symbiotique mutualiste.

Par exemple, le P-symbionte de la punaise du lit est une souche de *Wolbachia* ayant acquis les gènes capables de synthétiser certaines vitamines B (Hosokawa *et al.*, 2010). Ce parasite manipulateur de la reproduction est ainsi devenu un P-symbionte, illustrant parfaitement l'impact des transitions de stratégies évolutives sur le génome des symbiontes.



Si la coévolution symbiotique impacte le génome des symbiontes, elle a également des conséquences sur le génome des hôtes. Il faut garder à l'esprit qu'au sein de chaque symbiose, qu'elle soit parasitaire ou mutualiste, les partenaires « cherchent » à augmenter leur valeur sélective de manière égoïste. Dans le cadre d'une symbiose mutualiste obligatoire par exemple, des mutations augmentant la valeur sélective chez un P-symbionte peuvent alors se produire, mais se révéler néfastes pour l'hôte (Bennett and Moran, 2015). Ce dernier sera ainsi soumis à de fortes pressions de sélections pour réduire l'impact négatif des mutations du P-symbionte conduisant à une évolution rapide du génome de l'hôte. La coévolution au sein de l'hologénome peut donc entraîner

d'importantes mutations dans le génome de l'hôte, pouvant ainsi créer des incompatibilités génétiques entre différentes lignées d'holobiontes, donc un phénomène de spéciation (Figure 4) (Bennett and Moran, 2015). Ce processus est en quelque sorte un cercle vicieux évolutif, appelé « *rabbit hole* » par Bennett et Moran (2015), comparable à la « classique » course à l'armement entre un parasite et son hôte, se caractérisant par un cycle ininterrompu d'adaptations et contre-adaptations (Combes, 2010 ; Svensson and Råberg, 2010). Dans ce contexte, ces interactions mutualistes peuvent se définir comme du « mutualisme à double tranchant », procurant effectivement un avantage sélectif aux partenaires mais conduisant irréversiblement vers une interdépendance « dangereuse » d'un point de vue évolutif. De même chez certaines symbioses, il a été documenté des TH de gènes du génome du symbionte vers le génome nucléaire de l'hôte (Husnik *et al.*, 2013 ; Ioannidis *et al.*, 2014 ; Nikoh and Nakabachi, 2009). Ces TH peuvent avoir des conséquences non négligeables sur l'évolution de l'hôte allant jusqu'à lui apporter de nouvelles fonctions (bonnes ou mauvaises). Par exemple, certains pucerons ont de manière surprenante acquis, *via* TH de gènes fongiques, la capacité de synthétiser des caroténoïdes qui joueraient un rôle hypothétique dans la production énergétique (Moran and Jarvik, 2010 ; Valmalette *et al.*, 2012). A l'opposé, une insertion génomique récente de *Wolbachia* dans le génome du cloporte induit la féminisation des mâles génétiques en l'absence de parasite de la reproduction. Cette insertion, appelée « élément f », est même considérée comme à l'origine de l'apparition d'un nouveau chromosome sexuel : le chromosome W (Chebbi *et al.*, 2019 ; Cordaux and Gilbert, 2017 ; Juchault *et al.*, 1993 ; Leclercq *et al.*, 2016). Ces exemples illustrent parfaitement à quel point les génomes des hôtes peuvent être influencés par une relation symbiotique.

Symbiotic Coevolution as a Source of Genetic Incompatibility

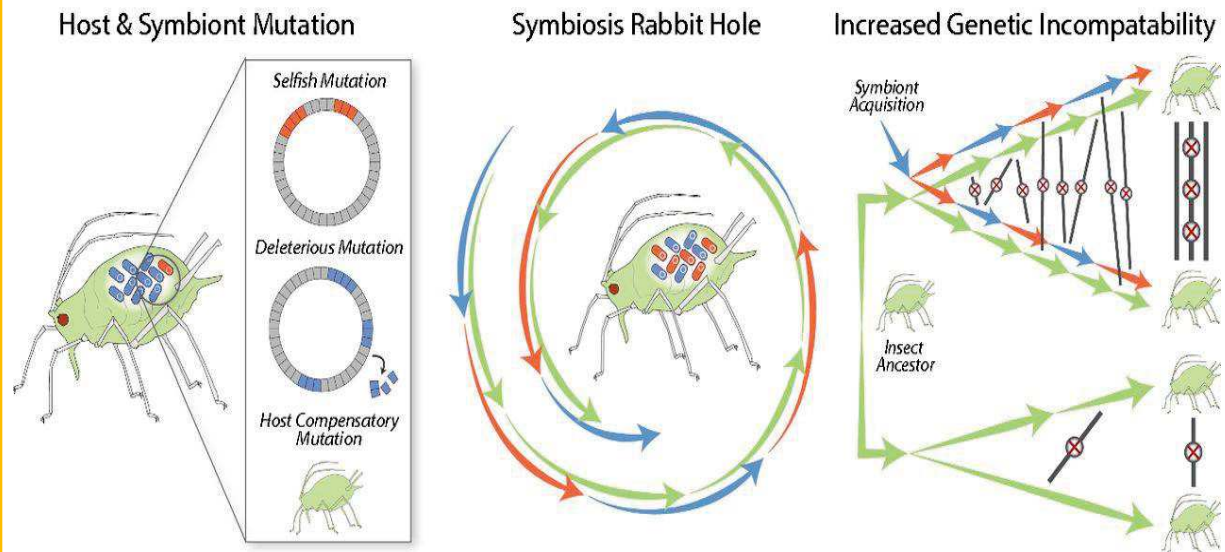


Figure 4 : Causes et conséquences de la coévolution hôte-symbionte primaire.

Les mutations qui ont un impact négatif sur la symbiose peuvent être fixées par dérive génétique due à la clonalité et à la petite taille des populations de symbionte (en bleu) ou par la sélection au sein de l'hôte de symbiontes égoïstes qui favorisent leur propre valeur sélective au détriment de celle de l'hôte (en rouge). En réponse, l'hôte est sélectionné pour amortir ces mutations (en vert), menant à ce fameux « rabbit hole » évolutifs. Cette coévolution peut entraîner l'accumulation rapide d'incompatibilités génétiques entre les lignées d'hôtes, et entre les hôtes et les souches de symbionte. Ce processus peut conduire à une accélération de l'isolement reproductif et de la spéciation, ce qui pourrait réduire davantage la taille effective des populations hôtes génétiquement compatibles. D'après Bennet and Moran (2015)

1.3 Facteurs structurants

Bien que la symbiose au sein d'un holobionte soit soumise à des processus évolutifs, il est également nécessaire de prendre en compte les mécanismes écologiques sous-jacents à la structuration de la symbiose afin de démêler au mieux cette dernière. Comme nous l'avons déjà évoqué dans la partie contexte général de ce manuscrit, la structuration des microbiomes est soumise à de nombreux facteurs écologiques, tant

biotiques qu'abiotiques. Ces facteurs changeront et n'auront pas le même pouvoir structurant en fonction de l'échelle résolutive considérée. Ces échelles de variabilités sont classiquement divisées en trois catégories, allant d'une résolution fine à une résolution plus globale : intra-individuelle, ou dans le contexte de cette thèse intra-holobionte, inter-holobionte (ou intra-spécifique) et inter-spécifique. Ainsi, plus la résolution sera fine, plus les facteurs structurants seront de nature écologique. A l'inverse, plus l'échelle est grande, plus les phénomènes coévolutifs deviennent importants pour expliquer les variations des communautés symbiotiques. Par conséquent, à l'échelle inter-spécifique, il existe chez une multitude d'holobiontes une corrélation entre microbiomes et espèces hôtes, que nous avons déjà définis comme phylosymbiose (Brooks *et al.*, 2016). La coévolution et les patrons phylogénétiques sont donc aussi importants que des facteurs écologiques, tels que le régime alimentaire de l'hôte, pour expliquer la variabilité symbiotique entre espèces d'hôte (Figure 5) (Groussin *et al.*, 2017 ; Ley *et al.*, 2008 ; Muegge *et al.*, 2011). **Cette thèse se concentre d'ailleurs sur les processus coévolutifs et écologiques qui surviennent à cette échelle.** Toutefois, il est important d'aborder les processus de structuration à l'œuvre à des échelles plus faibles pour appréhender dans son ensemble le contexte écologique régissant les holobiontes.

La variabilité de la composante microbienne au niveau intra-holobionte ou inter-holobionte a été essentiellement étudiée dans le contexte de la santé humaine. Globalement, cette variabilité semble dépendre en grande partie de facteurs écologiques, qu'ils soient endogènes (liés à l'hôte) ou exogènes (liés à l'environnement). Il existe, par exemple, une très grande différence de compositions et de diversités des communautés microbiennes inter ou intra-organes chez un humain (Grice and Segre, 2011 ; Hilty *et al.*, 2010 ; Spor *et al.*, 2011). Cette labilité peut s'expliquer par des différences de niches écologiques qui impactent le processus de « *species sorting* » : un concept de l'écologie des

communautés qui regroupe les facteurs abiotiques d'un environnement et les interactions biotiques pour expliquer le tri des taxons microbiens s'opérant dans un milieu donné (Costello *et al.*, 2012 ; Leibold *et al.*, 2004). Par exemple, les différences de microbiomes cutanés chez un même humain semblent dépendre des zones du corp considérées et seraient principalement structurées par des différences de pH, de température et d'humidité (Oh *et al.*, 2014). Ainsi cet exemple illustre parfaitement l'importance des niches écologiques, où des différences abiotiques forment des micro-environnements aux conditions physico-chimiques particulières, appelées filtres environnementaux, dans lesquels ne survivront que des micro-organismes possédant des exigences écologiques compatibles (Costello *et al.*, 2012 ; Kraft *et al.*, 2015).

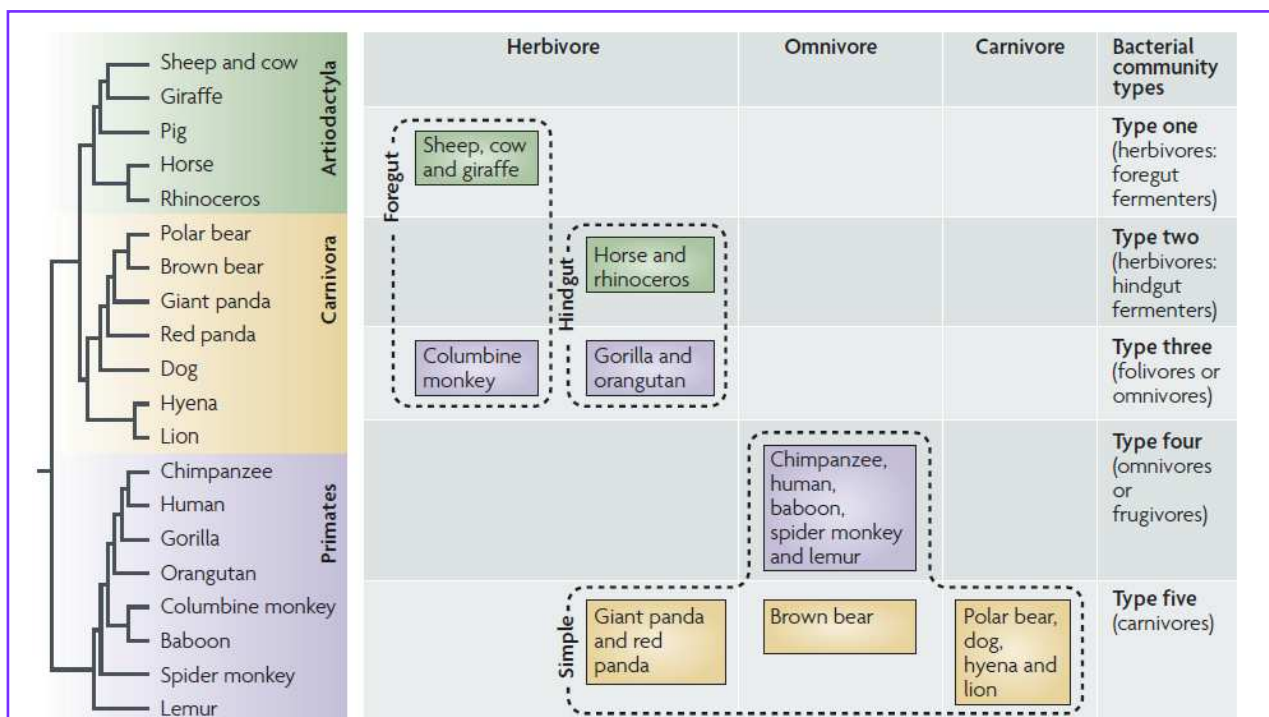


Figure 5 : Facteurs structurants du microbiome intestinal à une échelle inter-spécifique Représentation schématique de l'impact de la phylogénie de l'hôte, de la morphologie intestinale et du régime alimentaire sur la composition des communautés bactériennes fécales. La phylogénie montre l'impact des relations évolutives d'un ensemble représentatif de mammifères dont la couleur est codée selon le régime alimentaire dominant du clade phylogénique de l'hôte. Les morphologies intestinales des mammifères sont indiquées ainsi que leurs catégories alimentaires et le type de communauté bactérienne qui a été retrouvé. Les animaux phylogéniquement apparentés ont généralement le même type d'intestin, de régime alimentaire et de communauté bactérienne intestinale (par exemple, l'ordre des Artiodactyles). D'autres clades de mammifères contiennent des espèces singulières, comme l'ordre des primates où les singes « columbine » qui présentent une physiologie intestinale (élargissement de l'intestin) et un régime alimentaire (herbivore) inhabituels pour leur lignée. D'après Ley *et al.* (2008).

A ces facteurs abiotiques, s'ajoute bien entendu le panel d'interactions biotiques existant au sein d'un holobionte. Pour commencer, en fonction de la nature de l'association entre un symbionte et son hôte, il existera une interaction cellulaire différente : un agent pathogène ou mutualiste ne déclenchera pas, entre autres, les mêmes réactions immunitaires chez leur hôte. Il a même été démontré que la reconnaissance des organismes commensaux du microbiome intestinal par les récepteurs de type Toll-like était requise pour maintenir l'homéostasie de l'environnement intestinal humain (Rakoff-Nahoum *et al.*, 2004). Ensuite, en plus des interactions symbiontes-hôtes, les différents micro-organismes d'un holobionte vont interagir entre eux. À l'instar des communautés de macro-organismes, des phénomènes de compétition, de prédation ou de coopération existent entre micro-organismes. Ainsi de récentes études ont montré l'impact de ces interactions sur le microbiome d'une part et sur l'hôte d'autre part (Bauer *et al.*, 2018 ; Coyte *et al.*, 2015 ; Coyte and Rakoff-Nahoum, 2019 ; Gould *et al.*, 2018 ; Rakoff-Nahoum *et al.*, 2016). Il a, par exemple, été attesté que la compétition entre micro-organismes au sein des différentes niches écologiques que représente l'intestin humain était indispensable au maintien de l'homéostasie : en réduisant la diversité microbienne, la compétition augmente la stabilité du système (Bauer *et al.*, 2018; Coyte and Rakoff-Nahoum, 2019). À l'inverse, il a été découvert qu'un symbionte intestinal prédominant (*Bacteroides ovatus*) digère les polysaccharides au détriment de lui-même mais au bénéfice d'une autre espèce (*B. vulgatus*) qui, en « échange », désintoxique des substances inhibitrices ou produit des facteurs favorisant la croissance de *B. ovatus* (Rakoff-Nahoum *et al.*, 2016). De manière générale, une étude chez la drosophile a mis en exergue que les différentes interactions au sein du microbiome façonnent la valeur sélective de leur hôte en modifiant des aspects fondamentaux de la physiologie de ce dernier (Gould *et al.*, 2018). Enfin, l'abondance relative des taxons microbiens au sein

d'une communauté microbienne peut également varier suite à des événements stochastiques appelés « *ecological drift* » et pouvant entraîner l'extinction locale de taxons faiblement abondants (Costello *et al.*, 2012). Ainsi, Burns *et al.* (2016) ont démontré l'importance des événements stochastiques dans la structuration du microbiome digestif des alevins du poisson-zèbre. Toutefois, au fur et à mesure du développement de l'hôte, la composition de son microbiome était de plus en plus influencée par le processus de *species sorting* : la maturation du système immunitaire et l'apparition de niches écologiques au sein du tube digestif de l'hôte expliquent majoritairement la structuration du microbiome (Burns *et al.*, 2016).

Ces différents exemples et processus écologiques montrent qu'il semble difficile d'appréhender l'ensemble des facteurs structurants des microbiomes à des échelles intra et inter-holobiontes, sans l'étude d'organismes modèles comme le poisson-zèbre ou la souris. Cependant, comme cette thèse repose sur l'étude d'holobiontes non modèles, à savoir les tiques de Guyane et leur microbiome, il apparaît plus abordable d'étudier ces facteurs à des échelles interspécifiques où les processus stochastiques et écologiques sont moins déterminants pour expliquer la structuration des microbiomes.

2. Les tiques et leurs microbiomes

2.1 Les tiques : généralités

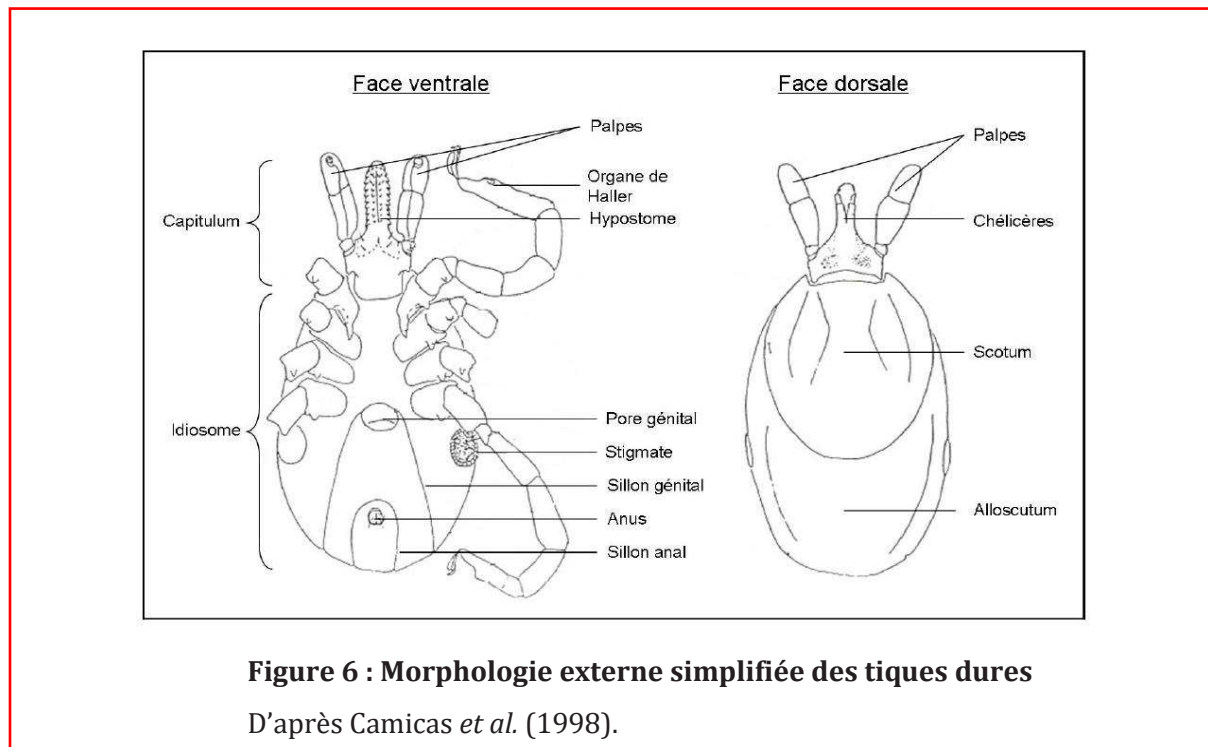
2.1.1 Taxonomie et morphologie

Les Ixodida, appelées couramment tiques, sont des arthropodes ectoparasites se nourrissant exclusivement du sang des vertébrés. Leur nom, Ixodida, provient du grec *ixôdês* signifiant « gluant » : la glue était à l'époque obtenue à partir des baies de gui, et

comme certaines tiques gorgées de sang ressemblent à une baie de gui, « engluée » à la peau, cette dénomination leur a été attribuée. On distingue classiquement trois familles de tiques : les Argasidae ou tiques « molles », les Nuttalliellinea qui ne comportent qu'une seule espèce (*Nuttalia namaqua*), et les Ixodidae (tiques « dures ») famille principalement étudiée dans cette thèse (Guglielmone *et al.*, 2010). Le terme de tique dure provient des zones du tégument chitinisées dures, présentes chez les Ixodidae et absentes chez les Argasidae. Du fait qu'elles ne possèdent pas d'antennes ni de mandibules, mais une paire de chélicères et de pédipalpes, les tiques appartiennent au sous-embranchement des Chélicérates. Au même titre que les araignées, les tiques comportent quatre paires de pattes (excepté au stade larvaire) et font donc partie de la classe des Arachnida. Elles sont rangées avec les acariens dans la sous-classe des Acari, dans laquelle les tiques représentent d'ailleurs les plus grands organismes (3 à 6 mm au stade adulte). Enfin, les tiques dures sont comprises dans le super ordre des Parasitiformes, ordre des Ixodida, et dans la famille des Ixodidae.

Comme tous les acariens, le corps des tiques est segmenté en deux parties : le capitulum (tête en latin) et l'idiosome (Figure 6) (Camicas *et al.*, 1998). Le capitulum porte les pièces buccales composées de deux pédipalpes qui ont une vocation sensorielle, d'une paire de chélicères permettant de dilacérer la peau de l'hôte et d'un hypostome muni de dents permettant l'ancrage à l'hôte (Figure 6). Les chélicères et l'hypostome forment ce que l'on appelle le rostre vulnérant. L'idiosome, quant à lui, porte entre autres le pore génital, l'anus et les quatre paires de pattes (trois au stade larvaire), dont la première paire comporte l'organe de Haller. Cet organe permet à la tique de percevoir son environnement en détectant la concentration en vapeur d'eau par exemple (Gaede and Knülle, 1997), tout en participant activement à la recherche d'hôte, en détectant entre

autres le CO₂ et des molécules odorantes (phénols et lactones) émises par ceux-ci (Leonovich, 2004).



2.1.2 Cycle de développement

Il existe trois phases de développement chez les tiques dures : une fois la femelle complètement gorgée de sang, elle se décrochera de son hôte pour digérer et pondre sur la végétation des milliers d'œufs desquels éclore des larves. Ces larves prendront à leur tour un repas sanguin pour pouvoir se métamorphoser en nymphes qui vont à leur tour prendre un repas sanguin pour se métamorphoser en femelles ou en mâles fonctionnels (Figure 7). Il existe alors une alternance entre phase parasitaire sur un hôte et phase libre au sol. Le nombre d'alternances que va réaliser une tique durant son cycle de vie va dépendre du nombre nécessaire d'hôtes à son bon développement. En effet, on distingue trois types de cycles : triphasique lorsqu'une espèce de tique va prendre ses trois repas sanguins sur trois hôtes différents, ce cycle est le plus courant chez les tiques

dures (Figure 7) ; biphasique et monophasique lorsqu'une espèce se nourrit sur deux ou un seul hôte au cours de son développement, respectivement.

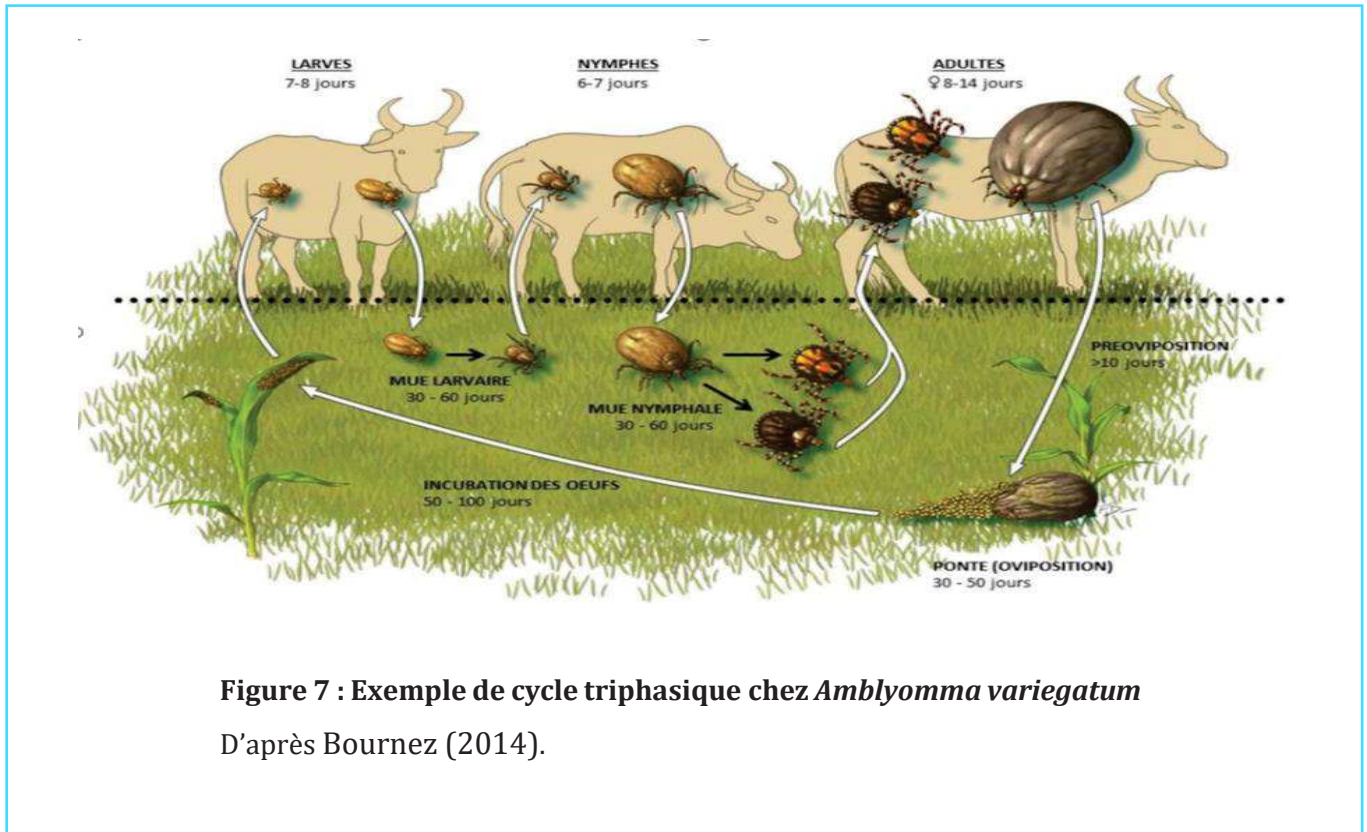


Figure 7 : Exemple de cycle triphasique chez *Amblyomma variegatum*
D'après Bournez (2014).

La recherche d'hôte va s'effectuer durant les phases libres par différentes stratégies en fonction de l'espèce de tique considérée. Alors que certaines espèces sont endophiles et vont donc se retrouver dans des lieux propres à l'hôte comme un nid ou un terrier, d'autres sont exophiles et rencontrent leur hôte dans des environnements ouverts. Ces dernières peuvent rester à l'affut sur la végétation à attendre qu'un hôte vienne à leur rencontre ou mener une « chasse » active de leur hôte. De même, on distingue différentes préférences trophiques, appelées spectre d'hôte, chez les tiques. Ainsi, il existe des espèces se nourrissant de manière optimale sur une seule espèce d'hôte que l'on dénomme spécialistes. D'autres peuvent prendre leur repas sanguin sur plusieurs espèces d'hôtes appartenant à un même ordre ou classe de vertébrés, on parle alors de

sélectivité parasitaire. Enfin certaines espèces sont dites généralistes puisqu'elles peuvent se nourrir avec succès sur différents ordres ou classes de vertébrés.

Une fois sur leur hôte, les tiques vont se mettre en quête d'une zone du corps optimale pour leurs repas sanguin, généralement un endroit où la peau est fine. Le rostre va ensuite leur permettre de pénétrer la peau de l'hôte et de s'ancrer à ce dernier (Richter *et al.*, 2013). Les glandes salivaires de la tique prennent par la suite une importance capitale pour le bon déroulement du repas sanguin. En effet, elles permettent la sécrétion du ciment, une colle biologique qui renforce la fixation de la tique à son hôte (Kemp *et al.*, 1982). Par la suite, les glandes salivaires vont libérer la salive qui va lyser les tissus, formant ainsi une poche hémorragique, permettant à la tique d'ingurgiter par aspiration le sang libéré des microcapillaires sanguins. En plus de sa propriété cytolytique, la salive de la tique a également des propriétés vasodilatatrices, anticoagulantes, anti-inflammatoires et immunosuppressives (Šimo *et al.*, 2017). La salive va donc être libérée, sporadiquement, pendant toute la durée du repas sanguin pour agrandir la poche de lyse et contrer le système immunitaire de l'hôte. Ainsi, c'est également *via* la salive que les tiques vont transmettre de nombreux agents pathogènes à leurs hôtes vertébrés.

2.1.3 Le rôle de vecteur et le cas des tiques de Guyane

Le rôle de vecteur de maladie des tiques est en effet au centre de la majorité des études sur ces arthropodes. Le mode d'alimentation de ces derniers, couplé au mode de transmission typiquement horizontale des micro-organismes responsables de maladies chez les vertébrés, font des tiques un des principaux groupes de vecteur connus (de la Fuente *et al.*, 2008 ; Jongejan and Uilenberg, 2004). La liste de potentiels agents pathogènes vectorisés par les tiques ne cesse effectivement d'augmenter et comprend

virus, bactéries et parasites eucaryotes (de la Fuente *et al.*, 2017). Le plus connu du public et certainement le plus étudié est la bactérie *Borrelia burgdorferii*, responsable de la maladie de Lyme, causant plusieurs tableaux cliniques chez l'humain. Cette pathologie est d'ailleurs la maladie à transmission vectorielle la plus fréquente de l'hémisphère nord, et ne semble pas ou peu sévir dans l'hémisphère sud où ses vecteurs ne sont pas présents. Néanmoins, la moitié sud du globe n'échappe pas aux maladies bactériennes transmises par les tiques. Par exemple, en Amérique du Sud, les différentes rickettsioses (causées par certaines *Rickettsia*) chez les humains, et anaplasmoses ou ehrlichioses (causées par les *Anaplasma* et *Ehrlichia*, respectivement) chez le bétail font partie des bactéries les plus étudiées dans un contexte de santé humaine et vétérinaire (Guglielmone *et al.*, 2006, 2003).

Toutefois, comme nous l'avons vu dans la partie contexte général de ce manuscrit, une dichotomie existe entre l'étude des tiques de l'hémisphère nord et celles du sud. Ce phénomène est parfaitement illustré par le cas des tiques de Guyane : aucune étude, ou presque, n'a été entreprise sur la faune acarologique de ce département français depuis une cinquantaine d'années. Paradoxalement, la Guyane est sujette à une épidémie de fièvre Q, causée par une souche très virulente de la bactérie intracellulaire *Coxiella burnetii* (Mahamat *et al.*, 2013). Cet agent pathogène a été associé, sans preuves formelles, avec une transmission potentielle par les tiques guyanaises du paresseux (*Amblyomma geayi*) (Davoust *et al.*, 2014 ; Eldin *et al.*, 2014 ; Epelboin *et al.*, 2016). De plus, depuis quelques années maintenant des syndromes de Baggio-Yoshinari, aussi nommés Brazilian Lyme-like disease, sévissent dans le pays voisin, le Brésil, et seraient potentiellement présents en Guyane (Basile *et al.*, 2017 ; Yoshinari *et al.*, 2010). Cette maladie serait causée par des formes atypiques de *B. burgdorferii* qui auraient évolué pour s'adapter à de nouveaux vecteurs comme les *Amblyomma* ou *Rhipicephalus* (Miziara *et al.*, 2018).

Néanmoins, le manque de preuves (convaincantes) et d'études à disposition sur ces maladies émergentes démontrent la nécessité d'appréhender le risque acarologique en Guyane par l'étude des tiques de ce département et de leurs agents pathogènes associés. **C'est d'ailleurs l'un des objectifs de cette thèse, sur lequel nous reviendrons dans le chapitre 1 pour la diversité des tiques présentes en Guyane, et dans le chapitre 4 pour l'étude des cortèges parasitaires hébergés par ces dernières.**

2.2. Le microbiome des tiques

2.2.1 Diversité, interactions et rôles

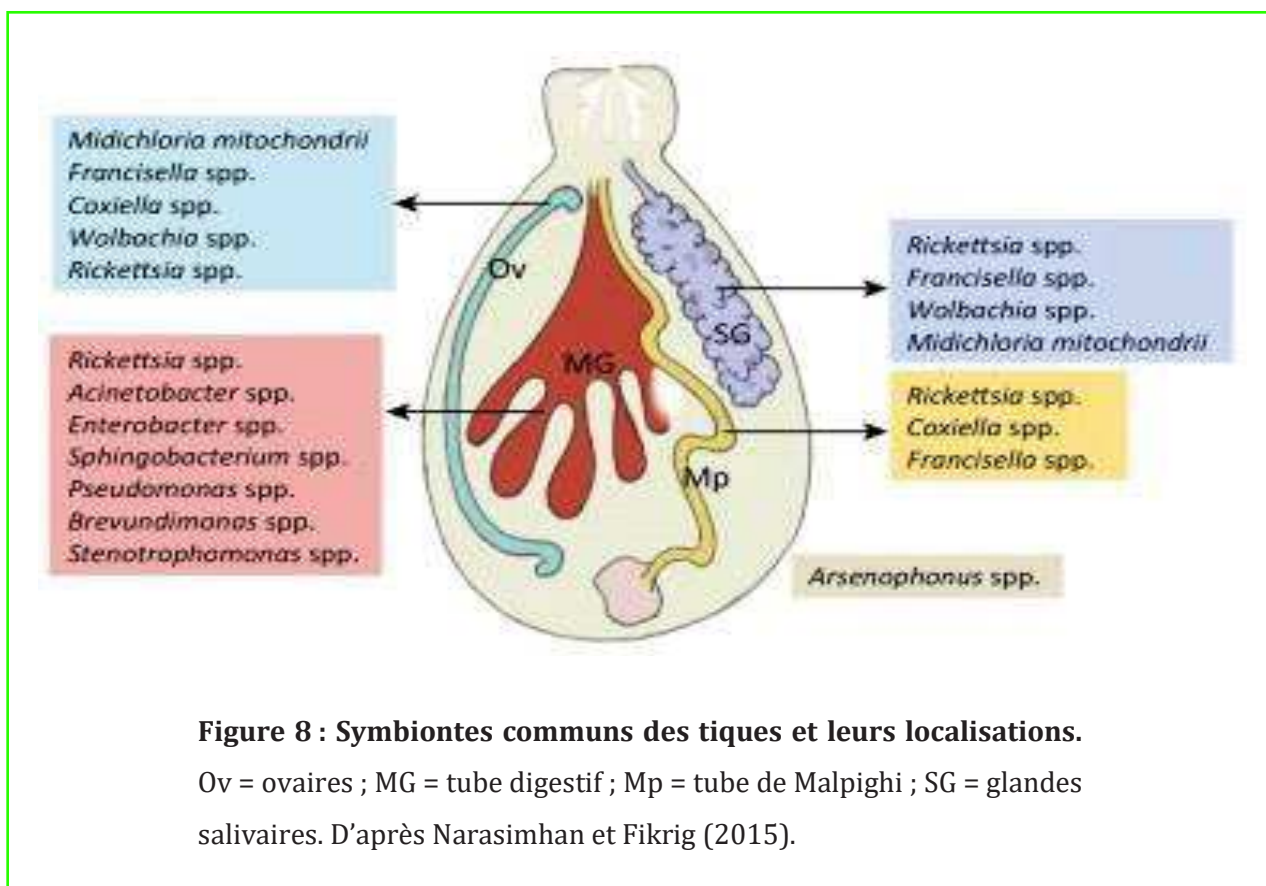
Les métazoaires et les arthropodes n'hébergent pas que des agents pathogènes mais des consortiums de microbes très variés, et les tiques ne dérogent pas à cette règle (Hanning and Diaz-Sanchez, 2015 ; Narasimhan and Fikrig, 2015). Ainsi, les tiques abritent par exemple l'une des plus grandes diversités de bactéries intracellulaires non pathogènes répertoriées : l'endosymbiose tiques/bactéries intracellulaires est décrite pour au moins dix genres bactériens, phylogénétiquement distants (Ahantarig *et al.*, 2013 ; Duron *et al.*, 2017). De manière surprenante, alors qu'une complémentation nutritionnelle par un P-symbionte a été prouvée chez la majorité des arthropodes hématophages stricts (Akman *et al.*, 2002 ; Allen *et al.*, 2007 ; Boyd *et al.*, 2014 ; Hosokawa *et al.*, 2010 ; Manzano-Marín *et al.*, 2015 ; Nikoh *et al.*, 2014 ; Rio *et al.*, 2016), ce n'était pas le cas jusqu'à récemment chez les tiques. En effet, bien que certains symbiontes comme *Coxiella*-like, *Francisella*-like présentent des preuves génomiques de synthèses de vitamine B (Duron *et al.*, 2018 ; Gerhart *et al.*, 2016 ; Gottlieb *et al.*, 2015 ; Smith *et al.*, 2015), aucune preuve physiologique de la symbiose mutualiste tique/bactérie n'avait été avancée. **Ainsi, dans une étude à laquelle j'étais associé durant cette thèse, il a été**

prouvé que la tique *Ornithodoros moubata* était incapable de satisfaire ses besoins en vitamines B *via* ses repas sanguins sans un P-symbionte bactérien intracellulaire : une *Francisella*-like (Duron *et al.*, 2018). En effet, une fois la tique privée de la bactérie *Francisella*, elle cesse son développement mais reprend une croissance normale grâce à une supplémentation artificielle en vitamines B, ce qui démontre le rôle de *Francisella* dans la nutrition des tiques (Duron *et al.*, 2018). De plus, différentes études portant sur le microbiome des tiques ont mis en exergue une remarquable diversité de procaryotes dominée par des bactéries extracellulaires non pathogènes. Ces micro-organismes représentent la majorité de la diversité taxonomique du microbiome et se répartissent dans la plupart des grands groupes bactériens (Andreotti *et al.*, 2011 ; Carpi *et al.*, 2011 ; Nakao *et al.*, 2013 ; Qiu *et al.*, 2014 ; Williams-Newkirk *et al.*, 2014).

Les micro-organismes qui habitent les tiques ne sont pas seulement taxonomiquement diversifiés, mais aussi écologiquement diversifiés. Cette diversité est clairement illustrée par le grand panel de stratégies évolutives que les symbiontes utilisent pour infecter et persister dans les populations de tiques. Les interactions tiques-micro-organismes occupent tout le continuum parasitisme/mutualisme : les agents pathogènes se propageant habituellement *via* TH lors du repas sanguin de la tique, et les symbiotes intracellulaires hautement spécialisés qui dépendent presque exclusivement de la transmission maternelle (TV) pour assurer leur persistance dans les populations de tiques (Klyachko *et al.*, 2007 ; Machado-Ferreira *et al.*, 2011 ; Lalzar *et al.*, 2014 ; Duron *et al.*, 2015 ; Duron *et al.*, 2018). De plus, des preuves de transmission mixte ont été mises en évidence chez certains symbiontes de tiques. Ainsi, grâce à des résultats obtenus lors de mon stage de Master 2, **nous avons pu réaliser lors de cette thèse, une étude phylogénétique sur la bactérie *Spiroplasma ixodetis*** indiquant une TV chez les tiques mais également des TH entre les tiques et d'autres arthropodes à une échelle évolutive

(Annexe A) (Binetruy *et al.*, 2019). De même, tout un panel de micro-organismes, dont des microbes environnementaux, colonise également les tiques. Ces dernières peuvent donc être « contaminées » par les organismes présents sur la peau de leurs hôtes ou par ceux présents dans le sol ou la végétation lors de leur phase libre (Narasimhan et Fikrig, 2015).

Grâce au développement des nouvelles technologies de séquençage, la localisation chez la tique de certains de ces symbiotes est en partie connue (Figure 8) (Narasimhan and Fikrig, 2015). Néanmoins, les interactions au sein du microbiome, le rôle des symbiotes sur la transmission d'agents pathogènes et leur impact sur le phénotype étendu des tiques restent très peu compris. **C'est dans ce contexte que j'ai co-écrit, au début de ma thèse, la review ci-après, faisant l'état des connaissances sur le microbiome des tiques et de son implication dans les aspects mentionnés avant** (Bonnet *et al.*, 2017).





The Tick Microbiome: Why Non-pathogenic Microorganisms Matter in Tick Biology and Pathogen Transmission

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Ticks are among the most important vectors of pathogens affecting humans and other animals worldwide. They do not only carry pathogens however, as a diverse group of commensal and symbiotic microorganisms are also present in ticks. Unlike pathogens, their biology and their effect on ticks remain largely unexplored, and are in fact often neglected. Nonetheless, they can confer multiple detrimental, neutral, or beneficial effects to their tick hosts, and can play various roles in fitness, nutritional adaptation, development, reproduction, defense against environmental stress, and immunity. Non-pathogenic microorganisms may also play a role in driving transmission of tick-borne pathogens (TBP), with many potential implications for both human and animal health. In addition, the genetic proximity of some pathogens to mutualistic symbionts hosted by ticks is evident when studying phylogenies of several bacterial genera. The best examples are found within members of the *Rickettsia*, *Francisella*, and *Coxiella* genera: while in medical and veterinary research these bacteria are traditionally recognized as highly virulent vertebrate pathogens, it is now clear to evolutionary ecologists that many (if not most) *Coxiella*, *Francisella*, and *Rickettsia* bacteria are actually non-pathogenic microorganisms exhibiting alternative lifestyles as mutualistic ticks symbionts. Consequently, ticks represent a compelling yet challenging system in which to study microbiomes and microbial interactions, and to investigate the composition, functional, and ecological implications of bacterial communities. Ultimately, deciphering the relationships between tick microorganisms as well as tick symbiont interactions will garner invaluable information, which may aid in the future development of arthropod pest and vector-borne pathogen transmission control strategies.

Keywords: tick, tick symbionts, tick borne pathogens, microbiome, microbial interactions

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INTRODUCTION

Over the last few decades, considerable research efforts have focused on the diversity, distribution, and impact of tick-borne pathogens (TBP). The list of known or potential TBP is constantly evolving, and includes a variety of viruses, bacteria, and parasites afflicting humans and many other animals worldwide (de la Fuente et al., 2008; Heyman et al., 2010; Dantas-Torres et al., 2012; Rizzoli et al., 2014). Less well studied or understood are whole microbial communities hosted by

ticks, which attract much less attention, yet are equally important. These communities include TBP, but also non-pathogenic microorganisms such as commensal and mutualistic microbes that are also abundant in ticks (Andreotti et al., 2011; Carpi et al., 2011; Williams-Newkirk et al., 2014; Duron et al., 2015a, 2017). Until recently, these non-pathogenic microorganisms were generally overlooked by scientists working with ticks and TBP. Before 1990, their existence was largely ignored and all bacteria found in ticks were usually considered to be potential TBP without necessarily undergoing rigorous health risk assessment. Toward the end of the 1990s, the advent of simple PCR assays led to a growing understanding that a few intracellular bacteria, such as the *Coxiella*-like endosymbiont and the *Francisella*-like endosymbiont (*Coxiella*-LE and *Francisella*-LE hereafter), are actually non-pathogenic microorganisms hosted by ticks (Niebylski et al., 1997a; Noda et al., 1997). Deeper investigation of microbial biodiversity through high-throughput sequencing and DNA barcoding led to another leap in understanding: non-pathogenic microorganisms from many different clades are present in ticks, and can generally coexist with TBP (Clay et al., 2008; Andreotti et al., 2011; Carpi et al., 2011; Lalzar et al., 2012; Vayssier-Taussat et al., 2013; Qiu et al., 2014; Williams-Newkirk et al., 2014; Narasimhan and Fikrig, 2015; Abraham et al., 2017). In addition, current available data on tick microbiomes suggest that non-pathogenic microorganisms exhibit higher taxonomic diversity than TBP since they encompass most major bacterial and Archaea groups (Andreotti et al., 2011; Carpi et al., 2011; Nakao et al., 2013; Qiu et al., 2014; Williams-Newkirk et al., 2014). Altogether, it is now clear that ticks carry complex microbial communities that are largely dominated by non-pathogenic microorganisms. Most importantly, this implies that both ticks and TBP are commonly engaged in interactions with non-pathogenic microorganisms.

The composition of these microbial communities is highly variable: environmental constraints are key drivers of their structure as shown by differences in bacterial diversity observed between laboratory-reared and wild ticks (Heise et al., 2010; Zolnik et al., 2016). It was further reported that bacterial community structures could vary depending on the examined tick species (Lalzar et al., 2012), the season during which ticks were collected (Lalzar et al., 2012), the examined geographical regions (van Overbeek et al., 2008; Carpi et al., 2011; Williams-Newkirk et al., 2014), the examined tick life stage (Moreno et al., 2006; Clay et al., 2008; Williams-Newkirk et al., 2014; Zolnik et al., 2016), and between different feeding statuses (Heise et al., 2010; Menchaca et al., 2013; Zhang et al., 2014). Furthermore, bacterial community structures may differ depending of the presence of pathogens (Steiner et al., 2008; Abraham et al., 2017). Overall, the quantity of potential variations highlights the lability of microbial communities hosted by ticks, and future studies should focus on understanding how these variations impact tick biology. Below, we will discuss the interesting hypothesis that the inherent flexibility of microbial communities may help ticks adapt to environmental stresses, such as TBP presence.

Microorganisms inhabiting ticks are not only taxonomically diverse, they are also ecologically diverse. This diversity is

clearly illustrated by the large panel of lifestyle strategies that microorganisms use to infect and persist within tick populations (Figure 1). As vertebrate pathogens, TBP normally spread via infectious (horizontal) transmission through tick bite and blood feeding. A few TBP can also be vertically transmitted in ticks, and thus be maintained throughout each generation as observed for *Babesia* species (Chauvin et al., 2009), *Rickettsia rickettsii* (Burgdorfer et al., 1981), or viruses (Xia et al., 2016). Other tick microorganisms are highly specialized intracellular symbionts depending almost exclusively on maternal (transovarial) transmission to ensure their persistence in tick populations (Niebylski et al., 1997b; Lo et al., 2006; Sasser et al., 2006; Klyachko et al., 2007; Felsheim et al., 2009; Machado-Ferreira et al., 2011; Lalzar et al., 2014; Duron et al., 2015a; Kurtti et al., 2015). Tick microorganism diversity is further augmented due to the fact that environmental microorganisms can also colonize ticks: microbes present on vertebrate skin surfaces may colonize ticks during blood feeding, while those present in the soil or vegetation can colonize ticks on the ground, once they have dropped off their vertebrate hosts (Narasimhan and Fikrig, 2015). Overall, the diverse range of microbial lifestyle strategies creates a complex web of interactions offering excellent opportunities to tackle questions about the impact of whole microbial communities on tick biology and TBP transmission. In spite of this, the direct effects of pathogens and other microbes on tick physiology and activity has received much less attention than their effects on vertebrate hosts. In most cases, the function of tick endosymbionts in relation to their host has not been determined. Many of these endosymbionts have obligate intracellular life cycles or are difficult to cultivate, which may explain the gaps in current knowledge (Tully et al., 1995; Kurtti et al., 1996; Niebylski et al., 1997a; Duron et al., 2015a). However, for some bacteria, tissue-specific localization has been defined, which may aid us to understand bacterial impact on both tick biology and pathogen transmission (Noda et al., 1997; Klyachko et al., 2007; Lalzar et al., 2014; Narasimhan and Fikrig, 2015). Similarly, the use of microarray or RNASeq technologies to analyze induced tick microbiome expression patterns and varying composition following a variety of conditions, may also further elucidate their roles (Rodriguez-Valle et al., 2010). This

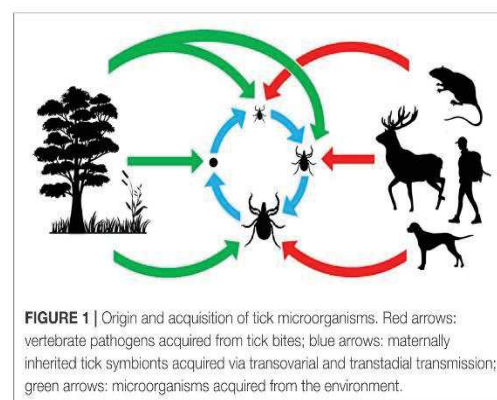


FIGURE 1 | Origin and acquisition of tick microorganisms. Red arrows: vertebrate pathogens acquired from tick bites; blue arrows: maternally inherited tick symbionts acquired via transovarial and transtadial transmission; green arrows: microorganisms acquired from the environment.

knowledge is of both medical and veterinary interest since it may enable the reassessment of tick-associated health risks, but also of ecological and evolutionary importance by highlighting co-evolutionary processes acting between ticks and their microbes. Indeed, some symbionts, but not all (Weller et al., 1998), have a joint evolutionary history of several million years with their tick hosts (Almeida et al., 2012; Duron et al., 2017), suggesting that complex interactions may have evolved in these associations. If biologists aim to fully understand the ecological and evolutionary processes involved in tick biology and the emergence of tick-borne diseases, a thorough examination of non-pathogenic microorganisms is also required.

Maternally-inherited symbionts are well-known to use specific adaptive strategies to spread and persist within arthropod populations, either providing fitness benefits to female hosts or manipulating host reproduction (Moran et al., 2008; Ferrari and Vavre, 2011). Two categories of widespread endosymbionts are usually recognized in arthropods, although intermediates and transition forms are also frequent:

- The first category consists of obligate (primary) mutualistic symbionts required to support normal host development, thus assisting their host in various essential functions. This includes nutritional diet upgrades by providing biosynthetic pathways absent from their hosts (Moran et al., 2008; Wernegreen, 2012). Indeed, most blood-feeding insects such as bed bugs, lice, and tsetse flies harbor obligate symbionts that provide B vitamins and cofactors not readily obtainable in sufficient quantities from a uniquely blood-based diet (Akman et al., 2002; Hosokawa et al., 2010; Boyd et al., 2013; Nikoh et al., 2014).
- The second category consists of facultative (secondary) symbionts not required for host survival. Some are defensive symbionts conferring protection against natural enemies or heat (Oliver et al., 2010; Ferrari and Vavre, 2011), while others are reproductive parasites that manipulate host reproduction through the induction of parthenogenesis, feminization of genetic males, male-killing, and cytoplasmic incompatibility (conditional sterility between infected and uninfected specimens) (Engelstadter and Hurst, 2009; Cordaux et al., 2011).

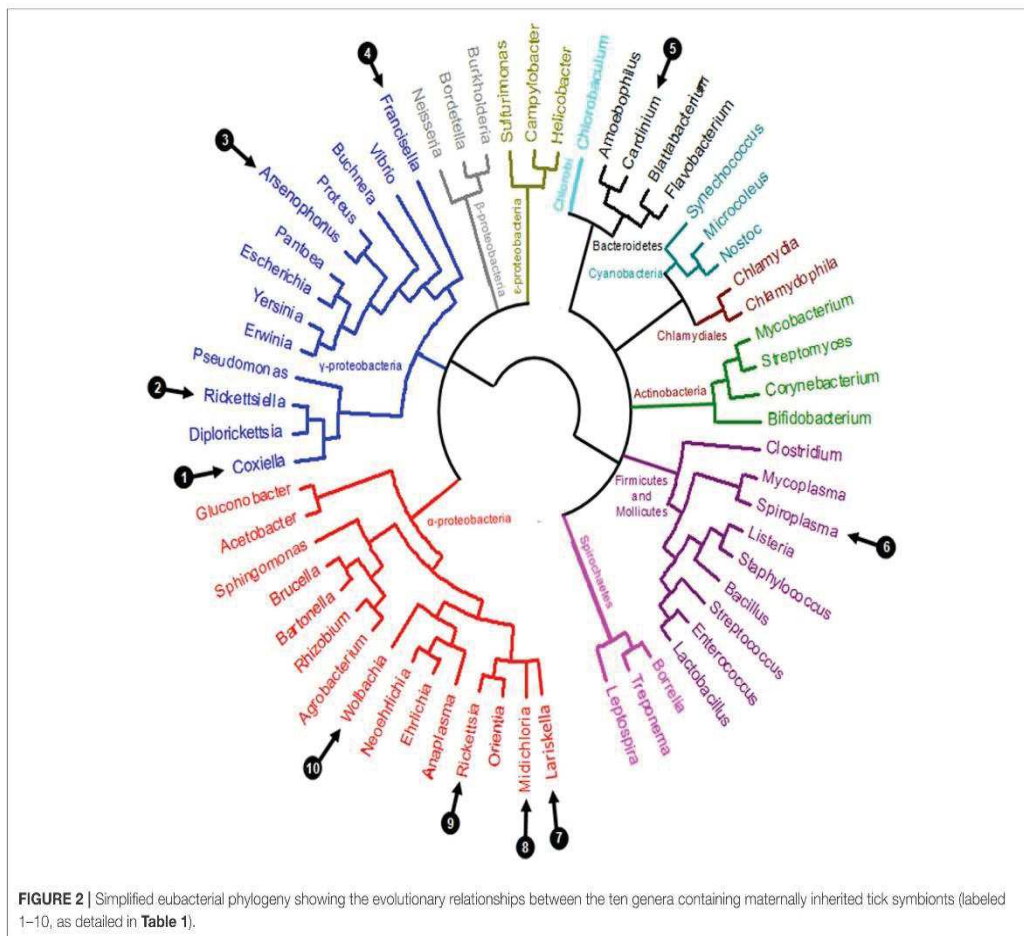
In this article we review four major biological aspects where our views on tick microbes have undergone substantial change over the last decade. Firstly, we must emphasize that non-pathogenic microorganisms have much more complex effects on ticks than previously thought. Indeed, it is now evident that several maternally-inherited symbionts are required for tick survival and reproduction, while other symbionts can have multiple effects on tick life history traits. Secondly, whilst tick TBP transmission modes have been studied for decades, we now understand that certain non-pathogenic microorganisms may also interfere with TBP transmission. Thirdly, although microorganisms are often categorized as “TBP,” “commensals,” or “maternally-inherited symbionts,” both intermediate and transitional states frequently occur. In this context, it thus appears vital to not overlook the full range of potential effects, as have been recently described in microbiome studies. And finally, bacterial phylogeny

demonstrates that several infectious agents have close genetic proximity with mutualistic tick symbionts. This indicates that some bacterial genera (eg. *Rickettsia*, *Francisella*, and *Coxiella*) have the capacity to frequently undergo evolutionary shifts between pathogenic and non-pathogenic forms, a process that may lead to the emergence of novel infectious diseases.

THE EFFECT OF NON-PATHOGENIC MICROORGANISMS ON TICK BIOLOGY

Perhaps the most remarkable observation of recent times is the pivotal role of symbiotic interactions in normal tick biology, including ecological specialization to an exclusive blood diet. Symbionts—i.e., microorganisms engaged in close and long-term interactions with their tick hosts—are exceptionally diverse in ticks: at least 10 distinct genera of maternally-inherited bacteria have been reported in ticks over the last decade (listed in **Table 1** and **Figure 2**) (Duron et al., 2017). Three of these symbionts are only found in ticks: *Coxiella*-LE, which infects at least two thirds of tick species, *Midichloria*, which inhabits the mitochondria of some tick species, and *Francisella*-LE, which has only been reported in a few tick species (**Table 1**). The seven remaining symbiont genera are more- or less-frequently found in other arthropod groups, including several well-studied insects. Five symbionts, *Wolbachia*, *Cardinium*, *Arsenophonus*, *Spiroplasma*, and *Rickettsia*, are commonly identified in some arthropod groups, while two others, *Rickettsiella* and *Lariskella*, have only been reported in a few other arthropod taxa in addition to ticks (**Table 1**).

Coxiella-LE has been reported as essential for tick survival and reproduction in the *Amblyomma americanum* lone star tick (Zhong et al., 2007). As an obligate symbiont, *Coxiella*-LE is, by definition, present in most individuals of a given tick species (Clay et al., 2008; Machado-Ferreira et al., 2011; Lalzar et al., 2012; Duron et al., 2015b, 2017): thus their mutualistic relationship is required for the survival of both organisms. Remarkably, some *Coxiella*-LE may form evolutionarily stable associations with their tick hosts that last for millions of years (Duron et al., 2017). These associations typically exhibit strict co-cladogenesis, resulting in congruent host-symbiont phylogenies as recently observed between members of the *Rhipicephalus* genus and their associated *Coxiella*-LE (Duron et al., 2017). The discovery of *Coxiella*-LE in numerous other tick groups (Jasinskas et al., 2007; Clay et al., 2008; Machado-Ferreira et al., 2011; Almeida et al., 2012; Lalzar et al., 2012; Duron et al., 2014, 2015a, 2017), indicates that it is the most widespread and biologically relevant tick symbiont. An examination of *Coxiella*-LE intra-host localization revealed pronounced tissue tropism in all examined tick species. This symbiont typically infects the ovaries (to ensure maternal transmission) and the distal part of Malpighian tubules, suggesting a possible role in nutrition, osmoregulation, or excretion (Klyachko et al., 2007; Machado-Ferreira et al., 2011; Lalzar et al., 2014). Examination of eggs from several tick species confirmed that *Coxiella*-LE is transmitted to >99% of tick progeny, demonstrating highly efficient maternal transmission (Machado-Ferreira et al., 2011; Lalzar et al., 2014;



Duron et al., 2015a). Remarkably, the *Coxiella*-LE genome was shown to encode major B vitamin synthesizing pathways such as biotin (B7 vitamin), folic acid (B9), riboflavin (B2), and their cofactors, that are not usually obtainable in sufficient quantities from a uniquely blood-based diet (Gottlieb et al., 2015; Smith et al., 2015). By ensuring nutritional upgrading of the blood diet, *Coxiella*-LE has enabled ticks to utilize an unbalanced dietary resource and thus become hematophagy specialists.

Recent studies have suggested that alternative obligate symbionts other than *Coxiella*-LE may also exist. Around one third of examined tick species lack *Coxiella*-LE or harbor *Coxiella*-LE at much lower frequencies than expected for an obligate symbiont (Duron et al., 2014, 2015a, 2017). A large survey of 81 tick species showed that in almost all tick species without *Coxiella*-LE infection, another maternally-inherited symbiont was usually present (Duron et al., 2017). Among these alternative obligate symbionts were *Francisella*-LE, *Rickettsia*, and *Rickettsiella*, which are often present in all specimens of the infected tick species (Duron et al., 2017). Although formal testing with nutritional and physiological experiments is now required to validate their role as alternative obligate symbionts,

recent bacterial genome data suggest that these bacteria have highly-evolved adaptive mechanisms enabling tick survival. Indeed, their genomes encode functions suggesting that they have—at least partially as for *Coxiella*-LE—a genetic capability for *de novo* B vitamin synthesis. Indeed, the *Francisella*-LE genomes from the fowl tick *Argas persicus* and the Gulf Coast tick *Amblyomma maculatum* contain complete genetic pathways for biotin, folic acid, and riboflavin biosynthesis (Sjodin et al., 2012; Gerhart et al., 2016). Similarly, recent metabolic reconstructions of *Rickettsia* genomes indicated that all genes required for folic acid biosynthesis are present in *Rickettsia* symbiont genomes obtained from both the black-legged tick *Ixodes scapularis* and the Western black-legged tick *I. pacificus* (Hunter et al., 2015). Worthy of note here is that laboratory findings directly corroborate the existence of beneficial *Rickettsia* symbionts since they exert a significant effect on larval motility of *A. americanum*, *Dermacentor variabilis*, and *I. scapularis* ticks (Kagemann and Clay, 2013). Overall, these maternally-inherited symbionts are thus of ecological and evolutionary importance to the tick species they infect, and potentially mediate tick adaptation to hematophagy. In addition, it should be notice

TABLE 1 | List of the ten maternally inherited bacteria found in ticks and relevant (non-exhaustive) references.

Maternally inherited bacteria	Distribution in ticks and other arthropods	Major properties (non-exhaustive references)
1- <i>Coxiella</i> -LE	Very common in ticks, not found in other arthropods (Noda et al., 1997; Jasinskis et al., 2007; Clay et al., 2008; Carpi et al., 2011; Machado-Ferreira et al., 2011; Almeida et al., 2012; Lalzar et al., 2012; Duron et al., 2014, 2015a, 2017)	Obligate symbiont in most tick species (Zhong et al., 2007; Gottlieb et al., 2015; Smith et al., 2015). Closely related to the agent of Q fever, <i>C. burnetii</i> (Duron et al., 2015a)
2- <i>Rickettsiella</i>	Scattered distribution in arthropods (Tsuchida et al., 2010, 2014; Bouchon et al., 2012; lasur-Kruh et al., 2013), common in ticks (Kurtti et al., 2002; Vilcins et al., 2009; Anstead and Chilton, 2014; Duron et al., 2015a, 2016, 2017)	Unknown effect in ticks. Facultative mutualist in aphids (Tsuchida et al., 2010, 2014) and likely in other insects (lasur-Kruh et al., 2013). Some strains are entomopathogenic (Bouchon et al., 2012)
3- <i>Arsenophonus</i>	Common in arthropods (Duron et al., 2008a; Novakova et al., 2009), present in ticks (Clay et al., 2008; Dergousoff and Chilton, 2010; Reis et al., 2011; Clayton et al., 2015; Duron et al., 2017)	Male-killer in parasitoid wasps (Werren et al., 1986; Duron et al., 2010), putative obligate symbiont in bat flies and louse flies (Duron et al., 2014), facultative symbiont in other insects (Novakova et al., 2009; Jousset et al., 2013). In the sheep tick, <i>Ixodes ricinus</i> , detection of <i>Arsenophonus</i> may be due to contamination by a hymenopteran parasitoid (Bohacsova et al., 2016)
4- <i>Francisella</i> -LE	Rare in ticks, not found in other arthropods (Niebylski et al., 1997a; Noda et al., 1997; Scoles, 2004; Goethert and Telford, 2005; Clayton et al., 2015; Gerhart et al., 2016; Duron et al., 2017)	Unknown effect in most cases but alternative obligate symbiont in some tick species (Gerhart et al., 2016; Duron et al., 2017); closely related to the agent of tularaemia (<i>F. tularensis</i>) (Sjodin et al., 2012)
5- <i>Cardinium</i>	Common in arthropods (Zchori-Fein and Perlman, 2004; Duron et al., 2008a,b), present in ticks (Kurtti et al., 1996; Benson et al., 2004; Duron et al., 2017)	Unknown effect in ticks. Reproductive manipulator in diverse insect species (Engelstadter and Hurst, 2009)
6- <i>Spiroplasma</i>	Common in arthropods (Weinert et al., 2007; Duron et al., 2008a), present in ticks (Tully et al., 1981, 1995; Henning et al., 2006; Duron et al., 2017)	Unknown effect in ticks. Male-killer in diverse insect species (Engelstadter and Hurst, 2009)
7- <i>Lariskella</i>	Rare and with a scattered distribution in arthropods (Matsuura et al., 2012; Toju et al., 2013), rarely reported in ticks (Qiu et al., 2014; Duron et al., 2017)	Unknown effect
8- <i>Mitochondria</i>	Present in ticks, not found in other arthropods (Lo et al., 2006; Epis et al., 2008; Venzal et al., 2008; Reis et al., 2011; Najm et al., 2012; Qiu et al., 2014; Williams-Newkirk et al., 2014; Paul et al., 2016; Bonnnet et al., 2017; Duron et al., 2017)	Unknown effect; inhabits tick mitochondria (Epis et al., 2013)
9- <i>Rickettsia</i>	Common in arthropods (Perlman et al., 2006; Weinert et al., 2009), present in ticks (Niebylski et al., 1997b; Clayton et al., 2015; Kurtti et al., 2015; Duron et al., 2017)	Unknown effect in ticks. Reproductive manipulator in diverse insect species (Engelstadter and Hurst, 2009) and defensive symbiont in other insects (Lukasik et al., 2013) closely related to pathogenic strains, often tick-borne, infecting vertebrates (Perlman et al., 2006; Darby et al., 2007; Weinert et al., 2009)
10- <i>Wolbachia</i>	Very common in arthropods (Duron et al., 2008b; Zug and Hammerstein, 2012), present in ticks (Andreotti et al., 2011; Carpi et al., 2011; Reis et al., 2011; Duron et al., 2017)	Unknown effect in ticks. Reproductive manipulation in many arthropods (Engelstadter and Hurst, 2009), facultative mutualist (defensive symbiosis) in others such as mosquitoes (Brownlie and Johnson, 2009; Hamilton and Perlman, 2013), obligate symbiont in bed bugs (Hosokawa et al., 2010; Nikoh et al., 2014). At least in the case of the sheep tick, <i>Ixodes ricinus</i> , it has been demonstrated that the detection of <i>Wolbachia</i> was due to a contamination by a hymenopteran parasitoid (Plantard et al., 2012)

Adapted from Duron et al. (2017).

that some tick-borne pathogenic Anaplasmataceae bacteria (including *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, and *Neorickettsia sennetsu*) are also able to synthesize all major vitamins, suggesting that they may also confer a beneficial role in ticks when present (Dunning Hotopp et al., 2006).

Many *Rickettsia* species are well-known TBP, therefore a comment on the true ecological diversity existing within the

Rickettsia genus is appropriate. Indeed, most of the novel *Rickettsia* species or strains discovered in recent years are found exclusively in arthropods and never in vertebrates (Perlman et al., 2006; Darby et al., 2007; Weinert et al., 2009). In ticks, as for many other arthropods, these *Rickettsia* are not pathogenic but are actually maternally-inherited symbionts with poorly known effects on tick biology. This is the case for the *Rickettsia*

species identified in the black-legged tick *I. scapularis* (*Rickettsia buchneri*, formerly known as *Rickettsia* REIS; Kurtti et al., 2015), the American dog tick *Dermacentor variabilis* (*Rickettsia peacockii*; Felsheim et al., 2009), and likely the tree-hole tick *Ixodes arboricola* (*Rickettsia vini*; Duron et al., 2017). The fact that ticks carry both pathogenic and non-pathogenic *Rickettsia* that may interact (as early reported by Burgdorfer et al., 1981), underscores the need to be able to clearly distinguish between the two in further studies on these bacteria.

Along with obligate symbionts, ticks commonly harbor facultative symbionts belonging to a variety of bacterial genera (listed in Table 1). Examination of a representative collection of 81 tick species (i.e., approximately 10% of known tick species and including both soft and hard ticks) illustrated facultative symbiont diversity, since it revealed the presence of maternally-inherited bacteria in almost all species (79 of 81) (Duron et al., 2017). Remarkably, many of these tick species (44) hosted more than one symbiont. In multi-infected tick species, symbionts assembled in communities which could reach high levels of complexity. Indeed, six distinct genera of maternally-inherited symbionts co-exist in sheep tick *I. ricinus* populations (*Midichloria*, *Spiroplasma*, *Coxiella*-LE, *Rickettsia*, *Wolbachia*, and *Rickettsiella*) and in African blue tick *Rhipicephalus decoloratus* populations (*Midichloria*, *Coxiella*-LE, *Francisella*-LE, *Rickettsia*, *Cardinium*, and *Spiroplasma*) (Duron et al., 2017). It should be noted that detecting a heritable bacterium can sometimes be due to cross-contamination as for several *I. ricinus* studies. Some recorded *Wolbachia* and *Arsenophonus* infections were actually due to the cryptic presence of a *Wolbachia*- and *Arsenophonus*-infected endoparasitoid wasp, *Ixodiphagus hookeri*, within tick tissues (Plantard et al., 2012; Bohacsova et al., 2016). As a result, the presence of at least some of these tick symbionts must be treated with caution.

Although the role of these facultative symbionts is not yet clearly elucidated, one study suggested that *Arsenophonus* sp. can affect host-seeking success by decreasing *A. americanum*, *I. scapularis*, and *D. variabilis* tick motility when *Rickettsia* symbionts increased such mobility (Kagemann and Clay, 2013). Recent sequencing and analysis of the *Midichloria mitochondrii* genome led to the hypothesis that the bacteria could serve as an additional ATP source for the host cell during oogenesis (Sassera et al., 2011). In addition, this symbiont has been ascribed a possible helper role in tick molting processes (Zchori-Fein and Bourtzis, 2011). As mentioned above, there is no evidence to date showing that the *Wolbachia* detected in ticks are “true” tick symbionts (Plantard et al., 2012). Interestingly, in insects, *Wolbachia* is known to act as defensive endosymbionts (reviewed by Brownlie and Johnson, 2009), or as manipulator of host reproduction (review in Engelstadter and Hurst, 2009; Cordaux et al., 2011), suggesting that similar effect may exist in ticks.

The same questioning are required regarding *Arsenophonus nasoniae*: known to be responsible for sex-ratio distortion in diverse arthropod species (Werren et al., 1986; Duron et al., 2010) but of unknown effect in ticks. Finally and interestingly, a *Rickettsiella* symbiont has been observed in a parthenogenetic laboratory colony of the tick *I. woodi* (Kurtti et al., 2002). This tick species is generally known to be bisexual, suggesting that *Rickettsiella* infection may induce asexuality which represents

to date the only demonstration of sex ratio distortion in ticks possibly due to a symbiont.

High symbiont infection frequency is rarely observed within each tick species, and an intermediate infection frequency is much more common (Noda et al., 1997; Clay et al., 2008; Lalzar et al., 2012; Duron et al., 2017). Interestingly, infection frequencies of each maternally-inherited symbiont are often variable between geographic populations of a given tick species (Clay et al., 2008; Lalzar et al., 2012; Duron et al., 2017). This is the case for the soft tick *Ornithodoros sonrai*, with *Midichloria* and *Rickettsia* reaching high infection frequencies in some populations, but remaining absent from others (Duron et al., 2017). Similar geographical patterns were observed for many other tick species and for several symbionts such as *Rickettsiella* and *Spiroplasma* in the tree-hole tick *I. arboricola* and the polar seabird tick *I. uriae* (Duron et al., 2016, 2017). These patterns strongly suggest that maternally-inherited symbiont infection dynamics are variable across tick populations and species. Such infection frequency variations may be driven by costs and benefits associated with harboring maternally-inherited symbionts, and may maintain intermediate frequencies in tick populations, as is commonly observed in other arthropod species (Oliver et al., 2010). However, even if the nature of these costs and benefits has been well-studied in insects, they remain to be determined in ticks. Another important parameter in our understanding of this infection dynamics may be variation of biological features between tick males and females: indeed, adult males from *Ixodes* species do not blood-feed, contrary to females, which may imply that adult males do not need nutritional symbionts. The importance of this “sex” parameter to symbiont infection dynamics remains also to be demonstrated. Interestingly, this pattern was observed for *Midichloria* with males less commonly infected than females in *I. ricinus* (Lo et al., 2006). This suggests that *Midichloria* may be an important nutritional symbiont for *I. ricinus*, as recently proposed (Duron et al., 2017).

Along with maternally-inherited symbionts, other non-pathogenic microbes are present in ticks (Andreotti et al., 2011; Carpi et al., 2011; Narasimhan et al., 2014; Qiu et al., 2014; Williams-Newkirk et al., 2014; Abraham et al., 2017). Most are likely to inhabit the tick gut, while others may also colonize the tick surface cuticle. Overall, the biological effects of these non-pathogenic microbes on ticks remain entirely unknown, but it is likely that those inhabiting the tick gut could participate in blood meal digestion (Narasimhan and Fikrig, 2015) and complex interactions with TBP as we further detailed.

NON-PATHOGENIC MICROORGANISMS INTERACT WITH TBP IN DIFFERENT WAYS

An alternative fascinating aspect is that non-pathogenic microorganisms can also interfere with TBP replication and transmission by influencing TBP abundance and diversity in tick populations, as well as their transmission to vertebrate hosts. All aforementioned non-pathogenic bacteria present in ticks could have the potential to impact TBP infection processes in different ways. One can assume that TBP and non-pathogenic

microorganisms may neutralize each other because they are in direct competition for limited resources, such as a particular nutrient or tissue, or because they stimulate the same immune system function. Alternatively, non-pathogenic microorganisms may excrete molecules directly inhibiting the growth of a TBP competitor, or, inversely, facilitate TBP development by immunosuppressing the tick host. As a result, non-pathogenic microorganisms may facilitate, limit, or block TBP transmission, depending on the nature of tick microbial interactions.

Equally pertinent in this regard is the role of the microbiota inhabiting the tick gut (Narasimhan et al., 2014; Abraham et al., 2017). The tick gut represents the TBP entry point, therefore gut microbiota can directly mediate TBP colonization and influence their early survival (Narasimhan and Fikrig, 2015). This finding has been perfectly illustrated in a recent study which manipulated the gut microbiota of the black-legged tick *I. scapularis*: specimens reared in sterile containers (i.e., thus preventing external bacterial contamination) showed increased engorgement weights, and decreased colonization by the causative agent of Lyme disease, *Borrelia burgdorferi*, when compared to normal specimens (Narasimhan et al., 2014). Similarly, ticks fed on antibiotic-treated mice exhibited modified gut microbiota that also resulted in increased feeding and low *B. burgdorferi* colonization rates (Narasimhan et al., 2014). Altering the gut microbiota was actually found to decrease production of a glycoprotein from the tick peritrophic matrix, which separates the gut lumen from the epithelium. This peritrophic matrix is pivotal for *Borrelia* colonization success, as it protects *B. burgdorferi* colonizing the gut epithelial cells from toxic gut lumen compounds. Compromised peritrophic matrix due to altered gut microbiota will thus impede *B. burgdorferi* colonization. However, the reverse is true for another TBP, the anaplasmosis agent, *A. phagocytophilum* (Abraham et al., 2017). Remarkably, this bacterium manipulates the gut microbiota of *I. scapularis* to favor its establishment. By inducing tick glycoprotein production, *A. phagocytophilum* partially blocks bacterial biofilm formation, and thus reduces peritrophic matrix integrity, rendering the tick more susceptible to infection (Abraham et al., 2017). Altogether, these observations have uncovered a “Dr. Jekyll and Mr. Hyde”-like role of the tick gut microbiota, so that an unaltered gut microbiota will favor colonization by some TBP, such as *Borrelia*, whereas it may also block colonization by other TBP, such as *Anaplasma*. This antagonistic effect of tick gut microbiota on TBP may explain the rarity of *Borrelia-Anaplasma* co-infections in ticks collected from the field (Abraham et al., 2017).

Other interaction mechanisms may also exist. In well-studied animals, such as insects, antagonistic interactions arise when horizontally-transmitted parasites and vertically-transmitted microorganisms co-infecting the same host have conflicting evolutionary interests (Haine et al., 2005; reviewed by Haine, 2008; Ben-Ami et al., 2011; Hamilton and Perlman, 2013). Vertically-transmitted microorganisms, such as maternally-inherited symbionts, are under strong selection pressure to enhance the reproductive success of the hosts they infect (Moran et al., 2008). Conversely, parasites are typically transmitted between unrelated hosts and are therefore not directly affected by altered host fecundity. This conflict of interest has favored

the emergence of defensive symbionts in insects, including maternally-inherited symbionts that protect their insect host against a variety of pathogens (reviewed by Haine, 2008; Brownlie and Johnson, 2009). For instance, some maternally-inherited symbionts such as *Wolbachia* may interfere with the replication and transmission of a wide range of pathogens (including viruses, bacteria, protozoa, nematodes, and parasitoids), and protect insects from parasite-induced mortality, possibly by up-regulating the insect's immune system (Brownlie and Johnson, 2009; Gross et al., 2009). Recently, some of these findings have been applied to the development of parasite control methods, where *Wolbachia* infection has been used to limit the vector competence of mosquitoes (Hoffmann et al., 2011). In comparison, very little was known about the existence of defensive symbionts in ticks. However, Burgdorfer et al. were the first to report the presence of defensive symbionts in the Rocky Mountain wood tick *Dermacentor andersoni*. In this tick, a maternally-inherited symbiont, *Rickettsia peacockii*, hampered the multiplication and transovarial transmission of the spotted fever agent, *Rickettsia rickettsii* (Burgdorfer et al., 1981). Similarly, resistance of the ovaries of *D. variabilis* to co-infection with *Rickettsia montana* and *Rickettsia rhipicephali* has been reported (Macaluso et al., 2002). Other experiments on *D. andersoni* further showed that *A. marginale* infection density was negatively correlated to the infection density of another maternally-inherited symbiont, *Rickettsia belli* (Gall et al., 2016). In *I. scapularis*, it has also been reported that male ticks infected by the maternally-inherited symbionts *Rickettsia buchneri* have significantly lower rates of *B. burgdorferi* infection than symbiont-free males (Steiner et al., 2008). Overall, these observations suggested that the maternally-inherited *Rickettsia* symbionts may be major defensive symbionts protecting ticks against TBP colonization. As a result, *Rickettsia* symbionts may be a key factor influencing TBP abundance and diversity in tick populations.

Conversely, maternally-inherited symbionts may not always protect ticks against pathogens: the presence of one maternally-inherited symbiont, *Francisella*-LE, in *D. andersoni* was positively associated with pathogenic *Francisella novicida* infection (Gall et al., 2016). Because these results were only obtained following laboratory manipulations, they should be treated with caution as *F. novicida* is not considered to be a TBP, as the majority of people infected with *F. novicida* contract the pathogen after ingesting infected water or ice, and not via tick bites. This study thus relies on an artificial *F. novicida* tick infection that is unlikely to happen in the field, and most importantly, using a pathogen that has not co-evolved with tick symbionts. This naturally raises the question of whether *Francisella*-LE can actually protect *D. andersoni* against TBPs that naturally occur in this tick species.

TICK SYMBIONTS CAN BE OPPORTUNISTIC VERTEBRATE PATHOGENS

Although biologists often classify host-microbe relationships as either “mutualism,” “commensalism,” or “parasitism,” there are difficulties in defining the boundaries of these definitions. Rather,

host-microbe relationships should be best described as a broad continuum, as intermediate states and transitions between states occur frequently. Several maternally-inherited tick symbionts are remarkable examples of this continuum, as recent literature has reported that certain symbionts may be transmitted to vertebrates following tick bite, as will be detailed further in this section (Shivaprasad et al., 2008; Woc-Colburn et al., 2008; Vapniarsky et al., 2012; Bazzocchi et al., 2013; Edouard et al., 2013; Angelakis et al., 2016; Seo et al., 2016a; Bonnet et al., 2017). Most importantly, some of these symbionts have the potential to opportunistically infect vertebrate hosts, including humans.

Maternally-inherited arthropod symbionts are commonly thought to be exclusively domesticated by their arthropod hosts: they cannot invade naïve hosts and have evolved to be dependent on arthropod-based transmission mechanisms through transovarial inheritance (Moran et al., 2008; Wernegreen, 2012). However, some tick symbionts, such as certain *Coxiella*, *Midichloria* and *Arsenophonus* strains, are not actually completely dependent on ticks. Rather than strictly maternal, their transmission may be partially horizontal, i.e., infectious, thus presenting a substantial infection risk to vertebrates (Shivaprasad et al., 2008; Woc-Colburn et al., 2008; Vapniarsky et al., 2012; Bazzocchi et al., 2013; Edouard et al., 2013; Angelakis et al., 2016; Seo et al., 2016a). Among these symbionts, *Coxiella*-LE are the most commonly found microorganisms in vertebrates. Indeed, tick-transmitted *Coxiella*-LE has recently been reported to cause mild infectious symptoms in humans from Europe (Angelakis et al., 2016). These microorganisms were notably detected in human skin biopsy samples and may be a common causative agent of scalp eschar and neck lymphadenopathy. *Coxiella*-LE infections have also been occasionally reported in pet birds such as psittacines and toucans reared in North America (Shivaprasad et al., 2008; Woc-Colburn et al., 2008; Vapniarsky et al., 2012). These latter *Coxiella*-LE can cause fatal disease: infected birds exhibited lethargy, weakness, emaciation, and progressive neurologic signs for several days prior to death. Conversely, another *Coxiella*-LE was identified in several South Korean horse blood samples, but none of the horses showed apparent symptoms of infection (Seo et al., 2016b).

The ability of *Coxiella*-LE to infect vertebrates through tick biting is at least partially explained by their tissue tropism within the tick body. Aside from tick ovaries and Malpighian tubules, examination of tick internal organs also revealed substantial *Coxiella*-LE concentrations within the salivary glands of some tick species (Klyachko et al., 2007; Machado-Ferreira et al., 2011; Qiu et al., 2014) but not in others (Liu et al., 2013; Lalar et al., 2014). This tissue tropism may enable *Coxiella*-LE release into the vertebrate during tick bite, thus favoring opportunistic infections (Duron et al., 2015a). The overall likelihood of such *Coxiella*-LE tick-to-vertebrate transfers seems high since (i) ticks are found worldwide and feed on many different vertebrate species, (ii) at least two thirds of tick species are infected by *Coxiella*-LE, and (iii) when present in a given tick species, *Coxiella*-LE are usually present in almost all specimens (Duron et al., 2015a). Overall, these observations suggest that, through tick parasitism, vertebrates are often exposed to *Coxiella*-LE, and probably at

a higher rate than TBP. However, despite this, *Coxiella*-LE infections are very rare in vertebrates, and most strains described to date have only been identified from ticks (Duron et al., 2015a). It is thus thought that these bacteria pose a low infection risk to vertebrates because their genome seems to be extremely reduced and is devoid of known virulence genes (Gottlieb et al., 2015; Smith et al., 2015). Nonetheless, *Coxiella*-LE have the potential to cause rare infections in vertebrates and should always be considered in future studies on tick-borne diseases.

In comparison, vertebrate infections by symbionts other than *Coxiella*-LE are clearly less common. This includes the maternally-inherited symbiont *Arsenophonus*, present in approximately 5% of terrestrial arthropods, including some tick species (Duron et al., 2008a, 2017). *Arsenophonus* is actually unique among maternally-inherited symbionts because it is able to grow outside arthropod cells, in extracellular environments (Huger et al., 1985; Werren et al., 1986). This ability enhances the likelihood of successful opportunistic *Arsenophonus* infection, as was recently observed in a woman who was bitten by a tick during a trip to Southeast Asia. This patient presenting with a rash and an eschar developed a co-infection with *Arsenophonus* and *Orientia tsutsugamushi* (the causative agent of scrub typhus) (Edouard et al., 2013). In this context, it is likely that rash and eschar development following *Orientia* infection may have favored a secondary, opportunistic, *Arsenophonus* infection.

In other cases, the identification of symbionts as opportunistic vertebrate pathogens is more difficult and remains speculative. This is the case for *Midichloria*, an intra-mitochondrial symbiont of the sheep tick *I. ricinus* and a few other tick species. Several lines of evidence have recently suggested that vertebrate hosts can be inoculated with *Midichloria* during a tick bite. Indeed, most *Midichloria* are localized in the tick ovaries, where they are transmitted to the progeny, but some have also been detected in the salivary glands and saliva of *I. ricinus* (Di Venere et al., 2015). In addition, *Midichloria* DNA, as well as antibodies against a *Midichloria* antigen, were detected in the blood of vertebrates exposed to tick bites (Bazzocchi et al., 2013). However, whether *Midichloria* can cause a true infection and pathological alteration in mammalian hosts remains to be determined.

PATHOGENS AND TICK SYMBIONTS ARE OFTEN PHYLOGENETICALLY RELATED

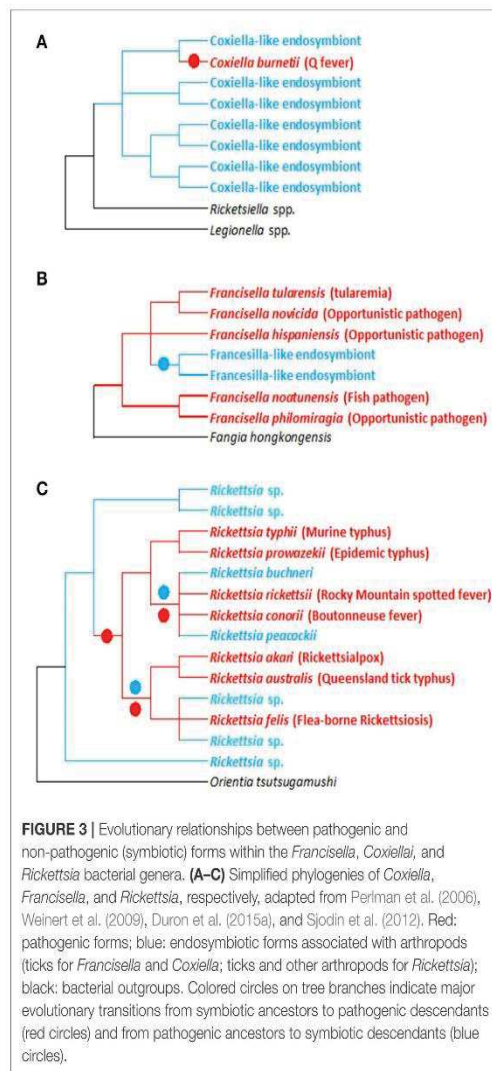
The vast range of intracellular bacteria in ticks is particularly illustrative of their propensity to evolve extreme and contrasting phenotypes. Certain species, such as *Rickettsia* spp. and *Coxiella* spp., have taken eukaryote associations to the extreme by completely abandoning any semblance of a free-living phase and replicating solely within the host cell. However, they do use a large panel of lifestyle strategies to spread and persist within host populations: while some are extremely virulent pathogens, others behave as subtle mutualistic symbionts (Perlman et al., 2006; Darby et al., 2007; Weinert et al., 2009; Sjodin et al., 2012; Duron et al., 2015a, 2017; Gerhart et al., 2016). Although both strategies require high degrees of lifestyle specialization, they are not fixed endpoints along the bacterium-eukaryote interaction spectrum;

rather, parasitism and mutualism may shift through repeated evolutionary transitions. This explains why both pathogenic and mutualistic forms of several major bacterial genera commonly hosted by ticks are abundantly represented.

The foremost examples of these transitions are found in three major intracellular bacteria genera: *Coxiella*, *Francisella*, and *Rickettsia* (Figure 2), which are all commonly identified in ticks. In medical and veterinary research, these intracellular bacteria are traditionally recognized as highly virulent vertebrate pathogens, as they have evolved specific mechanisms to penetrate into the host cytosol, appropriate nutrients for replication, subvert host immune responses, and ultimately enable infectious transmission to a new host individual (Darby et al., 2007; Celli and Zahrt, 2013; van Schaik et al., 2013; Jones et al., 2014). In humans, major intracellular pathogens have been identified from these bacterial groups, as exemplified by the agent of Q fever, *Coxiella burnetii*, the agent of tularaemia, *Francisella tularensis*, the agent of epidemic typhus, *Rickettsia prowazekii*, the agent of Rocky Mountain spotted fever, *Rickettsia rickettsia*, or the causative agent of Mediterranean spotted fever, *Rickettsia conorii* (Figure 2). All of these organisms are extremely infectious and some are currently classified as potential weapons for biological warfare (Darby et al., 2007; Celli and Zahrt, 2013; van Schaik et al., 2013; Jones et al., 2014). In addition, several species of tick-borne bacteria as typified by rickettsiae that were considered non-pathogenic for decades are now associated with human infections (Parola et al., 2013; Bonnet et al., 2017). However, as we have detailed above, novel intracellular bacteria engaged in endosymbiotic associations with arthropod hosts have also recently been discovered within each of these groups (Figure 2).

Phylogenetic investigations have revealed rapid and repeated evolutionary shifts within these three genera between pathogenic (associated with vertebrates and, in some cases, vectored by arthropods) and endosymbiotic forms (specifically linked to arthropods). However, the evolutionary shifting pattern varies among genera (Figure 2). In *Coxiella*, complementary lines of argument indicate a recent emergence of the Q fever agent, *C. burnetii*, from a *Coxiella*-LE strain hosted by soft ticks (Duron et al., 2015a). The *Coxiella* genus displays extensive genetic diversity, with at least four highly divergent clades (Duron et al., 2015a). While *Coxiella*-LE strains hosted by ticks are found in all these clades, all *C. burnetii* strains cluster within one of these clades, delineating an embedded group among soft tick *Coxiella*-LE (Figure 3A). This phylogenetic pattern indicates that the ancestor of *C. burnetii* was a tick-associated *Coxiella* which succeeded in infecting vertebrate cells (Duron et al., 2015a). The remarkably low genetic diversity of *C. burnetii* indicates unique and recent emergence of this highly infectious vertebrate pathogen (Duron et al., 2015a). Interestingly, this hypothesis was initially raised a decade ago from observations of the profound differences in *C. burnetii* genome content relative to other pathogenic intracellular bacteria (Seshadri et al., 2003).

Similarly, in *Rickettsia* spp., recent evidence revealed that human pathogens—vectored by blood feeding arthropods such as ticks—emerged relatively late in the evolution of this genus (Figure 3C; Perlman et al., 2006; Darby et al., 2007; Weinert et al., 2009). Phylogenetic investigations taking into account



the entire *Rickettsial* diversity (i.e., including pathogenic and non-pathogenic forms) clearly indicate that switching between hosts (invertebrates, vertebrates, and even plants) has been a common feature of *Rickettsia* evolution (Perlman et al., 2006; Darby et al., 2007; Weinert et al., 2009). Based on current data, it is difficult to estimate how often vertebrate pathogenesis has evolved within *Rickettsia*. But as intracellular adaptation to arthropods is a feature of all current *Rickettsia*, it suggests that their most common recent ancestor was adapted to arthropod endosymbiosis. Surprisingly, comparing human pathogens with closely related non-pathogens showed no relationships between pathogenicity and the acquisition of novel virulence genes: vertebrate virulence seems to occur rather as result of lost or malfunctioning replication systems (Darby et al., 2007).

Conversely, in *Francisella*, the evolutionary pattern is substantially different, since most of the diversity found in this genus is due to pathogenic or opportunistic species (Sjodin et al., 2012). Very little is known about the evolution and origin of tick *Francisella*-LE (Michelet et al., 2013; Gerhart et al., 2016; Duron et al., 2017). However, the few *Francisella*-LE species identified to date delineate a unique monophyletic clade that clearly originates from pathogenic forms (Figure 3B; Duron et al., 2017). Interestingly, the *Francisella*-LE genome is similar in size to pathogenic *Francisella* species' genomes, but about one-third of the protein-coding genes are pseudogenized and are likely non-functional (Gerhart et al., 2016). This suggests that *Francisella*-LE is undergoing a global process of genome reduction, an evolutionary development typically observed in maternally-inherited symbionts (Moran et al., 2008). Interestingly, *Francisella*-LE has conserved intact most of its genes involved in B vitamin biosynthesis, highlighting the pivotal role these genes play in adaptation to its current endosymbiotic lifestyle (Gerhart et al., 2016).

Overall, these two very different phenotypes (symbiosis vs. pathogenesis), along with two contrasting transmission modes (vertical vs. horizontal), and variable host specificity (ticks vs. vertebrates), make the *Coxiella*, *Francisella*, and *Rickettsia* genera especially fascinating. They thus offer an unusual opportunity to answer questions about the origins and mechanisms of symbiosis and pathogenesis. Further studies characterizing host range and infectivity of different genera members would be invaluable to obtaining such results, as would the characterization of tick symbiotic strain genomes. However, research efforts to date have invariably tended to concentrate on their medically important relatives, and so we know comparatively little about the biology of maternally-inherited symbionts. This neglect is unfortunate because fully understanding the whole scope of *Coxiella*, *Francisella*, and *Rickettsia* phenotypes linked to genome sequences, will provide an excellent system to test hypotheses on the importance of genome content and plasticity in the emergence and reversibility of extreme phenotypes such as symbiosis and pathogenesis.

CONCLUSION AND PERSPECTIVES

Extensive literature studies have now made it clear that TBP are not alone: an appreciable range of diverse non-pathogenic microorganisms has also been detected in almost all tick species examined so far. Perhaps the most important consideration for the future is not the incidence of these non-pathogenic microorganisms, but their phenotypes. The varied collection of non-pathogenic microorganisms includes intracellular maternally-inherited symbionts and microbes inhabiting the tick gut, and each could strongly influence—in very different ways—the biology of their tick hosts as well as TBP infection dynamics. Recent findings have shown that several maternally-inherited symbionts such as *Coxiella*-LE are important drivers

of evolutionary change in ticks, as clearly shown by their role in driving their tick hosts to adapt to a strict hematophagous diet (Gottlieb et al., 2015; Smith et al., 2015; Duron et al., 2017). Other non-pathogenic microorganisms such as maternally-inherited *Rickettsia* symbionts and gut microbiota are also likely to substantially contribute to the acquisition of ecologically important traits, such as TBP resistance (Burgdorfer et al., 1981; Steiner et al., 2008; Narasimhan et al., 2014; Abraham et al., 2017). It is therefore vital to establish the nature of the interactions between non-pathogenic microorganisms, their tick hosts, and co-infecting TBP. To achieve this goal, it is essential to understand how ticks acquire their microbiota, and how microbial community structures are shaped by various environmental and host factors, and also by microbial interactions within these communities. This knowledge is a key step toward using non-pathogenic microorganisms to limit TBP transmission and persistence. Similarly, whether tick symbionts have the potential to opportunistically infect humans and other vertebrates should be investigated in depth. Lastly, we would like to emphasize that the study of some non-pathogenic microorganisms, such as members of the *Coxiella*, *Francisella*, and *Rickettsia* genera, can advance our understanding of many infectious diseases including Q fever, tularemia, and rickettsial diseases. The broad phenotypic diversity present in these three bacterial genera make them perfect models to study the evolutionary emergence of pathogenicity and adaptations to living in vertebrate cells. Owing to their medical importance, the pathogenic species of these genera have been the targets of several genome sequencing projects, which have provided insights into the mechanisms and consequences of their specialized lifestyles (Darby et al., 2007; van Schaik et al., 2013). Conversely, the symbiotic forms adapted to tick hosts have received much less attention and a lot of things remain to be elucidated (but see Gillespie et al., 2012, 2015; Clark et al., 2015 for genomics insights about *Rickettsia*). In this context, comparative genomic approaches will be highly valuable in enhancing our understanding of the evolutionary ecology of both pathogenic and non-pathogenic intracellular bacteria, and in identifying novel candidate genes contributing to virulence and persistence in vertebrate cells.

AUTHOR CONTRIBUTIONS

SB, AH, FB, and OD conducted the literature research, wrote the paper and prepared the figures and tables. All authors provided critical review and revisions.

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2.2.2 Structuration des microbiomes

La composition totale des microbiomes de tiques, bien qu'elle reste encore non élucidée, semble très variable et assujettie à des facteurs structurants différents en fonction des études et des échelles d'analyses. Néanmoins très peu de choses sont connues sur ces phénomènes de structuration chez les tiques, d'autant que la majorité des études sur le microbiome des tiques reste cloisonnée aux échelles intra-holobiontes ou intra-spécifiques. Ainsi, à ces échelles, les communautés bactériennes semblent varier en fonction de différentes contraintes abiotiques (Couper *et al.*, 2019 ; Heise *et al.*, 2010 ; Zolnik *et al.*, 2016) comme par exemple, la saison au cours de laquelle les tiques ont été collectées (Lalzar *et al.*, 2012), la température (Thapa *et al.*, 2019a) ou encore les régions géographiques examinées (Carpi *et al.*, 2011 ; Thapa *et al.*, 2019b ; van Overbeek *et al.*, 2008 ; Williams-Newkirk *et al.*, 2014).

Ces microbiomes semblent également soumis à des contraintes biotiques, comme par exemple : le stade de développement ou le sexe de la tique (Clay *et al.*, 2008 ; Moreno *et al.*, 2006 ; Ruiling *et al.*, 2019 ; Williams-Newkirk *et al.*, 2014 ; Zhang *et al.*, 2018, 2019 ; Zolnik *et al.*, 2016), le gorgement ou non de la tique (Heise *et al.*, 2010 ; Landesman *et al.*, 2019 ; Lee *et al.*, 2019 ; Menchaca *et al.*, 2013 ; Zhang *et al.*, 2018, 2014), l'hôte sur lequel la tique s'est nourrie (Swei and Kwan, 2017) ou encore la présence d'agents pathogènes (Abraham *et al.*, 2017 ; Couper *et al.*, 2019 ; Narasimhan *et al.*, 2017 ; Steiner *et al.*, 2008).

Pour aller plus loin, Couper *et al.* (2019) ont montré que le microbiome d'*Ixodes pacificus* (un vecteur de la maladie de Lyme sur la côte ouest des USA) semblait être soumis au processus de *species sorting* à une échelle intra-holobionte. Ils ont, premièrement, montré que la diversité des symbiontes internes de la tique était majoritairement acquise et structurée *via* TV et que l'environnement local de la tique

impactait très peu cette diversité. Deuxièmement, ils ont également suggéré que le microbiome était majoritairement structuré de façon non aléatoire par les différentes niches écologiques à l'intérieur de la tique elle-même. Enfin, il semblerait que les interactions biotiques au sein de la communauté microbienne n'impactent pas ou peu la structuration de ce microbiome.

Un nombre bien moins important d'études a essayé d'appréhender les facteurs structurants des microbiomes à une échelle inter-spécifique. Ainsi, il a été montré que les communautés symbiotiques variaient en fonction de l'espèce de tique étudiée et donc en fonction de sa phylogénie (Lalzar *et al.*, 2012 ; Portillo *et al.*, 2019 ; Wang *et al.*, 2018 ; Zhang *et al.*, 2019). Pourtant, il n'existe qu'une seule étude, à ma connaissance, à évoquer un patron de phylosymbiose au sein de l'holobionte que représentent les tiques et leur microbiome (Díaz-Sánchez *et al.*, 2019). En effet, à l'instar des patrons de phylosymbioses existant chez les vertébrés, cette étude semble montrer que la composition des microbiomes chez le genre de tique *Ixodes* est très fortement assujettie à la phylogénie des tiques (Figure 9). Dans l'ensemble, il semble évident que la majorité des espèces de tiques (sinon toutes) hébergent des communautés bactériennes complexes pouvant être en partie structurées par un réseau complexe d'interactions entre la tique et son microbiome, l'environnement de la tique, sa phylogénie, mais aussi son spectre d'hôtes.

Néanmoins, les précédents résultats sont à prendre avec précaution, car comme nous allons le voir dans les futurs chapitres (2 et 3), et en discussion, il existe de nombreux biais et résultats contradictoires dans les études portant sur le microbiome des tiques. De ce fait, il n'est pas étonnant que la structuration des cortèges de micro-organismes chez les tiques soit encore loin d'être élucidée.

Topological congruence

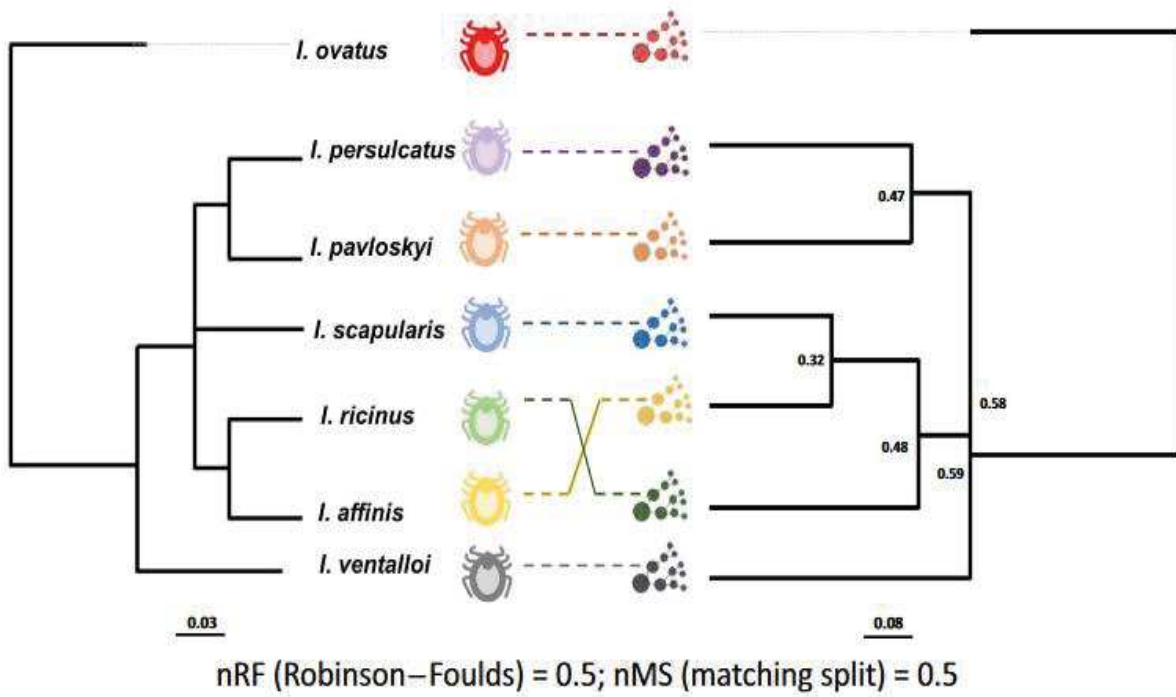


Figure 9 : Patron de phylosymbiose entre des *Ixodes* et leur microbiome.
D'après Díaz-Sánchez et al. (2019).

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Chapitre I

Chapitre I

Diversité et spectres d'hôtes des tiques de Guyane

Article 2 : Binetruy F, Chevillon C, de Thoisy B, Garnier S & Olivier Duron. Survey of ticks in French Guiana. *Ticks and Tick-borne Diseases*, 2019.

Contexte

Ce chapitre correspond à l'objectif 1 de cette thèse qui consistait à mettre à jour la diversité des tiques retrouvées en Guyane. En effet, depuis l'inventaire de Floch & Fauran en 1958, aucun suivi de la diversité acarologique n'a été réalisé en Guyane. Il en découle une ignorance totale du risque acarologique dans ce département, malgré le contexte épidémiologique particulier de la Guyane. Il s'agit d'un vaste territoire équatorial d'environ 83 000 km² couvert en grande partie de forêts tropicales denses. La population humaine de la Guyane (environ 300 000 habitants) est concentrée principalement dans une poignée de villes réparties le long de la côte et de deux fleuves principaux, tandis que l'intérieur est largement inhabité. De plus, de nombreux élevages bovins sont disséminés le long de la côte, entre les villes « fortement peuplées », et au contact de la faune sauvage. Ce patron d'occupation du territoire est un terreau propice à l'apparition de zoonose et autres maladies émergentes (Epelboin, 2017). Ceci est illustré par l'épidémie particulière de fièvre Q sévissant en Guyane, une zoonose dont la transmission ni les réservoirs ne sont toujours pas appréhendés dans ce contexte Guyanais (Eldin *et al.*, 2014). Ce chapitre et l'article qui en découle sont donc primordiaux dans le but d'appréhender le risque acarologique en Guyane. Cette première étude est également indispensable pour les autres chapitres de la thèse puisqu'elle nous a permis de constituer un échantillonnage robuste pour étudier la structuration des microbiomes de tique.



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Original article

Survey of ticks in French Guiana

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ABSTRACT

In this study, we examine the current pattern of tick diversity and host use in French Guiana, South America, from 97 sampling localities encompassing peri-urban, rural and natural habitats. We collected 3395 ticks, including 1485 specimens from 45 vertebrate species (humans, domestic and wild animals) and 1910 questing specimens from vegetation. Morphological examinations identified 22 species belonging to six genera: *Amblyomma* (16 species), *Rhipicephalus* (two species), *Ixodes* (one species), *Dermacentor* (one species), *Haemaphysalis* (one species), *Ornithodoros* (one species). To facilitate future identification, we produced a bank of pictures of different stages for all these species. Taxonomic identification was then confirmed by molecular characterization of two mitochondrial genes, cytochrome c oxidase *COI* and 16S rDNA. Eleven of the 22 reported species were collected on humans, six on domestic animals and 12 on wild animals. The most widespread tick species collected were *A. cajennense* sensu stricto and, to a lesser extent, *A. oblongoguttatum*; both of these species were frequently found on humans. We used these results to discuss the tick-associated risks for human and animal health in French Guiana.

1. Introduction

Considerable research efforts are focusing on the impacts of ticks and tick-borne pathogens (TBPs) on humans and livestock. The specific diversity of ticks, their distribution and host-use patterns have been extensively described in very diverse geographic zones with the aim to understand the circulation and infection dynamics of TBPs (e.g., Labruna et al., 2005a; Szabó et al., 2013). However, knowledge on tick fauna remains scarce in some regions such as French Guiana, a vast equatorial land covering ca. 83,000 km² located on the north-east coast of South America and mostly covered by dense rainforests. French Guiana's human population (ca. 245,000 inhabitants) is concentrated principally in a handful of towns spread along the coastline and two main rivers, while the interior is largely uninhabited. No extensive survey of ticks has been conducted in this territory over the past 60 years, since the publication of Floch and Fauran's (1958) review. This last inventory to date was mainly based on early studies from Floch and Abonnenc (1940, 1941, 1942) and counted 28 indigenous tick species which were mostly associated with wildlife (Table 1). Since this early work, only two other tick species were reported (Davoust et al., 2016; Labruna et al., 2005b; Tahir et al., 2016), raising to 30 the tick species

reported in French Guiana (Table 1). Nonetheless, during the past 60 years, urbanisation has modified the ecological conditions in some zones of French Guiana (principally along the coastline) and has thus probably impacted the tick fauna. Therefore, an updated inventory based on recent tick collections from French Guiana is needed to respond to these changes.

The present study aimed at documenting the current pattern of tick diversity and host use in French Guiana. We conducted a wide survey across sites with distinct ecological conditions (including anthropogenic, agricultural and natural ecological conditions), sampling ticks either in seabird nests, on different hosts (including humans, domestic animals and wildlife) or as questing ticks found on vegetation. We further identified, both morphologically and genetically, the specimens collected. Since the morphological similarities between some species (especially at the larval stage) make their identification a difficult task, we produced a series of photos as well as a bank of molecular data to facilitate future identifications of ticks collected in French Guiana. We also discuss the potential health risk associated with the tick species currently present in French Guiana.

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Table 1
List of tick species collected in this study or reported in early studies conducted in French Guiana.

Ticks Species	Floch and Fauran, 1958	Labruna et al., 2005b	Davoust et al., 2016; Tahir et al., 2016	This study
Ixodidae (hard ticks):				
<i>Amblyomma aureolatum</i> (Pallas, 1772)	+	-	-	-
<i>A. auricularium</i> (Conil, 1878)	+	-	-	-
<i>A. cajennense</i> sensu stricto (Fabricius, 1787)	+	-	-	+
<i>A. calcaratum</i> Neumann, 1899	+	-	-	-
<i>A. coelebs</i> Neumann, 1899	+	-	-	+
<i>A. dissimile</i> Koch, 1884	+	-	-	+
<i>A. geayi</i> Neumann, 1899	+	-	-	+
<i>A. goeldii</i> Neumann, 1899	+	-	-	+
<i>A. humerale</i> Koch, 1844	+	-	-	+
<i>A. incisum</i> Neumann, 1906	+	-	-	-
<i>A. latepunctatum</i> Tonelli-Rondelli, 1939	-	+	-	+
<i>A. longirostre</i> (Koch, 1844)	+	-	-	+
<i>A. naponense</i> (Packard, 1869)	+	-	-	+
<i>A. oblongoguttatum</i> Koch, 1844	+	-	-	+
<i>A. ovale</i> Koch, 1844	+	-	-	+
<i>A. pacae</i> Aragão, 1911	-	-	-	+
<i>A. parvum</i> Aragão, 1908	+	-	-	-
<i>A. pictum</i> Neumann, 1906	+	-	-	-
<i>A. pseudoconcolor</i> Aragão, 1908	+	-	-	-
<i>A. romitti</i> Tonelli-Rondelli, 1939	+	-	-	+
<i>A. rotundatum</i> Koch, 1844	+	-	-	+
<i>A. scalpturatum</i> Neumann, 1906	+	-	-	+
<i>A. tigrinum</i> Koch, 1844	+	-	-	-
<i>A. varium</i> Koch, 1844	+	-	-	+
<i>Dermacentor nitens</i> Neumann, 1897	-	-	-	+
<i>Haemaphysalis juxtakochi</i> Cooley, 1946	+	-	-	+
<i>Ixodes luciae</i> Senevet, 1940	+	-	-	+
<i>Rhipicephalus microplus</i> (Canestrini, 1888)	+	-	-	+
<i>R. sanguineus</i> sensu lato (Latreille, 1806)	+	-	-	+
Argasidae (soft ticks):				
<i>Argas persicus</i> (Oken, 1818)	+	-	-	-
<i>Ornithodoros capensis</i> sensu stricto Neumann, 1901	-	-	-	+
<i>O. hasei</i> (Schulze, 1935)	-	-	+	-
<i>O. talaje</i> (Guérin-Méneville, 1849)	+	-	-	-

2. Material and methods

2.1. Tick collection

Most ticks ($n = 3129$ or 92% total) were collected in French Guiana in 2016 and 2017 while a few others were sporadically collected between 1994 and 2014 ($n = 266$; Table S1 and Fig. 1). Questing ticks were collected on vegetation by a drag-flag method over 17 sites distributed on an East-West transect and covering three types of ecological conditions (peri-urban, agricultural and natural). Ticks were also directly collected in seabird nests of Grand Connétable Island or on hosts (including humans, four domestic animal species and 40 wild animal species). Some of these ticks were also obtained through a broadcast advertisement to scientists from various fields (e.g. botanists, ecologists, microbiologists, epidemiologists, etc.) as well as wildlife managers working in French Guiana. This advertisement allowed us to obtain ticks collected in remote areas which otherwise remain difficult to visit. All ticks were stored in 75% ethanol until examination.

2.2. Morphological identification and photography collection

All tick specimens were examined under a Leica Z16 APO-A microscope and categorised by stage and sex. Adults were morphologically identified to species using the study of Labruna et al. (2005b) and the dichotomous keys from Floch and Fauran (1958) and Jones et al. (1972). Nymphs were morphologically determined using the identification key by Martins et al. (2010). To the best of our knowledge, there is no identification key for South American tick larvae. Larvae were identified to genus based on morphological comparisons with adults and identification keys (at the genus level; Clifford et al., 1961), before being partitioned into distinct morphological groups: larvae sharing the

same morphological characteristics (e.g. average body size, gnathosoma and scutum shape and size, presence of cornua or number of spurs on each coxa, etc.) were assigned to same group before to be investigated for a further molecular confirmation (see below). One representative per species, sex and stage or per morphological group was photographed. Ventral and dorsal views of these specimens were photographed using a Leica DFC425 camera coupled with a Leica Z16 APO-A zoom system.

2.3. Molecular typing

A subset of tick specimens (for each sex, stage and species or for each larval morphological group) was used for molecular typing in order to confirm morphological identification. Total DNA from nymphs and larvae or DNA from one adult leg were extracted using the DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's instructions. For each tick DNA sample, mitochondrial gene fragments of the 16S rDNA (ca. 450 bp) and the cytochrome c oxidase *COI* (655–680 bp) were amplified by polymerase chain reaction (PCR) using specific primers (listed in Table 2). PCRs were performed in a 25 μ L reaction volume containing 12.85 μ L of H₂O, 2.5 μ L of 10x PCR buffer (Roche), 5 μ L of 2.5 mM dNTP mix (Thermo Scientific), 2 μ L of 25 mM MgCl₂ (Roche), 0.7 μ L of each primer (10 μ M), 0.25 μ L of Taq Roche polymerase (5U/ μ L) and 1 μ L of DNA. The amplification cycle involved a denaturation step at 94 °C for 3 min followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, 1 min at 72 °C and a final 5 min elongation step of at 72 °C. The amplified products were detected by electrophoresis on 1.5% agarose gel in TAE 0.5X buffer and stained with EZ-Vision (Amresco). Positive amplifications were sequenced by Eurofins (Ebersberg, Germany). Sequence chromatograms were manually cleaned with Chromas Lite (http://www.technelysium.com.au/chromas_lite.html) and aligned

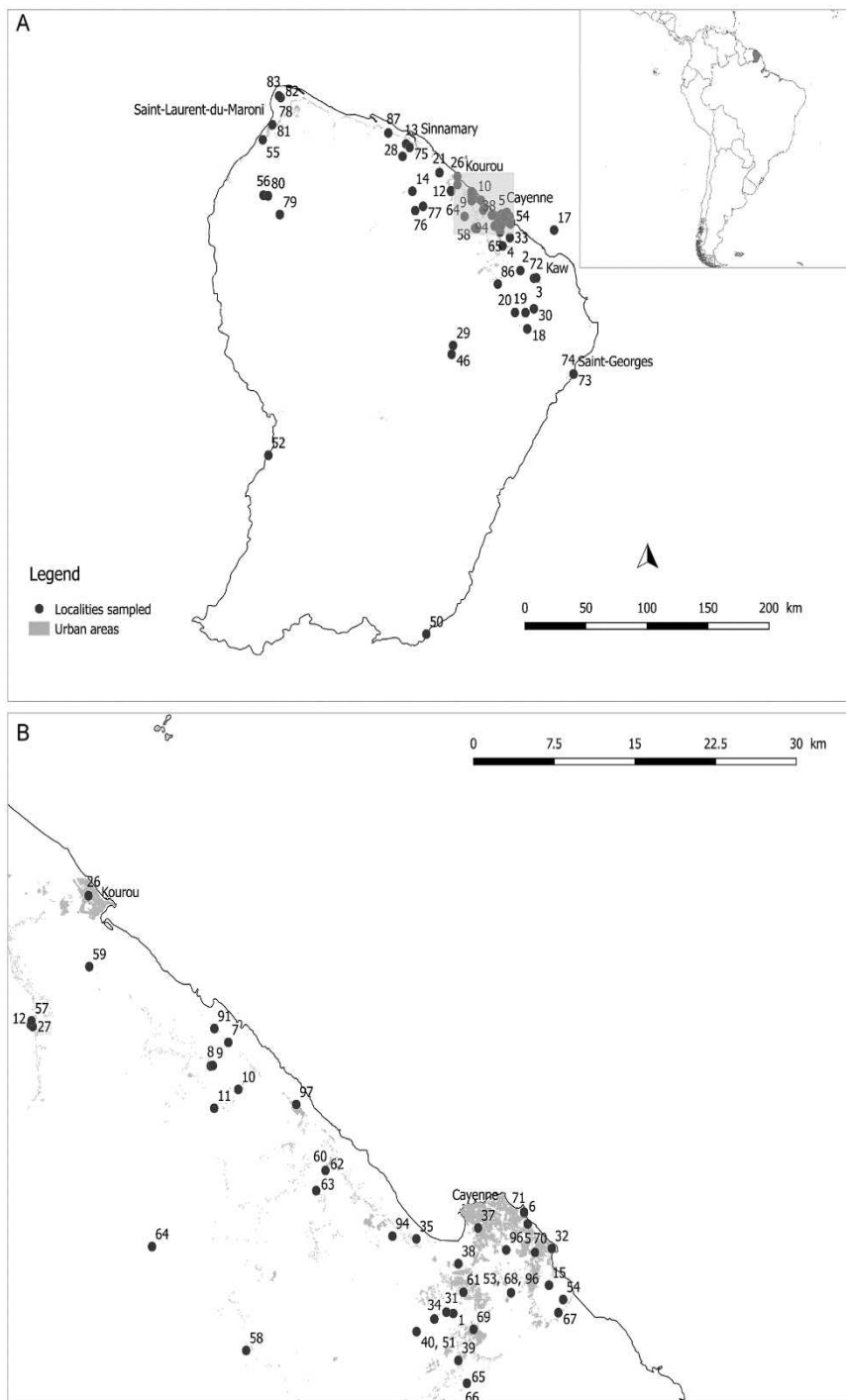


Fig. 1. Localization of sampling sites. Localities are represented by dark dots and urban areas by grey areas. Numbers correspond to the population number given in Table S1. Figure B is the magnification of the area bounded by the translucent grey rectangle in Figure A. Localities with no GPS coordinates are not indicated in this map (see #22–25, 36, 41–50, 75, 84, 85, 88–90, 92 and 93 in Table S1).

Table 2
Primers used for characterising tick species.

Genes	Primers (5'-3')	Amplified species	References
16S rRNA	Tm16S+1 (CTGCTCAATGATTTTTAAATTGC) Tm16S-1 (CCGGTCTGAACCTCAGATCATGTA)	All species (Tm16 + 1-Tm16S-1: 450bp)	Fukunaga et al., 2001
CO1	TickCO1 F (ATTTTACCGGATGAHTWTTT) TickCO1R (CWGGRTGRCCAARAATCAAATA)	<i>A. cajennense</i> s.s., <i>A. coelebs</i> , <i>A. dissimile</i> , <i>A. geayi</i> , <i>A. latepunctatum</i> , <i>A. longirostre</i> , <i>A. oblongoguttatum</i> , <i>A. scalpturatum</i> , <i>A. varium</i> , <i>D. nitens</i> , <i>H. juxtakochi</i> , <i>O. capensis</i> s.s., <i>R. microplus</i> (TickCO1 F-TickCO1R: 676bp)	This study
	AmbCO1 F (TACCGGATGAATATACTC)	<i>A. humerale</i> , <i>A. naponense</i> , <i>A. ovale</i> , <i>A. pacae</i> , <i>R. sanguineus</i> s.l. (AmbCO1 F-TickCO1R: 675bp)	This study
	Lco1490 (GGTCAACAATGATAAAGATATTGG) Hco2198 (TAAACTTCAGGGTGACCAAAAAATCA)	<i>A. goeldii</i> , <i>A. rotundatum</i> (Lco1490-Hco2198: 660bp); <i>A. romitii</i> , <i>I. luciae</i> (Lco1490-TickCO1R: 654bp)	Folmer et al., 1994

with MEGA 7 (<http://www.megasoftware.net>). The DNA sequences obtained were compared with sequences referenced in Genbank nucleotide database using the BLAST algorithm (megablast) (Table S2). All 16S rDNA and CO1 gene sequences obtained in this study were deposited on Genbank (accession numbers are listed in Table S2).

2.4. Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the French code of practice for the care and use of animals for scientific purposes, established by articles R214-87 to R214-137 of the French rural code. The capture of ticks in the Grand Connétable protected area was authorized by the Prefecture of French Guiana by the prefectural decree R03-2016-09-23-003. The use of the genetic resources was declared to the French Ministry of Environment under the reference #150401230100, in compliance with the Access and Benefit Sharing procedure implemented by the Loi pour la Reconquête de la Biodiversité.

3. Results

A total of 3395 tick specimens (2,026 Larvae, 687 nymphs and 682 adults) were collected from 97 localities in French Guiana, mostly located along the coast line, including 16 peri-urban sites, four agricultural sites and 76 natural sites (Table S1, Fig. 1). Of the 3395 ticks, 1910 (56%) were collected on vegetation (1,588 Larvae, 248 nymphs and 74 adults) while 1485 (44%) were collected on vertebrate hosts (437 larvae, 435 nymphs and 613 adults). The sampling origin is further detailed in the Table S1.

The 3395 tick specimens were morphologically identified to 22 species (Table 3). Pictures of representative specimens (including larvae, nymphs and adults; Table 3) are shown in Supplementary File 1. Molecular typing based on 16S rDNA and CO1 gene sequences corroborated most of these morphological identifications: sequences from 21 of the 22 species showed a satisfactory percentage of identities (96–100%) with referenced sequences of the same tick species already available in GenBank (Table S2). It is worth to noting that 16S rDNA appears as a better molecular tool for *Amblyomma* identification since only a few *Amblyomma* CO1 sequences are currently available in GenBank. Nonetheless one species, *A. latepunctatum*, could not reach proper molecular identification since no 16S rDNA and CO1 gene sequences were available in GenBank for this species.

Among the 22 species identified, 17 belong to the *Amblyomma* genus, two to the *Rhipicephalus* genus and four others to *Dermacentor*, *Haemaphysalis*, *Ixodes* and *Ornithodoros* genera. Among the 3395 tick specimens, the most abundant species collected were *A. dissimile* ($n = 735$; 22%), *A. cajennense* s.s. ($n = 557$; 16%) and *D. nitens* ($n = 493$; 15%) whereas the least abundant species were *A. ovale* ($n = 4$; 0.1%), *A. romitii* ($n = 4$; 0.1%) and *O. capensis* s.s. ($n = 11$; 0.3%) (Table 3). Across the 97 Localities sampled, *A. cajennense* and *A. oblongoguttatum* were the most widespread tick-species, being observed in 48 and 19 Localities, respectively. By contrast, *A. ovale*, *A. pacae*, *A.*

romitii, *D. nitens* and *O. capensis* s.s. were only collected in one locality each (Table 3).

Ticks were collected on 45 different vertebrate species (24 mammals including humans, 14 birds, six reptiles and one amphibian) (Table 3). The host range varied greatly depending on tick species and stages. Indeed, all stages of *D. nitens* and *R. sanguineus* s.l. were found on only one host species (horses and dogs) whereas *A. longirostre*, *A. cajennense* s.s. and *A. dissimile* were generalist species that were respectively collected on 14 passerines, 11 mammals and 10 vertebrate species (six mammals, three reptiles and one amphibian). The greatest tick diversity was found on humans with 11 tick species collected: *A. cajennense* s.s. ($n = 259$ collected on humans), *A. coelebs* (53), *A. dissimile* (1), *A. humerale* (3), *A. latepunctatum* (3), *A. naponense* (9), *A. oblongoguttatum* (89), *A. pacae* (13), *A. scalpturatum* (45), *A. varium* (6) and *Haemaphysalis juxtakochi* (4).

4. Discussion

With the report of 22 tick species (21 Ixodidae and one Argasidae; Table 1, Table 3), the present survey represents the largest update of the tick fauna in this Amazonian region that has been conducted over the past 60 years. To facilitate future identification for all potential users, including taxonomists, medical and veterinary practitioners, we also produced a bank of pictures of different tick stages and species combined with CO1 and the 16S rDNA molecular barcoding.

Compared with the previous inventory (Floch and Fauran, 1958), three additional tick species are reported here (*A. pacae*, *Dermacentor nitens* and *Ornithodoros capensis* s.s.), bringing the total number of tick species recorded in French Guiana to 33 (Table 1). It is noteworthy that the presence of *A. pacae* and *D. nitens* in French Guiana was strongly presumed because both species are widely distributed in the Neotropical region and because their main hosts (respectively paca and horses) are present in French Guiana (Guglielmo et al., 2003; Jones et al., 1972; Nava et al., 2017). We confirm the previously reported presence of *A. latepunctatum* in French Guiana (Labruna et al., 2005b). This is likely to indicate that, as suggested by Labruna et al. (2005b), *A. latepunctatum* could have been misidentified with *A. incisum* and/or *A. scalpturatum* in the early surveys reviewed by Floch and Fauran (1958). The lack of 16S rRNA and CO1 referenced sequences of *A. latepunctatum* in GenBank complicated the taxonomic identification. However, we are confident in our identification to *A. latepunctatum* since (i) the morphological diagnostic characteristics described by Labruna et al. (2005b) were unambiguously observed in our specimens, and that (ii) the 16S rDNA and CO1 sequences obtained from these specimens show a low level of identity with those retrieved from any other *Amblyomma* species (< 91%). By contrast to these Ixodidae species, the presence of *O. capensis* s.s. in South America had long remained controversial or speculative (Dupraz et al., 2016; Nava et al., 2017). In that context, our sampling of several specimens in the seabird colony of Grand Connétable Island (Table S1) along with that of Muñoz-Leal et al (2017) in Queimada Grande Island, Brazil, both confirm the presence of *O. capensis* s.s. on the Atlantic coast of South America.

Table 3
List of tick species collected in this study with their abundances (number of populations where each tick species was found), sampling sizes and specific identity of the hosts on which they were collected. Relevant (non-exhaustive) references for the main host usages of the ticks collected are also indicated.

Ticks Species	Number of collections		n total	n adult	n nymph	n larvae	n questing ticks on vegetation		n on hosts	Hosts (this study)	Main hosts (literature)	
	n	total					n	on vegetation				
<i>A. cajemense sensu stricto</i> (Fabricius, 1787)	41	557	313	2	45	116	106	259	298	Humans [138 F; 92 M; 19 N; 9 L], horse (<i>Equus caballus</i>) [3 M; 1 N], jaguar (<i>Panthera onca</i>) [2 N], white-striped peccary (<i>Tayassu pecari</i>) [4 F; 4 M], nine-banded armadillo (<i>Dasypus novemcinctus</i>) [11 M; 1 N], southern tamandua (<i>Tamandua tetradactyla</i>) [1 F], southern naked-tailed armadillo (<i>Cabassous unicinctus</i>) [1 M], South American tapir (<i>Tapirus terrestris</i>) [1 F; 2 N], pale-throated sloth (<i>Bradypus tridactylus</i>) [4 M; 1 N], Guianan white-eared opossum (<i>Didelphis imperfecta</i>) [1 N], common opossum (<i>Didelphis marsupialis</i>) [3 N]	Mammals (Estrada-Peña et al., 2004; Floch and Fauran, 1958; Guglielmine et al., 2006; Lopes et al., 1998; Martins et al., 2011)	
<i>A. coetzi</i> Neumann, 1899	16	163	2	45	116	106	259	298	57	Humans [1 M; 25 N; 28 L], South American tapir (<i>Tapirus terrestris</i>) [1 F], common opossum (<i>Didelphis marsupialis</i>) [2 N]	Mammals (Floch and Fauran, 1958; Garcia et al., 2015; Guglielmine et al., 2006; Martins et al., 2014, 2011)	
<i>A. dissimile</i> Koch, 1884	16	735	30	18	687	674	674	674	61	Human [1 F], horse (<i>Equus caballus</i>) [2 L], common opossum (<i>Didelphis marsupialis</i>) [1 N], woolly mouse opossum (<i>Micoureus demerarae</i>) [1 N; 1 L], linnaeus's mouse opossum (<i>Marmosa murina</i>) [1 L], gray four-eyed opossum (<i>Philander opossum</i>) [2 N], smooth machete savane (<i>Chironomus scaritoides</i>) [2 F; 2 M], pseudoboa (<i>Pseudoboa</i> sp.) [1 N], green iguana (<i>Iguana iguana</i>) [3 F; 4 M; 7 N; 1 L], cane toad (<i>Rhinella marina</i>) [3 F; 12 M; 6 N; 11 L], Pale-throated sloths (<i>Bradypus tridactylus</i>) [5 M]	Human [1 F], horse (<i>Equus caballus</i>) [2 L], common opossum (<i>Didelphis marsupialis</i>) [1 N], woolly mouse opossum (<i>Micoureus demerarae</i>) [1 N; 1 L], linnaeus's mouse opossum (<i>Marmosa murina</i>) [1 L], gray four-eyed opossum (<i>Philander opossum</i>) [2 N], smooth machete savane (<i>Chironomus scaritoides</i>) [2 F; 2 M], pseudoboa (<i>Pseudoboa</i> sp.) [1 N], green iguana (<i>Iguana iguana</i>) [3 F; 4 M; 7 N; 1 L], cane toad (<i>Rhinella marina</i>) [3 F; 12 M; 6 N; 11 L], Pale-throated sloths (<i>Bradypus tridactylus</i>) [5 M]	Mammals (Floch and Fauran, 1958; Garcia et al., 2015; Guglielmine et al., 2006; Martins et al., 2014, 2011) Reptiles and amphibians (Floch and Fauran, 1958; Guglielmine and Nava, 2010)
<i>A. geayi</i> Neumann, 1899	6	13	5	0	8	8	8	8	5	Southern tamanduas (<i>Tamandua tetradactyla</i>) [5 F; 30 M]	Sloths (Floch and Fauran, 1958; Labruna et al., 2009b)	
<i>A. goeldii</i> Neumann, 1899	3	35	35	0	0	0	0	0	35	Human [3 L], southern tamandua (<i>Tamandua tetradactyla</i>) [1 N], red acouchi (<i>Myoprocta acouchy</i>) [1 N], rodents (<i>Euryoryzomys</i> sp.) [1 N], gray four-eyed opossum (<i>Philander opossum</i>) [12 N; 12 L], common opossum (<i>Didelphis marsupialis</i>) [3 N; 11 L], opossum (<i>Marmosops</i> sp.) [2 L], white-flanked antwren (<i>Myrmotherula axillaris</i>) [1 N], Werner's false lancehead (<i>Xenodon werneri</i>) [1 N], tortoise (<i>Chelonoidis</i> sp.) [7 M]	Anteaters (Floch and Fauran, 1958; Martins et al., 2015) Tortoises (Floch and Fauran, 1958; Labruna et al., 2002b)	
<i>A. humerale</i> Koch, 1844	17	116	7	24	85	61	61	61	55	Human [2 N; 1 L], South American tapir (<i>Tapirus terrestris</i>) [3 F; 1 M] White-bearded manakin (<i>Manacus manacus</i>) [1 N; 6 L], buff-throated saltator (<i>Saltator maximus</i>) [1 L], crimson-hooded manakin (<i>Pipra aureola</i>) [2 N; 28 L], barred antshrike (<i>Thamnophtus delatus</i>) [6 L], silver-backed tanager (<i>Amphispiza bilineata</i>) [2 L], yellow-olive flatbill (<i>Troglodytes sulphureus</i>) [1 L], straight-billed woodcreeper (<i>Dendroptix picus</i>) [1 L], golden-spangled piculet (<i>Picumnus exilis</i>) [1 L], blue-backed manakin (<i>Chiroxiphia pareola</i>) [1 N; 48 L], wedge-billed woodcreeper (<i>Glyphorynchus spirurus</i>) [34 L], McConnell's flycatcher (<i>Mionectes macconnelli</i>) [4 L], white-necked thrush (<i>Turdus albicollis</i>) [1 N; 1 L], white-flanked antwren (<i>Myrmotherula axillaris</i>) [1 N] and golden-headed manakin (<i>Pipra erythrocephala</i>) [1 L]	Tapirs and peccaries (Labruna et al., 2010, 2005b; Martins et al., 2014) Porcupines, passerines (immatures) (Floch and Fauran, 1958; Jones et al., 1972; Nava et al., 2010; Ogzevsvalska et al., 2010, 2009)	
<i>A. laepunctatum</i> Tonelli-Rondelli, 1939	5	12	4	7	1	5	5	5	7	Human [2 N; 1 L], South American tapir (<i>Tapirus terrestris</i>) [3 F; 1 M] White-bearded manakin (<i>Manacus manacus</i>) [1 N; 6 L], buff-throated saltator (<i>Saltator maximus</i>) [1 L], crimson-hooded manakin (<i>Pipra aureola</i>) [2 N; 28 L], barred antshrike (<i>Thamnophtus delatus</i>) [6 L], silver-backed tanager (<i>Amphispiza bilineata</i>) [2 L], yellow-olive flatbill (<i>Troglodytes sulphureus</i>) [1 L], straight-billed woodcreeper (<i>Dendroptix picus</i>) [1 L], golden-spangled piculet (<i>Picumnus exilis</i>) [1 L], blue-backed manakin (<i>Chiroxiphia pareola</i>) [1 N; 48 L], wedge-billed woodcreeper (<i>Glyphorynchus spirurus</i>) [34 L], McConnell's flycatcher (<i>Mionectes macconnelli</i>) [4 L], white-necked thrush (<i>Turdus albicollis</i>) [1 N; 1 L], white-flanked antwren (<i>Myrmotherula axillaris</i>) [1 N] and golden-headed manakin (<i>Pipra erythrocephala</i>) [1 L]	Tapirs and peccaries (Labruna et al., 2010, 2005b; Martins et al., 2014) Porcupines, passerines (immatures) (Floch and Fauran, 1958; Jones et al., 1972; Nava et al., 2010; Ogzevsvalska et al., 2010, 2009)	
<i>A. longirostre</i> (Koch, 1844)	18	142	0	7	135	2	2	2	140	Human [1 M; 8 N]	Suina (Floch and Fauran, 1958; Labruna et al., 2002a)	

(continued on next page)

Table 3 (continued)

Ticks Species	Number of collections	n total		n adult		n nymph		n larvae		n questing ticks on vegetation	n on hosts	Hosts (this study)	Main hosts (literature)
		n	n	n	n	n	n						
<i>A. oblongoguttatum</i> Koch, 1844	19	118	100	18	0	18	0	0	0	18	100	Humans [36 F; 38 M; 15 N], cat (<i>Felis silvestris catus</i>) [1 F], jaguar (<i>Panthera onca</i>) [3 F], cougar (<i>Puma concolor</i>) [4 F], white-lipped peccary (<i>Tayassu pecari</i>) [1 F], South American tapir (<i>Tapirus terrestris</i>) [1 F], gray four-eyed opossum (<i>Philander opossum</i>) [1 N], South American tapir (<i>Tapirus terrestris</i>) [3 F; 1 M]	Mammals (Fairchild et al., 1966; Floch and Fauran, 1958; Guglielmine et al., 2006; Martins et al., 2017; Nuttall, 1912)
<i>A. ovale</i> Koch, 1844	1	4	4	0	0	0	0	0	0	0	4	Human [13 L]	Mammals (Aragão, 1936; Floch and Fauran, 1958; Guglielmine et al., 2006; Martins et al., 2012)
<i>A. paucae</i> Aragão, 1911	1	13	0	0	13	0	0	0	0	0	13	Human [13 L]	Paca (Jones et al., 1972; Guglielmine et al., 2003)
<i>A. romilli</i> Tonelli-Rondelli, 1939	1	4	4	0	0	0	0	0	0	0	4	Capybara (<i>Hydrochoerus hydrochaeris</i>) [4 F]	Capybaras (Barros-Hartesi et al., 2007; Floch and Fauran, 1958; Jones et al., 1972; Landulfo et al., 2016)
<i>A. rotundatum</i> Koch, 1844	2	127	1	0	126	126	0	0	0	126	1	Red-footed tortoise (<i>Chelonia carbonaria</i>) [1 F]	Amphibians and reptiles (Floch and Fauran, 1958; Guglielmine and Nava, 2010; Jones et al., 1972; Labruna et al., 2005; Rodrigues et al., 2010)
<i>A. scalpturatum</i> Neumann, 1906	7	49	3	7	39	3	0	0	0	3	46	Human [1 M; 5 N; 39 L], jaguar (<i>Panthera onca</i>) [1 N]	Tapirs (Floch and Fauran, 1958; Jones et al., 1972; Labruna et al., 2005b, 2010)
<i>A. varium</i> Koch, 1844	11	95	6	4	85	81	0	0	0	81	14	Humans [1 N; 5 L], pale-throated sloth (<i>Bradypus tridactylus</i>) [2 F; 4 M; 1 N], wedge-billed woodcreeper (<i>Glyphorynchus spirurus</i>) [1 N]	Sloths (Floch and Fauran, 1958; Jones et al., 1972; Marques et al., 2002)
<i>Dermacentor nitens</i> Neumann, 1897	1	493	72	259	162	0	0	0	0	0	493	Horses (<i>Equus caballus</i>) [37 F; 40 M; 254 N; 162 L]	Equines (Labruna et al., 2001; Rodrigues et al., 2017)
<i>Haemaphysalis juxtakochi</i> Cooley, 1946	5	13	0	9	4	9	0	0	0	9	4	Human [4 L]	Deer (Floch and Fauran, 1958; Guglielmine et al., 1992; Jones et al., 1972)
<i>Ixodes luciae</i> Senevet, 1940	8	50	12	38	0	0	0	0	0	0	50	Large-headed rice rat (<i>Hyalomys megacephalus</i>) [3 N], Guianan oecomys (<i>Oecomys auyantepui</i>) [2 N], common opossum (<i>Didelphis marsupialis</i>) [1 M], woolly mouse opossum (<i>Micoureus dennerae</i>) [3 F; 27 N], Linnaeus's mouse opossum (<i>Marmosa murina</i>) [2 F; 6 N], gray four-eyed opossum (<i>Philander opossum</i>) [5 F; 1 M]	Metatherians and small rodents (Díaz et al., 2009; Floch and Fauran, 1958; Hoogstraal and Aeschlimann, 1982; Labruna et al., 2009a; Luz et al., 2013)
<i>Ornithodoros capensis</i> sensu stricto Neumann, 1901	1	11	8	3	0	11	0	0	0	11	0	In laughing gull nest (<i>Leucophaeus atricilla</i>)	Seabirds (Dietrich et al., 2011; Hoogstraal, 1985)
<i>Rhipicephalus macropus</i> (Canestrini, 1888)	3	392	24	1	367	367	0	0	0	367	25	Brahmans (<i>Bos taurus indicus</i>) [18 F; 6 M; 1 N]	Cattle (Floch and Fauran, 1958; Jones et al., 1972)
<i>R. sanguineus</i> sensu lato (Latreille, 1806)	2	51	51	0	0	0	0	0	0	0	51	Dogs (<i>Canis lupus familiaris</i>) [28 F; 23 M]	Dogs (Dantas-Forges, 2010; Dantas-Forges et al., 2010; Estrada-Peña and Jongejans, 1999; Floch and Fauran, 1958)

Despite an intensive sampling effort, the present study has failed to detect 11 tick species reported in Floch and Fauran's (1958) inventory (Table 1; Table 3). This could be explained by sampling differences: the present survey gave some preference to coastal habitats and used sampling strategies that were not perfectly suited to collecting nidicolous soft tick species (e.g. *Argas persicus* and *O. talaje* reported by Floch and Fauran, 1958, but not detected in the present study). Nevertheless, the wide geographical coverage of the present survey allows us to report *A. cajennense* s.s. as the most prevalent tick species from French Guiana. Interestingly, this conclusion is consistent with that of Floch and Fauran (1958). Moreover, other tick species which were described as widely distributed by Floch and Fauran (1958) such as *A. oblongoguttatum*, *A. humerale* and *A. dissimile* also remain very common in the present survey (Table 3). *A. cajennense* s.s. appears to be far more prevalent in human-disturbed environments or at the edge of primary forest than in deep rainforest (Table 3; Table S1), a result congruent with the literature regarding *A. cajennense* s.l. in South-America (Martins et al., 2016; Szabó et al., 2013). It is worth noting that half of the tick species collected (11/22) were found at least once on humans in the present survey (Table 3). In addition, *A. oblongoguttatum*, *A. coelebs* and *A. scalpturatum* are the most common species found on humans after *A. cajennense* (Table 3). These species were commonly sampled on large mammals (e.g. tapirs) and have also been frequently found on humans in other South American countries (Guglielmo et al., 2006).

In French Guiana, TBPs circulating in tick populations are largely overlooked. A few cases have nevertheless been reported, including an outbreak of Q fever due to a very virulent strain of the intracellular bacterium *Coxiella burnetii* (Mahamat et al., 2013). Remarkably, this *C. burnetii* strain was recently detected in *A. geayi* specimens engorged on a dead sloth found in the Cayenne peri-urban area after a Q fever outbreak (Davoust et al., 2014; Eldin et al., 2014; Epelboin et al., 2016). These findings suggest that sloths play a role as a reservoir for *Coxiella burnetii* and a putative role for the ticks feeding on sloths in the transmission of this TBP. While *A. geayi* is not known to feed on humans (Guglielmo et al., 2006), other species feeding on sloth such as *A. cajennense* s.s. and *A. varium* (the sloth tick) could play a role in the transmission of Q fever to human and others vertebrates. This hypothesis has also been considered in Argentina where *Amblyomma* spp. specimens were found to be infected by *C. burnetii* (Pacheco et al., 2013). In this context, it was suggested that ticks may play a pivotal role in the spread of Q fever in South America, while it is instead an airborne disease in the Northern hemisphere (Duron et al., 2015). Further surveys in French Guiana are needed to elucidate the role of ticks in the transmission of this particular virulent strain of Q fever agent.

Complementarily, the TBP infection risks involving *A. cajennense* s.s. deserve attention since this tick species was found to display the widest distribution in French Guiana as well as the highest abundance across local anthropogenic landscapes. It is noteworthy indeed that, in South-America, *A. cajennense* s.l. is considered as an important vector of tick-borne rickettsiosis to human and domestic animals (da Silva et al., 2015; Labruna, 2009; Szabó et al., 2013) and may potentially transmit the Brazilian borreliosis agent (Basile et al., 2017). The present survey also reports three other Ixodidae species which, although less widely distributed than *A. cajennense*, may account for certain infection risks in French Guiana: *A. oblongoguttatum*, *A. coelebs*, and *R. sanguineus* s.l. have been associated with TBPs (mainly *Rickettsia* spp.) in South-America (da Silva et al., 2015; Labruna, 2009; Parola et al., 2013; Szabó et al., 2013). Finally, some soft ticks may also be a threat to human or animal health in French Guiana. Recently, *O. hasei* collected from bats in unoccupied buildings in Saint-Jean-du-Maroni were found infected by a new *Rickettsia* species and two potential new *Bartonella* species but risks to human health remain currently unknown (Davoust et al., 2016; Tahir et al., 2016). Other soft tick species described in French Guiana (i.e. *A. persicus*, *O. capensis* s.s and *O. talaje*) are also thought as potential vector of TBPs (Dietrich et al., 2011; Manzano-Román et al., 2012).

In conclusion, the present survey showed that French Guiana currently harbours substantial diversity in tick species including some known as TBP vectors in South America. However, we have likely underestimated this diversity since our sampling regime suffered from an important geographical bias: most of the samples came from the coastline where French Guiana's population is concentrated while the inner territory remains largely underexplored. Future surveys will be necessary to fully describe the tick fauna in French Guiana in these remote areas. Overall, the results reported herein also highlight the potential acarological risk in French Guiana for both human and animal health, highlighting the need to document the local prevalence and diversity of TBP there.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.ttbdis.2018.09.002>.

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Discussion

Dans cette étude nous avons donc collecté 3395 tiques appartenant à 22 espèces différentes. Cet article illustre parfaitement le manque de connaissances sur les tiques en Guyane, puisque nous avons décrit pour la première fois en Guyane deux nouvelles espèces (*Ornithodoros capensis* et *Amblyomma pacae*). Il est fort à parier que malgré un échantillonnage qui se veut le plus exhaustif possible, et les anciens inventaires, la diversité totale des tiques en Guyane ne soit pas encore totalement appréhendée. En effet, à l'heure où j'écris ces lignes, nous avons désormais une collection qui comprend pas loin de 5000 individus ainsi qu'une espèce de plus d'identifiée : *A. calcaratum*. Le seul individu de cette espèce a été collecté sur un passereau (manakin casse-noisette, *Manacus manacus*). Cette espèce avait déjà été décrite en Guyane et elle est connue pour se nourrir sur les xénarthres et occasionnellement sur les passereaux (Floch & Fauran, 1958). La diversité totale des tiques de Guyane apparaît largement dominée par le genre *Amblyomma* (17 espèces / 23 au total). Ce genre de tique est le troisième plus important de la famille des Ixodidae, occupant principalement les zones tropicales de tous les continents. Le centre de diversité de ce genre se trouve en Amérique du Sud, où la moitié de ces espèces sont présentes (Guglielmone, Estrada-Peña, Keirans, & Robbins, 2003). Ces tiques présentent de manière générale des cycles triphasiques où tous les vertébrés terrestres (amphibiens, reptiles, oiseaux et mammifères) peuvent servir d'hôtes (Guglielmone *et al.*, 2003). De manière intéressante, les espèces retrouvées en Guyane présentent une large variété de spectres d'hôtes, allant d'une espèce spécialiste des tortues terrestres (*A. humerale*) à des espèces généralistes comme *A. cajennense* ou *A. oblongoguttatum*. Ces dernières, de par leur caractère généraliste, sont certainement les plus « intéressantes » d'un point de vue santé humaine ou animale. En effet, *A. cajennense* est par exemple, l'espèce la plus commune de notre collection et celle qui est le plus

souvent associée à l'homme en Amérique du Sud (Guglielmone *et al.*, 2006). Sa capacité à se nourrir sur la faune sauvage ou domestique, puis sur l'homme, fait potentiellement de cette tique un des principaux moteurs à l'émergence de zoonose chez l'humain mais également chez l'animal.

Il est facile de comparer le rôle de vecteur de *A. cajennense* à celui de sa proche cousine d'Amérique du Nord, *A. americanum* : elle aussi généraliste, abondante et très fréquemment associée à l'homme. En effet, depuis le début des années 90, cette espèce fait l'objet de plus en plus d'attentions à cause de son rôle dans l'émergence de maladies comme l'ehrlichiose (Childs & Paddock, 2003). Le dernier exemple en date, est son rôle dans l'émergence chez l'humain de différents virus ayant comme réservoir la faune sauvage (les virus Heartland et Bourbon) (Brault, Savage, Duggal, Eisen, & Staples, 2018 ; Savage *et al.*, 2017, 2016 ; Schweon, 2016). Enfin, *A. americanum* serait également l'un des principaux responsables de la très surprenante allergie à la viande rouge chez l'humain, causée par l'inoculation, *via* la salive de la tique, du sucre alpha-gal normalement présent dans la viande rouge (Crispell *et al.*, 2019 ; Wuerdeman & Harrison, 2014). Le rôle de vecteur de zoonose d'*A. americanum* n'est plus à prouver, et cette comparaison montre une fois de plus l'importance d'études approfondies sur les *Amblyomma*, et dans une plus grande mesure sur les tiques de Guyane. Dans ce contexte de hotspot de biodiversité, les risques de maladies émergentes véhiculées par les tiques apparaissent donc comme sérieux.

Finalement, grâce à cet important échantillonnage nous avons pu : appréhender une partie du risque infectieux associé aux tiques en Guyane que nous développerons dans le chapitre 4 ; caractériser et étudier la structuration des microbiomes d'*Amblyomma* (chapitre 3) ; et dans le chapitre 2, mettre en avant les biais méthodologiques existant dans les études du microbiome des tiques.

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Chapitre II

Chapitre II

Les biais inhérents à l'étude du microbiome des tiques

Article 3 : Binetruy F, Dupraz M, Buysse M & Duron O. Surface sterilization methods impact measures of internal microbial diversity in ticks. *Parasites & Vectors*, 2019.

Contexte

Récemment, l'explosion du séquençage haut débit ainsi que des techniques de barcoding moléculaires ont permis de découvrir et d'appréhender la diversité et la composition des microbiomes chez une multitude d'espèces. Ainsi, dans ce chapitre correspondant à l'objectif 2 de cette thèse, nous avons utilisé ces techniques pour caractériser les cortèges microbiens abrités par les tiques de Guyane.

Bien que le microbiome comprenne l'ensemble des communautés microbiennes hébergées à l'intérieur et à l'extérieur par un hôte, la quasi-totalité des études sur le microbiome des tiques s'intéressent au microbiome interne de celles-ci. Toutefois, en regardant plus attentivement les protocoles utilisés dans ces études, il apparaît que la majorité possède, au moins, un biais conséquent. En effet, dans le but de se débarrasser du microbiome externe (ou cuticulaire) des tiques, la majorité de ces études utilisent des bains d'éthanol avant la phase d'extraction de l'ADN. Or, bien que l'éthanol soit extrêmement efficace pour tuer les micro-organismes, il ne l'est pas pour dénaturer l'ADN puisqu'il est largement utilisé durant les procédures d'extractions d'ADN pour sa capacité à faire précipiter l'ADN et à le « fixer ». Nous nous sommes donc interrogés sur l'efficacité d'un tel traitement pour enlever toute trace d'ADN du microbiome cuticulaire, et l'avons comparé avec un traitement à la Javel. Effectivement, avant de caractériser le microbiome interne de nos 23 espèces de tiques *via* un sous-échantillonnage de 1000 tiques, il nous a

paru primordial de s'assurer de la non-contamination de ce microbiome par des micro-organismes cuticulaires.

RESEARCH

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Surface sterilization methods impact measures of internal microbial diversity in ticks



Florian Binetruy^{*} , Marlène Dupraz, Marie Buysse and Olivier Duron

Abstract

Background: Ticks are obligate blood feeders transmitting major pathogens worldwide. Over the past few years, considerable research efforts have focused on the diversity, distribution and impact of gut and intracellular bacterial symbionts on tick development and tick-borne pathogen transmission. The study of this internal microbiome requires the use of a sterilization method to remove external (i.e. cuticular) microbes present on the tick's surface and to avoid any further contamination. Several sterilization methods exist, including ethanol- or bleach-based treatments that are both effective in killing microbes but with different potential effects on DNA denaturation.

Methods: We examined how these different sterilization methods impact the measure of internal microbial diversity hosted by the Cayenne tick *Amblyomma cajennense* (*sensu stricto*). Bacterial barcoding investigations based on 16S rRNA gene sequences were conducted on two batches of 50 individuals each: Ticks of the first batch were sterilized with bleach diluted at 1% and the second batch with 70% ethanol. Tick external microbiome was also determined from cuticle smearing and water samples used for tick washing.

Results: Bacterial barcoding investigations showed major differences between ethanol- and bleach-treated specimens. Both methods led to the detection of major intracellular bacteria associated with *A. cajennense* (*s.s.*) but ethanol-treated ticks always harbored a higher bacterial diversity than bleach-treated ticks. Further examinations of tick gut and tick external microbiome revealed that ethanol-based surface sterilization method is inefficient to eliminate the DNA of external bacteria.

Conclusions: We herein provide evidence that studies investigating the internal microbiome of ticks should consider bleach as the gold standard to efficiently remove cuticular bacterial DNA. Indeed, this method does not impact the internal bacterial diversity hosted by ticks and is thus a better method than the ethanol-based one for studying the internal microbiome.

Keyword: 16S rRNA, Bacterial communities, Tick microbiome, Metabarcoding, *Amblyomma*

Background

Over the past 15 years, advances in genomics and microbiology have shown that metazoans commonly harbor complex microbial communities living inside and on their body, i.e. the microbiome. In arthropods, some studies have focused on cuticular (i.e. external) microbiomes leading to the description of symbiotic bacteria either protecting their hosts against superficial infections

or modulating host intraspecific recognition [1–4]. However, most studies on arthropods have instead focused on the diversity and the biological importance of their internal microbiome, including the microbes living within the gut but also those living within their own cells such as maternally inherited intracellular bacteria [5–9]. It is now clear that the internal microbes of arthropods contribute to a variety of ecological and evolutionary processes, driving pivotal nutritive, reproductive and immunity functions [10–13]. Recently, some of these findings have also been discussed in the context of an eventual use of the internal microbes to limit the transmission of

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pathogens by blood-feeding arthropods, especially mosquitoes and ticks [14–20].

Ticks are major vectors of pathogens and especially well known for the part they play in spreading Lyme and other emerging diseases. Unlike mosquitoes, ticks feed exclusively on blood at all stages in their development and exhibit a unique internal microbiome with a diversity of non-pathogenic extracellular and intracellular bacteria [21–30]. Recent case studies have demonstrated the evolutionary and ecological importance of these internal microbes for ticks. For instance, some resident microbes of the tick gut can influence tick immune responses and then modulate their resistance to pathogens [14, 17]. Other examples include maternally inherited bacterial symbionts that are essential for ticks' growth and survival to adulthood: the vertebrate blood ingested by ticks is limited in B vitamins, and symbionts supply these missing nutrients to them [31–33]. A deeper investigation of the biodiversity of the internal microbes of ticks is now ongoing, as shown by the increasing number of metagenomics studies using high-throughput sequencing [23, 34–36].

These case studies require the use of a sterilization protocol of the cuticles to eliminate surface microbes and exogenous DNA before investigating internal microbe diversity. Ticks are especially prone to harboring external microbes since they can be contaminated either by the skin microbiome of their vertebrate hosts during blood-feeding and, when they are “off-host,” by environmental microbes from the soil or plants [26, 37]. For these reasons, sterilization methods are commonly employed before investigating internal microbiomes, but these methods differ greatly between studies: while most studies used ethanol solutions for this purpose [14, 23, 36], a few used sodium hypochlorite (bleach) solutions instead [38, 39]. Ethanol and bleach are both effective at killing microbes but only bleach will denature DNA [40, 41]. One can thus assume that the DNA of external microbes may remain present on the tick's cuticle after an ethanol-based sterilization. This may be an important source of contamination for internal microbial communities, and external microbes will then be misidentified as internal microbes. In addition, abundant cuticular microbes may also limit the effectiveness of next-generation microbe community profiling by masking less abundant internal microbes. However, how different sterilization methods of the tick cuticle (i.e. ethanol- versus bleach-based methods) impact the difference in the diversity of internal microbial communities remains entirely unknown. It is noteworthy that studies using ethanol-based sterilization methods tend to show more diverse bacterial communities [14, 17, 30, 42–46] than studies using bleach-based sterilization methods [39, 47, 48]. However,

this comparison is only partly relevant since these studies did not use standardized protocols: they differ in regard to the tick species examined, but also the stages and sexes of the ticks, the sampling localities, as well as the molecular and analytical approaches. The importance of these potential biases means that a definitive comparison between ethanol- and bleach-based sterilization methods was not possible.

In this study, we thus evaluated the accuracy of the two commonly used sterilization methods for the tick cuticle: ethanol- and bleach-based. We tested the impact of these two methods on the measure of the internal microbiome of ticks, and further estimated the diversity of the cuticular microbiome through swab samples and water washes. For this purpose, 100 field adult females of the Cayenne tick *Amblyomma cajennense* (*sensu stricto*) were used as a case study. This South American tick species is restricted to the Amazonian region and its microbiome has not been investigated to date. Previous molecular investigations have, however, shown that members of the *A. cajennense* species complex commonly harbor intracellular bacteria of the *Rickettsia* and *Anaplasma* genera, which are both potential pathogens for humans and animals [49, 50]. Recent investigations have further shown that all *A. cajennense* (*s.s.*) individuals are also infected by a maternally inherited bacterium, the *Coxiella*-like endosymbiont (hereafter *Coxiella*-LE), which is assumed to be the B vitamin-providing symbiont required for tick survival [51–53].

Methods

Tick sampling and processing

Unfed (“questing”) adult females of *A. cajennense* (*s.s.*) were collected in October 2017 from a single locality in French Guiana (4°51'48"N, 52°20'1"W; Piste de La Mirande). All individuals were obtained during one session, through drag-flagging on vegetation along a 100-m transect. Individuals were identified using morphological keys [54] and kept alive in sterile 50-ml Falcon tubes until their dissection or extraction of their DNA.

We randomly divided 100 *A. cajennense* (*s.s.*) female ticks into two batches of 50 individuals each. Ticks of the first batch were processed with commercial bleach diluted at 1% for 30 s and then rinsed for 1 min in three successive baths of DNA-free water. Ticks of the second batch were processed with 70% ethanol for 30 s and then rinsed for 1 min in three successive baths of DNA-free water. Directly after the baths, 25 ticks of each batch were stored in 1.5 ml of 70% ethanol prior to DNA extraction. The 25 remaining ticks from each batch were carefully dissected in a sterilized Petri dish under a stereomicroscope. Sterile scalpel blades and 21-gauge needles were used to remove cuticles, and sterile forceps were used to

carefully recover the midgut that was stored in 1 ml of 70% ethanol. The rest of the tick carcass (i.e. the whole body without the gut) were also stored in 1 ml of 70% ethanol prior to DNA extraction. Between each dissection, new needles and Petri dishes were used, and scalpel blades and forceps were sterilized by washing two times in sterile water and commercial bleach. Water washes of the dissection tools were further used as negative dissection controls.

To investigate and control the composition of the external microbiome, 27 additional *A. cajennense* (*s.s.*) females were subjected to a cuticle smear (ventral and dorsal faces) with sterile swabs. Furthermore, 25 other females were individually washed by vortexing for 1 min in 1.5-ml tubes full of DNA-free water, which was kept for DNA extraction. All these samples were stored at -20°C prior to DNA extraction.

DNA of tick samples, swabs, and water used for tick washing were extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Three negative extraction controls were included in all extraction series.

PCR amplification and high-throughput sequencing

A 251-bp portion of the V4 variable region of the bacterial 16S rDNA was amplified using the universal primer pair modified by Galan et al. [55] 16S-V4F (5'-GTG CCA GCM GCC GCG GTA A-3') and 16S-V4R (5'-GGA CTA CHV GGG TWT CTA ATC C-3'). Polymerase chain reaction (PCR) was performed in a total volume of 25 μl containing 12.5 μl of Multiplex PCR Kit (Qiagen), 10 μM of each primer, 9.5 μl of DNA-free water and 1 μl of genomic DNA. PCR amplifications for each tick sample were performed in duplicate to evaluate amplification and sequencing consistency. Each PCR product was tagged with a combination of two different barcodes designed by a genomic platform (GenSeq, Montpellier University) that allows for the identification of 384 different PCR products loaded onto the same MiSeq flow cell. 16S rDNA amplicons from external microbiome control samples were amplified, prepared and sequenced separately from tick amplicons. All PCR products were pooled and purified, and the library was constructed and sequenced by the GenSeq platform using Illumina paired-end 2×300 -bp technology with V3 chemistry.

16S rRNA data processing and taxonomic assignment

Sequence filtering criteria were applied through Illumina's quality control procedure. All bioinformatics analyses were conducted using the pipeline FROGS [56] implemented on a Galaxy workbench [57]. First, paired-end reads were merged into contigs with the FLASH algorithm [58]; sequences not included in the size range

of 200–300 bp were considered as sequencing errors and discarded. Then, chimeras were removed with the VSEARCH tool [59] and remaining sequences were clustered using SWARM [60]. Sequences with 97% similarity were clustered together and identified as an operational taxonomic unit (OTU). Each representative OTU sequence was aligned and taxonomically assigned using the Silva132 16S database (<https://www.arb-silva.de/>). Sequences that did not align to reference genes with a minimum of 80% similarity threshold were assumed to be non-bacterial 16S rDNA and removed from further analysis. OTUs having a maximal abundance in negative controls were discarded, as described by Birer et al. [61]. False-positive OTUs were removed by filtering OTU representing less than 0.005% of the OTU total abundance [62]. Whole-tick and tick-organ sequences (guts and carcasses) were considered as different data sets and OTUs were filtered separately to maximize the probability of discarding contaminants and false-positives specific to each data set.

Bacterial diversity and statistical analysis

To explore the difference in bacterial diversity according to the different parameters of our study, OTU sequences were used to build a phylogenetic tree using FastTree [63]. The resulting tree was used to assess beta-diversity matrices using the generalized UniFrac ($\alpha=0.5$) index with the *GUniFrac* package in R [64]. Bar plots, non-metric multidimensional scaling (NMDS) plots, and heatmaps were generated using different FROGSTAT tools on a Galaxy workbench [56].

Amplification and sequencing repeatability were evaluated by comparing the distance matrix between PCR duplicates using permutational multivariate analysis of variance (PERMANOVA) implemented in the *vegan* package in R, and performed on the generalized UniFrac ($\alpha=0.5$) dissimilarity matrix. To determine whether the sterilization methods statistically influence the bacterial diversity of ticks, OTU sequences of whole ticks were compared as described before. To assess (i) the potential impact of the sterilization method on the internal microbiome, and (ii) the relative importance of the sterilization method vs tick organs in shaping the internal microbiome, a pairwise PERMANOVA was performed with all the category pairs possible between the two parameters: sterilization treatments (ethanol and bleach) and tick body parts (guts and carcasses). The pairwise PERMANOVA was conducted using the R function *pairwiseAdonis* [65] (<https://github.com/pmartinezarbizu/pairwiseAdonis>) and *P*-values were corrected for multiple comparisons using Holm's method [66]. To control and determine the external microbiome of ticks,

a pairwise PERMANOVA was performed with swabs, water used for tick washing, and ethanol-sterilized whole-tick samples. In order to compare external samples with those of ticks in a meaningful way, all known types of internal bacteria of *A. cajennense* (*s.s.*) (members of the *Coxiellaceae* and *Rickettsiaceae* bacterial families) were removed from tick samples, duplicates were merged (duplicates one + duplicates two = number of reads), and sequence data were rarefied at 2698 reads per sample (i.e. the minimal number of reads obtained here for one sample). Except for the particular case mentioned above, we performed analyses with both non-rarefied and rarefied data. All statistical tests were conducted with R version 3.5.0.

Results

DNA contaminants and repeatability controls

We generated 16S rRNA gene sequences from a total of 150 samples of 100 specimens of *A. cajennense* (*s.s.*), including 50 samples from 50 whole specimens (25 ethanol-treated whole bodies and 25 bleach-treated whole bodies) and 100 samples from 50 dissected ticks (25 guts from ethanol-treated ticks, 25 guts from bleach-treated ticks, 25 carcasses of ethanol-treated ticks and 25 carcasses of bleach-treated ticks). Additional 16S rRNA gene sequences were also generated from 27 swabs used for cuticle smearing and 25 water samples used for tick washing. The rarefaction curves confirmed that bacterial diversities were sufficiently sampled (in almost all samples). After filtration of false-positives OTUs, 4,504,538 reads distributed in 320 OTUs were obtained in the data sets of the 50 whole ticks and 6,097,046 reads distributed in 373 OTUs were obtained in the 100 tick organs samples (50 guts and 50 carcasses of ticks). We also identified 59 and 41 contaminant OTUs that had a maximum abundance in negative controls. These OTUs corresponded, respectively, to 3 and 1.7% of the total number of reads after false-positive filtration. The two most abundant contaminants were affiliated to chloroplasts and *Streptococcus* bacteria.

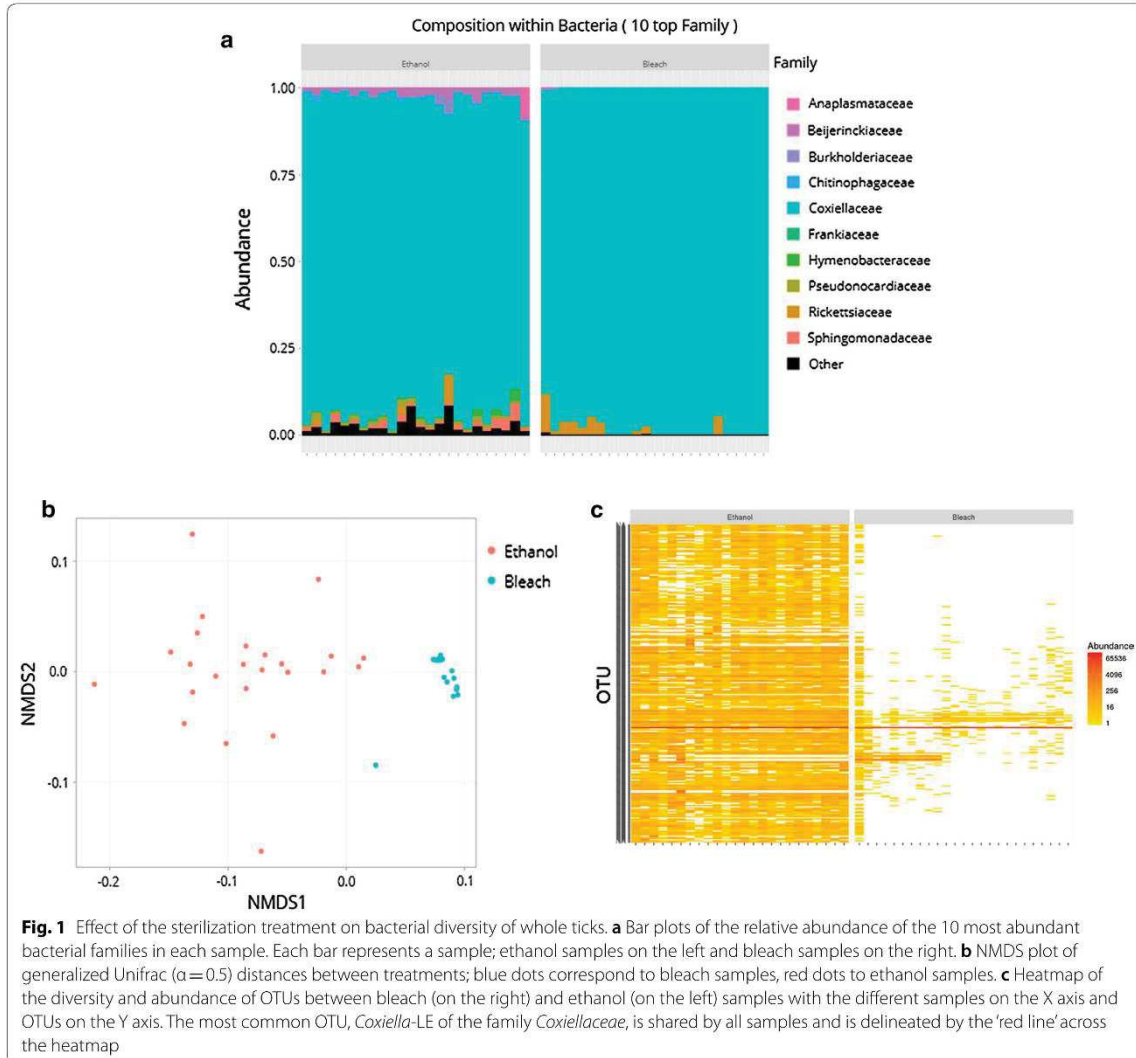
No difference in bacterial composition and diversity was observed between PCR duplicates (Additional file 1: Figure S1; PERMANOVA whole ticks, $R^2=0.006$, $P=0.59$; tick organs, $R^2=0.004$, $P=0.51$). PCR duplicates of the same sample were thus pooled for further analyses. The vitamin B-providing symbiont *Coxiella*-LE was consistently observed in all *A. cajennense* (*s.s.*) samples, while the putative pathogen *Rickettsia* was only observed in some of them as further detailed below. These two intracellular bacteria were not detected from swab and water samples.

Comparison of surface sterilization methods on bacterial diversity from tick whole bodies

We first compared the results of the 25 ethanol-treated whole bodies and of the 25 bleach-treated whole bodies of *A. cajennense* (*s.s.*) females (Fig. 1a–c). Ethanol-treated ticks produced more than twice as many reads (mean \pm SE: $58,218 \pm 17,817$ reads) as bleach-treated ticks ($26,877 \pm 20,477$; Wilcoxon two-tailed test, $W=363$, $P=9.9e-10$; Additional file 2: Table S1). Each ethanol- and bleach-treated whole-body sample was highly dominated by one taxon with more than 90% (from 93% to 100%) of the reads assigned to a member of the family *Coxiellaceae*, *Coxiella*-LE (Fig. 1a). The second most abundant taxon was a member of the family *Rickettsiaceae*, *Rickettsia*, although it was heterogeneously distributed within the samples and detected in four and ten ethanol- and bleach-treated samples, respectively (Fig. 1a). However, bacterial diversity patterns clearly differed between the two different sterilization methods. There was significant variation in the bacterial diversity between ethanol-treated samples while only a few variations were apparent between the bleach-treated samples, with most observations clustering together on the NMDS plot (Fig. 1b). This difference was also consistent in the heatmap: many OTUs, most showing a low-to-medium abundance such as *Sphingomonadaceae* or *Beijerinckiaceae* were widely present in ethanol-treated samples while only very few OTUs were present in bleach-treated samples (Fig. 1c). PERMANOVA analysis further confirmed that the sterilization method explains most of the bacterial diversity variation between samples ($R^2=0.57$, $P=0.001$). Overall, this makes clear that bleach-treated whole-body samples exhibited a lower bacterial diversity than ethanol-treated samples.

Characterization of internal microbial diversity

The bacterial diversity observed suggests that bleach is more effective for removing external microbes than ethanol. However, one may also assume that bleach was internalized during the sterilization process and then denatured the DNA of internal microbes. This may explain why bleach-treated samples exhibited a lower bacterial diversity than ethanol-treated samples. To examine this possibility, we further assessed the bacterial diversity present in the guts of ethanol- and bleach-treated *A. cajennense* (*s.s.*) females. To this aim, we used 25 guts from ethanol-treated ticks, 25 guts from bleach-treated ticks, 25 carcasses of ethanol-treated ticks and 25 carcasses of bleach-treated ticks (Fig. 2a, b; Additional file 3: Table S2). Guts consistently harbored a higher diversity than carcasses in the bleach- (pairwise PERMANOVA, $R^2=0.327$, adjusted P for

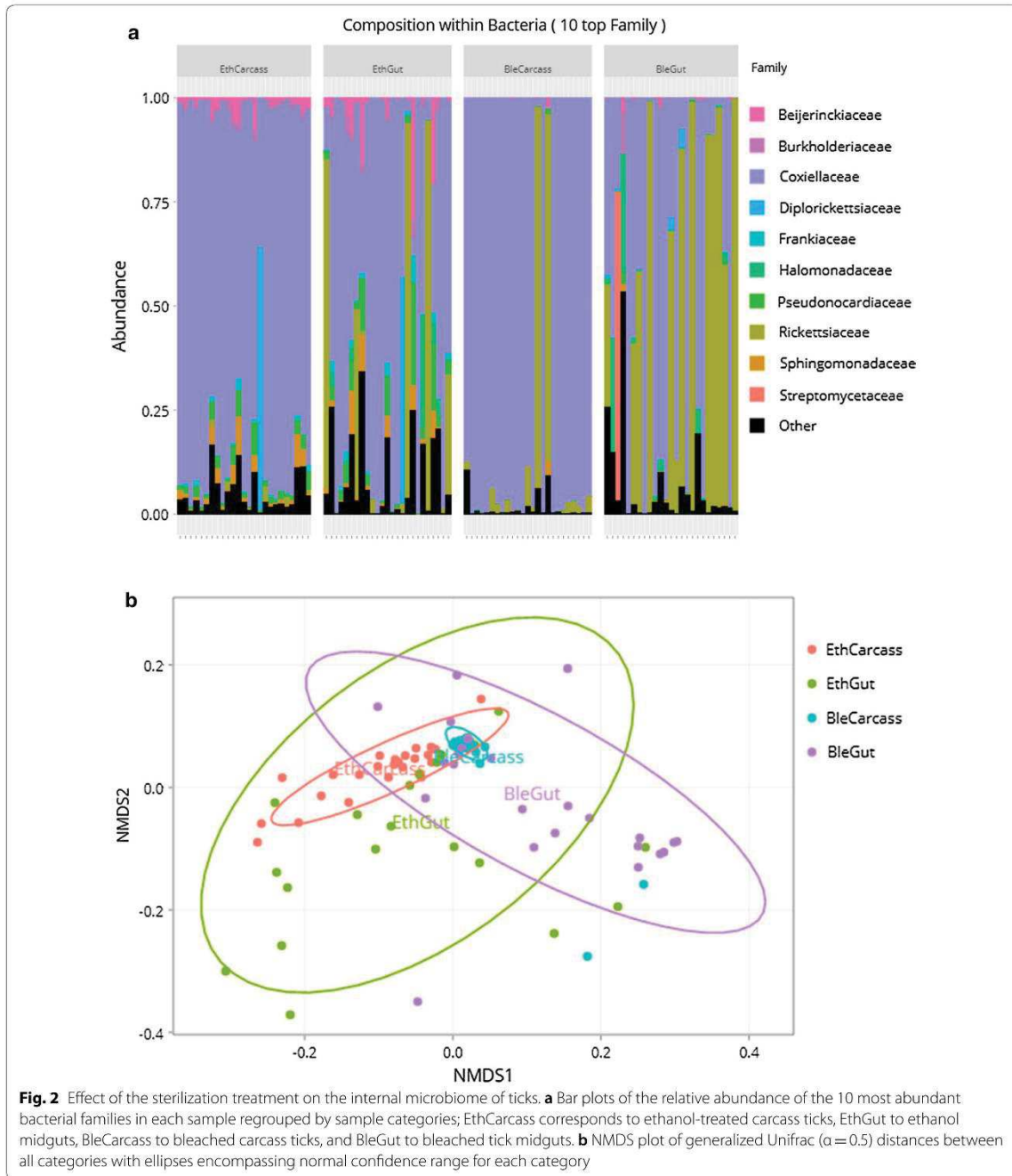


multiple comparisons = 0.0006) and ethanol-treated specimens (pairwise PERMANOVA, $R^2=0.09$, adjusted P for multiple comparisons = 0.0308; Fig. 2). Carcasses of bleach-treated ticks showed a lower bacterial diversity than carcasses of ethanol-treated ticks (pairwise PERMANOVA, $R^2=0.368$, adjusted P for multiple comparisons = 0.0006; Fig. 2a, b), thereby corroborating our previous observations on tick whole bodies. While there was a significant structural change between the gut communities between guts from ethanol- and bleach-treated ticks ($F=40$, $P<0.0001$), no significant difference in bacterial diversity was observed (pairwise PERMANOVA, $R^2=0.03$, adjusted P for multiple comparisons = 0.18;

Fig. 2a, b), showing that surface sterilization protocols impact the gut bacterial diversity in the same way.

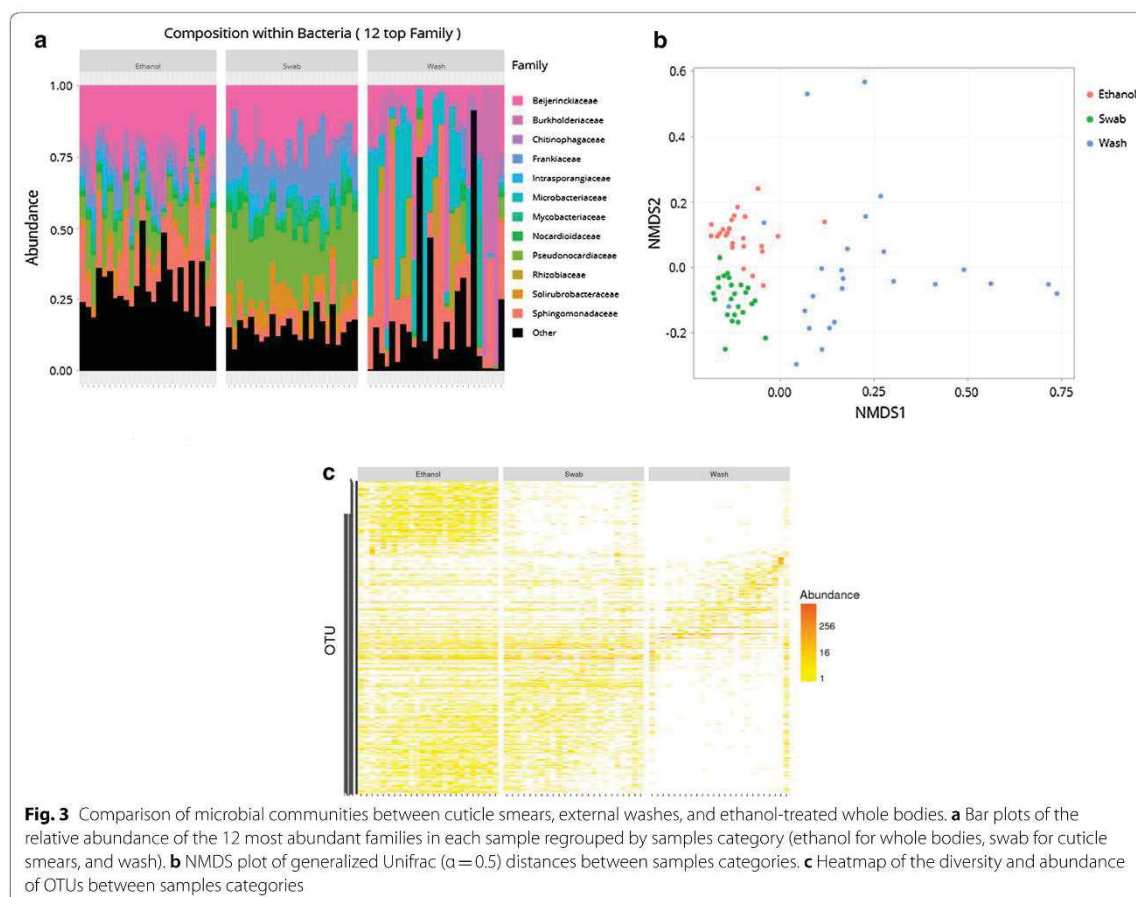
Detection of external microbes

To characterize the external microbes of *A. cajennense* (*s.s.*) females, we characterized the microbial communities of 27 cuticular smears and 25 water samples used for external cleaning (Fig. 3a–c). We obtained 1,433,868 reads leading to the identification of 859 OTUs after filtration of false-positive OTUs. We further compared this diversity with the one observed in the 25 ethanol-treated whole bodies described earlier. All known intracellular symbionts (e.g. *Coxiella*, *Rickettsia*) of ticks were



discarded from the whole-tick data, resulting in only ethanol-treated ticks being used for this analysis since intracellular symbionts represented over 98% of the microbial community in bleached ticks (Additional file 4: Table S3).

The bacteria community retrieved in external washes was clearly different to the two others observed in swabs and ethanol-treated whole bodies (Fig. 3a–c). Indeed, the taxa composition was far more heterogeneous across samples



of the external wash categories than those of the other two (Fig. 3a). The microbial diversity of the wash samples was dominated by *Burkholderiaceae*, *Mycobacteriaceae*, *Sphingomonadaceae* and *Beijerinckiaceae* (Fig. 3a). These taxa were also present in ethanol and swab samples, but only the *Beijerinckiaceae* and *Sphingomonadaceae* families were additionally highly abundant (Fig. 3a). However, in contrast to ethanol, swab samples were also dominated by *Frankiaceae* and *Pseudonocardiaceae* families (Fig. 3a). These results are clearly illustrated in the NMDS plot, where wash samples are widely distributed reflecting the heterogeneity of bacteria diversity across samples (Fig. 3b). By contrast, the ethanol and swab data set are clustered in the left of the X axis and differ only by small amounts in the Y axis (Fig. 3b). A similar pattern is observed in the heatmap (Fig. 3c): wash samples differ greatly from ethanol samples and swab samples, which differ by a cluster of OTUs only present in the ethanol samples. The three pairs tested with the pairwise PERMANOVA show a significant difference in all categories:

ethanol vs swab ($R^2=0.31$, adjusted P for multiple comparisons = $3e-04$), ethanol vs wash ($R^2=0.50$, adjusted P for multiple comparisons = $3e-04$) and swab vs wash ($R^2=0.56$, adjusted P for multiple comparisons = $3e-04$).

Discussion

In the present study, we evaluated the effect of the two most common methods, one based on ethanol and the other on bleach, used to remove microbe contaminants present on tick cuticles. Although most previous studies on the internal microbiome of ticks commonly used an ethanol-based method [14, 17, 30, 42–46], we observed systematic differences between ethanol- and bleach-treated specimens. Both methods led to the detection of the B vitamin-providing symbiont *Coxiella*-LE in all *A. cajennense* (s.s.) samples, and of a putative pathogen *Rickettsia* in some of them, as expected from previous studies [50–53]. However, despite these obvious similarities, ethanol-treated ticks consistently harbored a higher bacterial diversity than bleach-treated ticks. In this context,

further observations are particularly relevant: (i) there was no difference in bacterial diversity between ethanol- and bleach-treated ticks, showing that surface sterilization methods impact the internal microbiome in the same way; (ii) the bacterial diversity of cuticle smears was very similar to the one found in ethanol-treated ticks, but not to the one of bleach-treated ticks. Most of the taxa retrieved in the cuticle smears and ethanol-treated ticks are known to be environmental bacteria associated with soil and plants (i.e. *Beijerinckiaceae* [67]) or to be cuticular symbionts of arthropods (i.e. *Pseudonocardiaceae* [68]). Overall, these findings prove that the ethanol-based surface sterilization method is not efficient to eliminate DNA of external bacteria and could lead to DNA contamination from the cuticle during tick dissection. By contrast, the bleach-based surface sterilization method can denature the DNA of external bacteria and is thus a better practice for studies aiming to characterize the internal microbiome of ticks.

The low bacterial diversity observed here in bleach-treated *A. cajennense* (*s.s.*) ticks, along with previous studies using bleach-treated specimens of other tick species [39, 47, 48], supports the recent finding that ticks harbor a rather simple internal microbiome dominated by maternally inherited symbionts [69, 70]. Indeed, *Coxiella*-LE alone represents the quasi-totality of the internal microbiome of *A. cajennense* (*s.s.*) females. This suggests that only few other internal bacteria are present but, alternatively, one can also assume that the abundance of *Coxiella*-LE 16S rDNA reads masks the presence of less abundant bacteria. The rarefaction curves of our samples and the conclusions of previous studies [39, 69, 70] indicate that such a low bacterial diversity, highly dominated by intracellular symbionts, is a biological reality in ticks. On the other hand, in a study of the Australian tick *Ixodes holocyclus* [38], the authors successfully eliminated a maternally inherited symbiont, *Midichloria*, using blocking primers and showed a significant increase of bacterial diversity in *Midichloria*-free samples. However, all these studies confirm that maternally inherited endosymbionts are the major bacterial partner of ticks.

Conclusions

In conclusion, we herein provide evidence that studies investigating the internal microbiome of ticks should consider commercial bleach as the gold standard to efficiently remove cuticular bacterial DNA. We used a standardized 30 second bleach treatment, sufficient to remove external microbes, although it is obvious that a shorter or longer time may affect the result. As such, prior studies investigating the microbiome without bleach surface sterilization should be reconsidered in light of our results. Moreover, this study contributes

evidence supporting the new paradigm that a highly diversified and complex gut microbiome is not shared by all arthropods [69–72]. Interestingly, this lack of complex gut microbiome seems to be shared by arthropods specialized in a restricted diet, such as blood or plant sap: these arthropods commonly harbor one or two types of maternally inherited symbionts able to satisfy most of the nutritional requirements of their hosts [6, 8, 13, 73–76]. Such maternally inherited symbionts may render facultative the presence of other internal microbes, leading to an internal microbiome of low complexity. This suggests a role of these nutritive symbionts in shaping the gut microbiome of arthropods specialized in a restricted diet.

Additional files

Additional file 1: Figure S1. Effect of PCR duplicates on bacterial diversity. Nonmetric multidimensional scaling (NMDS) plot of generalized UniFrac ($\alpha = 0.5$) distances between PCR duplicates of samples: **a** Whole ticks, **b** tick organs. Blue dots correspond to first duplicates (D1), red dots to second (D2). **c** Heatmap plot showing abundance of OTUs across whole-tick samples and **d** abundance of OTUs across tick-organ samples. X and Y axes show the different samples and OTUs, respectively. D1 heatmaps correspond to first duplicates while D2 corresponds to the second ones.

Additional file 2: Table S1. OTU abundance without contaminants retrieved in whole-tick samples.

Additional file 3: Table S2. OTU abundance without contaminants retrieved in tick-organ samples (guts and carcasses).

Additional file 4: Table S3. OTU abundance without contaminants and intracellular endosymbiont retrieved in cuticle smears, wash samples, and ethanol-treated whole-tick samples.

Abbreviations

LE: like endosymbiont; OTU: operational taxonomic unit; NMDS: non-metric multidimensional scaling; PERMANOVA: permutational multivariate analysis of variance.

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Authors' contributions

FB and OD conceived and designed the study. FB, MD, MB and OD performed the experiments. FB analyzed the data. FB and OD wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the additional files and raw sequencing data are available in the GenBank database under the Accession Number PRJNA530927.

Ethics approval and consent to participate

The use of the genetic resources was declared to the French Ministry of the Environment under reference #150401230100, in compliance with the Access and Benefit Sharing procedure implemented by the Loi pour la Reconquête de la Biodiversité.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Discussion

Les résultats de cette étude montrent clairement que le traitement à l'éthanol n'est pas efficace pour enlever l'ADN du microbiome cuticulaire. Nous recommandons d'utiliser à la place un traitement à la Javel qui, en plus de dénaturer efficacement l'ADN du microbiome externe, ne semble pas impacter le microbiome interne. De manière plus importante, cette étude démontre qu'il est nécessaire d'être précautionneux en considérant les résultats des études n'utilisant pas un tel protocole. En effet, bien que dans un contexte descriptif, la différence entre microbiomes interne et externe ne soit pas très importante, cela devient une nécessité de premier ordre pour appréhender les facteurs structurants des cortèges microbiens internes. Comme nous l'avons vu en introduction, aux échelles intra-holobionte et intra-spécifique, les microbiomes sont majoritairement soumis à des processus écologiques dépendant du principe des niches écologiques. Il apparaît alors évident, que les microbiomes interne et externe d'une tique ne partageront pas les mêmes niches écologiques et ne seront donc pas soumis aux mêmes pressions de sélection. Bien qu'aucune étude, à proprement parler, ne se soit intéressée aux facteurs structurants des microbiomes cuticulaires des tiques, il n'est pas illogique de s'attendre à observer des variations dans ces microbiomes, en fonction des localités ou de l'hôte (et à quel endroit de l'hôte) où la tique s'est nourrie, par exemple. **Ainsi, les conclusions sur la structuration du microbiome interne, avancées par les études qui, par un biais technique, ne différencient pas le microbiome interne de l'externe, sont à prendre avec précaution.**

Pour aller plus loin, il est même périlleux de comparer les résultats de deux études sur les microbiomes de tiques. En effet, en plus du biais mentionné dans l'article et le paragraphe précédent, il existe énormément de paramètres de techniques et d'analyses

pouvant influencer la diversité et la composition retrouvées dans un microbiome. Pour éviter des résultats biaisés et des surinterprétations, il est alors nécessaire d'utiliser des études partageant les mêmes paramètres techniques. Or, à la lumière de la littérature, cela apparaît compliqué, voire impossible, avec les études traitant du microbiome des tiques. Effectivement, il n'existe aucune standardisation de protocole pour de telles études. Ainsi, outre l'utilisation ou non de Javel, les différences dans les protocoles sont nombreuses et influencent les résultats de composition ou de diversité microbiennes (revue par Greay *et al.*, 2018 ; Hornung, Zwartink, & Kuijper, 2019). Par exemple, la diversité et la composition du microbiome pour un même hôte seront différentes en fonction : de la région hypervariable (V1-V9) amplifiée du gène bactérien 16S rRNA (*e.g.* Sperling *et al.*, 2017) ; des amorces de PCR utilisées (Klindworth *et al.*, 2013 ; Tremblay *et al.*, 2015) ; des différents kit d'extractions (*e.g.* Glassing, Dowd, Galandiuk, Davis, & Chiodini, 2016 ; Lejal *et al.*, 2019) ; de la technologie de séquençage utilisée (Liu *et al.*, 2012), ou encore du pipeline bio-informatique suivi pour l'analyse des données de séquençages (Escudié *et al.*, 2018 ; Sun *et al.*, 2012) ; etc...

En plus de souligner le manque de standardisation dans l'étude des microbiomes internes de tiques, notre étude semble confirmer le nouveau paradigme existant sur ces microbiomes. En effet, comme discuté dans l'étude, la diversité microbienne hébergée par les tiques apparaît très faible et largement dominée par la seule présence du P-symbionte de la tique (dans notre cas : *Coxiella*). Ainsi, la structuration aux échelles intra-holobionte et intra-spécifique des microbiomes chez les tiques « javélisées » apparaît comme majoritairement expliquée par la présence des symbiontes à TV, et très peu (ou pas) impactée par l'environnement de la tique. De manière intéressante, ces résultats concordent avec ceux de Couper *et al.* (2019) présentés en introduction et concernant l'impact de la TV sur la structuration des microbiomes chez *I. pacificus*. Ce patron de

microbiome n'est pas isolé au seul cas de l'espèce utilisée dans cette étude (*A. cajennense*), mais à l'ensemble des espèces d'*Amblyomma* investiguées comme nous allons le voir, en partie, dans l'article en préparation du chapitre suivant.

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Chapitre III

Chapitre III

Structuration des microbiomes des tiques à l'échelle inter-spécifique

Article 4 : Binetruy F, Buysse M, Barosi R & Duron O. Microbial community structure reveals instability of nutritional symbiosis during evolutionary radiation of *Amblyomma* ticks. En préparation.

Contexte

L'objectif principal de cette thèse était, à l'origine, de comprendre la structuration des microbiomes aux différentes échelles résolutive et notamment à l'échelle inter-spécifique. Nous voulions, entre autres, tester des hypothèses de phylosymbiose entre la phylogénie des tiques et la structure de leur microbiome. Néanmoins, la très faible diversité microbienne caractérisée chez nos tiques, qui plus est dominée par la présence d'un P-symbionte, rend notre idée originelle superfétatoire.

En effet, puisque la présence et l'identité des P-symbiontes sont les principales variables influençant la diversité et la composition microbiennes à une échelle inter-spécifique, nous avons décidé de nous concentrer sur ces P-symbiontes qui composent donc le core-microbiome des tiques (microbiome retrouvé chez toutes les espèces de tiques). Ainsi, comme nous l'avons vu en introduction, la coévolution au sein d'un holobionte est l'un des principaux facteurs expliquant les communautés microbiennes à une échelle inter-spécifique. Ce processus va également structurer en partie l'hologénome d'un holobionte, ce qui est d'autant plus vrai concernant les arthropodes et leurs P-symbiontes. Connaissant cela, nous avons essayé d'appréhender les effets structurants du core-microbiome de nos tiques en utilisant des données moléculaires et phylogénétiques. Grâce à ces données, nous avons notamment testé de possibles effets phylogéographiques

de l'hôte et du spectre d'hôtes des espèces de tiques sur la structure des core-microbiomes. Pour ce faire, nous nous sommes concentrés sur le genre de tique *Amblyomma*. En effet, mis à part pour ce genre, notre collection de Guyane n'est pas assez représentative de la diversité inter-générique des autres genres (*Ixodes*, *Dermacentor*, *Haemaphysalis* et *Ornithodoros* : 1 espèce par genre ; *Rhipicephalus* : 2 espèces ; voir chapitre 1) et donc biaisée pour une telle étude.

Microbial community structure reveals instability of nutritional symbiosis during evolutionary radiation of *Amblyomma* ticks

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Abstract

Mutualistic interactions with microbes have facilitated the adaptation of major eukaryotic lineages to restricted diet-niches. Hence, ticks are associated with intracellular bacterial symbionts strictly required for the ticks' blood feeding lifestyle through a pivotal B vitamin supplementation. In this study, the examination of the whole bacterial diversity in 25 tick species of the *Amblyomma* genus shows that three intracellular bacteria, *Coxiella*-LE, *Francisella*-LE and *Rickettsia*, are remarkably common. No other bacterium is so uniformly present in *Amblyomma* ticks. Almost all *Amblyomma* species were found to harbour a nutritive obligate symbiont, *Coxiella*-LE or *Francisella*-LE, both able to synthesize B vitamins. However, despite the co-evolved and obligate nature of these mutualistic interactions, the structure of microbiomes does not mirror the *Amblyomma* phylogeny with a clear exclusion pattern between *Coxiella*-LE and *Francisella*-LE across tick species. *Coxiella*-LE, but not *Francisella*-LE, forms evolutionarily stable associations with ticks leading commonly to co-cladogenesis. We further evidenced symbiont replacements during radiation of *Amblyomma*, with recent, likely ongoing, and repeated invasions by *Francisella*-LE and subsequent replacements of ancestral *Coxiella*-LE through transient coinfections. Nutritional symbiosis in *Amblyomma* ticks is thus not a stable evolutionary state, but rather arises from conflicting origins between unrelated but competing symbionts with similar metabolic capabilities.

Introduction

Macroorganisms harbour complex microbial communities living inside and on their body [1, 2]. These microbial communities, known as the microbiomes, can determine pivotal phenotypic traits of their hosts, contributing to a variety of ecological and evolutionary processes, driving important nutritive, reproductive and immunity functions [3–7]. As hosts vary in the microbiomes they harbor, an associated functionally important phenotypic variation exists within host populations [8–12]. In arthropods, these microbiomes notably include highly specialized intracellular bacteria depending almost exclusively on maternal (transovarial) transmission to ensure their persistence in host populations [13, 14]. Some of these maternally inherited symbionts are essential for the life cycle of their arthropod hosts: they are obligate mutualists able to synthesize biochemical products favoring the specialization of arthropods to novel habitats or on particular feeding niches such as strict haematophagy or phloemophagy [13, 14]. Overall, these mutualistic interactions have facilitated the radiation of major arthropod lineages, leading to remarkable host-symbiont phylogenetic congruence with strict cocoladogenesis pattern in many cases [15–19].

Arthropods and beneficial maternally inherited symbionts can form evolutionary stable associations lasting for millions of years, but that are not necessarily permanent [13, 14, 20, 21]. Recent phylogenetic reconstructions suggest that beneficial symbiotic relationships can break down: recently acquired symbionts can replace ancestral beneficial symbionts and provide similar benefits to the host [20, 22]. New beneficial symbionts often originate from microbes abundant in the host environment, potentially including entomopathogens, parasites vectored by arthropods or other maternally inherited symbionts, primarily facultative (ie, not essential) for host survival [20, 23–25]. These facultative symbionts however determine important traits in arthropods: protection against natural enemies, adaptation to changing

environments or reproductive traits [8, 12, 13, 26]. Contrary to beneficial obligate symbionts, facultative symbionts undergo occasional horizontal transfers (HT) across arthropod species, resulting in limited phylogenetic congruence between hosts and symbionts [27–29]. Overall, the diverse range of microbial lifestyle strategies creates a complex web of interactions mediating dynamics of beneficial symbioses in arthropods [20].

Coexistence of symbionts within microbial communities is expected to involve interactions ranging from cooperation to competition and which can, in turn, determine aggregation and exclusion patterns [8, 13, 30]. Exclusion patterns have been recently detected between maternally inherited symbionts of ticks, suggesting that replacements of beneficial symbionts occur in this system [16]. Among arthropods, ticks (Arachnida: Ixodidea) are well known to engage in symbiotic associations with at least 10 different genera of maternally inherited bacteria [16, 31]. Ticks are specialized on an exclusive diet of vertebrate blood, and have evolved intimate interactions with beneficial symbionts which provide essential B vitamins and cofactors deficient in the blood diet [16, 32–39]. Approximately two third of tick species harbor a *Coxiella*-like endosymbiont (*Coxiella*-LE hereafter) which is required for tick survival and reproduction [34, 35, 37, 40]. *Coxiella*-LE genomes encode pathways for the biosynthesis of major B vitamins and cofactors that fit closely with the expected nutritional complements required for strict hematophagy [34, 35, 37]. Due to their maternal inheritance and beneficial nature, these symbionts are present in most individuals within a given host species. In the *Rhipicephalus* tick genus, the acquisition of *Coxiella*-LE was followed by codiversification resulting in deeply congruent *Rhipicephalus*–*Coxiella*-LE phylogenies [16].

In few tick species, *Coxiella*-LE are however present at much lower frequencies than expected for obligate nutritional symbionts, suggesting that they are rather facultative symbionts in these

hosts [41]. Phylogenetic evidences corroborate this hypothesis since closely related *Coxiella*-LE may infect distantly related tick species suggesting recurrent HT of some *Coxiella*-LE [41]. In other tick species, no *Coxiella*-LE was even detected, but alternative obligate beneficial symbionts have been identified or hypothesized [16, 32, 33, 38, 42]. Indeed, *Francisella*-like endosymbionts (*Francisella*-LE) are commonly found in tick species lacking *Coxiella*-LE. A recent analysis of endosymbiotic communities in 81 tick species also showed that there is a significant exclusion pattern between *Francisella*-LE and *Coxiella*-LE [16]. As *Coxiella*-LE, *Francisella*-LE is pivotal for tick nutrition: ticks deprived of their *Francisella*-LE completely ceases development but resumes normal growth upon supplementation with B vitamins [32]. Genomes of *Francisella*-LE contain roughly the same biosynthesis pathways of B vitamins and cofactors than observed in *Coxiella*-LE genomes [32, 33]. Although *Francisella*-LE and *Coxiella*-LE are distantly related, they have thus converged toward an analogous nutritional mutualism with ticks. While *Coxiella*-LE symbioses are likely ancestral in ticks, replacements by *Francisella*-LE [32, 33], having recently transitioned to an endosymbiotic lifestyle, appear across the tick phylogeny [16]. Yet, the factors favouring *Francisella*-LE over *Coxiella*-LE in this evolutionary dynamic are not well understood. In this study, we examine the evolutionary dynamic of tick microbiomes, with a focus on beneficial nutritional symbioses, in an ecologically diverse genus of hard ticks, *Amblyomma*. This genus is the third largest in the family Ixodidae, with its species primarily occupying the tropical zones. The centre of species diversity is on the American continent, where half of all the 130 *Amblyomma* species occur [43]. The *Amblyomma* genus includes major vectors of tick-borne disease agents, including the lone star tick, *A. americanum*, which is the primary vector of *Ehrlichia* spp. [44]. Only a few studies have examined the microbial diversity in *Amblyomma* species, showing that they are infected either by *Coxiella*-LE or by *Francisella*-LE, in addition to other maternally inherited bacteria [16, 33, 45–47]. Here we investigate variation in tick microbial communities at

different geographic and phylogenetic scales using a representative collection of specimens covering *ca.* 20% of *Amblyomma* species diversity. First, we reconstruct the *Amblyomma* phylogeny through the sequencing of large nuclear rDNA sequences (18S, ITS1, 5.8S, ITS2, and 28S rDNA). Second, we extensively characterize bacterial communities in *Amblyomma* species through a DNA barcoding approach targeting the 16S rDNA. Third, we trace the evolutionary histories of *Coxiella*-LE and *Francisella*-LE using multilocus sequence typing (MLST) systems. While the *Coxiella*-LE MLST already exists [41], we develop in this study a specific *Francisella*-LE MLST. Finally, we compare *Amblyomma* phylogeny with microbiome structure, and further use cophylogenetics, through the contrast of *Amblyomma*, *Coxiella*-LE and *Francisella*-LE phylogenies, to reveal the global dynamics of symbiotic interactions.

Materials and methods

Tick collection and processing

Specimens belonging to 25 *Amblyomma* species were collected from a variety of field sites in America and Africa, or from laboratory colonies (Supplementary Table 1). From 1 to 11 specimens per *Amblyomma* species were processed individually and a total of 144 specimens were used (details of each individual's sex, development stage and population are listed in Table S1). Samples were preserved in 70% ethanol until use. To eliminate external (ie, cuticular) microbes, tick specimens were surface cleaned with bleach prior to DNA extraction following a published protocol [45] specimens of *A. loculosum* ($n=4$), *A. sculptum* ($n=1$) and *A. sp.* ($n=1$), obtained from a previous study [16], were however not bleach-treated but ethanol-treated prior to DNA extraction (Table S1). All tick DNA was individually extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following manufacturer instructions.

Molecular typing of Amblyomma ticks

To reconstruct *Amblyomma* phylogeny, we used almost complete rDNA sequences, including 5' end of 18S, entire ITS1, 5.8S and ITS2 sequences, and 3' end of 28S rDNA. This fragment was amplified by long-range PCR for one to four specimens of each of the 25 *Amblyomma* species, using primers specifically designated for this study (Amb18SF1: 5'-GCTCGCGTTGATTACGTCCC -3' and Amb28SR3: 5'-CTAGTTGCTTCGGCAGGTGAG -3'). La Taq DNA polymerase kits (TaKaRa Bio, Osaka, Japan) were used in PCR reactions in a total volume of 50 μ L and following manufacturer protocol. PCR conditions were: a first denaturation step of 96°C for 1 min, followed by 35 cycles of 96°C for 40 s, an annealing step of 58°C for 1 min, an elongation step of 72°C for 6 min and a final elongation of 72°C for 12 min. Depending of the tick species, the size of the rDNA amplicon obtained varies from 4,270 bp to 5,054 bp. PCR products were purified using the kit Cleanpcr (CleanNA, Waddinxveen, Netherlands) following manufacturer protocol, fragmented to a size of 300bp and further used to construct libraries with the kit Nextera XT (Illumina, San Diego, California, USA). Then, these libraries were pooled together, indexed and pair-end sequenced on a Miseq (Illumina) sequencer using a flow cell equipped with a V3, 600-cycle reagent cartridge.

We obtained approximately 24 million reads after quality filtering and adaptors removing, using Cutadapt tool on a Galaxy workbench [48, 49]. Paired-end reads were *de novo* assembled using metaSPAdes v3.11 with k-mer sizes of 21, 33, 55, and 73 bp [50]. Resulting metaSPAdes contigs for each *Amblyomma* specimen were binned into tick nuclear DNA and non-tick nuclear DNA groups through megablast into Genbank nucleotide collection [51]. Resulting one or two contigs depending on *Amblyomma* species, corresponding to the almost complete rDNA sequences amplified by long-range PCR. These contigs were scaffolded, when applicable, by aligning to the complete sequences of the rRNA genes operon of *A. americanum* (GenBank:

AF291874), *A. hebraeum* (GenBank: KY457489) and partial sequence of *A. marmoreum* (GenBank: KY457492) using MEGA 7 [52].

Bacterial 16S rDNA metabarcoding

A 251-bp portion of the V4 variable region of the bacterial 16S rRNA gene was amplified individually for each DNA sample, processed for MiSeq (Illumina) sequencing, and further analysed as previously described [45]. One step of operational taxonomic units (OTUs) affiliation post-process was additionally performed through Frogs pipeline on a Galaxy workbench [48, 53]. This step consists to aggregate OTUs sharing 97% of identity within 99% of amplicon length and reduced the probabilities of keeping artefactual OTUs by resolving multi-hit ambiguities. Following this procedure, the microbiome was determined individually for almost all *Amblyomma* specimen ($n=142/143$) but the amount of DNA for the single specimen of *A. sculptum* was however not sufficient to perform this approach along with other molecular typing done in this study.

Tick specimens were considered infected by a symbiont when the percentage of reads assigned to the symbiont was greater than or equal to 2% of the total number of reads of each specimen. Below that threshold: From 0.1% to 1.99%, the presence or absence of *Coxiella*-LE and *Francisella*-LE were tested by PCR during MLST approach (described above); below 0.1% the reads assigned to any symbiont were considered as artefactual.

To determine difference of bacterial diversity between *Amblyomma* species and test if the presence of symbionts drives this difference, a phylogenetic tree using OTUs sequences and beta-diversities matrices based on this tree was assessed with FastTree and GUnifrac packages in R [54, 55], using the computational procedure described in [45]. Then, multidimensional

scaling (MDS) plots were generated using the package ggplot2 in R [56]. Finally, permutational multivariate analysis of variance (PERMANOVA) implemented in the vegan package in R or pairwise PERMANOVA [57], were performed on the generalized UniFrac ($\alpha = 0.5$) dissimilarity matrix to evaluate the potential impact of the presence of symbionts and coinfection on bacterial diversity. The *P*-values of the pairwise PERMANOVA were corrected for multiple comparisons using Holm's method [58]. Mantel test was used to test the association between microbial diversity (GUniFrac distance) and *Amblyomma* phylogeny using the R software and the ecodist package [59] with 9999 permutations.

Multilocus typing of Coxiella-LE and Francisella-LE

Coxiella-LE of *Amblyomma* spp. were genotyped through nested or semi-nested PCR amplification and sequencing of three housekeeping genes (16S rRNA, *rpoB* and *groEL*) previously developed for the *Coxiella* MLST methodology [41]. *Coxiella*-LE gene features, primers and PCR conditions are detailed in Table S2. No *Francisella*-LE MLST was previously developed and we thus used the published genome of *Francisella*-LE F-Om strain (isolated from the soft tick *Ornithodoros moubata*; [32]; GenBank accession number: QAPC00000000) as reference to design specific PCR primers for multi-locus typing. We selected five genes (16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB*) present in a single copy and physically distant within the *Francisella*-LE F-Om genome. Fragments of these five *Francisella*-LE genes were amplified using nested or semi-nested PCR assays (Table S2). The efficiency of our typing method was ascertained through positive PCR amplification and clear sequences for the five loci from DNA samples of *O. moubata* infected by *Francisella*-LE.

Nested and semi-nested PCR amplifications were performed as follows: the first PCR run with the external primers was performed in a 10 μ L volume containing ca. 20 ng of genomic DNA,

3 mm of each dNTP (Thermo Scientific, Waltham, Massachusetts, États-Unis), 8 mm of MgCl₂ (Roche Diagnostics, Rotkreuz, Switzerland), 3 μm of each primer, 1 μL of 10 × PCR buffer (Roche Diagnostics), and 0.5 U of Taq DNA polymerase (Roche Diagnostics). A 1-μL aliquot of the PCR product from the first reaction was then used as a template for the second round of amplification. The second PCR was performed in a total volume of 25 μL and contained 8 mm of each dNTP (Thermo Scientific), 10 mm of MgCl₂ (Thermo Scientific), 7.5 μm of each of the internal primers, 2.5 μL of 10 × PCR buffer (Thermo Scientific), and 1.25 U of Taq DNA polymerase (Roche Diagnostics). All PCR amplifications were performed under the following conditions: initial denaturation at 93 °C for 3 min, 35 cycles of denaturation (93 °C, 30 s), annealing (T_m = 52–56 °C, depending on primers, cf. Table S2, 30 s), extension (72 °C, 1 min) and a final extension at 72 °C for 5 min. Known positive and negative individuals were used as controls in each PCR assay. The use of nested or semi-nested PCR was efficient at decreasing the probability of contamination from unwanted amplification products (false positives). To prevent possible contamination, different parts of this process have been physically separated from one another, in entirely separate rooms. All amplicons from semi-nested PCR assays were also sequenced to control for false positive amplifications. Positive PCR products were purified and sequenced in both directions by Eurofins (Ebersberg, Germany). Sequence chromatograms were manually cleaned with Chromas Lite (http://www.technelysium.com.au/chromas_lite.html) and aligned with CLUSTALW implemented in MEGA 7 [52, 60].

Phylogenetic analyses

Phylogenetic relationships were assessed using *Amblyomma* (18S, ITS1, 5.8S, ITS2, and 28S rDNA), *Coxiella*-LE (16S rRNA, *rpoB* and *groEL*) and *Francisella*-LE (16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB*) sequences. In addition to sequences produced in this study, additional ticks, *Coxiella*-LE and *Francisella*-LE sequences available in GenBank were also used. The GBLOCKS program [61] with default parameters was used on each gene data set to remove poorly aligned positions and to obtain unambiguous sequence alignments. All sequence alignments were also checked for putative recombinant regions using the RDP3 computer analysis package [62]. The best fitting evolutionary models were determined using Akaike information criterion and Bayesian information criterion with MEGA 7 [52]. Phylogenetic analyses were based on Bayesian inferences (BI) with MrBayes v3.2.7 [63]. Two replicate analyses were run for 1 million generations. For each replicate, we ran one cold chain and three hot chains of the Markov Chain Monte Carlo method, using a random starting tree and sampling trees every 100 generations and discarding 25% as burn-in. The remaining trees were used to calculate 50% majority rule consensus trees. Posterior probabilities (pp) were summarized accordingly.

The BI phylogenies of *Amblyomma*, *Coxiella*-LE and *Francisella*-LE were then used to conduct cophylogenetic analyses using the Procrustean Approach to Cophylogeny (PACo) package in R [64, 65]. The significance of cophylogenetic tests were established by 100,000 random permutations of the two-association matrix.

Ethics statement

The use of the genetic resources was declared to the French Ministry of the Environment under reference TREL19028117S/156 and #150401230100, in compliance with the Access and Benefit Sharing procedure implemented by the *Loi pour la Reconquête de la Biodiversité*.

Results

Phylogeny of Amblyomma ticks

We first reconstructed the phylogenetic relationships between *Amblyomma* species using BI analyses based on large fragments of nuclear rDNA sequences (18S, ITS1, 5.8S, ITS2, and 28S rDNA; 3 312 bp unambiguously aligned; best-fit approximation for the evolutionary model: GTR+G+I). One-to-two specimens per *Amblyomma* species (45 specimens in total), were sequenced and analyzed (Figure 1). There is strong support for relationships among most *Amblyomma* species which can be split into different subclades. Indeed, the BI phylogeny supports a monophyletic origin of the five African *Amblyomma* species (AF group): they cluster together in a robust clade nested among the 20 New World *Amblyomma* species (NW group), suggesting an American origin of these African species (Figure 1). Moreover, the phylogeny of *Amblyomma* also parallels the tick host-range at least in the NW group: closely related, *Amblyomma* species often share the same host species, as *A. dissimile* and *A. rotundatum* feeding on poikilotherms (reptiles and amphibians) or *A. latepuctatum*, *A. scapularum* and *A. naponense* feeding mainly on tapirs and Suidae (Figure 1). However, in another cases, closely related, *Amblyomma* species use very different host species, as *A. ovale*, a generalist species feeding on a diversity of domestic and wild animals, vs. *A. geayi*, *A. longirostre* and *A. varium* which are specialized species feeding mainly on arboreal vertebrates (passerine birds and arboreal mammals).

Microbial diversity and symbiont prevalence in Amblyomma species

We further examined the whole bacterial diversity of 24 out of the 25 *Amblyomma* species ($n=142$ specimens) through high-throughput 16S rDNA sequencing. The amount of DNA for the 25th *Amblyomma* species, *A. sculptum* ($n=1$), was not sufficient to perform bacterial barcoding along with other molecular typing done in this study (this specimen of *A. sculptum* was already typed for its *Coxiella*-LE and *Francisella*-LE infections, as further detailed). After filtration of false-positives OTUs and contaminants [45, 66], 4,363,589 reads distributed in 187 OTUs were obtained in the data set from the 142 *Amblyomma* specimens. Overall, *Coxiella*-LE and *Francisella*-LE are the most abundant bacterial genera representing 33.6% and 33.1% of the total number of reads, respectively (Figure 2). Other bacterial genera were found: in most cases each represented very few of the 16S rDNA reads when present. A remarkable exception of this pattern is the presence of abundant intracellular bacteria belonging to the *Rickettsia* genus in 16 *Amblyomma* species (Figure 2). These *Rickettsia* could be either tick-borne pathogens or maternally inherited endosymbionts since both types have been documented in this genus [67, 68]. Other exceptions include the *A. romitii* samples for which no *Coxiella*-LE and *Francisella*-LE 16S rDNA reads were detected: most reads were assigned to a tick-borne pathogen, *Ehrlichia* sp., which may limit the effectiveness of bacterial community profiling by masking other bacteria. In *A. loculosum* and *Amblyomma* sp. samples, *Coxiella*-LE 16S rDNA reads were detected but reads of other bacteria were more abundant. Since the *A. loculosum* and *Amblyomma* sp. samples were not bleach-treated prior to DNA extraction (as done for the other samples), the abundant presence of these bacteria is due cuticular bacteriome, as recently observed in another *Amblyomma* species [45].

We further examined how bacterial diversity varies between *Amblyomma* species. Microbial structure is globally not impacted by the phylogenetic proximity between *Amblyomma* species:

dendrogram of microbial diversity does not parallel the *Amblyomma* phylogeny (Mantel two tailed test, $R=0.16$, $P=0.19$) (Figure S1). However, MDS plot highlight a clear difference in bacterial diversity between all tick species suggesting a strong effect of the tick species on the microbiome diversity (Figure S2). PERMANOVA analysis further confirmed this result ($R^2=0.51$, $P=0.001$). However, tick species is not sufficient to fully explain the two major clusters appearing on the right and on the left of the MDS plot (Figure S2). To elucidate these clusters, we have tested a possible effect of the presence of both *Coxiella*-LE and *Francisella*-LE on bacterial diversity. The resulting MDS plot show a clear separation between *Coxiella*-LE-infected tick species/specimens and *Francisella*-LE-infected ones (Figure 3a), corresponding to the two clusters observed previously (Figure S2). This result is supported by a pairwise PERMANOVA analysis performed with the three categories pairs possible: *Coxiella*-LE vs. *Francisella*-LE ($R^2=0.31$, adjusted P for multiple comparisons= 0.0003); *Coxiella*-LE vs. none symbiont ($R^2=0.11$, adjusted P for multiple comparisons= 0.0028); *Francisella*-LE vs. none symbiont ($R^2=0.10$, adjusted P for multiple comparisons= 0.0036) and showing that the identity of symbiont and the presence or not of the last influences the bacterial diversity in *Amblyomma* ticks. In a same way, the presence or absence of *Rickettsia* also impacts the bacterial diversity but at a lesser extent than *Coxiella*-LE and *Francisella*-LE (Figure 3b; PERMANOVA, $R^2=0.11$, $P=0.001$). To go further, the effect of *Rickettsia* coinfection with *Coxiella*-LE or *Francisella*-LE vs. single *Rickettsia* infection on bacterial diversity produced similar MDS plot and statistical result than observed with the presence/absence pattern of *Rickettsia* (Figure 3b, 3d; PERMANOVA, $R^2=0.13$, $P=0.001$). Although *Rickettsia* has a limited impact on the grouping of bacterial diversity in two major clusters, it seems to structure the bacterial diversity within each of the two clusters. Indeed, the MDS plots of bacterial diversity shows that the *Coxiella*-LE and *Francisella*-LE clusters are structured into two sub-clusters each, fitting with the presence of *Rickettsia* (Figure 3a, 3b, 3d). Pairwise PERMANOVA analysis supporting

these results: *Coxiella*-LE vs. *Francisella*-LE ($R^2=0.46$, adjusted P for multiple comparisons=0.0015); *Coxiella*-LE vs. *Coxiella*-LE-*Rickettsia* ($R^2=0.2$, adjusted P for multiple comparisons=0.0015); *Francisella*-LE vs. *Francisella*-LE-*Rickettsia* ($R^2=0.22$, adjusted P for multiple comparisons=0.0015).

Of the 25 *Amblyomma* species (including *A. sculptum*) examined for the presence of *Coxiella*-LE and *Francisella*-LE, 24 were found infected by one or both of these symbionts: 11 *Amblyomma* species harbour only *Coxiella*-LE, 13 species only *Francisella*-LE and three species both (Figure 2, Table S1). Only *A. romitii* was found not infected by *Coxiella*-LE and *Francisella*-LE but this may be explained by the presence of *Ehrlichia* sp. as detailed above. In the 24 infected *Amblyomma* species, *Coxiella*-LE and *Francisella*-LE were not randomly associated (Fisher's exact test, $P=0.001$): these two symbionts co-occurred in the same tick species less frequently than expected by chance (exclusion pattern), meaning that their distribution across tick species was strongly dependent each other. Conversely, neither *Coxiella*-LE nor *Francisella*-LE shows a non-random association with *Rickettsia* (Fisher's exact tests, $P=0.99$ and 0.68, respectively). *Coxiella*-LE and *Francisella*-LE has however a patchy and quite uniform distribution along the *Amblyomma* phylogeny with no clear structure: while some closely related *Amblyomma* species are infected by the same symbiont genus (eg, *A. dissimile* and *A. rotundatum* by *Francisella*-LE, or *A. latepunctatum*, *A. sculpturum* and *A. naponense* by *Coxiella*-LE), other are not (eg, *A. americanum*, infected by *Coxiella*-LE, and *A. oblongoguttatum* by *Francisella*-LE) (Figure 2). This distribution pattern thus also parallel partly the tick host-range: indeed, *A. dissimile* and *A. rotundatum* which are related specialized on poikilotherms are both infected by *Francisella*-LE (Figure 1, 2).

At the species level, *Coxiella*-LE and *Francisella*-LE are present in most specimens examined, as expected for obligate nutritive symbionts (Figure 2, Table S1). However, a more contrasted

pattern is apparent in the three *Amblyomma* species which are co-infected by *Coxiella*-LE and *Francisella*-LE: (i) in *A. geayi*, of the 10 examined specimens, seven are infected by *Coxiella*-LE but, in other localities, two specimens are infected by *Francisella*-LE and one is co-infected by *Coxiella*-LE and *Francisella*-LE; (ii) in *A. latepunctatum*, of the four examined specimens, three specimens of the same locality are co-infected by *Coxiella*-LE and *Francisella*-LE but one specimen from another locality is only infected by *Coxiella*-LE; (iii) in *A. sculptum*, the single examined specimen is co-infected by *Coxiella*-LE and *Francisella*-LE (Figure 2, Table S1). Contrasting to *Coxiella*-LE and *Francisella*-LE, the prevalence of *Rickettsia* is heterogeneous, with infection frequencies ranging from 14% to 100% depending on tick species (Figure 2, Table S1). In all cases, *Rickettsia* is found with either *Coxiella*-LE (seven *Amblyomma* species) or *Francisella*-LE (seven species) or both (two species) (Figure 2, Table S1). Similarly, to *Coxiella* and *Francisella*, no *Rickettsia* were detected in the two specimens of *A. romitii*.

Evolutionary history of Coxiella-LE and Francisella-LE symbioses

The phylogenetic diversity of *Coxiella*-LE was examined in a subset of 32 specimens representing the 14 infected *Amblyomma* species (one-to-four specimens per species were used; Table S1). Sequencing of three *Coxiella* MLST genes (16S rRNA, *rpoB* and *groEL*) led to the identification of 14 genetically different *Coxiella*-LE. The association of *Coxiella*-LE diversity with *Amblyomma* species is highly specific: each *Amblyomma* species is infected by a genetically distinct *Coxiella*-LE and no variation of *Coxiella*-LE was observed between specimens belonging to the same *Amblyomma* species. We further reconstructed the phylogenetic relationships between *Coxiella*-LE using BI analyses based on their 16S rRNA, *rpoB* and *groEL* nucleotidic sequences. We observed no sign of recombination in the *Coxiella*-LE data set (all $P > 0.05$ for the GENECONV and RDP recombination-detection tests) and we thus used the 16S rRNA, *rpoB* and *groEL* concatenated sequences for analyses (2045

unambiguously aligned bp; best-fit approximation for the evolutionary model: GTR+G+I). Comparisons with other *Coxiella*-LE sequences available on GenBank shown that the *Coxiella*-LE of *Amblyomma* are polyphyletic: they are scattered into different well supported clusters among *Coxiella*-LE from other tick species (Figure S3). Indeed, the *Coxiella*-LE of *A. variegatum*, *A. tholloni* and *A. splendidum* form a monophyletic clade that is more closely related to the *Coxiella*-LE found in various *Ixodes* tick species than to the *Coxiella*-LE found in the other *Amblyomma* species. Similarly, the *Coxiella*-LE clade of *A. cajennense*, *A. sculptum* and *A. americanum* is more closely related to the *Coxiella*-LE found in *Dermacentor* tick species. This pattern is suggestive of recurrent HT events of *Coxiella*-LE between tick species. However, the *Coxiella*-LE clusters of *Amblyomma* species can be gathered into two main groups, one with all the *Coxiella*-LE of NW *Amblyomma* species (along with those of *Dermacentor* tick species) and the other with all the *Coxiella*-LE of AF *Amblyomma* species (along with those of *Ixodes* tick species) (Figure S3). This pattern suggests an effect of phylogeographic drivers in structuring the evolution of *Coxiella*-LE. This is strongly supported by the cophylogeny analysis between *Coxiella*-LE and *Amblyomma* phylogenies: there is a significant topological congruence between their phylogeny (PACo analysis, $P=0.0001$; Figure 4a). This shows that cocladogenesis with *Coxiella*-LE also happened during the radiation of *Amblyomma*.

The phylogenetic diversity of *Francisella*-LE was examined in a subset of 28 specimens representing the 13 infected *Amblyomma* species (one-to-four specimens per species were used; Table S1). Sequencing of five *Francisella* MLST genes (16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB*) led to the identification of 15 genetically different *Francisella*-LE (Figure S4, Table S3). There was only one *Francisella*-LE in each *Amblyomma* species, but not in *A. pacae* and *A. rotundatum* in which 2 and 3 genetically distinct *Francisella*-LE, respectively, are present in

specimens from different localities. Each *Amblyomma* species harbours a genetically distinct *Francisella*-LE, excepted *A. geayi* and *A. latepunctatum* which harbour identical *Francisella*-LE on the basis of their 16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB* nucleotidic sequences (Figure S4, Table S3). Due to the lack of *Francisella*-LE *rpoB*, *groEL*, *ftsZ* and *gyrB* genes sequences available in Genbank prior this study (with the exception of two published *Francisella*-LE genomes), the BI phylogenetic analysis between the *Francisella*-LE of *Amblyomma* and those of other tick species (with sequences available on GenBank) was only examined using their 16S rRNA nucleotidic sequences (Figure S5). The *Francisella*-LE of ticks, including those harboured by *Amblyomma*, form a robust monophyletic clade well differentiated to other *Francisella* species. No *Francisella*-LE subclade specific to *Amblyomma* are evident along 16S rRNA phylogenetic tree: the *Francisella*-LE of *Amblyomma* are rather scattered among *Francisella*-LE of other tick genera suggesting recurrent HT events between unrelated tick species (Figure S5). However, the inner topology of the *Francisella*-LE clade based on 16S rRNA gene sequences remains too poorly resolved in many cases (as shown by low support values of inner branches) for inferring the exact relatedness between all *Francisella*-LE from different tick species (Figure S5). We thus further reconstructed the phylogenetic relationships between *Francisella*-LE using BI analyses based on their 16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB* nucleotidic sequences. We observed no sign of recombination in the *Francisella*-LE data set (all $P > 0.05$ for the GENECONV and RDP recombination-detection tests) and we thus used the 16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB* concatenated sequences for analyses (3506 unambiguously aligned bp; best-fit approximation for the evolutionary model: GTR+G+I). Conversely to *Coxiella*-LE, there is no apparent cocladogenesis or phylogeographic pattern along the *Francisella*-LE phylogeny (Figure S4). Indeed, the *Francisella*-LE of *A. sculptum* (NW group) is closely related to the *Francisella*-LE of an unrelated *Amblyomma* species, *A. paulopunctatum* (AF group). In addition, the *Francisella*-LE of *A. sculptum* and *A.*

paulopunctatum are more closely related to the *Francisella*-LE of the soft tick *O. moubata* than to the *Francisella*-LE of other *Amblyomma* species (Figure S4). The cophylogeny analysis also showed no significant signal of congruence between the *Francisella*-LE and *Amblyomma* phylogenies (PACo analysis, $P = 0.06$): no co-cladogenesis event is apparent along the *Francisella*-LE phylogeny, and only HT events have impacted the distribution *Francisella*-LE across *Amblyomma* phylogeny (Figure 4b). Interestingly, while the association of *Rickettsia* along the *Coxiella*-LE phylogeny seems random (Figure S6a), this is apparently not the case along the *Francisella*-LE phylogeny (Figure S6b): the co-infections with *Rickettsia* are present in certain *Francisella*-LE subclades but not in others. In these cases of co-infections, *Rickettsia* prevalence is often high (>60% of *Amblyomma* specimens are infected; Figure S6b).

Discussion

We examined 25 species of *Amblyomma* for their whole bacterial diversity, and found that three intracellular bacterial genera, *Coxiella*-LE, *Francisella*-LE and *Rickettsia*, are widespread across these tick species. Only a few other bacteria have been detected and none is so uniformly present in the 25 *Amblyomma* species. However, the structure of microbiomes does not mirror the *Amblyomma* phylogeny and closely related *Amblyomma* commonly harbour divergent microbiomes. As expected, almost all *Amblyomma* species were found to harbour a nutritive obligate symbiont, *Coxiella*-LE or *Francisella*-LE, both able to synthesize B vitamins but with a clear exclusion pattern between them: *Coxiella*-LE was found as single infection in 11 *Amblyomma* species, *Francisella*-LE in 13 species while co-infection was only found in three species. Despite the co-evolved and obligate interactions of ticks with these mutualistic partners, we detected evidences of symbiont replacements during radiation of *Amblyomma*, raising questions regarding the ecological and evolutionary factors underlying replacements.

The contrast of symbiont and tick phylogenies reveals that the *Coxiella*-LE symbiosis is ancient and arose in early evolution of the *Amblyomma* genus. Hence, some *Coxiella*-LE are specialized on their *Amblyomma* hosts, with an ancient acquisition followed by codiversification, meaning that the persistence of *Coxiella*-LE through vertical transmission is stable over the timing of *Amblyomma* species diversification. A very similar codiversification pattern has also been reported from the *Coxiella*-LE symbiosis in the *Rhipicephalus* tick genus with a minimal date of 14Mya for the original *Coxiella*-LE infection [16]. However, the spread of *Coxiella*-LE was more complex in *Amblyomma*: the infections found in some *Amblyomma* species are distantly related and do not form an *Amblyomma*-specific clade. Rather, phylogenetics shows that *Coxiella*-LE of *Amblyomma* are actually scattered among *Coxiella*-LE of other tick genera as *Ixodes* and *Dermacentor*. Only extensive HT of *Coxiella*-LE between these tick genera may explain these phylogenetic incongruences. Since obligate, but not facultative, symbionts cannot undergo HT between host species [13, 14, 20, 21], this suggests that some *Coxiella*-LE are facultative symbionts of ticks. Interestingly, *Coxiella*-LE is a facultative symbiont in some *Ixodes* species, such as the castor bean tick *I. ricinus* and the polar seabird tick *I. uriae*, with only a few specimens infected within each of these species [16, 41, 69]. The phylogenetic proximity of *Coxiella*-LE of *Ixodes* spp. with the *Coxiella*-LE of *A. variegatum*, *A. splendidum* and *A. tholloni* thus suggests that a facultative *Coxiella*-LE has early jump to the *Amblyomma* ancestor of these species before to replace the ancestral obligate symbiont and evolve obligate symbioses with current species. Overall, the *Coxiella*-LE symbiosis in *Amblyomma* has a complex dynamic of depending on two distinct evolutionary strategies: (1) some *Coxiella*-LE are nutritional obligate symbiont which undergo a stable vertical transmission allowing their long term maintenance through host radiation, and (2) some other *Coxiella*-LE are facultative symbionts experimenting HT with host turnovers and potentially further evolving nutritional symbiosis with ticks.

The infection dynamics of *Francisella*-LE is different to the one of *Coxiella*-LE. While the *Francisella*-LE of ticks form a monophyletic clade within the *Francisella* genus [16, 32], there is frequent HT events between unrelated tick species. Contrary to *Coxiella*-LE, no cocladogenesis signal with *Amblyomma* is apparent along the phylogenies, meaning that *Francisella*-LE only recently arose in this genus. That unrelated *Francisella*-LE are present in *Amblyomma* further indicate that several independent acquisitions of *Francisella*-LE has happened during the radiation of this tick genus. These acquisitions have come at the expense of the *Coxiella*-LE with their ultimate replacements by *Francisella*-LE. The AF *Amblyomma* group is illustrative of this process: most species (*A. splendidum*, *A. variegatum*, *A. tholloni* and *A. loculosum*) are infected by *Coxiella*-LE with a strong cocladogenesis pattern, but one species (*A. paulopunctatum*) is infected by *Francisella*-LE. This pattern suggests that *Francisella*-LE arose recently in the AF *Amblyomma* group and has ultimately replaced the *Coxiella*-LE primarily present in the *A. paulopunctatum* ancestor. Other remarkable examples include the monophyletic group formed by *A. americanum*, *A. oblongoguttatum*, *A. cajennense* and *A. sculptum* and cocladogenesis pattern of *Coxiella*-LE with most of these species: here *Francisella*-LE has eliminated the *Coxiella*-LE primarily present in the *A. oblongoguttatum* ancestor. In addition, *A. sculptum* is co-infected at the individual level by an ancestral *Coxiella*-LE (*ie*, showing a cocladogenesis pattern with the *A. sculptum* relatives) and a recently acquired *Francisella*-LE (*ie*, not related to the *Francisella*-LE of *A. oblongoguttatum*). This pattern suggests that HT of *Francisella*-LE within *Amblyomma* communities are recent, likely ongoing, and that coinfections with ancestral *Coxiella*-LE are only transitory. The close relatedness between of *Francisella*-LE from unrelated *Amblyomma* species, as *A. dissimile*, *A. geayi* and *A. latepunctatum*, clearly indicates that *Francisella*-LE is currently invading the *Amblyomma* genus and replacing the ancestral *Coxiella*-LE.

Some biological traits of these symbioses are indicative of how and why *Francisella*-LE and some *Coxiella*-LE are both eliminating ancestral *Coxiella*-LE in *Amblyomma* ticks. *Francisella*-LE or *Coxiella*-LE were occasionally detected in the salivary glands of several tick species [46, 70, 71] and one may thus assume that ticks can inject part of their symbionts during feeding. Ticks, unlike other arthropod vectors, often attach and aggregate to the host for several days to obtain a meal, a process termed co-feeding. The spatiotemporal proximity of ticks during co-feeding may favour the HT of *Francisella*-LE and *Coxiella*-LE between conspecific but also between different tick species, as commonly observed for tick-borne pathogens [72, 73]. This process may lead to local or systemic infections to vertebrates since a few cases of opportunistic *Coxiella*-LE infections have been reported after tick feeding [74–76]. This mode of transmission may be particularly significant for HT of *Francisella*-LE and *Coxiella*-LE, further leading to coinfection with ancestral *Coxiella*-LE in ticks. Interestingly, we found two *Amblyomma* species, *A. geayi* and *A. latepunctatum*, that are coinfecting by their respective ancestral *Coxiella*-LE but that also share the same *Francisella*-LE which is also closely related to the *Francisella*-LE of *A. dissimile*. The genetic proximity of *Francisella*-LE in these three unrelated *Amblyomma* species is indicative of recent HT events possibly through co-feeding: while these three *Amblyomma* species feed on very different vertebrate hosts, hampering then the possibility of co-feeding, the immature stages of *A. dissimile* are commonly found on diverse mammals or birds [77–79], suggesting that they may be ecological bridges driving HT of *Francisella*-LE across tick species.

By replacing the ancestral *Coxiella*-LE, a symbiont (*Francisella*-LE or *Coxiella*-LE) colonizes a pre-adapted tick physiological environment that requires the B vitamin provisioning, so it must be able to synthesize these compounds as the ancestral *Coxiella*-LE did. The presence of several B vitamin biosynthesis pathways is ancestral in the *Coxiella* and *Francisella* genera,

and all members of these genera, which are also all intracellular, have conserved these abilities through their radiation [32, 33, 35, 80–82]. In *Francisella*, the biotin (vitamin B7) pathway is pivotal for the replication of *Francisella* pathogens in vertebrates' macrophages [83, 84], it may have primarily evolved for *Francisella* pathogenesis in vertebrates before being secondarily retained for nutritional use in *Francisella*-LE in ticks [32]. So, all *Francisella*-LE and *Coxiella*-LE are already preadapted to nutritional symbioses with ticks. Beyond B vitamins, the replacement of ancestral *Coxiella*-LE suggests that the new symbiont can supply an additional benefit that the ancestral *Coxiella*-LE was unable to supply to ticks, out-competing them. The genomes of ancient beneficial endosymbionts have lost most of their gene contents from their ancestor being usually small in size and dense in gene content but also suffering Muller's ratchet, leading to genetic drift and fixation of deleterious mutations [20, 85–87]. This evolution toward massive genomic reduction is obvious for the *Coxiella*-LE of *A. americanum* which have a genome of only 0.66Mb [35] but not for the *Francisella*-LE of *A. maculatum*: its genome is 1.56Mb, and although half is pseudogenized, it suggests a higher biosynthetic capability [33]. Similar variation is also reported between *Coxiella*-LE with some genomes reaching ca. 1.5Mb [34, 88], suggesting that some *Coxiella*-LE have greater biosynthetic capabilities than others with reduced genomes. Other processes may however act on the *Coxiella*-LE replacement. Indeed, ancestral *Coxiella*-LE may have evolved too reduced (degraded) genomes and become maladapted, opening the road to replacement by a new symbiont. Such degeneration–replacement model has been proposed for other arthropods as cicadas [25, 89, 90], but replacements are expected to be transient making them difficult to observe [20]. In *Amblyomma*, the observations of three species with co-infections by ancestral *Coxiella*-LE and recently acquired *Francisella*-LE may correspond to this transient state leading to extinction of *Coxiella*-LE.

That ecological specialization to strict hematophagy is driven by nutritional symbiotic interactions is beyond doubt for ticks. The present study nevertheless shows that nutritional symbiosis in the *Amblyomma* genus is not stable state, being often born from conflicting origins between *Coxiella*-LE and *Francisella*-LE or between *Coxiella*-LE themselves. The *Francisella*-LE invasion arose recently, but repeatedly, during the radiation of *Amblyomma* and this process is likely ongoing leading to evolutionary unstability of nutritional symbioses. Ticks march on with their recently acquired *Francisella*-LE, at the expense of *Coxiella*-LE, but the precise mechanisms providing the advantage to *Francisella*-LE remain now to be determined, including endosymbiosis intrinsic factors, such as competition between symbionts with similar metabolic capabilities and differential degree of genome reduction.

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Conflict of interest

The authors declare that they have no conflict of interest.

Figure legends

Figure 1. Phylogenetic relationship of 25 *Amblyomma* species with indication of their host range. The phylogenetic tree was inferred using Bayesian inferences (BI) from a 3312 bp nuclear rDNA sequences (18S, ITS1, 5.8S, ITS2, and 28S rDNA), the number of each node represent the BI probability. The reconstructed phylogeny is divided into two main group of *Amblyomma*: The AF (African) group, corresponding to ticks from Africa, represented by white dots; and the NW (New World) group corresponding to ticks from America, represented by black dots.

Figure 2. Diversity of the microbiome and prevalence of symbiont by *Amblyomma* species. The heatmap is representing the abundance and diversity of the nine most abundant OTUs characterized in the 16S rRNA bacterial data set for each tick species. The number at the right of the heatmap correspond to the number of specimens by species used to construct the heatmap. The presence / absence and prevalence of the three most common symbiont is illustrated by the coloured squares (*Coxiella*-LE=blue square; *Francisella*-LE=green square and *Rickettsia*=red square), a white square indicate the absence of a symbiont and a full square illustrate a prevalence of 100%. Intermediate prevalence are indicate by the numbers nearby squares. The the amount of DNA for the single specimen of *A. sculptum* was not sufficient to perform the 16S rRNA bacterial sequencing, therefore the heatmap for this specie indicate N/A.

Figure 3. Structuration of the *Amblyomma* microbiome. A- Multidimensional scaling plot (MDS) of the microbial diversity of each *Amblyomma* species depending on the presence of both *Coxiella*-LE (red dots) and *Francisella*-LE (green dots) or the absence of obligate symbiont (blue dots). B- MDS plot of the microbial diversity depending on the presence (red dots) / absence (blue dots) of *Rickettsia*. C- MDS plot of the microbial diversity depending on

symbionts coinfection (red dots), multi-infections (blue dots) and single infection (green dots). D- MDS plot of the microbial diversity depending on the association / no association between the three symbionts (*Coxiella*-LE, *Francisella*-LE and *Rickettsia*): C_R (red dots) correspond to *Amblyomma* specimens coinfecting by *Coxiella*-LE and *Rickettsia*; C (brownish dots) to specimens only infected by *Coxiella*-LE; F (green dots) to specimens only infected by *Francisella*-LE; F_R to specimens coinfecting by *Francisella*-LE and *Rickettsia*; C_F_R (sky-blue dots) to specimens multi-infected by *Coxiella*-LE, *Francisella*-LE and *Rickettsia*; dark blue dots to specimens not infected by these symbionts. Each MDS plot is based on the generalized Unifrac ($\alpha = 0.5$) distance matrix.

Figure 4. Evolutionary histories between *Amblyomma* and their nutritional symbionts. A- Cophylogeny between the phylogenies of *Amblyomma* (in the left) and *Coxiella*-LE (in the right): tick phylogeny is based on a 3312 bp nuclear rDNA sequences (18S, ITS1, 5.8S, ITS2, and 28S rDNA); *Coxiella*-LE phylogeny was reconstructed using a concatenated gene sequence (16S rRNA, *rpoB* and *groEL* concatenated sequences, 2045 unambiguously aligned bp). B- Cophylogeny between the phylogenies of *Amblyomma* (in the left) and *Francisella*-LE (in the right): tick phylogeny is based on a 3312 bp nuclear rDNA sequences (18S, ITS1, 5.8S, ITS2, and 28S rDNA); *Francisella*-LE phylogeny was reconstructed using a concatenated gene sequence (16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB* concatenated sequences, 3506 unambiguously aligned bp). Black dots represents NW *Amblyomma* and white dots AF ones. Each phylogeny was reconstructed using BI and each node number represent the BI probability (only values >70% are shown).

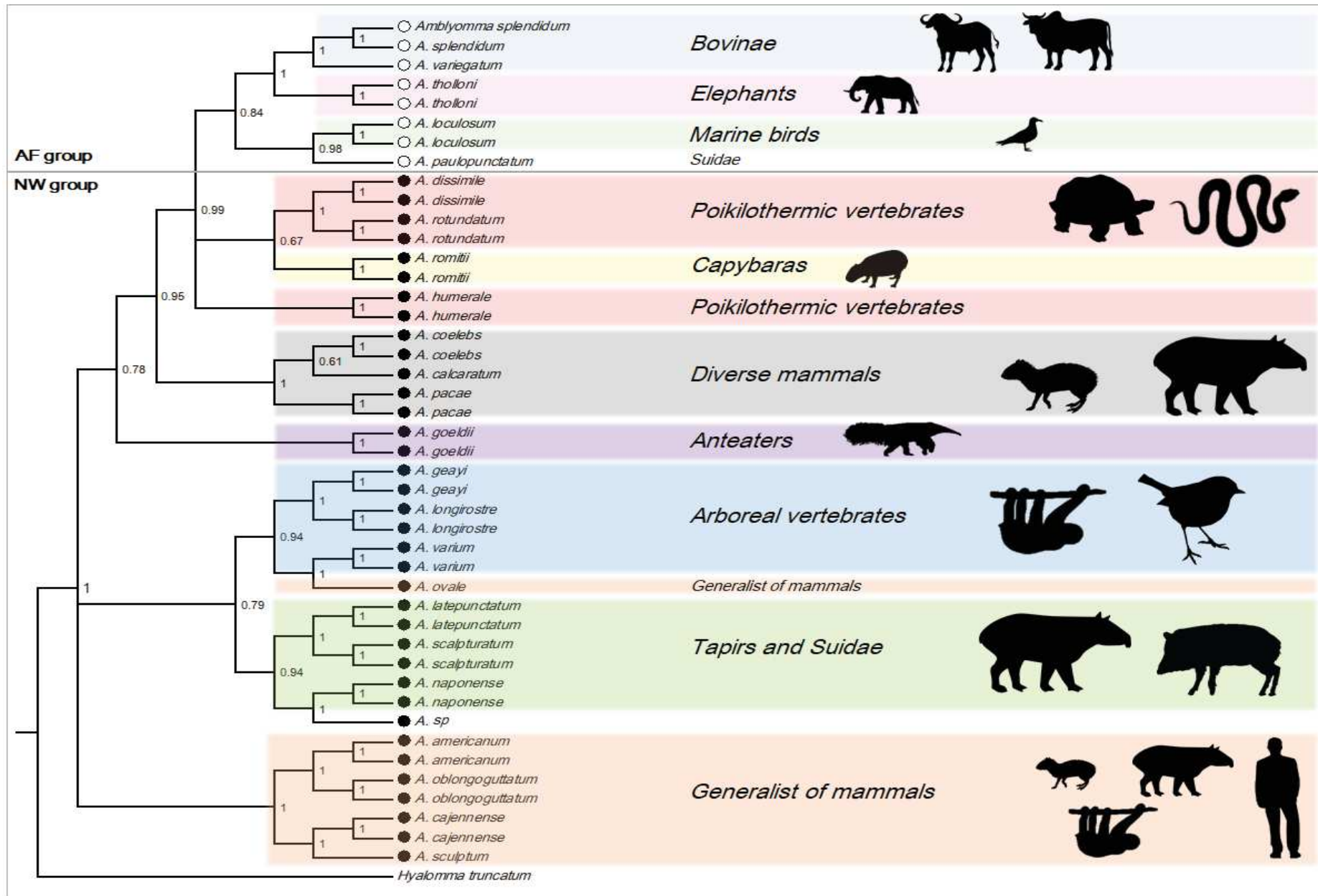


Figure 1.

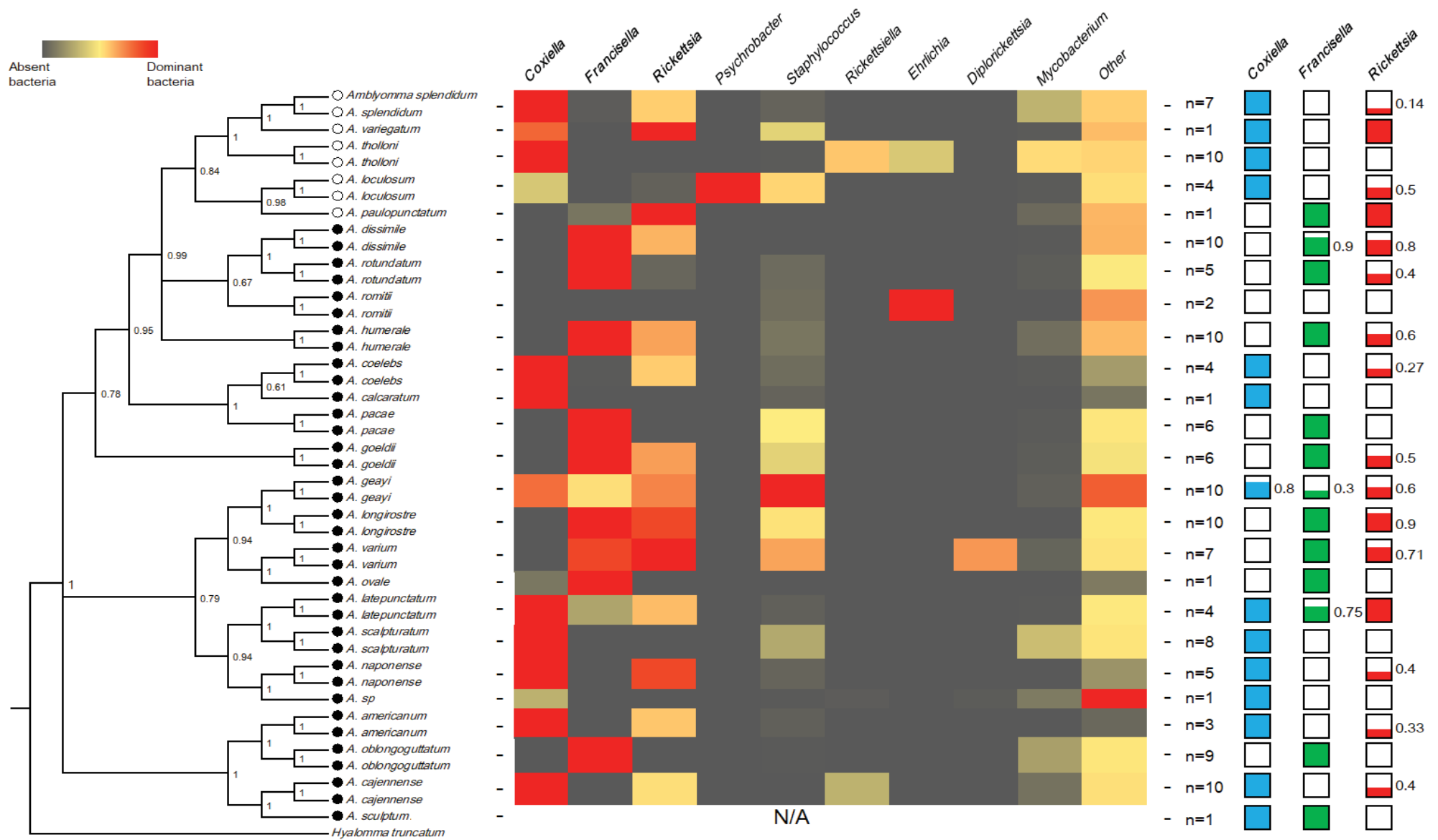


Figure 2.

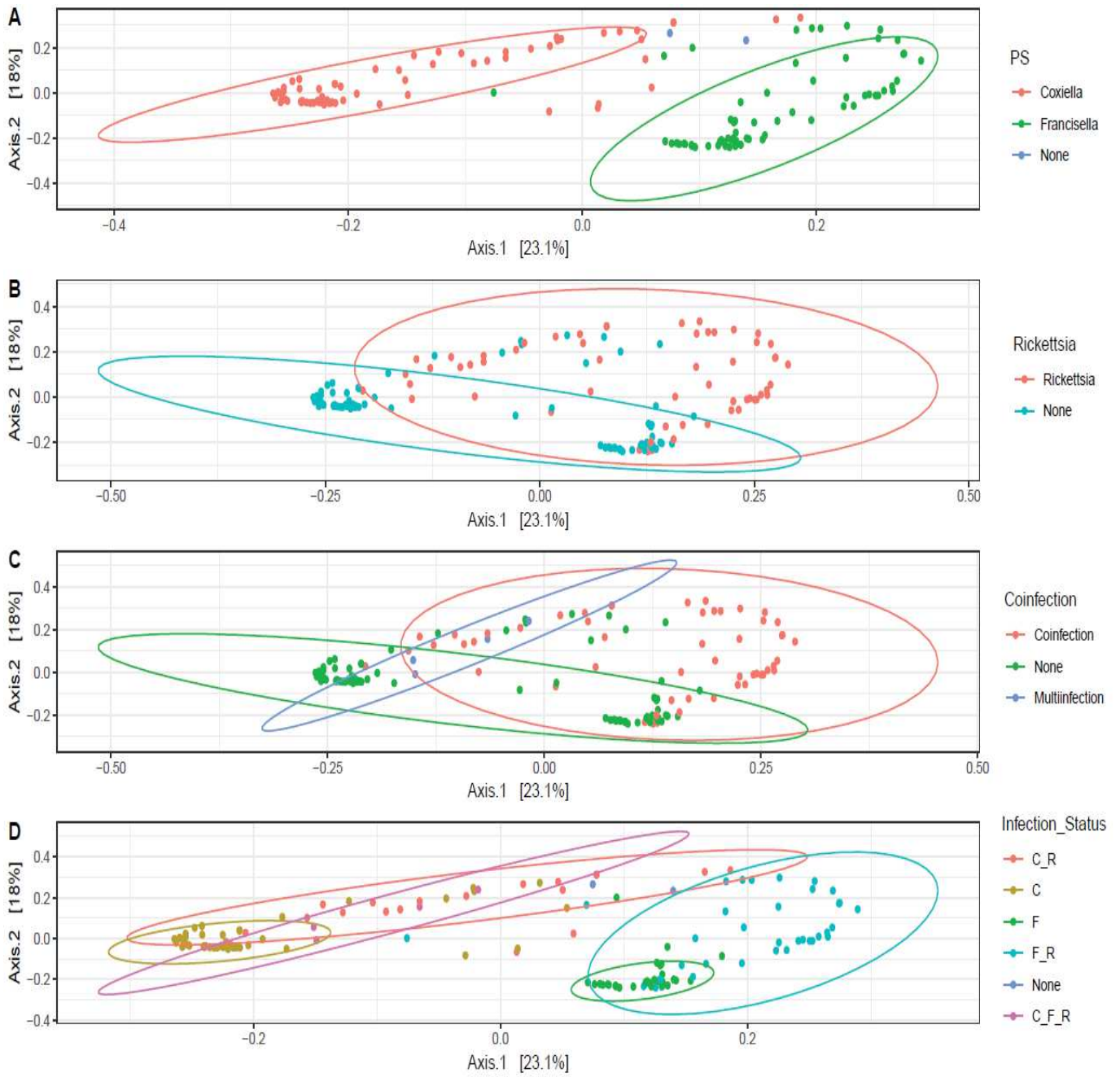


Figure 3.

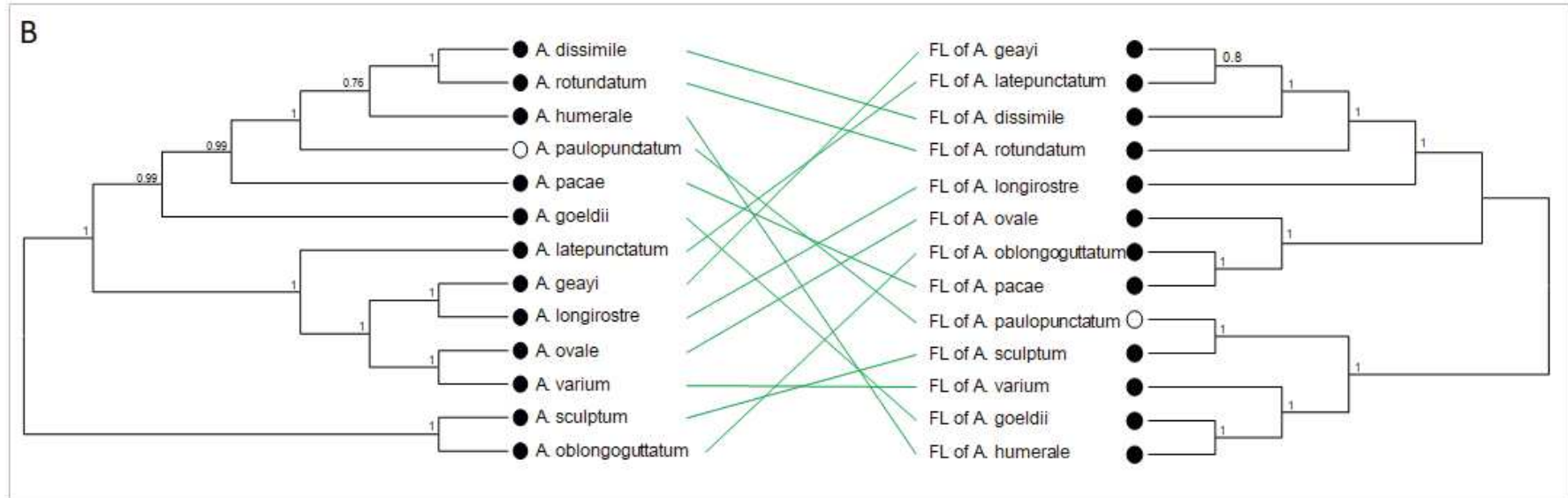
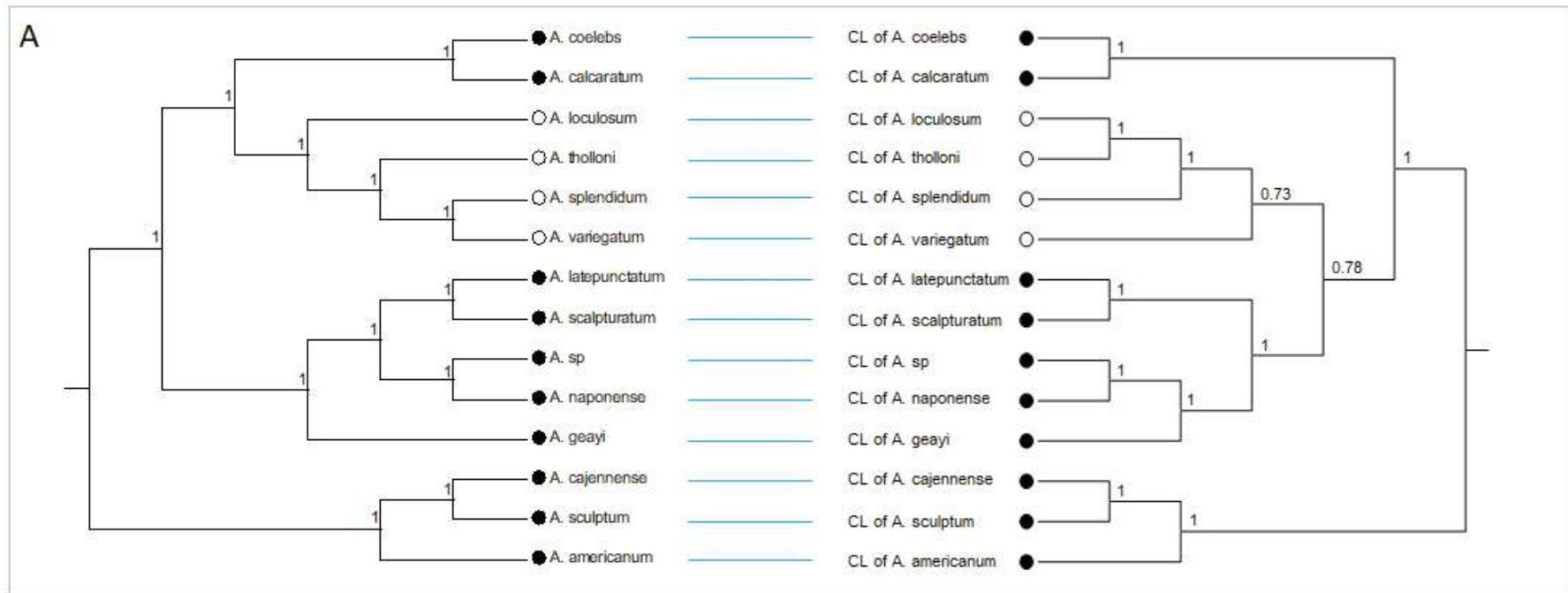


Figure 4.

Supplementary

Figure legends

Figure S1. Relationship between the phylogeny of *Amblyomma* and the structure of their microbiome. The *Amblyomma* phylogeny is the same used in Figure 1 and 2; the dendrogram of the microbial diversity was reconstructed based on the generalized Unifrac ($\alpha = 0.5$) distance matrix, using complete-linkage clustering.

Figure S2. MDS plot of the microbial diversity depending on the *Amblyomma* species, based on the generalized Unifrac ($\alpha = 0.5$) distance matrix.

Figure S3. Phylogenetic relationship between *Coxiella*-LE of *Amblyomma* and CLE of other tick species. The phylogeny was reconstructed by BI using a concatenated gene sequence (16S rRNA, *rpoB* and *groEL* concatenated sequences, 2045 unambiguously aligned bp). Black dots represents NW *Amblyomma* and white dots AF ones. The gene sequences for CLE without dots were recovered from NCBI Genbank and each node number represent the BI probability (only values $>70\%$ are shown).

Figure S4. Phylogenetic relationship between *Francisella*-LE of *Amblyomma*. *Francisella*-LE phylogeny was reconstructed by BI using a concatenated gene sequence (16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB* concatenated sequences, 3506 unambiguously aligned bp). Black dots represents NW *Amblyomma* and white dots AF ones. The gene sequences for FLE without dots were recovered from NCBI Genbank and each node number represent the BI probability (only values $>70\%$ are shown).

Figure S5. Phylogenetic relationship between *Francisella*-LE of *Amblyomma* and FLE of other tick species. *Francisella*-LE phylogeny was reconstructed by BI using 16S rRNA gene sequences (914 unambiguously aligned bp). Black dots represents NW *Amblyomma* and white dots AF ones. The gene sequences for FLE without dots were recovered from NCBI Genbank and each node number represent the BI probability (only values >70% are shown).

Figure S6. Association between *Rickettsia* and both *Coxiella*-LE (A) and *Francisella*-LE (B) from *Amblyomma*. *Amblyomma* CLE and FLE phylogenies were reconstructed using the same process as phylogenies in Figure 4. The prevalence of *Rickettsia* characterized in each *Amblyomma* species is represented by red circles, and the prevalence of *Rickettsia*-infected tick species by CLE- or FLE-clade is illustrated by gradient of red (white = no specie infected by *Rickettsia* in this clade, light reds = less than 90% of the tick species of a clade are infected by *Rickettsia*, dark red = 100% of a clade is infected by *Rickettsia*).

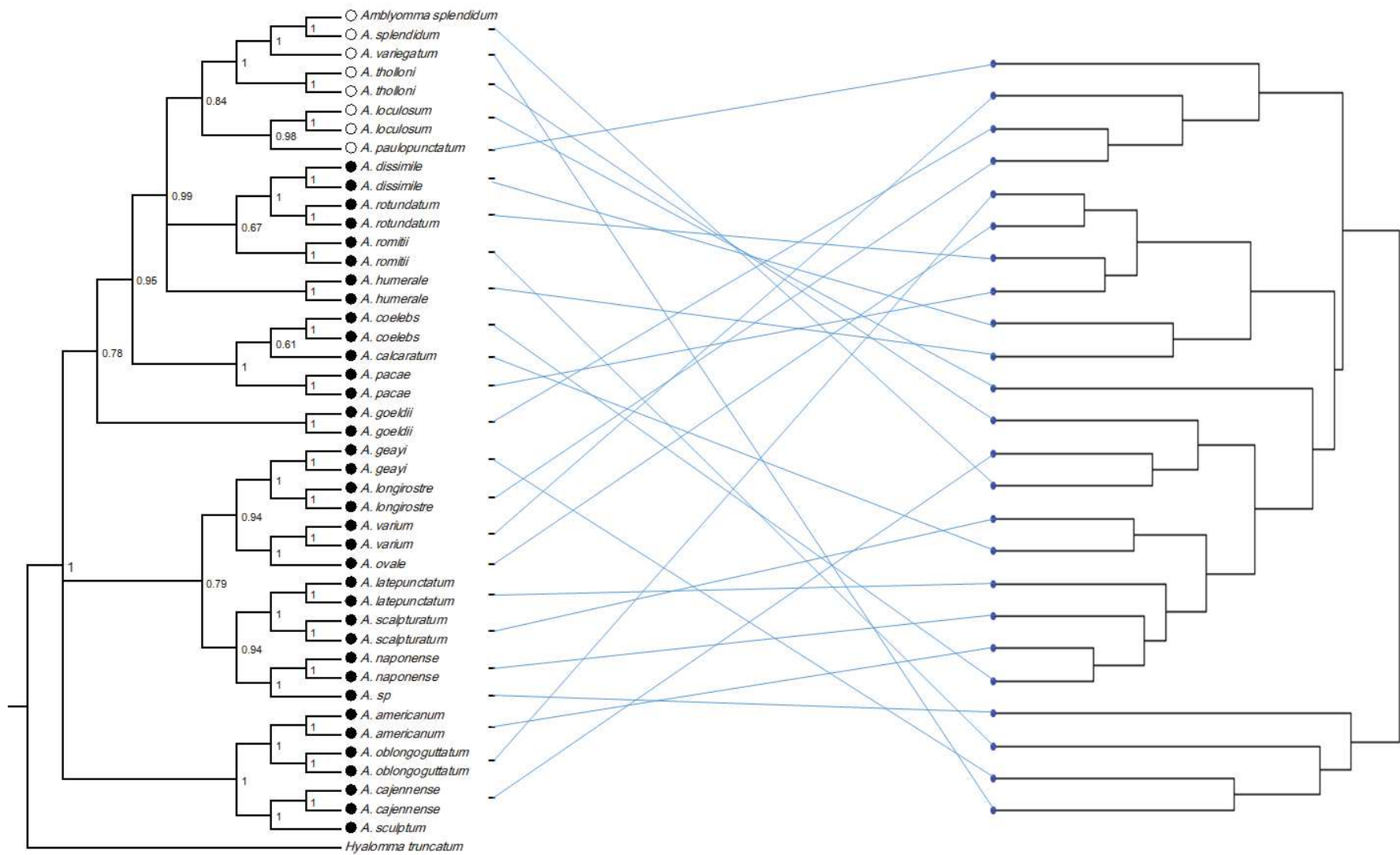


Figure S1.

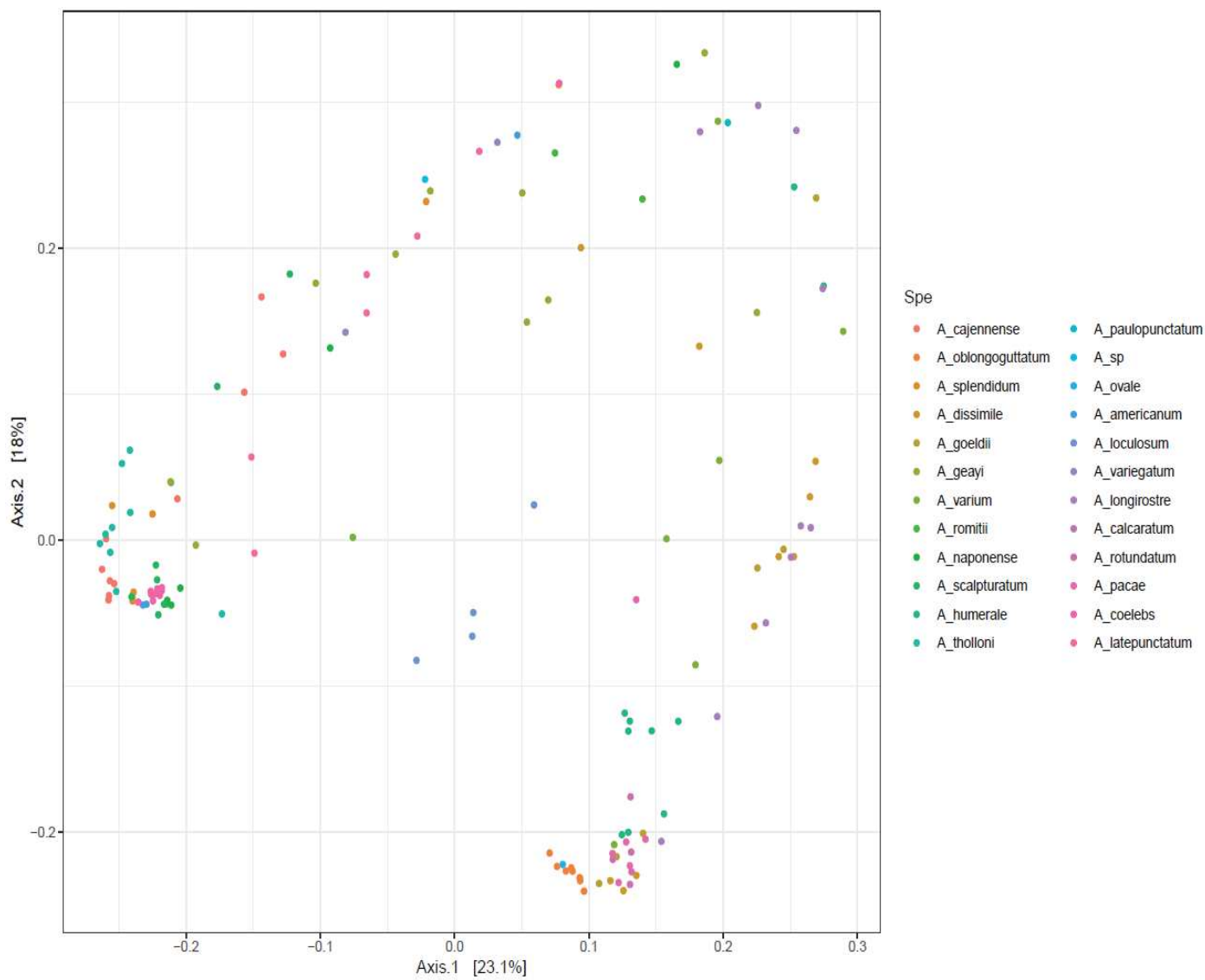


Figure S2.

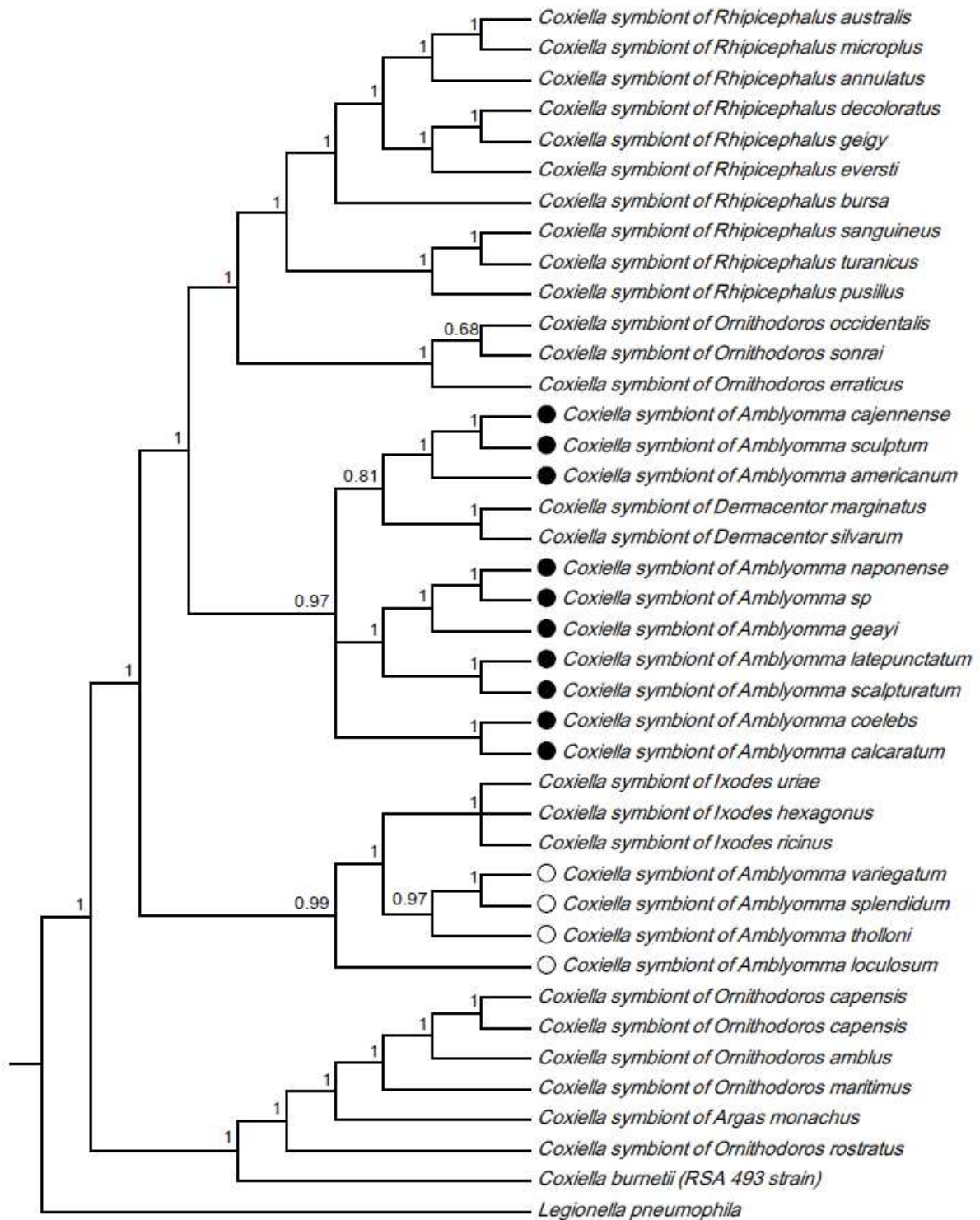


Figure S3.

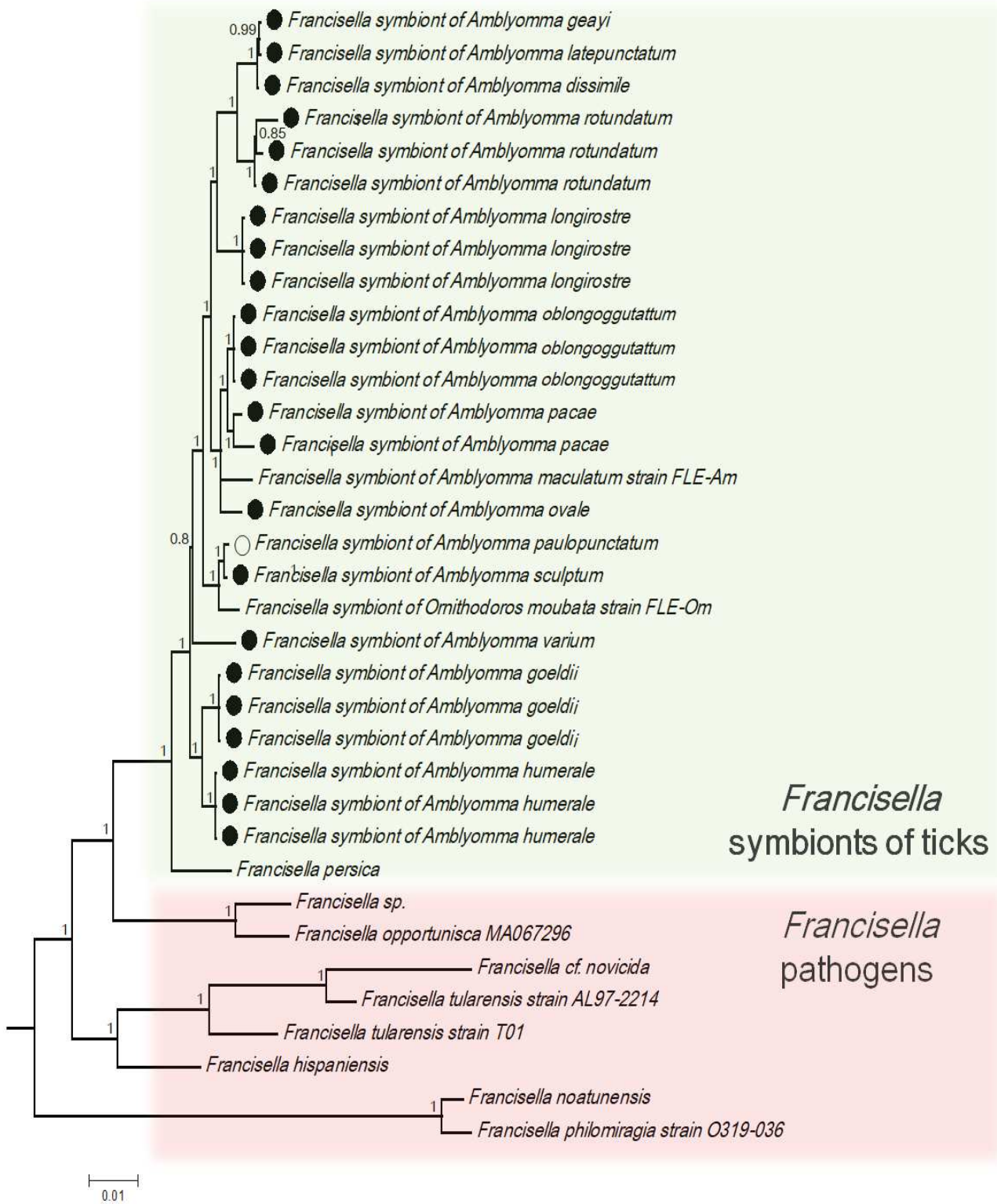


Figure S4.

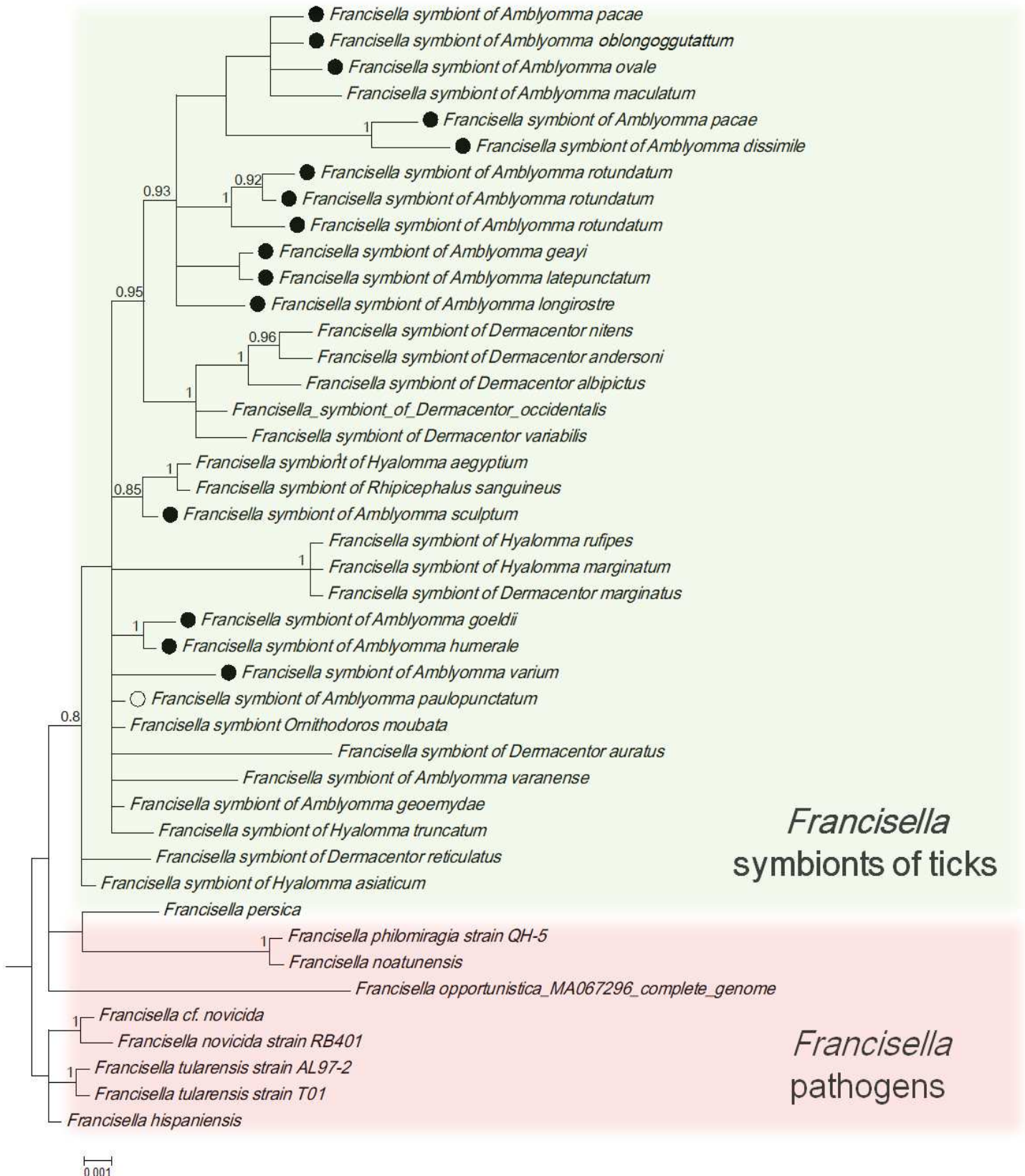


Figure S5.

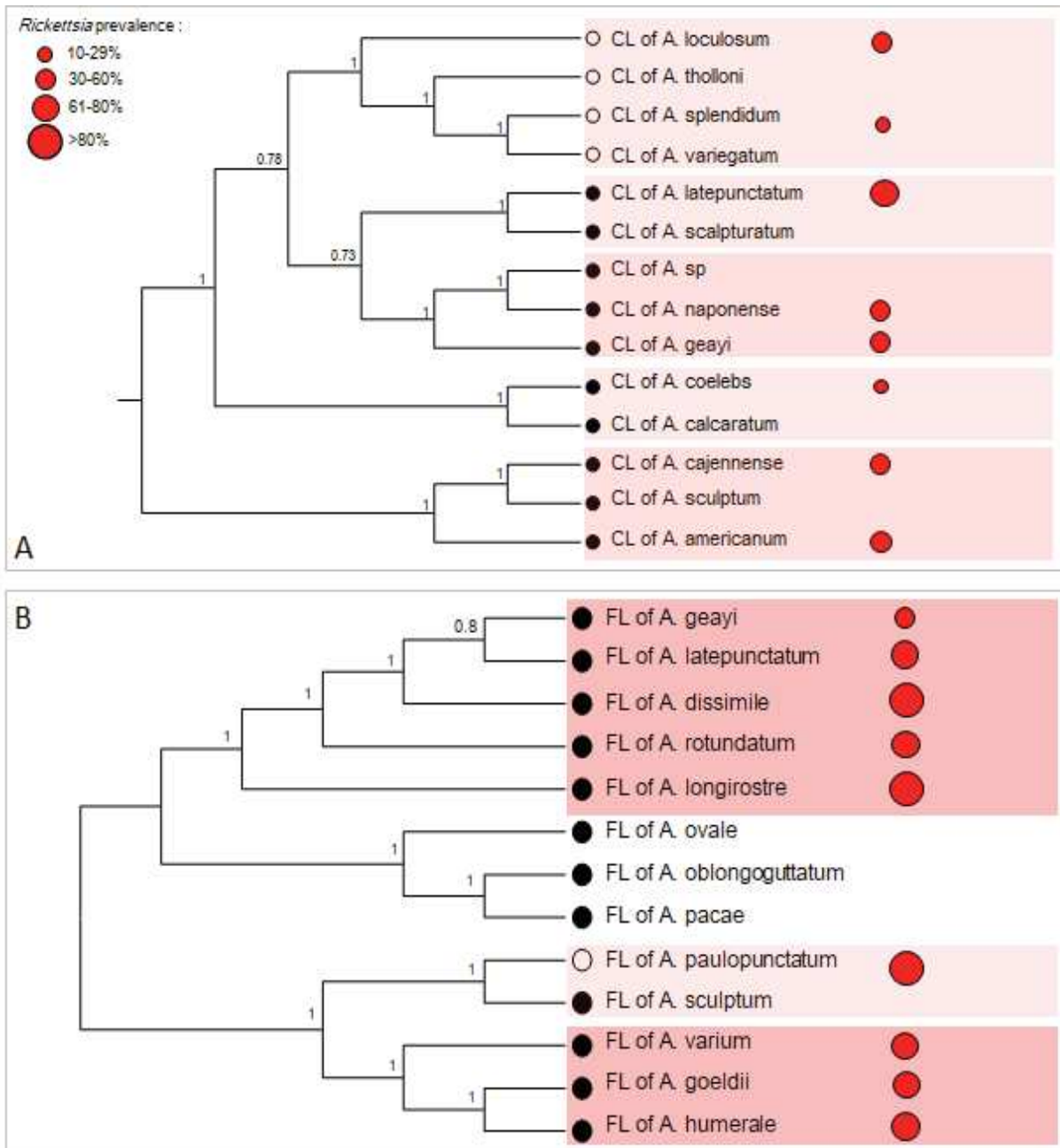


Figure S6.

Table S1

Sample	Species	Stases	Sex	Locality	Country	n reads	% reads <i>Coxiella</i>	% reads <i>Francisella</i>	% reads <i>Rickettsia</i>	Infection <i>Coxiella</i>	Infection <i>Francisella</i>	Infection <i>Rickettsia</i>
AameriWp4_S294_L001	<i>A. americanum</i>	Eggs	-	Lab colony, Oklahoma	USA	73082	99,99	0,00	0,00	1	0	0
AameriWp1_S293_L001	<i>A. americanum</i>	Eggs	-	Lab colony, Oklahoma	USA	51232	99,96	0,00	0,00	1	0	0
AameriTick18E_S295_L001	<i>A. americanum</i>	Adult	Female	Unknown	USA	40856	34,86	0,00	63,66	1	0	1
01-01T8_S134_L001	<i>A. cajennense</i>	Adult	Female	Matoury	French Guiana	33754	87,93	0,01	7,76	1	0	1
01-01T6_S132_L001	<i>A. cajennense</i>	Adult	Female	Matoury	French Guiana	22558	94,34	0,01	0,00	1	0	0
01-01T3_S129_L001	<i>A. cajennense</i>	Adult	Female	Matoury	French Guiana	24497	96,33	0,01	0,00	1	0	0
01-01T10_S136_L001	<i>A. cajennense</i>	Adult	Female	Matoury	French Guiana	18799	50,48	0,01	16,06	1	0	1
01-01T7_S133_L001	<i>A. cajennense</i>	Adult	Female	Matoury	French Guiana	25666	93,69	0,00	0,00	1	0	0
01-01T1_S127_L001	<i>A. cajennense</i>	Adult	Female	Matoury	French Guiana	26650	71,82	0,00	24,34	1	0	1
01-01T2_S128_L001	<i>A. cajennense</i>	Adult	Female	Matoury	French Guiana	27696	96,82	0,00	0,00	1	0	0
01-01T5_S131_L001	<i>A. cajennense</i>	Adult	Female	Matoury	French Guiana	18752	95,47	0,00	0,00	1	0	0
01-01T4_S130_L001	<i>A. cajennense</i>	Adult	Female	Matoury	French Guiana	31745	89,65	0,00	0,00	1	0	0
01-01T9_S135_L001	<i>A. cajennense</i>	Adult	Female	Matoury	French Guiana	21604	76,25	0,00	19,16	1	0	1
L0758T4_S142_L001	<i>A. calcaratum</i>	Larva	-	Kourou	French Guiana	42338	97,45	0,00	0,00	1	0	0
N2514T8_S11_L001	<i>A. coelebs</i>	Nymph	-	Petit-Saut	French Guiana	28118	99,65	0,07	0,02	1	0	0
N2514T6_S9_L001	<i>A. coelebs</i>	Nymph	-	Petit-Saut	French Guiana	36867	98,88	0,04	0,00	1	0	0
N2514T9_S12_L001	<i>A. coelebs</i>	Nymph	-	Petit-Saut	French Guiana	20185	97,48	0,01	0,00	1	0	0
N2514T1_S4_L001	<i>A. coelebs</i>	Nymph	-	Petit-Saut	French Guiana	24230	98,74	0,00	0,01	1	0	0
N2514T7_S10_L001	<i>A. coelebs</i>	Nymph	-	Petit-Saut	French Guiana	30845	26,01	0,00	73,89	1	0	1
N2514T2_S5_L001	<i>A. coelebs</i>	Nymph	-	Petit-Saut	French Guiana	43829	99,60	0,00	0,00	1	0	0
N12103_S266_L001	<i>A. coelebs</i>	Nymph	-	Rémire-Montjoly	French Guiana	35527	99,49	0,00	0,00	1	0	0
N2514T4_S7_L001	<i>A. coelebs</i>	Nymph	-	Petit-Saut	French Guiana	35819	99,29	0,00	0,00	1	0	0
N2514T3_S6_L001	<i>A. coelebs</i>	Nymph	-	Petit-Saut	French Guiana	38533	99,19	0,00	0,00	1	0	0
N2514T5_S8_L001	<i>A. coelebs</i>	Nymph	-	Petit-Saut	French Guiana	27670	64,84	0,00	34,97	1	0	1
N2514_S3_L001	<i>A. coelebs</i>	Nymph	-	Petit-Saut	French Guiana	18458	41,74	0,00	57,15	1	0	1
853T2_S20_L001	<i>A. dissimile</i>	Adult	Female	Talhuen	French Guiana	38067	0,00	99,35	0,19	0	1	1
853T1_S19_L001	<i>A. dissimile</i>	Adult	Female	Talhuen	French Guiana	36506	0,00	98,54	0,32	0	1	1
8110T1_S13_L001	<i>A. dissimile</i>	Adult	Female	Macouria	French Guiana	38858	0,00	98,01	0,00	0	1	0
8110T2_S14_L001	<i>A. dissimile</i>	Adult	Female	Macouria	French Guiana	35918	0,00	74,75	15,35	0	1	1
8110T4_S24_L001	<i>A. dissimile</i>	Adult	Female	Macouria	French Guiana	46148	0,00	67,19	25,26	0	1	1
8111T1_S25_L001	<i>A. dissimile</i>	Adult	Female	Rémire-Montjoly	French Guiana	31212	0,00	65,83	34,03	0	1	1
8111T2_S26_L001	<i>A. dissimile</i>	Adult	Female	Rémire-Montjoly	French Guiana	37852	0,00	58,20	41,41	0	1	1
8111T3_S27_L001	<i>A. dissimile</i>	Adult	Female	Rémire-Montjoly	French Guiana	34175	0,00	53,91	44,18	0	1	1

8110T3_S23_L001	A.dissimile	Adult	Female	Macouria	French Guiana	25529	0,00	28,41	1,46	0	1	1
10579T1_S28_L001	A.dissimile	Adult	Male	Awala-Yalimapo	French Guiana	32228	0,00	0,05	0,00	0	0	0
10969T2_S43_L001	A.geayi	Adult	Male	Cayenne	French Guiana	20207	0,23	27,77	32,52	0	1	1
L0715O2T2_S77_L001	A.geayi	Larva	-	Rémire-Montjoly	French Guiana	11086	49,61	1,80	33,19	1	1	1
10969T1_S42_L001	A.geayi	Adult	Male	Cayenne	French Guiana	24836	0,00	0,35	0,31	0	1	1
L04106T4_S47_L001	A.geayi	Larva	-	Rémire-Montjoly	French Guiana	2742	35,63	9,26	14,08	1	0	1
L04106T3_S46_L001	A.geayi	Larva	-	Rémire-Montjoly	French Guiana	4932	87,23	3,28	0,00	1	0	0
L04106T1_S44_L001	A.geayi	Larva	-	Rémire-Montjoly	French Guiana	11952	81,95	0,00	0,00	1	0	0
L04106T2_S45_L001	A.geayi	Larva	-	Rémire-Montjoly	French Guiana	4819	56,75	0,00	5,00	1	0	1
L0715O3T2_S80_L001	A.geayi	Larva	-	Rémire-Montjoly	French Guiana	3770	21,78	0,00	0,00	1	0	0
L0715O3T5_S83_L001	A.geayi	Larva	-	Rémire-Montjoly	French Guiana	25926	4,07	0,00	0,00	1	0	0
L5157_S134_L001	A.geayi	Larva	-	Saint-Laurent-du-Maroni	French Guiana	15879	1,42	0,00	67,27	1	0	1
10888T2_S52_L001	A.goeldii	Adult	Male	Régina	French Guiana	31387	0,00	98,59	0,00	0	1	0
1089T1_S247_L001	A.goeldii	Adult	Male	Régina	French Guiana	34761	0,01	94,82	0,02	0	1	0
10888T1_S51_L001	A.goeldii	Adult	Male	Régina	French Guiana	28031	0,00	89,29	0,02	0	1	0
1288T2_S49_L001	A.goeldii	Adult	Female	Régina	French Guiana	19830	0,00	67,87	25,06	0	1	1
1288T1_S48_L001	A.goeldii	Adult	Female	Régina	French Guiana	41883	0,00	64,37	35,21	0	1	1
1288T3_S50_L001	A.goeldii	Adult	Female	Régina	French Guiana	32779	0,00	15,53	82,68	0	1	1
N4432_S54_L001	A.humerale	Nymph	-	Matoury	French Guiana	26775	0,00	93,62	0,21	0	1	1
N4632_S55_L001	A.humerale	Nymph	-	Matoury	French Guiana	30897	0,00	93,36	5,18	0	1	1
11785T4_S61_L001	A.humerale	Adult	Male	Nouragues	French Guiana	21326	0,00	90,50	0,00	0	1	0
N4435_S56_L001	A.humerale	Nymph	-	Matoury	French Guiana	14176	0,00	81,58	1,71	0	1	1
11785T2_S59_L001	A.humerale	Adult	Male	Nouragues	French Guiana	33891	0,00	73,69	0,01	0	1	0
11785T1_S58_L001	A.humerale	Adult	Male	Nouragues	French Guiana	30817	0,00	71,70	0,00	0	1	0
11785T3_S60_L001	A.humerale	Adult	Male	Nouragues	French Guiana	36712	0,00	70,94	0,00	0	1	0
21108_S57_L001	A.humerale	Adult	Female	Trinité	French Guiana	39467	0,00	69,66	2,62	0	1	1
11785T5_S62_L001	A.humerale	Adult	Male	Nouragues	French Guiana	40557	0,00	28,69	70,27	0	1	1
N2012_S53_L001	A.humerale	Nymph	-	Kourou	French Guiana	38277	0,00	12,29	86,40	0	1	1
N2414T2_S73_L001	A.latepunctatum	Nymph	-	Petit-Saut	French Guiana	24849	88,13	6,41	4,22	1	1	1
N2314_S71_L001	A.latepunctatum	Nymph	-	Petit-Saut	French Guiana	10927	64,59	2,05	15,49	1	1	1
N2414T1_S72_L001	A.latepunctatum	Nymph	-	Petit-Saut	French Guiana	20497	82,70	1,32	2,04	1	1	1
N2309_S74_L001	A.latepunctatum	Nymph	-	Macouria	French Guiana	29105	55,95	0,00	43,79	1	0	1
AlocF20N3_S300_L001	A.loculosum	Nymph	-	Petite île	La Réunion Island	62170	4,01	0,00	0,00	1	0	0
AlocF20F1_S290_L001	A.loculosum	Adult	Female	Petite île	La Réunion Island	58841	0,51	0,00	0,12	1	0	1
AlocF20F2_S291_L001	A.loculosum	Adult	Female	Petite île	La Réunion Island	56693	0,30	0,00	0,31	1	0	1
AlocF20M2_S292_L001	A.loculosum	Adult	Male	Petite île	La Réunion Island	56838	0,20	0,00	0,00	1	0	0
L0760O6T3_S153_L001	A.longirostre	Larva	-	Kourou	French Guiana	57401	0,00	88,89	0,00	0	1	0

L076006T1_S151_L001	A.longirostre	Larva	-	Kourou	French Guiana	54675	0,00	83,77	15,55	0	1	1
L075503T1_S105_L001	A.longirostre	Larva	-	Rémire-Montjoly	French Guiana	42563	0,00	73,63	25,97	0	1	1
L075503T2_S106_L001	A.longirostre	Larva	-	Rémire-Montjoly	French Guiana	31881	0,00	65,85	33,67	0	1	1
L075503T3_S107_L001	A.longirostre	Larva	-	Rémire-Montjoly	French Guiana	31076	0,00	61,23	38,36	0	1	1
L076007T2_S155_L001	A.longirostre	Larva	-	Kourou	French Guiana	50399	0,00	60,30	28,73	0	1	1
L075503T4_S108_L001	A.longirostre	Larva	-	Rémire-Montjoly	French Guiana	34347	0,00	28,22	70,82	0	1	1
L076007T1_S154_L001	A.longirostre	Larva	-	Kourou	French Guiana	45172	0,00	7,37	88,44	0	1	1
L075502T2_S104_L001	A.longirostre	Larva	-	Rémire-Montjoly	French Guiana	10004	0,00	2,11	32,94	0	1	1
L076006T2_S152_L001	A.longirostre	Larva	-	Kourou	French Guiana	32420	0,00	0,85	98,72	0	1	1
N0202I2_S230_L001	A.naponense	Nymph	-	Kaw	French Guiana	26962	98,59	0,01	0,00	1	0	0
22108_S233_L001	A.naponense	Adult	Female	Trinité	French Guiana	39565	70,93	0,01	28,08	1	0	1
11475_S232_L001	A.naponense	Adult	Male	Saint-Georges	French Guiana	66581	7,80	0,00	92,10	1	0	1
N0202I3_S231_L001	A.naponense	Nymph	-	Kaw	French Guiana	22336	97,95	0,00	0,00	1	0	0
N0202I1_S229_L001	A.naponense	Nymph	-	Kaw	French Guiana	17277	95,91	0,00	0,00	1	0	0
02-21T7_S243_L001	A.oblongoguttatum	Adult	Female	Kourou	French Guiana	35570	0,00	98,41	0,00	0	1	0
02-21T6_S242_L001	A.oblongoguttatum	Adult	Female	Kourou	French Guiana	44552	0,00	97,21	0,00	0	1	0
02-21T3_S239_L001	A.oblongoguttatum	Adult	Female	Kourou	French Guiana	33501	0,00	96,84	0,00	0	1	0
02-21T9_S245_L001	A.oblongoguttatum	Adult	Female	Kourou	French Guiana	41016	0,01	96,56	0,00	0	1	0
02-21T8_S244_L001	A.oblongoguttatum	Adult	Female	Kourou	French Guiana	36056	0,00	96,01	0,00	0	1	0
02-21T11_S247_L001	A.oblongoguttatum	Adult	Female	Kourou	French Guiana	41562	0,00	95,22	0,00	0	1	0
02-21T2_S238_L001	A.oblongoguttatum	Adult	Female	Kourou	French Guiana	48166	0,00	94,97	0,00	0	1	0
02-21T1_S237_L001	A.oblongoguttatum	Adult	Female	Kourou	French Guiana	27448	0,00	94,61	0,00	0	1	0
02-21T4_S240_L001	A.oblongoguttatum	Adult	Female	Kourou	French Guiana	29328	0,01	92,85	0,00	0	1	0
AO2e2_S297_L001	A.ovale	Adult	Female	Unknown	Brazil	49827	1,64	96,38	0,00	0	1	0
L5275I3_S236_L001	A.pacae	Larva	-	Saint-Georges	French Guiana	27151	0,00	97,16	0,00	0	1	0
L5275I2_S235_L001	A.pacae	Larva	-	Saint-Georges	French Guiana	12317	0,00	94,43	0,01	0	1	0
L5275I1_S234_L001	A.pacae	Larva	-	Saint-Georges	French Guiana	27780	0,02	93,93	0,01	0	1	0
L5275I6_S239_L001	A.pacae	Larva	-	Saint-Georges	French Guiana	21991	0,02	92,78	0,00	0	1	0
L5275I5_S238_L001	A.pacae	Larva	-	Saint-Georges	French Guiana	23619	0,01	87,88	0,00	0	1	0
L5275I4_S237_L001	A.pacae	Larva	-	Saint-Georges	French Guiana	5905	0,00	47,57	0,00	0	1	0
109_S105_L001	A.splendidum	Adult	Male	Bakoumba	Gabon	17822	95,12	0,00	0,00	1	0	0
11101T1I2_S241_L001	A.romitii	Adult	Female	Macouria	French Guiana	5618	0,00	0,02	0,02	0	0	0
11101T1I1_S240_L001	A.romitii	Adult	Female	Macouria	French Guiana	39389	0,00	0,00	0,01	0	0	0
L1512I1_S242_L001	A.rotundatum	Larva	-	Kourou	French Guiana	35967	0,00	98,97	0,46	0	1	1
L1512I2_S243_L001	A.rotundatum	Larva	-	Kourou	French Guiana	27586	0,00	96,14	0,13	0	1	0
L1512I3_S244_L001	A.rotundatum	Larva	-	Kourou	French Guiana	28941	0,00	95,46	0,58	0	1	1
L1512I4_S245_L001	A.rotundatum	Larva	-	Kourou	French Guiana	1443	0,00	94,73	0,00	0	1	0

L1512I5_S246_L001	A.rotundatum	Larva	-	Kourou	French Guiana	17512	0,00	87,24	0,11	0	1	0
L5385T2I3_S262_L001	A.scalpturatum	Larva	-	Nouragues	French Guiana	17085	66,89	0,02	0,01	1	0	0
L5385T2I1_S260_L001	A.scalpturatum	Larva	-	Nouragues	French Guiana	15054	92,19	0,01	0,02	1	0	0
718_S255_L001	A.scalpturatum	Adult	Female	Régina	French Guiana	32270	95,36	0,01	0,00	1	0	0
N2921I1_S257_L001	A.scalpturatum	Nymph	-	Kourou	French Guiana	24558	94,21	0,01	0,00	1	0	0
N2921I2_S258_L001	A.scalpturatum	Nymph	-	Kourou	French Guiana	29889	99,10	0,01	0,00	1	0	0
11678_S259_L001	A.scalpturatum	Adult	Male	Petit-Saut	French Guiana	47548	99,54	0,00	0,00	1	0	0
712_S254_L001	A.scalpturatum	Adult	Female	Kourou	French Guiana	48540	99,52	0,00	0,00	1	0	0
L5385T2I2_S261_L001	A.scalpturatum	Larva	-	Nouragues	French Guiana	9405	45,98	0,00	0,01	1	0	0
AO1e2_S296_L001	A.sp	Adult	Female	Unknown	Brazil	24318	10,13	0,00	0,00	1	0	0
110_S106_L001	A.splendidum	Adult	Male	Bakoumba	Gabon	18542	82,53	0,00	0,00	1	0	0
103_S99_L001	A.splendidum	Adult	Female	Bakoumba	Gabon	30773	27,05	0,00	72,90	1	0	1
102_S98_L001	A.splendidum	Adult	Male	Bakoumba	Gabon	15276	8,39	0,00	0,00	1	0	0
228_S204_L001	A.paulopunctatum	Adult	Male	Bateke Plateau National Park	Gabon	34510	0,00	2,25	71,12	0	1	1
25_S23_L001	A.splendidum	Adult	Female	Moukalaba-Doudou	Gabon	42199	97,42	0,00	0,00	1	0	0
100_S95_L001	A.splendidum	Adult	Female	Moukalaba-Doudou	Gabon	33247	96,94	0,00	0,00	1	0	0
17_S15_L001	A.tholloni	Adult	Female	Moukalaba-Doudou	Gabon	41259	94,71	0,00	0,00	1	0	0
23_S21_L001	A.tholloni	Adult	Female	Moukalaba-Doudou	Gabon	39249	88,64	0,00	0,00	1	0	0
3_S2_L001	A.tholloni	Adult	Female	Moukalaba-Doudou	Gabon	30044	87,03	0,00	0,00	1	0	0
5_S4_L001	A.tholloni	Adult	Female	Moukalaba-Doudou	Gabon	26358	84,89	0,00	0,00	1	0	0
13_S11_L001	A.tholloni	Adult	Female	Moukalaba-Doudou	Gabon	28406	81,00	0,00	0,00	1	0	0
20_S18_L001	A.tholloni	Adult	Female	Moukalaba-Doudou	Gabon	23949	74,05	0,00	0,00	1	0	0
27_S25_L001	A.tholloni	Adult	Female	Moukalaba-Doudou	Gabon	23575	72,99	0,00	0,00	1	0	0
19_S17_L001	A.tholloni	Adult	Female	Moukalaba-Doudou	Gabon	31120	69,16	0,00	0,00	1	0	0
11_S9_L001	A.tholloni	Adult	Female	Moukalaba-Doudou	Gabon	21176	67,25	0,00	0,00	1	0	0
24_S22_L001	A.tholloni	Adult	Female	Moukalaba-Doudou	Gabon	38622	0,10	0,00	0,00	1	0	0
112_S108_L001	A.splendidum	Adult	Female	Tchibanga	Gabon	32784	87,38	0,00	0,02	1	0	0
AvarieTick19E_S298_L001	A.variegatum	Adult	Female	Unknown	La Réunion Island	32949	31,27	0,00	62,02	1	0	1
AvF1_S299_L001	A.variegatum	Adult	Female	Unknown	La Réunion Island	11907	27,57	0,00	0,00	1	0	0
L0101I3_S269_L001	A.varium	Larva	-	Matoury	French Guiana	16382	0,00	92,99	0,00	0	1	0
L0101I2_S268_L001	A.varium	Larva	-	Matoury	French Guiana	8273	0,00	55,63	2,73	0	1	1
11039I1_S263_L001	A.varium	Adult	Male	Matoury	French Guiana	38359	0,00	51,92	0,00	0	1	0
L0101I1_S267_L001	A.varium	Larva	-	Matoury	French Guiana	5570	0,00	44,52	8,74	0	1	1
N12104_S270_L001	A.varium	Nymph	-	Cayenne	French Guiana	47778	0,00	35,91	64,03	0	1	1
11039I2_S264_L001	A.varium	Adult	Male	Matoury	French Guiana	44136	0,00	6,85	0,24	0	1	1
2069TT1_S265_L001	A.varium	Adult	Female	Cayenne	French Guiana	53086	0,00	0,54	98,03	0	1	1

Table S2

Genes	Hypothetical product	Primers (5'-3')	Tm	Fragment size	References
<i>rpoB</i>	DNA-directed RNA polymerase beta chain	CoxrpoBF2-	56°C	Nested PCR assay: 1st round PCR: CoxrpoBF2/CoxrpoBR1: 607-610bp 2nd round PCR: CoxrpoBF3/CoxrpoBR3: 539-542bp	[41]
		CoxrpoBR1-			
		CoxrpoBF3-			
		CoxrpoBR3-			
<i>GroEL</i>	Chaperone protein GROEL	CoxGrF1 -	56°C	Nested PCR assay: 1st round PCR: CoxGrF1/CoxGrR2: 655bp 2nd round PCR: CoxGrF2/CoxGrR1: 619bp	
		CoxGrR2 -			
		CoxGrF2 -			
		CoxGrR1 -			
<i>16S rRNA</i>	Small ribosomal subunit	Cox16SF1-	56°C	Semi-nested PCR assay: 1st round PCR: Cox16SF1/Cox16SR2: 1315-1320bp 2nd round PCR: Cox16SF1/Cox16SR1: 719bp 2nd round PCR: Cox16SF2/Cox16SR2: 596-601bp	
		Cox16SR2-			
		Cox16SR1-			
		Cox16SF2-			
<i>16S rRNA</i>	Small ribosomal subunit	Francis16SF1-	52°C	Semi-nested PCR assay: 1st round PCR: Francis16SF1/Francis16SR2: 1250bp 2nd round PCR: Francis16SF1/Francis16SR1: 745bp 2nd round PCR: Francis16SF2/Francis16SR2: 505bp	This study
		Francis16SR2-			
		Francis16SR1-			
		Francis16SF2-			
<i>GroEL</i>	Chaperone protein GROEL	FrancisgroELF1-	50°C	Semi-nested PCR assay: 1st round PCR: FrancisgroELF1/FrancisgroELR2: 1138bp 2nd round PCR: FrancisgroELF1/FrancisgroELR1: 954bp	This study
		FrancisgroELR2-			
		FrancisgroELR1-			
<i>ftsZ</i>	Cell division protein FtsZ	FrancisftsZF1-	52°C	Semi-nested PCR assay: 1st round PCR: FrancisftsZF1/FrancisftsZR2: 1110bp 2nd round PCR: FrancisftsZF1/FrancisftsZR1: 1001bp	This study
		FrancisftsZR2-			
		FrancisftsZR1-			
<i>gyrB</i>	DNA gyrase subunit B	FrancisgyrBF1-	54°C	Semi-nested PCR assay: 1st round PCR: FrancisgyrBF1/FrancisgyrBR2: 1109bp 2nd round PCR: FrancisgyrBF1/FrancisgyrBR1: 999bp	This study
		FrancisgyrBR2-			
		FrancisgyrBR1-			
<i>rpoB</i>	DNA-directed RNA polymerase beta chain	FRCLF2-	56°C	Semi-nested PCR assay: 1st round PCR: FRCLF2/FRCLR2: 420bp 1st round PCR: FRCLF2/FRCLR1: 379bp	[32]
		FRCLR2-			
		FRCLR1-			

Table S3

	n=	<i>Coxiella</i> frequencies by species	<i>Francisella</i> frequencies by species	<i>Rickettsia</i> frequencies by species	n complete <i>Coxiella</i> MLST	n <i>Coxiella</i> strains	n complete <i>Francisella</i> MLST	n <i>Francisella</i> strains
Amblyomma americanum	3	1	0	0.33	2	1	N/A	N/A
A. cajennense	10	1	0	0.4	4	1	N/A	N/A
A. calcaratum	1	1	0	0	1	1	N/A	N/A
A. coelebs	11	1	0	0.27	2	1	N/A	N/A
A. dissimile	10	0	0.9	0.8	N/A	N/A	1	1
A. geayi	10	0.8	0.3	0.6	2	1	1	1*
A. goeldii	6	0	0.5	0.5	N/A	N/A	N/A	N/A
A. humerale	10	0	1	0.6	N/A	N/A	3	1
A. latepunctatum	4	1	0.75	1	3	1	1	1*
A. loculosum	4	1	0	0.5	2	1	N/A	N/A
A. longirostre	10	0	1	0.9	N/A	N/A	3	1
A. naponense	5	1	0	0.4	3	1	N/A	N/A
A. oblongoguttatum	9	0	1	0	N/A	N/A	3	1
A. ovale	1	0	1	0	N/A	N/A	1	1
A. pacae	6	0	1	0	N/A	N/A	2	2
A. paulopunctatum	1	0	1	1	N/A	N/A	1	1
A. romitii	2	0	0	0	N/A	N/A	N/A	N/A
A. rotundatum	5	0	1	0.4	N/A	N/A	3	3
A. sculpturatum	8	1	0	0	3	1	N/A	N/A
A. sp	1	1	0	0	1	1	N/A	N/A
A. splendidum	7	1	0	0.14	3	1	N/A	N/A
A. tholloni	10	1	0	0	3	1	N/A	N/A
A. variegatum	2	1	0	1	2	1	N/A	N/A
A. varium	7	0	1	0.71	N/A	N/A	1	1
A. sculptum	N/A	N/A	N/A	N/A	1	1	1	1

Discussion

Comme le montre cette étude, l'association entre un P-symbionte et son hôte n'est pas figée dans le temps évolutif, mais possède une dynamique rythmée par des phénomènes de remplacement symbiotique. Les facteurs structurants de ce processus sont ainsi discutés dans cet article et, d'une manière plus globale, brillamment revus par McCutcheon *et al.* (2019). Toutefois, si les résultats de notre étude sont présentés sous l'angle des événements de remplacement entre P-symbiontes chez les tiques, ils démontrent également l'impact de ces phénomènes sur la structuration des core-microbiomes. Pour commencer, l'article et particulièrement la Figure 2a montrent bien que le peu de diversités microbiennes retrouvées chez nos tiques est très fortement structuré par la présence et l'identité du P-symbionte (*Coxiella* ou *Francisella*) ainsi que par la présence ou l'absence de *Rickettsia*. Ces trois bactéries sont très bien connues pour ce TV chez les tiques, ce qui indique un fort effet structurant du mode de TV sur le microbiome des tiques. Ce résultat est en accord avec l'étude portant sur la structuration du microbiome d'*I. pacificus* (Couper, Kwan, Ma, & Swei, 2019) et ce, malgré des échelles différentes. De plus, il semble exister une forte compétition entre *Coxiella* et *Francisella*, expliquant ces phénomènes de remplacement. Ces symbiontes sont en compétition pour les ressources et avantages (la TV par exemple) que leur fournissent une ou plusieurs niches écologiques au sein de leur hôte acarien. Par ailleurs, nos résultats suggèrent également qu'il existe une interaction de « coopération » ou de compétition et d'exclusion en fonction des souches de *Francisella* et de *Rickettsia* (des tests statistiques sont en cours à l'heure où j'écris ces lignes pour vérifier cette hypothèse). Les interactions biotiques au sein du core-microbiome apparaissent donc comme primordiales dans sa structuration. Pour aller plus loin, **le core-microbiome des tiques semble donc être assujéti au processus écologique de *species sorting***. Ce processus regroupe, comme facteurs structurants d'une communauté, les interactions biotiques existantes au sein d'un

holobionte avec les filtres environnementaux, formées par les niches écologiques à l'intérieur de l'hôte. Ce résultat est d'autant plus surprenant que le processus de *species sorting* n'est généralement pas considéré comme impactant les microbiomes à une échelle inter-spécifique. Effectivement, à une telle échelle, les microbiomes sont majoritairement structurés par la coévolution et les patrons de phylosymbiose. Cette observation est néanmoins à mesurer. En effet, une telle observation est essentiellement basée sur l'étude du microbiome intestinal de différentes espèces de vertébrés qui sont, de par leur nature et structure, bien différents du core-microbiome des tiques, composé d'une ou deux bactéries intracellulaires.

Au-delà du processus de *species sorting*, **il apparaît clairement que le core-microbiome des tiques est également structuré par un processus coévolutif.** Premièrement, comme nous l'avons précédemment vu, la coévolution entre les P-symbiontes et leurs hôtes arthropodes va impacter le génome des P-symbiontes. Ce faisant, la coévolution va directement structurer les phénomènes de compétition et de remplacement entre ces derniers, et donc structurer la composition des core-microbiomes. Ceci est parfaitement illustré par les différentes histoires (co)évolutives de *Coxiella* et de *Francisella* chez les *Amblyomma*. Deuxièmement, les tiques et leurs core-microbiomes semblent également former des patrons de « phylosymbiose ». La composition du core-microbiome se montre effectivement influencée par la phylogénie des *Amblyomma* puisque certains clades d'*Amblyomma* sont infectés par un P-symbionte préférentiel. De manière intéressante, ces clades sont typiquement composés d'espèces partageant des spectres d'hôtes très similaires. En étant un peu audacieux, nous pouvons même comparer ces patrons, toute proportion gardée, aux patrons des communautés microbiennes intestinales retrouvées chez les mammifères appelés « entérotypes », et dépendant du régime alimentaire et de la phylogénie de l'hôte (Groussin *et al.*, 2017 ; Ley *et al.*, 2008 ; Wu *et al.*, 2011). Il est tout de même nécessaire de prendre des précautions

en parlant de phylosymbiose dans notre cas. Les tiques apparaissent effectivement comme des holobiontes à part entière, peu comparables aux vertébrés et soumis à des processus structurants qu'ils partagent, sans doute, avec d'autres arthropodes « P-symbiotiques ». Il me paraît donc délicat de contextualiser les processus de structuration affectant un microbiome dominé par la TV avec ceux des microbiomes de vertébrés dominés par un mode de transmission mixte.

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Chapitre IV

Chapitre IV

Le risque acarologique en Guyane : cas d'étude des *Rickettsia* et *Borrelia*

Article 5 : Binetruy F, Buysse M, Barosi R & Duron O. Novel *Rickettsia* genotypes, species and group in ticks of French Guiana, South America. Soumis à Scientific Reports.

Article 6 : Binetruy F, Noël V, Garnier S, Boulanger N, Buysse M, Barosi R & Duron O. A novel *Borrelia* species, divergent to Lyme disease and relapsing fevers groups, in passerine-transported ticks of French Guiana, South America. En préparation

Contexte

Comme nous l'avons vu en introduction, la Guyane représente une zone favorable à l'émergence de maladies. Ces pathologies peuvent être à transmission vectorielle, à l'image du virus Zika, ou transmis par les moustiques du genre *Aedes* qui a récemment défrayé la chronique en Guyane et dans le monde (Enfissi, Codrington, Roosblad, Kazanji, & Rousset, 2016 ; Laval *et al.*, 2016). En ce sens, la faune sauvage est considérée comme le principal réservoir d'agents pathogènes pouvant provoquer des maladies émergentes chez les humains (Jones *et al.*, 2008). Ainsi, l'incroyable biodiversité faunistique et le grand nombre d'espèces de tiques que renferme le territoire guyanais donnent sens à l'étude des cortèges de parasites hébergés par ces acariens. Le spectre d'hôte généraliste des tiques, les plus abondantes en Guyane et les plus souvent associées à l'homme, comme *A. cajennense* (voir chapitre I), renforce également cette notion de risque d'émergence.

Dans le but d'appréhender ce risque, il est alors nécessaire de commencer par identifier et caractériser de potentiels agents pathogènes au sein du microbiome des tiques de Guyane. Pour cela, les données du barcoding bactérien obtenues *via* les précédents objectifs ont été utilisées pour identifier les bactéries d'intérêts médical et vétérinaire. Certaines de ces bactéries ont ensuite été caractérisées moléculairement

(séquençage de plusieurs loci, ou séquençage de génomes complets) et étudiées dans un contexte phylogénétique ; les deux articles ci-après présentent justement les résultats pour deux de ces genres : *Borrelia* et *Rickettsia*. Au-delà des *Borrelia* et des *Rickettsia*, nous avons également identifié la présence de *Ehrlichia* et *Anaplasma*, ces deux bactéries étant connues pour être des parasites intracellulaires de vertébrés. Un screening PCR a également permis de mettre en évidence des parasites eucaryotes comme *Babesia* et des filaires chez des tiques non gorgées. Néanmoins, l'étude des *Ehrlichia* et *Anaplasma* (non discutée dans les articles suivants) est toujours en phase de mise au point ; aussi n'aborderai-je pas ces derniers qui feront l'objet de prochaines publications.

Novel *Rickettsia* genotypes, species and group in ticks in French Guiana, South America

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Abstract

Rickettsia are obligate intracellular bacteria often associated with ticks and best known for causing human diseases (rickettsiosis), including typhus fever and sporadic cases of serious infection. In this study, we conducted a large survey of ticks in French Guiana to understand the overall diversity of *Rickettsia* in this remote area largely covered by dense rainforests. Out of 819 individuals (22 tick species in six genera), 252 (30.8%) samples were positive for *Rickettsia* infection. Multilocus typing and phylogenetic analysis identified 19 *Rickettsia* genotypes, but none was 100% identical to already known *Rickettsia* species or strains. Among these 19 genotypes, we identified two validated *Rickettsia* species, *Rickettsia amblyommatis* (spotted fever group) and *Rickettsia bellii* (bellii group), and characterized two novel species, *Candidatus Rickettsia matouryensis* (spotted fever group) and *Candidatus Rickettsia guianensis* (which belongs to a novel and divergent group, the guiana group), and four unclassified *Rickettsia*. While some tick hosts of these *Rickettsia* genotypes are among the most common ticks to bite humans in French Guiana, their potential pathogenicity remains entirely unknown. However, we found a strong association between *Rickettsia* genotypes and their host tick species, suggesting that most of these *Rickettsia* genotypes may be nonpathogenic forms maintained through transovarial transmission.

Introduction

Members of the *Rickettsia* genus are obligate intracellular bacteria of eukaryotes¹⁻³. The best known *Rickettsia* species are major human pathogens that include the etiological agents of the epidemic typhus, *R. prowazekii*, the Rocky Mountain spotted fever, *R. rickettsii*, and the flea-borne spotted fever, *R. felis*^{1,4,5}. Most of these pathogenic *Rickettsia* species have a zoonotic life cycle and are transmitted by blood-feeding arthropods such as ticks, mites, lice and fleas, which commonly serve as ecological bridges for transmission from wildlife to humans and domestic animals^{1,4,5}. However, members of the *Rickettsia* genus are actually more widespread than previously recognized: ecological surveys are uncovering substantial *Rickettsia* diversity associated with blood-feeding arthropods but also with non-blood-feeding arthropods, protozoa, algae and plants¹⁻³.

There are currently more than 30 recognized *Rickettsia* species but the advent of multilocus sequence typing (MLST) and molecular phylogenetics has recently led to the description of several new putative species^{1,2,4,6,7}. Historically, *Rickettsia* were classified into a few major groups based on serological characteristics, but subsequent DNA sequencing led to classification of at least 10 distinct phylogenetic groups^{1,2,6}. Three *Rickettsia* groups – the spotted fever group, the typhus group and the transitional group – are the subject of intensive study since they all include major pathogenic species and are commonly found in blood-feeding arthropods¹. In addition, another *Rickettsia* group widely found in ticks but also in many other arthropods, the bellii group, is also commonly studied. This group is thought to be basal to the three other major *Rickettsia* groups and is largely composed of nonpathogenic *Rickettsia* species and strains^{1-3,7}.

Despite considerable research effort, the diversity of *Rickettsia* remains largely unknown in most remote geographic regions and in undersampled arthropod taxa. Interestingly, a recent

survey reported the presence of 22 tick species in French Guiana⁸, a vast equatorial land located on the northeast coast of South America and mostly covered by dense rainforests including old-growth forests, which are biodiversity hotspots. French Guiana's human population (ca. 250,000 inhabitants) is concentrated principally in a handful of towns spread along the coastline and main rivers, while the interior is largely uninhabited. We know little on the presence, diversity and effect of *Rickettsia* on ticks in this region. Only one *Rickettsia* species of the spotted fever group, *R. wissemanii*, has been documented in bat soft ticks *Ornithodoros hasei* caught in French Guiana⁹. Some of the tick species of French Guiana are also present in adjacent countries such as Brazil, but the presence of *Rickettsia* has been investigated there in a few of these species including the Cayenne tick *Amblyomma cajennense* and the arboreal tick *A longirostre*^{7,10-14}.

In this study, we conducted a wide molecular survey of *Rickettsia* in ticks in French Guiana. This survey included 819 field specimens belonging to 22 tick species of the 33 known from French Guiana⁸. We further used MLST, including *gltA*, *coxA*, *atpA*, *ompB* and 16S rRNA gene sequences, and phylogenetics for the description of these *Rickettsia* infections. Lastly, we examined and discussed their genetic proximity with known *Rickettsia* species and strains.

Results

Detection of *Rickettsia*. We assayed for the presence of *Rickettsia* in 819 individual ticks collected in French Guiana and belonging to six genera and 22 species: *Amblyomma* (16 species, 686 specimens), *Rhipicephalus* (2 species, 16 specimens), *Ixodes* (1 species, 6 specimens), *Dermacentor* (1 species, 97 specimens), *Haemaphysalis* (1 species, 8 specimens) and *Ornithodoros* (1 species, 6 specimens) (Figure 1 and Table 1). Using total tick DNA extracts, we applied a high-throughput 16S rDNA sequencing approach to characterize their whole bacterial diversity and then to detect the presence of *Rickettsia* in each tick specimen.

Of the 819 specimens, 252 (30.8%) exhibited *Rickettsia* 16S rDNA reads and were thus considered as *Rickettsia*-positive specimens. The 567 remaining specimens (69.2%) were devoid of any of *Rickettsia* 16S rDNA reads but had satisfactory DNA template quality, as shown by the positive amplification of other bacterial 16S rDNA reads commonly detected in ticks (including *Coxiella*- and *Francisella*-like endosymbionts¹⁵⁻¹⁸); these specimens were therefore considered as uninfected. Within tick populations, species and genera, *Rickettsia* was detected at diverse frequencies, ranging from 0% to 100% (Tables 1 and S1), as detailed below.

Of the 22 tick species examined, 10 species (6/16 *Amblyomma* species, 1/1 of *Dermacentor*, 2/2 of *Rhipicephalus* and 1/1 of *Ornithodoros*) were not infected by *Rickettsia* (Tables 1 and S1). The 12 other tick species (10/16 *Amblyomma* species, 1/1 *Haemaphysalis* and 1/1 *Ixodes*) were *Rickettsia*-positive for at least one of the examined specimens (Figure 1, Tables 1 and S1). The detection rate of *Rickettsia* did not co-vary with the screening effort, i.e., the number of examined specimens per tick species (Spearman's rank correlation, $n=22$, $r_s=0.20$, $p=0.37$): the tick species observed with *Rickettsia* infections were not those for which we examined more specimens. This is best exemplified by (1) *Rickettsia*-positive tick species for which we examined few specimens, such as *I. luciae* ($n=6$ examined specimens and all were *Rickettsia*-positive) and *A. goeldii* ($n=5$ examined specimens and 4 were *Rickettsia*-positive), and (2) *Rickettsia*-negative tick species for which we examined a large number of specimens, such as *A. oblongoguttatum* ($n=95$ examined specimens but none positive) and *D. nitens* ($n=97$ examined specimens but none positive) (Figure 1, Tables 1 and S1).

The prevalence of *Rickettsia* differed substantially between the 12 infected tick species (Fisher's exact test, $p=2.10^{-16}$): while prevalence is low to moderate in some tick species (e.g., 4 infected specimens of the 14 examined in *A. coelebs*; 29%), it is significantly higher in other species (e.g., 106 infected specimens of the 130 examined in *A. longirostre*; 82%) (Tables 1 and S1). Prevalence of *Rickettsia* varied significantly between populations of two tick species, *A. cajennense* (7 populations, with prevalence ranging from 0 to 48%; Fisher's exact test, $p=3.10^{-5}$) and *A.*

longirostre (14 populations, with prevalence ranging from 0 to 100%; Fisher's exact test, $p=4.10^{-6}$) (Table S1). However, prevalence of *Rickettsia* did not vary significantly between populations of the nine other tick species for which distinct geographic populations were examined (Fisher's exact tests, all $p>0.05$) (Table S1).

Diversity of *Rickettsia*. The diversity of *Rickettsia* in French Guiana ticks was examined using sequences from one to five genetic markers (*gltA*, 16S rRNA, *atpA*, *ompB* and *coxA*). Overall, the examination of this multilocus data set led to the identification of 19 distinct *Rickettsia* genotypes (FG019a–FG019s hereafter; Table 2), as detailed below.

First, *Rickettsia* sequences from the *gltA* gene were taken from a subsample of 92 infected specimens from of the 12 infected species (one to 47 specimens per infected species were examined; Table 2). On the basis of DNA sequencing, 12 distinct *gltA* genotypes with 84.9–99.8% pairwise nucleotide identity were characterized from the 92 specimens examined. Six tick species of the 12 infected species harbored each only one *gltA* genotype: *A. cajennense*, *A. coelebs*, *A. longirostre*, *A. geayi*, *A. naponense* and *I. luciae*. In each of the six other tick species, two to four distinct *gltA* genotypes were found. We characterized four *gltA* genotypes from the seven sequenced *A. dissimile* specimens (Table 2).

Second, we amplified four additional bacterial markers (16S rRNA, *atpA*, *ompB* and *coxA*) from 44 representative tick samples infected by the 12 *Rickettsia gltA* genotypes (Table 2). We then obtained 10 genotypes of 16S rRNA (97.7–99.9% pairwise nucleotide identity), 10 *atpA* genotypes (85.4–99.8%), 10 *ompB* genotypes (78.1–99.8%) and eight *coxA* genotypes (86.1–99.8%). While the 16S rRNA and *atpA* gene fragments were amplified from the 44 samples, the *ompB* and *coxA* were only amplified from 41 and 38 samples, respectively (Table 2). The diversity at the 16S rRNA, *atpA*, *ompB* and *coxA* gene fragments was consistent with the results

inferred from the *gltA* sequences: *Rickettsia* infections with distinct *gltA* sequences have distinct sequences at the other gene markers. No 16S rRNA, *atpA*, *ompB* and *coxA* sequence variation was observed within tick species in which only *gltA* genotype was detected (i.e., *A. cajennense*, *A. coelebs*, *A. longirostre*, *A. geayi*, *A. naponense* and *I. luciae*). However, the combined use of these five markers allowed the distinction of additional *Rickettsia* genetic variation not detected with the single *gltA* gene sequences. Indeed, while one of the *Rickettsia* infections of *A. dissimile* and one of *A. humerale* shared the same *gltA* sequence (the *gltA* sequence type #d in Table 2), their *atpA*, *ompB* and *coxA* (but not 16S rRNA) gene sequences were different, showing that they were thus two distinct *Rickettsia* genotypes. The examination of *gltA*, 16S rRNA, *atpA*, *ompB* and *coxA* gene sequences thus led to the identification of 19 *Rickettsia* genotypes (FG019a–FG019s; Table 2). Only one of these *Rickettsia* genotypes, FG019c, was shared by several tick species (*A. dissimile*, *A. geayi* and *A. latepunctatum*). Each of the 18 other *Rickettsia* genotypes (FG019a, FG019b and FG019d–FG019s) was found in only one tick species (Table 2).

Phylogeny of *Rickettsia*. The phylogenetic relationships between the *Rickettsia* infections were first estimated using the 92 *gltA* sequences from the 12 infected tick species found in this study, as well as *gltA* sequences from representative *Rickettsia* species and strains available in GenBank (Figure 2). The closest relatives of the *Rickettsia* found in French Guiana were also included in the analyses. No recombination events were detected for the *gltA* data set using both the RDP and GENCONV methods (all $p > 0.23$). The ML phylogenetic analysis based on the *gltA* sequences showed that the 19 *Rickettsia* genotypes (FG019a–FG019s) found in this study consisted of three distinct groups (Figure 2):

- (1) Nine *Rickettsia* genotypes clustered with known members of the spotted fever group, including *R. amblyommatis* (*Rickettsia* FG019a and FG019m of *A. cajennense* and *A. longirostre*, respectively), *R. tamurae*, *R. buchneri* and *R. monacensis* (FG019j, FG019k

and FG019r of *A. humerale*, *A. latepunctatum* and *H. juxtakochi*, respectively), *R. aeschlimannii* and *R. rhipicephali* (FG019h of *A. goeldii*) and *Rickettsia* sp. strains AL (FG019l and FG019p of *A. latepunctatum* and *A. varium*, respectively). The *Rickettsia* FG019b of *A. coelebs* also belonged to the spotted fever group within a cluster including *R. raoultii*, *R. aeschlimannii* and *R. rhipicephali*.

(2) Nine other *Rickettsia* genotypes clustered with known members of the *R. bellii* group previously found in other tick species. These genotypes included FG019c (of *A. dissimile*, *A. geayi* and *A. latepunctatum*), FG019d (*A. dissimile*), FG019e (*A. dissimile*), FG019f (*A. dissimile*), FG019g (*A. goeldii*), FG019i (*A. humerale*), FG019o (*A. varium*), FG019q (*H. juxtakochi*) and FG019s (*I. luciae*).

(3) The last *Rickettsia* genotype, FG019n of *A. naponense*, was genetically distant to all other known *Rickettsia* species and groups. It showed a low level of *gltA* nucleotide identity (<88%) with all other *Rickettsia* species and groups. This *Rickettsia* genotype was the single member of a novel and highly divergent group, here provisionally called the guiana group (Figure 2). It is noteworthy that this strain is also distantly related to the *Rickettsia* sp. clone Tapirape1 (canadensis group; Figure 2), which was previously found in *A. naponense* from Brazil¹⁹.

However, none of the *Rickettsia* genotypes found in this study is closely related to the single species already reported from French Guiana, *R. wissemanii* (Figure 2).

A second analysis was performed to refine the intrageneric phylogeny of *Rickettsia*. For this, we used the *Rickettsia* 16S rRNA, *atpA*, *ompB* and *coxA* sequences from the 19 *Rickettsia* genotypes identified in the present work, as well as sequences of representative *Rickettsia* species and strains available in GenBank. The analysis of single and concatenated gene sequences did not detect significant recombination events in the data set using both RDP and GENCONV methods (all $p > 0.08$). When the sequences were examined separately for each

gene, we obtained the same phylogenetic pattern as observed with the ML analysis based on *gltA* gene sequences with the partitioning of the 19 *Rickettsia* genotypes into the same three different groups (i.e., spotted fever, *bellii* and *guiana*) (Figures S1–S4). Indeed, the examination of the 16S rRNA and *atpA* gene sequences of *Rickettsia* FG019n genotype of *A. naponense* (neither *ompB* nor *coxA* could be amplified from this *Rickettsia* strain; see Table 2) corroborated the existence of the *guiana* group: the 16S rRNA, *atpA* and *gltA* single-gene phylogenies (Figures 2, S1 and S2) and the *gltA*, 16S rRNA and *atpA* concatenated phylogeny (Figure 3) showed that the FG019n genotype is highly divergent from all other known *Rickettsia* groups, species and strains. On account of the distinct genetic and phylogenetic traits of the *Rickettsia* FG019n of *A. naponense*, we propose the designation *Candidatus Rickettsia guianensis* (referring to the Guianas, a region in northeastern South America that includes French Guiana where this *Rickettsia* was found) for this organism.

Analyses of a multilocus data set further showed that the nine *Rickettsia* genotypes (FG019c–g, FG019i, FG019o, FG019q and FG019s) belonging to the *bellii* group always clustered together with the *R. bellii* strains previously found in other American tick species, such as *A. neumanni* (Argentina) and *D. variabilis* (USA) (Figures 3 and S1–4). These nine *Rickettsia* genotypes can therefore be considered as members of the *R. bellii* species. None of these nine *Rickettsia* genotypes were 100% identical to already known *R. bellii* members.

The multilocus data set also showed that the nine *Rickettsia* genotypes (FG019a, FG019b, FG019h, FG019j–m, FG019p and FG019r) belonging to the spotted fever group can be split into two subgroups:

- (1) The first subgroup included the *Rickettsia* FG019j (*A. humerale*), FG019k (*A. latepunctatum*) and FG019r (*H. juxtakochi*), which clustered with *R. tamurae*, *R. buchneri* and *R. monacensis* although remaining substantially divergent from them at

each gene marker (Figures 2, 3 and S1–4). On account of the distinct genetic and phylogenetic traits of the spotted fever *Rickettsia* FG019j, FG019k and FG019r, we propose the designation *Candidatus Rickettsia matouryensis* (referring to Matoury, the town in French Guiana where this *Rickettsia* was primarily found in *A. humerale*) for this organism.

(2) The second subgroup included the *Rickettsia* FG019a (*A. cajennense*), FG019m (*A. longirostre*), FG019b (*A. coelebs*), FG019h (*A. goeldii*), FG019l (*A. latepunctatum*) and FG019p (*A. varium*), which all clustered with *R. amblyommatis* on the basis of multilocus analyses (Figure 3). However, only FG019a and FG019m consistently clustered with *R. amblyommatis* at each gene marker (Figures 2 and S1–4), showing that these two genotypes can be considered as members of the *R. amblyommatis* species. None of these two *Rickettsia* genotypes were 100% identical to already known *R. amblyommatis* members, however. The four other *Rickettsia* genotypes (FG019b, FG019h, FG019l and FG019p) cannot be classified into specific species due to a lack of consensus between the phylogenetic trees (Figures 2, 3 and S1–4). Indeed, while the 16S rDNA genotype of FG019b is more closely related to *R. amblyommatis* (Figure S1), its *atpA* genotype is more closely related to *R. montanensis* (Figure S2). Although these last four *Rickettsia* genotypes may each represent a novel species, additional gene sequencing is required to determine their precise phylogenetic proximity with other members of the spotted fever group.

Discussion

We found here that *Rickettsia* infections are common in French Guiana ticks, a pattern also observed among ticks of other South American regions^{7,10–13,20}. The incidence of infection varied between tick species and populations of French Guiana: 12 of the 22 tick species examined, including *Amblyomma*, *Haemaphysalis* and *Ixodes* species, harbored *Rickettsia*, and

when present, prevalence ranged from 10 to 100%, with significant variations between populations of some species. We further found that these *Rickettsia* infections are remarkably diverse. Multilocus strain typing revealed the presence of 19 *Rickettsia* genotypes of different phylogenetic origins. The single *Rickettsia* species known from this region, *R. wissemanii*⁹, was not detected in the present study. Altogether, this means that at least 20 different *Rickettsia* genotypes are circulating in ticks in French Guiana.

None of the 19 *Rickettsia* genotypes we identified in French Guiana had been documented before this study. Multilocus typing showed that 11 of these *Rickettsia* genotypes can be assigned to two validated *Rickettsia* species, namely *R. amblyommatis* (two genotypes) and *R. bellii* (nine genotypes). These two *Rickettsia* species are widely present among Central and South American ticks, each infecting more than 10 species^{7,11–13,21–26}. Their presence in French Guiana was therefore expected, but the observation of novel genotypes there indicates the presence of important geographic variability: *R. amblyommatis* and *R. bellii* have probably radiated within their respective regions, including French Guiana, into different genotypes. Overall, this confirms that *R. amblyommatis* and *R. bellii* have the widest host range and the broadest geographic distribution among all *Rickettsia* species reported from South America, as suggested in early studies^{7,25}. Besides the *R. amblyommatis* and *R. bellii* genotypes, the eight other *Rickettsia* genotypes are rarer, since they are apparently endemic to French Guiana and cannot be assigned to formerly validated species. While a few genotypes remain unclassified within the spotted fever group, we described two novel species in this study: *Candidatus Rickettsia guianensis*, which belongs to a novel and divergent group, the guiana group, and *Candidatus Rickettsia matouryensis* within the spotted fever group. It is noteworthy that *Candidatus Rickettsia guianensis*, and thus the guiana group, has an intermediate phylogenetic position between the spotted fever and *bellii* *Rickettsia* groups, since it is more related on the

basis of its *gltA* sequence to the rare species *R. mendelii*, which was found only in Europe^{27,28}. To date, *Candidatus Rickettsia guianensis* is the single member of the guiana group.

Most of human pathogenic *Rickettsia* species are vectored by hard ticks^{4,29}. This leads to the question of the pathogenicity of the 19 *Rickettsia* genotypes we found in French Guiana and the associated health risk. Since none of the 19 *Rickettsia* genotypes was previously described before this study, no evidence of their pathogenicity currently exists, even for those belonging to validated *Rickettsia* species. Indeed, although *R. amblyommatis* and *R. bellii* are commonly found in the ticks of French Guiana, and more broadly in American ticks^{7,11–13,21–26}, they have never been found in vertebrate hosts, suggesting that they are nonpathogenic *Rickettsia* species. Yet, some tick species such as the Cayenne tick *A. cajennense* show a combination of traits that may facilitate tick-to-vertebrate transfers of tick-borne pathogens: *A. cajennense* is one of the most common ticks found in French Guiana, blood-feeding on many different hosts, including humans^{8,30,31}, but *R. amblyommatis* (infecting here 25% of the *A. cajennense* specimens examined) seems confined to ticks. In addition, while low prevalence is more consistent with vertebrate pathogens, *R. amblyommatis* and *R. bellii* are at medium to high prevalence in French Guiana, which is atypical of vertebrate pathogens. Another intriguing point is the apparent specificity of *Rickettsia* genotypes to tick species: 18 out of 19 *Rickettsia* genotypes were detected in only a single tick species. Even generalist tick species, such as *A. cajennense* and *A. dissimile*, feeding on (and sharing) a variety of vertebrate hosts^{8,30,31}, did not share the same *Rickettsia* genotype. These observations may indicate that at least some of the *Rickettsia* genotypes in French Guiana are present in ticks but not in vertebrate hosts.

The persistence means of the 19 *Rickettsia* genotypes remain unknown in French Guiana. As pointed out in a recent study³², the current view in rickettsiology has a strong anthropocentric bias and tends to describe all novel *Rickettsia* species as pathogenic forms. However, most of

the novel *Rickettsia* species or strains discovered in recent years are also found exclusively in arthropods and never in vertebrates^{1-3,33,34}. In ticks, as for many other arthropods, some *Rickettsia* are maternally inherited endosymbionts with poorly known effects on tick biology. This is the case for *R. buchneri* in the black-legged tick *I. scapularis*³⁵, *R. peacockii* in the American dog tick *D. variabilis*³⁶, and *R. vini* in the tree-hole tick *I. arboricola*^{16,37}. These nonpathogenic *Rickettsia* may interact with a variety of tick-borne pathogens³⁴, including *Anaplasma marginale*³⁸, *Borrelia burgdorferi*³⁹ and also other *Rickettsia*^{40,41}. Indeed, the endosymbiont *R. peacockii* hampered the multiplication of the spotted fever agent, *R. rickettsii*⁴⁰, and blocked transovarial transmission colonization of *R. rickettsii*, *R. montana* and *R. rhipicephali*^{40,41}. In French Guiana, further studies are needed to test this hypothesis of endosymbiosis by observing transstadial and transovarial transmission in ticks.

To conclude, this study revealed substantial diversity of *Rickettsia*, including novel genotypes, species and group, in ticks in French Guiana. This underlines the need to better document *Rickettsia* diversity in diverse regions, and more especially in remote regions. A recent meta-analysis suggests that more than 20% of terrestrial arthropods may be infected by *Rickettsia*, with ticks hosting most of this bacterial diversity, as observed in this study²⁹. In many arthropods other than ticks, *Rickettsia* are nonpathogenic, undergo exclusive maternal transmission to offspring, and may function as both a mutualist and reproductive manipulator^{2,3,42,43}. Overall, adaptations of *Rickettsia* to this diversity of hosts encompass an array of parasitic, but also mutualistic, interactions¹⁻³. In French Guiana, the effect of the 19 *Rickettsia* genotypes on human and animal health as well as on tick physiology and reproduction remains to be elucidated. Perhaps both vertical transmission and horizontal (infectious) transfers are epidemiologically dominant factors that facilitate their maintenance and global incidence within tick communities.

Materials and Methods

Tick collection. A collection of 819 specimens from 22 tick species, collected in French Guiana mostly in 2016 and 2017, was used (Table 1). Questing ticks were collected on vegetation using a drag-flag method over sites covering three types of ecological conditions (periurban, agricultural and natural). Ticks were also directly collected in nests or on hosts (including humans, four domestic animal species and wild animal species; see Table 1). All ticks were stored in 75% ethanol until examination. For each tick specimen, species were formally identified through morphological examination and DNA sequencing in a previous study⁸.

Detection of *Rickettsia*. To avoid external bacterial contaminants, ticks were processed with commercial bleach diluted at 1% for 30 s and then rinsed for 1 min in three successive baths of DNA-free water following a published protocol⁴⁴. For each tick specimen, total DNA was further extracted from whole body using a genomic DNA extraction kit according to the manufacturer's instructions (Qiagen). The presence of *Rickettsia* within each DNA template was investigated through high-throughput 16S rDNA sequencing. To this aim, a 251-bp portion of the V4 variable region of the bacterial 16S rDNA was amplified from whole-body DNA samples using the universal forward and reverse primers listed in Table S4. Each PCR product from individual samples was tagged with a unique 35-base barcode using the Nextera Index Kit (Illumina, San Diego, CA, USA). PCR amplifications were performed in duplicates for each sample. PCR reactions were conducted using a Multiplex PCR Kit (Qiagen). Amplified bacterial 16S rDNA products were purified and sequenced on an Illumina MiSeq platform (GenSeq, Montpellier University) and 250-bp end sequence reads were obtained. All bioinformatic analyses were conducted using the pipeline Frogs (<https://github.com/geraldinepascal/FROGS>) as follows⁴⁵: primers were removed from paired-end sequences with Cutadapt⁴⁶, and these sequences were merged into contigs with FLASH⁴⁷ before filtering by length ($251\text{bp} \pm 10\text{bp}$). Chimaeras were removed with VSEARCH⁴⁸, then

sequences were clustered using SWARM⁴⁹. We obtained an average number of 29,206 bacterial 16S rDNA reads per tick specimen. Sequences with 97% similarity were clustered together and identified as an operational taxonomic unit (OTU). Each representative OTU sequence was aligned and taxonomically assigned using the Silva database (<https://www.arb-silva.de/>). To eliminate the possibility of contamination, we included four mock DNA extractions under identical conditions using water, buffers and kits utilized for the experimental samples followed by Illumina Miseq analysis of 16S rDNA reads. The negative controls provided only a handful of reads that did not correspond to the bacterial genera found in the tick samples.

Molecular typing of *Rickettsia*. A subset of DNA templates for which *Rickettsia* reads were obtained through high-throughput 16S rDNA sequencing were used for *Rickettsia* multilocus typing. These *Rickettsia* infections were genotyped using independent PCR assays based on *gltA*, *coxA*, *ompB*, *atpA* and 16S rRNA, using semi-nested or nested PCR assays (Table S2). To prevent possible contamination, different parts of this process were physically separated from one another, in entirely separate rooms. All amplicons were also sequenced to control for false-positive amplifications. Gene features, primers and PCR conditions are detailed in Table S2.

Seminested and nested PCR amplifications were performed as follows: the first PCR run with the external primers was performed in a 10- μ L volume containing approximately 20 ng of genomic DNA, 3 mM of each dNTP (Thermo Scientific), 8 mM of MgCl₂ (Roche Diagnostics), 3 μ M of each primer, 1 μ L of 10 \times PCR buffer (Roche Diagnostics) and 0.5 U of Taq DNA polymerase (Roche Diagnostics). A 1- μ L aliquot of the PCR product from the first reaction was then used as a template for the second round of amplification. The second PCR was performed in a total volume of 25 μ L and contained 8 mM of each dNTP (Thermo Scientific), 10 mM of MgCl₂ (ThermoScientific), 7.5 μ M of each of the internal primers, 2.5 μ L of 10 \times PCR buffer

(Thermo Scientific) and 1.25 U of Taq DNA polymerase (Thermo Scientific). All PCR amplifications were performed under the following conditions: initial denaturation at 93°C for 3 min, 35 cycles of denaturation (93°C, 30 s), annealing ($T_m=52-56^\circ\text{C}$, depending on primers, 30 s), extension (72°C, 1 min), and a final extension at 72°C for 5 min. Known positive and negative individuals were used as controls in each PCR assay. All PCR products were visualized through electrophoresis in a 1.5% agarose gel. Positive PCR products were purified and sequenced in both directions (EUROFINS). The chromatograms were manually inspected and cleaned with CHROMAS LITE (http://www.technelysium.com.au/chromas_lite.html) and sequence alignments were done using CLUSTALW⁵⁰, both implemented in MEGA7⁵¹. Novel nucleotide sequences were deposited in the GenBank nucleotide database (Accession numbers: pending).

Molecular phylogenetics. The GBLOCKS⁵² program with default parameters was used to remove poorly aligned positions and to obtain unambiguous sequence alignments. All sequence alignments were also checked for putative recombinant regions using the RDP3 computer analysis package⁵³. Given a set of aligned nucleotide sequences, RDP3 can rapidly analyze these with a range of powerful nonparametric recombination detection methods, including the GENECON⁵⁴ and RDP⁵⁵. Phylogenetic relationships were evaluated between *Rickettsia* strains using *gltA*, *coxA*, *ompB*, *atpA* and 16S rRNA gene sequences. The evolutionary models most closely fitting the sequence data were determined using Akaike information criterion with the MEGA7 program⁵¹. Phylogenetic analyses were based on maximum likelihood (ML) analyses. A ML heuristic search, using a starting tree obtained by neighbor-joining, was conducted, and clade robustness was further assessed by bootstrap analysis using 1000 replicates in MEGA7⁵¹.

Ethics approval. The use of the genetic resources was declared to the French Ministry of the Environment under reference TREL19028117S/156 and #150401230100, in compliance with the Access and Benefit Sharing procedure implemented by the Loi pour la Reconquête de la

Biodiversité. The capture of ticks in the Grand Connétable protected area was authorized by the Prefecture of French Guiana by prefectoral decree R03-2016-09-23-003. All animals were handled in strict accordance with good animal practices as defined by the French code of practice for the care and use of animals for scientific purposes, established by articles R214-87 to R214-137 of the French rural code.

Data availability

Nucleotide sequences of *Rickettsia* were deposited in the GenBank nucleotide database (Accession numbers: 16S rRNA: [pending]; *gltA*: [pending]; *ompB*: [pending]; *atpA*: [pending]; *coxA*: [pending]).

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Author contribution

F.B. and O.D. designed the study and wrote the manuscript. F.B., M.B. and O.D. collected the samples. F.B., M.B. and R.B. performed the molecular typing. F.B. and O.D. performed the phylogenetic analyses. All authors agreed to the final version of the manuscript.

Additional information

Supplementary information accompanies this paper.

Competing interests

The authors declare no competing interests.

Table 1. List of tick species and populations included in the analysis, with details on their origin, the population sample size (*n*), and the prevalence of *Rickettsia*.

Ticks Species	Locality (# on Figure 1)	Ecological habitat	Collected on host/habitat	Stage	<i>n</i> examined	<i>n</i> <i>Rickettsia</i> -positive (%)
Ixodidae (hard ticks):						
1- <i>Amblyomma cajennense</i> sensus stricto (Fabricius, 1787)	7 localities (#1,2,6,7,11,13,43)	Degraded forests	Vegetation	Nymphs and adults	351	88 (25%)
2- <i>A. calcaratum</i> Neumann, 1899	1 locality (#22)	Old-growth forests	White-bearded Manakin (<i>Manacus manacus</i>)	Larva	1	0 (0%)
3- <i>A. coelebs</i> Neumann, 1899	4 localities (#2,6,7,34)	Old-growth forests	Vegetation	Nymphs	14	4 (29%)
4- <i>A. dissimile</i> Koch, 1884	5 localities (#1,17,36,44,45)	Degraded forest and peri-urban areas	Cane toad (<i>Rhinella marina</i>), Green Iguana (<i>Iguana iguana</i>) and vegetation	Nymphs and adults	24	16 (67%)
5- <i>A. geayi</i> Neumann, 1899	5 localities (#8,19,21,32,38)	Old-growth forests, degraded forests and peri-urban areas	Sloth (<i>Bradypus tridactylus</i>), a variety of passerine species (cf. Table S1 for species list) and vegetation	Larvae	16	10 (62%)
6- <i>A. goeldii</i> Neumann, 1899	1 locality (#38)	Degraded forests	Tamandua (<i>Tamandua tetradactyla</i>)	Adults	5	4 (80%)
7- <i>A. humerale</i> Koch, 1844	4 localities (#13,15,42,47)	Old-growth forests and degraded forests	Gray Four-eyed Opossum (<i>Philander opossum</i>), Human and vegetation	Nymphs and adults	10	5 (50%)
8- <i>A. latepunctatum</i> Tonelli-Rondelli, 1939	2 localities (#7,23,33)	Old-growth forests and pastures	Vegetation	Nymphs	4	4 (100%)
9- <i>A. longirostre</i> (Koch, 1844)	14 localities (#8,19,31)	Old-growth forests, degraded forests and peri-urban areas	A variety of passerine species (cf. Table S1 for species list) and vegetation	Larvae	130	106 (82%)
10- <i>A. naponense</i> (Packard, 1869)	3 localities (#2,3,42)	Old-growth forests and degraded forests	Human and vegetation	Nymphs and adults	5	1 (20%)
11- <i>A. oblongoguttatum</i> Koch, 1844	3 localities (#6,7,11)	Old-growth forests and degraded forests	Human and vegetation	Nymphs and adults	95	0 (0%)
12- <i>A. pacae</i> Aragão, 1911	2 localities (#11,31)	Old-growth forests and degraded forests	Human and vegetation	Larvae and nymphs	7	0 (0%)
13- <i>A. romitii</i> Tonelli-Rondelli, 1939	1 locality (#41)	Peri-urban area	Capybara (<i>Hydrochoerus hydrochaeris</i>)	Adults	2	0 (0%)
14- <i>A. rotundatum</i> Koch, 1844	2 localities (#5,39)	Old-growth forests and degraded forests	Red-footed tortoise (<i>Chelonoidis carbonaria</i>) and vegetation	Larvae and adults	6	0 (0%)
15- <i>A. scalpturatum</i> Neumann, 1906	5 localities (#5,10,11,35,37)	Old-growth forests	Human and vegetation	Larvae, nymphs and adults	8	0 (0%)
16- <i>A. varium</i> Koch, 1844	5 localities (#1,16,32,43,44)	Degraded forests	Human, sloth (<i>Bradypus tridactylus</i>) and vegetation	Larvae, nymphs and adults	8	5 (63%)
17- <i>Dermacentor nitens</i> Neumann, 1897	1 locality (#4)	Pastures	Horses (<i>Equus caballus</i>)	Larvae, nymphs and adults	97	0 (0%)
18- <i>Haemaphysalis juxtakochi</i> Cooley, 1946	3 localities (#5,7,11)	Old-growth forests	Human and vegetation	Nymphs	8	3 (38%)
19- <i>Ixodes luciae</i> Senevet, 1940	3 localities (#18,40,46)	Degraded forest	Gray Four-eyed Opossum (<i>Philander opossum</i>) and Linnaeus's Mouse Opossum (<i>Marmosa murina</i>)	Larvae, nymphs and adults	6	6 (100%)
20- <i>Rhipicephalus microplus</i> (Canestrini, 1888)	1 locality (#3)	Pastures	Cattle (<i>Bos taurus indicus</i>) and vegetation	Larvae and adults	10	0 (0%)
21- <i>R. sanguineus</i> sensus lato (Latreille, 1806)	2 localities (#12,14)	Peri-urban	Dog (<i>Canis lupus familiaris</i>)	Adults	6	0 (0%)
Argasidae (soft ticks):						
22- <i>Ornithodoros capensis</i> sensus stricto Neumann, 1901	1 locality (#9)	Seabird colony	Laughing gull nest (<i>Leucophaeus atricilla</i>)	Nymphs and adults	6	0 (0%)

Table 2. Sequence profiles of the five genes in the 19 *Rickettsia* genotypes (FG019a–FG019s) identified in this study. Letters a–k represent the different alleles at each gene locus. Dash indicates an absence of PCR product. *n*, number of specimens by each *Rickettsia* genotype (on the basis of multilocus typing of 44 representative tick samples).

Ticks Species	<i>Rickettsia</i> MLST genotypes	Genes					<i>n</i>
		<i>gltA</i>	16S rDNA	<i>atpA</i>	<i>ompB</i>	<i>coxA</i>	
Ixodidae (hard ticks):							
<i>Amblyomma cajennense</i> sensus stricto (Fabricius, 1787)	FG019a	a	a	a	a	a	13
<i>A. coelebs</i> Neumann, 1899	FG019b	c	b	b	c	b	3
<i>A. dissimile</i> Koch, 1884	FG019c	d	c	c	d	c	2
	FG019d	e	c	c	d	–	1
	FG019e	f	d	d	e	–	1
	FG019f	g	c	c	d	c	2
<i>A. geayi</i> Neumann, 1899	FG019c	d	c	c	d	c	1
<i>A. goeldii</i> Neumann, 1899	FG019g	d	f	e	f	d	1
	FG019h	h	e	f	g	e	1
<i>A. humerale</i> Koch, 1844	FG019i	d	c	e	i	d	2
	FG019j	i	g	g	h	–	1
<i>A. latepunctatum</i> Tonelli-Rondelli, 1939	FG019c	d	c	c	d	c	1
	FG019k	i	g	g	j	f	1
	FG019l	j	e	a	e	g	1
	FG019m	b	a	a	b	a	4
<i>A. longirostre</i> (Koch, 1844)	FG019n	k	h	h	–	–	1
<i>A. naponense</i> (Packard, 1869)	FG019o	d	k	i	f	d	1
<i>A. varium</i> Koch, 1844	FG019p	l	i	j	e	h	2
	FG019q	d	c	e	–	–	1
<i>Haemaphysalis juxtakochi</i> Cooley, 1946	FG019r	i	c	g	–	–	1
<i>Ixodes luciae</i> Senevet, 1940	FG019s	g	k	e	f	d	3

Figure legends

Figure 1. Location of sampling sites in French Guiana. Localities are represented by dots and numbers correspond to the population number given in Table S1. Figure 1B is the magnification of the area bounded by the translucent grey rectangle in Figure 1A. Red and black dots indicate populations infected by *Rickettsia* or not infected, respectively.

Figure 2. Phylogeny of *Rickettsia* constructed using maximum-likelihood (ML) estimations based on *gltA* gene sequences (589 unambiguously aligned nucleotide sites; best-fit approximation for the evolutionary model: GTR + G+I). Sequences from *Rickettsia* characterized in this study are shown in red. Only one *gltA* sequence per *Rickettsia* genotype and per tick species is shown. Sequences from representative *Rickettsia* groups, species and strains available in GenBank were also added to the analysis. The grey boxes delineate the different *Rickettsia* groups (their names are indicated in upper case), including the novel guiana group described in this study. Bacterial name, host species and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values >70% are shown). The scale bar is in units of substitution/site.

Figure 3. Phylogeny of *Rickettsia* constructed using maximum-likelihood (ML) estimations based on concatenated 16S rDNA, *gltA* and *atpA* sequences (1886 unambiguously aligned nucleotide sites; best-fit approximation for the evolutionary model: GTR + G+I). Sequences from *Rickettsia* characterized in this study are shown in red. Only one 16S rDNA, *gltA* and *atpA* concatenated sequence per *Rickettsia* genotype and per tick species is shown. Sequences from representative *Rickettsia* groups, species and strains available in GenBank were also added to the analysis. The grey boxes delineate the different *Rickettsia* groups (their names are indicated in upper case), including the novel guiana group described in this study. Bacterial name, host

species and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values >70% are shown). The scale bar is in units of substitution/site.

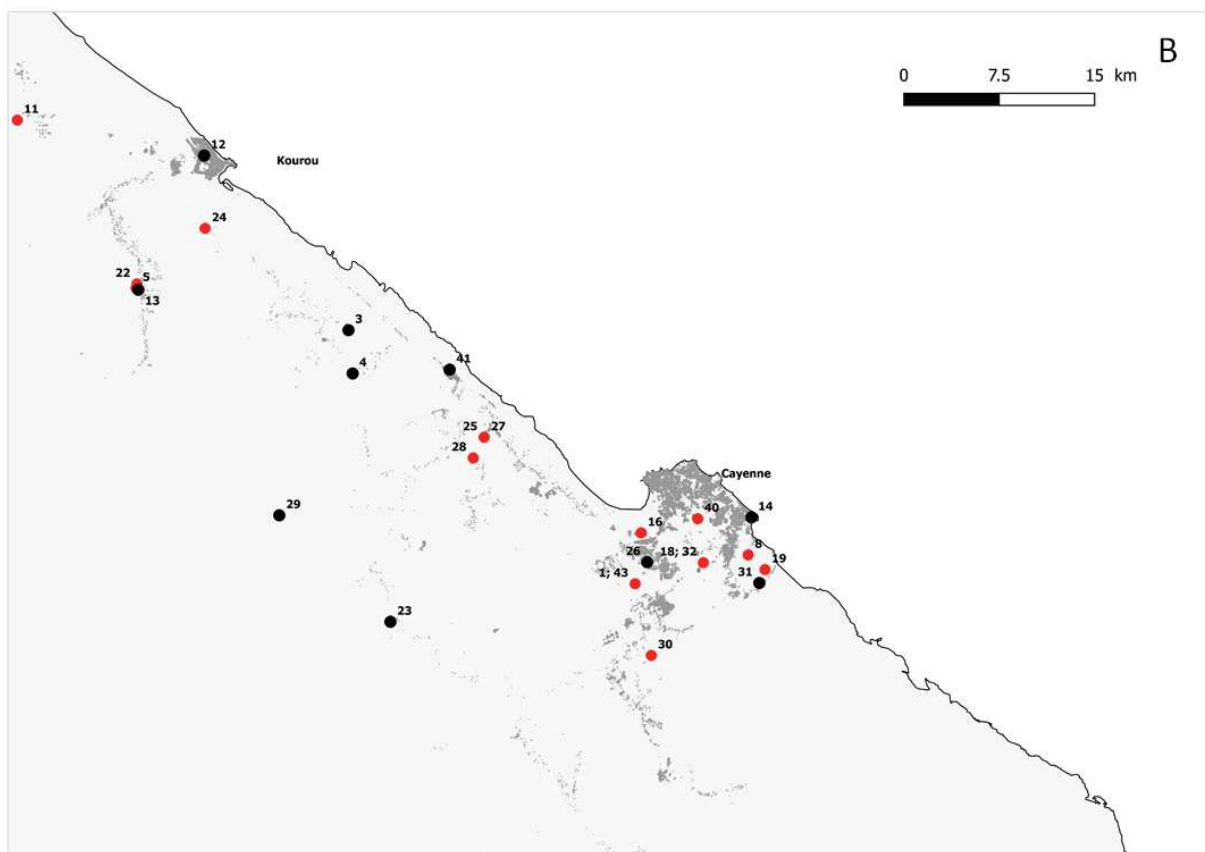
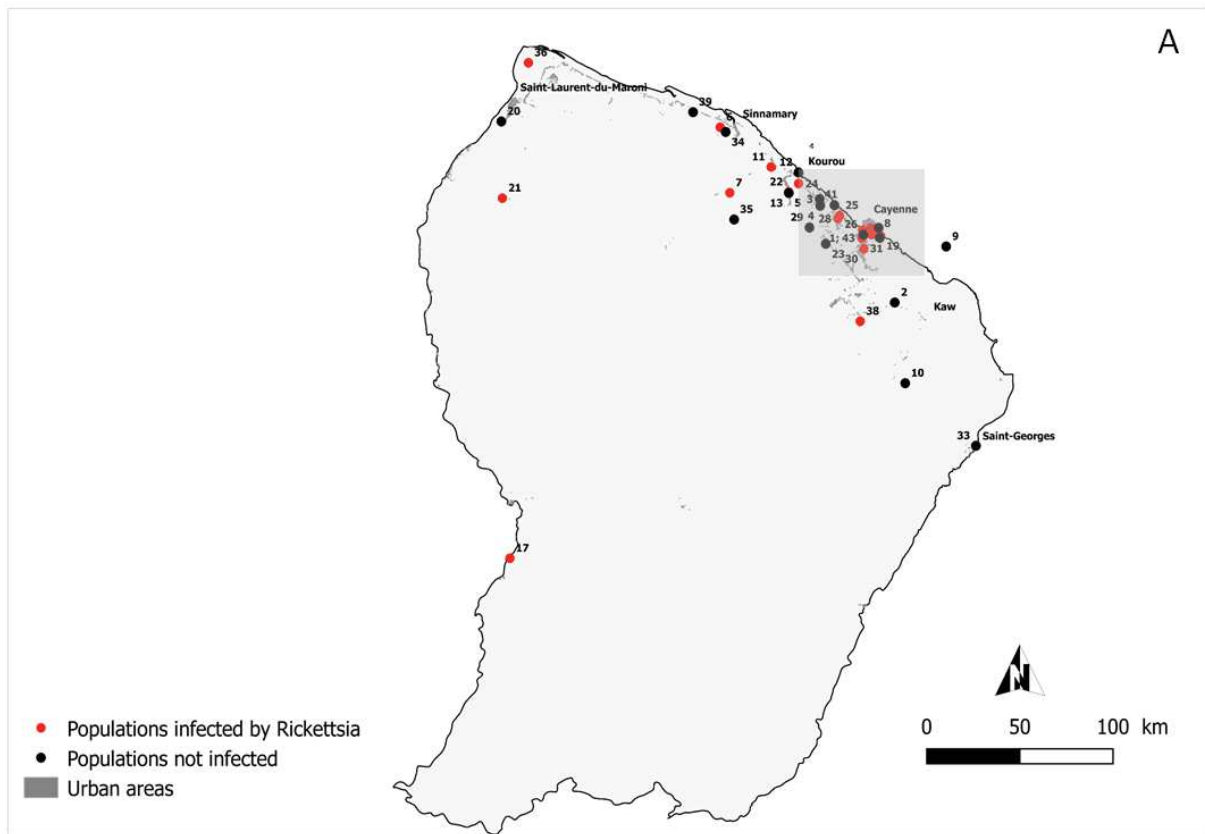


Figure 1.

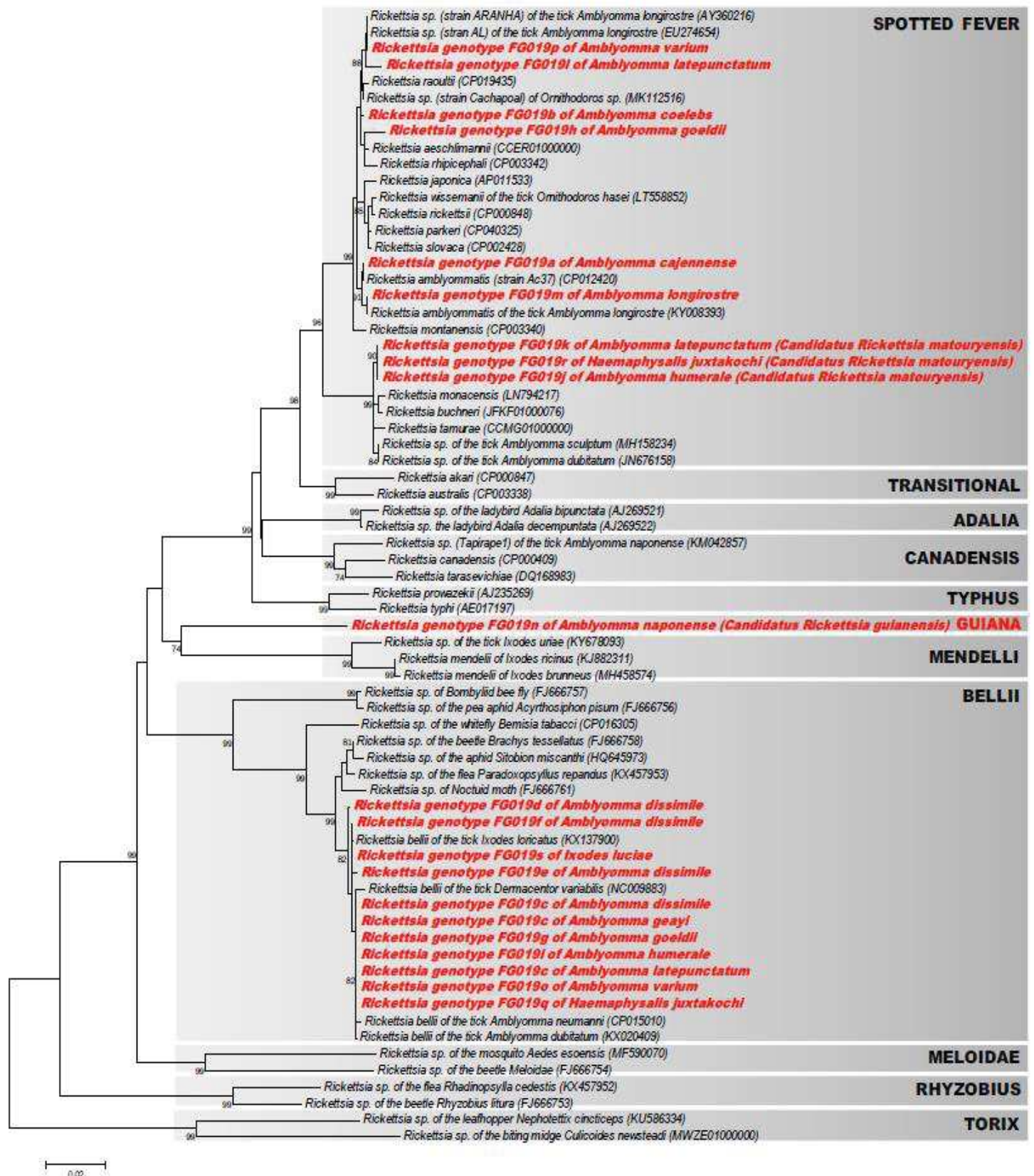


Figure 2.

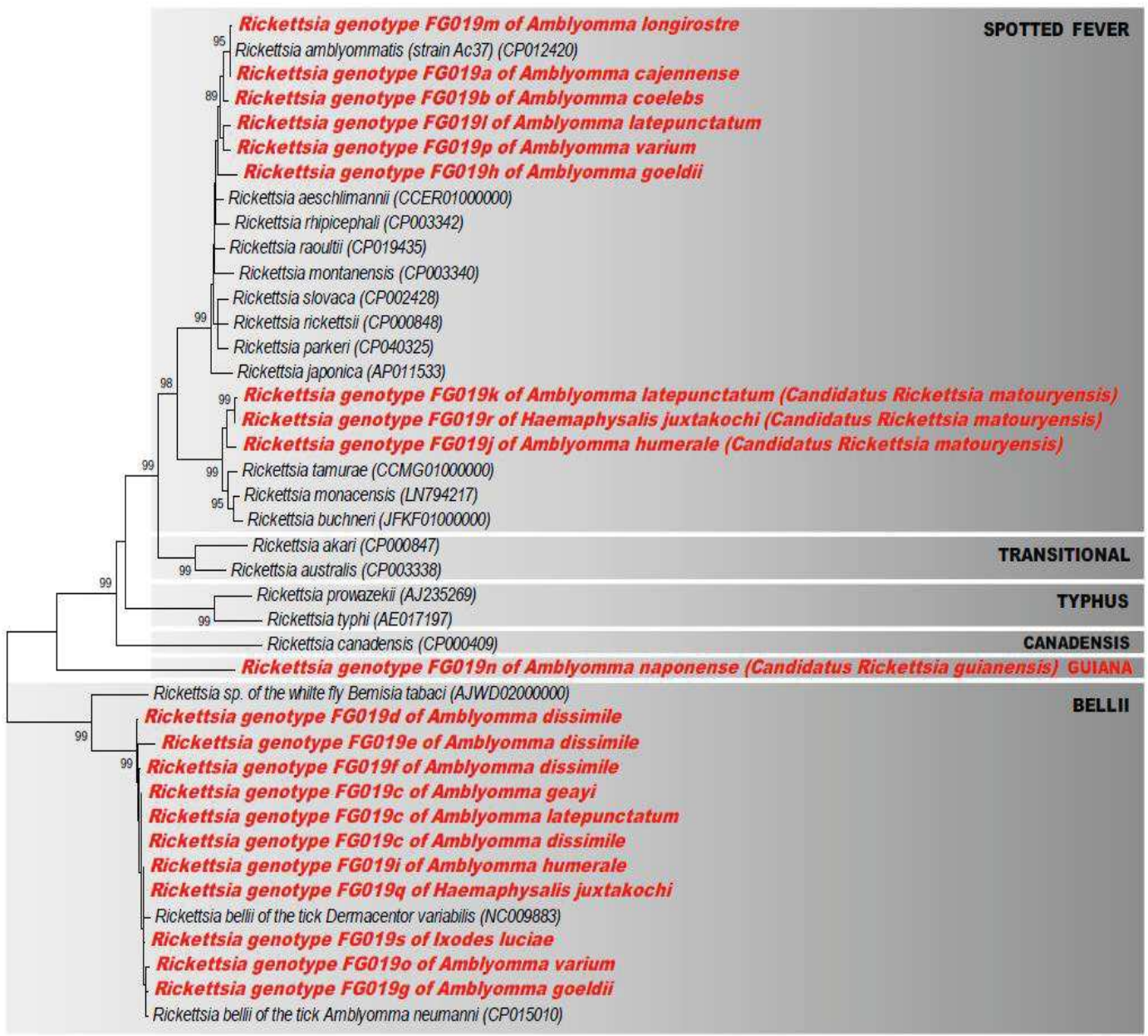


Figure 3.

SUPPLEMENTARY MATERIALS

Novel *Rickettsia* genotypes, species and group in ticks in French Guiana, South America

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Table S1. Detailed list of tick species and populations included in the analysis, with details on their origin, the population sample size (*n*), and the prevalence of *Rickettsia*.

Ticks Species	Locality (# on Figure 1)	Ecological habitat	Collected on host/habitat	Stage	<i>n</i> examined	<i>n</i> <i>Rickettsia</i> -positive (%)	
Ixodidae (hard ticks):							
1	<i>Amblyomma cajennense</i> sensus stricto (Fabricius, 1787)	A - Sinnamary, Piste Saint-Elie, 2016 (#6)	Degraded forest	Vegetation	Nymphs	5	2 (40%)
		B - Kourou, CSG, 2016 (#11)	Old-growth forests	Vegetation	Nymphs and adults	72	11 (15%)
		C - Kourou, Montagne des Singes, 2016 (#13)	Old-growth forests	Vegetation	Nymphs	12	0 (0%)
		D - Matoury, Piste de la Mirande, 2016 (#1)	Degraded forest	Vegetation	Nymphs and adults	64	31 (48%)
		E - Matoury, Piste de la Mirande, 2017 (#43)	Degraded forest	Vegetation	Adults	150	33 (22%)
		F - Kaw, Montagne de Kaw, 2016 (#2)	Old-growth forests	Vegetation	Nymphs	28	9 (32%)
		G - Petit-Saut, Montagne Plomb, 2016 (#7)	Old-growth forests	Vegetation	Nymphs	20	2 (10%)
2	<i>A. calcaratum</i> Neumann, 1899	Kourou, Montagne des Singes, 2016 (#22)	Old-growth forests	White-bearded Manakin (<i>Manacus manacus</i>)	Larva	1	0 (0%)
3	<i>A. coelebs</i> Neumann, 1899	A - Petit-Saut, Montagne Plomb, 2016 (#7)	Old-growth forests	Vegetation	Nymphs	11	4 (36%)
		B - Kaw, Montagne de Kaw, 2016 (#2)	Old-growth forests	Vegetation	Nymph	1	0 (0%)
		C - Sinnamary, Piste Saint-Elie, 2016 (#6)	Degraded forest	Vegetation	Nymph	1	0 (0%)
		D - Sinnamary, Crique Verte, 2016 (#34)	Old-growth forests	Vegetation	Nymph	1	0 (0%)
4	<i>A. dissimile</i> Koch, 1884	A - Awala-Yalimapo, Ayawande, 2016 (#36)	Peri-urban	Cane toad (<i>Rhinella marina</i>)	Nymphs and adults	4	1 (25%)
		B - Rémire-Montjoly, 2017 (#44)	Peri-urban	Green Iguana (<i>Iguana iguana</i>)	Nymphs and adults	14	11 (79%)
		D - Rémire-Montjoly, 2018 (#45)	Peri-urban	Green Iguana (<i>Iguana iguana</i>)	Adults	3	3 (100%)
		E - Matoury, Piste de la Mirande, 2016 (#1)	Degraded forest	Vegetation	Nymph	1	0 (0%)
		F - Talhuen, Village, 2016 (#17)	Peri-urban	Vegetation	Nymphs	2	1 (50%)
5	<i>A. geayi</i> Neumann, 1899	A - Cayenne, Montagne Tigre, 2017 (#40)	Peri-urban	Vegetation	Larvae	4	2 (50%)
		B - Rémire-Montjoly, Sentier du Rorota, 2014 (#19)	Degraded forest	Crimson-hooded Manakin (<i>Pipra aureola</i>) and Woodcreeper (<i>Glyphorhynchus spirurus</i>)	Larvae	3	2 (67%)
		C - Rémire-Montjoly, Sentier du Rorota, 2016 (#8)	Degraded forest	Crimson-hooded Manakin (<i>Pipra aureola</i>) and Barred Antshrike (<i>Thamnophilus doliatus</i>)	Larvae	5	2 (40%)
		D - Rémire-Montjoly, Vidal, 2016 (#32)	Degraded forest	Sloth (<i>Bradypus tridactylus</i>)	Adults	2	2 (100%)

		E - Saint-Laurent-du-Maroni, Piste Voltaire, 2013 (#21)	Old-growth forests	White-bearded Manakin (<i>Manacus manacus</i>) and White-necked Thrush (<i>Turdus albicollis</i>)	Larvae	2	2	(100%)
6	<i>A. goeldii</i> Neumann, 1899	Régina, N1 road PK63, 2017 (#38)	Degraded forest	Tamandua (<i>Tamandua tetradactyla</i>)	Adults	5	4	(80%)
7	<i>A. humerale</i> Koch, 1844	A - Kourou, Montagne des Singes, 2016 (#13)	Old-growth forests	Human	Nymph	1	0	(0%)
		B - Station des Nouragues, 2017 (#47)	Old-growth forests	Vegetation	Adults	5	1	(20%)
		C - Matoury, RN, 2016 (#15; no GPS data)	Degraded forest	Gray Four-eyed Opossum (<i>Philander opossum</i>)	Nymphs	3	3	(100%)
		D - Mana, Trinité, 2017 (#42)	Old-growth forests	Human	Adult	1	1	(100%)
8	<i>A. latepunctatum</i> Tonelli-Rondelli, 1939	A - Saint-Georges, Crique Gabaret 2016 (#33)	Pasture	Vegetation	Nymph	1	1	(100%)
		B - Petit-Saut, Montagne Plomb, 2016 (#7)	Old-growth forests	Vegetation	Nymphs	3	3	(100%)
9	<i>A. longirostre</i> (Koch, 1844)	A - Montsinéry, Bagne des Annamites, 2014 (#23)	Degraded forest	McConnell's flycatcher (<i>Mionectes macconnelli</i>)	Larvae	2	0	(0%)
		B - Macouria, lotissement Maillard, 2012 (#25)	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>) and Blue-backed Manakin (<i>Chiroxiphia pareola</i>)	Larvae	18	13	(72%)
		C - Macouria, lotissement Maillard, 2013 (#27)	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>)	Larvae	3	3	(100%)
		D - Macouria, lotissement Maillard, 2014 (#28)	Degraded forest	Blue-backed Manakin (<i>Chiroxiphia pareola</i>) and Crimson-hooded Manakin (<i>Pipra aureola</i>)	Larvae and nymph	6	6	(100%)
		E - Kourou, Montagne des Pères, 2014, (#24)	Old-growth forests	Blue-backed Manakin (<i>Chiroxiphia pareola</i>) and Crimson-hooded Manakin (<i>Pipra aureola</i>)	Larvae and nymph	12	10	(83%)
		F - Kourou, Montagne des Singes, 2014 (#22)	Old-growth forests	Woodcreeper (<i>Glyphoryncus spirurus</i>), White-bearded Manakin (<i>Manacus manacus</i>), White-flanked Antwren (<i>Myrmotherula axillaris</i>) and Golden-headed Manakin (<i>Pipra erythrocephala</i>)	Larvae and nymph	5	2	(40%)
		G - Rémoire-Montjoly, Sentier du Rorota, 2014 (#19)	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>), Blue-backed Manakin (<i>Chiroxiphia pareola</i>) and Crimson-hooded Manakin (<i>Pipra aureola</i>)	Larvae	29	29	(100%)
		H - Rémoire-Montjoly, Sentier du Rorota, 2016 (#8)	Degraded forest	Crimson-hooded Manakin (<i>Pipra aureola</i>), Barred Antshrike (<i>Thamnophilus doliatus</i>), Buff-throated Saltator (<i>Saltator maximus</i>), Silver-beaked Tanager (<i>Ramphocelus carbo</i>), Yellow-olive Flatbill (<i>Tolmomyias sulphurescens</i>), Straight-billed Woodcreeper (<i>Dendroplex picus</i>) and Golden-spangled Piculet (<i>Picumnus exilis</i>)	Larvae	18	15	(83%)
		I - Matoury, Mont Paramana, 2014 (#30)	Degraded forest	Crimson-hooded Manakin (<i>Pipra aureola</i>) and White-bearded Manakin (<i>Manacus manacus</i>)	Larvae and nymph	14	11	(79%)
		J - Saint-Laurent-du-Maroni, Piste Voltaire, 2013 (#21)	Old-growth forests	Grey-breasted Sabrewing (<i>Campylopterus largipennis</i>)	Larva	1	1	(100%)
		K - Matoury, PK7, 2012 (#26)	Peri-urban	White-bearded Manakin (<i>Manacus manacus</i>)	Larva	1	0	(0%)
		L - Montsinéry, Chemin de Risquetout, 2014 (#29)	Old-growth forests	McConnell's flycatcher (<i>Mionectes macconnelli</i>) and White-necked Thrush (<i>Turdus albicollis</i>)	Larva and nymph	2	0	(0%)
		M - Saint-Laurent-du-Maroni, Saint-Jean, 2013 (#20)	Degraded forest	McConnell's flycatcher (<i>Mionectes macconnelli</i>) and Woodcreeper (<i>Glyphoryncus spirurus</i>)	Larvae	2	0	(0%)
		N - Rémoire-Montjoly, Vidal, 2013 (#31)	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>), Blue-backed Manakin (<i>Chiroxiphia pareola</i>) and Crimson-hooded Manakin (<i>Pipra aureola</i>)	Larvae and nymph	17	16	(94%)
10	<i>A. naponense</i> (Packard, 1869)	A - Kaw, Montagne de Kaw, 2016 (#2)	Old-growth forests	Vegetation	Nymphs	3	0	(0%)
		B - Saint-Georges, crique Gabaret, 2016 (#33)	Degraded forest	Vegetation	Adult	1	0	(0%)
		C - Mana, Trinité, 2017 (#42)	Old-growth forests	Human	Adult	1	1	(100%)
11	<i>A. oblongoguttatum</i> Koch, 1844	A - Sinnamary, Piste Saint-Elie, 2016 (#6)	Degraded forest	Vegetation	Nymph	24	0	(0%)

		B - Kourou, CSG, 2016 (#11)	Old-growth forests	Human		Adults	70	0	(0%)
		C - Petit-Saut, Montagne Plomb, 2016 (#7)	Old-growth forests	Vegetation		Nymph	1	0	(0%)
12	<i>A. pacae</i> Aragão, 1911	A - Saint-Georges, crique Gabaret, 2016 (#31)	Degraded forest	Vegetation		Larvae	6	0	(0%)
		B - Kourou, CSG, 2016 (#11)	Old-growth forests	Human		Nymph	1	0	(0%)
13	<i>A. romitii</i> Tonelli-Rondelli, 1939	Macouria, Tonate, 2017 (#41)	Peri-urban	Capybara (<i>Hydrochoerus hydrochaeris</i>)		Adults	2	0	(0%)
14	<i>A. rotundatum</i> Koch, 1844	A - Iracoubo, Counamie, 2017 (#39)	Degraded forest	Red-footed tortoise (<i>Chelonoidis carbonaria</i>)		Adult	1	0	(0%)
		B - Kourou, Montagne des Singes, 2016 (#5)	Old-growth forests	Vegetation		Larvae	5	0	(0%)
15	<i>A. sculpturatum</i> Neumann, 1906	A - Petit-Saut, montagne Plomb, 2016 (#35)	Old-growth forests	Human		Adult	1	0	(0%)
		B - Kourou, CSG, 2016 (#11)	Old-growth forests	Human		Nymph	2	0	(0%)
		C - Kourou, Montagne des Singes, 2016 (#5)	Old-growth forests	Vegetation		Adult	1	0	(0%)
		D - Station des Nouragues, 2017 (#37; No GPS data)	Old-growth forests	Human		Larvae	3	0	(0%)
		E - Régina, Savane-Roche Virginie, 2016 (#10)	Old-growth forests	Vegetation		Adult	1	0	(0%)
16	<i>A. varium</i> Koch, 1844	A - Rémire-Montjoly, Sentier Loyola, 2017 (#48)	Degraded forest	Vegetation		Nymph	1	1	(100%)
		B - Matoury, Piste de la Mirande, 2016 (#1)	Degraded forest	Vegetation		Larvae	3	2	(67%)
		C - Matoury, Piste de la Mirande, 2017 (#43)	Degraded forest	Human		Nymph	1	0	(0%)
		D - Matoury, Larivot, 2016 (#16)	Degraded forest	Sloth (<i>Bradypus tridactylus</i>)		Adults	2	1	(50%)
		E - Rémire-Montjoly, Vidal, 2016 (#32)	Degraded forest	Sloth (<i>Bradypus tridactylus</i>)		Adult	1	1	(100%)
17	<i>Dermacentor nitens</i> Neumann, 1897	Macouria, N1 road PK 34 (#4)	Pasture	Horses (<i>Equus caballus</i>)		Larvae, nymphs and adults	97	0	(0%)
18	<i>Haemaphysalis juxtakochi</i> Cooley, 1946	A - Kourou, CSG, 2016 (#11)	Old-growth forests	Human		Nymph	1	0	(0%)
		B - Kourou, Montagne des Singes, 2016 (#5)	Old-growth forests	Vegetation		Nymphs	2	1	(50%)
		A - Petit-Saut, montagne Plomb, 2016 (#7)	Old-growth forests	Vegetation		Nymphs	5	2	(40%)
19	<i>Ixodes luciae</i> Senevet, 1940	A - Cayenne, Camp du tigre, 2017 (#46)	Degraded forest	Gray Four-eyed Opossum (<i>Philander opossum</i>) and Linnaeus's Mouse Opossum (<i>Marmosa murina</i>)		Larva and adult	2	2	(100%)
		B - Cayenne, Montagne Tigre, 2017 (#40)	Degraded forest	Gray Four-eyed Opossum (<i>Philander opossum</i>)		Adults	3	3	(100%)
		C - Rémire-Montjoly, Vidal, 2016 (#18)	Degraded forest	Large-headed rice rat (<i>Hylaeamys megacephalus</i>) and Gray Four-eyed Opossum (<i>Philander opossum</i>)		Nymph	1	1	(100%)
20	<i>Rhipicephalus microplus</i> (Canestrini, 1888)	Macouria, Matiti, 2016 (#3)	Pasture	Cattle (<i>Bos taurus indicus</i>) and vegetation		Larvae and adults	10	0	(0%)
21	<i>R. sanguineus</i> sensus lato (Latreille, 1806)	A - Kourou, rue Maurice Ravel, 2016 (#12)	Peri-urban	Dog (<i>Canis lupus familiaris</i>)		Adults	3	0	(0%)

		B - Rémire-Montjoly, Montravel, 2016 (#14)	Peri-urban	Dog (<i>Canis lupus familiaris</i>)		Adults	3	0	(0%)
Argasidae (soft ticks):									
22	<i>Ornithodoros capensis</i> stricto Neumann, 1901	Grand Connétable island, 2016, (#9)	Seabird colony	Laughing gull nest (<i>Leucophaeus atricilla</i>)		Nymphs and adults	6	0	(0%)

Table S2. Genes and primers used for *Rickettsia* sequencing.

Genes	<i>Rickettsia</i> specie	Hypothetical product	Primers (5'-3')	Tm	Fragment size	References		
<i>16S rRNA</i>	All	Small ribosomal subunit	R16SF1-	CGTGGGAATCTGCCATCAG	55°C	Semi-nested PCR assay: 1st round PCR: R16SF1/R16SR2: 1015bp 2nd round PCR: R16SF2/Cox16SR1: 869bp	This study	
			R16SR1-	CTCTGCGATCCGCRACCACC				
			R16SF2-	CGCTGATGGATGAGCCCGCGTC				
<i>gltA</i>	All	Citrate synthase	RickF1-	GTTCTCTTTCKGCATTTTATCC	56°C	Semi-nested PCR assay: 1st round PCR: RickF1/RickR1: 672bp 2nd round PCR: RickF2/RickR1: 645bp	1	
			RickF2-	GTTCTCTTTCKGCATTTTATCC				
			RickR1-	CATCTTTAAGAGCGATAGCTTCAAG				
<i>coxA</i>	All	Cytochrome c oxidase subunit 1	RcoxAF2-	CCYGATATGGCATTCCACGCC	55°C	Semi-nested PCR assay: 1st round PCR: RcoxAF2/RcoxaAR2: 849bp 2nd round PCR: RcoxAF2/RcoxaAR1: 568bp	This study	
			RcoxAR2-	AAGCACCGAGCGACATCGTA				
			RcoxAR1-	ACATATGGTGAGCCATACGAT				
<i>atpA</i>	Spotted fever group species	ATP synthase subunit alpha	RatpAF1-	ATCGGTGATAGRCAAACCGGA	55°C	Semi-nested PCR assay: 1st round PCR: RatpAF1/RatpAR2: 624bp 2nd round PCR: RatpAF2/RatpAR2: 600bp	This study	
			RatpAR2-	CGAACCTGACYACCTGCTTCATAG				
			RatpAF2-	ACGGCGATAGCSGTTGATACT				
	Bellii group species			RatpAbelliiF1-	GCTCCTGGTATAATCGATAG	55°C	Semi-nested PCR assay: 1st round PCR: RatpAbelliiF1/RatpAR2: 730bp 2nd round PCR: RatpAbelliiF1/RatpAR1: 699bp	This study
				RatpAR2-	CGAACCTGACYACCTGCTTCATAG			
				RatpAR1-	GTGCTGCAGAACCAACACGGCT			
<i>ompB</i>	Spotted fever group species	Outer membrane protein B precursor	RompBF1-	GGCTGGACCTGAAGCTGGAGC	52°C	Nested PCR assay: 1st round PCR: RompBF1/RompBR2: 792bp 2nd round PCR: RompBSFF2/RompBR1: 644bp	This study	
			RompBR2-	GCATCAGGTCTTATGCTTGACAC				
			RompBSFF2-	GCTTACGGTATATGGGCA				
	Bellii group species			RompBR1-	GTCCATCTAACTGAGACTGAG	53°C	Semi-nested PCR assay: 1st round PCR: RompBbelliiF1/RompBbelliiR2: 1373bp 2nd round PCR: RompBbelliiF2/RompBbelliiR2:: 859bp	This study
				RompBbelliiF1-	CAGTGGTGCAGCAGAAG			
				RompBbelliiR2-	GGCGGACTTTAACAGTACCCTG			
RompBbelliiF2-	GGCATCAAGTGAAGGCGGTG							

Reference

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Figure legends

Figure S1. Phylogeny of *Rickettsia* constructed using maximum-likelihood (ML) estimations based on 16S rDNA sequences (730 unambiguously aligned nucleotide sites; best-fit approximation for the evolutionary model: TN93+G). Sequences from *Rickettsia* characterized in this study are shown in red. Only one 16S rDNA sequence per *Rickettsia* genotype and per tick species is shown. Sequences from representative *Rickettsia* groups, species and strains available in GenBank were also added to the analysis. Bacterial name, host species and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values >70% are shown). The scale bar is in units of substitution/site.

Figure S2. Phylogeny of *Rickettsia* constructed using maximum-likelihood (ML) estimations based on *atpA* gene sequences (567 unambiguously aligned nucleotide sites; best-fit approximation for the evolutionary model: GTR+G). Sequences from *Rickettsia* characterized in this study are shown in red. Only one *atpA* sequence per *Rickettsia* genotype and per tick species is shown. Sequences from representative *Rickettsia* groups, species and strains available in GenBank were also added to the analysis. Bacterial name, host species and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values >70% are shown). The scale bar is in units of substitution/site.

Figure S3. Phylogeny of *Rickettsia* constructed using maximum-likelihood (ML) estimations based on *ompB* gene sequences (644 unambiguously aligned nucleotide sites; best-fit approximation for the evolutionary model: GTR+G). Sequences from *Rickettsia* characterized in this study are shown in red. Only one *ompB* sequence per *Rickettsia*

genotype and per tick species is shown. Sequences from representative *Rickettsia* groups, species and strains available in GenBank were also added to the analysis. Bacterial name, host species and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values >70% are shown). The scale bar is in units of substitution/site.

Figure S4. Phylogeny of *Rickettsia* constructed using maximum-likelihood (ML) estimations based on *coxA* gene sequences (546 unambiguously aligned nucleotide sites; best-fit approximation for the evolutionary model: GTR+G). Sequences from *Rickettsia* characterized in this study are shown in red. Only one *coxA* sequence per *Rickettsia* genotype and per tick species is shown. Sequences from representative *Rickettsia* groups, species and strains available in GenBank were also added to the analysis. Bacterial name, host species and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values >70% are shown). The scale bar is in units of substitution/site.

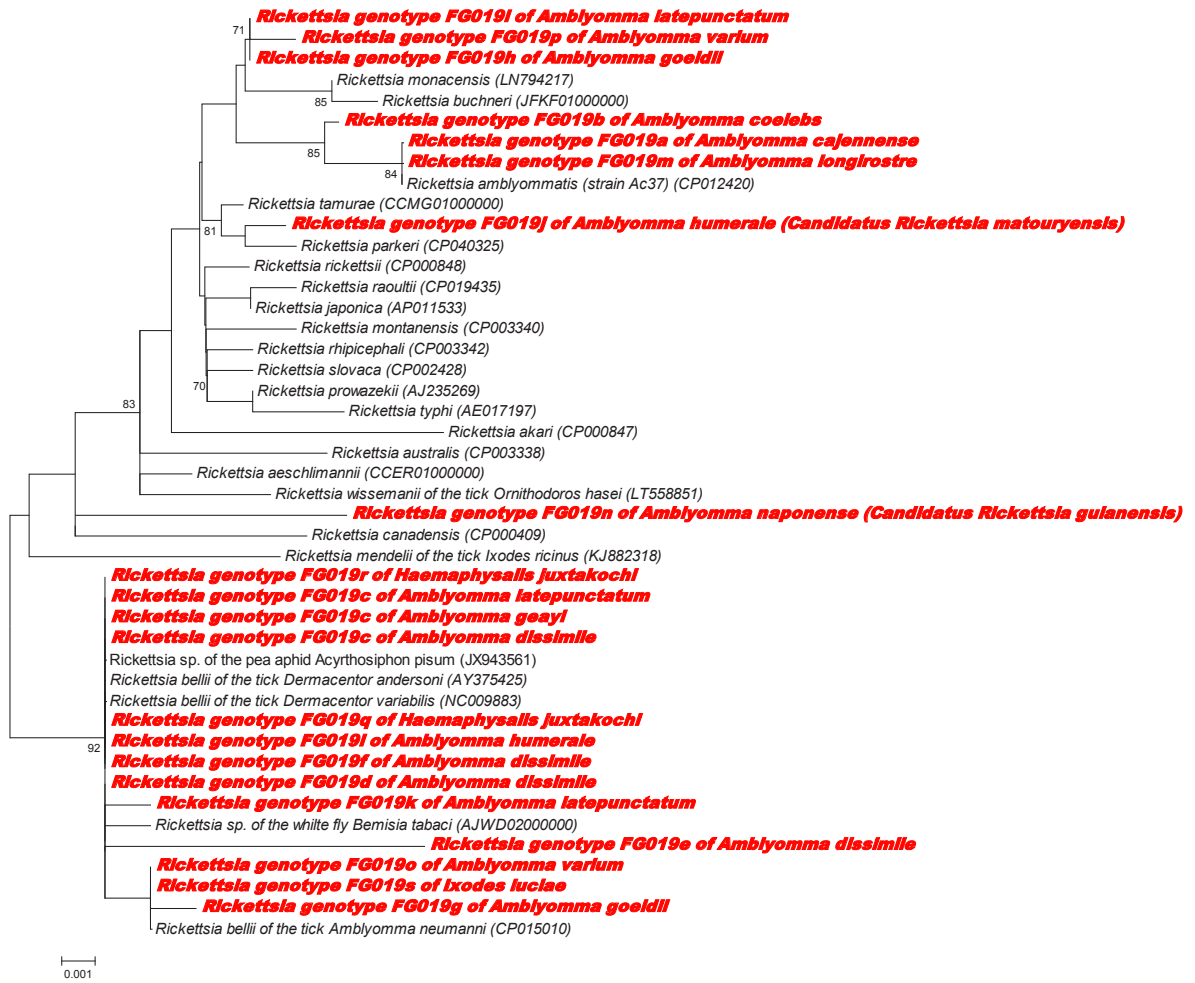
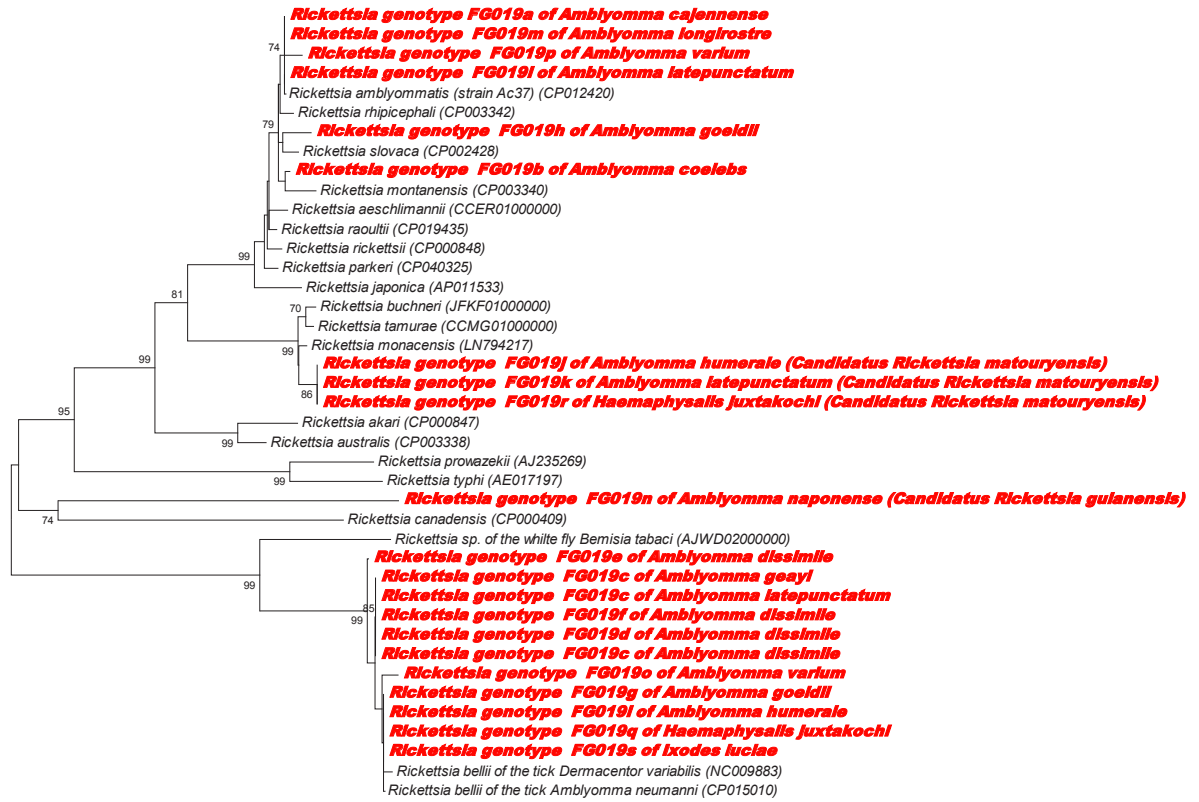


Figure S1



0.01

Figure S2

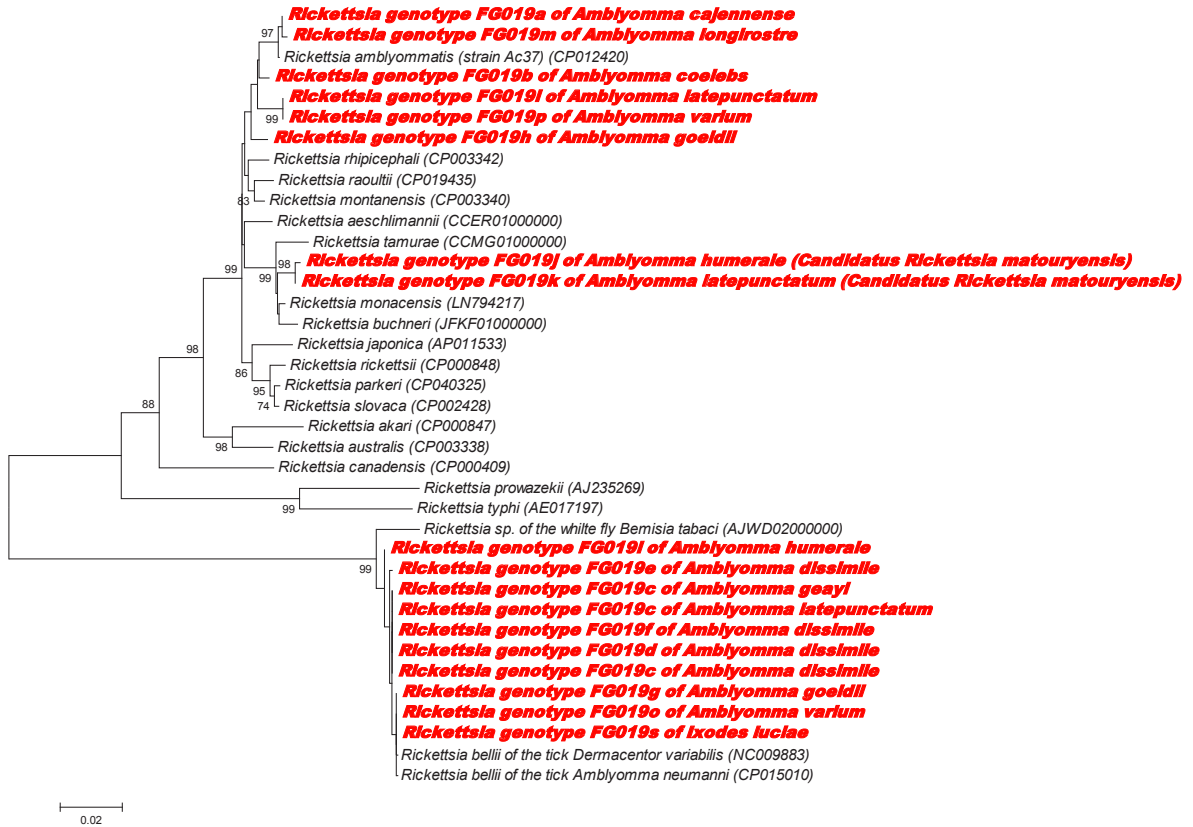


Figure S3



Figure S4

A novel *Borrelia* species, divergent to Lyme disease and relapsing fevers groups, in passerine-transported ticks of French Guiana

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Abstract

Lyme disease (LD) and relapsing fevers (RF) are vector-borne diseases caused by bacteria of the *Borrelia* genus. We report here on the widespread infection by a non-described *Borrelia* species in passerine-transported ticks in tropical rainforests of French Guiana, South America. This novel *Borrelia* species is common in two tick species, *Amblyomma longirostre* and *A. geayi*, which feed on a broad variety of neotropical mammal and bird species, including migratory species moving to North America. This novel *Borrelia* species is divergent to the LD and RF species, being more related to the reptile- and echidna-associated *Borrelia* group which was recently described. Genome sequencing showed that this novel *Borrelia* have a RF-like genome but with a unique mixture of LD- and RF-specific genes, including genes used by RF *Borrelia* for multiphasic antigen-switching system and a number of immune-reactive protein genes used for the LD diagnosis. Overall, this indicates that this novel *Borrelia* is an intermediate form to the LD and RF species which may impact a very large host spectrum. The designation ‘Candidatus *Borrelia mahuryensis*’ is proposed for this species.

Introduction

Bacteria of the genus *Borrelia* (Spirochaetes) are the causative agents of major vector-borne diseases including Lyme disease (LD), the most important tick-borne disease from the northern hemisphere, and relapsing fevers (RF) which are quite commonly transmitted by ticks and lice worldwide^{1,2}. While RF epidemics occurred repeatedly in past centuries², LD is nowadays one of the fastest-expanding infectious diseases with more than 300,000 new human cases each year^{3,4}. Most LD and RF *Borrelia* species are maintained in enzootic cycles and their presence in humans and domestic animals are mostly incidental to their usual wildlife reservoir hosts^{1,2,5}. These enzootic cycles, sometimes complex, make *Borrelia* difficult to track especially in wildlife in remote regions where their detection may present considerable challenges.

LD and RF species were usually thought to be the only two groups within *Borrelia*. The LD and RF *Borrelia* species form two separate sister groups^{6,7}, and are sometimes referred as two sister genera depending on authors^{8,9}. However, recent surveys are uncovering new *Borrelia* species and strains that do not fall into the LD and RF groups¹⁰⁻¹⁹. They form a third group, sometimes referred as the reptile clade, encompassing only two designated species, *B. turcica* and ‘*Candidatus Borrelia tachyglossi*’ (*B. tachyglossi* hereafter), and a few strains not taxonomically described¹⁰⁻¹⁹. The only two genomes sequenced to date confirmed that these *Borrelia* are substantially different to the LD and RF *Borrelia*¹⁹. Members of the third *Borrelia* group have been detected worldwide, but not in Western Europe^{10-14,17}. They have been detected in hard ticks (*Amblyomma*, *Hyalomma*, *Bothriochroton* and *Ixodes* species) which are associated mostly with reptiles, but also with a few mammals as echidna¹⁰⁻¹⁴. Recently, a few studies reported the presence of new *Borrelia* strains in ticks feeding on ground-dwelling birds (for immature

stages) and mammals (for adult stages) in Brazil¹⁶, Argentina¹⁷ and the USA^{15,17,18}. All these new American *Borrelia* strains are closely related each other and may belong to the third *Borrelia* group^{15,18}. However, we actually know little on these new American *Borrelia* strains: a small fragment of the flagelin *flaB* gene has been used as an exclusive marker for their description^{15,18}. However, this marker is inadequate for inferring a reliable *Borrelia* phylogeny since the inner topology is poorly resolved at many nodes because of insufficient sequence polymorphism on this short DNA fragment.

In this study, we described a novel species, belonging to the third *Borrelia* group, in passerine-transported ticks in tropical rainforests of French Guiana. This remote territory is a vast equatorial land located on the north-east coast of South America, mostly covered by dense rainforests and with a low people density. The novel *Borrelia* species we found there is remarkably common in two tick species feeding on a variety of mammal and bird species. We further sequenced its complete genomes and showed that this novel *Borrelia* is in-between LD and RF *Borrelia*, sharing common features with both of them.

Results

A novel *Borrelia* species in passerine-transported ticks. A total of 290 tick specimens were collected from 19 sites in French Guiana, mostly located along the coast line (Table 1 and Figure 1). Most of the specimens were larvae ($n=274$), with only few nymphs ($n=16$), and no adults (Table 1). The 290 ticks were collected on 26 bird species, mainly on the Blue-backed Manakin, *Chiroxiphia pareola* ($n=98$ ticks; 34%), the Wedge-billed Woodcreeper, *Glyphorynchus spirurus* ($n=75$; 26%) and the Crimson-hooded Manakin, *Pipra aureola* ($n=33$; 11%). Morphological and genetic examination of tick specimens led to the identification of six tick species, all belonging to the *Amblyomma*

genus: *A. longirostre* (n=230), *A. geayi* (n=52), *A. varium* (n=4), *A. cajennense* (n=2), *A. calcaratum* (n=1) and *A. humerale* (n=1). *A. longirostre* was found on all bird species but one, *A. geayi* was found on 11 bird species, and the four other *Amblyomma* species were found in only one or two bird species (Table 1).

Examination of the 290 *Amblyomma* spp. specimens through high-throughput 16S rDNA sequencing showed that *Borrelia* was present in 20 specimens (6.9%): infection was detected in 12 of the 230 *A. longirostre* specimens (5.2%) and eight of the 52 *A. geayi* (15.4%) specimens, but not in *A. varium*, *A. cajennense*, *A. calcaratum* and *A. humerale* (Table 1 and Figure 1). Infection was found mostly in larvae, but also in nymphs (Table 1). This *Borrelia* was detected in 6 sampling sites of the 19 examined, and in ticks collected on eight bird species: the Woodcreeper *G. spirurus* (three sites), the Crimson-hooded Manakin *P. aureola* (two sites), the Blue-backed Manakin *C. pareola* (one site), the Pale-breasted Thrush *Turdus leucomelas* (one site), the Silver-beaked Tanager *Ramphocelus carbo* (one site), the Straight-billed Woodcreeper *Dendroplex picus* (one site), the Black-headed Antbird *Pernostola rufifrons* and the White-throated Xenops *Xenops minutus* (Table 1). The prevalence of *Borrelia* in ticks vary from 0 to 28.5% depending on sites, but did not vary significantly between the localities (Fisher's exact test, $p=0.63$).

Specific PCR surveys on the *Borrelia* 16S rDNA (710 bp), *flaB* (540 bp), *gyrB* (1172 bp), *groEL* (600 bp) and *glpQ* (784 bp) genes further confirmed this infection pattern. On the basis of these five gene sequences, no nucleotide variation was observed in the *Borrelia* from the *A. longirostre* and *A. geayi* positive specimens, showing that only one *Borrelia* species was present in the study area. For each gene sequence, higher pairwise nucleotide identities were observed with members of the third *Borrelia* groups, but lower values with

members of the LD and RF groups. None of the 16S rDNA, *gyrB*, *groEL* and *glpQ* gene sequences observed in this study is 100% identical to other *Borrelia* sequences available on GenBank (Table S1). However, based on partial *flaB* gene sequences, this *Borrelia* strain is 100% identical to the *Borrelia* sp. clone 2T found in *A. longirostre* from Brazil¹⁶. A maximum-likelihood (ML) analysis based on the *flaB* gene sequences (323 bp) was further used to examine the phylogenetic relationship of these *Borrelia* with other *Borrelia* species available on GenBank. We found a robust clustering of the new *Borrelia* of French Guiana with the *Borrelia* sp. clone 2T of Brazil, along with several other *Borrelia* sp. isolates from *A. maculatum* of Texas¹⁸, suggesting that all these *Borrelia* belong to the same species (Figure 2). The ML phylogeny places this *Borrelia* cluster among members of the third *Borrelia* group, but the inner topology of the *flaB* tree remains too poorly resolved in most cases (as shown by low support values of inner branches) for inferring the exact relatedness of this *Borrelia* cluster with other *Borrelia* species and groups (Figure 2).

Cultivation of *Borrelia*. Five freshly-molted tick specimens were primarily used for cultivation of *Borrelia*. After eight weeks of cultivation, only one tick specimen has produced a viable *Borrelia* isolate (A-FGy1 hereafter). The *Borrelia* A-FGy1 isolate has a typical Spirochaetes helical shaped structure of ca. 1 μm wide and 10-25 μm long (Movie S1). The growth rate is however slow, and the yields of cultures are low. No sequence variation of the *flaB*, *gyrB*, *groEL* and *glpQ* genes was observed between the *Borrelia* A-FGy1 isolated in cultivation and the *Borrelia* typed from tick DNA described above, showing that they belong to the same species. There was however one single nucleotide polymorphism (SNP) at the 16S rDNA sequence, suggesting that two very closely related strains are present in this *Borrelia* species.

Genomic features of the *Borrelia* A-FGy1 isolate. [Please note that this part is still under analysis. Only partial and preliminary results are shown below]. The sequencing of the *Borrelia* A-FGy1 isolate led to the production of 10 contigs with a mean coverage of 300x. The final *Borrelia* A-FGy1 genome is >1Mb in size (30.8% mean GC content) and consists of a 918,483 bp linear chromosome with several plasmids. Phylogenetic analysis based on single copy orthologous genes present in the linear chromosome showed that the *Borrelia* A-FGy1 isolate clusters within a robust clade with the two known species of the third *Borrelia* group, *B. turcica* and *B. tachyglossi* (Figure 2). The closest relative of this novel *Borrelia* species is *B. turcica* which was primarily reported from reptiles and reptile-transported ticks. The third *Borrelia* group, including the *Borrelia* A-FGy1 isolate, clearly forms an independent *Borrelia* lineage which shares a common origin with LD and RF groups (Figure 2).

The *Borrelia* A-FGy1 chromosome shows an extensive synteny with chromosomes of other *Borrelia* species and, compared to LD, RF and third group *Borrelia* chromosomes, it exhibits a roughly similar size and organization (Figure 3). The single exception concerns a specific 8.5 kb inversion at the 5' end of the *B. turcica* chromosome¹⁹ but that is absent in *Borrelia* A-FGy1 chromosome as in other *Borrelia* chromosomes (Figures 4). The *Borrelia* A-FGy1 genome contains >900 predicted protein-coding genes, of which >100 are located on plasmids. There are a number of previously described genes encoding immune-reactive proteins. The *Borrelia* A-FGy1 chromosome also contain genes that are present in *B. turcica* and *B. tachyglossi* but not in LD or RF *Borrelia*, including >5 hypothetical proteins with unknown functions. The *Borrelia* A-FGy1 chromosome also contains a dozen unique orthologs with *B. tachyglossi*, *B. turcica* and RF *Borrelia* that

are absent in LD species. These include genes involved in important cellular functions such as DNA replication and repair (RecF, RecN, RecR, DinG-type ATP-dependent helicase), glycerophospholipid metabolism (glpT and glpQ), copper homeostasis (CutC) and transcriptional regulation (anti-sigma factor antagonist protein). Additionally, the *Borrelia* A-FGy1, as *B. tachyglossi* and *B. turcica*, harbors a conserved RF-like gene architecture of the rRNA operon, with only one copy each of the 23S rRNA and 5S rRNA genes (duplicated in LD *Borrelia*), and a horizontally acquired set of three purine salvaging pathway genes (purA, purB, and htp) that intersect the 23S rRNA and 16S rRNA open reading frames. Finally, the *Borrelia* A-FGy1 chromosome also contains genes found only in *B. tachyglossi*, *B. turcica* and LD chromosomes and that are absent from all RF species.

Two immunogenic alpha subfamily variable large proteins (vlp) were identified on *Borrelia* A-FGy1 plasmids, which were also found on *B. tachyglossi*, but not on *B. turcica*, plasmids. Several vlp delta subfamily (vlpD) genes were identified on *Borrelia* A-FGy1 plasmids, as well as at least one variable small protein (vsp1).

Proposal of candidate name. On account of those distinct and coherent microbiological, phylogenetic and evolutionary traits described above, we propose the designation ‘Candidatus *Borrelia mahuryensis*’ for this novel bacterium associated with the passerine-transported ticks *A. longirostre* and *A. geayi*. The specific name refers to the Mount Mahury, French Guiana, which was the first sampling site where we detected the presence of this bacterium.

Discussion

We found here that a novel *Borrelia* species, *Cand. Borrelia mahuryensis*, divergent to the LD and RF *Borrelia* species, is common in passerine-transported ticks in tropical rainforests of French Guiana. We obtained a pure culture isolate of *Cand. Borrelia mahuryensis* (A-FGy1 isolate), with morphology similar to those of other *Borrelia* species. This novel *Borrelia* species is more related, although distinct, to the two known species of the third *Borrelia* group, *B. turcica* and *B. tachyglossi*. At the five loci extensively examined, only one SNP was observed between all our *Cand. Borrelia mahuryensis* DNA samples, showing that at least two very closely related strains are circulating in French Guiana. The recent detection of *Borrelia* isolates in Brazil¹⁷ sharing the same *flaB* sequence with *Cand. Borrelia mahuryensis*, along with the presence of closely related strains in Texas¹⁸, suggest that *Cand. Borrelia mahuryensis* have actually a broad geographic distribution across American countries with a substantial intraspecific variation. Finally, examination of the *Cand. Borrelia mahuryensis* A-FGy1 genome confirmed its difference with other *Borrelia* species, but revealed the presence of shared features with either LD or RF or both *Borrelia* species.

The repeated detection of *Cand. Borrelia mahuryensis* from 2012 to 2018 confirms that infection durably persists in French Guiana through its circulation in at least two tick species, *A. longirostre* and *A. geayi*. Infected tick specimens were collected from 26 passerine species, suggesting that these vertebrates can be reservoir hosts for *Cand. Borrelia mahuryensis*. Interestingly, the related *Borrelia* isolates detected in Brazil were also detected in *A. longirostre*¹⁶ collected from ground-dwelling birds and those detected in the USA were detected in *A. maculatum*^{15,18}, a species also using birds as hosts^{20,21}. All these evidences support the hypothesis of birds as reservoir hosts for *Cand. Borrelia mahuryensis*. However, *A. longirostre*, *A. geayi* and *A. maculatum* are also known to be

associated with a variety of vertebrate species with immature stages feeding usually on passerine birds and adults feeding rather on arboreal mammals as porcupines and sloths (for *A. longirostre* and *A. geayi*)^{22,23} or large mammals as cattle (for *A. maculatum*)^{20,21}. Natural hosts of *Cand. Borrelia mahuryensis* can thus be either passerine birds, or mammals or both. The observation that tick larvae are commonly infected suggests the existence of two non-exclusive scenarios: (i) passerine birds are the hosts with tick larvae acquiring *Borrelia* through feeding on infected passerine birds; (ii) mammals are the hosts with tick females acquiring *Borrelia* through feeding on infected mammals and further transmitting transovarially infection to their larvae. Transovarial transmission, however, is not a common feature in the genus since it has been only demonstrated for RF, but rarely for LD, *Borrelia* species^{24–27}. Interestingly, the isolation of *Cand. Borrelia mahuryensis* in culture from a freshly-molted tick demonstrates that infection is maintained through moulting to subsequent developmental stages in tick host. Through this transstadial transmission, infected ticks have the potential to infect all animals on which they further feed. A variety of mammals can be then exposed to *Cand. Borrelia mahuryensis*, including humans on which *A. longirostre* and *A. maculatum* can occasionally feed^{20,21,28,29}. Furthermore, while both *A. longirostre* and *A. geayi* are native to South and Central America^{22,30–32}, migratory birds also regularly introduced *A. longirostre* to North America^{30,33–37}. Bird migration (for *A. longirostre*, *A. geayi* and *A. maculatum*) and livestock transportation (for *A. maculatum*) may be two important factors affecting the distribution of *Cand. Borrelia mahuryensis* over long distances and across geographical barriers such as oceans and deserts, as observed with other tick-borne bacteria³³.

The genome of *Cand. Borrelia mahuryensis* is more similar to those of the *Borrelia* third group, *B. tachyglossi* and *B. turcica*, which were also recently sequenced¹⁹. Members of

these groups shared almost the same gene content, including a dozen of specific genes and diagnostic insertion within the rRNA operon not present in LD or RF *Borrelia*. When compared with LD or RF *Borrelia* species, the genome of *Cand. Borrelia mahuryensis* show more similarities with RF *Borrelia* species, with a linear chromosome and several linear and circular plasmids, and harbour genes not found in LD *Borrelia*. It however also harbours LD-specific genes, showing that *Cand. Borrelia mahuryensis* exhibits intermediate features between the RF and LD groups, as also observed in *B. tachyglossi* and *B. turcica*¹⁹. Their genomes encode several immunogenic vlpD and vlp proteins (used in the multiphasic antigen variation system of RF *Borrelia* to evade detection by the host immune system³⁸) as well as a number of immune-reactive proteins, including flaB, BmpA, outer membrane protein OspC homolog, p66, and p58, which are all used for the diagnosis of LD³⁹. Altogether, these genomic features suggests that *Cand. Borrelia mahuryensis* and members of the third *Borrelia* group form a continuum of *Borrelia* species between LD and RF groups, affecting our ability to clearly distinguish between these two opposite groups.

To conclude, the description of *Cand. Borrelia mahuryensis* reveals that the third *Borrelia* group is both widespread and biologically diverse. Most of the previously members of this group were found in association with reptile hosts^{10,13,14}, with the exception of one echidna host^{11,12}. However, the description of *Cand. Borrelia mahuryensis* now suggests that members of the third *Borrelia* group have a large host spectrum which may also include a variety of birds and mammals as reservoirs. Noting the genomics features of members of *Cand. Borrelia mahuryensis*, *B. turcica* and *B. tachyglossi*, it is perhaps not unexpected to find other members of the third *Borrelia* group infecting a wide diversity of vertebrates. They have RF-like genomes with megaplasmids, several housekeeping

and macronutrient metabolism genes only present in RF *Borrelia* species. Paralogous vlp and vsp proteins playing key roles in the RF multiphasic antigenic variation system and pathogenicity are also conserved, but *Cand. Borrelia mahuryensis*, *B. turcica* and *B. tachyglossi* also have LD-specific orthologs. Overall, it underlines how members of the third *Borrelia* group are intermediate to the LD and RF species, and represents currently understudied components of Spirochaetes biology. Additional studies are now needed to determine what their transmission cycles and to establish whether these bacteria are pathogenic for humans.

Material and methods

Study area and tick sampling. Birds were captured using mist nets during the dry season (2012-2018) at 19 sites within forest patches (Table 1 and Figure 1). Before bird releases, ticks were collected and stored in 75% ethanol until examination. All ticks were morphologically identified to species using morphological and genetic diagnosis criteria²³.

Molecular screening and typing. Tick whole body DNA was extracted using an extraction kit according to the manufacturer's instructions (Qiagen). The presence of *Borrelia* was further examined through DNA barcoding involving the production of PCR amplicons from a 251-bp portion of the V4 variable region of the bacterial 16S rDNA using a Multiplex PCR Kit (Qiagen) and universal primers (16SV4F: 5'-GTGCCAGCMGCCGCGGTAA-3' and 16SV4R: 5'-GGACTACHVGGGTWTCTAATCC-3')⁴⁰ as previously described⁴¹. Amplified bacterial 16S rDNA products were purified and sequenced by an Illumina MiSeq platform (GenSeq, Montpellier University) and 250-bp end sequence reads were obtained. All

bioinformatic analyzes were conducted using the pipeline Frogs⁴² as previously described⁴¹. Sequences with 97% similarity were clustered together and identified as an operational taxonomic unit (OTU). Each representative OTU sequence was aligned and taxonomically assigned using the Silva database (<https://www.arb-silva.de/>).

Independent PCR assays for *Borrelia* species identification were performed through the amplification of *flaB*, *gyrB*, *groEL*, *glpQ* and 16S rRNA gene fragments using specific primers (Table S1). All PCR products were visualized through electrophoresis in a 1.5% agarose gel. Positive PCR products were purified and sequenced in both directions (Eurofins). Sequence chromatograms were manually cleaned with CHROMAS LITE (http://www.technelysium.com.au/chromas_lite.html), and alignments were performed using CLUSTALW⁴³, implemented in the MEGA V7 software⁴⁴.

***Borrelia* cultivation.** Engorged tick specimens were brought alive to the laboratory for blood meal digestion and molting. Freshly-molted ticks were further surface-sterilized with a bleach solution before to be individually crushed and then prepared for cultivation of *Borrelia*. For each tick sample, ca. 5 mm³ of crush was incubated at 34°C in BSK-II medium⁴⁵ and, after several weeks, examined under dark field microscopy.

Genome sequencing and analyses. One *Borrelia*-positive culture was used to prepare DNA-seq libraries using the Illumina Nextera DNA Flex sample preparation kit. Library validity was assessed by quantification using a Fragment Analyzer and a Qubit (Invitrogen). DNA-seq experiments have been performed on Illumina Miniseq by a platform (MGX, Montpellier) using Illumina MiniSeq Mid Output Reagent Cartridge with paired-end read length of 2x150 bp. A total of 6Gb data was obtained. The quality

of Illumina reads was analyzed by FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and reads were further cleaned and trimmed using Trimmomatic⁴⁶. The remaining reads were then assembled into contigs then scaffolds with SPAdes v3.8 assembler⁴⁷. The annotation was performed by the NCBI prokaryotic genome annotation pipeline⁴⁸.

Molecular phylogenetic analyses. For analyses of single gene sequences, the GBLOCKS program⁴⁹ with default parameters was used to remove poorly aligned positions and to obtain nonambiguous sequence alignments. Closely related organisms obtained from GenBank were also included in the analyses. The evolutionary models that best fit the sequence data were determined using the Akaike information criterion with the program MEGA 7⁴⁴. Tree-based phylogenetic analyses were performed using maximum-likelihood (ML) analyses. ML heuristic searches using a starting tree obtained by neighbor joining was conducted in MEGA 7⁴⁴. Clade robustness was assessed by bootstrap analysis using 2,000 replicates.

Ethics approval. All animals were handled in strict accordance with good animal practice as defined by the French code of practice for the care and use of animals for scientific purposes, established by articles R214-87 to R214-137 of the French rural code. All captures were performed by competent people without causing avoidable pain, suffering, distress or lasting harm to birds. The use of the genetic resources was declared to the French Ministry of the Environment (reference TREL1902817S/156), in compliance with the Access and Benefit Sharing procedure implemented by the Loi pour la Reconquête de la Biodiversité.

Data availability

The genome of *Cand. Borrelia mahuryensis* A-FGy1 has been deposited at DDBJ/ENA/GenBank under the accession [pending].

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Author contribution

F.B. and O.D. designed the study and wrote the manuscript. F.B., S.G. and O.D. collected the samples. F.B., M.B. and R.B. performed the molecular typing. F.B., E.L. and O.D. performed the phylogenetic and genomic analyses. All authors agreed to the final version of the manuscript.

Additional information

Supplementary information accompanies this paper.

Competing interests

The authors declare that they have no competing interests.

Table 1. List of tick species and populations included in the analysis, with details on their origin, the population sample size (*n*), and the prevalence of *Cand. Borrelia mahuryensis*.

Tick species	Locality (# on Figure 1)	Locality name	Ecological habitats	Hosts	Tick stage	<i>n</i> ticks examined	<i>n</i> <i>Borrelia</i> -positive ticks (%) / hosts on which <i>Borrelia</i> -positive ticks were collected (<i>n</i>)
1- <i>Amblyomma longirostre</i> (Koch, 1844)	#1	A - Montsinéry, Bagne des Annamites, 2014	Degraded forest	McConnell's flycatcher (<i>Mionectes macconnelli</i>)	Larvae	2	0
	#2	B - Macouria, lotissement Maillard, 2012	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>) and Blue-backed Manakin (<i>Chiroxiphia pareola</i>)	Larvae	18	2 (11%) / Woodcreeper (2)
	#3	C - Macouria, lotissement Maillard, 2013	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>)	Larvae	3	0
	#4	D - Macouria, lotissement Maillard, 2014	Degraded forest	Blue-backed Manakin (<i>Chiroxiphia pareola</i>) and Crimson-hooded Manakin (<i>Pipra aureola</i>)	Larvae	6	0
	#5	E - Kourou, Montagne des Pères, 2014,	Old-growth forests	Blue-backed Manakin (<i>Chiroxiphia pareola</i>) and Crimson-hooded Manakin (<i>Pipra aureola</i>)	Larvae and Nymph	12	0
	#6	F - Kourou, Montagne des Singes, 2014	Old-growth forests	Woodcreeper (<i>Glyphoryncus spirurus</i>), White-bearded Manakin (<i>Manacus manacus</i>), White-flanked Antwren (<i>Myrmotherula axillaris</i>) and Golden-headed Manakin (<i>Pipra erythrocephala</i>)	Larvae and Nymph	5	0
	#7	G - Rémire-Montjoly, Sentier du Rorota, 2014	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>), Blue-backed Manakin (<i>Chiroxiphia pareola</i>) and Crimson-hooded Manakin (<i>Pipra aureola</i>)	Larvae and Nymph	29	1 (5.3%) / Crimson-hooded Manakin (1)
	#8	H - Rémire-Montjoly, Mont Mahury, 2016	Degraded forest	Crimson-hooded Manakin (<i>Pipra aureola</i>), Barred Antshrike (<i>Thamnophilus doliatus</i>), Buff-throated Saltator (<i>Saltator maximus</i>), Silver-beaked Tanager (<i>Ramphocelus carbo</i>), Yellow-olive Flatbill (<i>Tolmomyias sulphurens</i>), Straight-billed Woodcreeper (<i>Dendroplex picus</i>) and Golden-spangled Piculet (<i>Picumnus exilis</i>)	Larvae	20	3 (15%) / Silver-beaked Tanager (1) and Straight-billed Woodcreeper (2)
	#9	I - Matoury, Mont Paramana, 2014	Degraded forest	Crimson-hooded Manakin (<i>Pipra aureola</i>) and White-bearded Manakin (<i>Manacus manacus</i>)	Larvae and Nymph	13	0

#10	J - Saint-Laurent-du-Maroni, Piste Voltaire, 2013	Old-growth forests	Grey-breasted Sabrewing (<i>Campylopterus largipennis</i>)	Larva	1	0
#11	K - Matoury, PK7, 2012	Peri-urban	White-bearded Manakin (<i>Manacus manacus</i>)	Larva	1	0
#12	L - Montsinéry, Chemin de Risquetout, 2014	Old-growth forests	McConnell's flycatcher (<i>Mionectes macconnelli</i>) and White-necked Thrush (<i>Turdus albicollis</i>)	Larvae and Nymph	2	0
#13	M - Saint-Laurent-du-Maroni, Saint-Jean, 2013	Degraded forest	McConnell's flycatcher (<i>Mionectes macconnelli</i>) and Woodcreeper (<i>Glyphoryncus spirurus</i>)	Larvae	3	0
#14	N - Rémire-Montjoly, Vidal, 2013	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>), Blue-backed Manakin (<i>Chiroxiphia pareola</i>) and Crimson-hooded Manakin (<i>Pipra aureola</i>)	Larvae and Nymph	19	2 (10.5%) / Woodcreeper (1) and Crimson-hooded Manakin (1)
#15	O - Rémire-Montjoly, Sentier du Rorota, 2018	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>), Crimson-hooded Manakin (<i>Pipra aureola</i>), Blue-backed Manakin (<i>Chiroxiphia pareola</i>), Northern Slaty Antshrike (<i>Thamnophilus punctatus</i>), Ochre-bellied Flycatcher (<i>Mionectes oleagineus</i>) and Pale-breasted Thrush (<i>Turdus leucomelas</i>)	Larvae and Nymph	60	3 (5%) / Blue-backed Manakin (1) and Pale-breasted Thrush (2)
#16	P- Matoury, Mont Grand Matoury, 2018	Old-growth forests	Woodcreeper (<i>Glyphoryncus spirurus</i>), Blue-backed Manakin (<i>Chiroxiphia pareola</i>), Cocoa Thrush (<i>Turdus fumigatus</i>), Ferruginous-backed Antbird (<i>Myrmoderus ferrugineus</i>), Chestnut-rumped Woodcreeper (<i>Xiphorhynchus pardalotus</i>) and Cinereous Antshrike (<i>Thamnomanes caesius</i>)	Larvae	18	0
#17	Q - Rémire-Montjoly, Mont Mahury, 2018	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>) and Black-headed Antbird (<i>Percnostola rufifrons</i>)	Larvae and Nymph	8	0
#18	R- Tonate-Macouria, Matiti, 2018	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>), Black-headed Antbird (<i>Percnostola rufifrons</i>) and White-throated Xenops (<i>Xenops minutus</i>)	Larvae	6	2 (33.3%) / Black-headed Antbird (1) and White-throated Xenops (1)
#19	S- Saint-Georges-de-l'Oyapock, Îlet Galion, 2018	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>), Black-headed Antbird (<i>Percnostola rufifrons</i>) and Blue-black Grosbeak (<i>Cyanoloxia cyanooides</i>)	Larvae and Nymph	4	0
#7	A - Rémire-Montjoly, Sentier du Rorota, 2014	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>) and Crimson-hooded Manakin (<i>Pipra aureola</i>)	Larvae	3	2 (66.7%) / Crimson-hooded Manakin (2)

2- *A. geayi* Neumann, 1899

	#8	B - Rémire-Montjoly, Mont Mahury, 2016	Degraded forest	Barred Antshrike (<i>Thamnophilus doliatus</i>) and Crimson-hooded Manakin (<i>Pipra aureola</i>)	Larvae	4	0
	#9	C - Matoury, Mont Paramana, 2014	Degraded forest	White-bearded Manakin (<i>Manacus manacus</i>)	Larva	1	0
	#10	D - Saint-Laurent-du-Maroni, Piste Voltaire, 2013	Old-growth forests	White-bearded Manakin (<i>Manacus manacus</i>) and White-necked Thrush (<i>Turdus albicollis</i>)	Larvae	2	0
	#15	E - Rémire-Montjoly, Sentier du Rorota, 2018	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>), Blue-backed Manakin (<i>Chiroxiphia pareola</i>), Chestnut-rumped Woodcreeper (<i>Xiphorhynchus pardalotus</i>) and Pale-breasted Thrush (<i>Turdus leucomelas</i>)	Larvae	30	6 (20%) / Woodcreeper (1), Blue-backed Manakin (1) and Pale-breasted Thrush (4)
	#16	F - Matoury, Mont Grand Matoury, 2018	Old-growth forests	Woodcreeper (<i>Glyphoryncus spirurus</i>), Bright-rumped Attila (<i>Attila spadiceus</i>), Black-headed Antbird (<i>Pernostola rufifrons</i>), White-bearded Manakin (<i>Manacus manacus</i>), Chestnut-rumped Woodcreeper (<i>Xiphorhynchus pardalotus</i>) and Cinereous Antshrike (<i>Thamnomanes caesius</i>)	Larvae	12	0
3- <i>A. varium</i> Koch, 1844	#4	A - Macouria, lotissement Maillard, 2014	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>)	Nymph	1	0
	#15	B - Rémire-Montjoly, Sentier du Rorota, 2018	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>)	Larva	1	0
	#16	C - Matoury, Mont Grand Matoury, 2018	Old-growth forests	Woodcreeper (<i>Glyphoryncus spirurus</i>)	Larva	1	0
	#18	D - Tonate-Macouria, Matiti, 2018	Degraded forest	Woodcreeper (<i>Glyphoryncus spirurus</i>)	Larva	1	0
4- <i>A. cajennense</i> sensus stricto (Fabricius, 1787)	#15	A - Rémire-Montjoly, Sentier du Rorota, 2018	Degraded forest	Pale-breasted Thrush (<i>Turdus leucomelas</i>) and Blue-backed Manakin (<i>Chiroxiphia pareola</i>)	Larva and Nymph	2	0
5- <i>A. calcaratum</i> Neumann, 1899	#6	A - Kourou, Montagne des Singes, 2014	Old-growth forests	White-bearded Manakin (<i>Manacus manacus</i>)	Larva	1	0
6- <i>A. humerale</i> Koch, 1844	#5	A - Kourou, Montagne des Pères, 2014,	Old-growth forests	White-flanked Antwren (<i>Myrmotherula axillaris</i>)	Nymph	1	0

Figure legends

Figure 1. Location of sampling sites in French Guiana. Localities are represented by dots and numbers correspond to the population number given in Table 1. Figure 1B is the magnification of the area bounded by the translucent grey rectangle in Figure 1A. Red and black dots indicate populations infected by *Cand. Borrelia mahuryensis* or not infected, respectively.

Figure 2. Phylogenic relationship of *Cand. Borrelia mahuryensis* A-FGy1 with other *Borrelia*. The phylogenetic tree was inferred using maximum likelihood from a 323 bp sequence of the *flab* gene. The numbers on each node represent the support of 1,000 bootstrap replicates (only values >70% are shown).

Figure 3. Phylogenetic relationship of XX *Borrelia* genomes, including the *Cand. Borrelia mahuryensis* A-FGy1 genome. The phylogenetic tree was inferred using maximum likelihood from a concatenated alignment of XXX single-copy orthologous genes (XXX,XXX amino acids). The numbers on each node represent the support of 1,000 bootstrap replicates.

Figure 4. Synteny of *Borrelia* genomes. Alignment between *Cand. Borrelia mahuryensis* A-FGy1, *B. turcica* (GenBank accession number: CP028884), *B. tachyglossi* (CP025785), *B. miyamotoi* (CP006647) and *B. burgdorferi* (AE000783) chromosomes shows high conservation of global gene order with only one genomic rearrangements at the end of the *B. turcica* chromosome (arrow). Each contiguously colored locally collinear block (LCB) represents a region without rearrangement of the homologous backbone sequence estimated with the Mauve aligner. LCBs below the center in *Borrelia*

genomes represent blocks in the reverse orientation. The height of color bars within LCBs corresponds to the average level of similarity (conservation) profile in that region of the genome sequence.

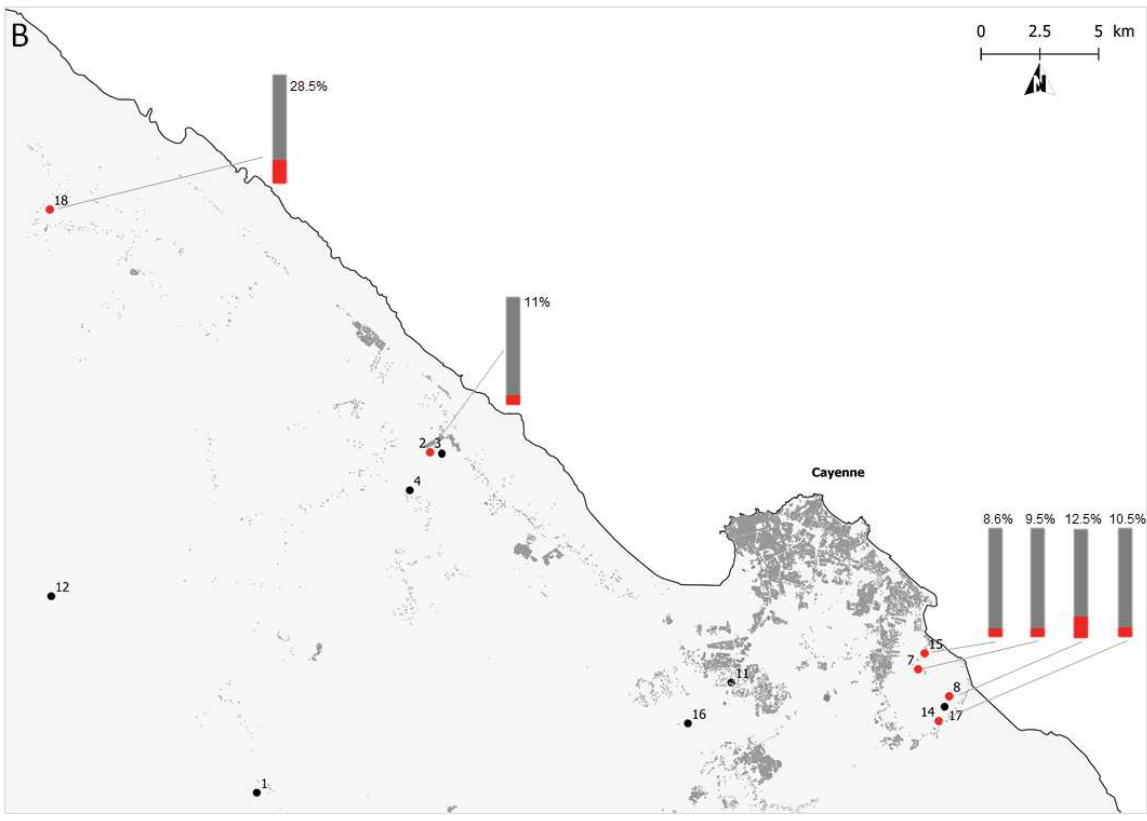
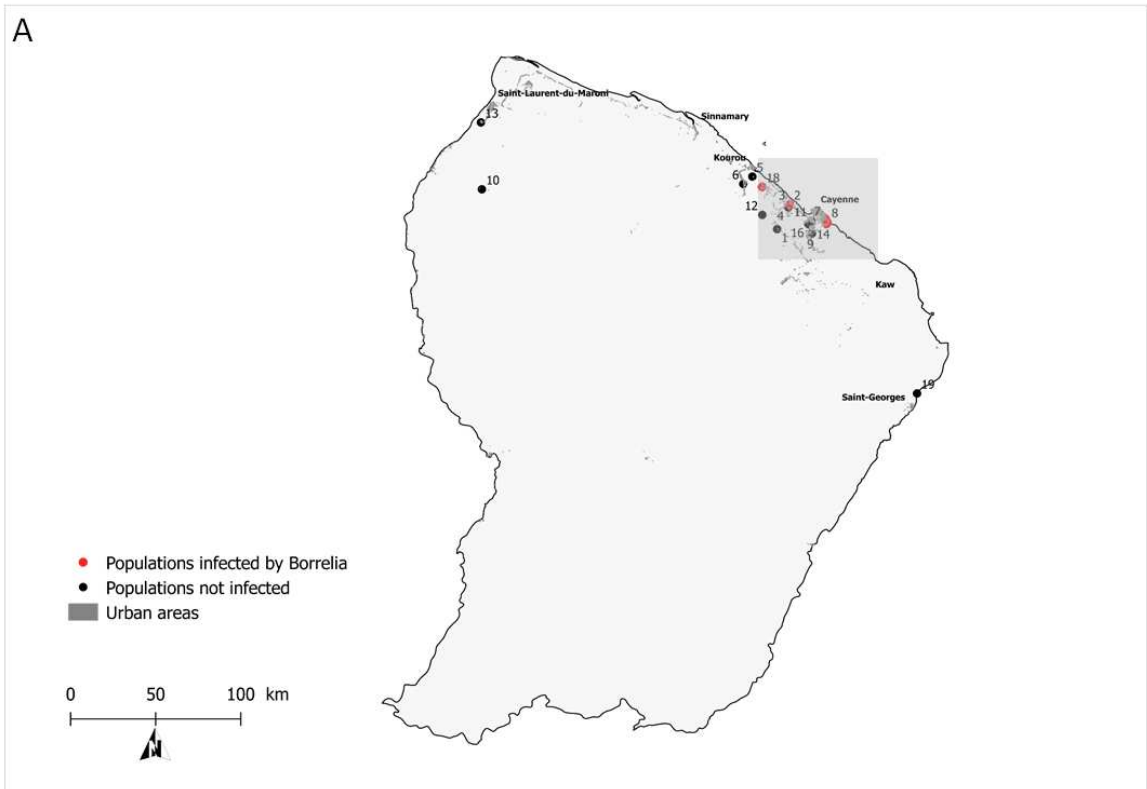


Figure 1.

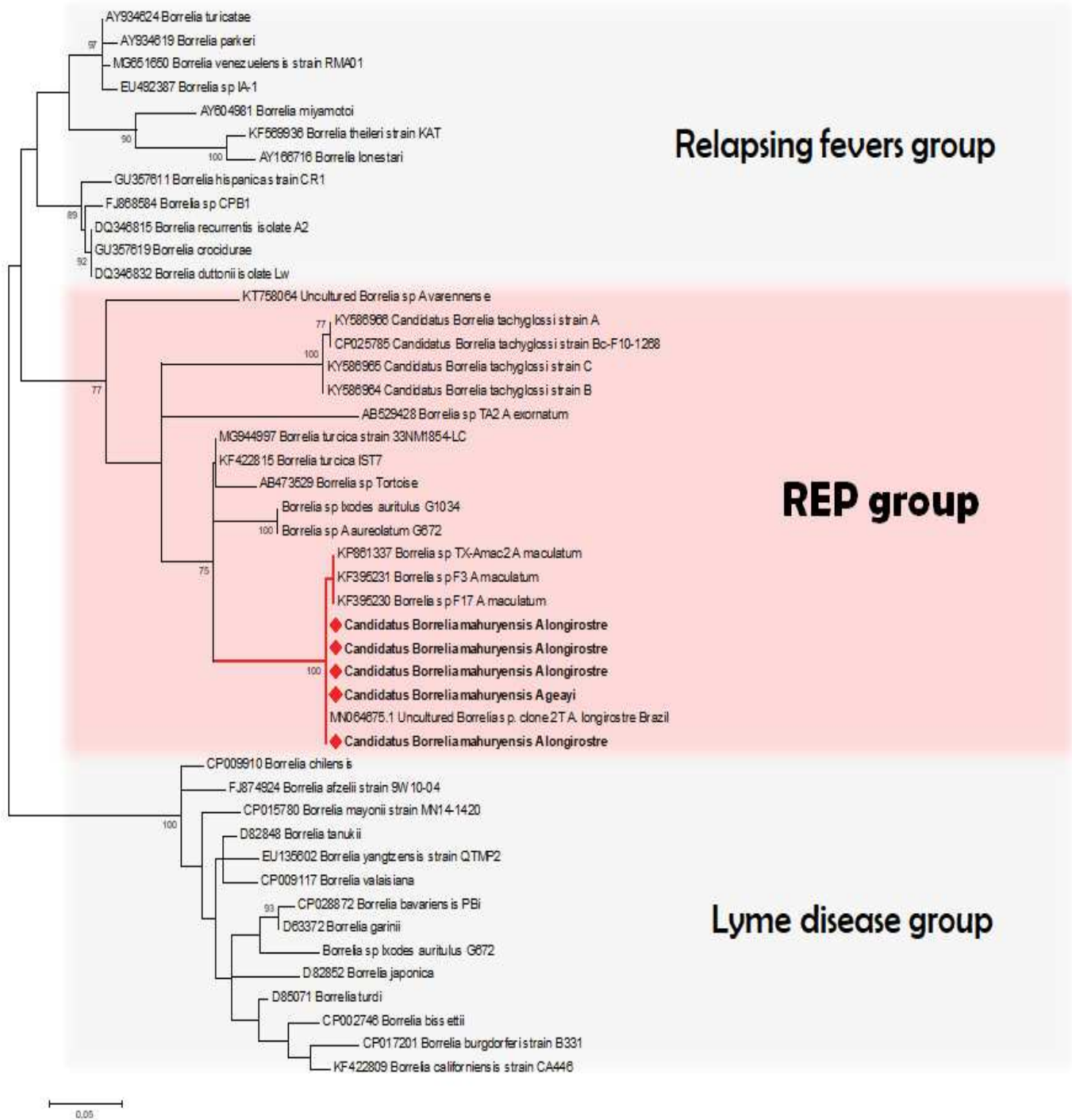


Figure 2.

Figure 3. Pending

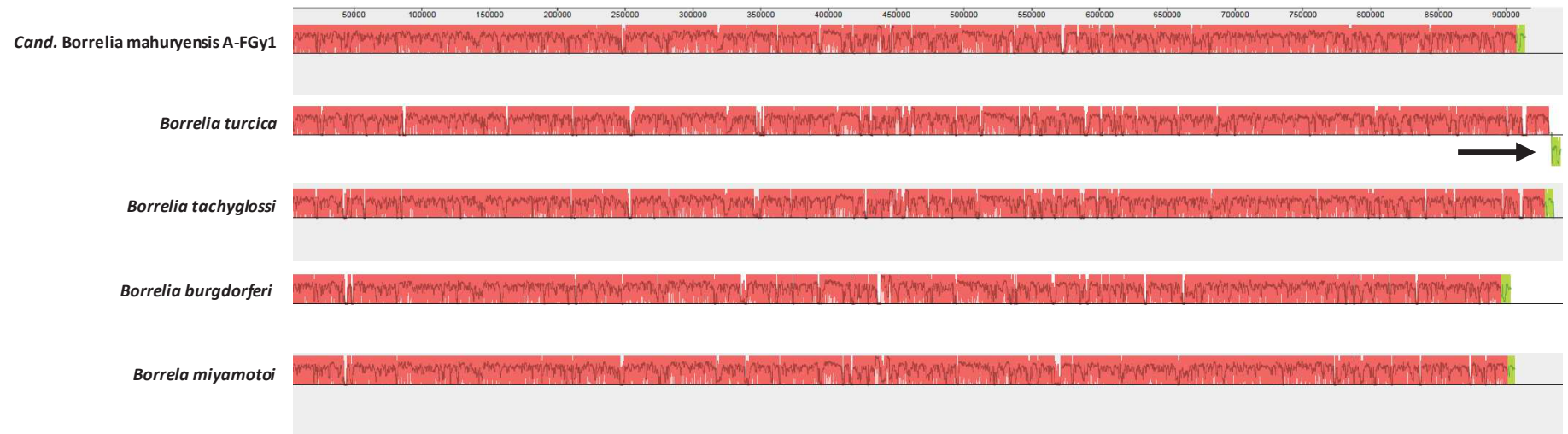


Figure 4.

SUPPLEMENTARY MATERIALS

Table S1. Sequence similarity of the 16S rDNA, *flaB*, *gyrB*, *groEL* and *glpQ* gene sequences of *Cand. Borrelia mahuryensis* to representative sequences of representative *Borrelia* species available in GenBank. The list is not exhaustive and only include the threeof best BLAST matches.

<i>Borrelia</i> genes	Highest similar sequences available on GenBank (% identity)
<i>16S rRNA</i> (710bp)	<i>B. turcica</i> IST7, CP028884 (98.17%); Uncultured <i>Borrelia</i> sp. clone P12, MH628250 (98.17%); <i>Borrelia</i> sp. tAG158M, AB529425 (98.17%)
<i>flaB</i> (540bp)	Uncultured <i>Borrelia</i> sp. clone 2T; MN064675 (100%); <i>B. turcica</i> IST7, CP028884 (93.22%); <i>B. turcica</i> 33NM1854-LC, MG944997 (93.01%)
<i>glpQ</i> (784bp)	<i>B. turcica</i> KZ11, KP067812 (92.32%); <i>B. turcica</i> KZ14, KP067814 (92.18%); <i>B. turcica</i> KZ112, KP067807 (92.18%)
<i>groEL</i> (600bp)	<i>B. turcica</i> IST7, CP028884 (93.12%); <i>B. miyamotoi</i> CA17-2241, CP021872 (88.35%); <i>B. miyamotoi</i> Yekat, CP037471 (87.88%)
<i>gyrB</i> (1172bp)	<i>B. sp.</i> Tortoise14H1; AB473537.1 (90.98%); <i>B. turcica</i> IST7, CP028884 (89.91%); <i>Borrelia</i> sp. tAG66M, AB529349 (89.82%)

Table S2. Genes and primers used for *Borrelia* screening and multilocus typing.

Genes	Hypothetical product	Primers (5'-3')	Tm	Fragment size	References
<i>16S rRNA</i>	Small ribosomal subunit	Borr16SF1- Borr16SR1- Borr16SR2-	54°C	Semi-nested PCR assay: 1st round PCR: Borr16SF1/Borr16SR2: 1506bp 2nd round PCR: Borr16SF1/Borr16SR1: 757bp	This study
<i>gyrB</i>	DNA gyrase subunit B	BorrgyrBF1- BorrgyrBR1- BorrgyrBR2-	48°C	Semi-nested PCR assay: 1st round PCR: BorrgyrBF1/BorrgyrBR2: 1208bp 2nd round PCR: BorrgyrBF1/BorrgyrBR1: 1172bp	This study
<i>glpQ</i>	Glycerophosphodiester phosphodiesterase	BorrglpQF1- BorrglpQR1- BorrglpQF2-	48°C	Semi-nested PCR assay: 1st round PCR: BorrglpQF1/BorrglpQR1: 808bp 2nd round PCR: BorrglpQF2/BorrglpQR1: 784bp	This study
<i>groEL</i>	Chaperone protein GROEL	BorrgroELF1- BorrgroELR2- BorrgroELR1-	50°C	Semi-nested PCR assay: 1st round PCR: RatpAF1/RatpAR2: 624bp 2nd round PCR: RatpAF2/RatpAR2: 600bp	This study
<i>flaB</i>	Flagellin B	BorrflaBF1- BorrflaBR2- BorrflaBR1-	54°C	Nested PCR assay: 1st round PCR: BorrflaBF1/BorrflaBR2: 545bp 2nd round PCR: BorrflaBF1/BorrflaBR1: 518bp	This study

Movie S1. Microscopy movie of the culture of *Cand. Borrelia mahuryensis* A-F (not available for this thesis)

Discussion

L'étude du risque acarologique en Guyane est encore loin d'être complétée, il est ainsi impossible de statuer d'une manière définitive. Néanmoins, pour les deux cas présentés dans les articles il apparaît modéré, tout du moins pour l'humain. En effet, dans le cas de la nouvelle souche de *Borrelia*, cette dernière semble pour l'instant se cantonner à un cycle arboricole, se retrouvant uniquement chez des tiques peu anthropophiles avec un spectre d'hôtes spécialisé sur des animaux arboricoles (passereaux, porc-épic arboricole ou encore paresseux). Ces caractéristiques semblent induire un risque de zoonose humaine due à cette *Borrelia*, relativement faible, mais toutefois possible. En ce qui concerne les *Rickettsia*, il est difficile d'émettre des hypothèses sur leurs risques infectieux pour les raisons avancées dans l'article correspondant.

Comme nous l'avons évoqué précédemment, la Guyane est sujette à une épidémie de fièvre Q et à une potentielle présence du syndrome de Baggio–Yoshinari. Il est alors intéressant de noter qu'aucune des bactéries associées à ces pathologies (*Coxiella burnetii* et *B. burgdorferi*, respectivement) n'a été mise en évidence dans notre collection. Pourtant notre échantillonnage comprend les espèces supposées vectrices de ces maladies en Guyane. Ainsi, si ces pathologies sont effectivement transmises par les tiques (bien que nos études suggèrent l'inverse), il existe une très faible prévalence des agents pathogènes associés chez les tiques de Guyane et/ou une transmission extrêmement localisée. Le risque de contracter ces maladies sur le territoire guyanais suite à la piqûre d'une tique, apparaît ainsi quasi-nul. Dans tous les cas, pour clairement définir un risque infectieux, il est également nécessaire de rechercher la présence d'agents pathogènes transmis par les tiques chez leurs hôtes vertébrés. Cette partie de l'étude est en cours sur une vaste

collection d'extraits sanguins et d'organes de différents vertébrés, obtenue grâce à la collaboration avec différents acteurs du LabEx CEBA. Cette collection est en majorité composée de matériel biologique de passereaux, d'opossums et de paresseux. La recherche par exemple de *C. burnetii* chez les paresseux permettra ainsi de clairement statuer sur leur hypothétique rôle de réservoir de la fièvre Q en Guyane.

Enfin, au-delà des parasites procaryotes ou eucaryotes, les tiques peuvent également transmettre une grande variété de virus (Mansfield, Jizhou, Phipps, & Johnson, 2017). L'étude des virus transmissibles par une piqûre de tique est encore à un stade peu avancé, néanmoins de plus en plus de cas d'émergence chez l'humain sont documentés (Mansfield *et al.*, 2017). Dans le contexte guyanais, il serait intéressant de s'interroger sur l'incidence de ces émergences. En effet, de nombreuses arboviroses (dengue, fièvre jaune, maladie du virus Zika, etc...) circulent dans les populations humaines *via* les moustiques sur ce territoire. Il serait alors très surprenant de ne pas retrouver des virus transmis à l'homme ou aux animaux domestiques par les tiques. Ainsi, à l'instar de ce qui se fait pour les moustiques, il est nécessaire, dans un futur proche, de caractériser le virome (l'ensemble des virus) chez les tiques de Guyane. Cela permettra d'avoir une idée précise du risque acarologique global sur ce territoire tout en offrant la possibilité de documenter de potentielles nouvelles souches de virus.

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Discussion

L'objectif principal de cette thèse était d'appréhender les facteurs structurants des microbiomes. Pour ce faire, nous avons utilisé comme modèle les communautés microbiennes hébergées par les tiques de Guyane que nous avons principalement étudiées à l'échelle interspécifique chez le genre de tique *Amblyomma*. Il est apparu que ces microbiomes présentent une structuration influencée par des facteurs différents de ceux des microbiomes hébergés par des organismes modèles (comme l'humain ou la souris ; plus généralement les vertébrés). Ces différences de facteurs peuvent en majeure partie s'expliquer par : 1- des différences fondamentales dans la diversité et la composition des microbiomes : le microbiome interne des humains exhibe par exemple une diversité remarquable comparé à celui des tiques. 2- des stratégies évolutives différentes du compartiment microbien : au sein de l'holobionte que représente une tique, l'ensemble de « ses » symbiotes vont majoritairement se transmettre par TV. Chez l'holobionte humain, une transmission mixte du microbiome est majoritaire. Cette différence fondamentale va entraîner une structure des microbiomes des tiques bien différente de ceux des vertébrés. Elle ne présente ainsi pas de patron de phylosymbiose clair, comme chez les vertébrés ou d'autres animaux. A la place, des patrons évolutifs typiques des symbiotes intracellulaires d'arthropodes sont observés, comme par exemple des signaux de cocladogénèse entre *Coxiella* et les *Amblyomma*. De plus, les bactéries intracellulaires vont permettre d'expliquer en grande partie la structuration du microbiome des tiques, ou tout du moins celui de leur core-microbiome. Toutefois, comme nous allons le discuter, ces core-microbiomes apparaissent comme le seul microbiome stable chez les tiques, représentant la majorité de la diversité de ces derniers. Ainsi, les interactions biotiques au sein des core-microbiome jouent très probablement un rôle important dans sa structuration mais également sur la circulation des agents pathogènes (et *vice-versa*). Cette thèse a également permis de souligner les nombreux biais existant

dans les études des microbiomes des tiques, rendant, à mon avis, difficile de conduire une méta-analyse sur la structuration de ces derniers. Enfin, ce travail contribue grandement aux connaissances associées aux tiques de Guyane, qu'elles soient de l'ordre de la biodiversité ou du risque acarologique.

1. La phylosymbiose : des résultats contradictoires chez les tiques ?

Récemment le terme phylosymbiose a été proposé pour décrire le patron de corrélation entre la structure d'un microbiome et la phylogénie de son hôte qui semble exister chez une multitude d'hôte (Brooks, Kohl, Brucker, Opstal, & Bordenstein, 2016 ; Brucker & Bordenstein, 2012, 2013). Ainsi, sous une hypothèse de phylosymbiose, les communautés microbiennes associées à l'hôte se forment, en partie, à la suite d'interactions avec l'hôte plutôt que par des processus purement associés à l'environnement. Toutefois, ce patron ne semble pas dû à des processus de coévolution, cospéciation ou de cocladogénèse de l'ensemble de la communauté microbienne, et ne présume donc pas nécessairement que les membres de la communauté microbienne sont constants, stables ou transmis verticalement de génération en génération (Brooks *et al.*, 2016). Les patrons de phylosymbioses sont reconnus aujourd'hui comme l'un des facteurs principaux expliquant la structure des microbiomes à une échelle inter-spécifique (Brooks *et al.*, 2016 ; Groussin *et al.*, 2017 ; Mazel *et al.*, 2018). En ce sens, les tiques et leur microbiome ont été récemment décrits comme formant également des patrons de phylosymbioses (Figure 9) (Díaz-Sánchez, Estrada-Peña, Cabezas-Cruz, & de la Fuente, 2019). Néanmoins, nous avons vu que nos résultats semblent contredire le caractère principal des patrons de phylosymbioses pour expliquer la structure des microbiomes chez les tiques (Figure 10).

En effet, à la différence de l'étude ci-avant, qui semble montrer une forte structuration des microbiomes par un patron de phylosymbiose chez des *Ixodes* (Díaz-Sánchez *et al.*, 2019), les microbiomes des *Amblyomma*, apparaissent essentiellement structurés par la présence de bactéries endosymbiotiques intracellulaire, qu'elles soient des P-symbiontes (*Coxiella* et *Francisella*) ou de phénotypes inconnus (*Rickettsia*). Néanmoins, cette dichotomie peut s'expliquer de différentes manières.

Premièrement, nous pouvons simplement imaginer que les *Amblyomma* et les *Ixodes* ne soient pas soumis au même processus éco-évolutifs expliquant la structuration de leur microbiome. Les *Ixodes* forment effectivement une sous-famille au sein des Ixodidae (tiques dures), les Ixodinae ou Prostriata, qui est bien différenciée du reste des tiques dures (Metastriata) (Burger, Shao, Beati, Miller, & Barker, 2012). La phylogénie des tiques dures peut alors suggérer que les *Ixodes* et les *Amblyomma* ne partagent globalement pas les mêmes pressions sélectives ni les mêmes niches écologiques. Ainsi, ces différences pourraient expliquer l'apparition d'un patron de phylosymbiose qui semble bien défini chez les Ixodinae mais non chez les *Amblyomma*. Pour tester cette hypothèse, il faudrait conduire des études sur d'autres genres de tiques dures comme les *Rhipicephalus*. Toutefois, à la vue de l'apparente ubiquité des patrons de phylosymbioses dans le règne animal (Brooks *et al.*, 2016 ; Groussin *et al.*, 2017; Ley *et al.*, 2008 ; Mazel *et al.*, 2018), il serait extrêmement surprenant que seuls les Metastriata ou *Amblyomma* échappent à ce phénomène.

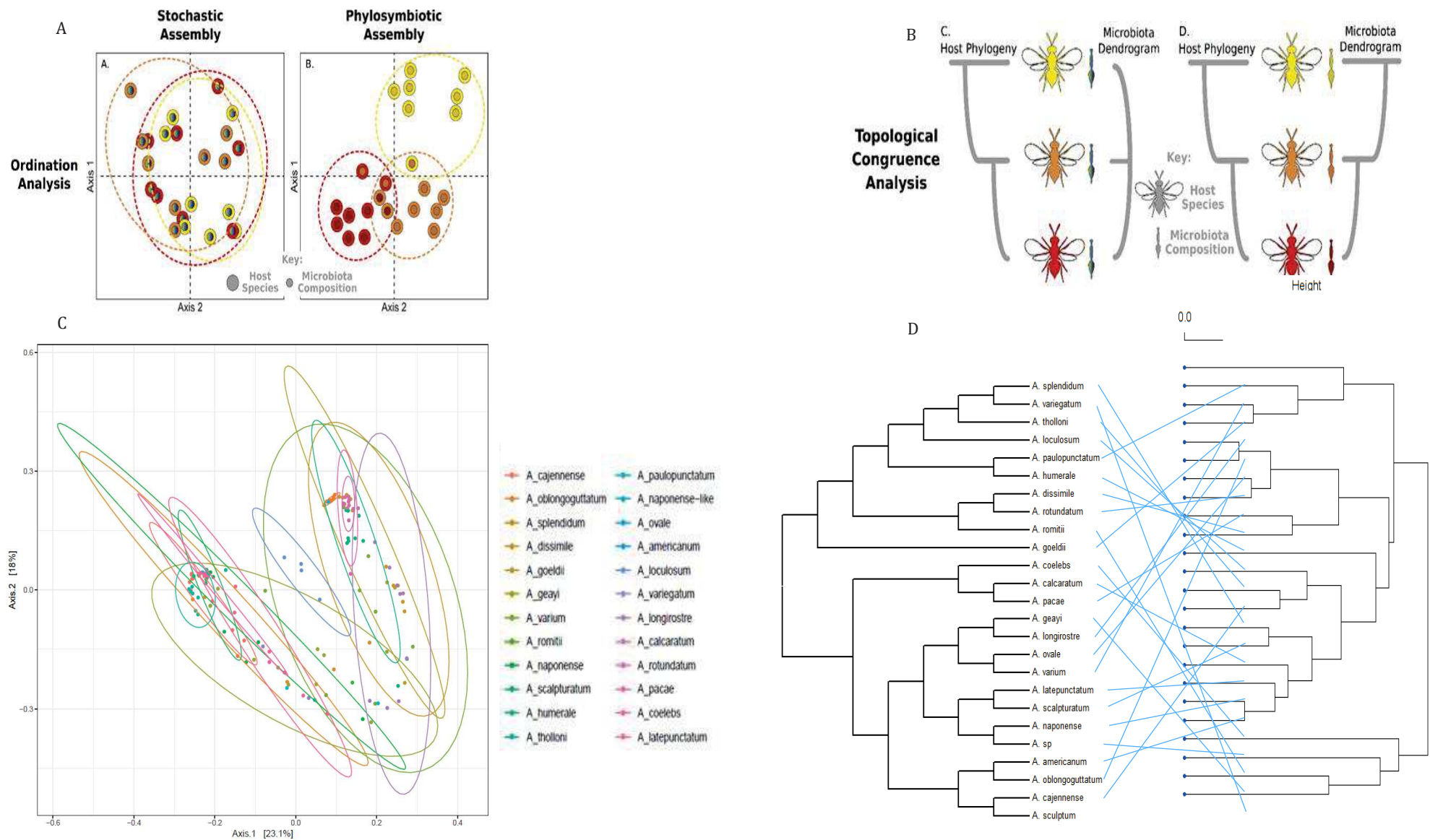


Figure 10 : Les patrons de phylosymbioses : de la théorie aux *Amblyomma*.

Pour déterminer les patrons de phylosymbioses, des diagrammes d'ordination bidimensionnelle (A et C) et des analyses de congruence topologique entre la phylogénie de l'hôte (parenté évolutive) et le dendrogramme des communautés microbiennes (parenté écologique) (B et D), sont classiquement utilisés.

En Théorie : [A] illustre la similitude hypothétique des microbiomes sous (à gauche) les modèles stochastiques et (à droite) les modèles phylosymbiotiques. Les lignes en pointillés représentent les clusters de microbiome spécifiques aux hôtes. [B] décrit le modèle attendu pour (à gauche) l'assemblage stochastique *versus* (à droite) l'assemblage sous phylosymbiose. D'après Brooks *et al.* (2016)

En pratique : [C] Les clusters délimités par les lignes colorées montrent clairement une structuration qui ne dépend qu'en partie de l'espèce de tique (adaptée de l'article du chapitre III). [D] L'analyse de congruence entre la phylogénie des *Amblyomma* (à gauche, adaptée de l'article du chapitre III) et le dendrogramme des microbiomes (à droite, obtenu par regroupement hiérarchique) ne semblent montrer aucun patron clair de phylosymbiose.

Dans un second temps, il est possible que notre étude ne comprenne pas assez d'individus par espèce d'*Amblyomma*. La véritable structure des microbiomes de chaque espèce ne serait ainsi pas bien représentée, conduisant à la non-détection d'un patron de phylosymbiose équivoque. En effet, les espèces de notre étude ne sont représentées que par un maximum de 10 individus, certaines étant même définies par un seul individu. Ce biais était, malheureusement, difficilement évitable du fait de la rareté de certaines espèces d'*Amblyomma* et du coût associé à une telle étude. Toutefois, si les microbiomes internes des *Amblyomma* étaient bien principalement soumis à un patron de phylosymbiose, un signal équivoque de cette phylosymbiose aurait dû tout de même apparaître. Or, nous avons vu que ce n'était pas le cas, et qu'à la place se dessinait un patron de structuration majoritairement expliqué par les bactéries intracellulaire (Figure 10 et article chapitre III). Au vu de la faible diversité bactérienne hébergée par les *Amblyomma* et largement dominée par les P-symbiontes, ce résultat était peu surprenant.

Ainsi, la différence de diversité microbienne qui semble exister entre les *Ixodes* et les *Amblyomma* apparaît comme la raison principale de la dichotomie concernant l'impact des patrons de phylosymbioses chez les tiques. Comment alors expliquer une telle différence ? Par une contrainte phylogénétique chez les tiques comme supposé précédemment ? Une plus faible abondance du P-symbionte chez les *Ixodes* ? Ces deux potentielles explications sont réfutées par de récentes études portant sur le microbiome de certaines espèces d'*Ixodes* (e.g. Couper, Kwan, Ma, & Swei, 2019 ; Gofton *et al.*, 2015 ; Ross *et al.*, 2018). Ainsi, ces études ont démontré que le microbiome des *Ixodes* était extrêmement comparable à celui des *Amblyomma*, à savoir très peu diversifié et largement dominé par un P-symbionte (pouvant généralement être des *Rickettsia*, *Rickettsiella*, *Midichloria* ou *Coxiella*, dans le cas des *Ixodes*, Duron *et al.*, 2017). Il apparaît alors une controverse entre les précédentes études et l'étude démontrant un fort patron

de phylosymbiose *Ixodes* / microbiome (Díaz-Sánchez *et al.*, 2019). Toutefois, ces différences dans les résultats de diversités microbiennes entre études peuvent facilement s'expliquer par des variations dans les protocoles de ces études.

En effet, comme nous l'avons discuté dans le chapitre II, il existe d'innombrables paramètres techniques / méthodologiques pouvant influencer les jeux de données impliquant des microbiomes. Il est alors intéressant de noter que l'étude de Díaz-Sánchez *et al.* (2019) repose sur une méta-analyse de cinq études différentes (Carpi *et al.*, 2011 ; Díaz-Sánchez *et al.*, 2018 ; Kurilshikov *et al.*, 2015 ; Qiu, Nakao, Ohnuma, Kawamori, & Sugimoto, 2014 ; Van Treuren *et al.*, 2015). Or, les protocoles de ces études varient entre eux de plusieurs manières : par la technologie de séquençage, par la région hypervariable du 16S rRNA bactérien séquencée, ou encore par les organes investigués (glandes salivaires vs tiques entières), mais également (et surtout) par le protocole de stérilisation de la cuticule des tiques. Ainsi, une seule étude a eu recours à une stérilisation par Javel (Van Treuren *et al.*, 2015) tandis que les autres ont utilisé au mieux de l'éthanol, rien dans le pire des cas... Nous avons prouvé lors du chapitre II que l'éthanol n'était pas suffisant pour se débarrasser efficacement du microbiome externe. Ce biais, couplé aux autres variations de protocoles recensés dans ces études, est connu pour induire des biais dans la structure des microbiomes (voir chapitre II). Il n'est alors pas étonnant qu'une méta-analyse sur ces jeux de données produise un tel patron de « phylosymbiose ». La majorité de la diversité observée dans une telle étude va effectivement résulter du microbiome cuticulaire. Les différences dans la structure bactérienne entre espèces de tiques seront donc principalement expliquées par des facteurs écologiques de l'environnement de la tique (comme la localité ou le type de végétation par exemple), et renforcées par la variabilité des protocoles utilisés. Cette accumulation de biais peut alors conduire à la

création d'un patron de phylosymbiose artificielle, résultant de l'interaction écologique entre une espèce de tique et son environnement, elle-même biaisée par un signal méthodologique entre une étude donnée et son modèle biologique.

Au vu de ces arguments, il semble aujourd'hui nécessaire que la communauté scientifique travaillant sur le microbiome des tiques s'accorde à l'avenir sur un protocole d'étude défini et borné. Il est certainement utopique de considérer cela, du fait des différences budgétaires, technologiques et préférentielles des différents laboratoires travaillant sur cette thématique. Néanmoins, nonobstant ces différences, il apparaît tout à fait réalisable de standardiser certains aspects des protocoles, comme par exemple la javellisation des tiques ou l'utilisation d'une même région hypervariable du 16S rRNA. Cet effort de « gold standardisation » ne pourra qu'être bénéfique pour appréhender finement les processus de structuration des communautés microbiennes chez les tiques.

2. Le microbiome interne des tiques : une diversité cachée ?

L'image d'une incroyable diversité est profondément ancrée dans l'idée que l'on se fait d'un microbiome. Ce regard anthropocentré est notamment dû à la diversité bactérienne foisonnante dans les intestins des vertébrés. Néanmoins, cette idée n'est peut-être pas transposable à l'ensemble du règne animal. En effet, ces travaux de thèse ont révélé que la diversité bactérienne hébergée par les tiques de Guyane était (très) faible et largement dominée par un P-symbionte. Ces résultats ne concordent pas avec la majorité des études s'intéressant aux microbiomes des tiques, qui semblent démontrer l'opposé (*e.g.*, Abraham *et al.*, 2017 ; Budachetri, Gaillard, Williams, Mukherjee, & Karim,

2016 ; Clay *et al.*, 2008 ; Díaz-Sánchez *et al.*, 2018 ; Kurilshikov *et al.*, 2015 ; Landesman *et al.*, 2019 ; Narasimhan *et al.*, 2017). Nous avons toutefois établi que l'importante diversité bactérienne retrouvée dans ces études était très certainement un reliquat du microbiome cuticulaire (chapitre II). Cette conclusion repose bien évidemment sur nos résultats, mais également sur diverses études, ayant suivi un protocole de stérilisation externe des tiques similaire au nôtre (*e.g.*, Clayton, Gall, Mason, Scoles, & Brayton, 2015 ; Couper *et al.*, 2019 ; Gall, Scoles, Magori, Mason, & Brayton, 2017 ; Lalzar, Harrus, Mumcuoglu, & Gottlieb, 2012). Néanmoins dans ce paysage controversé, une étude apporte des résultats intrigants sur la diversité bactérienne interne des tiques. En effet, les travaux de Gofton *et al.* (2015) semblent suggérer que la diversité bactérienne des tiques est totalement masquée par la présence d'un P-symbionte. Pour arriver à cette conclusion, les auteurs ont réussi à inhiber, avant la phase de séquençage haut-débit, l'amplification du P-symbionte (*Midichloria*) chez des *Ixodes* javellisées. Ils ont par la suite comparé les résultats de cette diversité avec des microbiomes « non-inhibés » et observé une différence significative. Ainsi, cette étude suggère que la présence des P-symbiontes est l'arbre qui cache la forêt microbienne chez les tiques, allant même jusqu'à masquer la présence d'agents pathogènes comme des *Borrelia*. Démêler la controverse sur la réelle diversité bactérienne interne des tiques est donc primordiale d'un point de vue écologique mais également dans un intérêt de santé publique.

En ce sens, de récentes études semblent apporter des réponses convaincantes à cette problématique. Effectivement, *via* une approche par hybridation fluorescente *in situ* (FISH), les travaux de Ross *et al.* (2018) et Wang *et al.* (2018) avancent l'hypothèse que les tiques n'hébergent pas de microbiome stable dans leur tube digestif. En effet, bien que de rares individus présentent une abondante « flore » bactérienne, la majorité des tiques étudiées n'hébergeaient aucune bactérie ou, à la rigueur, un ou des symbiontes

intracellulaires communs des tiques (voir également, Clayton *et al.*, 2015; Gall *et al.*, 2017). Cela concorde avec nos résultats montrant une faible diversité bactérienne dans les tubes digestifs d'*A. cajennense* javellisées (voir article du chapitre II). Ainsi, à l'inverse de nombreux animaux, les tubes digestifs des tiques ne semblent pas héberger (le cas échéant) de flore bactérienne diversifiée, sauf cas isolés. Cette constatation fait directement écho à un nouveau paradigme qui suggère que tous les animaux n'abritent pas un microbiome intestinal complexe et hautement diversifié. C'est par exemple le cas de nombreuses espèces de lépidoptères qui, lorsqu'ils sont encore au stade de chenille, n'hébergent quasiment aucune trace de microbiome intestinal (Hammer, Janzen, Hallwachs, Jaffe, & Fierer, 2017). Ce phénomène s'observe également, dans une moindre mesure, chez une grande variété d'insectes se nourrissant exclusivement de sève (Jing *et al.*, 2014), mais également chez des arthropodes strictement hématophages comme la glossine (Griffith *et al.*, 2018). De manière générale, le manque de diversité bactérienne intestinale s'avère être fréquent chez les arthropodes aux régimes alimentaires ultraspecialisés comme l'hématophagie stricte ou la phloèmophagie. Comme nous l'avons déjà vu en introduction et au long de ce manuscrit de thèse, ce type d'arthropodes est connu pour héberger des P-symbiontes qui complètent leurs manques nutritionnels associés à un tel régime alimentaire. Maintenir un microbiome interne diversifié apparaît alors redondant avec le maintien de P-symbionte, et certainement plus néfaste que bénéficiaire à la valeur sélective de l'hôte. Cela est en parfait accord avec nos résultats sur le rôle majeur des P-symbiontes dans la structuration du microbiome interne des tiques (voir chapitre III), et suggère donc un phénomène similaire chez d'autres arthropodes aux régimes alimentaires biaisés. Toutefois, un tel processus n'explique pas les observations de Gofton *et al.* (2015) qui montrent l'apparition d'une importante diversité microbienne une fois le P-symbionte des tiques « enlevé ». Il est alors important de noter que cette

étude a été conduite sur des tiques entières, et que les microbiomes internes ne se limitent pas au tube digestif, mais englobent les communautés microbiennes de tous les autres organes. Par conséquent, si la diversité microbienne « cachée » ne se situe pas dans les tubes digestifs de tiques, elle pourrait bien se localiser dans les autres organes de la tique comme les glandes salivaires. Il existe peu d'études ayant essayé de caractériser la diversité bactérienne en fonction des différents organes de la tique *via* une approche de barcoding du 16S rRNA bactérien (*e.g.* Clayton *et al.*, 2015 ; Gall *et al.*, 2017 ; Qiu *et al.*, 2014). De plus, ces études se concentrent essentiellement sur deux organes : le tube digestif (dont nous avons déjà discuté) et les glandes salivaires. Chez ces dernières, le peu d'études existantes semble montrer un patron de diversité similaire au tube digestif. En effet, en s'intéressant aux résultats des études de Clayton *et al.* (2015), de Gall *et al.* (2017) et de Qiu *et al.* (2014), il apparaît que les glandes salivaires présentent généralement une faible diversité bactérienne, dominée encore une fois par des bactéries intracellulaires connues pour infecter les tiques (*Arsenophonus*, *Spiroplasma*, *Rickettsia*, *Francisella* ou encore *Coxiella*). Dans certains cas, des agents pathogènes de vertébrés (autres que *Rickettsia*) sont également caractérisés dans les glandes salivaires (notamment *Ehrlichia*), ce qui n'est pas surprenant et cohérent avec la stratégie évolutive de tels organismes (Qiu *et al.*, 2014). De plus, l'excellent article de Wang *et al.* (2018) démontre chez plusieurs espèces de tiques que le microbiome des glandes salivaires, mais aussi des autres organes généralement considérés chez une tique, à savoir les ovaires, les testicules ou le tube de Malpighi, sont très peu diversifiés et majoritairement composés de bactéries intracellulaires (Figure 11). Pour cela, les auteurs ont utilisé une approche d'hybridation fluorescente *in situ* (FISH) : dans un premier temps en se basant sur une sonde fluorescente universelle des bactéries, puis dans un second temps, sur des sondes spécifiques à certains symbiontes intracellulaires comme *Rickettsia* ou *Coxiella*. Ainsi, les

résultats de diversité microbienne retrouvés dans les différents organes des tiques abondent avec ceux des tubes digestifs et avec nos résultats, et pointent tous vers le paradigme que les tiques hébergent, de manière globale, une diversité bactérienne réduite aux bactéries intracellulaires.

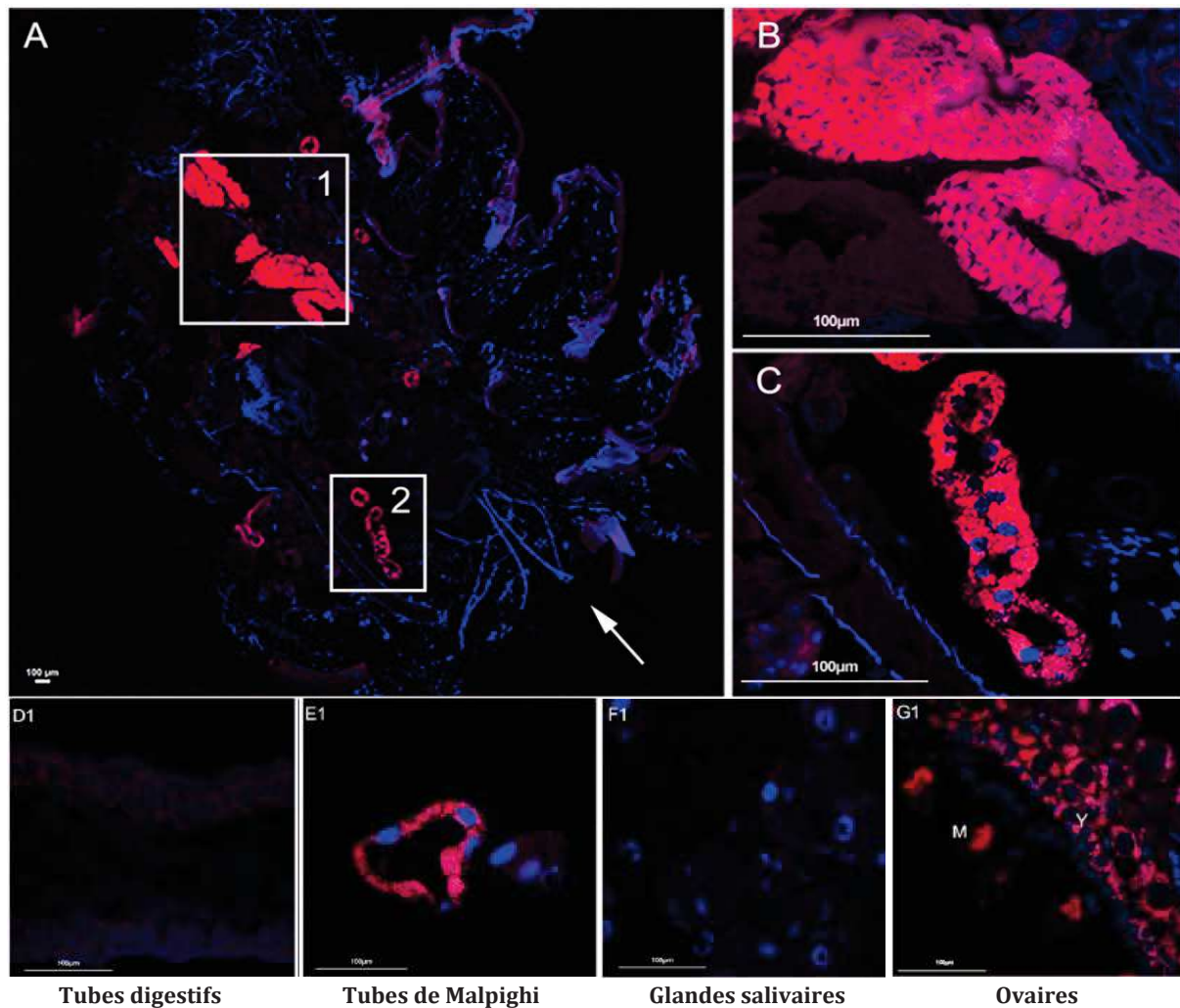


Figure 11 : Localisation des bactéries chez les tiques

Localisation du microbiome dans le corps entier (A à C) et dans différents tissus (D1 à G1) de femelle *Haemaphysalis longicornis* par analyse FISH avec une sonde universelle des eubactéries ciblant le 16S rDNA (rouge). Les noyaux ont été colorés avec DAPI (bleu).

(A) Signaux détectés dans des sections entières du corps ; la flèche indique la direction de la tête vers le bas ; (B) vue rapprochée de l'encadré 1 montrant une importante concentration de bactéries dans les ovaires ; (C) vue rapprochée de l'encadré 2 montrant que des bactéries colonisent également intensément les tubes de Malpighi.

(D1) et (F1) : pas de trace de bactérie dans les tubes digestifs et glandes salivaires vs (E1) et (G1) : concentration bactérienne dans les tubes de Malpighi et les ovaires (Y= jeunes ovocytes et M = ovocytes matures).

Toutes les images sont représentatives d'au moins 5 spécimens différents et de trois groupes indépendants d'expériences. D'après Wang *et al.* (2018).

L'avantage de la technique FISH est de permettre la localisation intra-organe des bactéries, tout en s'affranchissant des nombreux biais liés aux techniques moléculaires. Effectivement, plusieurs études démontrent l'existence de phénomènes de contamination bactérienne provenant des différents et nombreux tampons utilisés tout au long des études moléculaires des microbiomes (*e.g.*, Glassing, Dowd, Galandiuk, Davis, & Chiodini, 2016 ; Lejal *et al.*, 2019 ; Salter *et al.*, 2014). Ces contaminants peuvent alors fausser les distributions taxonomiques et les fréquences relatives dans les jeux de données des microbiomes, et contribuer à des interprétations et des identifications erronées (Glassing *et al.*, 2016 ; Salter *et al.*, 2014). Les problèmes de contaminations par les tampons ou par l'environnement du laboratoire impactent d'autant plus fortement les échantillons avec une faible biomasse bactérienne et contenant de l'ADN de l'hôte (Dahlberg *et al.*, 2019 ; Glassing *et al.*, 2016 ; Salter *et al.*, 2014). Ainsi, pour déterminer les taxons contaminants il est important de faire des témoins négatifs ne comprenant que ces tampons. Néanmoins, du fait des biais propres à la technique de PCR, ces tampons ne sont pas toujours représentatifs de l'ensemble des contaminants possiblement retrouvés dans les échantillons d'intérêts. D'après les résultats discutés tout au long de cette partie, les tiques apparaissent également comme des échantillons avec une faible biomasse bactérienne. Cette constatation est encore plus pertinente dans le contexte où les P-symbiotes sont inhibés pendant la phase de PCR. La diversité microbienne ainsi observée et masquée par les P-symbiotes ne serait-elle pas, au final, artificielle ? Bien que les résultats de Wang *et al.* (2018), en s'affranchissant des biais discutés ci-avant, soient très convaincants, des études spécialement conçues dans le but de tester cette question et de statuer sur le sujet de la diversité microbienne chez les tiques devront être entreprises.

Ainsi, à la suite de ma thèse, notre équipe va tenter d'appréhender la très hypothétique diversité bactérienne cachée des tiques. Pour ce faire, 40 femelles *A. cajennense* provenant d'une même localité seront disséquées vivantes après avoir été javellisées, dans le but de collecter les glandes salivaires, les ovaires, les tubes de Malpighi, les tubes digestifs et les restes de carcasses. Puis la diversité microbienne de chaque organe, pour une trentaine de spécimens, sera investiguée de manière classique par barcoding du 16S rRNA bactérien ; parallèlement, une approche par FISH sera conduite sur le reste des spécimens. Enfin, la biomasse bactérienne contenue dans chaque organe sera déterminée par PCR quantitative avec des amorces généralistes puis comparée à la biomasse de P-symbionte mesurée grâce à des amorces spécifiques (dans ce cas *Coxiella*). Ainsi, nous aurons une vision moins biaisée et plus fine de la diversité bactérienne hébergée par les tiques. Néanmoins, pour aller plus loin, il serait nécessaire d'utiliser une technique de barcoding non biaisée comme le séquençage en temps réel d'une seule molécule d'ADN (SMRT) (Rhoads & Au, 2015). Or, de telles technologies sont pour l'instant encore trop coûteuses et présentent un taux d'erreurs extrêmement conséquent (Ardui, Ameer, Vermeesch, & Hestand, 2018). Finalement, il faut garder à l'esprit qu'une grande diversité microbienne chez les tiques existe peut-être sous la forme de micro-organismes eucaryotes, ou même de procaryotes non amplifiés par les amorces communes et composant la « microbial dark matter » (Rinke *et al.*, 2013). En ce sens, une collaboration est en cours pour la recherche d'archées bactéries chez les tiques, qui est un groupe souvent ignoré dans la caractérisation d'un microbiome. Quoi qu'il en soit, l'impact des bactéries intracellulaires, et notamment des P-symbiontes sur ces microbiomes, est indéniable.

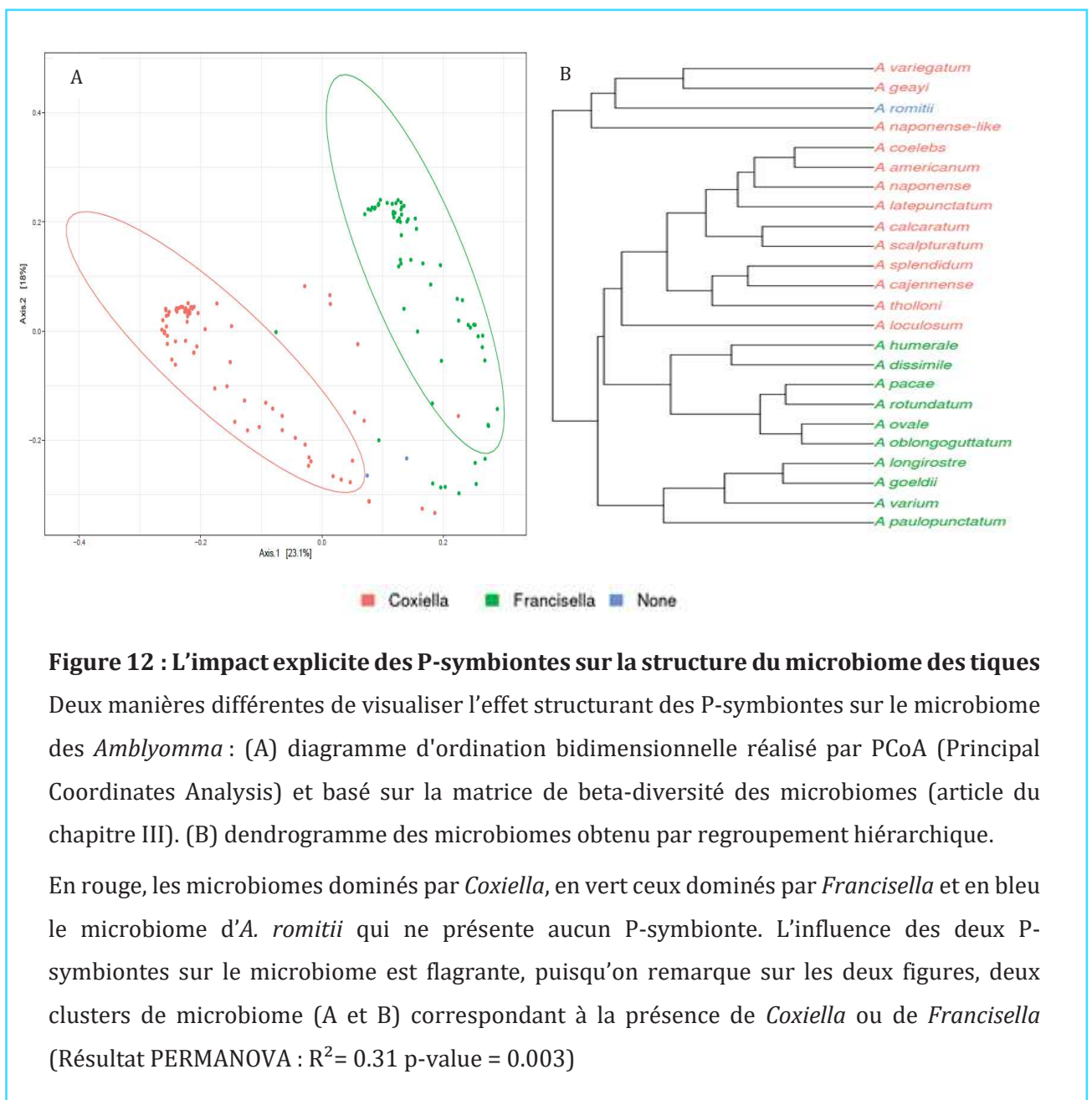
3. Structuration du microbiome : les interactions biotiques comme moteur ?

Comme nous l'avons vu en introduction, les interactions biotiques sont un facteur essentiel dans la structuration des microbiomes à une échelle intra-holobionte, voire intra-spécifique. Néanmoins, à une échelle inter-spécifique, l'influence des interactions inter-microbiomes se fait plus ténue, pour laisser place à une structuration majoritairement dominée par des mécanismes éco-évolutifs liés à l'hôte. Dans l'article et la discussion du chapitre III, nous avons toutefois montré que la structure des microbiomes des *Amblyomma* est majoritairement expliquée par la présence et l'identité du P-symbionte (Figure 12), et par conséquent avons mis en évidence l'importance des interactions biotiques dans la structuration de ces microbiomes à une échelle inter-spécifique. En effet, cela est parfaitement illustré par la compétition et les phénomènes de remplacements occurrents entre les P-symbiontes des *Amblyomma*, que sont *Coxiella* et *Francisella*. Il est important néanmoins de garder à l'esprit que ces mécanismes de remplacements restent des événements rares à une échelle générationnelle, et ne sont mis en évidence que sur des temps évolutifs (McCutcheon, Boyd, & Dale, 2019). Les interactions biotiques entre les P-symbiontes sont donc elles-mêmes soumises à une structuration dépendante de processus coévolutifs avec les tiques, voire de facteurs écologiques comme le spectre d'hôte de l'espèce de tique. Ce dernier point reste à prouver de façon plus convaincante que dans notre étude, bien que le véritable spectre d'hôte d'une espèce de tique soit difficile à cerner, particulièrement chez des espèces peu étudiées. Malgré cela, le spectre d'hôte fait très certainement partie des facteurs expliquant le « succès » de *Francisella* dans sa faculté à remplacer *Coxiella* chez les

Amblyomma de Guyane. Toutefois, ce sont bien les capacités biosynthétiques supérieures du génome des *Francisella* sur celui des *Coxiella* qui sont très probablement la principale composante permettant à *Francisella* d'exclure compétitivement *Coxiella* dans la course à la niche écologique réservée au P-symbionte chez les *Amblyomma* (Gerhart, Moses, & Raghavan, 2016 ; Gottlieb, Lalzar, & Klasson, 2015). Il apparaît alors important de considérer le patron d'exclusion existant entre ces deux bactéries dans la détermination des facteurs structurants des microbiomes des *Amblyomma*. Nous avons déjà discuté de la nature de l'interaction entre ces deux symbiontes ; néanmoins dans le but d'aller plus loin dans la compréhension de ce phénomène, de futures études sont nécessaires.

Bien que *Coxiella* soit le P-symbionte le plus commun des tiques (Duron *et al.*, 2017), ces travaux de thèse démontrent que *Francisella* est en pleine expansion chez ces dernières. Pour appréhender dans son ensemble le processus sous-jacent à la diffusion des *Francisella*, il est nécessaire de caractériser moléculairement un maximum de souches de *Francisella* associées aux tiques. Ainsi, à l'image d'une précédente étude sur *Coxiella* (Duron *et al.*, 2015), un MLST des souches de *Francisella* existantes au sein de l'ensemble de notre collection de tiques va être entrepris. Cela permettra d'appréhender l'histoire évolutive des *Francisella* associée avec les tiques, et de comparer les différences de patrons évolutifs existant entre différents genres de tiques, notamment les *Amblyomma* de Guyane qui sont les plus fréquemment infectées au sein des tiques dures, au même titre que les *Hyalomma* (Duron *et al.*, 2017). De plus, cette étude permettra sans doute de renforcer l'hypothèse que les *Francisella* mutualistes des tiques sont issues de l'évolution d'une *Francisella* pathogène de mammifères, sans doute très similaire à *F. tularensis*, l'agent de la tularémie (Duron *et al.*, 2018 ; Gerhart *et al.*, 2016). Enfin, cette étude

apportera également de précieuses données pour étudier l'hypothétique relation entre certaines *Francisella* et certaines *Rickettsia*.



En effet, pour développer cette hypothèse (qu'il faut, je le rappelle, considérer avec précaution puisque faisant encore l'objet de tests statistiques), il est intéressant de remarquer que certaines souches de *Francisella*, comme celle hébergée par *A. longirostre* ou par *A. dissimile*, sont souvent associées avec une souche spécifique de *Rickettsia*, en fonction de l'espèce de tique (dans ce cas : *R. amblyommatis* et *R. bellii*, respectivement).

De plus, nos résultats semblent suggérer que les *Rickettsia* pourraient jouer un rôle clé dans l'histoire évolutive des *Francisella* en impactant la phylogénie de ces dernières. Cette hypothèse est pour le moins intrigante, considéré la nature diverse des souches de *Rickettsia* caractérisées rien que chez les tiques de Guyane (19 au total). Cela suggère une possible convergence phénotypique de certaines *Rickettsia*, impactant l'évolution des *Francisella* P-symbiotique de tiques et permettant le maintien de ces co-infections. L'étude phylogénétique du MLST de nombreuses souches de *Francisella* permettra ainsi de statuer sur la validité de l'hypothèse suggérant un impact des *Rickettsia* sur l'évolution des *Francisella*.

Pour aller plus loin, il serait très intéressant de caractériser les génomes des *Francisella* et des *Rickettsia* présentant une forte prévalence chez une espèce de tique (par exemple : *A. longirostre* ou *A. dissimile* ; 82% et 67% d'individus infectés par *Rickettsia*) et de les comparer avec les génomes des souches de *Francisella* d'espèces de tiques rarement (voire jamais) infectées par *Rickettsia* (par exemple : *A. oblongogutatum* et *Dermacentor nitens* ; 0 individus infectés / n=95 et 97 respectivement). Au vu de l'importance des P-symbiontes dans la structuration du microbiome des tiques, ces prévalences opposées peuvent en effet suggérer l'existence de patrons d'agrégation ou d'exclusion entre *Francisella* et *Rickettsia*, dépendant de la souche de *Francisella* étudiée. Ainsi, l'étude des génomes de ces différentes *Francisella* permettra peut-être de déceler les raisons de ces patrons. Nous pouvons supposer que les *Francisella* « sans *Rickettsia* » (exclusives) ont été plus récemment acquises par leur hôte que les *Francisella* « avec *Rickettsia* » (agrégatives), et de ce fait possèdent un génome moins dégradé que les *Francisella* « sans *Rickettsia* », leur permettant d'être de meilleurs compétiteurs pour la niche écologique

liée à la TV. En effet, avec des prévalences supérieures à 50%, certaines souches de *Rickettsia* semblent présenter clairement un phénotype lié avec l'exploitation de la TV. Par conséquent, elles sont directement en compétition pour ce mode de transmission avec les *Francisella* de leur hôte, mais semblent pourtant se maintenir dans les populations hôtes. Ceci est cohérent avec les théories et la littérature sur les multi-infections. En effet, les interactions entre symbiotes à TV sont structurées par les pressions de sélection liées à ce mode de transmission (revue par Vautrin & Vavre, 2009). Effectivement, en « liant » les différents partenaires, la TV créerait des situations privilégiées pour les interactions symbiotiques, en particulier pour la coopération et la dépendance entre symbiotes à TV. De plus, le maintien d'infections multiples à TV au sein des populations hôtes exige que la production moyenne de femelles multi-infectées soit supérieure à la production moyenne de femelles infectées par un seul symbiote ou non infectées, ce qui semble être le cas au sein de certaines populations de tiques de Guyane. Cette condition peut être obtenue de la même manière que pour une simple infection : soit par l'apport d'un avantage sélectif à l'hôte, soit par la manipulation de la reproduction. Quoi qu'il en soit, il faut que les différents symbiotes induisent des effets différents ou synergétiques sur l'hôte : dans le cas contraire un hôte multi-infecté sera défavorisé par rapport à sa valeur sélective et par rapport à un hôte présentant une infection simple (Vautrin & Vavre, 2009). Dans notre contexte, cela suggère que les *Rickettsia* peuvent se maintenir pour différentes raisons : 1- ces *Rickettsia*, se maintiennent chez certaines espèces de tiques par TH à partir d'un hôte vertébré réservoir, ce qui apparaît très peu probable pour les *Rickettsia* avec de fortes prévalences. 2- Les *Rickettsia* avec de fortes prévalences se maintiennent en étant des manipulateurs de la reproduction à l'image de *Rickettsia* chez des parasitoïdes présentant un phénotype induisant la parthénogénèse (Hagimori, Abe, Date, & Miura, 2006) ; des S-symbiotes apportant un bénéfice à la valeur sélective de leur hôte

arthropode sous certaines conditions écologiques, comme *R. peacockii* qui, en se localisant dans les tissus ovariens, empêcherait *R. rickettsii*, l'agent de la fièvre pourprée, d'envahir les ovaires de l'espèce de tique *D. andersoni* et de diminuer ainsi leur valeur sélective (Burgdorfer, Hayes, & Mavros, 1980) ; enfin il peut s'agir de P-symbionte co-complémentant leur hôte avec *Francisella* (phénotypes synergétiques), faisant écho à l'interaction entre les *Buchnera* et d'autres symbiontes complémentant conjointement certains pucerons (Meseguer *et al.*, 2017).

L'impact hypothétique des *Rickettsia* sur la phylogénie des *Francisella* va dans le sens de cette dernière théorie, puisqu'il suggère un patron d'agrégation (ou d'exclusion) stable dans le temps entre les deux bactéries, pouvant se manifester phénotypiquement par de la co-complémentation. De plus, le mode de TV contraint les mêmes génotypes bactériens à se transmettre sur des générations d'hôtes, et favorise donc l'émergence de patrons d'agrégations mais surtout de patrons de coopération interspécifique (Sachs, Mueller, Wilcox, & Bull, 2004 ; Vautrin & Vavre, 2009). Cette coopération sera d'autant plus probable que les symbiontes partagent une même niche écologique, comme par exemple les ovocytes durant les événements de TV (Vautrin & Vavre, 2009). Il serait alors intéressant, de reproduire l'étude portant sur les organes de *A. cajennense*, avec des espèces infectées par *Francisella* et *Rickettsia* pour comparer le tropisme (le cas échéant) de chacune de ces bactéries, et ainsi de tester l'hypothèse d'un possible patron de coopération. De même, ces études permettraient d'hypothétiser sur les phénotypes des *Rickettsia* associés aux tiques de Guyane : une forte présence dans les glandes salivaires et le tube digestif suggérera un phénotype d'agent pathogène de vertébré, tandis qu'un tropisme dans les ovaires et les tubes de Malpighi évoquera plutôt un phénotype de S-symbionte.

Toutefois, bien que les coopérations inter-symbiotiques peuvent prendre la forme d'une association pour augmenter la valeur sélective de l'hôte (co-complémentation), elles peuvent également être plus simplement le résultat d'une convergence phénotypique favorisant une TV conjointe, pouvant même conduire à des phénomènes « d'auto-stop génétique » (ou dans notre cas : symbiotique) (Vautrin & Vavre, 2009). Ainsi les *Francisella* étant devenues des P-symbiontes relativement récemment, leur génome pourrait encoder des capacités biosynthétiques supérieures aux symbiontes remplacés. Il serait alors très surprenant que certains des gènes conférant cet avantage aux *Francisella* aient déjà subi un processus de pseudogénéralisation, nécessitant la complémentation d'une *Rickettsia*, pour continuer à fournir un avantage sélectif à leur hôte, donc à se maintenir chez les populations de ces derniers. De même, ce phénomène de co-complémentation ne semble pas exister chez les espèces de tiques infectées par *Coxiella*, qui présentent pourtant une association plus ancienne et donc « normalement » un génome plus dégradé que ceux des *Francisella*. Il serait alors plus logique d'observer des patrons évolutifs et d'agrégations entre les *Coxiella* et les *Rickettsia*. Enfin l'hypothèse d'une co-complémentation *Francisella* / *Rickettsia* semble peu probable au vu des prévalences de *Rickettsia* : aucune souche n'apparaît fixée chez une espèce de tique, à moins que des problèmes de détection liés au statut de gorgement (inhibition PCR par le sang des vertébrés) ou liés au sexe d'un individu (contrairement aux femelles, les P-symbiontes sont inutiles à la valeur sélective des mâles et peuvent donc résoudre l'infection) n'expliquent que *Rickettsia* ne soit « que » quasi-fixée chez certaines espèces comme *A. longirostre*... De plus, il est important de garder à l'esprit que ces différents symbiontes sont, dans tous les cas, en compétition pour les ressources de l'hôte, ce qui peut conduire à l'apparition d'interactions asymétriques : un symbionte va prendre le dessus sur un autre (e.g. interaction entre *Spiroplasma* et *Wolbachia* chez les drosophiles, Goto, Anbutsu,

& Fukatsu, 2006 ; interaction entre *Rickettsia* et *Buchnera*, Sakurai, Koga, Tsuchida, Meng, & Fukatsu, 2005). De même, lors de multi-infections, les hôtes sont souvent perçus comme des niches écologiques partagées, avec des ressources limitées dans lesquelles une compétition inter-symbiotique pour leurs exploitations fait rage. Ce phénomène est appelé « la tragédie des biens communs » (Hardin, 1968). Le résultat de ce phénomène peut être l'apparition de patrons d'exclusion entre divers micro-organismes, ce qui rend impossible le maintien de multi-infections interspécifiques et qui semble être le cas entre *Francisella* et *Coxiella*, mais aussi entre certaines souches de *Francisella* et de *Rickettsia* chez les *Amblyomma* de Guyane (Vautrin & Vavre, 2009). Les interactions biotiques entre les différents symbiontes à TV chez les tiques apparaissent donc complexes et peuvent conduire à l'apparition de différents patrons interactionnels (agrégation et exclusion). Ces patrons vont eux-mêmes être typiquement structurés par l'impact des multi-infections sur deux paramètres principaux des associations hôte-symbiontes : l'efficacité de la TV de chaque symbionte et le coût de l'infection pour l'hôte (Vautrin & Vavre, 2009). Toutefois, nous avons précédemment vu qu'il est dans l'intérêt des symbiontes à TV de favoriser la bonne santé de leurs hôtes, puisque leur valeur sélective dépend directement de celle de leurs hôtes. Ainsi, pour résumer, le maintien d'infections multiples par des symbiontes à TV nécessite des conditions spécifiques, notamment que les symbiontes présentent des phénotypes différents ou synergiques pour l'hôte, et que la balance avantages / coûts associée à leurs infection et transmission sur la valeur sélective de l'hôte soit positive (Vautrin & Vavre, 2009). Dans tous les cas, l'étude moléculaire et génomique de *Rickettsia* et de *Francisella* chez les tiques de Guyane sera précieuse pour permettre la discrimination de l'interaction liant potentiellement ces deux bactéries. De plus, cela permettra de mieux comprendre les phénomènes de multi-infections, qui semblent

fréquentschez les tiques (*e.g.* Duron *et al.*, 2017).

Pour aller plus loin, l'étude approfondie des cortèges d'agents pathogènes de vertébrés abrités par les tiques de Guyane, qui continuera après cette thèse (voir chapitre IV), permettra, en plus d'appréhender le risque acarologique global de ce territoire, de potentiellement caractériser des patrons d'exclusions entre ces agents pathogènes et les symbiontes à TV. En théorie, il est effectivement attendu qu'un agent infectieux à TH qui exploite au maximum les ressources de son hôte, et un symbionte à TV qui augmente (généralement) la valeur sélective de son hôte partagent une relation antagoniste (Vautrin & Vavre, 2009). Ceci est classiquement observé entre des S-symbiontes mutualistes (à TV) et des agents pathogènes de leurs hôtes : chez la drosophile par exemple, *Wolbachia* protège son hôte de virus entomopathogènes (Teixeira, Ferreira, & Ashburner, 2008), alors que *Spiroplasma* le protège de nématodes entomopathogènes (Jaenike & Brekke, 2011). Toutefois, ce phénomène a été surtout rendu célèbre par l'infection expérimentale de moustiques (*Aedes aegypti*) par une souche de *Wolbachia*, qui a pour effet de réduire de manière conséquente les titres viraux de différents virus (notamment DENV et CHIKV), l'abondance de bactéries entomopathogènes (notamment *Erwinia*) et les parasitémies (pour certains *Plasmodium* ou filaires) (Eleftherianos, Atri, Accetta, & Castillo, 2013 ; Moreira *et al.*, 2009). Cet exemple souligne l'importance de caractériser la totalité des cortèges microbiens hébergés par les tiques (virus, archées, bactéries et eucaryotes) pour appréhender l'ensemble des interactions biotiques du microbiome des tiques et mieux comprendre leur impact dans la structuration de ces derniers. Enfin, comme nous l'avons développé dans l'article de l'introduction, il a été apparemment démontré que certaines bactéries pathogènes comme *Borrelia burgdorferi*

ou *Anaplasma marginale* pouvaient modifier le microbiome du tube digestif des tiques par différents mécanismes pour faciliter leur invasion de la tique (Abraham *et al.*, 2017 ; Narasimhan *et al.*, 2017). Les agents pathogènes pourraient donc, au même titre que les P- ou S-symbiotes, influencer la structure du microbiome des tiques, démontrant encore un peu plus l'importance des interactions biotiques dans la structuration des microbiomes. Néanmoins, ce phénomène est à modérer, puisque les travaux cités ci-avant semblent contredits par les récentes études décrivant l'absence de microbiome intestinal chez les tiques (Ross *et al.*, 2018 ; Wang *et al.*, 2018). Ainsi notre prochaine étude sur la structure microbienne par organe des tiques sera un premier pas vers la compréhension des patrons interactionnels et de leur complexité existant réellement chez ces arthropodes.

4. Conclusion

Dans cette thèse, nous nous sommes attachés à étudier l'holobionte constitué par les tiques et leurs symbiotes, et plus précisément comment se structurent les communautés symbiotiques chez leur hôte. Ce travail est original dans le sens où nous avons essayé d'aborder ici, non seulement les interactions hôte/symbiote, mais également les interactions entre les différents symbiotes. Nous avons démontré au long de ce manuscrit que les microbiomes des tiques étaient très peu diversifiés et majoritairement soumis à des facteurs dépendants des bactéries intracellulaires non pathogènes. En effet, que ce soit des P-symbiotes ou de potentiels S-symbiotes, les différents patrons interactionnels et évolutifs entre ce core-microbiome sont des sources majeures de la variabilité des communautés microbiennes hébergées par les tiques. De plus, c'est également la première étude suggérant que les patrons de phylosymbiose entre la structure microbienne et la phylogénie des tiques semblent plus ténus que ce qui a été

précédemment avancé dans la littérature. Ainsi nous apportons un point de vue différent au paradigme actuel, qui suggère que tous les animaux, ou presque, forment des patrons clairs de phylosymbiose avec leur microbiome interne. Cette thèse démontre donc l'impossibilité de généraliser à tous les holobiontes du règne animal des processus de structuration des microbiomes internes à une échelle inter-spécifique.

Toutefois, suite à ces travaux de thèse, il apparaît que la structuration des microbiomes chez les tiques est encore loin d'être comprise. De nombreuses études seront encore nécessaires pour démêler les différentes controverses sur la réalité biologique que représentent les microbiomes hébergés par les tiques. Idéalement, cela passera par un effort d'uniformisation des protocoles servant à caractériser ces cortèges microbiens. Les études découlant de cette standardisation permettront la réalisation de méta-analyses robustes, aidant à n'en point douter à une élucidation plus fine des facteurs structurants des microbiomes. En effet, de telles études sont nécessaires à la caractérisation globale des interactions biotiques régissant les holobiontes, tout en permettant de les replacer à une échelle inter-spécifique et dans un contexte éco-coévolutif.

Finalement, bien que le risque acarologique globale associé aux tiques de Guyane ne soit pas encore appréhendé, il apparaît comme faible concernant les bactéries. C'est notamment le cas pour la nouvelle souche de borrelie potentiellement pathogène pour l'homme ou la faune domestique (voir chapitre IV). En effet, bien que les espèces de tiques infectées par cette *Borrelia* soient sporadiquement retrouvées sur l'homme (Guglielmone *et al.*, 2006), l'aléa de rencontre entre ces dernières et le compartiment domestique (homme et faune) apparaît comme très faible puisque ces tiques sont spécialistes

d'animaux sauvages arboricoles, ce qui limite le risque de zoonose. De ce fait, le risque d'apparition de zoonose chez l'homme sera donc majoritairement lié aux aléas de rencontres avec des espèces de tiques généralistes et anthropophiles, comme *A. cajennense* ou *A. oblongoguttatum* (voir chapitre I et IV). Or, à l'exception de *Rickettsia*, ces différentes espèces n'hébergaient pas ou peu de bactéries connues pour être pathogènes de vertébrés, limitant ainsi le risque acarologique associé à ces tiques. De plus, bien que nous ne puissions pas (encore) déterminer le phénotype des souches de *Rickettsia* circulant chez les tiques de Guyane, nos résultats semblent tendre vers des phénotypes de S-symbiontes. Bien évidemment, nous ne pouvons pas écarter le risque de contracter une rickettsiose après la piqûre d'une tique guyanaise ; toutefois ce risque nous apparaît comme faible. En effet, les *Rickettsia* sont en fait plus répandues qu'on ne le pensait auparavant : une importante diversité de *Rickettsia* a été associée à des arthropodes hématophages mais également à d'autres arthropodes ainsi qu'à des protozoaires, des algues et même des plantes (Perlman, Hunter, & Zchori-Fein, 2006 ; Weinert, 2015 ; Weinert, Werren, Aebi, Stone, & Jiggins, 2009). Il apparaît donc que le risque infectieux des *Rickettsia* hébergées par les tiques est peut-être (trop) largement survendu par les « tiquologues » et « rickettsiologues », alors que cette bactérie se retrouve chez une multitude d'autres arthropodes (et organismes) sans risque de zoonose associé... Ces *Rickettsia* se retrouvent par exemple, chez les arthropodes phloémophages qui ont la particularité d'héberger la plus grande diversité de bactéries hérissables connues... avec les tiques (Duron *et al.*, 2017 ; Moran, McCutcheon, & Nakabachi, 2008). A l'instar des *Rickettsia* associées aux tiques, celles hébergées par les phloémophages sont capables de TV et de TH (Gottlieb *et al.*, 2006 ; Perlman *et al.*, 2006 ; Weinert *et al.*, 2009), suggérant des convergences phénotypiques chez des hôtes, certes phylogénétiquement distants mais liés par un régime alimentaire biaisé. Ceci évoque ainsi l'existence de facteurs

favorisant l'apparition d'une telle diversité de bactéries intracellulaires qui semble propre aux organismes avec une alimentation extrême. Certains de ces facteurs pourraient même être partagés entre organismes écologiquement et phylogénétiquement distants, puisque de nombreux genres bactériens intracellulaires sont communs des tiques et des arthropodes phloémophages (*e.g. Rickettsia, Wolbachia, Arsenophonus, Cardinium, Rickettsiella, Spiroplasma, etc...*) (Duron *et al.*, 2017 ; Moran *et al.*, 2008). Les conditions éco-évolutives permettant les TH symbiotiques entre les tiques et les phloémophages (*e.g. S. ixodetis*, Binetruy *et al.*, 2019) ne sont pas encore élucidées et représentent ainsi une question aussi passionnante et stimulante que difficile à appréhender.

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Annexe A

Annexe A

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Original article

Phylogenetics of the *Spiroplasma ixodetis* endosymbiont reveals past transfers between ticks and other arthropods



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ABSTRACT

The bacterium *Spiroplasma ixodetis* is a maternally inherited endosymbiont primarily described from ticks but also found widespread across other arthropods. While it has been identified as a male-killing agent in some insect species, the consequences of infection with *S. ixodetis* in ticks are entirely unknown, and it is unclear how this endosymbiont spreads across tick species. Here, we have investigated this aspect through the examination of the diversity and evolutionary history of *S. ixodetis* infections in 12 tick species and 12 other arthropod species. Using a multi-locus typing approach, we identified that ticks harbor a substantial diversity of divergent *S. ixodetis* strains. Phylogenetic investigations revealed that these *S. ixodetis* strains do not cluster within a tick-specific subclade but rather exhibit distinct evolutionary origins. In their past, these strains have undergone repeated horizontal transfers between ticks and other arthropods, including aphids and flies. This diversity pattern strongly suggests that maternal inheritance and horizontal transfers are key drivers of *S. ixodetis* spread, dictating global incidence of infections across tick communities. We do not, however, detect evidence of *S. ixodetis*-based male-killing since we observed that infections were widely present in both males and females across populations of the African blue tick *Rhipicephalus decoloratus*.

1. Introduction

Ticks commonly harbor maternally inherited bacterial endosymbionts (Bonnet et al., 2017; Duron et al., 2017). Some of these endosymbionts, such as *Coxiella* and *Francisella*, are essential for the life cycle of ticks: they are obligate nutritional mutualists able to synthesize B vitamins that are deficient in the blood meal of ticks (Duron et al., 2018; Gerhart et al., 2016; Gottlieb et al., 2015; Smith et al., 2015). The experimental elimination of these symbionts results in decreased tick survival, fecundity and egg viability, as well as in some physical abnormalities (Duron et al., 2018; Guizzo et al., 2017; Li et al., 2018; Zhang et al., 2017; Zhong et al., 2007), which can be fully restored with an oral supplement of B vitamins (Duron et al., 2018). These obligate symbionts are, by definition, present in most individuals of a given tick species. However, many other maternally inherited bacteria, for instance *Arsenophonus* or *Rickettsiella*, are facultative endosymbionts: they are not essential for the life cycle of ticks and exhibit variable infection

frequencies across tick populations (Cafiso et al., 2016; Clay et al., 2008; Duron et al., 2017, 2016; Lalzar et al., 2012). Facultative endosymbionts are expected to play various roles in tick fitness, reproduction or immunity. Their effects remain largely unstudied yet (Bonnet et al., 2017; Narasimhan and Fikrig, 2015).

Spiroplasma ixodetis (Mollicutes: Entomoplasmatales: Spiroplasmataceae) is one of the most intriguing facultative endosymbionts of ticks. The *Spiroplasma* genus includes a variety of commensal, pathogenic and mutualistic species of arthropods and plants (Gasparich, 2002). In 1981, a new *Spiroplasma* was isolated from extracts of Western black-legged ticks, *Ixodes pacificus* (Tully et al., 1981). This bacterium was morphologically and genetically distinct from known *Spiroplasma* species (Tully et al., 1983), and was formally described as *S. ixodetis* (Tully et al., 1995). Since its initial discovery, membership of the species *S. ixodetis* has expanded, and it is now known to be widespread in ticks: in addition to *I. pacificus*, it was found in *I. ricinus*, *I. arboricola*, *I. frontalis*, *I. uriae*, *I. ovatus*, *I. persulcatus*,

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several other undetermined *Ixodes* species, *Dermacentor marginatus*, *Rhipicephalus annulatus*, *R. decoloratus*, *R. pusillus* and *R. geigy* (Bell-Sakyi et al., 2015; Duron et al., 2017; Henning et al., 2006; Hornok et al., 2010; Qiu et al., 2014; Taroura et al., 2005; Van Oosten et al., 2018).

Surveys of microbial diversity have further uncovered *S. ixodetis* in other arthropod groups, including spiders, mites, butterflies, leafhoppers, phasmids, aphids, various flies and coleopterans (Duron et al., 2008; Fukatsu et al., 2001; Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 2000, 1999; Martin et al., 2012; Martin et al., 2013; Sanada-Morimura et al., 2013; Simon et al., 2011; Tinsley and Majerus, 2006; DiBlasi et al., 2011; Pérez-Ruiz et al., 2015; Weinert et al., 2007). In all examined cases, *S. ixodetis* showed stable maternal (transovarial) transmission through successive host generations (Fukatsu et al., 2001; Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 2000, 1999; Tinsley and Majerus, 2006). In most insects, such as the African queen butterfly *Danaus chrysippus* and a variety of ladybird species, *S. ixodetis* acts as a male-killer: the viability and fertility of infected females is usually either not, or only mildly, affected, but *S. ixodetis* can be strongly virulent in males, often killing them (Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 2000, 1999; Sanada-Morimura et al., 2013; Tinsley and Majerus, 2006). The bacterium typically induces the death of male hosts during embryonic development and results in (almost) all-female broods from infected mothers (Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 2000, 1999; Sanada-Morimura et al., 2013; Tinsley and Majerus, 2006). Insect species harboring *S. ixodetis* thus show a sex bias in infection prevalence, with some females found infected while infection is never (or rarely) observed in males (Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 2000, 1999; Sanada-Morimura et al., 2013; Tinsley and Majerus, 2006). Male hosts being “dead ends” for transmission, male-killing benefits *S. ixodetis* transmission by favoring reproduction of infected female hosts through the reallocation of resources from males to females (e.g. by reducing sibling competition for food), or through the reduction of inbreeding depression (Engelstadter and Hurst, 2009). However, not all *S. ixodetis* members are male-killers since there is no evidence of sex bias in infection in spiders, leafhoppers or crane flies (Duron et al., 2008). The tree-hole tick *I. arboricola* is the single tick species examined for sex bias in *S. ixodetis* infection but no evidence of male-killing was found (Van Oosten et al., 2018).

How *S. ixodetis* infections have spread across tick species is still unclear. Phylogeny can be used to this aim by estimating proximity of current infections and retracing their evolutionary histories. To date, the 16S rRNA gene sequence has been used as an exclusive marker for the *S. ixodetis* description, with all known strains represented only by this gene (e.g. Bell-Sakyi et al., 2015; Duron et al., 2008; Fukatsu et al., 2001; Jiggins et al., 2000). However, this marker is inadequate for inferring a reliable intraspecific phylogeny since the inner topology is poorly resolved because of insufficient 16S rRNA sequence polymorphism. Therefore, in the present study, we characterized *S. ixodetis* variation in ticks and other arthropods at five genes (16S rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG*). We further attempted to characterize overall *S. ixodetis* diversity and to infer the evolutionary processes shaping this diversity. We additionally tested the African blue tick, *Rhipicephalus decoloratus* for possible evidence of male-killing activity, by assessing sex bias in *S. ixodetis* infection.

2. Material and methods

2.1. Tick collection

The multi-locus typing of *S. ixodetis* was conducted using a collection of DNA templates from 12 tick infected species (Table 1). Of the 12 tick species examined, seven belong to the *Ixodes* genus, four to *Rhipicephalus* and one to *Dermacentor*. Most specimens were collected on taxonomically diverse vertebrate hosts or their environment (Table 1). In addition, another collection of DNA templates from 12 arthropod

species infected by *S. ixodetis* was used: it includes a parasitic mite species and 11 insect species (five species of Hemiptera and six of Diptera) (Table 1). For each DNA template, *S. ixodetis* infection had been previously confirmed through sequencing of the 16S rRNA gene (Duron et al., 2017, 2008), or of intergenic ribosomal spacer and adjacent regions (Martin et al., 2012, 2013). Within each examined species, there were one to four *S. ixodetis*-infected individuals separately investigated for the multi-locus typing analysis (Table 1).

The consequences of *S. ixodetis* infection for tick reproduction was examined using field-caught adult specimens of the African blue tick *R. decoloratus* sampled from three localities in South Africa and one in Benin (Table 2). All specimens were sexed on the basis of their morphological characters and further examined for a sex bias in *S. ixodetis* infection prevalence.

2.2. Molecular screening and typing

Each DNA template was obtained from individual extraction of tick whole body using the DNeasy Blood and Tissue Kit (QIAGEN) following manufacturer instructions. To design specific PCR primers for multi-locus typing, we used the draft genome of *S. ixodetis* (*S. ixodetis* Y32 type strain, early isolated from *I. pacificus* extracts by Tully et al., 1981, and further sequenced by X. Bailly, unpublished data) as a reference. Along with the 16S rRNA gene, we selected four other genes (*rpoB*, *dnaK*, *gyrA* and *EpsG*) present in a single copy within the *S. ixodetis* Y32 draft genome. Gene features, primers and PCR conditions are detailed in Table 3. Fragments of these five genes were amplified using nested or semi-nested PCR assays which were efficient at increasing sensitivity and specificity for *S. ixodetis* detection. The efficiency of our typing method was ascertained through positive PCR amplification and clear sequences for the five loci from DNA samples of *I. ricinus* infected by *S. ixodetis* as we further detailed in the Results section. The nested PCR assay targeting the 16S rRNA gene was further used to examine the specimens of *R. decoloratus* for sex bias in *S. ixodetis* infection prevalence.

Nested and semi-nested PCR amplifications were performed as follows: the first PCR run with the external primers was performed in a 10 μ L volume containing ca. 20 ng of genomic DNA, 3 mM of each dNTP (Thermo Scientific), 8 mM of MgCl₂ (Roche Diagnostics), 3 μ M of each primer, 1 μ L of 10 \times PCR buffer (Roche Diagnostics), and 0.5 U of Taq DNA polymerase (Roche Diagnostics). A 1 μ L aliquot of the PCR product from the first reaction was then used as a template for the second round of amplification. The second PCR was performed in a total volume of 25 μ L and contained 8 mM of each dNTP (Thermo Scientific), 10 mM of MgCl₂ (ThermoScientific), 7.5 μ M of each of the internal primers, 2.5 μ L of 10 \times PCR buffer (Thermo Scientific), and 1.25 U of Taq DNA polymerase (Thermo Scientific). All PCR amplifications were performed under the following conditions: initial denaturation at 93 °C for 3 min, 35 cycles of denaturation (93 °C, 30 s), annealing (T_m = 49–56 °C, depending on primers, 30 s), extension (72 °C, 1 min), and a final extension at 72 °C for 5 min. To prevent possible contamination, first and second PCR runs have been physically separated from one another, in entirely separate rooms. Known positive and negative individuals were used as controls in each PCR assay. All PCR products were visualized through electrophoresis in a 1.5% agarose gel. All amplicons from nested and semi-nested PCR assays were purified and sequenced in both directions (EUROFINS). The chromatograms were manually inspected and cleaned with CHROMAS LITE (http://www.technelysium.com.au/chromas_lite.html) and sequence alignments were done using CLUSTALW (Thompson et al., 2002), both implemented in MEGA (Tamura et al., 2007). Alleles were determined on the basis of sequence identity (i.e. the number of characters which match exactly between two different sequences). If two sequences differed by one or more bases, they were described as two different alleles. Nucleotide sequences of *S. ixodetis* have been deposited in the GenBank nucleotide database (Accession numbers: 16S rRNA: MK248055-MK248068, MK241976,

Table 2
Populations of *Rhipicephalus decoloratus* examined for sex bias in infection prevalence of *Spiroplasma ixodensis*.

Population	Vertebrate host	n	Number of infected specimens				
			males	females	males and females	males and females	
Vaalwater, South Africa, 2010	Zebu (<i>Bos indicus</i>)	10	9	19	2 (0.20%)	0 (0%)	2 (0.11%)
Sandvelt, South Africa, 2011	Blue Wildebeest (<i>Connochaetes taurinus</i>), Greater Kudu (<i>Tragelaphus strepsiceros</i>) and Southern Eland (<i>Taurotragus oryx</i>)	10	16	26	4 (0.40%)	9 (0.56%)	13 (0.50%)
Hopetown, South Africa, 2012	Zebu (<i>Bos indicus</i>)	10	13	23	0 (0%)	0 (0%)	0 (0%)
Gogonou, Benin, 2012	Zebu (<i>Bos indicus</i>)	0	6	6	0 (0%)	0 (0%)	0 (0%)
All populations	-	30	44	74	6 (0.20%)	9 (20.5%)	15 (20.3%)

Table 3
List of genes and primers used for *Spiroplasma ixodensis* typing.

Gene	Hypothetical product	Primers (5'-3')	Tm and Fragment size	References
16S rDNA	Small ribosomal subunit	Spix0_F2- TTAGGGGCTCAAGCCCTAACCGC	First PCR run: 56 °C, Spix0_F2/Spix0_R2: 865 bp Second PCR run: 56 °C, Spix0_F2/Spix0_R1: 822bp	This study (modified from Duron et al., 2008)
		Spix0_R2- CCTGCTTTGGGAGGTCGGTCC		
		Spix0_F2- TTAGGGGCTCAAGCCCTAACCGC		
rpoB	DNA-directed RNA polymerase β chain	Spix0_R1- TCTGGCATTCGCAACTCTGTGG	First PCR run: 52 °C, Spix0_rpoB_F1/Spix0_rpoB_R2: 984 bp Second PCR run: 54 °C, Spix0_rpoB_F2/Spix0_rpoB_R1: 628bp	This study
		Spix0_rpoB_F1- TTGTTGTAAGGGGTGTGAG		
		Spix0_rpoB_R2- CATCAAAAACGAGERTTGTCT		
		Spix0_rpoB_F2- GGAGCTTCAATGAAAGATGG		
		Spix0_rpoB_R1- CAACGGGTTCCATCTCT		
		Spix0_dnaK_F1- GCTGCCATGCTTAAGTTT		
dnaK	Chaperone protein DnaK	Spix0_dnaK_R2- ACACCTCTTGGTGGTTC	First PCR run: 52 °C, Spix0_dnaK_F1/Spix0_dnaK_R2: 871bp Second PCR run: 49 °C, Spix0_dnaK_F2/Spix0_dnaK_R1: 515bp	This study
		Spix0_dnaK_F2- TCGTGTGAAAAGCAAAA		
gyrA	DNA gyrase subunit A	Spix0_dnaK_R1- ACAGCGGTTGATTTCTTC	First PCR run: 54 °C, Spix0_gyrA_F1/Spix0_gyrA_R: 573bp Second PCR run: 54 °C, Spix0_gyrA_F2/Spix0_gyrA_R: 561bp	This study
		Spix0_gyrA_R- TCCACTGTAGGAAAGTCAGG		
EpsG	Transmembrane protein EpsG	Spix0_gyrA_F2- CCAGATGCAAGAGATGGATTG	First PCR run: 51 °C, Spix0_EpsG_F1/Spix0_EpsG_R2: 676bp Second PCR run: 54 °C, Spix0_EpsG_F2/Spix0_EpsG_R2: 505bp	This study
		Spix0_EpsG_F1- CAAGCAACGATCAAAATCA		
		Spix0_EpsG_R2- AGCGAATGCTATGTCATTC		
		Spix0_EpsG_F2- CANTGACCAAAATGTGGGTAAA		

Table 4

Allelic profile of the five polymorphic genes in *Spiroplasma ixodetis* from the 12 tick species and 12 other arthropod species examined in this study. Letters a–q represent the different alleles at each *S. ixodetis* locus. Dashes indicate an absence of gene PCR amplification. Letters A–V represent the seven *S. ixodetis* haplotypes identified on the basis of 16S rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG* allelic profiles.

Host species of <i>Spiroplasma ixodetis</i>	<i>Spiroplasma ixodetis</i> genes					<i>Spiroplasma ixodetis</i> haplotypes
	16S rDNA	<i>rpoB</i>	<i>dnaK</i>	<i>gyrA</i>	<i>EpsG</i>	
<i>Ixodes pacificus</i>	a	a	a	a	a	A (<i>S. ixodetis</i> Y32 type strain)
<i>Ixodes arboricola</i>	b	b	-	-	-	B
<i>Ixodes frontalis</i>	c	c	b	b	b	C
<i>Ixodes ricinus</i>	d	c	b	b	b	D
<i>Ixodes uriae</i>	d	c	b	b	-	E
<i>Ixodes</i> sp. (#1)	e	d	c	c	c	F
<i>Ixodes</i> sp. (#3)	d	c	b	b	-	E
<i>Dermacentor marginatus</i>	f	-	-	-	d	G
<i>Rhipicephalus annulatus</i>	g	e	d	d	b	H
<i>Rhipicephalus decoloratus</i>	g	e	d	d	b	H
<i>Rhipicephalus geigy</i>	g	e	d	d	b	H
<i>Rhipicephalus pusillus</i>	h	-	-	-	-	I
<i>Dermanyssus gallinae</i> (1)	i	f	e	e	e	J
<i>Dermanyssus gallinae</i> (2)	i	f	f	f	e	K
<i>Acyrtosiphon pisum</i>	a	g	-	g	f	L
<i>Cinara confinis</i>	j	-	-	-	-	M
<i>Cinara maritimae</i>	g	h	-	h	b	N
<i>Cicadella viridis</i>	k	i	g	i	g	O
<i>Notostira elongata</i>	l	j	h	j	h	P
<i>Ceratinostoma ostiorum</i>	m	k	i	k	-	Q
<i>Scathophaga stercoraria</i>	n	l	j	-	-	R
<i>Cinocera</i> sp.	o	m	k	l	-	S
<i>Ethromyia chalybea</i>	a	n	l	m	b	T
<i>Poecilobothrus chrysozygus</i>	p	o	m	m	b	U
<i>Thrypticus tarsalis</i>	q	c	b	n	i	V

peaks, indicating a confident degree of primer specificity for *S. ixodetis* nested and semi-nested PCR amplifications. All these sequences belong unambiguously to the *S. ixodetis* species as described below. Further characterizations were thus based on 1 to 5 *S. ixodetis* gene markers per DNA template (Table 4).

On the basis of DNA sequencing, we characterized 9 to 17 distinct alleles depending on *S. ixodetis* gene (Table 4), including 17 16S rRNA alleles (97.4–99.9% pairwise nucleotide identity), 16 *rpoB* alleles (86.6–99.8%), 13 *dnaK* alleles (85.3–99.8%), 14 *gyrA* alleles (94.2–99.8%) and 9 *EpsG* alleles (94.5–99.8%). The 16S rRNA alleles we observed here showed a 98.3–100% pairwise nucleotide identity with the *S. ixodetis* Y32 type strain, suggesting that all these bacteria belong to the *S. ixodetis* species. Overall, the allelic variation at the five gene markers led to the identification of 22 genetically different *S. ixodetis* haplotypes (A–V hereafter, cf. Table 4) allied to 12 tick species (9 haplotypes) or to the 12 other arthropod species (13 haplotypes). In the six arthropod species for which more than one individual was examined, no sequence variation at any *S. ixodetis* gene was observed between DNA templates from the same arthropod species, excepted for the poultry mite *Dermanyssus gallinae* for which two *S. ixodetis* haplotypes were found (Table 4).

The *S. ixodetis* A haplotype from our *I. pacificus* sample showed 100% nucleotide identity at the five gene markers with the *S. ixodetis* Y32 type strain. All the other tick and arthropod species were infected by *S. ixodetis* haplotypes differing from the *S. ixodetis* Y32 type strain (Table 4). Most of the arthropod species harbored a different *S. ixodetis*

haplotype with the exception of a few tick species, which were each infected by a genetically identical *S. ixodetis* haplotype on the basis of 16 rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG* typing. These tick species are *I. uriae*/*Ixodes* sp. 3 (*S. ixodetis* haplotype E) and *R. annulatus*/*R. decoloratus*/*R. geigy* (*S. ixodetis* haplotype H) (Table 4).

3.2. Phylogenetics of *Spiroplasma ixodetis*

We further examined the evolutionary relationships between the different *S. ixodetis* haplotypes, including the *S. ixodetis* Y32 type strain. A comparison with other *S. ixodetis* strains reported from previous studies was however restricted to the 16S rRNA gene sequences since no *S. ixodetis rpoB*, *dnaK*, *gyrA* and *EpsG* gene sequences were available in GenBank. A maximum-likelihood (ML) analysis based on 16S rRNA gene sequences was first used to examine the phylogenetic relationship of our 32 *S. ixodetis* 16S rRNA gene sequences with 16 other *S. ixodetis* sequences (including male-killer strains of the butterfly *Danaus chrysippus*, the planthopper *Laodelphax striatellus* and the ladybirds *Anisosticta novemdecimpunctata*, *Harmonia axyridis*, *Adalia bipunctata*) available in GenBank, as well as sequences of 16 other *Spiroplasma* species (Fig. 1). We recovered the clustering of all *S. ixodetis* 16S rRNA gene sequences within a robust monophyletic clade, and the *S. ixodetis* characterized in this study are phylogenetically closely related to the *S. ixodetis* found in other arthropod species. All other *Spiroplasma* species (including other endosymbionts of insects such as *S. poulsonii*) cluster outside the *S. ixodetis* clade (Fig. 1).

No *S. ixodetis* subclade specific to ticks are evident along 16S rRNA gene tree: the *S. ixodetis* found in ticks are rather scattered among *S. ixodetis* of parasitic mites, spiders and insects (Fig. 1). Indeed, the *S. ixodetis* of *I. ricinus* and *I. uriae* are closely related to the *S. ixodetis* male-killers of the ladybird *Anisosticta novemdecimpunctata* and of the planthopper *Laodelphax striatellus*. Another example includes the *S. ixodetis* of *R. pusillus*, *R. geigy*, *R. annulatus* and *R. decoloratus* which all cluster together in the same clade with the *S. ixodetis* of the aphid *Cinara maritimae*. However, the inner topology of the *S. ixodetis* clade based on 16S rRNA gene sequences remains too poorly resolved in many other cases (as shown by low support values of inner branches) for inferring the exact relatedness between all *S. ixodetis* from different arthropod species (Fig. 1).

Novel ML analyses based on *rpoB*, *dnaK*, *gyrA* and *EpsG* gene sequences were conducted to refine the *S. ixodetis* intrageneric phylogeny using our *S. ixodetis*-positive DNA templates, the *S. ixodetis* Y32 type strain genome sequences and other *Spiroplasma* genomes available in GenBank. When the sequences were examined separately for each gene, ML analyses confirmed that all *S. ixodetis* described in this study unambiguously belong to the *S. ixodetis* species (Fig. 2A–D). We observed no sign of recombination in the data set: the analysis of concatenated sequences did not detect significant recombination events between *S. ixodetis* strains (all $P > 0.05$ for the GENECONV and RDP recombination-detection tests). An additional ML analysis was conducted using 16S rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG* *S. ixodetis* concatenated dataset from 18 of our 32 DNA templates (at least one of the genes was not amplified from the 14 other DNA templates, see Table 4). It also confirms that all the strains characterized in this study belong to the *S. ixodetis* species (Fig. 3).

The single *rpoB*, *dnaK*, *gyrA* and *EpsG* gene phylogenies and concatenated phylogeny consistently showed that ticks harbor divergent *S. ixodetis* strains (Figs. 2A–D and 3). The *S. ixodetis* strain of *Ixodes pacificus* (including the Y32 type strain) was markedly different to other *S. ixodetis* strains on the basis of the *dnaK* and *EpsG* gene phylogeny (Fig. 2B and D). Similar observations were also obvious for the *S. ixodetis* strains of *Ixodes arboricola* for the *rpoB* gene (Fig. 2A), *Ixodes* sp.1 (*rpoB*, *dnaK*, *gyrA* and *EpsG*, Figs. 2A–D and 3) and *D. marginatus* (*EpsG*, Fig. 2D). Closely related *S. ixodetis* strains can also be found in phylogenetically divergent arthropod taxa: the *S. ixodetis* strains of *I. ricinus*, *I. uriae*, *Ixodes* sp. 3, *R. annulatus*, *R. decoloratus* and *R. geigy* all

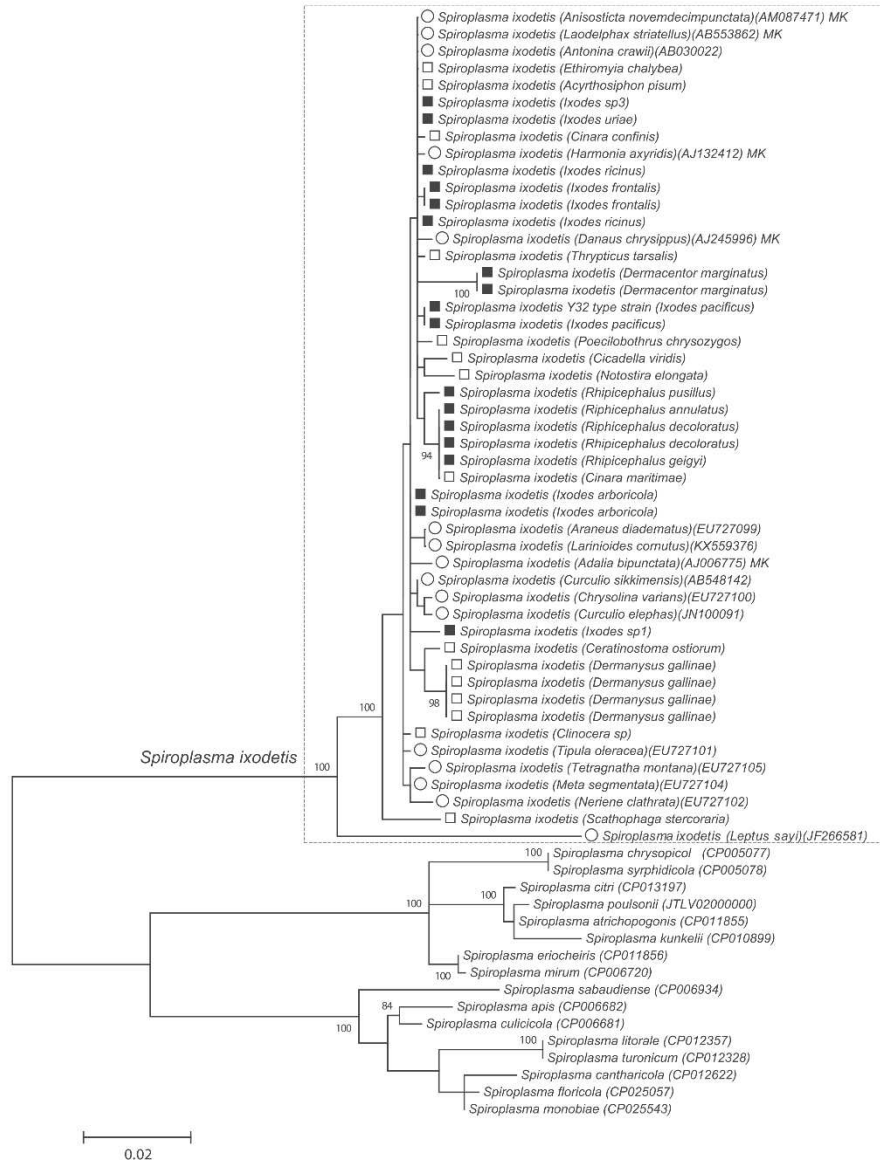


Fig. 1. Phylogeny of *Spiroplasma ixodetis* constructed using maximum-likelihood (ML) estimations based on 16S rRNA gene sequences (763 unambiguously aligned bp; best-fit approximation for the evolutionary model: GTR + G + I). Sequences from other *S. ixodetis* and *Spiroplasma* species available in GenBank were added to the analysis. The box delineates the *S. ixodetis* species. Black and white symbols indicate *S. ixodetis* strains found in ticks and other arthropods, respectively. Square symbols indicate *S. ixodetis* strains characterized in this study while circle symbols show other *S. ixodetis* strains already available in GenBank. MK indicates known *S. ixodetis* male-killer strains of insects. Bacterial name, host species (if applicable) and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values > 70% are shown). The scale bar is in units of substitution/site.

clustered together with the *S. ixodetis* strains of insects, including flies (e.g. the long-legged fly *Poecilobothrus chrysozygos*), aphids (the pea aphid *Acyrtosiphon pisum*), leafhoppers (*Cicadella viridis*) or mirid bugs (*Notostira elongata*) (Figs. 2A–D and 3). Overall, these phylogenies

revealed that *S. ixodetis* strains that were present in ticks did not cluster together within the same *S. ixodetis* subclade. However, it is noteworthy that the *S. ixodetis* strains of *Rhipicephalus* species clustered together (Figs. 1, 2A–D and 3), showing that related tick species may also harbor

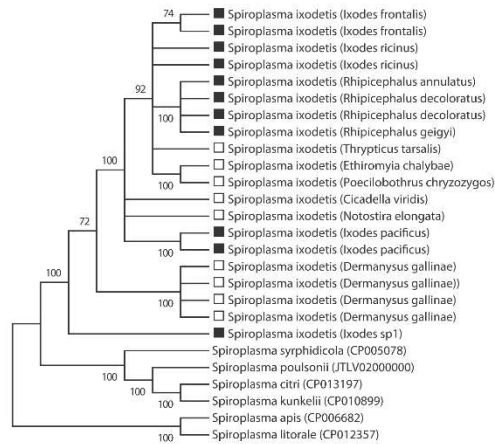


Fig. 3. Cladograms depicting the 70% majority-rule consensus of *Spiroplasma ixodetis* phylogenetic trees constructed using maximum-likelihood (ML) estimations based on concatenated 16S rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG* sequences (2805 unambiguously aligned bp; best-fit approximation for the evolutionary model: GTR + G + I). The box delineates the *S. ixodetis* species. Black and white squares indicate *S. ixodetis* strains found in ticks and other arthropods, respectively. Square symbols indicate *S. ixodetis* strains characterized in this study. Bacterial name, host species (if applicable) and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values > 70% are shown).

related *S. ixodetis* strains.

3.3. Sex bias in infection prevalence in *Rhipicephalus decoloratus*

We examined 74 *R. decoloratus* specimens (30 males and 44 females) from four populations for sex bias in *S. ixodetis* infection prevalence, as an indication of potential male-killing activity. Through the 16S rRNA nested PCR assay, we detected *S. ixodetis* infection in 15 specimens (20.3%) (Table 2). Infected specimens were found in two of the four populations, i.e. the Vaalwater ($n = 2$ infected specimens of 19) and the Sandvelt ($n = 13$ infected specimens of 26) populations. However, *S. ixodetis* showed no evidence of sex-biased prevalence in adult *R. decoloratus*: 6 of 30 males (20%) and 9 of 44 females (20.5%) were infected showing that there was no evidence to reject the null hypothesis of equal prevalence of *S. ixodetis* in male and females (Fisher's exact test, $p = 0.76$).

4. Discussion

We examined 12 tick species and 12 other arthropod species for the diversity of *S. ixodetis*, a maternally inherited endosymbiont primarily described from the Western black-legged tick, *I. pacificus* (Tully et al., 1981). Our multi-locus typing scheme confirmed the presence of *S. ixodetis* infections in diverse tick species, as shown by recent studies using the 16S rRNA gene sequence as an exclusive marker (Bell-Sakyi et al., 2015; Duron et al., 2017; Henning et al., 2006; Hornok et al., 2010; Qiu et al., 2014; Taroura et al., 2005; Van Oosten et al., 2018). Importantly, the examination of *rpoB*, *dnaK*, *gyrA* and *EpsG* gene sequences revealed a wider diversity of *S. ixodetis* than previously known from 16S rRNA gene sequences.

Phylogenetic analyses based on the multi-locus data set consistently showed that ticks harbor divergent *S. ixodetis* strains. The structure revealed was complex, showing that evolution of *S. ixodetis* has been

shaped by both maternal inheritance and horizontal transfers (HT) with frequent host turnover. Because of maternal inheritance, persistence of infection in a tick species over long periods of time should result in diversification of *S. ixodetis* alongside its tick host, that is to say in co-cladogenesis. This process may explain why the four *Rhipicephalus* species examined in this study harbor closely related *S. ixodetis* strains. Nevertheless, in most cases, our results rather revealed extensive HT since the *S. ixodetis* strains found in ticks do not form a tick-specific subclade. The *S. ixodetis* strains of ticks have different evolutionary origins as shown by their phylogenetic proximity with strains found in insects. The best examples are found in the *Ixodes* genus: the *S. ixodetis* strains found in *I. ricinus*, *I. uriae* and *Ixodes* sp. 3 are more closely related to strains found in aphids, leafhoppers and flies than the *S. ixodetis* strains found in *I. pacificus* (including the *S. ixodetis* Y32 type strain), *I. arboricola* and *Ixodes* sp. 1. The rarity of co-cladogenesis suggests that the long term maintenance of *S. ixodetis* infections is mainly possible through repeated interspecies transfers between ticks and other arthropods. A few case studies have demonstrated the key importance of ectoparasitic mites, which feed on arthropod haemolymph, for HT. Indeed, mites can serve as interspecific vectors of *Spiroplasma* spp. by picking up the endosymbiont from infected arthropods, and subsequently transferring the infection to recipient arthropods (Jaenike et al., 2007; DiBlasi et al., 2011). Specific adaptations facilitating HT are also likely present since *S. ixodetis* is one of the rare endosymbionts of arthropods able to grow outside the host cells (Tully et al., 1995). This ability may enhance the likelihood of successful HT between arthropod species, as empirically shown for another endosymbiont, *Arsenophonus* (Duron et al., 2010; Skinner, 1985).

Spiroplasma ixodetis infections in ticks showed the typical features of secondary endosymbionts: the patchy distribution within Ixodidae suggests that ticks are not dependent upon infection for survival or reproduction (Bell-Sakyi et al., 2015; Duron et al., 2017; Henning et al., 2006; Hornok et al., 2010; Qiu et al., 2014; Taroura et al., 2005; Van Oosten et al., 2018). The maternal transmission of *S. ixodetis* however implies that its transmission success should broadly depend on its effect on arthropod fitness (Engelstadter, Hurst, 2009). While the *S. ixodetis* strains found in ticks are related to the male-killer strains found in insects, we did not observe evidence for this in *R. decoloratus*, a result also previously observed in *I. arboricola* (Van Oosten et al., 2018). In this context, the effects of *S. ixodetis* infection on ticks may thus be more complex than previously considered in other arthropods. One can assume that *S. ixodetis* may confer multiple benefits to ticks with diverse roles in development, reproduction, defense against environmental stress or immunity. It is worthy of interest that some *Spiroplasma* spp. are defensive symbionts that can protect their insect hosts from their natural enemies, strongly reducing individual mortality, e.g. in pea aphids versus pathogenic fungi, or in *Drosophila* versus parasitic nematodes (Lukasik et al., 2013; Jaenike et al., 2010). Another possibility is that detection of *S. ixodetis* in ticks results from cross-contamination due to the presence of infected parasitic wasp eggs: although *S. ixodetis* has not been found in parasitic wasps of ticks so far, the presence of the endosymbiont *Wolbachia* in parasitic wasp eggs contained in the bodies of tick samples caused incidental detection of symbiont DNA (Tijssse-Klasen et al., 2011; Plantard et al., 2012). Anyway, the precise nature of how *S. ixodetis* affects tick biology remains to be characterized, suggesting a need for further investigations.

In conclusion, the *S. ixodetis* endosymbiosis represents an important but insufficiently studied component of tick biology. An important diversity of *S. ixodetis* is certainly present in ticks, yet what remains unclear is what possible role they may play in shaping tick biology, or the ecological processes that govern their distribution and abundance. Interesting in this regard is the intriguing pattern of *S. ixodetis* distribution within ticks: while infection is common in hard ticks (Ixodidae), the symbiont has never been detected in soft ticks (Argasidae) despite intensive screening efforts (Duron et al., 2017). Why soft ticks do not seem to harbour *S. ixodetis* is not currently

understood, but suggests that some so far unknown degree of host specificity may exist.

Competing interests

The authors declare that they have no competing interests.

Ethic statements

Some of the tick specimens were removed from wild and domestic animals, in accordance with the recommendations on the French National charter on the ethics of animal experimentation. The capture of animals was also carried out in strict accordance with good animal care practices recommended by the European guidelines. All captures were performed by competent people without causing avoidable pain, suffering, distress or lasting harm to animals.

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Résumé : Le microbiome désigne les communautés écologiques de micro-organismes hébergés par un hôte. De nos jours, les microbiomes sont perçus comme de véritables génomes accessoires déterminant le phénotype étendu de leurs hôtes. Les microbiomes, à l'image de leurs hôtes, sont extrêmement divers et structurés par tout un panel de facteurs biotiques, abiotiques et stochastiques. Ces facteurs varient en fonction de l'échelle résolutive concernée : la structure des microbiomes ne sera pas impactée par les mêmes facteurs au niveau de l'individu hôte ou de la métacommunauté d'hôtes. Dans ce contexte fondamental de l'écologie des communautés, ma thèse a notamment essayé de déterminer **quels sont les facteurs structurants d'un microbiome à différentes échelles résolutes**. Pour répondre à cette question, nous avons étudié les cortèges microbiens abrités par les tiques de Guyane. Ce modèle est particulièrement pertinent pour deux raisons : 1/ Il est admis que les tiques hébergent des communautés microbiennes complexes potentiellement assujetties à des facteurs structurants complexes. 2/ Il n'existe aucune donnée récente sur la diversité, la biologie ou le risque infectieux associés aux tiques de Guyane. Par conséquent, ces travaux de thèses ont, premièrement permis, de décrire des espèces de tiques jamais observées en Guyane et de caractériser des souches inconnues de bactéries potentiellement pathogènes, dont une nouvelle borrelie proche de celles responsables de la maladie de Lyme et de fièvres récurrentes. Deuxièmement, nous avons démontré qu'il existait de nombreux biais dans les études de microbiomes des tiques et que cela impactait l'interprétation des données de structuration microbienne. Cela se traduit notamment par l'observation artéfactuelle d'une forte diversité microbienne liée essentiellement au microbiome cuticulaire. En réalité, les microbiomes des tiques apparaissent majoritairement structurés par l'identité, la présence/absence et les interactions entre des bactéries intracellulaires mutualistes des tiques, que ce soit à une échelle intra-spécifique ou inter-spécifique. Enfin, nous avons mis en évidence que l'association entre les tiques et leur microbiome était également soumise à des patrons coévolutifs typiques des symbiotes intracellulaires d'arthropodes, ainsi qu'à des facteurs phylogéographique et bioécologique dépendant de l'hôte. Toutefois, il apparaît que la structuration des microbiomes chez les tiques est encore loin d'être totalement comprise, et que de futures études seront encore nécessaires pour démêler les différentes controverses sur la réalité biologique des microbiomes hébergés par les tiques.

Mots-clés : microbiome, tiques, Guyane, structuration microbienne, coévolution, endosymbiose, agent pathogène transmis par les tiques

Abstract: Microbiome refers to the ecological communities of microorganisms hosted by a host. Microbiomes are now perceived as true accessory genomes that determine the extended phenotype of their hosts. They are, like their hosts, extremely diverse and structured by a wide range of biotic, abiotic and stochastic drivers. These factors vary according to the resolution scale: the structure of microbiomes will not be impacted by the same factors at an individual scale or an interspecific scale. In this fundamental context of community ecology, my thesis tried to determine **what the microbiome drivers at different resolution scales are**. To answer this question, we studied the microbial community sheltered by ticks in French Guiana. This model is particularly relevant for two reasons: 1/ It is recognized that ticks harbour complex microbial communities that appear to be subject to a diversity of potential drivers. 2/ There are no recent data on the diversity, biology or infectious risks associated with ticks in French Guiana. As a result, in this thesis we described tick species which were never observed in French Guiana and characterized unknown strains of potentially pathogenic bacteria, including a new borrelia related to Lyme disease and to relapsing fever ones. Then, we demonstrated the ubiquity of biases in tick microbiome studies and their impact on the interpretation of microbiome data by confusing cuticular and internal microbes. Tick microbiomes actually appear mainly structured by the identity, the presence/absence and the interactions between mutualistic intracellular bacteria of ticks, whether on an intra- or interspecific scale. Finally, we have shown that the association between ticks and their microbiome is also subject to co-evolutionary patterns, typical of intracellular symbionts of arthropods, as well as phylogeographic and bio-ecological factors depending of the tick hosts. However, the microbiome structure in ticks, is still far from being fully understood and future studies are necessary to resolve the various controversies on the biological reality of microbiomes hosted by ticks.

Key words: microbiome, ticks, French Guiana, microbial structuring, coevolution, endosymbiosis, tick-borne pathogen