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Caractérisation des espèces aphylls du genre *Vanilla* endémiques de Madagascar en vue de leur conservation

Felambinintsoa Cathucia Andriamihaja

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UNIVERSITÉ DE LA RÉUNION

ÉCOLE DOCTORALE SCIENCES, TECHNOLOGIES ET SANTÉ

UNITÉ MIXTE DE RECHERCHE PEUPELEMENTS VÉGÉTAUX ET BIOAGRESSEURS EN MILIEU
TROPICAL - UMR PVBMT

THÈSE

pour l'obtention du titre de

DOCTEUR DE L'UNIVERSITÉ DE LA RÉUNION

Spécialité : Génétique

Par : ANDRIAMIHAJA Felambinintsoa Cathucia

CARACTÉRISATION DES ESPÈCES APHYLLES DU GENRE *Vanilla*
ENDÉMIQUES DE MADAGASCAR EN VUE DE LEUR
CONSERVATION

Le 15 juillet 2021

Jean-Yves DUBUISSON	Professeur	Sorbonne Université	Rapporteur
Achille RASELIMANANA	Professeur	Université d'Antananarivo	Rapporteur
Bakolimalala RAKOUTH	Professeure	Université d'Antananarivo	Examinatrice
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Aro Vonjy RAMAROSANDRATANA	MCF	Université d'Antananarivo	Co-encadrant
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UNIVERSITÉ D'ANTANANARIVO

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LABORATOIRE DE PHYSIOLOGIE VÉGÉTALE

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A ma fille

Duc in Altum....

(Explorer ! Aller dans les plus grandes profondeurs...)

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(La solidarité est une force...)*

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LISTE DES ABRÉVIATIONS

Abréviations scientifiques

- ADN** – Acide désoxyribonucléique
AMOVA - Analysis of molecular variance
ARN – Acide ribonucléique
BEAST - Bayesian Evolutionary Analysis Sampling Trees
BIC - Bayesian Information Criterion
BSC – Biological species concept
CAM – Crassulacean acid metabolism
cpDNA – Chloroplast DNA
dbRDA - Distance-based redundancy analysis with randomization
DNA- Deoxyribonucleic acid
DT - Decision-theoretic performance-based approach
EcSC - Ecological species concept
ESU - Evolutionary Significant Unit
EvSC - Evolutionary species concept
GBS - Genotyping by sequencing
GSC - Genotypic species concept
HadCM3 - Hadley Center for Coupled Model 3
HW – Hardy Weinberg
HWE – Hardy –Weinberg equilibrium
IBA - Isolation by adaptation (isolement par adaptation)
IBD – Isolation by distance (isolement par la distance)
IBE - Isolation by environmental factors (isolement par les facteurs environnementaux)
ISSR – Inter Simple Sequence Repeat
ITS – Internal Transcribed Spacer (ribosomal RNA genes)
LD – Linkage disequilibrium
MANOVA - Multivariate analysis of variance
matK – Maturase K gene
MCMC - Monte Carlo Markov Chain
ML - Maximum Likelihood
ML – Maximum Likelihood (maximum de vraisemblance)
MMRR – Multiple matrix regression with randomization
MP - Maximum Parsimony
MU – Management Unit
NGS - Next generation sequencing
NJ - Neighbor-joining
PC - Principal component
PCA - Principal Component Analysis
PCR – Polymerase Chain Reaction
-

PheSC - Phenetic species concept
PhySC - Phylogenetic species concept
psaB - Photosystem I P700 chlorophyll a apoprotein A2 gene
psbB - Photosystem II CP47 reaction center protein gene
psbC - Photosystem II CP43 reaction center protein gene
RAD-seq – Restriction-site associated DNA
rbcL - Ribulose biphosphate carboxylase large chain (RuBisCO) gene
SDM - Species distribution modelling
SNP - Single nucleotide polymorphism
SSR - Simple Sequence Repeats
TCG - Taxonomically Complex Group

Autres abréviations

BRC - Biological Resource Center
CBNF - Fédération des Conservatoires Botaniques Nationaux
CBNM – Conservatoire Botanique National Mascarin
CCCM - Canadian Center for Climate Modeling and Analysis
CITES - Convention on International Trade in Endangered Species
CR - Critically Endangered IUCN Red List (en danger critique, liste rouge UICN)
CRB – Centre de Ressources Biologiques
CSIRO - Commonwealth Scientific and 255 Industrial Research Organisation
DD - Data Deficient IUCN Red List
EN - Endangered IUCN Red List (en danger, liste rouge UICN)
EW - Extinct in the Wild IUCN Red List (éteinte à l'état sauvage, liste rouge UICN)
EX - Extinct IUCN Red List (éteinte, liste rouge UICN)
IPCC - Intergovernmental Panel on Climate Change
IUCN - International Union for Conservation of Nature
LC - Least Concern IUCN Red List (préoccupation mineure, liste rouge UICN)
LGM - Last Glacial Maximum
Mya (Ma) - Million years ago (millions d'années)
MNHN - Muséum National d'Histoire Naturelle
NE - Not Assessed IUCN Red List
NT – Near Threatened IUCN Red List (quasi menacée, liste rouge UICN)
SOOI - Sud-Ouest de l'océan Indien
SWIO – South-West Indian ocean
UICN - Union internationale pour la conservation de la nature
UMR PVBMT – Unité Mixte de Recherche Peuplements Végétaux et Bioagresseurs en milieu tropical
VU – Vulnerable IUCN Red List (vulnérable, liste rouge UICN)
WCSPF - World checklist of selected plant families

Unités

bp - basepairs, (**pb** - paire de bases)

ha - hectare, **Kha** - kilohectare

g - gramme, **ng** - nanogramme, **µg** - microgramme, **mg** - milligramme

µl - microlitre, **ml** - millilitre

m - mètre, **mm** - millimètre, **cm** - centimètre, **km** - kilomètre

M - molaire, **µM** micromolaire

s - seconde, **min** - minute, **h** - heure

°C - degré Celsius

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CHAPITRE 1

CHAPITRE 1

Introduction

1.1. La biodiversité : processus évolutifs et spéciation

*"L'homme est devenu la plus grande force évolutive du monde."
Palumbi*

Introduite pour la première fois sous le terme « diversité biologique » en 1968 (Dasmann, 1968), la biodiversité est devenue aujourd'hui un concept populaire au cœur de tous les débats, que ce soit sur le plan scientifique, social, économique ou même politique. La biodiversité fait partie des termes scientifiques qui ont reçu plusieurs définitions au fil du temps (DeLong, 1996). Mais en général, elle désigne la variabilité des organismes vivants et regroupe trois niveaux : i) la diversité intraspécifique mesurée en termes de diversité génétique ou phénotypique¹ au sein d'une espèce, ii) la diversité des espèces, iii) la diversité des communautés et des écosystèmes ainsi que des processus écologiques qui s'y opèrent (Dirzo and Raven, 2003; United Nations, 1992). Au-delà de constituer un sujet principal largement étudié en biologie, elle est également le garant de la durabilité des écosystèmes et de ses composantes, le fondement de la sécurité alimentaire et un facteur clé du développement économique à l'échelle mondiale (Chivian, 2002). Le grand ensemble des organismes vivants sur la terre est le fruit d'un long processus évolutif ayant opéré depuis plusieurs millions d'années. L'évolution, terme désignant le passage d'un état à un autre, est un phénomène commun à plusieurs disciplines d'études. En géologie et en géographie, la théorie de la dérive des continents suggère, par exemple, un déplacement progressif des plaques continentales tandis que le changement climatique en météorologie admet l'évolution des paramètres liés au climat dont principalement la précipitation et la température (Frankel, 1988; Nooten et al., 2014; Tadross et al., 2008). Néanmoins, la notion d'« évolution » est surtout connue comme le phénomène biologique à l'origine de la transformation des organismes primitifs, de l'extinction des espèces ou de la formation de nouvelles espèces (Burkhardt, 2013; Losos et al., 2014; Wright, 1931). Au cours de l'histoire, plusieurs théories ont vu le jour pour expliquer le processus évolutif. Lamarck (1800) fut le premier à apporter une théorie, dite transformiste, sur l'évolution en affirmant que les organismes développent de nouvelles habitudes pour s'adapter à leur environnement, les maintiennent pendant de longues périodes et les transmettent à leurs descendants (Burkhardt, 2013). Cependant, les idées de Lamarck furent longtemps critiquées, car elles n'expliquaient pas clairement le fondement de l'hérédité ou la transmission des caractères acquis (Burkhardt, 2013). Cinquante ans plus tard, Darwin a proposé une théorie de l'évolution basée sur la sélection naturelle qu'il a essayé de résumer

¹ Phénotype : ensemble des caractères apparents d'un individu

sous forme d'un arbre ramifié (Darwin, 1859). Dans son célèbre ouvrage « *On the origin of species* », Darwin expose plusieurs principes (Darwin, 1859). Premièrement, il explique l'idée que les espèces ne sont pas des entités fixes, mais présentent des variations naturelles plus ou moins importantes. Ensuite, il affirme que les organismes produisent plus de descendants que nécessaire pour compenser leur disparition à la génération suivante. Néanmoins, les populations naturelles conservent généralement des tailles à peu près constantes régulées par le succès reproductif différent entre les individus du fait de la sélection naturelle (Darwin, 1859). En d'autres termes, certains individus porteraient des caractères favorables qui leur permettraient de survivre dans leur environnement contrairement à d'autres. Le quatrième principe de Darwin évoque la théorie de la « descendance avec modification » en stipulant que tous les organismes proviennent de la modification d'un ancêtre commun sous l'effet de la sélection naturelle. Tout comme celle de Lamarck, la théorie de Darwin sur la formation des espèces (spéciation) a fait l'objet de vives critiques comme elle ne permettait pas d'expliquer complètement l'origine des variations et de définir la notion d'espèce (Mallet, 2008). Avec l'essor de la génétique grâce aux travaux de Mendel (1866), la théorie néodarwinienne combinant plusieurs branches de la biologie dont les idées de Darwin et la génétique fit son apparition (Ayala and Fitch, 1997; Ho and Saunders, 1979; Mayr, 1942; Stebbins, 1950). En plus de conserver le concept de la sélection naturelle, le néodarwinisme précise que l'évolution s'opère graduellement soit par anagenèse (remplacement d'une ancienne lignée par une nouvelle) soit par cladogenèse (séparation de deux nouvelles lignées à partir d'une lignée ancestrale) via des mutations génétiques (Dajoz, 2012; Ho and Saunders, 1979). Après 1960, d'autres courants d'idées sont apparus parmi lesquels : i) la « théorie des équilibres ponctués » qui suggère une évolution des espèces par périodes ponctuelles séparées par des périodes stagnantes, et non de manière graduelle (Ahad, 2019; Gould and Chaline, 1983), ii) la théorie neutraliste de Kimura qui accorde plus d'importance à l'effet combiné de la dérive génétique, des mutations neutres et des migrations dans les processus évolutifs (Kimura, 1979), iii) l'évolution réticulée qui est le processus par lequel deux organismes éloignés phylogénétiquement échangent du matériel génétique via des mécanismes comme le transfert horizontal de gènes ou l'hybridation (Linder and Rieseberg, 2004; Richardson and Palmer, 2006; Vriesendorp and Bakker, 2005). Ainsi, jusqu'à ce jour, il y a une quête incessante pour essayer de comprendre l'origine de la diversité du vivant. Même si les théories évolutionnistes se sont beaucoup perfectionnées au fil du temps, elles suscitent encore des controverses (Dajoz, 2012). Néanmoins, elles représentent des jalons de connaissances essentiels pour expliquer les mécanismes de la formation des espèces ou « spéciation ».

La spéciation est un processus évolutif complexe. Comme le présentait Darwin, les processus (microévolution) qui régissent l'évolution des populations représentent un continuum avec ceux (macroévolution) à l'origine des espèces. La spéciation débute par l'accumulation de diversité intraspécifique dans les populations sous l'influence des forces évolutives comme la dérive

génétique², la sélection naturelle, la mutation³ et la migration (Darwin, 1859; Gandon and Nuismer, 2009; Johnson et al., 2018; Wright, 1931) qui peut aboutir au cours du temps à la formation de nouvelles espèces distinctes, sans intermédiaires, adaptées à des niches spécifiques (Darwin, 1859; Mallet, 2008). La complexité du processus de spéciation est attribuée tout d'abord à la notion d'espèce qui pose de sérieux problèmes dans la classification du vivant. En effet, il existe aujourd'hui plusieurs concepts d'espèces basés sur différents critères (Figure 1) (Aldhebiani, 2018; De Queiroz, 2007, 2005, 1998).

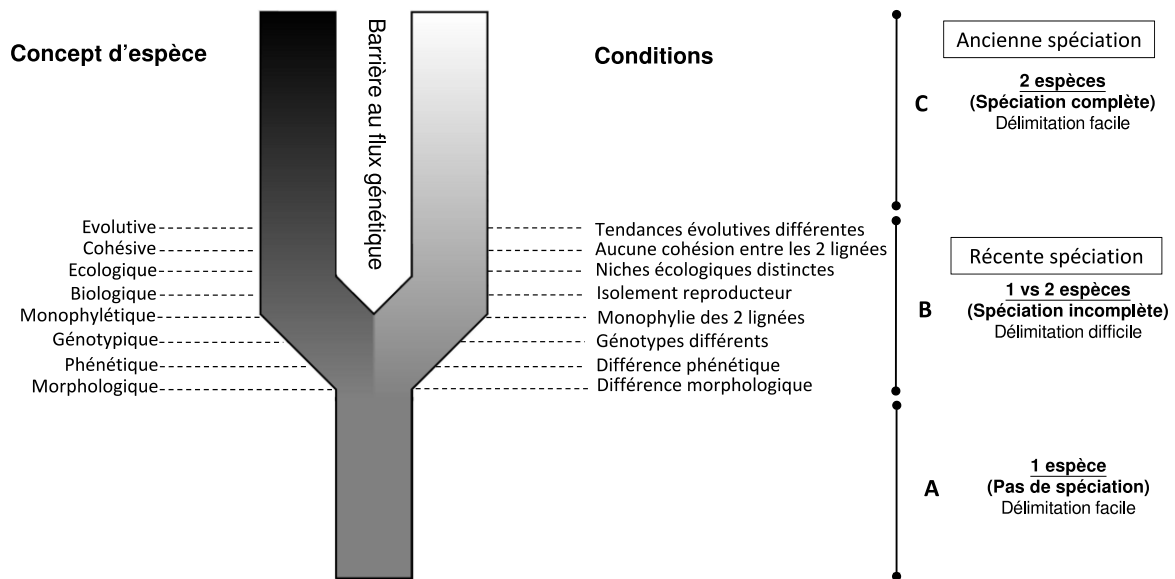


Figure 1. Concepts d'espèce et mécanismes de séparation et de divergence (spéciation).

Figure adaptée de De Queiroz, (2007) et Shaffer and Thomson (2007). La figure représente la formation de deux nouvelles lignées à partir d'un ancêtre commun. Le changement graduel des couleurs grises représente l'évolution continue de la lignée ancestrale en deux lignées filles divergentes à travers le temps. A—Avant la division, tous les individus se ressemblent, il n'y a pas de spéciation et la délimitation de l'espèce est facile. B— Au cours de la spéciation, les individus des deux nouvelles lignées acquièrent progressivement des caractéristiques différentes (morphologiques, génétiques, écologiques, etc.). Dans cet intervalle, il est difficile de définir la notion d'espèce, car les concepts alternatifs entrent en conflit. C'est le cas des espèces récentes dont les processus de spéciation ne sont pas encore achevés. Les mécanismes (concepts) ne sont pas représentés suivant un ordre sur la figure. C—Une fois que toutes les caractéristiques distinctives sont acquises par chacune des deux espèces, la spéciation est complète. La délimitation est facile.

Dans le cas d'une spéciation ancienne, la notion d'espèce est claire (Shaffer and Thomson, 2007), car tous les concepts sont applicables (Figure 1). Par contre, si la spéciation est récente, la délimitation des espèces est difficile (Shaffer and Thomson, 2007), car les critères d'identification des espèces (concepts) ne sont pas tous satisfaits et dépendront des propriétés biologiques du groupe étudié (De Queiroz, 2007) (Figure 1). Ainsi, le temps est un facteur important dans les processus de spéciation. Selon Coyne et Orr (2004), l'intensité de l'isolement reproducteur entre les populations divergentes augmente généralement avec le nombre de générations apparues depuis le début du processus. D'après Nosil et al. (2009), la spéciation se

² Dérive génétique : variation aléatoire des fréquences alléliques au sein d'une population et au cours des générations

³ Mutation : modification rare, accidentelle ou provoquée, de l'information génétique (séquence d'ADN ou ARN) dans le génome

fait d'une manière progressive le long d'un continuum allant de l'initiation, où l'isolement reproducteur est absent, les populations appartiennent au même clusteur génétique et à la même lignée et se rencontrent dans un milieu géographique et/ou écologique plus ou moins similaire, jusqu'au stade final caractérisé par l'isolement reproducteur total, la différenciation génotypique, la séparation des lignées et des changements abrupts de leurs environnements (Figure 2A).

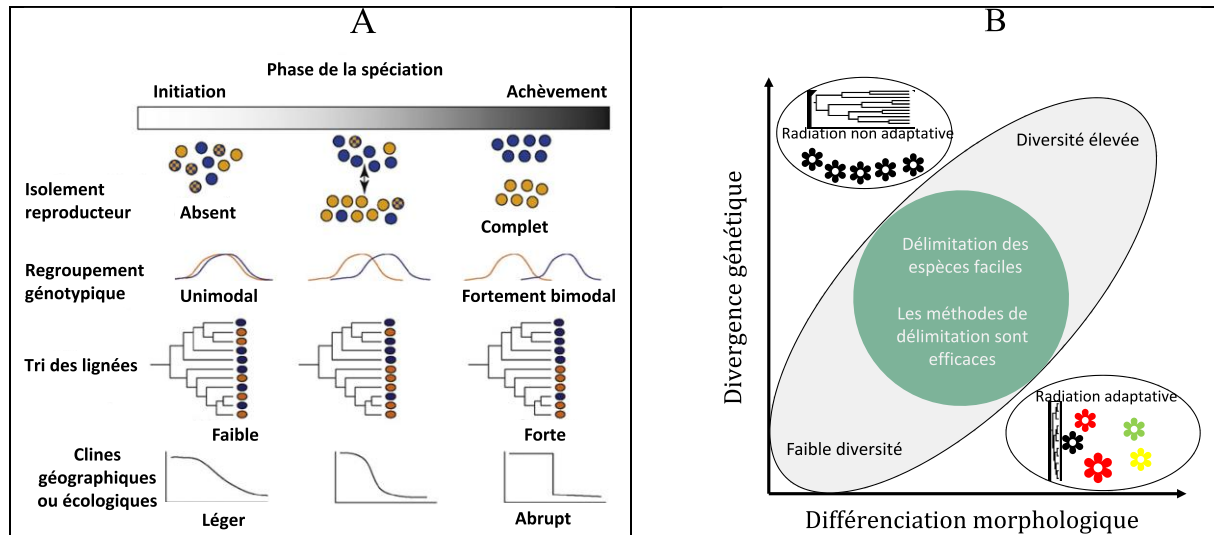


Figure 2. Processus de spéciation et axes hypothétiques divergence génétique-morphologie

A—Processus de spéciation. Figure modifiée d'après Nosil et al. (2009). La spéciation est représentée par trois phases successives allant de l'initiation à l'achèvement total. Au cours de ces trois phases, l'isolement reproducteur passe d'un état absent à un état complet. La distribution des fréquences alléliques varie d'une forme unimodale à une forme bimodale. Les lignées sont initialement regroupées puis sont fortement séparées lorsque la spéciation est complète. La pente des clines géographiques ou écologiques de la fréquence des gènes peut passer de léger en début de spéciation à abrupt en fin de spéciation. B— Axes hypothétiques montrant la correspondance entre la divergence génétique et la différenciation morphologique. Figure modifiée à partir de Barley et al. (2013). La zone gris clair délimitée par l'ellipse noire au centre représente les systèmes où la délimitation des espèces est normalement simple. Les deux zones blanches délimitées par une ellipse noire au niveau des deux extrémités représentent les systèmes où la délimitation des espèces est compliquée.

Dans le cas des radiations adaptatives, la différenciation morphologique est très prononcée, mais ne s'accompagne pas d'une divergence génétique conséquente (Figure 2B). Si au contraire la divergence génétique est forte, mais ne reflète pas la différenciation morphologique, comme dans le cas des espèces cryptiques, il y a un phénomène de radiation non adaptative (Figure 2B) (Barley et al., 2013).

Même si le concept morphologique semble le plus opérationnel, car défini à partir de caractéristiques visibles, le concept biologique fondé sur l'isolement reproducteur entre deux espèces différentes semble le plus accepté actuellement (Aldhebiani, 2018). En effet, les traits morphologiques peuvent varier au sein d'une espèce en fonction de son environnement sans pour autant modifier le matériel génétique, phénomène connu sous le nom de « plasticité phénotypique » (Sultan, 2021). L'isolement reproducteur est le mécanisme qui empêche deux espèces de s'hybrider assurant ainsi le maintien des deux lignées différentes (Baack et al., 2015).

Les mécanismes d'isolement reproducteur peuvent être classés en deux catégories, prézygotique et post-zygotique. L'isolement prézygotique peut être de nature géographique (séparation géographique), phénologique ou saisonnière (ex : différence de la période de reproduction) et écologique (différences des niches écologiques, ex : spécificité aux pollinisateurs) (Baack et al., 2015). L'isolement post-zygotique, quant à lui, se traduit par l'incompatibilité des deux espèces après la fécondation, donnant naissance à des descendants non viables ou des hybrides stériles (Baack et al., 2015). Deux principaux modes de spéciation, basés sur la répartition géographique des populations en divergence, peuvent conduire à l'isolement génétique ou reproducteur des populations. La spéciation allopatrique se produit lorsque les populations d'une même espèce sont séparées les unes des autres par des barrières géographiques telles que les océans et les montagnes, ou lorsqu'elles sont très distantes géographiquement (Fitzpatrick et al., 2009; Sobel, 2016; Wright, 1943). En l'absence de flux génétique entre elles, ces populations isolées vont évoluer indépendamment les unes des autres sous l'action de la sélection et/ou de la dérive génétique (Ramírez-Barrera et al., 2019; Sobel, 2016; Sobel et al., 2009; Wright, 1943). Elles vont éventuellement former des nouvelles espèces distinctes lorsque la divergence sera suffisante. Ce type de spéciation allopatrique pourrait être à l'origine des modèles d'équilibre ponctué (Ahad, 2019). La séparation spatiale peut être le résultat de divers phénomènes géologiques comme la dérive des continents. Selon la théorie de Wegener (1912), les continents d'aujourd'hui sont issus de l'évolution d'un bloc unique appelé « Supercontinent » ou « Pangée » qui s'est morcelé en plusieurs continents en mouvement sous l'effet de forces horizontales (Frankel, 1988; Wilson, 1963). Par la suite, certaines études ont permis d'établir un lien entre l'histoire géologique des continents, la distribution des espèces et le niveau de biodiversité dans les différentes zones géographiques (Cracraft and Prum, 1988; Leprieur et al., 2016; Magri et al., 2007; Pellissier et al., 2017). Lorsque la capacité de dispersion des individus de deux populations et leur distance géographique ne leur permettent pas de se reproduire, par conséquent limite le brassage génétique entre elles, une spéciation allopatrique (parfois appelée spéciation parapatrique) peut aussi se produire (Sobel, 2016; Wright, 1943). Cet isolement par la distance géographique (IBD) est un schéma évolutif couramment exploré dans la bibliographie (Jenkins et al., 2010). La spéciation sympatrique, contrairement au mécanisme précédent, survient en l'absence de barrières géographiques (Butlin et al., 2008). Ce mode de spéciation est le résultat de processus spéciaux tels que la polyploïdie, les réarrangements chromosomiques et la modification des comportements alimentaires (spéciation éthologique) ou des comportements reproducteurs (spéciation allochronique) (Dajoz, 2012). Il peut également être le fruit d'un isolement par les facteurs écologiques (Wang and Bradburd, 2014), dans le cas d'une spéciation écologique. Pour cette dernière, les barrières aux flux génétiques entre les populations sont la conséquence d'une sélection divergente basée sur l'écologie (Rundle and Nosil, 2005). Cependant, il est important de souligner que la spéciation écologique peut également se produire dans le cas des populations allopatriques (Rundle and Nosil, 2005). L'isolement écologique, plutôt connu dans sa traduction anglaise « Isolation by Ecology » (Shafer and Wolf, 2013) est un mécanisme issu de plusieurs types de processus. Il peut résulter de la sélection naturelle contre des populations migrantes (Wang and Bradburd, 2014) qui ne

sont pas adaptées à l'écologie des populations autochtones du fait, par exemple, des interactions écologiques comme la compétition, la prédation et les mutualismes (Doebeli and Dieckmann, 2000). La sélection sexuelle divergente contre les migrants qui limite le succès reproducteur de ces derniers peut également générer un isolement écologique tout comme la sélection divergente réduisant la valeur sélective des hybrides provenant de deux populations différentes (Wang and Bradburd, 2014). Les variables écologiques ayant déclenché la spéciation écologique sont souvent d'ordre climatique ou édaphique. Les oscillations climatiques passées comme les périodes de glaciation ont, en effet, contribué à la formation de nouvelles espèces aussi bien dans les continents que dans les îles (Cowling et al., 2001; Rand, 1948; Vences et al., 2009; Wallis et al., 2016). Des cas de différenciation résultant de l'hétérogénéité édaphique (structure et/ou composantes du sol) ont également été rapportés chez plusieurs espèces de plantes (Kruckeberg, 1986; Savolainen et al., 2006; Tuomisto, 2006). Il existe plusieurs approches pour tester la spéciation écologique (Mallet, 2014; Nosil, 2012) dont la plus utilisée est celle basée sur les flux génétiques testant la corrélation entre les distances génétiques et les distances écologiques (Garot et al., 2019; Liu et al., 2018; Mallet et al., 2014; Nogueras et al., 2016; Ogden and Thorpe, 2002; Ramirez-Barrera et al., 2019; Shafer and Wolf, 2013) (Figure 3).

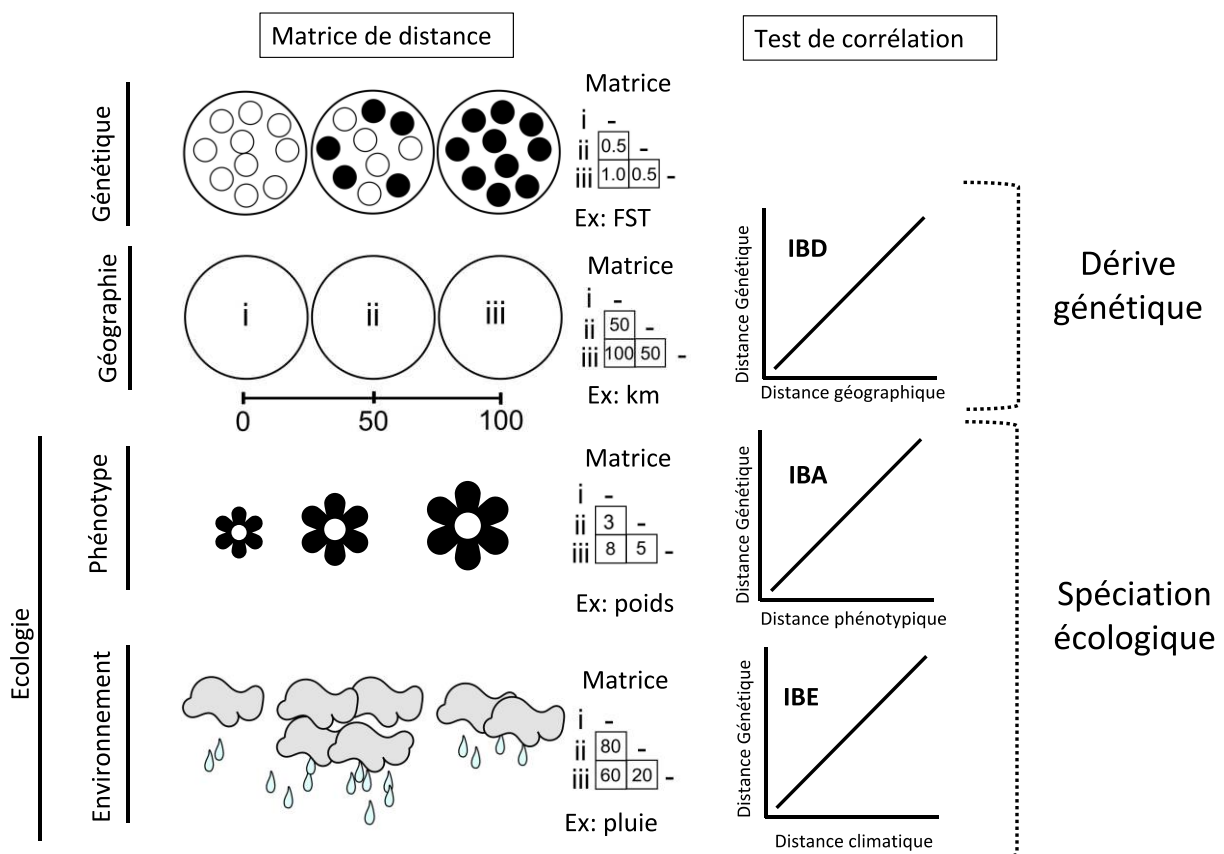


Figure 3. Résumé conceptuel de l'isolement par la distance (IBD), de l'isolement par adaptation (IBA) et de l'isolement par les facteurs environnementaux (IBE)

Figure modifiée de Ramirez-Barrera et al. (2019) et Shafer et Wolf (2013)). L'IBD entre deux populations peut être généralement testé en vérifiant la corrélation entre la distance géographique (ex : distance en km) et la distance génétique (ex : indice de différenciation génétique FST calculé à partir de séquences d'ADN tels que les

microsatellites). L'IBA et l'IBE sont deux composantes de l'isolement écologique. L'IBA peut être vérifié à partir d'un test de corrélation entre la distance phénotypique (ex : différences entre la taille des fleurs) et la distance génétique. Le test de corrélation entre la distance génétique et la distance environnementale (ex : différence de température) permet de tester l'IBE.

Dans la pratique, l'isolement écologique peut être vérifié soit en testant la corrélation entre la distance phénotypique et la distance génétique (Isolement par adaptation, IBA), soit en testant la corrélation entre la distance environnementale (ex : distance climatique, distance édaphique, etc.) et la distance génétique (Isolement par les facteurs environnementaux, IBE) (Figure 3) (Ramírez-Barrera et al., 2019; Shafer and Wolf, 2013). En ce qui concerne l'isolement géographique (Isolement par la distance, IBD), il peut être détecté en vérifiant la corrélation entre la distance géographique et la distance génétique (Figure 3) (Ramírez-Barrera et al., 2019; Shafer and Wolf, 2013).

La biodiversité est le résultat de plusieurs processus dynamiques. Elle reflète les phénomènes historiques et continue à évoluer, en réponse notamment aux modifications du milieu telles que le changement du climat ou des propriétés du sol (Clark et al., 2009; Frankel, 1988; Médail and Diadema, 2009; Steffen et al., 2006; Vitousek, 1992). Or, les activités anthropiques sont devenues une force supplémentaire majeure de changement environnemental, et par conséquent de changement de la biodiversité, au cours des derniers siècles (Bellard et al., 2014; Steffen et al., 2006; Tylianakis et al., 2008). Les activités anthropiques concernent notamment l'intensification agricole, l'exploitation minière, la surexploitation des espèces et les constructions des infrastructures (Sanderson et al., 2002). Conscients de ce changement global caractérisé par une empreinte forte et omniprésente de l'homme (Sanderson et al., 2002), plusieurs scientifiques reconnaissent aujourd'hui que la terre est entrée dans une nouvelle ère géologique appelée « Anthropocène » (Crutzen, 2002; Haraway et al., 2016; Steffen et al., 2011). Face à cette situation, les décideurs politiques de toutes les nations ont été alarmés par la communauté scientifique internationale sur la nécessité d'établir une stratégie commune ou mondiale de protection de la biodiversité. Ce n'est que depuis la signature de la convention sur la diversité biologique, lors du sommet de la Terre à Rio de Janeiro en 1992, que sa protection a commencé à être formalisée par le biais de politiques publiques nationales et internationales (United Nations, 1992). En réponse à ces enjeux, la biologie de la conservation, qui est une jeune science en développement, a émergé dans le but d'étudier et atténuer la crise de la biodiversité (Figure 4) (Pullin, 2002). Pour cela, elle utilise plusieurs disciplines pour approfondir les connaissances sur les éléments structurels de la biodiversité et contribuer à sa conservation (Figure 4) (Frankham et al., 2010; Pullin, 2002). Par exemple, la biologie de la conservation a recours à la socio-économie et l'écologie pour comprendre les impacts de la déforestation et de la fragmentation à l'échelle écosystémique (Pullin, 2002). Elle unit la biogéographie, l'écologie et la biologie pour identifier les zones de protection et déterminer les menaces pesant sur les espèces dans leurs habitats naturels (Pullin, 2002). Enfin, elle utilise la biologie moléculaire pour résoudre la taxonomie des espèces, déterminer le niveau de diversité génétique et établir les stratégies pour le maintien des processus évolutifs (Frankham et al., 2010; Pullin, 2002). Paradoxalement, la situation de la biodiversité, 20 ans plus tard, ne s'est pas améliorée. D'après les récentes évaluations dont

celles de Dirzo et Raven (2003) et de Sala et Knowlton (2006), les menaces qui pèsent sur la diversité biologique sont d'une telle intensité que nous nous dirigeons actuellement sur une trajectoire menant à une probable sixième extinction massive.

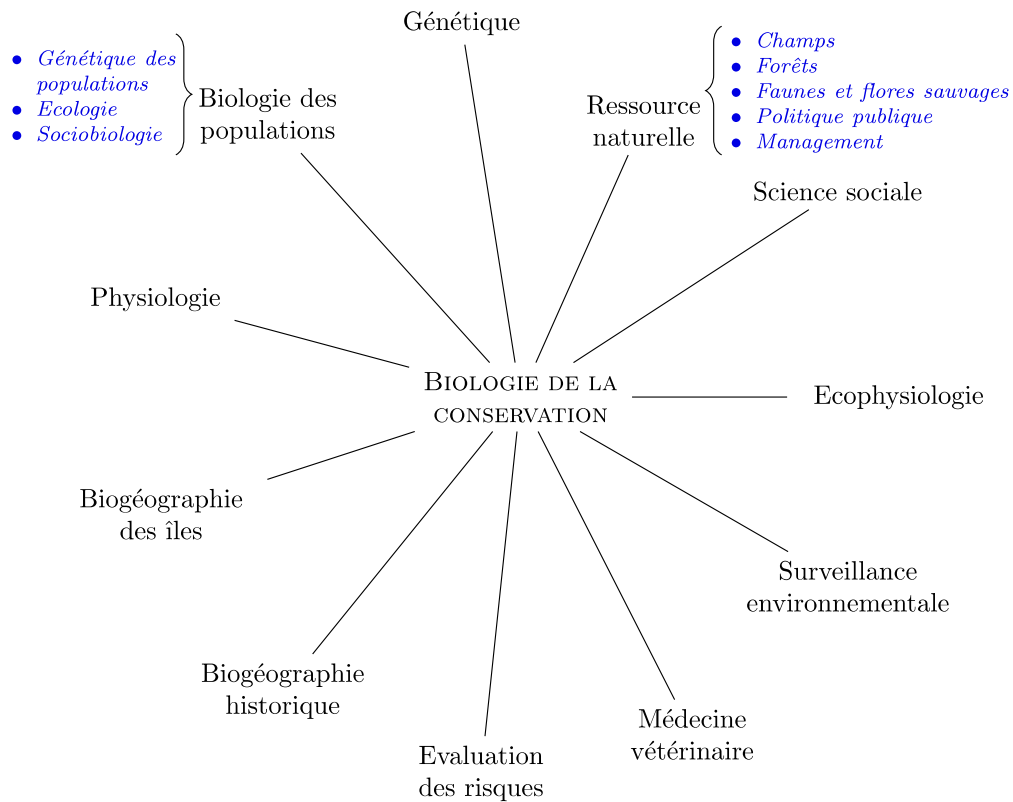


Figure 4: La biologie de la conservation et ses disciplines annexes (Figure modifiée de Frankham et al. (2010)). La biologie de la conservation fait intervenir plusieurs disciplines. La génétique permet de résoudre les questions liées à la taxonomie et à l'évolution. Elle est également utilisée dans les études à l'échelle populationnelle.

Cette thèse représente le premier examen détaillé d'un groupe d'orchidées aphyllées de Madagascar appartenant au genre *Vanilla* et s'inscrit dans la volonté de renseigner et d'améliorer l'état des populations au regard des enjeux de conservation et des processus évolutifs. Elle s'appuie sur des bases génétiques, écologiques, biologiques et biogéographiques pour enrichir nos connaissances sur leur évolution et sur l'état de leurs populations naturelles afin d'apporter les informations clés indispensables à l'élaboration des plans de conservation dans le contexte des changements globaux actuels. En préalable, ce travail passe en revue toutes les connaissances disponibles à ce jour sur ce complexe d'espèces endémiques de Madagascar et des espèces voisines de la zone Sud-Ouest de l'océan Indien (SOOI). Cette synthèse bibliographique permettra d'identifier les besoins urgents en termes de connaissances pour mieux appréhender la conservation de ces espèces.

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1.2. Etat des lieux sur la connaissance des vanilliers aphyllés du Sud-Ouest de l'océan Indien

« Les historiens des sciences observent souvent que poser la bonne question est plus important que produire la bonne réponse. La bonne réponse à une question triviale est également triviale, mais la bonne question, même lorsqu'elle est insoluble dans sa forme exacte, est un guide vers une découverte majeure. »

E. O. Wilson

Le genre *Vanilla* Plumier ex Miller est un membre de la famille des orchidées, l'une des familles les plus diversifiées des plantes à fleurs. Il est originaire d'Amérique et s'est différencié en Amérique, en Afrique et en Asie. Depuis la première description botanique d'une plante appartenant au genre, environ 132 espèces sont actuellement acceptées dans les bases de données mondiales des plantes, incluant celles réalisées par Kew Royal Botanic Gardens, Tropicos et International Plant Names Index. La première classification du genre réalisée par Rolfe divise les espèces en deux grands groupes morphologiques : les espèces munies de feuilles (feuillées) et les espèces dépourvues de feuilles pérennes (aphyilles). L'aphyllie est apparue au moins trois fois indépendamment dans l'évolution du genre *Vanilla*, en Afrique, en Asie et en Amérique, résultant de l'adaptation des ancêtres foliés aux conditions de sécheresse. Dans la région du Sud-Ouest de l'océan Indien (SOOI), les vanilliers aphyllés sont représentés par sept espèces formant un groupe monophylétique récent. Ces espèces sont impossibles à différencier à partir des marqueurs chloroplastiques classiques généralement efficaces pour l'identification des espèces. D'autre part, leur discrimination sur la base des caractères morphologiques est difficile. De plus, certaines espèces, notamment celles de Madagascar, ont des périodes de floraison et une distribution géographique chevauchantes, augmentant la probabilité d'hybridation. Les espèces aphyllés du genre *Vanilla* endémiques des îles du SOOI constituent donc un excellent modèle pour comprendre l'évolution des orchidées.

Toutes les informations disponibles dans la littérature concernant ces vanilliers aphyllés du SOOI seront présentées dans cette partie (Chapitre 1—1.2). Ces informations ont été synthétisées sous forme d'une revue bibliographique intitulée « *The leafless Vanilla species-complex from the South-West Indian ocean region: a taxonomic puzzle and a model for orchid evolution and conservation research* » publiée dans le journal *Diversity* en 2020.

Review

The Leafless *Vanilla* Species-Complex from the South-West Indian Ocean Region: A Taxonomic Puzzle and a Model for Orchid Evolution and Conservation Research

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Abstract: The pantropical genus *Vanilla* is a member of the Orchidaceae family, one of the largest and most diverse families of angiosperms in the world. It originated in America and differentiated in America, Africa, and Asia. About 132 species of *Vanilla* have now been accepted since the discovery of the genus. Within the *Vanilla* genus, leaflessness has appeared at least three independent times, in Africa, Asia, and America as an adaptation to drought conditions. The South-West Indian Ocean (SWIO) region, a biodiversity hotspot for orchids, is home to seven leafless species. Chloroplast markers failed to differentiate species within this recent SWIO clade. Morphological traits used for description are very similar between species and do not provide reliable identification. Moreover, some of the species have overlapping flowering periods and geographic distribution in Madagascar, increasing the probability of finding some sympatric species and, therefore, hybrids. Leafless *Vanilla* species from the SWIO islands are thus an excellent model for understanding the evolution of orchids. For their conservation, and to confirm the identity of these leafless species, an integrated approach with classical taxonomy using a large number of samples, intense fieldwork on biology and ecology, and molecular studies using variable markers is necessary.

Keywords: *Vanilla*; leafless; South West Indian Ocean; diversification; taxonomy; biology; ecology; diversity; ethnobotany

1. Introduction

With more than 24,000 species and 700 genera worldwide, Orchidaceae is one of the largest families of flowering plants [1,2], and originated around 83 Mya (Million years ago) [3,4]. Orchids are widely celebrated for their beautiful flowers, extraordinary diversity, and potential medicinal proprieties. For example, among the 494 terrestrial and epiphytic orchid species recorded in Southern Africa, 49 are used as traditional medicinal plants to treat a wide range of diseases [5]. The South-West Indian Ocean (SWIO) islands are one of the global hotspots for orchids. The Orchidaceae family is represented by 65 genera and over 1000 species in this region [6]. Madagascar is the richest, with approximately 862 species, of which more than 85% are endemics [7], and 57 genera [8]. The Orchidaceae genus *Vanilla* can be considered as a flagship genus in the SWIO region, because the genus is represented

there by wild, as well as cultivated, genetic resources. This region ranks first in terms of leafless *Vanilla* species richness [9,10]. It is also renowned worldwide for its *Vanilla* fruit production, with the islands of Madagascar, Comoros and La Réunion being amongst the first world producers [11].

This paper provides an updated bibliographical review about the *Vanilla* genus. It is enriched by our own observations, and is focused on leafless species, particularly those indigenous and endemic to the SWIO islands (Madagascar, Comoros, Mayotte, Seychelles) and the East coast of Southern Africa. Some elements about the complexity of their taxonomy will be discussed.

2. The *Vanilla* Genus and Leafless Species

2.1. Botany

Vanilla plants are perennial vines characterized by thick and fleshy stems [12], a monopodial growth habit, aerial roots growing at each node, absence of pseudobulbs, fleshy fruits and wingless seeds [9]. The inflorescence is axillary [13]. The aerial roots are hairless, while the underground roots are hairy [14,15]. Leafless *Vanilla* species share most vegetative characteristics with leafy species, but they are photosynthetic plants with green, sometimes reddish to glaucous stems, distinguished by the absence of leaves or the modification of the leaves into scale-like structures [10,16]. Also, as opposed to leafy species, green stems of leafless species do not have a sclerenchyma band separating cortex from ground tissue [14]. Two types of roots were identified in leafless species from Madagascar: short, flattened or filiform roots used for attachment to a supporting tree, and long aerial roots up to 15 m exploring the soil for nutrition [15]. The same study demonstrated that populations in the north, where rainfall is higher, have thinner aerial roots with smaller xylem vessels than those in the arid south [15]. This is probably one of the developed strategy of succulent plants to face the scarcity or abundance of water in the soil [17]. Floral morphology is diverse but, in general, flowers are composed of three sepals, two petals, and a third large petal called labellum [10,12] forming a funnel surrounding the column [13]. Pollinia and stigma are separated by a membrane called the “rostellum”. It usually prevents self-pollination within the same flower, although species are self-compatible [18,19]. The number of opened flowers per day varies from one to several, depending on the species. For Mexican leafy species, for example, it is generally one (e.g., *V. phaeantha*) to three (e.g., *V. calyculata*) [20]. In Madagascar, we observed that the number of opened flowers per inflorescence per day can go up to three in the leafless white-flower species (*V. madagascariensis*, *V. decaryana*, *V. bosseri*), whereas this number is on average one flower and rarely two in the yellow-flower populations of Montagne des Français (*V. humblotii*). This is also the case of *V. humblotii* from Mayotte, for which on average 1.84 flowers open per day and per individual [21]. Flowers usually open in succession and are short lived (one day) (e.g., in leafless *V. barbellata*, *V. claviculata*, *V. dilloniana* [22]) but can remain for two or three days for some species like the leafy species *V. inodora* [20]. Unfertilized flowers rapidly fall, otherwise they turn into fruits in the form of an elongated capsule, called a “pod”.

2.2. Taxonomy

The genus *Vanilla* Plum. ex Miller forms a monophyletic group (34.6 Mya) (Figure 1) in the large Orchidaceae family, the Vanilloideae sub-family, the Vanilleae tribe and the Vanillinae sub-tribe [1,10,16,23].

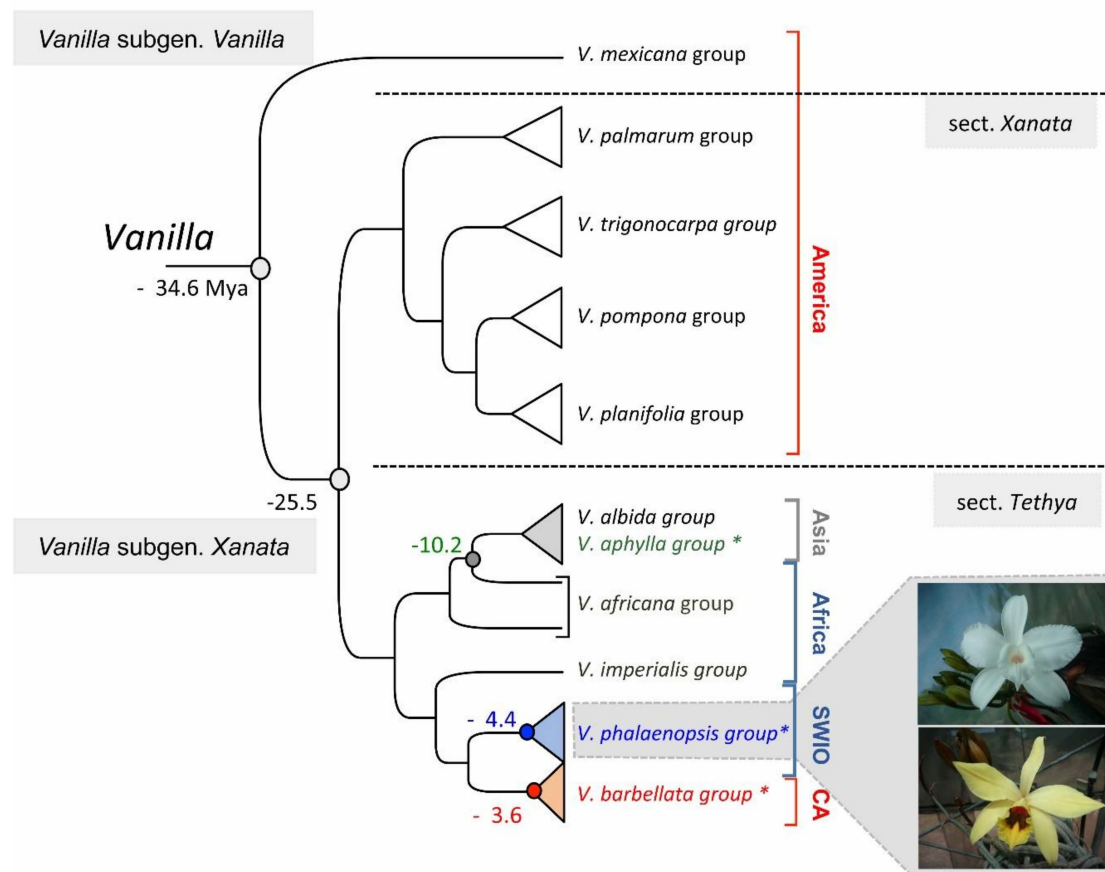


Figure 1. Representation of the (rbcL, psaB, psbB, psbC) phylogeny of Bouetard et al. in 2010 including species groups defined by Soto Arenas and Cribb in 2010, showing leafless *Vanilla* species names in color (green for Asia, blue for SWIO, red for Caribbean CA) [3,9]. Node dates in Mya correspond to datations obtained by Bouetard et al., with the datation of *Vanilla* node at -34.6 Mya estimated using 71 Mya as the minimum age for Vanilloideae published by Ramírez et al., 2007, based on fossil evidence of an orchid pollinarium (of *Meliorchis caribea*) attached to an extinct stingless bee (*Problebeia dominica*) recovered in Miocene amber [3,4].

The first *Vanilla* subgeneric classification was essentially based on morphological descriptions and distinguished leafless species (Aphyllae section) with scale-like leaves, from leafy species (Foliosae section) which have conspicuous and persisting leaves [10,23]. The latter was divided into three sub-sections by Portères on the basis of labellum and leaf characteristics: Membranaceae, Papillosae, and Lamellosae [10,16]. Since the development of molecular approaches, which are playing an important role in plant taxonomy by allowing reliable phylogenetic-based classifications [24], phylogenetic markers (matK, rbcL, psaB, psbB, psbC, ITS) and chemical analyses have increasingly been used in *Vanilla* taxonomy [3,9,16,20,25]. A revision of the genus was done in 2010 by Soto Arenas and Cribb, who confirmed that *Vanilla* is monophyletic but noticed that the sections and subsections defined by Portères in 1954 are polyphyletic and incongruent with cladistic analysis [9]. Consequently, they proposed a new *Vanilla* classification based on phylogenetic and morphological reconstructions with two subgenera and 20 morphological groups [9]. The *Vanilla* subgenus is composed by species with membranaceous leaves, inflorescences poorly differentiated from the vegetative axis, a labellum without penicillar callus, a column united to the labellum only at the base, a sub-perpendicular anther and a concave stigma [9]. It is represented by *V. mexicana*, the type specimen for the *Vanilla* genus (Figure 1) [23].

The leafless species are now included in the other subgenus, *Xanata*, together with species with coriaceous to fleshy leaves [9]. Their inflorescences are well distinct from the vegetative axis,

they have a column joined to the labellum at least on half of its length, and a parallel anther [9]. This second subgenus is further divided into two sections: the *Xanata* section comprising mostly the leafy neotropical species (American) and divided into six groups, and the *Tethya* section including the leafy and leafless paleotropical species (African and Asian) and Caribbean leafless species [9,16]. The same year, Bouetard et al. proposed a phylogeographic classification based on chloroplast DNA sequencing which was congruent with the revision by Soto Arenas and Cribb (Figure 1) [3,9]. It also showed that leaflessness is polyphyletic and has appeared at least three independent times, in Africa, Asia and America as a convergent adaptation to drought conditions following coastal establishments and island colonization [3]. The leafless species all belong to the subgenus *Xanata* section *Tethya*, except *V. penicillata* which is the only species included in section *Xanata* [9]. Also, this chloroplast phylogeny showed the surprising result that the Caribbean leafless species originated from an African ancestor, via transoceanic dispersion to America, rather than from an American leafy species [3]. This molecular data contradicts previous suggestions made by Portères in 1954, that leafless *Vanilla* species from the three geographical distributions (SWIO, Asia and Caribbean) originated from leafy species from neighboring continents [10].

2.3. Evolution

Vanilla is a transoceanic genus, found only in tropical forests between the 27th north and south parallels, but absent in Australia (Figure 2).

According to Rolfe, the diversification of the genus happened in Brazil and Guiana [23]. However, based on floral morphology, Portères suggested a primary diversification center in Indo-Malaysia that has evolved into two groups (Madagascar, Mascarene Islands, Africa versus Asia and Western Pacific islands), with the American species resulting from the migration of Asian species during the Tertiary (65.5–2.5 Mya) [10]. But the recent molecular phylogeny rejected this theory of Portères and suggested a South American center of diversity [3]. In 2005, Cameron stated that the differentiation of the genus *Vanilla* would have taken place before the dissolution of Gondwana [26], about 129 Mya [3]. However, following a fossil-based tree dating in Orchids [4], Bouetard et al. supported the alternative theory that *Vanilla* genus probably appeared 34 Mya (Figure 1), and its migration from the New World to the Old World occurred at the beginning of the Tertiary, about 25 Mya via a transoceanic dispersion, facilitated by the proximity of the continents and the presence of the series of islands of the mid-oceanic ridge of the Rio [3]. Three obvious cases of transoceanic migration events from Africa were also noted: (1) the dispersal of the genus from Africa to Asia; (2) from Africa to the SWIO islands and finally (3) from Africa to the Caribbean islands [3], the two last events concerning therefore the evolution of leafless groups in the SWIO and Caribbean islands.

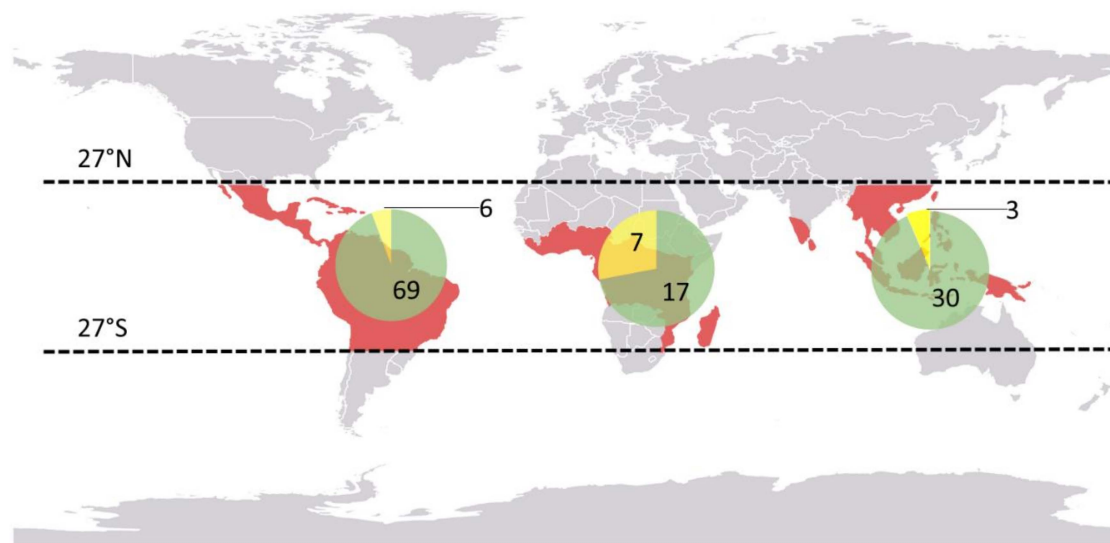


Figure 2. World distribution of *Vanilla* species: number of leafy *Vanilla* species (in green) and number of leafless species (in yellow) based on the World checklist of Selected Plant Families [27], the International Plant Name Index [28] and the database of Tropicos [29].

2.4. Ecology

Vanilla orchids are thermophilous [14]. These terrestrial, epiphytic or hemi-epiphytic plants are adapted to a variety of habitats [10,14,16,20], although they are scarce on mountain rain forest [14]. Lamellosae are found in fresh and wet places. They are mainly ombrophilous to mesophilous with various levels of heliophilia [10]. Papillosae species are also sciaphilous, living in rainforest formations, to heliophilous, sometimes growing in tropophilous and sub-xerophilous formations [10]. *V. organensis*, *V. palmarum*, *V. wrightii* and relatives without lamellae and with column fused to the labellum can be found in more mesophilic or even tropophilic formations in the area of Brazilian palm groves or wooded savannas [10]. Species with longitudinal lamellae and aromatic fruits are distributed in coastal or sublittoral forests [10]. However, some can be found in uncommon habitats like marshy forests (e.g., *V. arcuata* and *V. paludosa* [30]). While most species can climb to various substrates (trees and rocks), an epiphytic *Vanilla* (*V. palmarum*) is exclusively associated with nine species of palms in South America [31]. Some species can be found in different types of ecosystems like *V. inornata* [32]. There are species that thrive on particular substrates such as *V. martinezii* growing on an unusual peat-like soil [20], *V. planifolia* alliance in Central America restricted to calcareous or sedimentary substrate [14] and *V. insignis* found on seasonally dry volcanic areas [14]. *Vanilla* species are often associated with mycorrhizal fungi [15,16,33]. For example, studies in Puerto Rico, Cuba and Costa Rica recorded several species belonging to three genera of mycorrhizal fungi (*Ceratobasidium*, *Thanatephorus* and *Tulasnella*) in *V. potaei* and *V. planifolia* [33].

Aphyllae is the only group that contains species adapted to hot, xeric, full sun habitats [10,14,16]. They grow on coastal bushes (e.g., *V. roscheri* and *V. decaryana*), in very dry tropophilous forests (e.g., *V. madagascariensis*), on sandy soils (e.g., *V. perrieri*, *V. bosseri*, *V. aphylla*), on rocks (e.g., *V. humblotii*, *V. phalaenopsis* and *V. marmoreisense*), on coral reefs (*V. roscheri*) but also in mangrove swamps (e.g., *V. barbellata* and *V. dilloniana*) or in humid forests (*V. madagascariensis*) (Figure 3A,B) [8,10,34–36].



Figure 3. Ecology and usages of leafless *Vanilla* species: (A) *V. phalaenopsis* individual growing on a rock in Seychelles, (B) *V. decaryana* plant in thorny bushes in the south of Madagascar (Antandroka) and (C) leafless *Vanilla* stems for sale on the Tuléar market (Madagascar). (Photographies A, C: Pascale Besse, B: Cathucia F. Andriamihaja).

Leafless species are exclusively found on islands or on nearby littoral environment [10]. According to our observations in several populations in Madagascar, they are all hemi-epiphytes characterized by early germination and early stage in the soil, a root system exploring the ground and a long stem climbing along a support.

2.5. Species Diversity and Distribution

In the Vanilloideae sub-family, the *Vanilla* genus is the most species-rich [16]. However, the diversity of the *Vanilla* Plumier ex Miller genus is very complex. Indeed, as will be discussed later, *Vanilla* species delimitation is confused due to interspecific hybridization (e.g., *V. barbellata* × *V. claviculata* [22]) and phenotypic plasticity [18,37]. Furthermore, numerous synonymies still exist between species (Table 1) [9,10,27]. This diversity is constantly in revision thanks to new discoveries. In the last decade, for example, 28 novel species have been described [25,27,32,35,36,38–40]. The first extensive classification of the genus carried out by Rolfe in 1896, listed 50 species with six aromatic species [23]. Portères, in 1954, reported 110 species (18 aromatic) [10], this was reduced to 90 species by Cameron and Chase in 1999 [41]. The number of valid species was extended to 106 (35 aromatic) in 2010 [9,14]. Altogether, 231 *Vanilla* species, subspecies, varieties and synonymies are listed in the International Plant Names Index website [28] and 240 are registered in the Tropicos website [29]. The current world checklist of selected plant families (WCSPF) records 230 *Vanilla* names, of which 130 are accepted [27].

By adding the species *V. bosseri* discovered by Allorge-Boiteau in 2013 [35] and the last described species *V. armoriquensis* [40] to the list of 130 accepted species in WCSPF [27], the present number of *Vanilla* species would rise to 132. Most of these species are endemic to tropical America (75) (including *V. armoriquensis* found in Peru in 2020), where the Amazon Basin constitute the center of diversity [3,9,14], 24 occur in Africa (including *V. bosseri*), and 33 are distributed in Asia, New Guinea and the Pacific islands (Figure 2). Based on Rolfe's sections 116 *Vanilla* species among these 132 are leafy, the remaining species being leafless.

After the discovery of the first leafless species in 1799 (*V. claviculata*), 18 species have been recorded by Portères in 1854 [10]. This number is reduced to 16 at the present time due to several synonymies (Table 1). Leafless species are mainly distributed on islands and nearby coastal regions: along the east African coast, SWIO islands, southeast Asia and Caribbean islands [9,10], except *V. penicillata* which is found in South America (Table 1) [9].

Table 1. List of the 16 leafless *Vanilla* species accepted by Govaerts et al. [27]. Synonyms are in brackets. *V. bosseri* was not present in the accepted list of Govaerts et al., but is listed as an accepted name in Tropicos [29]. Morphological species groups are according to Soto Arenas and Cribb [9].

Species	Year	Continent	Group	Location
<i>V. aphylla</i> Blume. (<i>V. calopogon</i>)	1825	Asia	<i>V. aphylla</i>	Java, Malaysia, Philippines, Thailand
<i>V. barbellata</i> Rchb.f. (<i>V. articulata</i> , <i>V. bakeri</i> , <i>V. parishii</i>)	1865	America	<i>V. barbellata</i>	Caribbean Islands
<i>V. bosseri</i> L. Allorge. (<i>V. montagnacii</i>)	2013	Africa	<i>V. phalaenopsis</i>	Madagascar
<i>V. claviculata</i> Sw.	1799	America	<i>V. barbellata</i>	Caribbean Islands
<i>V. decaryana</i> H. Perrier.	1934	Africa	<i>V. phalaenopsis</i>	Madagascar
<i>V. dilloniana</i> Correll.	1946	America	<i>V. barbellata</i>	Caribbean Islands
<i>V. humblotii</i> Rchb.f.	1885	Africa	<i>V. phalaenopsis</i>	Comoros, Mayotte, Madagascar
<i>V. madagascariensis</i> Rolfe.	1896	Africa	<i>V. phalaenopsis</i>	Madagascar
<i>V. marmoreisense</i> Soto Calvo.	2019	America	<i>V. barbellata</i>	Cuba
<i>V. penicillata</i> Garay & Dunst.	1965	America	<i>V. penicillata</i>	Colombia, Suriname, Brazil
<i>V. perrieri</i> Schltr.	1924	Africa	<i>V. phalaenopsis</i>	Madagascar
<i>V. phalaenopsis</i> Rchb.f.	1867	Africa	<i>V. phalaenopsis</i>	Seychelles
<i>V. poitaei</i> Rchb.f. (<i>V. correllii</i> , <i>V. eggersii</i>)	1876	America	<i>V. barbellata</i>	Saint Domingue
<i>V. roscheri</i> Rchb.f.	1876	Africa	<i>V. phalaenopsis</i>	Tanzania, Kenya, Mozambique, South Africa
<i>V. walkeriae</i> Wight	1845	Asia	<i>V. phalaenopsis</i>	India, Sri Lanka
<i>V. wightii</i> Lindl. (<i>V. wightiana</i>)	1845	Asia	<i>V. aphylla</i>	India, Sri Lanka

2.6. Reproduction Biology

In most cases, *Vanilla* species combine vegetative and sexual reproduction, except for *V. mexicana*, *V. bicolor* and *V. palmarum* for which artificial clonal propagation was reported to be unsuccessful or difficult [18,37,42]. The vegetative reproduction is possible as soon as an individual or a sufficient fragment of stem cutting can take root in the soil [37], sprout vegetative buds [42] and climb to a support to create a new individual. This clonal propagation constitutes the predominant reproductive, colonization and survival strategy. Studies on leafless species also revealed their ability for clonal reproduction in natural habitats. In Puerto Rico, Nielsen and Siegismund used polymorphic enzymes to analyse genetic diversity [22,43]. The results showed a high clonality rate for *V. dilloniana* (38%) [22], a moderate rate for *V. barbellata* (25%) [22] and a low rate for *V. claviculata* (6%) [43]. In *V. humblotii* in Mayotte, microsatellite analyses showed a moderate clonality rate (12.5%), with an average clonal patch size of 4.6 (± 2.7) m, and a phalanx distribution [21].

The sexual reproduction, on the other hand, can occur only during the flowering period and only when the individual has attained maturity [20]. Also, it is common that some populations of a species fail to bloom for one or more successive years, as it was reported for *V. hartii* in tropical America [20]. There are, however, species that bloom throughout the year or during several months like *V. inornata* [32], *V. rivasii* [44] or *V. bicolor* [42]. Although some *Vanilla* species have an unusual high natural fruit set (>40%) (Figure 4), which suggests self-pollination [20,42,45,46], most are pollinator-dependent due to the presence of the rostellum membrane separating the female and male parts of the flower [10,19,21,23]. Some mechanisms were reviewed by Gigant et al., to explain self-pollination: it may be caused by either stigmatic leak and/or the presence of a dehydrated or reduced rostellum, or agamospermy (e.g., *V. bicolor* and *V. palmarum*), but some spontaneous self-pollinations remain unexplained (e.g., *V. planifolia* and *V. chamissonis*) [18]. In addition to these mechanisms, Householder et al. also noted the absence of a rostellum in *V. guianensis* in Peru, facilitating pollen transfer between the two sexual organs [42].

Pollinators visit flowers for various reasons, such as collecting nectar, oil, pollen, stigmatic exudates or floral scents [47–49]. In *Vanilla*, floral odor seems to play an important role in attracting pollinators as in the case of *V. trigonocarpa* or *V. pompona* in America [14,20,42]. Other strategies are also used by orchid species to attract pollinators such as deceptive systems [50], as described for *V. planifolia*,

V. odorata, and *V. insignis* in America [18]. In orchids, bee-pollination also requires a morphological association between floral characteristics and the insect traits [47,51–53].

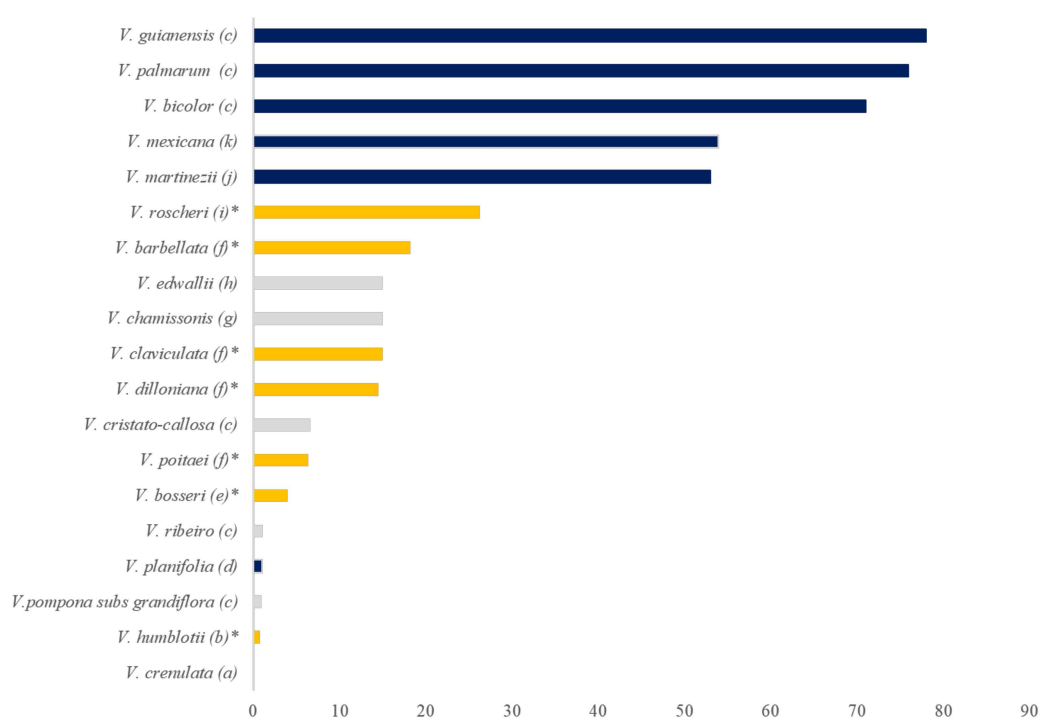


Figure 4. Rate of natural fruit set (%) of *Vanilla* species according to (a) Soto Arenas and Cameron [14], (b) Gigant et al. [21], (c) Householder et al. [42], (d) Ackerman et al. [54], (e) Petersson [53], (f) Tremblay et al. [45], (g) Macedo [55], (h) Pansarin, Aguiar, and Pansarin, 2014, (i) Gigant et al., 2014, (j) Soto Arenas and Dressler, 2010, (k) Gigant et al. [46]. Species that can self-pollinate spontaneously are in dark blue, leafless *Vanilla* species are in orange and indicated by *, and the grey bars represent the other species.

In most cases in America, euglossine bee species were proved to be the main *Vanilla* pollinators (Table 2) [14,42,56], probably alongside with other species such as *Trigona* bees, *Xylocopa* bees [42], birds (e.g., *V. abundiflora* and *V. griffithii* [20]) and even bat species (e.g., *V. insignis* [20]).

With regards to the leafless *Vanilla* species, the low natural fruit set of six studied species (<30%) testifies their dependence on pollinators (Figure 4). Soto Arenas and Cameron suggested that species in Puerto Rico would probably be pollinated by bees of the genus *Centris*, although it was also reported that they could be pollinated by euglossine bees [45]. In Africa, three leafless species have been studied for their reproduction biology, and the results show that they are most probably pollinated by female Xylocopinae (Allodapine) bees, although these bees might also act as pollen thieves [19,21,53]. Different Allodapine bee species were observed to move *V. bosseri* and *V. roscheri* pollen, but Meliponae and Anthophorine might be the true pollinators, respectively (Table 2). The presence of a rostellum that covers the entire stigmatic surface prevents self-pollination in *V. roscheri* [19]. Despite this, Sibaya populations (South Africa) have a high natural fruit set of up to 26.3% [19]. This can be explained by the large number of visiting insects recorded (about 11), of which three Apidae have been observed to move pollen probably for brood provisioning (Table 2) [19]. The observed natural fruit set of *V. humblotii* was very low (0.62 to 1.2%) in natural populations from Mayotte [21], possibly due to insufficient pollinators attributed to the highly fragmented landscape. Studies conducted on populations of *V. bosseri* in the CNRFEREF forest in Madagascar showed that this taxon is bee-pollinated, allogamous but self-compatible (like the two previous species) with a low flowering rate and a low natural fruit set of 3.96% [53]. Although several bee species belonging to four orders (*Hymenoptera*, *Lepidoptera*, *Orthoptera*, *Diptera*) as well as one Primate species

(*Prophithecus verreauxi*) were recorded visiting flowers [53], this low natural fruit set indicates a limited pollination. Indeed, only three bee species were found to carry pollen similar to the one of *V. bosseri* (Table 2) [53]. The author hypothesized that the pollinators may instead act as pollen thieves and reduce *V. bosseri* reproductive success [53].

Table 2. Potential pollinators of some *Vanilla* species. Leafless species are highlighted in bold.

<i>Vanilla</i> Species	Potential Pollinators	References
	America	
<i>V. insignis</i>	<i>Eulaema polychrome</i>	[20]
<i>V. planifolia</i>	<i>Euglossa</i> spp.	[20]
<i>V. pompona subs grandiflora</i>	<i>Eulaema</i> spp.	[42,56]
<i>V. cribbiana</i>	<i>Eulaema</i> sp.	[20]
<i>V. pompona</i>	<i>Eulaema cingulata</i>	[57]
<i>V. trigonocarpa</i>	<i>Euglossa asarophora</i>	[20]
<i>V. edwallii</i>	<i>Epicharis affinis</i>	[52]
	Asia	
<i>V. siamensis</i>	<i>Thrinchostoma</i> sp.	[58]
<i>V. cf kaniensis</i>	<i>Aegilopa</i> sp.	[14]
	Africa	
<i>V. bosseri</i>	<i>Macrogalea ellioti</i> , <i>Liotrigona mahafalya</i> , <i>Liotrigona madecassa</i>	[53]
<i>V. roscheri</i>	<i>Allodapula variegata</i> , <i>Allodapa rufogastra</i> , Anthophorini	[19]
<i>V. humblotii</i>	<i>Allodape obscuripennis</i> , <i>Nectarinia coquerelli</i>	[21]

2.7. *Vanilla*: A Taxonomic Complex Group

Since 1703, when the first three species were described by Plumier, the taxonomy of the genus has always been considered to be difficult [23].

Indeed, the botanical identification of *Vanilla* species is mainly based on the description of flowers which are often ephemeral, variable within species due to phenotypic plasticity (e.g., *V. barbellata*, *V. bakeri*, *V. parishii* [10]), or similar between cryptic species [20,59]. Sometimes, no type specimen or botanical collections were recorded (e.g., *V. guianensis* [42]). Besides, the number of individuals used for species description was often limited due to scarcity of flowers [9]. Vegetative characteristics also show considerable intra-specific variations and inter-specific similarities between related species [34,35,37].

Hybridization is also one of the factors that can blur the boundaries between species, leading to a mismatch between morphology and genetics. Prezygotic and postzygotic barriers can generally prevent or limit hybridization [60]. In the Orchid family, prezygotic barriers include differences in floral morphology, and phenological and ecological isolation [51,57,61]. Postmating barriers result from incompatibility mechanisms, hybrid inviability or sterility, as reported for some deceptive orchids [62,63]. In the *Vanilla* genus, differences in flowering period and pollinator specificity are probably the two main factors that can limit gene exchange between sympatric species [10,20,22,56]. On the other hand, postmating barriers were not observed following artificial crossing experiments between closely related species (e.g., *V. planifolia* × *V. pompona* [37]) or highly differentiated species (e.g., *V. aphylla* × *V. planifolia* [59,64]). Many *Vanilla* species occur in sympatry (Table 3), and cases of natural interspecific hybrids have been reported [22,43,65].

Table 3. Sympatric species recorded. Species for which hybridization was observed are highlighted in bold.

Sympatric Species	Location	References
Leafy Species		
<i>V. hartii</i> & <i>V. cribbiana</i>	Mexico	[20]
<i>V. hartii</i> & <i>V. insignis</i>	Mexico	[20]
<i>V. hartii</i> & <i>V. odorata</i>	Mexico	[20]
<i>V. hartii</i> & <i>V. inodora</i>	Mexico	[20]
<i>V. rivasii</i> & <i>V. dressleri</i>	Colombia	[44]
<i>V. javieri</i> & <i>V. appendiculata</i>	Colombia	[66]
<i>V. yanesha</i> & <i>V. mexicana</i>	Peru	[38]
Leafless Species		
<i>V. barbellata</i> & <i>V. claviculata</i>	Puerto Rico	[43]
<i>V. barbellata</i> & <i>V. dilloniana</i>	Puerto Rico	[22]
<i>V. dilloniana</i> & <i>V. claviculata</i>	Puerto Rico	[22]
<i>V. madagascariensis</i> & <i>V. perrieri</i>	Madagascar	[8] ¹
<i>V. madagascariensis</i> & <i>V. bosseri</i>	Madagascar	1

¹ Personal observation.

Thanks to an integrative taxonomic approach using ITS markers, the hybrid status of *V. × tahitiensis*, described as a new species in 1933, was recently proven [16,65]. It is a hybrid between *V. planifolia* and *V. odorata*, although it is still unclear where the hybridization took place. A study in Puerto Rico demonstrated the occurrence of hybrids between sympatric leafless species [22,43]. *V. dilloniana*, *V. claviculata* and *V. barbellata* are three leafless species endemic to the Caribbean islands [22]. They share similar vegetative traits but can be easily discriminated from each other by their labellum [9,10,22]. The three species grow in sympatry in the western/southwestern part of the Susua Forest Reserve (Puerto Rico). Populations of the two species, *V. barbellata* and *V. claviculata*, presented the same alleles but differed in allelic frequencies [22,43]. Genetic differentiation within *V. barbellata* and *V. claviculata* species (mean 14.1%) was low compared to the one between them (23.7%), which allowed individuals to be correctly assigned to their respective species, except for one locality (Susua Reserve Forest) where both species co-exist [22]. Indeed, a complementary study demonstrated the existence of some hybrid individuals in this locality, with intermediate morphological characteristics between the two original taxa, and a high heterozygosity [43]. Furthermore, cross-pollination experiments were successful between *V. barbellata* and *V. claviculata* with high seed quality [43].

The mixed reproductive system (vegetative and sexual) of *Vanilla* can lead to a complex mixture of sexual reproduction and uniparental lineages [67]. Furthermore, polyploidization is frequent in *Vanilla*, especially in African species, increasing intraspecific diversity and making species delimitation even more difficult [37,68,69]. As a result of the three characteristics previously developed, i.e., (a) uniparental reproduction (clonal reproduction or self-fertilization), (b) interspecific hybridization between sympatric species, and (c) polyploidization capacity, the *Vanilla* genus gathers the criteria defined by Ennos, French, and Hollingsworth in 2005 to designate “Taxonomically Complex Groups” (TCGs) [67].

2.8. Ethnobotanic and Commercial Importance

The first use of *Vanilla* plants dates back to the time of the Maya civilization where it was called “sisbic” [20,70]. *Vanilla* was probably used by Mayas to treat skin wounds and bites at least since the 14th century, and the Mayas were also the first to develop the methods for treating fruits [70]. Then, other uses of *Vanilla* were also reported, such as vermicide in Mexico and Cuba, and to flavor rum by Chiapas at the beginning of the 19th century [20]. Roots of *V. grandifolia* and *V. ovalis* serve as a material for making fishing nets and baskets [14]. *Vanilla* species that produce aromatic fruits belong exclusively to the *Vanilla* subgenus *Xanata* sect. *Xanata* [9,71]. Three *Vanilla* species are cultivated and commercialized: *V. planifolia* worldwide especially in the SWIO region, *V. × tahitiensis* in the Pacific Ocean region and *V. pompona* in central America, the French Caribbean and Guiana [14,68].

But *V. planifolia* represents the most economically important species. It was first introduced in Europe in 1510 and used for medicinal and flavoring purposes [23]. The discovery of the manual pollination technique by Charles Morren in 1836 and the simplification of the technique in Reunion island by Edmond Albius in 1841 has made the culture of commercial *Vanilla* plants easy and possible worldwide [72].

Although only *V. planifolia* is of major economic interest on the global scale, the other species represent an important gene pool for the future breeding programs of commercial varieties. Indeed, other species have interesting traits that *V. planifolia* may not always have, such as high fruit set [42], self-pollination [20,42,46], resistance to diseases [59,64,73], medicinal proprieties [10,23,70,74] and adaptation to drought [10,23].

The leafless group comprises *Vanilla* species with a high potential for the future. In addition to their drought adaptation, they were shown to be resistant to *Fusarium*, one of the most destructive diseases of the *V. planifolia* plantations [75]. For example, four leafless *Vanilla* species from the SWIO region (*V. phalaenopsis*, *V. humblotii*, *V. madagascariensis*, *V. roscheri*) and *V. aphylla* from Asia were resistant or only slightly sensitive to *Fusarium oxysporum* f.sp. *radices-vanillae* after in vitro inoculation tests [59,64]. Also, leafless species are valued in a variety of traditional uses and for their medicinal properties. Chemical analyses realized on *V. dilloniana* stems showed antimicrobial and antiproliferative activities [74]. In India, local people use the stem of *V. walkeriae* as fodder for cows having new calves to increase their milking capacity [76] and to treat fever in cattle [77]. *V. decaryana* is used in the south of Madagascar for its aphrodisiac proprieties (Figure 3C) [10]. Traditional uses of leafless *Vanilla* plants are dominated by the knowledge of ethnic groups in southern Madagascar. Stems are used as a “tonic” for men but also in “post-partum” and child cares [78]. Ethnobotanical surveys have also revealed that the sister species *V. madagascariensis* is widely used in Malagasy cosmopolitan cities to treat certain diseases and for its aphrodisiac properties [79–81]. Also, *V. roscheri* serves as a charm to protect homes in South Africa [5].

Taking into consideration these interesting specificities, leafless species constitute an important genetic pool for *Vanilla* breeding programs especially in the present context of global climate change.

2.9. Conservation Issues

As for all wildlife species, wild *Vanilla* populations are in great danger due to anthropogenic activities. According to the IUCN red list, eight *Vanilla* species (*V. hartii*, *V. inodora*, *V. insignis*, *V. odorata*, *V. phaeantha*, *V. planifolia*, *V. pompona*, *V. somai*) are listed as endangered, and one species (*V. cribbiana*) is categorized as critically endangered [82]. Some species have a very limited distribution (e.g., *V. denshikoira* in Colombia [39]) and are critically threatened by agricultural practices and deforestation (e.g., *V. paulista* and *V. dietschiana* in Brazil) [83–85]. Populations even consist, in extreme cases (e.g., *V. javieri* in Colombia), of a single known individual with a generally low fruit set [66]. In Malaysia, *V. montana* has been indicated as a rare species with the type specimen as the only known plant [9], and recently it has been reported to be a lost taxa [86]. Wild *V. madagascariensis* are also sometimes over-exploited to be used in traditional medicine [79].

Habitat destruction can also lead to the loss of genetic diversity or reduce the pollinator availability, which can impact the reproduction strategy of species. This was demonstrated for two leafless species from the SWIO: *V. roscheri* in South Africa and *V. humblotii* in Mayotte, respectively [19,21,87].

A study using microsatellite markers demonstrated a total absence of genetic diversity, i.e., a generalized homozygosity and monomorphic loci in populations of *V. roscheri* near lake Sibaya in South Africa [19]. Two scenarios have been put forward to explain this worrying situation. First, habitat fragmentation could have led to population bottleneck and isolation, which resulted in inbreeding [19]. Alternatively, the Sibaya populations could be the result of clonal reproduction of a single migrant from Northern regions [19]. Although widely distributed, this species is considered as endangered and rare in several countries. For example, it has been classified as a flagship species, the second rarest endemic and threatened species in the St Lucia Wetland Park and an endangered

species in South Africa [5,88]. *V. roscheri* is also one of the traditional African plants [5]. Given this lack of genetic diversity, anthropogenic pressures and potential uses, in situ and ex situ conservation efforts of *V. roscheri* in South Africa should be implemented urgently. Indeed, loss of genetic diversity is synonymous with an inability to evolve in response to environmental changes, and is often associated with inbreeding and reduction in reproductive fitness [89]. Thus, the current status of natural populations in other sites throughout the distribution range of *V. roscheri* should be monitored in order to prevent genetic erosion and preserve the species.

In Mayotte, *V. humblotii* is represented by nine populations spread over less than 20 km² [87]. It is part of the protected species list cited in a Prefectoral decree (Arrêté n°361/DEAL/SEPR/2018), establishing the list of protected plant species and regulating the use of endangered plant species in the French department of Mayotte. According to the report carried out jointly by the IUCN (International Union for Conservation of Nature), the CBNM (Conservatoire Botanique National de Mascarin), the CBNF (Fédération des Conservatoires Botaniques Nationaux) and the MNHN (Muséum National d' Histoire Naturelle), *V. humblotii* is classified as “Vulnerable” in Mayotte due to competition with invasive species, clearing and burning carried out for banana, cassava and yam crops. A conservation strategy is being implemented [87]. A study using microsatellite markers revealed that, surprisingly, the genetic diversity of *V. humblotii* in Mayotte is relatively high. It is probably a remnant diversity, maintained almost exclusively by clonal reproduction [21], given the low fruiting rate and the lack of pollinators observed. Nevertheless, the very high anthropogenic pressure on *V. humblotii* populations in Mayotte could lead to a bottleneck, reducing genetic diversity, increasing inbreeding and putting populations in danger.

Given the lack of information or alarming status of some natural populations, the implementation of in situ and ex situ conservation actions is urgent for *Vanilla* species, as proposed by Flanagan and Mosquera-Espinosa for *Vanilla* species in Colombia [90].

3. The Leafless *Vanilla* Clade from the SWIO Region: A Taxonomic Puzzle

SWIO leafless *Vanilla* species are an interesting model for evolutionary studies of the genus *Vanilla* as they represent a recent monophyletic group [3,91] evolving in a mosaic of environments [92,93], with a high potentiality for breeding programs due to drought adaptation capacity [10,23], disease resistance [64,73] and medicinal properties [10,79–81].

The SWIO islands are home for nine *Vanilla* species including two Malagasy leafy species belonging to the Papillosae sub-section of Portères and *Tethya* section of Soto Arenas and Cribb, and seven leafless species [8–10,34,35]. After describing the distribution and botanical differences between these leafless species, we will discuss the importance of this clade for evolutionary studies.

3.1. Distribution

Among the seven leafless species from the SWIO, *V. humblotii* is the only one that has been observed in several countries: Comoros, Mayotte and Madagascar [8,10,34], although it was described as endemic to the Comoros Archipelago (Figure 5) [10]. This species grows on rocks in the north of Madagascar and in the Comoros, whereas the closely related species *V. perrieri* is found in dry forests with sandy soils in the west and south of Madagascar (Figure 5) [8,10,34]. Three other species are indigenous to Madagascar, of which *V. madagascariensis* is the most widely distributed (Figure 5) [8,10]. Indeed, it occurs in the dry forests along the west coast and was also observed in the northeastern rainforests of Madagascar [8,29]. Given its large distribution, *V. madagascariensis* lives in sympatry with the other Malagasy leafless *Vanilla* species (Table 3, Figure 5) [8]. *V. decaryana* grows mainly in dry deciduous shrubs among Didieraceae in the south of Madagascar [10]. Finally, *V. bosseri* was observed in several localities in the west of Madagascar [35,53]. *V. roscheri* is found along the coast and islands of East Africa. The species has been observed mainly in Ethiopia, Somalia, Kenya, Tanzania, Zanzibar, Pemba Islands, Mozambique and South Africa [5,9,10,19,94]. *V. phalaenopsis* is described as endemic to Seychelles (Figure 5) [9,10].

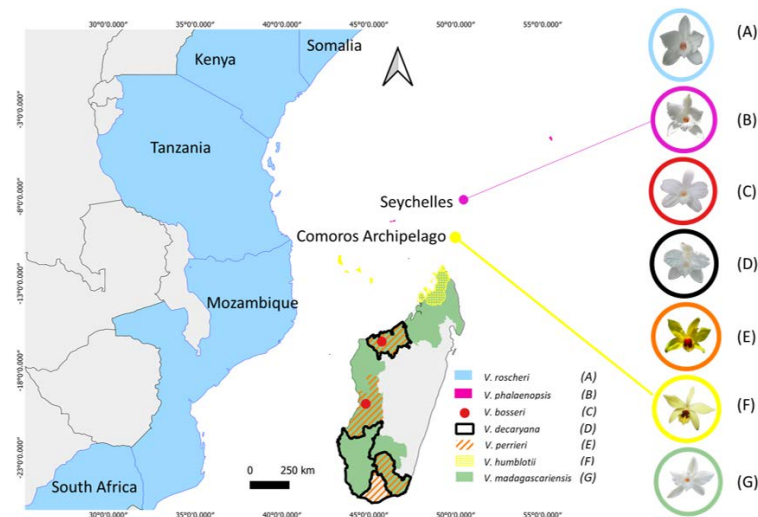


Figure 5. Distribution area of the seven leafless *Vanilla* species from the SWIO region according to Allorge-Boiteau [34], Allorge-Boiteau [35], Cribb and Hermans [8], Chinsamy, Finnie, and Van Staden [5], Luke [94], Portères [10]. Species are numbered from A to G. Images of their flowers are shown: photographs by (A) Rodolphe Gigant near Sibaya Lake (South Africa), (B) Thierry Pailler in Seychelles, (C) Cathucia F. Andriamihaja in Marosely (Madagascar), (D) Hoby Nomenjanahary in Mangily (Madagascar). (E) Cathucia F. Andriamihaja in Befandrama (Madagascar), (F) Johnson G. Andrianantenaina in Montagne des Français Park (Madagascar), and (G) Alemao Botomanga in Maromandia (Madagascar).

3.2. Botanical Description

Initially, Portères described seven leafless species in the SWIO [10], but one was invalidated by Soto Arenas and Cribb in 2010 in the absence of a Latin description and type specimen [9], and another one was recently added [35]. Seen for the first time in 2012 by Lucile Allorge-Boiteau in the Namoroka reserve [35], *V. bosseri* is one of the leafless species with white flowers endemic to Madagascar. Allorge-Boiteau describes it as a very fragrant species [35]. This species was also observed in the Morondava region by Bosser who identified it as *V. montagnacii* [35]. However, the latter was invalidated and Portère's description indicated the presence of a median hair in the basal position, which is not observed in *V. bosseri* [10,35].

Six of the SWIO species form, together with *V. walkeriae* from Asia, the *V. phalaenopsis* morphological group according to Soto Arenas and Cribb's classification [9]. This group is characterized by a flabellate labellum with two rows of hairs beside the adaxial line, obtuse and very wide petals [9]. However, the last species discovered in this group, *V. bosseri*, was not included in the list. Although similar to the others, *V. bosseri* does not completely satisfy the description of the *V. phalaenopsis* group due to the absence of hairs on its labellum (Figure 6) [35].

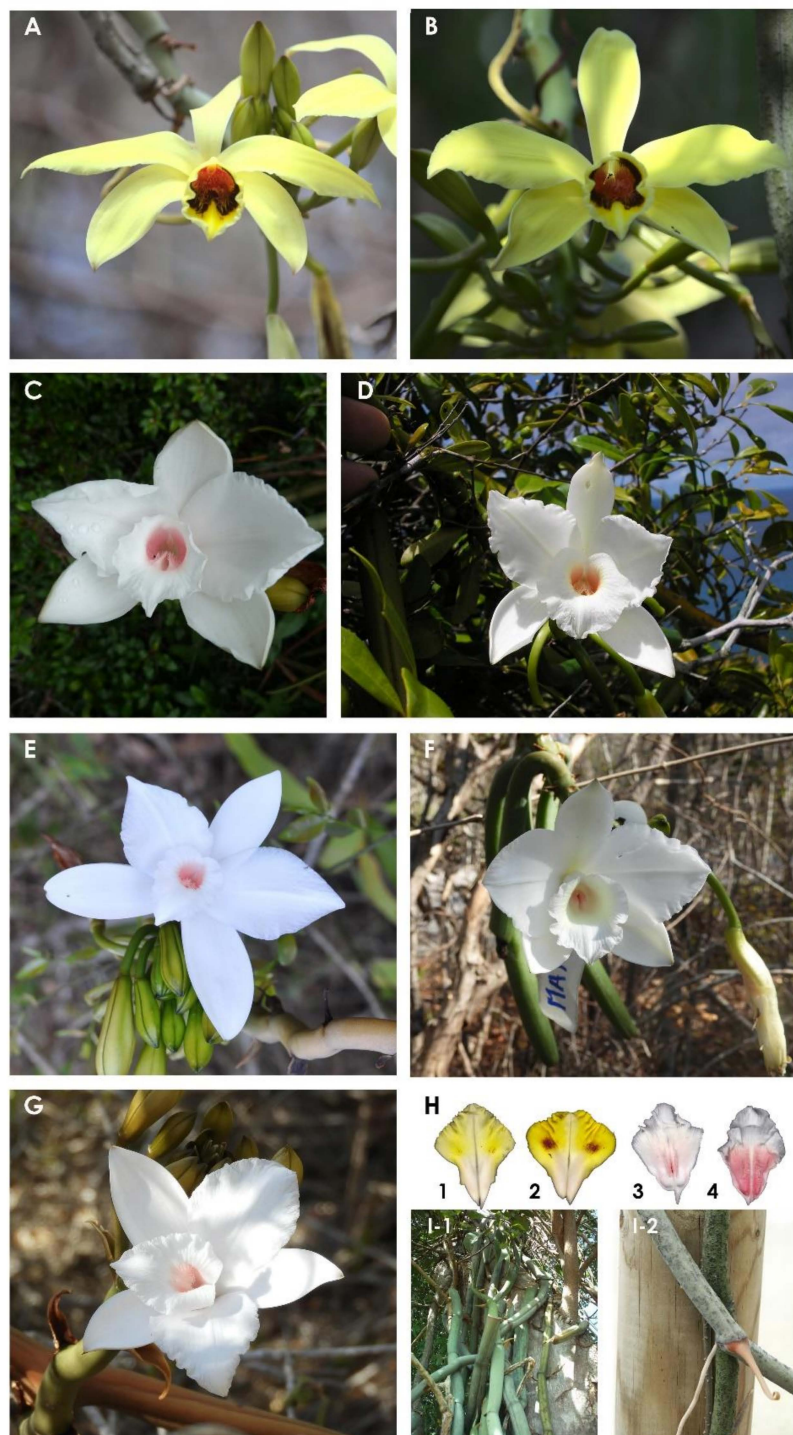


Figure 6. Morphological diversity of the seven leafless *Vanilla* species from the SWIO region. Flowers of (A): *V. humblotii* from Montagne des Français (Diego, Madagascar), (B): *V. perrieri* from Befandrama in the district of Analalava (Madagascar), (C): *V. roscheri* from Lake Sibaya in South Africa, (D): *V. phalaenopsis* from Seychelles, (E): *V. madagascariensis* from Maromandia in the district of Analalava (Madagascar), (F): *V. bosseri* from Marosely in the district of Port Bergé (Madagascar), (G): *V. decaryana* from Mangily in the district of Tuléar II (Madagascar). (H): scan of (1) the back side of *V. humblotii* labellum, (2) the back side of *V. perrieri* labellum, (3) the inside of *V. bosseri* labellum and (4) the inside of *V. madagascariensis* labellum. (I): Stems of (1) *V. bosseri* from Antsianitia (Madagascar), (2) *V. humblotii* CR0108 from the Comoros (BRC Vatel collection). (Photo A, B: Johnson G. Andrianantenaina, C: Rodolphe L. Gigant, D: Thierry Pailler, E: Alemao Botomanga, F,G,H: Cathucia F. Andriamihaja, I: Pascale Besse).

Nevertheless, the seven leafless *Vanilla* species from the SWIO region are very similar morphologically [9,10]. Following our own comparative observations in the SWIO region, the main discriminant morphological features between the various species were identified. The major distinctive features between the seven species are presented in Table 4 and flower images are shown in Figure 6.

Table 4. Leafless *Vanilla* species from the SWIO region: geographical distribution, flowering period and morphological characteristics of the flower and stem (C: color, LP: length of petal, LS: length of sepal, La: labellum, Fl: inflorescence, F: form, Tx: texture).

Distribution	Flowering Period	Flower	Stem
		<i>V. bosseri</i> L. Allorge, 2013	
Madagascar: Southwestern ^a Namoroka ^a Morondava ^a	September–November ^{b,c}	LS: 4.5 cm ^a C: white ^a La: pinkish throat with no pubescence and red protrusion between the crests ^{a,b}	C: grey in young stems and turns green in old stems ^a F: four longitudinal grooves ^a Tx: generally glabrous ^{a,c}
		<i>V. decaryana</i> H. Perrier, 1934	
Madagascar ^d : South ^d Southwestern ^e Andohahela ^e	January ^d	LP: 3 cm ^d LS: 2.5 cm ^d Fl: 20–40 flowers ^d La: pinkish throat with two pubescent lines ^d	C: greenish-brown ^d F: two longitudinal grooves ^e Tx: glabrous ^{c,d,e}
		<i>V. humblotii</i> Reichenbach f., 1885	
Comoros ^d , Mayotte ^{d,g} Madagascar ^f : North ^f Ankarana ^e Bemaraha ^e Andohahela ^e	November–April ^g	C: canary-yellow ^{d,g} LP: 5.5–7 cm ^d LS: 5.5–6.5 cm ^d La: very pubescent with velvety red or dark maroon throat ^{d,h} , papillosae ^h Fl: 40–50 flowers ^g	C: glaucous green ^d or bluish ^e F: one longitudinal groove ^e Tx: verrucose ^{c,d,e}
		<i>V. madagascariensis</i> Rolfe, 1896	
Madagascar ^d : North (Sava, Diana, Ankarana) ^a Isalo ^a Ivohibe ^a	June–October ^{a,d}	C: white ^d LP: 5.5–6.5 cm ^a LS: 5.5–6.5 cm ^d Fl: 10–20 flowers ^{a,d} La: pubescent with red throat ^a	C: glaucous green ^d F: one longitudinal groove ^e Tx: glabrous ^{a,d,e}
		<i>V. perrieri</i> Schlechter, 1925	
Madagascar: Ankarafantsika ^d Western coast (Majunga, Morondava, Fort Dauphin, Ambovombe) ^e	November–December ^c	C: sharp yellow ^d LP: 5.5 cm ^d LS: 6.5 cm ^d La: pubescent with bright red or dark brownish throat ^{d,h} not papillosae ^h , extension of the hair on the back ^d	C: dark green ^e F: two longitudinal grooves ^e Tx: glabrous ^{d,e} , similar to <i>V. madagascariensis</i> , but sometimes verrucose ^c
		<i>V. phalaenopsis</i> Reichenbach f., 1868	
Seychelles ^a	April ⁱ July–November ^j	C: white ^d LP: 5.5–6 cm ^d LS: 5.5–6 cm ^d Fl: corymbiform ^d La: two pubescent lines and yellow-orange desk ^d	C: green ⁱ F: one longitudinal groove ^d Tx: glabrous, similar to <i>V. madagascariensis</i> ⁱ
		<i>V. roscheri</i> Reichenbach f., 1877	
East African: Mozambique ^d Zanzibar ^d Pemba Island ^d Dar-es-Salam ^d South Africa ^k	November–March ^k	C: white ^d LP: 7.5–8 cm ^d LS: 7.5–8 cm ^d Bud: brownish ^d La: two pubescent lines desk and pink throat ^{d,k}	C: reddish-brown ^d Tx: glabrous similar to <i>V. madagascariensis</i> ⁱ

^a [35], ^b [53], ^c personal observation, ^d [10], ^e [34], ^f [8], ^g [21], ^h [9], ⁱ P. Besse, personal communication, ^j [6], ^k [19].

The stem texture and color are often different between species, with species showing glabrous or verrucose; grey, brown, red to green stems; with one to four longitudinal grooves [10,19,21,34,53]. But these characters are also highly variable within species. Based on flower color, species can be divided into two groups: species with yellow flowers and those with white flowers [8,10,34]. *V. humblotii* and *V. perrieri* compose the first group. The former has a verrucose stem and yellow canary

flowers, that allows to distinguish it from *V. perrieri* which exhibits a glabrous stem and sharp yellow flowers [10,34]. One of the particularities of *V. perrieri* is also the presence of an extension of the hairs outside the hulls on the back of the labellum (Figure 6) [10]. Five closely-related species compose the white-flower group, of which three are endemic to Madagascar [8–10,34,35]. All have a smooth stem and hairs on the inside of the labellum, except *V. bosseri* whose labellum is rather characterized by two pink crests and a short red protrusion between the crests (Figure 6) [34,35,53]. The five species also differ in the color of the inside of the labellum and flower size, *V. decaryana* being the one with the smallest flowers (Table 4) [9,10,34].

3.3. A Taxonomic Puzzle

When genetic differentiation is associated with consequent morphological differentiation, species delimitation is generally easy [95]. But in some cases, when species are highly differentiated morphologically but little divergent genetically (adaptive radiation), or when the morphological change is slow and does not reflect genetic divergence (nonadaptive radiation), species delimitation becomes a challenging task [95]. Often, adaptive radiation occurs among a related and recently diverged sympatric group [95–98], whereas nonadaptive radiation would be the result of prolonged geographical isolation of several populations of the same species, followed by subsequent slow speciation resulting from nonecological processes [98]. However, nonadaptive radiation can be followed by adaptive radiation when the environment of the two species becomes different [98]. Species delimitation is even harder when recent sympatric species can hybridize [97,99]. SWOI leafless *Vanilla* species are one of the most recently formed groups in the genus *Vanilla* (4.4 Mya) (Figure 1) [3]. Therefore, species divergence within the group has occurred recently. The observations we made on the vegetative and floral organs of individuals belonging to three species *V. roscheri*, *V. phalaenopsis* and *V. madagascariensis* present in the BRC (Biological resources center) Vatel (Reunion island) allowed us to confirm that they are closely related and difficult to differentiate. Flowers mainly show variations in size (Table 4), which is not always a sufficient criterion to discriminate species, especially in the *Vanilla* genus with high intra-specific variation [100]. Also, Cameron suggested that these three species could probably be morphological variations of a single species [101]. Given that these three species are supposedly endemic to three different geographical areas (East Africa, Madagascar, Seychelles), whose separation took place well beyond the formation of the leafless group (Figure 7), we suppose that they are probably separate populations of the same species that have recently diverged and are evolving via nonadaptive radiation.

On the other hand, the five described leafless species found in Madagascar can be differentiated by the size of the flower, and the color and the form of the labellum [8,34,35]. As previously discussed, these species are sympatric in several areas, and interspecific hybridization is possible between *Vanilla* species [18,43]. During our fieldwork in the north of Madagascar, we observed one individual with flowers showing intermediate colors between yellow and white, suggesting a possible hybridization between species. Unfortunately, we were unable to obtain images. The possible occurrence of five leafless species in Madagascar with some overlapping distribution and the possible hybridization raise numerous questions about the mechanisms of their evolution and speciation. For these Malagasy species, adaptive radiation and ecological speciation have probably occurred in the past via one of the five mechanisms reported in Madagascar by Vences et al. [92]. Concerning the remaining species, *V. humblotii*, supposed to be endemic from the Comoros archipelago, its presence in Madagascar and its great morphological similarity with the native Malagasy species, *V. perrieri*, suggest that they either form subspecies of the same species or are the result of colonization followed by differentiation long after the formation of the islands. Species movement between SWIO islands has already been demonstrated in various genera including birds (e.g., *Nectarinia*, *Hypsipetes* [102,103]), plants (e.g., genus of Coffeae tribe [104]) and more especially orchids (e.g., *Bulbophyllum*, *Angraecum* [105–107]).



Figure 7. Map showing the date (in Mya) of separation and formation of the SWOI islands adapted from Warren et al., 2003 [102].

Complexes of closely related species endemic to different islands provide an interesting model to better understand the different stages of speciation. In fact, islands own several characteristics that make them an excellent natural laboratory, including the presence of strict boundaries, the isolation, the high endemism, the small size relative to continent, the geographic history, and the environment heterogeneity providing natural ecological and evolutionary replicates [108]. This interest in islands goes back to Darwin, who used a variety of islands as a model to illustrate different patterns of species richness and evolution, of which the famous Galapagos islands largely studied until now [109]. All these interesting features are visible in the SWIO islands, which are a biodiversity hotspot [110]. At the scale of Madagascar, the oldest island of the SWIO region separated from Africa 130 Mya and from India 88 Mya (Figure 7), seven bioclimatic regions with unique vegetation are present, ranging from a humid tropical forest in the east to an arid biome in the far south (Figure 8) [93]. This Malagasy ecological contrast is the source of various speciations, particularly in the animal kingdom (e.g., lemur species [111,112]).

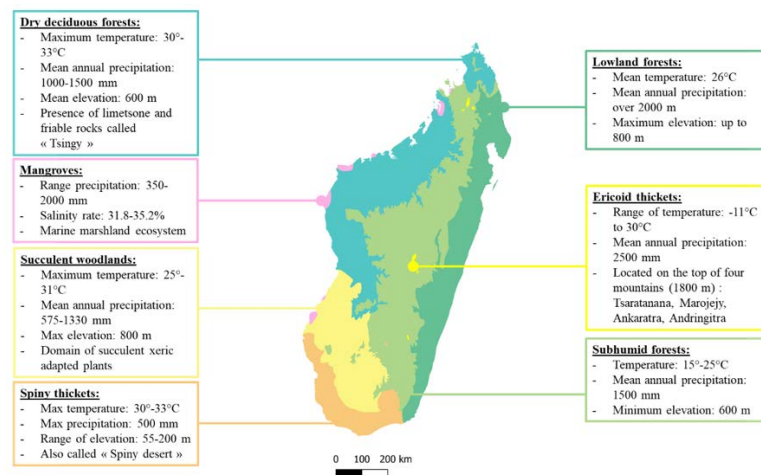


Figure 8. Map of the seven ecoregions of Madagascar adapted from WWF's ecoregion in Burgess et al. [93].

Moreover, SWIO islands have variable ages and different geographical histories (volcanic islands like Comoros vs. mainland islands such as Madagascar and Seychelles), allowing to test several speciation mechanism hypotheses. The presence of *Vanilla* sister-species, endemic to the African continent or to different islands (*V. roscheri*, *V. madagascariensis*, *V. phalaenopsis*) and the presence of sympatric *Vanilla* species in Madagascar offer a promising system for species diversification studies.

3.4. Conclusion and Prospects

Because of today's environmental crisis, biodiversity conservation has become a major global concern. Two kinds of strategies are generally recommended to achieve this goal: species-based conservation and conservation at the ecosystem scale [113]. Whatever the approach, efforts to catalogue and understand biodiversity form the basis for species and habitat effective conservation. However, correctly defining species belonging to taxonomically complex groups like *Vanilla*, where conventional classification fails, is not always evident. Yet, incorrect taxonomic classification could have important consequences for basic biological research and harm biodiversity conservation [114,115]. For example, *V. humblotii* is considered as endemic to the Comoros archipelago and is classified as endangered (EN) in Mayotte and therefore under protection status since 2006. However, this species has also been described as present in Madagascar, making its overall conservation status questionable. According to many authors, taxonomy often requires the comparison between several models based on different types of data and should incorporate molecular investigations [114,116,117]. In the case of the seven leafless *Vanilla* species native to the SWIO, classifications based on morphological characters in a very detailed manner [10,34,35], on a large sampling, as well as population genetics and phylogenetic analyses are needed. Chloroplast genes *rbcL*, *psaB*, *psbB*, and *psbC* have already been used for *Vanilla* phylogeny [3]. But these sequences are not variable enough to properly clarify the taxonomy in the recent SWIO clade [24,118]. As the available information on genetic structuring and phylogeny of these species is insufficient, the resolution of their taxonomy is problematic, which limits the development of conservation actions. Integrative taxonomy using phylogenetic analyses with more divergent regions of DNA such as ITS [24,118], genetic structuring analysis with highly polymorphic and rapidly evolving markers like microsatellites [97], completed by a morphological comparison of several individuals from the different species like what has been done for *Carappa spp.* [116] and *Coffea spp.* [119] will be a powerful approach to clarify their taxonomy. Such integrative approaches are currently becoming frequent in species delimitation [9,20,25,99,116] and mandatory for species complexes [97,99,116] or cryptic species [114,120,121]. These different markers associated with other environmental variables such as climatic or soil data would also help to understand the speciation processes at play [122–124].

Issues related to interspecific hybridization should also be addressed in taxonomic and phylogenetic studies [99] of these seven SWIO leafless species.

In addition, interspecific and intraspecific crossbreeding tests as done for *V. barbellata* and *V. claviculata* [43], *V. bosseri* [53], *V. humblotii* [21] and *V. roscheri* [19], the modeling of the geographical distribution and population genetic studies of the different species will greatly improve our knowledge about these interesting models and allow the implementation of effective and sustainable conservation programs.

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1.3. Problématique et objectifs de recherche

« Les défenseurs de la nature sont loin de pouvoir aider toutes les espèces menacées... D'où l'importance des priorités : comment soutenir le plus d'espèces à moindre coût ? L'une des solutions consiste à se focaliser sur les "points chauds de la biodiversité" où des concentrations exceptionnelles d'espèces endémiques subissent une perte exceptionnelle d'habitat. »
Myers

Madagascar fait partie des pays où la biodiversité est exceptionnelle, caractérisée par une richesse spécifique impressionnante, avec un taux d'endémicité estimé à 100% chez les amphibiens, 92% chez les reptiles, 44% chez les oiseaux, 74% chez les papillons et plus de 90 % chez les plantes (Vences et al., 2009). Bien qu'ayant une petite superficie, les îles abritent souvent une richesse spécifique équivalente, voire plus élevée que les continents (Kier et al., 2009; Myers et al., 2000; Stuart et al., 2012). En effet, le taux de renouvellement des espèces ou « species turnover » dans les îles est souvent plus élevé que celui des continents (Stuart et al., 2012). A Madagascar, la radiation des espèces est le résultat de plusieurs facteurs tels que son histoire d'isolement biogéographique, son histoire et sa diversité climatique, sa topographie et ses réseaux hydrographiques (Vences et al., 2009). Pour certains groupes à l'exemple des orchidées, cette richesse spécifique est plus accentuée dans les îles continentales⁴ comme Madagascar comparée aux îles océaniques⁵ (Taylor et al., 2019). En même temps, la richesse naturelle de Madagascar est dans un état critique, car menacée par un déclin considérable de la superficie des habitats naturels (Myers et al., 2000; Vieilledent et al., 2018). Par exemple, la couverture forestière de la forêt sèche malgache a régressé de près de 46%, celle des forêts épineuses du Sud a diminué d'environ 30% et celle des forêts humides a chuté d'environ 49% entre 1953 et 2014 (Vieilledent et al., 2018). A Madagascar, les communautés sont très dépendantes de la biodiversité pour leurs besoins quotidiens (Rakotomanana et al., 2013). Elles sont constituées majoritairement d'agriculteurs et utilisent principalement de l'énergie issue du charbonnage et du bois de feu. Durant les périodes de famine, la pratique de la chasse et les collectes dans les forêts permettent aux communautés riveraines des forêts de survivre. D'autre part, les activités minières sont nombreuses (Canavesio, 2014; Sarrasin, 2006; Stoudmann et al., 2021). Les évaluations réalisées à l'échelle mondiale ont montré que les impacts de l'activité humaine sont nettement supérieurs dans les îles que dans les continents, une situation qui risque d'empirer selon les projections futures (Kier et al., 2009; Myers et al., 2000). De ce fait, les pressions exercées sur les ressources naturelles de la grande île sont particulièrement élevées et les menaces sur la biodiversité de plus en plus ressenties. Cette situation paradoxale a valu à Madagascar sa place parmi les pays points chauds (hotspots) de la biodiversité mondiale (Myers et al., 2000). A Madagascar, les orchidées sont parmi les plantes préférées des naturalistes du fait de leur grande diversité, avec une richesse estimée à plus de 850 espèces réparties dans 57 genres (Callmander et al., 2011 ; Cribb et Hermans, 2009) parmi lesquels figure le genre emblématique *Vanilla*. Avec sept espèces, dont cinq aphylls (Chapitre 1—1.2),

⁴ Ile continentale : étendue de terre détachée du continent près duquel elle se trouve et dont elle est séparée.

⁵ Ile océanique : par exemple, île formée à partir de l'accumulation de matériaux, liée à l'activité volcanique.

Madagascar présente une riche diversité en vanilliers endémiques. Comme la plupart des espèces malgaches, les vanilliers aphyllés sont également victimes de la destruction de leurs habitats et de leur exploitation en tant que plantes médicinales (Randrianarivony et al., 2017; Vieilledent et al., 2018; Zizka et al., 2020). Malheureusement, les menaces dont font l'objet ces espèces ainsi que leur statut de conservation n'ont pas encore été bien définis pour de multiples raisons, dont la difficulté de l'identification taxonomique et l'absence ou l'insuffisance d'informations sur l'état des espèces dans leurs habitats naturels. En effet, l'intérêt porté à la conservation de la flore est, souvent, moins important comparé à celui dédié aux espèces animales emblématiques, un phénomène appelé « plant blindness » (Allen, 2003; Balding and Williams, 2016). A titre d'exemples, ces dernières années, il a fallu cibler des espèces à forte valeur ajoutée et menacées par le commerce illégal comme les bois précieux des genres *Dalbergia* et *Diospyros* pour recevoir des fonds de conservation conséquents. Dans le cas des vanilliers aphyllés malgaches, des projets se sont surtout développés grâce à la reconnaissance des potentialités médicinales et cosmétiques de ces espèces. A ce jour, les informations sur ces vanilliers aphyllés de Madagascar se limitent aux descriptions botaniques des espèces déjà inventoriées (Allorge-Boiteau, 2013, 2005; Cribb and Hermans, 2009; Portères, 1954; Soto-Arenas and Cribb, 2010), à une étude de la biologie de la reproduction dans la réserve de Kirindy pour l'espèce *V. bosseri* (Pettersson, 2015), et à une étude physiologique des racines (Botomanga, 2018). La taxonomie des cinq espèces de vanilliers aphyllés décrites à Madagascar reste encore extrêmement ambiguë, à cause de leur ressemblance morphologique et de leurs relations phylogénétiques qui ne peuvent être résolues à partir du seul recours aux séquences d'ADN chloroplastique (Chapitre 1—1.2).

Ce problème taxonomique est le point de départ de cette thèse. Il a engendré une série de questions relatives aux processus en jeu dans l'évolution de ces vanilliers aphyllés à Madagascar :

- Combien de groupes génétiques de vanilliers aphyllés existent à Madagascar ?
- Est-ce que les groupes génétiques correspondent à des espèces morphologiques décrites dans la littérature ?
- Les distances génétiques entre ces groupes s'expliquent-elles par de l'isolement géographique ou de l'isolement écologique ?

Les réponses à ces trois questions devraient permettre une meilleure compréhension des mécanismes de différenciation des populations et de spéciation de ces vanilliers aphyllés malgaches.

Une fois cet objectif résolu, il est important de résoudre les relations phylogénétiques entre les espèces malgaches et les espèces sœurs du groupe des aphyllés du SOOI. Une deuxième série de questions est alors soulevée :

- Quelles sont les relations phylogénétiques entre les espèces de vanilliers aphyllés de la zone Sud-Ouest de l'Océan Indien ?

- Quelles sont les dates approximatives de divergence des groupes phylogénétiques identifiés ?
- Est-ce que les groupes phylogénétiques correspondent aux espèces morphologiques décrites dans la littérature ?

Résoudre ces questions est essentiel afin de clarifier la taxonomie et la phylogénie des espèces de vanilliers aphyllés du SOOI et, si nécessaire, proposer une révision taxonomique.

Finalement, des questions relatives à l'état des populations naturelles de ces espèces sont posées dans le but de contribuer à leur conservation :

- Quel est le taux de fructification naturel de ces espèces à Madagascar ?
- Est-ce que les espèces peuvent s'hybrider entre elles ?
- Quelle est la distribution géographique de ces espèces à Madagascar ?
- Quel est le niveau de diversité génétique de chaque espèce à Madagascar ?
- Est-ce que la taille effective des populations des espèces est compatible avec leur survie à court et à long terme ?

A partir de toutes les informations découlant de ces questions, les menaces potentielles et le statut de conservation de chaque espèce pourront être identifiés.

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1.4. Stratégie d'étude

*« En préparant une bataille, j'ai toujours trouvé que les plans sont inutiles, mais que la planification est indispensable. »
Dwight D. Eisenhower*

Cette thèse entend ainsi contribuer à reconstituer l'histoire évolutive de ces vanilliers aphyllés et résoudre leur taxonomie pour une meilleure conception de futures stratégies de conservation, dans le contexte du changement global actuel. Néanmoins, répondre à toutes ces questions est un défi assez ambitieux. D'une part, la répartition de ces vanilliers aphyllés à Madagascar recouvre une large superficie représentant presque toutes les côtes malgaches. Ces zones de distribution sont parfois situées dans des localités très reculées et peu sécurisées. D'autre part, ces questions enveloppent plusieurs disciplines scientifiques : la biologie moléculaire, la systématique et l'écologie. Ces objectifs justifient, ainsi, la collaboration sous forme d'une cotutelle de thèse entre l'Université de La Réunion et l'Université d'Antananarivo, deux entités aux expertises complémentaires dans les domaines sus mentionnés.

Au début de la thèse, les échantillons d'ADN de vanilliers aphyllés de Madagascar à notre disposition étaient limités, représentant une soixantaine d'individus de la collection du CRB Vatel à La Réunion. Certains de ces échantillons avaient été extraits à partir d'individus provenant des collections de jardins botaniques avec très peu d'informations précises sur la région de collecte ou sur l'identité taxonomique des échantillons. D'autres échantillons étaient issus d'individus prélevés dans une forêt protégée du Nord-Est de Madagascar (Manompana) ou avaient été acquis auprès de tiseurs à Diego ou de commerçants à Tuléar et Antananarivo. Le reste provenait de l'échantillonnage réalisé dans le cadre de mon stage de master 2, dans le district d'Ambanja situé au Nord de Madagascar. Aussi, la première étape de ce travail de thèse a été de réaliser des prospections et un large échantillonnage dans les zones de répartition connues de toutes les espèces, en ciblant particulièrement les aires protégées (AP). Le choix de travailler dans les AP était délibéré. Ces zones sont, non seulement les plus sécurisées, mais également les mieux équipées en termes de personnels pour le guidage et d'infrastructures pour le camping. Ensuite, ces AP sont les mieux conservées, par conséquent abritent probablement la plus grande diversité génétique de ces espèces. Compte tenu de la délimitation taxonomique

problématique des cinq espèces connues, nous avons décidé d'adopter une stratégie d'échantillonnage à l'aveugle. En d'autres termes, la première mission a été effectuée sans à priori ni sur les identités des espèces, ni sur leur période de floraison. Les expéditions ont été réparties sur trois années successives. Une première mission a eu lieu du 20 Août au 25 Octobre 2017 dans 16 sites répartis le long de la côte Ouest de Madagascar, soit un parcours de plus de 4000 km. Deux autres missions se sont déroulées du 24 Février au 03 Mars 2018, puis du 04 Novembre 2018 au 27 Janvier 2019. Ces deux dernières missions avaient pour but d'explorer d'autres sites et de rééchantillonner dans certaines populations de la première mission. Les vanilliers aphyllés étaient présents dans tous les sites visités sauf dans la forêt humide du parc national Montagne d'Ambre. Au total, 900 échantillons de tiges de vanilliers aphyllés ont été collectés dans 22 sites de Madagascar durant cette thèse, les échantillons du 23^{ème} site (Manompana) collectés en 2012 (Lauri, 2012) provenant du CRB Vatel. Pour chaque échantillon collecté, les coordonnées géographiques, la texture de la tige, la couleur de la fleur pour ceux qui avaient fleuri, le diamètre et la longueur des sept premiers entre nœuds ont été notés. Les paramètres concernant la biologie de la reproduction des populations ont également été observés. Pour toutes les missions, un long processus a été nécessaire pour l'obtention d'une autorisation de permis de recherche dans les différents sites. Si les sites étaient gérés par un organisme ou une collectivité, des demandes d'autorisation spéciales leur ont été envoyées. C'est seulement ensuite qu'une demande officielle auprès de la direction générale de l'environnement et des forêts est déposée pour une autorisation finale. Si, par contre, les sites ne sont pas gérés, la demande d'autorisation est directement adressée au service public concerné. Les prélèvements et l'exportation des différents échantillons ont fait systématiquement l'objet d'obtention d'autorisations officielles des services forestiers et des gestionnaires des aires protégées de Madagascar. En parallèle, l'octroi des Lettres Officielles d'Autorisation (LOA) par les services publics français nous a permis d'importer les échantillons à La Réunion.

Au démarrage de la thèse, l'étude a été ciblée au niveau populationnel par l'utilisation de marqueurs microsatellites (plus variables que les marqueurs chloroplastiques ayant échoué à discriminer les espèces). L'approche a été réalisée sans *à priori* de l'appartenance supposée taxonomique ou géographique des individus, ce qui a permis de réaliser une analyse *à posteriori* de regroupement génotypique sur la base de la taille des allèles microsatellites. Pour cela, les ADN de 783 échantillons, sur les 900 collectés, ont été extraits puis amplifiés. Une telle approche a déjà été utilisée et s'est révélée être efficace pour le criblage des individus appartenant à différentes espèces récentes du genre *Carappa* (Duminil et al., 2006) et du genre *Coffea* (Razafinarivo et al., 2013). Cette stratégie a également permis de mettre en œuvre des tests d'isolement par la distance et d'isolement par les facteurs écologiques, en calculant les distances génétiques, géographiques et écologiques entre les populations.

À l'issue de cette première étape complètement exploratoire, une à trois populations pures et facilement accessibles de chaque groupe génétique, soit un total de huit populations, ont été choisies pour des analyses morphologiques complémentaires afin de résoudre l'identité

taxonomique des groupes génétiques identifiés. Pour cela, nous avons mesuré les fleurs des vanilliers localisés dans huit populations, cette fois-ci en considérant leur calendrier de floraison. Les travaux ont été réalisés en Décembre 2018 et du 11 Octobre au 27 Novembre 2019 pour sept populations. La dernière mission s'est effectuée en Décembre 2020 et en Janvier 2021 pour la population de Manompana. Les travaux consistaient à collecter 10 à 20 fleurs des populations et à les scanner afin d'estimer la variabilité florale intraspécifique et comparer les variations interspécifiques. En parallèle, nous avons réalisé des tests de pollinisation manuelle intraspécifique et des croisements interspécifiques dans certaines populations.

L'analyse de différenciation basée sur la longueur des séquences d'ADN, comme le cas des microsatellites, ne suffit pas pour confirmer le statut taxonomique d'une espèce. Par exemple, l'homoplasie, la présence d'allèles rares ou d'allèles privés résultant du taux de mutation élevé des microsatellites peuvent constituer un important facteur de confusion (Rouhan et Gaudeul, 2014). En d'autres termes, deux individus peuvent partager les mêmes tailles d'allèles sans pour autant provenir du même ancêtre, c'est-à-dire une homoplasie de taille. À l'inverse, un individu d'une espèce peut présenter des allèles rares ou privés qui le différencient des autres individus de cette même espèce. En général, les microsatellites sont surtout utilisés pour les analyses à l'échelle infraspécifique (Besse, 2014). Dans le genre *Vanilla*, l'analyse des régions ITS (Internal Transcribed Spacer Region) du génome a été démontrée comme étant une technique efficace pour la discrimination des espèces de vanilliers, même celles les plus proches génétiquement (Besse et al., 2021). Ainsi, les régions ITS des ADN de trois à cinq individus appartenant aux différents groupes génétiques identifiés à partir des microsatellites ont été amplifiées puis séquencées pour une analyse phylogénétique afin de répondre au deuxième objectif de cette thèse. Nous avons aussi utilisé les ADN de quelques individus provenant de l'Afrique de l'Est, des Seychelles et de l'Archipel des Comores disponibles dans la collection du CRB Vatel pour étudier les relations phylogénétiques entre toutes les espèces aphyllées du SOOI. La première approche de structuration génétique combinée aux nouvelles analyses morphologique et phylogénétique a permis de conduire une taxonomie intégrative, une approche utilisant plusieurs outils pour définir les espèces (Dayrat, 2005; Duminil et al., 2012; Duminil and Di Michele, 2009). Les analyses morphologiques complémentaires ont permis d'améliorer les clés d'identification des espèces.

Cette thèse est réalisée conjointement avec d'autres projets de recherche à l'Université d'Antananarivo utilisant le même modèle d'étude. Ces projets ont permis de mettre en place une collection de vanilliers aphyllés au sein de l'Université. Pour la dernière phase de la thèse, nous avons échantillonné dans cette collection pour compléter les échantillons d'ADN qui avaient déjà été analysés auparavant. Tous les échantillons extraits et amplifiés à partir des marqueurs microsatellites ont été réutilisés pour une analyse génétique globale par espèce. À partir de l'analyse spatiale de la répartition des différents groupes génétiques identifiés, une modélisation des niches écologiques potentielles de chaque espèce a été réalisée. L'analyse génétique globale, combinée aux données de la biologie de la reproduction et aux résultats de

la modélisation des habitats potentiels a été les outils utilisés pour évaluer le statut de conservation de ces vanilliers aphyllés à Madagascar.

Tableau 1: Liste des 23 sites étudiés dans le cadre de la thèse.

Code	Sites	Statut	Gestionnaire	Ecorégion
AMB	Ambanja	Forêt communautaire	Etat	Forêt subhumide
ANM	Analamerana	AP catégorie IV ^a	Madagascar National Park	Forêt décidue sèche
AND	Andringitra	Forêt sacrée	Communautés riveraines VOI ^b	Forêt subhumide
ANJ, AJA	Anja	Forêt communautaire	Communautés riveraines VOI	Forêt épineuse
AKF	Ankarafantsika	AP catégorie II ^c	Madagascar National Park	Forêt décidue sèche
ANK	Ankarana	AP catégorie IV	Madagascar National Park	Forêt décidue sèche
AKL	Ankililoaka	Forêt communautaire	Etat	Forêt décidue sèche
ATA, ATD	Antandroka	Forêt sacrée	Etat	Forêt épineuse
ATS, ANT	Antsianitia	Forêt privée	Hôtel et autres	Forêt décidue sèche
BBL	Baie de Baly	AP catégorie IV	Madagascar National Park	Forêt décidue sèche
BFD	Befandrama	Forêt communautaire	Etat	Forêt décidue sèche
BTM	Betara Mahamay	NAP ^d en cours	Bongolava Maitso	Forêt décidue sèche
CMK	Complexe Mahavavy Kinkony	NAP	ASITY	Forêt décidue sèche
KRD	Kirindy	AP	CNFEREF	Forêt claire succulente
KRM	Kirindy Mitea	AP catégorie IV	Madagascar National Park	Forêt claire succulente
MLT	Mahaleotse	Aucun	Etat	Forêt épineuse
MAN	Ambodiriana Manompana	NAP en cours	ADAFAM	Forêt humide
MMD, MND	Maromandia	Forêt communautaire	Etat	Forêt décidue sèche
MSL, MRL, MARO	Marosely	NAP	Bongolava Maitso	Forêt décidue sèche

Code	Sites	Statut	Gestionnaire	Ecorégion
MDF	Montagne des Français	NAP	SAGE	Forêt décidue sèche
NMK	Namoroka	AP catégorie IV	Madagascar National Park	Forêt décidue sèche
SAG	Saint Augustin	Forêt communautaire	Etat	Forêt épineuse
ZVB	Zombitse	AP catégorie II	Madagascar National Park	Forêt claire succulente

^a : Aire de gestion des habitats ou des espèces, ^b : Association paysanne locale appelée Communauté de base (COBA), ^c : Parc National, ^d : Nouvelles Aires Protégées.

Le manuscrit de thèse s'organise en cinq chapitres. Le contexte général a été présenté dans ce premier chapitre. Le second chapitre concerne l'analyse des facteurs de divergence des vanilliers aphyllés de Madagascar. Ce chapitre a fait l'objet d'un article scientifique intitulé « Drivers of population divergence and species differentiation in a recent group of indigenous orchids (*Vanilla spp.*) in Madagascar », publié dans le journal *Ecology and Evolution* au début de l'année 2021. Il a pour objectif d'identifier les processus et les unités évolutives en action. Le troisième chapitre porte sur les résultats de l'analyse phylogénétique des vanilliers aphyllés du Sud-Ouest de l'océan indien et de la taxonomie intégrative des espèces malgaches. Ce troisième chapitre intitulé « Integrative taxonomy and phylogeny of leafless *Vanilla* orchids from the South West Indian ocean region reveal two new Malagasy species » a été soumis à la revue *Journal of Systematics and Evolution*. Le quatrième chapitre, sous forme d'un article scientifique intitulé « Present and future status of native leafless *Vanilla* species in Madagascar », fait état du statut des espèces aphyllés de Madagascar sur la base d'analyses génétique, de la biologie de la reproduction et de la distribution spatiale. Ce quatrième chapitre sera soumis au journal *Global Ecology and Conservation*. À partir des résultats présentés dans les différents chapitres, les mécanismes de spéciation des orchidées à Madagascar seront discutés dans le dernier chapitre en comparant nos résultats à d'autres modèles. Des perspectives sur les futures études nécessaires et des recommandations sur la conservation seront également fournies.

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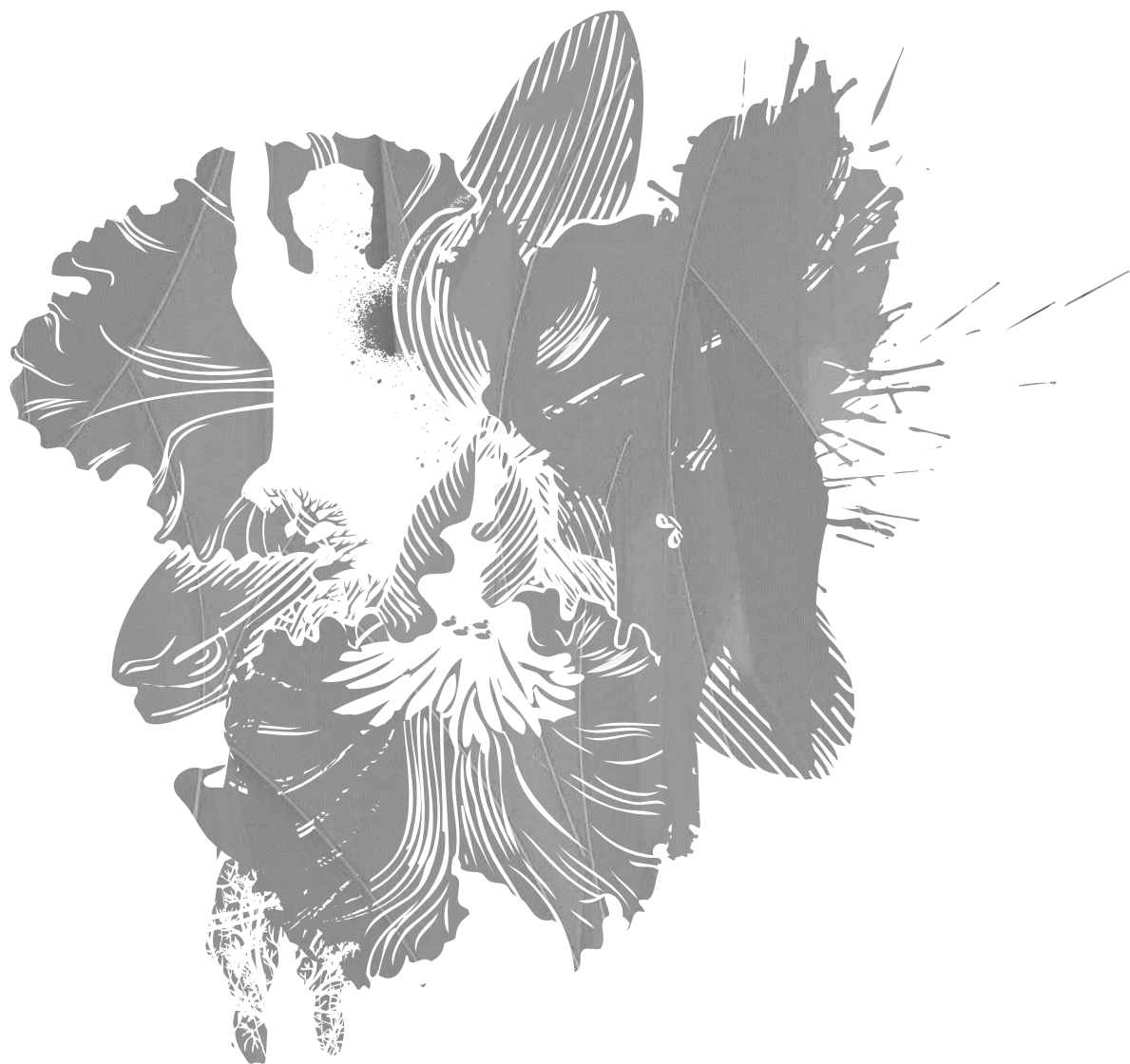
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CHAPITRE 2

CHAPITRE 2

Différenciation des espèces aphylls du genre *Vanilla* à Madagascar

« L'échiquier est le monde, les pièces sont les phénomènes de l'univers, les règles du jeu sont ce que nous appelons les lois de la nature, le joueur de l'autre côté nous est caché. » Thomas Henry Huxley

2.1. Présentation et résumé du chapitre

Avec plus de 25 000 espèces, les orchidées font partie des familles de plantes avec un taux remarquable de diversification. Depuis l'époque de Darwin, les avancées majeures ont attribué la diversité exceptionnelle des orchidées aux interactions plantes-pollinisateurs. Cependant, élucider les processus et les facteurs qui déterminent la variation phénotypique et génotypique des populations naturelles de cette grande famille reste encore un défi. Face à la difficulté de l'identification taxonomique des vanilliers aphylls de Madagascar (Chapitre 1), nous avons étudié la structuration génétique et morphologique des populations de ces espèces, en utilisant sept locus microsatellites et des variables morphologiques florales. En outre, des analyses ont été effectuées pour vérifier les différents mécanismes d'isolement qui auraient pu entraîner la différenciation génétique entre les populations, à savoir l'isolement par la distance géographique ou IBD, l'isolement par les facteurs environnementaux ou IBE et l'isolement par adaptation ou IBA. Les résultats ont montré un déficit d'hétérozygotes, donc une consanguinité, dans 22 populations, probablement du à la présence d'allèles nuls, à la géitonogamie et/ou à une sous-structuration des populations (zones de sympatrie). En revanche, un taux important de clonalité est révélé pour la seule population de haute altitude, ayant conduit à un excès d'hétérozygotes. La diversité génétique est maximale dans les populations occidentales, ce qui suggère une colonisation postglaciaire vers le nord et le sud. Les sept groupes génétiques identifiés à partir des analyses de regroupement génétique sont caractérisés par des traits floraux spécifiques, dont certains correspondent à des descriptions botaniques d'espèces déjà décrites. D'après les tests de corrélation réalisés, la différenciation de certaines populations serait le résultat d'un isolement par des barrières géographiques telles que les rivières. De plus, des effets combinés de l'IBD/IBE et de l'IBE/IBA sur la différenciation génétique ont été détectés. Sur la base de ces résultats, nous suggérons une contribution prépondérante des facteurs abiotiques (climat, sol) dans les processus de spéciation, bien que la sélection divergente résultant de la spécificité aux pollinisateurs ne puisse être exclue pour certaines des espèces. En somme, ce chapitre procure des connaissances supplémentaires sur la spéciation des orchidées, un groupe pour lequel Madagascar présente l'un des plus hauts niveaux d'endémisme au monde. Il confirme également l'importance de la biogéographie particulière de cette île qui façonne la différenciation des espèces.

2.2. Drivers of population divergence and species differentiation in a recent group of indigenous orchids (*Vanilla spp.*) in Madagascar

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Drivers of population divergence and species differentiation in a recent group of indigenous orchids (*Vanilla* spp.) in Madagascar

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Abstract

With over 25,000 species, orchids are among families with remarkable high rate of diversification. Since Darwin's time, major advances attributed the exceptional diversity of orchids to plant–pollinator interactions. However, unraveling the processes and factors that determine the phenotypic and genotypic variation of natural orchid populations remains a challenge. Here, we assessed genetic population structure and floral differentiation in recently diverged leafless *Vanilla* species in a world biodiversity hotspot, Madagascar, using seven microsatellite loci and 26 morphometric variables. Additionally, analyses were performed to test for the occurrence of any patterns of isolation by distance, isolation by environment, and isolation by adaptation and to detect possible physical barriers that might have caused genetic discontinuities between populations. Positive inbreeding coefficients detected in 22 populations were probably due to the presence of null alleles, geitonogamy and/or some admixture (sympatric species). In contrast, the only high-altitude population showed an important rate of clonality leading to heterozygote excess. Genetic diversity was maximum in western populations, suggesting a postglacial colonization to the north and south. Clustering analyses identified seven genetic groups characterized by specific floral traits that matched five botanical descriptions in the literature. A contribution of montane refugia and river barriers on population differentiation was detected. We also detected combined effects of IBD/IBE and IBE/IBA on genetic differentiation and suggested this pattern is more likely determined by ecological isolation, although pollinator-mediated divergent selection could not be ruled out for some of the species. Overall, this study provides further insights on speciation in orchids, a group for which Madagascar shows one of the world's highest level of endemism and confirms the importance of the peculiar biogeography of the island in shaping species differentiation.

KEYWORDS

ecological distances, flower traits, geographic distances, leafless, microsatellite, speciation, *Vanilla*

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1 | INTRODUCTION

Understanding how and at what rate phenotypic and genetic differentiation occurs across natural populations is one of the most fundamental questions in evolutionary biology (Ghalambor et al., 2007; Lekberg et al., 2012; Schmid & Guillaume, 2017). It is now well established that three mechanisms and their interactions shape the patterns of population diversity and structure, which are reduced gene flow, genetic drift, and natural selection (Gandon & Nuismer, 2009; Johnson et al., 2018; Wright, 1931). They are strongly influenced by many interrelating factors including historical events, human activities, distance and barriers, and ecological variations (Gomez et al., 2009; Wright, 1943; Zhang et al., 2014). However, the last two factors were identified as the most important ones (Mallet et al., 2014; Nogueras et al., 2016; Rundle & Nosil, 2005; Wright, 1931, 1943). First, geographic barriers can affect evolutionary processes by interrupting gene flow and therefore increasing population divergence (Capparella, 1987; Sobel, 2016). Nevertheless, in the absence of barriers, genetic differentiation can still occur. The pattern of isolation by distance (IBD) suggests a decrease of gene flow between populations as distance increases, triggering genetic differentiation via genetic drift and/or selection (Frankham et al., 2010; Ramírez-Barrera et al., 2019; Wright, 1943). On the other hand, selection is the primary factor for divergence in isolation by ecology or ecological speciation *lato sensu* (Orsini et al., 2013; Shafer & Wolf, 2013; Wang & Bradburd, 2014): In this case, gene flow between populations is reduced due to local adaptation to divergent environments (Shafer & Wolf, 2013).

Past climatic oscillations have been argued to be the major factors affecting current distributions and diversity of many plant species (Gomez et al., 2009; Médail & Diadema, 2009; Zhang et al., 2014). Climatic variations can threaten several plant species and reduce plant community diversity, as shown by the extinction of European tree species during the Plio-Pleistocene (Svenning, 2003). However, they can also be an important force driving genetic differentiation and speciation (Hewitt, 1996; Vences et al., 2009). For example, climatic variations along latitudinal gradients may affect organism functional traits such as phenology (Olsson & Ågren, 2002), physiology (Bognounou et al., 2010; De Frenne et al., 2012), and morphology (Feijó et al., 2019; Olsson & Ågren, 2002; Winn & Gross, 1993) and lead to plastic or genotypic shifts (Byars et al., 2007). Correlation analysis of neutral genetic variations versus geographic distances allows testing for IBD. On the other hand, a correlation between genetic divergence and morphological divergence can reveal isolation by adaptation (IBA) where barriers to gene flow between populations from different habitats arise following adaptive trait divergence (Nosil et al., 2008; Shafer & Wolf, 2013; Wang & Bradburd, 2014). When adaptive traits cannot be targeted, various cofactors may be used to test for isolation by ecology (Ramírez-Barrera et al., 2019; Shafer & Wolf, 2013; Wang & Bradburd, 2014). For this purpose, environmental data (excluding geographic data) may serve as a proxy in correlation analysis with neutral genetic data to assess isolation by environment (IBE). IBE is

caused by the reduction of exchanges between populations from ecologically divergent habitats due, for example, to the low recruitment in local environment. The roles of spatial isolation-by-distance and isolation-by-ecology patterns are complex in population differentiation and speciation processes, as they can be highly correlated and often act together as isolation mechanisms. Evolutionary patterns can be quite complex in plants (Lowry et al., 2008; Waser & Campbell, 2004), particularly in Orchidaceae. Indeed, orchid species evolve occasionally in sympatry (Barone Lumaga et al., 2012; Nielsen & Siegismund, 1999; Pansarin & Ferreira, 2019), produce numerous seeds with long-range dispersal ability (Arditti & Ghani, 2000), can reproduce clonally (Batygina et al., 2003), and hybridize (Arduino et al., 1996; Nielsen & Siegismund, 1999). Potential ecological drivers of orchid diversification are multiple, such as adaptation to pollinators (Ayasse et al., 2011; Dressler, 1982; Van der Niet et al., 2014), mycorrhizal specificity (Otero et al., 2007), differences in flowering phenology, and floral characteristics (color, form and fragrance) resulting from adaptation to different environmental conditions (Givnish et al., 2015; Pansarin & Ferreira, 2019; Sun et al., 2015) and leading to isolation by ecology. Although the various patterns namely IBD, IBE, and IBA have gained attention in the literature (e.g., Garot et al., 2019; Liu et al., 2018; Nogueras et al., 2016; Orsini et al., 2013; Shafer & Wolf, 2013), few studies have evaluated their joint contribution in orchid species differentiation (e.g., Jaros et al., 2016; Mallet et al., 2014), and no research efforts, to our knowledge, have been invested so far on this subject regarding *Vanilla* species divergence.

The *Vanilla* Plumier ex Miller (34.6 Ma, Bouetard et al., 2010) belongs to the Orchidaceae, one of the most diverse family of angiosperms (Bouetard et al., 2010; Gigant et al., 2011). It comprises about 132 species (Andriamihaja et al., 2020) distributed throughout tropical forests between the 27th north and south parallels, but are absent in Australia (Gigant et al., 2011; Portères, 1954). With nine wild *Vanilla* species described, the Southwest Indian Ocean (SWIO) region ranks fourth among the richest regions and first in terms of leafless species, most being located in Madagascar (Gigant et al., 2011; Portères, 1954). Leaflessness has evolved independently at least three times in the *Vanilla* genus (Bouetard et al., 2010). Within the SWIO region, seven leafless species can be found, ensuing from the adaptation of an African leafy ancestor to xeric conditions (Bouetard et al., 2010). As they are part of a recent monophyletic group (4.4 Ma) (Bouetard et al., 2010), these species retain numerous similarities in their morphology (Allorge-Boiteau, 2013; Andriamihaja et al., 2020; Cribb & Hermans, 2009; Portères, 1954; Soto-Arenas & Cribb, 2010), making their identification difficult. Chloroplast DNA phylogenetic analyses also failed to distinguish the various species from this group (Bouetard et al., 2010). Such recent and closely related species have always been recognized to be an interesting model for understanding speciation processes (Lowry, Modliszewski, et al., 2008; Van der Niet et al., 2014).

Our aim is to unravel wild leafless *Vanilla* species diversification processes in Madagascar. The island of Madagascar is a perfect place for studying evolution, thanks to a high level of endemism

attributed to a large heterogeneity of habitats, a small spatial scale, and a long history of geographic isolation (Vences et al., 2009; Warren et al., 2015; Wilmé et al., 2006). Actually, the level of endemism is more than 80% among plant species (Callmander et al., 2011; Goodman & Benstead, 2005; Phillipson et al., 2006) and even higher for animal taxa, ranging from 52% in birds to 100% in amphibians and lemurs (Goodman & Benstead, 2005). Habitat diversity is expressed by different biomes with unique vegetation composition and sharp borders, for instance, the long central highland plateau that rises along a latitudinal axis and separates west and east bioclimatic regions (Vences et al., 2009; Wilmé et al., 2006). Madagascar hosts more than 1,000 orchid species (Cribb & Hermans, 2007) including five leafless and two leafy *Vanilla* species (Allorge-Boiteau, 2005, 2013; Cribb & Hermans, 2009; Portères, 1954). Over 90% of Malagasy orchids are endemic to the island (Cribb & Hermans, 2007). In this study, we generated ecological, genotypic (using variable microsatellite markers), and morphological data for leafless *Vanilla* populations from Madagascar. We determined the genetic, morphological structure and differentiation of populations, to identify possible geographic barriers and to quantify the effects of isolation by distance (IBD), isolation by adaptation (IBA), and isolation by environment (IBE) in diversification processes.

2 | MATERIALS AND METHODS

2.1 | Study site

Fieldworks were performed in 23 sites (Table S1, Figure 1) within the distribution range of Malagasy leafless *Vanilla* species (Figure 1). Madagascar is located in the Indian ocean, southeast of Africa, around 20°S and 47°E, in the intertropical zone. A rugged chain of mountains separates the island from north to south, into dry forests (in the west) and rain forests (in the east) (Waeber et al., 2015). Several factors shaped the island climatic conditions such as topography, landform, maritime influence, and prevailing wind conditions (Cornet, 1973). During the dry season from May to October, the “Alizé” brings humid air masses from the Indian Ocean to the east coast causing precipitations (Cornet, 1973). These foggy masses move inland and drizzle in the elevated areas of the central domain. The winds become hot and dry beyond the mountains in the west, causing an accentuated dry season. During the wet season from November to April, on the other hand, the “Mousson” brings heavy rains over most of the island (Cornet, 1973). As a result, Madagascar is ecologically heterogeneous and displays five bioclimatic regions: dry, subhumid, humid, montane, and subarid (Vences et al., 2009). These ecoregions

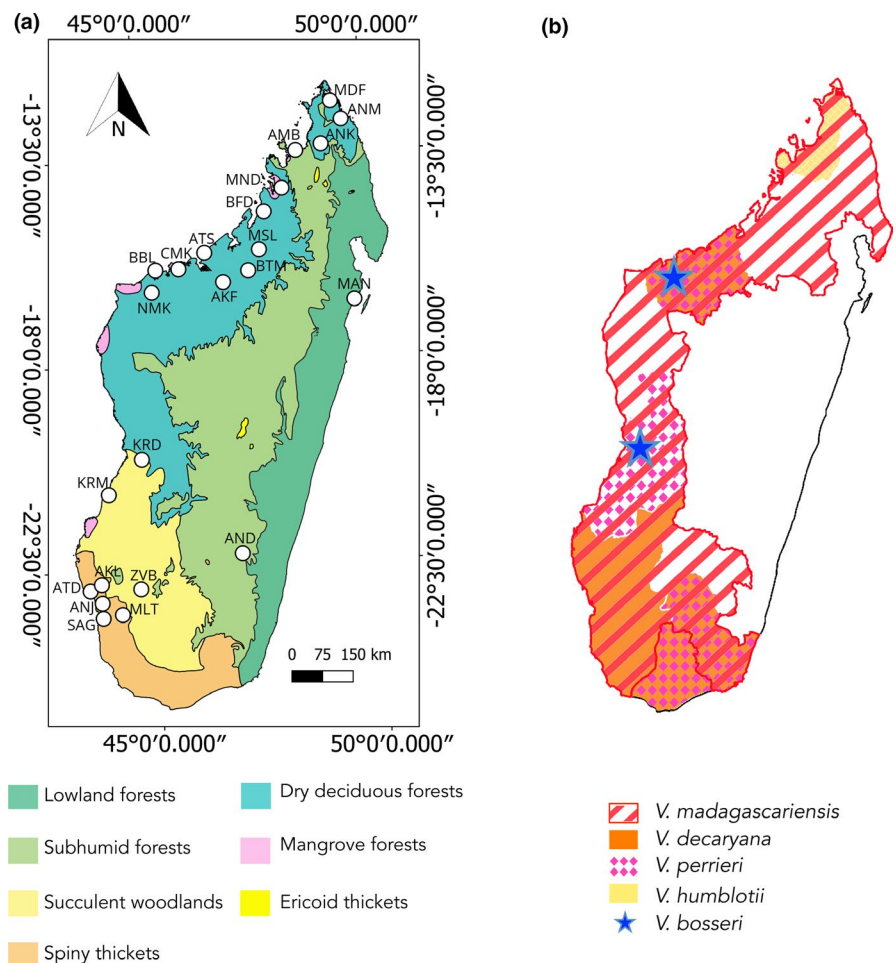


FIGURE 1 Map of studied populations, habitat types, and distribution of leafless *Vanilla* species in Madagascar. (a) Map shows the seven ecoregions of Madagascar based on WWF's ecoregions in Burgess et al. (2004). Circles indicate geographic positions of the 23 localities with *Vanilla* populations. (b) Map represents the distribution area of the five known leafless *Vanilla* species in Madagascar according to Portères (1954), Cribb and Hermans (2009) and Allorge-Boiteau (2013)

host seven typical vegetation formations: spiny thicket, succulent woodland, dry deciduous forest, subhumid forest, lowland (humid) forest, ericoid thicket, and mangrove forest (Figure 1a) (Burgess et al., 2004).

2.2 | Study species

Vanilla plants are long-lived semi-epiphytic lianas with both vegetative and sexual reproduction (Gigant et al., 2011). Stems are thick and fleshy reaching in some cases 30 m in length, with roots growing at nodes. They generally bloom once a year, and inflorescences are in form of clusters of ephemeral flowers with a large labellum (Bossier & Lecoufle, 2011; Portères, 1954). In this study, we focused on five indigenous Malagasy *Vanilla* species (Figure 1b), among the seven botanically described leafless species in the SWIO region comprising Madagascar, Seychelles, Comoros, Mayotte and the eastern Africa coast and islands (Allorge-Boiteau, 2005, 2013; Portères, 1954). Because of similarities regarding flower morphological traits, they are grouped within "*V. phalaenopsis*" by Soto-Arenas and Cribb (2010). Populations belonging to this group grow along the Malagasy west coast where the forests are dry or spiny (spiny thickets, dry deciduous forests, succulent woodlands) except *V. madagascariensis* Rolfe also found in the subhumid forests and humid northeastern forests (Figure 1b) (Bossier & Lecoufle, 2011; Cribb & Hermans, 2009; Portères, 1954).

2.3 | Population sampling

Between 2017 and 2020, 600 individuals from 22 populations were collected in Madagascar (Figure 1a). We used available occurrence data in the literature, including GBIF and Tropicos websites, but we have also prospected by interviewing local field botanists who are familiar with these species to choose these localities (Table S1). Fieldworks were done after a research permit (N° 201/17/MEEF/SG/DGF/DSAP/SCB.Re) was issued by the Ministry of environment and sustainable development granting an authorization to collect and export plant materials. Samples were cut from young stems from individuals at least 1 m apart, preserved in silica gel, and then lyophilized until DNA extraction. One living specimen per population was also stored in the *Vanilla* collection of the Department of Plant Biology and Ecology (University of Antananarivo). Eleven additional samples from the Manompana forest (MAN, Figure 1a) in the northeast were taken from the Biological Resource Center (BRC) Vatel collection in Reunion Island. In total, 611 individuals from 23 sites in Madagascar were studied (Figure 1a).

2.4 | Microsatellite genotyping

DNA was extracted using the DNEASY® plant kit from Qiagen. The 611 specimens were registered and stored as DNA samples in the

BRC Vatel collection (La Réunion). Each individual was amplified and genotyped using seven microsatellite markers developed by Gigant et al. (2012) (Table S2). Polymerase chain reaction (PCR) with fluorescently labeled primers was performed in a total volume of 25 μ l using Promega kit (Promega, 2007) by mixing 12.5 μ l of GoTaq Colorless Master Mix 2X, 2 μ l of DNA solution ($5 < \text{DNA} < 250$ ng), 0.4 μ M of each primer, and 8.5 μ l of nuclease-free water. Amplifications were carried out under the following conditions: an initial denaturation step at 95°C for 2 min followed by 45 serial cycles of denaturation step at 95°C for 30 s, annealing at 57°C for 45 s, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. Genotyping of PCR fragments was performed by capillary electrophoresis with an automated sequencer 3130XL ABI Genetic Analyzer (Applied Biosystems). Allele sizes were scored using GENEIOUS v.7.0 (Olsen et al., 2014).

2.5 | Genetic diversity

Genotypic diversity of each population was examined by dividing the number of unique genotypes G or "genets" by the total number of individuals N or "ramets" (Halkett et al., 2005). Clones were identified using the multilocus analysis of clonality in GenAIE 6.5 (Peakall & Smouse, 2006). Clones were, then, excluded from the dataset for genetic analysis. The multilocus exact test p -values based on Markov chain method (Guo & Thompson, 1992) in GENEPOP v.4.7.5 (Raymond, 1995; Rousset, 2008) were used to test for deviation from Hardy-Weinberg equilibrium (HWE) in each population, using the default parameters (1,000 dememorizations, 100 batches, and 1,000 iterations per batch). GENEPOP was also used to perform probability tests for assessing the linkage disequilibrium (LD) between all pairs of loci in each population. The Holm's sequential Bonferroni procedure (Abdi, 2010) or SB correction, feasible under the "p.adjust" function in R software V3.6.1 (R Core Team, 2019), was performed to correct the levels of statistical significance (p -value) for multiple hypothesis test of LD. The Waples method (Waples, 2015, 2018), examining the correlation between the mean squared correlation of allele frequencies at different gene locus (r^2) and the product of two fixation index (FST) for pairs of loci, was applied across all populations to test for a Wahlund or population structure effect on LD (Wahlund, 1928). GenAIE 6.5 was used to calculate the total number of alleles for all loci in each population (N_a), the mean observed number of alleles per locus (A_i), and the number of private alleles per population (P_a). As the number of alleles in a sample is highly dependent on sample size, the mean allelic richness (A_r) as suggested by Mousadik and Petit (1996) was computed using FSTAT 2.9.4. (Goudet, 1995). We determined the observed heterozygosity (H_o) and the mean expected heterozygosity under HWE (H_e) over all loci using the R package GENEPOP v.4.7.5 (Raymond, 1995; Rousset, 2008). The correlations between A_r, H_e, and latitude were tested by performing Pearson correlation tests using R. The inbreeding coefficient FIS was calculated as $FIS = 1 - (H_o/H_e)$. Null allele frequencies at each locus across all populations

were checked by INEst v.2.2 (Chybicki & Burczyk, 2009) using a Bayesian approach (IIM).

2.6 | Genetic structure and differentiation

The “genets” dataset was used to estimate the global differentiation index (FST_g) among populations (Weir & Cockerham, 1984) and the pairwise genetic differentiation (FST_p) between pairs of populations using the GENEPOP package under R software. Analysis of molecular variance (AMOVA) (Excoffier et al., 1992) implemented in GenAIEx 6.5 (Peakall & Smouse, 2006) was conducted to assess the genetic differentiation level among and within populations and among populations from different habitat types. Each population was associated to its corresponding ecoregion of Madagascar based on geographic location.

Assignment of multilocus genotypes to different clusters was inferred by conducting Bayesian clustering analyses implemented in STRUCTURE (Pritchard et al., 2000) on the “genet” dataset, with ten independent runs for each value of K (1 to 10 for the complete dataset), a burn-in period of 100,000 and 1,000,000 Markov chain Monte Carlo (MCMC) replications. To overcome sampling imbalance and thus correctly assign individuals to clusters, we used the model with admixture and independent allele frequencies and reduced the default alternative ancestry prior Alpha in STRUCTURE, as recommended by Wang (2017), with $\text{Alpha} = 1/K$. In our case, an Alpha equal to $1/10$ was chosen, as the number of K tested was 10. An individual was considered to belong to a particular genetic group when at least 80% of its genome was assigned to that genetic group. The summary likelihood statistics ΔK (Evanno et al., 2005), which is the most used method to find the best K, was plotted. As the ΔK method alone is not recommended by some authors to determine the best clustering (Janes et al., 2017; Wang, 2017), the posterior probabilities of K, called “ $\text{Ln } P(X|K)$,” implemented in STRUCTURE were also examined (Pritchard et al., 2000). The best K usually corresponds to the highest value of mean $\text{Ln } P(X|K)$ (Evanno et al., 2005; Pritchard et al., 2000; Rosenberg et al., 2001; Wang, 2017). Bar plot of genetic structuring obtained from STRUCTURE was generated using the POPHELPER package in R (Francis, 2017).

2.7 | Floral differentiation

Eight populations assumed to represent all clusters from genetic structuring analysis were targeted during their flowering season based on bibliographical information (Andriamihaja et al., 2020): MSL and MND (October), BFD and MDF (November), and AJA, ATD, MLT, and AND (December) (Table S1). One herbarium specimen of each genetic group was deposited at the herbarium of the Botanical and Zoological Park of Tsimbazaza (N°51/20/MESupRES/SG/DGRS/PBZT/Flore). Twenty to thirty mature flowers per population were randomly sampled. In total, 198 flowers were dissected

and classical taxonomic morphometric parameters were assessed (Gigant et al., 2014; Gigant, De Bruyn, et al., 2016; Petersson, 2015; Portères, 1954; Soto-Arenas & Cribb, 2010). We directly measured in situ ten parameters using a digital caliper and a precision balance, that is, total flower weight (TFW), ovary weight (OWE), ovary length (OL), ovary width (OW), column length (CL), column width (CW), rostellum length (RL), rostellum width (RW), anther length (AL), and anther width (AW) (Figure 2).

Thereafter, flower parts were digitalized using a canoScan LiDE 120. The resulting images were processed under IMAGEJ software (Abràmoff et al., 2004) in order to measure complementary traits, that is, adaxial sepal width (ASW), adaxial sepal length (ASL), right lateral sepal length (RLSL), right lateral sepal width (RLSW), labelum surface (LS), petal length (PL), and petal width (PW) (Figure 2). Scanned flower images were also transformed into CIE ($L^*a^*b^*$) color space according to the Kendal et al. (2013) script using the “convertColor” function in the grDevices package in R (R Core Team, 2019). The (L^*) dimension values vary between 0 (black) and 100 (white reference) and represent the luminance. The (a^*) and (b^*) dimension values vary between -100 and 100 and represent the colors between red and green, and between yellow and blue, respectively (Najjar & Zagrouba, 2012). Color variables estimated were L^* of inside of labellum (LIL), a^* of inside of labellum (AIL), b^* of inside of labellum (BIL), L^* of outside face of sepal (LOFS), a^* of outside face of sepal (AOFS), b^* of outside face of sepal (BOFS), L^* of inside face of petal (LIFP), a^* of inside face of petal (AIFP), and b^* of inside face of petal (BIFP). In total, 26 morphometric parameters were used in principal component analysis (PCA) using FACTOMINER (Lê et al., 2008) with R software. Three dimensions of PCA plot were generated with R package PCA3D (Weiner, 2019). PCA allows, by equal weighting of each character, reducing the number of variables in fewer dimensions and therefore investigating the overall morphological patterns. Means and standard deviations of the ten most explanatory variables from PCA were computed for each genetic group. Differences in these floral traits between groups were detected using a Bonferroni-corrected pairwise t test. Multivariate analysis of variance (MANOVA) was also conducted to test the statistical significance of overall floral differences based on a priori groupings. All analyses were done with R software V3.5.1.

2.8 | Geographic and environmental characterization of populations

Recorded GPS coordinates (Table S1) were used to calculate the geographic distances between *Vanilla* populations in Madagascar. Sample site elevation data were obtained from GPS (Table S1). To highlight the heterogeneity of habitats, 19 present-day bioclimatic variables (means over thirty years) downloadable from the WorldClim website (<https://www.worldclim.org/>) were extracted for each sampling sites in Madagascar (Tables S3 and S4). In addition, the annual

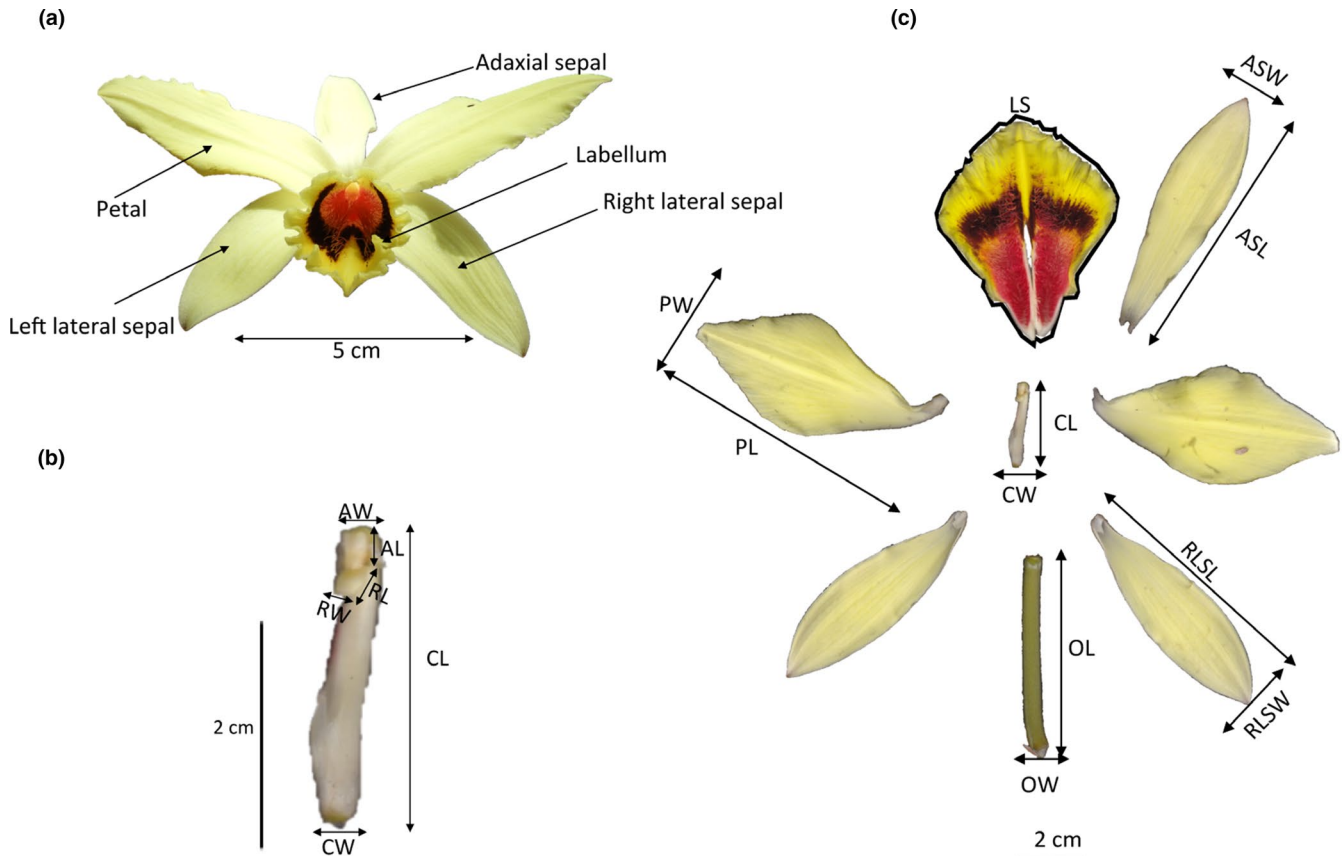


FIGURE 2 Floral measurements carried out on a *Vanilla* specimen with yellow flowers collected within the Montagne des Français (MDF, north) population. See text for morphological parameters abbreviations. Panel a: flower. Panel b: column. Panel c: flower parts

average values of maximum Normalized Difference Vegetation Index (NDVI) for each locality and four soil variables were also extracted from raster data at 250 m of resolution provided by GIMMS Global Agricultural Monitoring System (Becker-Reshef et al., 2010) and World Reference Base for Soil Resources (WRB) (Tables S3 and S4) (Ribeiro et al., 2018). Pearson's correlation tests were performed to detect collinearity between variables, and a combination of variables with a correlation of less than 0.3 was chosen. Habitat heterogeneity between the 23 populations was shown by applying a PCA to these uncorrelated variables. Environmental dissimilarity between each pair of populations was obtained by calculating the Euclidian distances of the PC scores of the three first principal components (PCs).

As the landscape discontinuity could be an important factor to differentiation processes, we performed Monmonier algorithm analysis using BARRIER 2.2. (Manni et al., 2004) to identify where genetic barriers might occur. GPS coordinates of each population were entered to construct Voronoï tessellation that determines which populations are neighbors and adjacent. The pairwise genetic distance (FST_p) was used to identify the top eleven potential barriers. Then, 100 resampled bootstrap Nei's standard genetic distances (D_n) matrices (Nei, 1972) were generated with MSA 4.05 (Dieringer & Schlötterer, 2003) for testing the robustness of each barrier (Manni et al., 2004).

2.9 | Effect of environmental, morphological, and geographic distances on genetic divergence

To detect possible patterns of IBD and IBE, we performed multiple matrix regression with randomization (MMRR) as implemented in the R function "MMRR" (Wang, 2013) on the 23 populations. This approach is similar to Mantel test but can incorporate several distance matrices, thus providing an interpretable result in the form of a multiple regression equation (Wang, 2013). In the case of MMRR, significance values for the regression coefficient (β) and coefficient of determination (R^2) were estimated by performing 9,999 randomized permutation procedure (Wang, 2013). MMRR was also applied for testing the correlation between geographic and environmental distances. Genetic differentiation between populations was primarily estimated using pairwise FST values (Weir & Cockerham, 1984) using the "genet dataset" after discarding null alleles using FreeNa (Chapuis & Estoup, 2007). The geographic distance matrix was obtained with the SP package in R (Pebesma & Bivand, 2020). For environmental parameters, we used the Euclidean distance calculated from PC scores as explained in the previous section. Additionally, one powerful approach called "distance-based redundancy analysis with randomization" (dbRDA), based on a linear ordination method that combines principal component analysis (PCA) and multiple linear regression (Legendre &

TABLE 1 Summary of genetic diversity at 7 microsatellite loci in 23 of leafless *Vanilla* populations from Madagascar

Pop	N	G/N	HWE	H_o	H_e	FIS	Na	AI	Pa	Ar
MDF	29	1.00	***	0.58	0.70	0.17	60	8.57 ± 1.65	5	5.57 ± 2.46
ANM	27	0.93	***	0.57	0.66	0.14	53	7.57 ± 1.46	1	5.52 ± 2.45
ANK	22	1.00	***	0.58	0.65	0.11	49	7.00 ± 1.11	2	5.04 ± 2.27
AMB	27	0.93	***	0.51	0.61	0.16	51	7.29 ± 1.34	2	4.95 ± 2.19
MND	41	0.93	***	0.52	0.67	0.22	84	12.00 ± 1.38	10	5.86 ± 2.17
BFD	24	1.00	***	0.62	0.80	0.22	73	10.43 ± 1.09	5	6.58 ± 1.39
ATS	27	0.93	***	0.58	0.82	0.30	80	11.43 ± 1.80	3	7.31 ± 2.65
MSL	41	0.93	***	0.61	0.82	0.26	95	13.57 ± 2.35	3	7.68 ± 2.86
CMK	26	1.00	***	0.57	0.79	0.28	78	11.14 ± 1.72	2	7.05 ± 2.55
BTM	34	1.00	***	0.63	0.74	0.15	75	10.71 ± 0.97	9	5.86 ± 1.28
BBL	26	1.00	***	0.59	0.82	0.28	92	13.14 ± 2.38	0	7.70 ± 3.10
AKF	24	1.00	***	0.68	0.81	0.16	96	13.71 ± 2.37	5	7.90 ± 3.05
NMK	24	0.96	***	0.68	0.78	0.13	93	13.29 ± 2.38	1	7.82 ± 3.17
KRD	33	1.00	***	0.67	0.83	0.20	112	16.00 ± 2.48	7	8.27 ± 2.70
KRM	29	0.90	***	0.41	0.53	0.22	51	7.29 ± 1.48	4	4.58 ± 2.52
AKL	25	0.80	***	0.61	0.81	0.25	57	8.14 ± 0.94	3	6.29 ± 1.57
ZVB	17	1.00	***	0.68	0.76	0.10	59	8.43 ± 1.31	0	6.39 ± 2.50
ATD	23	0.91	***	0.33	0.50	0.35	41	5.86 ± 1.18	3	4.07 ± 1.92
ANJ	24	0.83	**	0.23	0.35	0.32	16	2.29 ± 0.47	2	2.12 ± 1.02
MLT	38	0.82	***	0.45	0.61	0.27	55	7.86 ± 1.74	3	4.74 ± 2.53
SAG	9	1.00	*	0.29	0.43	0.34	25	3.57 ± 0.97	4	3.38 ± 2.35
MAN	11	0.82	***	0.49	0.62	0.21	33	4.71 ± 0.68	6	3.46 ± 1.58
AND	30	0.27	***	0.59	0.42	-0.40	20	2.86 ± 0.74	1	2.86 ± 1.95

Note: Pop, population; N, number of samples per population; G/N, genotypic diversity; HWE, results of test for departures from Hardy-Weinberg equilibrium (* $p < .05$, ** $p < .01$, *** $p < .001$); H_o , observed heterozygosity over all loci; H_e , expected heterozygosity over all loci; FIS, fixation index; Na, total number of alleles for all loci; AI, mean number of alleles per locus ± SD; Pa, total number of private allele; and Ar, mean allelic richness per locus ± SD (Mousadik & Petit, 1996).

Anderson, 1999), was applied using the “dbrda” function in the VEGAN package (Dixon, 2003) in R. Again, F_{ST} was tested against the geographic distances and population's PC scores from the PCA performed on the environmental data. Prior to IBD analysis, it was necessary to transform the geographic distances matrix into a continuous rectangular vector by principal coordinates analyses (PCoA) using the “pcnm” function included in the VEGAN package in R. Significance of the predictors was evaluated using multivariate F-statistics with 9,999 permutations using the “ANOVA” function in VEGAN. Three different types of dbRDA were performed: (a) a global dbRDA using all explanatory variables (geographic and environmental distances), (b) marginal tests evaluating independently the relationship between the genetic distance matrix and each of the two explanatory variables (geographic and environmental factors), and (c) a partial dbRDA (conditional test) estimating the relative contribution of one explanatory variable (geographic distance or environmental factors) to genetic differentiation, while controlling the effect of the other one. The amount of variance explained by the intersection of IBD and IBE was obtained by variance partitioning using the function “varpart” included in VEGAN package. The adjusted coefficient of determination R^2_{adj} was calculated to evaluate the quality of the model. MMRR and dbRDA were also conducted

on a reduced sampling of eight populations for which phenotypic data were available to test, in addition to IBD and IBE, a possible correlation between morphological distances and genetic distances (IBA). For that, a Pearson's correlation test on the 26 morphometric variables was carried out to choose a combination of uncorrelated variables ($cor < 0.3$). The PC scores of the first three principal components resulting from the PCA of these selected variables were, then, used to calculate the phenotypic distance between the eight populations in order to perform MMRR and dbRDA.

3 | RESULTS

3.1 | Genetic diversity

A total of 555 genets and 56 clones were identified among the 611 studied samples (ramets), representing approximately 9% of clonality. At the population level, the clonality rate was generally low ($G/N > 0.7$) except for AND population where genotypic diversity was only 0.27 (Table 1). The multilocus exact test p-values for each population showed a significant deviation from HWE (p -value $< .05$)

(Table 1). For 22 populations, the observed heterozygosity (H_o) was lower than the expected heterozygosity (H_e), with positive FIS values, while H_o was higher than H_e (negative FIS value) for the AND population (Table 1). Overall diversity was high with H_e values ranging from 0.42 to 0.83. The seven primers revealed a high level of polymorphism with 262 alleles across the 23 studied populations. The total number of alleles over all loci per population varied from 16 (ANJ) to 112 (KRD), with a mean of 9 alleles/locus/population. Two populations (BBL, ZVB) had no private alleles, while the highest proportion was attributed to MND with 10 private alleles across all loci (Table 1). The mean allelic richness per locus (A_r) was lowest (2.86) in AND and highest (8.27) in KRD (Table 1). The Pearson's correlation showed that allelic richness (A_r) and expected heterozygosity (H_e) increased slightly ($r_{A_r\text{-latitude}} = 0.43$, $r_{H_e\text{-latitude}} = 0.47$) but significantly ($p < .04$) with latitude, with southern populations showing the lowest diversity and western populations showing the highest diversity (Table 1).

Of the 21 combinations of loci tested for LD across the populations, two-locus combinations were significantly in LD after SB correction. LD analysis in each population revealed one-locus to seven-locus combinations in LD after SB correction in only 8 populations of the 23 studied, and with no pair of markers consistently in LD across all populations, as would be expected if LD resulted from physically linked loci (Slatkin, 2008). Therefore, all microsatellite loci were considered independent as shown by the original study of Gigant, De Bruyn, et al. (2016). A high frequency of null alleles ranging from 0.12 (HU03, HU07, HU08) to 0.26 (HU06) was detected.

3.2 | Genetic structure, differentiation, and barriers

The global F_{ST} value calculated (0.19) indicated a significant ($p < .001$) overall differentiation among populations. Pairwise F_{ST} was all significant ($p < .001$), with high amplitude values ranging from 0.01 between two northwestern populations (BBL and MSL)

up to 0.60 between AND (central highland) and ANJ (southwestern) (Table S5). A strong linear positive correlation (Pearson correlation coefficient = 0.81, r -squared = 0.63; $p < .001$) was obtained between r^2 and the product of two F_{ST} values for pairs of loci, suggesting a substructure of the sample (Figure 3).

AMOVA estimated that 79% of the total genetic variation was found within populations, a moderate proportion (15%) was found among populations, and only 6% was found among ecoregions. The distribution of ΔK values in the Bayesian assignment of STRUCTURE showed a modal value at $K = 4$ and the maximum value of the posterior probability " $\ln P(X|K)$ " corresponded to $K = 7$ (Figure S1). According to Wang (2017), with unbalanced sampling, it is preferable to choose the method of Pritchard et al. (2000) based on " $\ln P(X|K)$ " to find the best K . Consequently, seven distinct genetic clusters were retained as the optimal K in STRUCTURE analysis (Figure 4b, c). If most populations were rather homogeneous in terms of genetic structure, some showed evidence of admixture, of which the most obvious were AKL (southwest) and CMK (northwest) (Figure 4b, c).

F_{ST} -based analysis of genetic barriers revealed eleven boundaries. Six of them were well supported with bootstrap values exceeding 60%. These genetic barriers separated, in majority, the seven different genetic groups obtained from STRUCTURE (Figure 4b).

3.3 | Floral morphology differentiation

The first three principal components of PCA (Figure S2), explaining 80.6% of the total variation, were chosen to explain the total variation of 26 quantitative flowers traits, in relation with genetic clusters defined by STRUCTURE ($K = 7$). PCA1 explained 53.7% of the total variation and separated three clusters from three others by the weight and size of the flowers (Figure 5a, Figure S2). Color of the petals and of the inside face of labellum differentiated flowers from clusters 1 and 3 from the others on the second axis of the PCA with 19.3% of explained variance. Indeed, populations from these

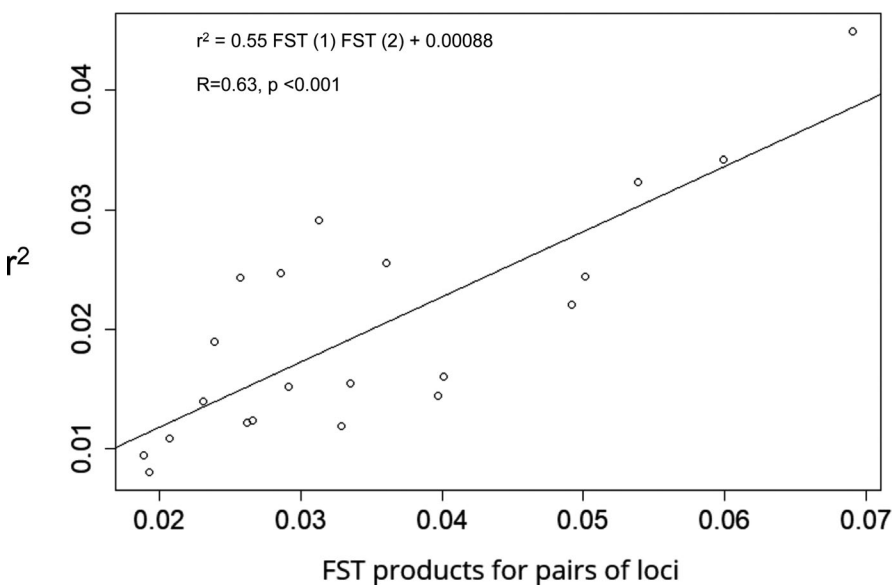


FIGURE 3 Scatterplot shows linear relationship between r^2 (Waples, 2006) and the product of two F_{ST} values for 21 pairs of microsatellite loci across 23 populations of leafless *Vanilla* species from Madagascar

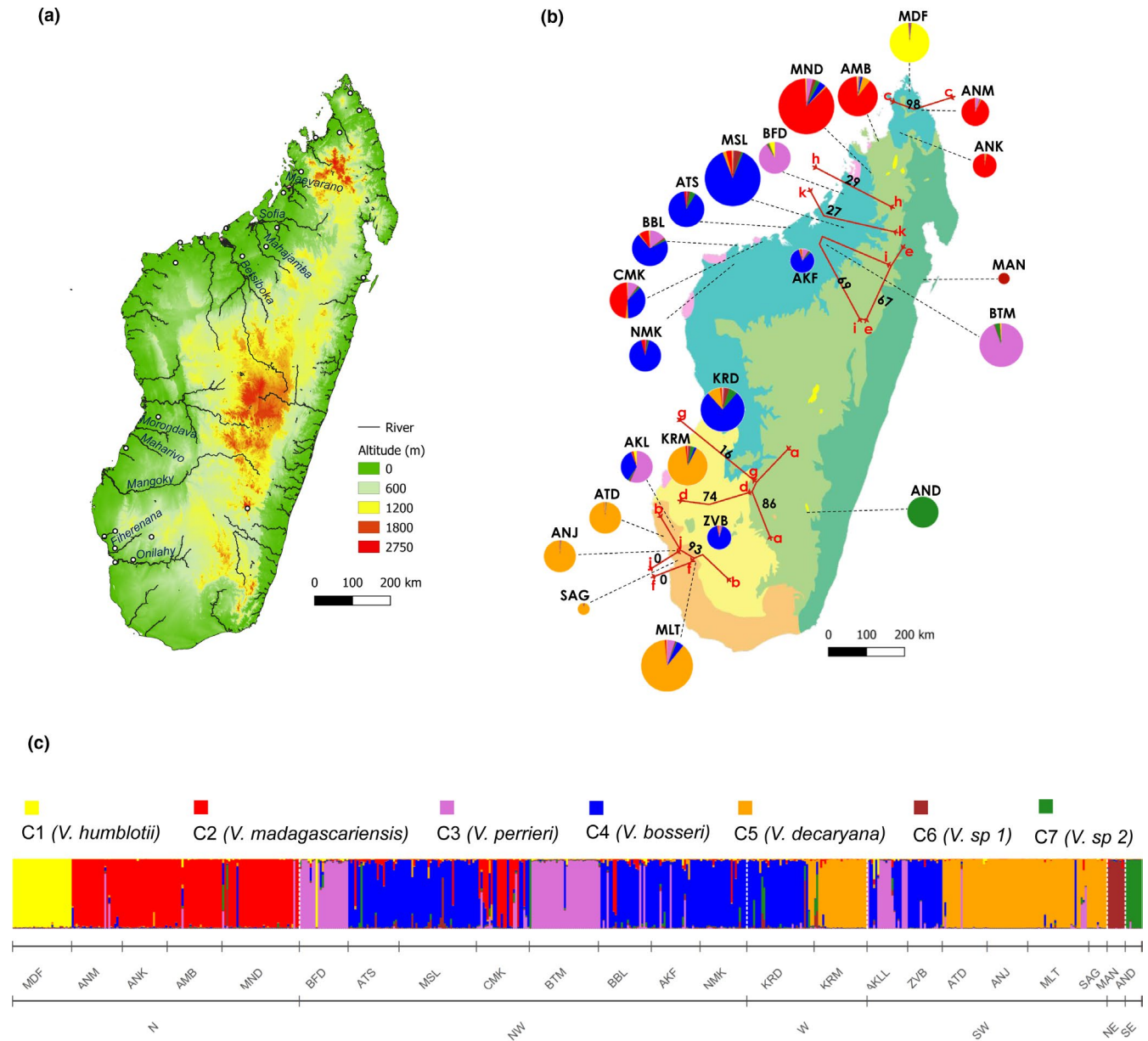


FIGURE 4 Genetic structuring and boundaries of leafless *Vanilla* populations from Madagascar. Panel a: topography of Madagascar with major rivers based on open street map data available on QGIS and USGS data (<https://earthexplorer.usgs.gov/>). The location of the 23 populations is indicated by circles. Panel b: distribution of leafless *Vanilla* genetic clusters in Madagascar based on Bayesian inference from STRUCTURE software and genetic barriers. Pie charts represent the mean proportion membership to $K = 7$ clusters and their sizes are proportional to sample sizes (N). Results of BARRIER analysis revealed regions of genetic discontinuity (in arrowed red lines) indicated by the letters a to k in descending order of F_{ST} values relative importance, with bootstrap values (in %) shown in black numbers calculated based on 100 Nei's standard genetic distances bootstrap matrices. Panel c: genetic assignment plot of individuals assessed by STRUCTURE program ($K = 7$). Each individual is represented by a vertical bar partitioned into K -colored segments that represent the individual's probability of belonging to the cluster with that color. Corresponding species are indicated, according to population morphological analyses (Figure 5)

two clusters have yellow flowers, while the others have white flowers (Figure 5c). Color of the outside face of sepals and size of flowers contributed most strongly to PCA3 (7.6%) and separated populations from the south of Madagascar (cluster 5) and those situated in the north and west of Madagascar (clusters 2 and 4) (Figure 5a). Clusters 2 and 4 overlapped on the PCA axes. However, flowers from cluster 4 could readily be differentiated from those in cluster 2 by the

absence of hairs inside the labellum, as detected during floral observation (Figure 5b). This morphological feature could not be included in the PCA analysis as it is a qualitative character. The MANOVA demonstrated significant differentiation of overall floral morphology between genetic groups ($p < .0001$). Means of the ten most explanatory variables of the first three PCA dimensions and results of pairwise t test are shown in Table 2. Seven to nine floral traits

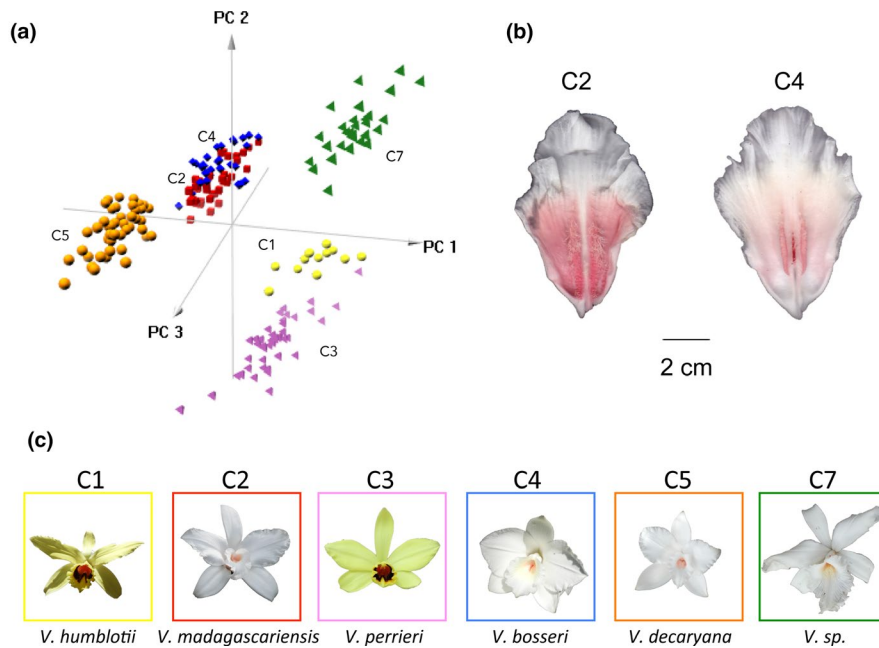


FIGURE 5 PCA of 26 quantitative floral morphometric characters and labellum hair qualitative character. (a) 3D plotting of the first three components of the PCA, with genetic groups from STRUCTURE analysis ($K = 7$) indicated by matching colors. C1: MDF; C2: MND; C3: BFD; C4: MSL; C5: ANJ, ATD, MLT; C7: AND. For the contribution of the 26 variables to the three axes of the PCA, see Figure S1. (b): Photograph of labellum of individuals from populations MND (C2) and MSL (C4) showing presence/absence of hairs. (c): Flower images representative of the 6 clusters: photographs by (C1) Johnson G. Andrianantenaina, (C2, C3, C4, C5): Cathucia F. Andriamihaja, (C7): Hoby Nomenjanahary

TABLE 2 Comparison of ten floral parameters (mean \pm SD) among six genetic groups derived from analysis with STRUCTURE. Means followed by the same letter in the same row are not significantly different (p -value $> .05$)

Floral traits	C1	C2	C3	C4	C5	C7
Species	VHUM	VMAD	VPER	VBOS	VDEC	VSP
TFW (g)	6.08 \pm 1.13 ^a	2.88 \pm 0.40 ^b	4.22 \pm 0.85 ^c	3.33 \pm 0.49 ^b	1.76 \pm 0.49 ^d	7.93 \pm 1.02 ^e
BIFP	41.42 \pm 5.86 ^a	-2.61 \pm 0.70 ^b	58.48 \pm 8.75 ^c	-2.25 \pm 0.78 ^b	-2.39 \pm 0.73 ^b	-1.80 \pm 0.64 ^b
AIFP	-8.07 \pm 1.06 ^a	1.83 \pm 0.35 ^b	-10.31 \pm 1.70 ^c	2.25 \pm 0.44 ^b	1.76 \pm 0.73 ^b	1.80 \pm 0.53 ^b
LS	16.63 \pm 2.28 ^a	7.27 \pm 0.83 ^b	13.74 \pm 1.94 ^c	9.36 \pm 1.23 ^d	4.82 \pm 0.36 ^e	21.44 \pm 2.50 ^f
PL	7.46 \pm 0.49 ^a	5.84 \pm 0.52 ^b	6.55 \pm 0.64 ^c	5.88 \pm 0.55 ^b	4.23 \pm 0.30 ^d	8.79 \pm 0.48 ^e
RLSL	7.18 \pm 0.36 ^a	5.74 \pm 0.51 ^b	6.42 \pm 0.60 ^c	5.87 \pm 0.54 ^b	4.14 \pm 0.20 ^d	8.57 \pm 0.40 ^e
ASL	7.08 \pm 0.57 ^a	5.92 \pm 0.59 ^b	6.61 \pm 0.70 ^a	5.93 \pm 0.51 ^b	4.21 \pm 0.30 ^c	8.48 \pm 0.52 ^d
BIL	40.53 \pm 1.97 ^a	2.48 \pm 0.88 ^b	39.81 \pm 6.64 ^a	2.21 \pm 1.32 ^b	-0.72 \pm 0.53 ^c	0.82 \pm 0.64 ^{b,c}
OW	4.92 \pm 0.52 ^a	3.64 \pm 0.26 ^b	4.11 \pm 0.46 ^c	3.76 \pm 0.29 ^b	3.32 \pm 0.30 ^d	5.85 \pm 0.58 ^e
OWE	1.25 \pm 0.43 ^a	0.54 \pm 0.11 ^b	0.80 \pm 0.23 ^c	0.59 \pm 0.10 ^b	0.49 \pm 0.12 ^b	1.71 \pm 0.29 ^d

Note: BIFP: b* of inside face of petal, AIFP: a* of inside face of petal, BIL: b* of inside of labellum

Abbreviations: TFW, total floral weight; LS, labellum surface; PL, petal length; RLSL, right lateral sepal length; ASL, adaxial sepal length; OW, ovary width; OWE, ovary weight; VHUM, *V. humblotii*; VMAD, *V. madagascariensis*; VPER, *V. perrieri*; VBOS, *V. bosseri*; VDEC, *V. decaryana*; and VSP, *V. sp.*

of the ten most discriminant variables varied significantly among all genetic groups, except for clusters 2 and 4, which presented many similarities as previously mentioned. The surface of the labellum was significantly different between all genetic groups.

3.4 | Geographic, environmental, and morphological effects on genetic differentiation

In general, PCA on the 14 uncorrelated environmental variables distributed samples between four types of habitat: east of Madagascar (MAN) with high NDVI and precipitation, north of Madagascar characterized by high annual temperature, west of Madagascar with high pH and temperature, and finally population

from AND (central highlands of Madagascar) that can be distinguished from the others by its altitude (mean elevation: 887 m) (Figure S3). Scores of the three principal components (PCs) explaining 85.6% of the environmental variance were used to calculate environmental distances between the 23 populations. The results of MMRR on 23 populations revealed that genetic differentiation was weakly positively correlated to geographic distances (IBD) ($\beta = 0.0001$, $p = .001$) and with a higher coefficient of determination ($R^2 = 0.29$) to environmental distances (IBE) ($\beta = 0.025$, $p = .001$) (Figure 6a, b). However, geographic and environmental distances showed autocorrelation (Figure 6c). Marginal tests of dbRDA showed significant effect of IBD and IBE on genetic distance, whereas partial dbRDA revealed no pure contribution of IBD ($p = .268$) but a weak independent pattern of IBE ($R^2_{\text{adj}} = 3.41\%$,

$p = .001$) (Table 3). The intersection IBD \cap IBE constituted the most important contributor to genetic divergence explaining 42.8% of the total variation (Figure 6d). Analyses carried out on the reduced sample (eight populations) indicated no contribution of geographic distance on genetic distance (no IBD) (Table 3) and eliminated its correlation with environmental distance. However, MMRR and dbRDA revealed a significant association between genetic distance and morphological distance (PC1) and between genetic distance and environmental distance (PC1) (Figure 6e,f) (Table 3). The PC1 axis from PCA of morphological data grouped together several variables related to flower size: TFW, PL, RLSL, ASL, OW, OWE, CL, and AL (Figure S4). The PC1 axis from PCA on environmental data assembles a pool of ecological variables related to temperature (BIO8, BIO10, BIO5), elevation, pH, and percentage of silt (Figure S5). However, environmental (PC1) and morphological predictors (PC1) were not significant after accounting for the influence of each other in partial dbRDA tests (Table 3), as they were highly intercorrelated (Figure 6g). The intersection IBE \cap IBA constituted the most important contributor to genetic divergence explaining 45% of the total variation (Figure 6h).

4 | DISCUSSION

4.1 | Genetic diversity of leafless *Vanilla* populations

Numerous mechanisms can maintain genetic diversity, the most important of which are mutation, migration and balancing selection where population sizes play a major role (Frankham et al., 2010). Genetic diversity organization in plants is also highly dependent on the life cycle, reproduction system, pollination, and seed dispersion mechanisms. For example, long-lived, allogamous taxa, with wind or ingested dispersed seeds, are more variable within populations than between, with high H_e (0.61–0.73) and lower F_{ST} (0.12–0.26) values, with an opposite organization for annual autogamous plants with gravity-dispersed fruits (Nybom, 2004; Ronfort et al., 2005). AMOVA results suggested that genetic variation was mainly (79%) allocated within *Vanilla* populations in Madagascar. The levels of genetic diversity were relatively high for 20 populations out of the 23 ($0.42 < H_e < 0.84$, $3.45 < A_r < 7.65$), with an overall low genetic differentiation ($F_{ST} = 0.19$). These results are compatible with what was expected for *Vanilla* species, which are long-lived thanks to vegetative growth, mostly allogamous (Gigant et al., 2014; Gigant, De Bruyn, et al., 2016; Gigant, Rakotomanga, et al., 2016; Petersson, 2015), with most likely bee-mediated pollination and suspected zoochorous seed dispersion (by bees or bats) (Gigant et al., 2011; Gigant, De Bruyn, et al., 2016). These results also agree with expectations for orchids species for which high H_e ranging from 0.3 up to 0.9 is reported (Chen et al., 2014; Soliva & Widmer, 2003; Stone et al., 2012; Swarts et al., 2009). Significant deviation from HW equilibrium was detected for all populations with a strong significant heterozygote deficit ($F_{IS} > 0$) for 22 populations. This could be due to the high frequency of null

alleles detected for all loci (0.12–0.26), although the substructure of some populations with different sympatric species (as will be further discussed), as well as possible inbreeding due to geitonogamy (as demonstrated for a *V. humblotii* Rchb. f. population in Mayotte, (Gigant, De Bruyn, et al., 2016), could be other factors explaining these deficits.

4.2 | Genetic and morphological differentiation

At the scale of Madagascar, a strong significant population substructure was detected by the Wahlund effect test for overall individuals as suggested by Waples (2015, 2018) (Figure 3). The microsatellite-based structuring corroborated this, by revealing seven well differentiated genetic groups (Figure 4b, 4c). Morphological analysis of Madagascar leafless *Vanilla* flowers revealed a similar grouping pattern to that found using microsatellite markers (STRUCTURE). Indeed, flower morphology was clearly specific for each of six STRUCTURE clusters (Figure 5), with size of flowers and particularly size of labellum as the determining feature of this differentiation (Table 2, Figure S2). In *Vanilla* plants, the difference in flower sizes is often a discriminating trait. For example, in the taxonomic review done by Soto-Arenas and Cribb (2010), petal size was the determination key between *V. mexicana* and *V. ovata*, two closely related species from South America. Unfortunately, no flowers belonging to the genetic group C6 MAN could be observed. This morphometric pattern, combined to geographic distribution of known species (Figure 1b), allowed to clearly link five of the STRUCTURE clusters to the five botanical species already described in Madagascar (Allorge-Boiteau, 2005, 2013; Cribb & Hermans, 2009; Portères, 1954). Flowers from clusters 1 and 3 matched the *V. humblotii* and *V. perrieri* Schltr. descriptions, respectively. Their weights and the amount of yellow in their flowers were significantly different according to our results (Figure 5, Table 2) and also as noted by Portères (1954). The presence of these two species in Madagascar has already been confirmed (Cribb & Hermans, 2009), although *V. humblotii* is supposedly endemic to the Comoros archipelago (Portères, 1954). The genetic groups with white flowers (C2, C4, C5, C7) could be differentiated mainly by the variation in their flower sizes (Table 2). Cluster 5 (ATD, MLT, KRD, SAG) flowers, which were the smallest ($PL \approx 4$ cm), were identified as morphologically close to the *V. decaryana* H. Perrier description by Allorge-Boiteau (2005) in southern Madagascar. *V. bosseri* L. Allorge and *V. madagascariensis* Rolfe descriptions matched with clusters C4 and C2, respectively. Clusters 2 and 4, well differentiated on STRUCTURE analysis, are relatively close morphologically on the PCA, but they can be readily separated by the presence/absence of hairs on the labellum (Figure 5b). The absence of hairs is an originality of *V. bosseri* (C4) compared to all the other species (Allorge-Boiteau, 2013; Petersson, 2015). Finally, the genetic group constituted by AND (cluster 7) was well differentiated and showed morphological traits different from the others that, however, have never been described in the literature.

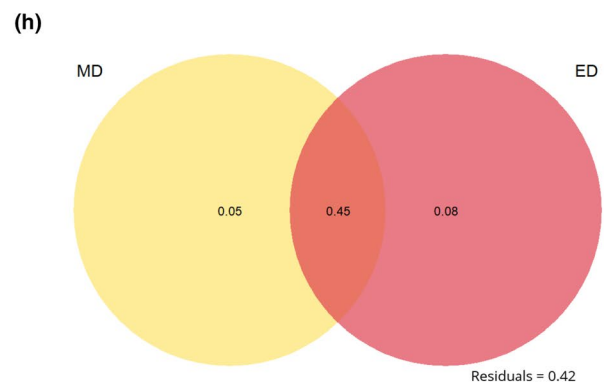
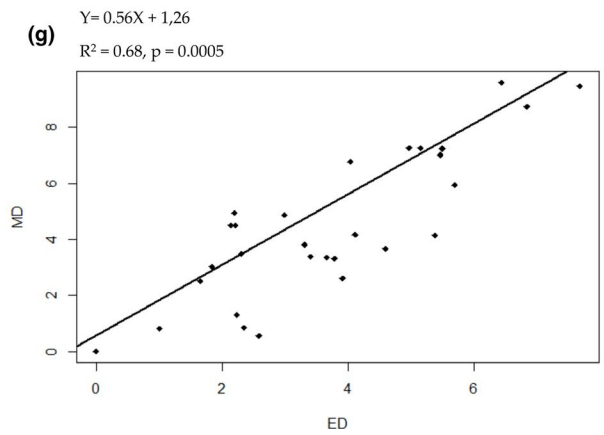
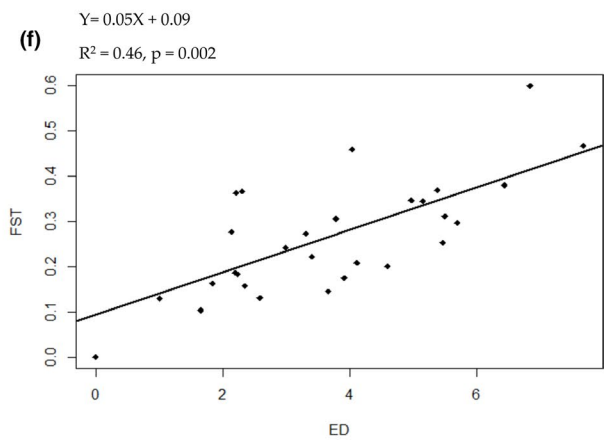
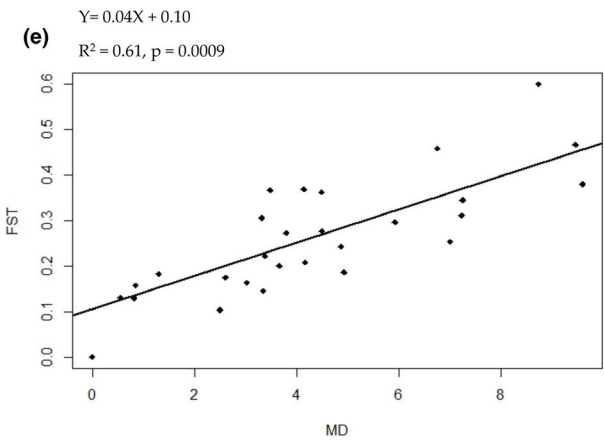
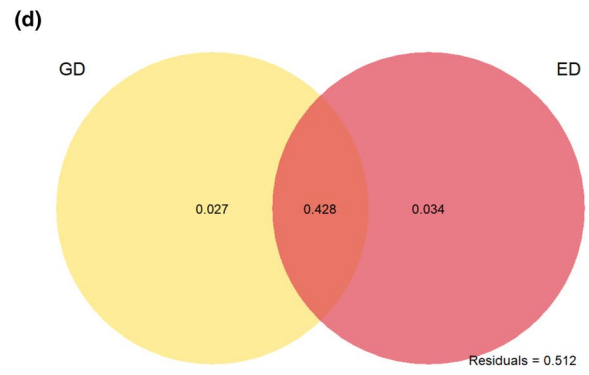
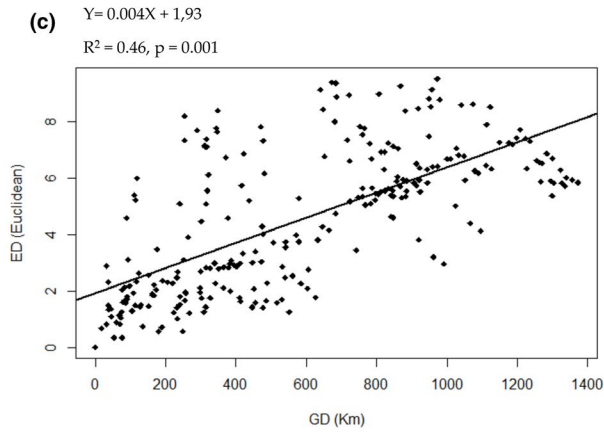
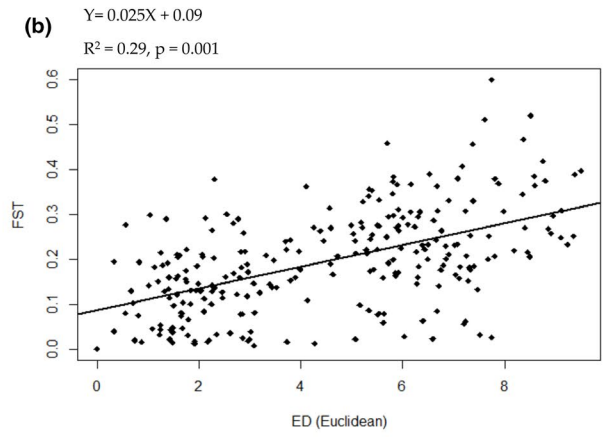
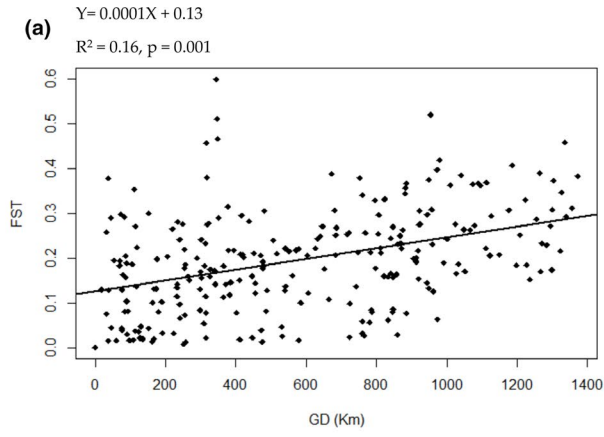


FIGURE 6 Multiple matrix regression with randomization (MMRR) analysis and distance-based redundancy analysis (dbRDA) based on 23 populations (a, b, c, d) and 8 populations (e, f, g, h). (a) MMRR between genetic distance (FST) and geographic distance (GD), (b) MMRR between genetic distance (FST) and environmental distance (ED), (c) MMRR between environmental distance (ED) and geographic distance (GD). Regression lines are drawn for the three models a, b, c. (d) shows variance partitioning results of dbRDA on GD and ED. Geographic distance (GD) comprises eleven continuous rectangular vectors from principal coordinates analyses. Environmental factors include PC scores of three first components of the PCA (Figure S3). The overlapping zone represents the intersection between IBD and IBE. (e) MMRR between morphological distance (MD) and FST. (f) MMRR between ED and FST. (g) MMRR between MD and ED. Regression lines are drawn for the three models e, f, g. (h) shows variance partitioning results of dbRDA on MD and ED. Morphological factors include PC scores of the first three components of the PCA based on selected uncorrelated variables (Figure S4). Environmental factors include PC scores of three first components of the PCA (Figure S5). The overlapping zone represents the intersection between IBA and IBE

TABLE 3 Results of distance-based redundancy analyses (dbRDA) testing the effects of geographic distance (GD), environmental distance (ED), and morphological distance (MD) on genetic differentiation (FST) among 23 populations and 8 populations of leafless *Vanilla* species from Madagascar

dbRDA (23 populations)				dbRDA (8 populations)			
Marginal tests							
Variables	F	p	% of variables	Variables	F	p	% of variables
FST-GD-ED	2.91	0.007	48.83	FST-GD	0.57	0.855	0
FST-GD	2.66	0.006	45.42	FST-ED (PC1) ^c	8.74	0.001	52.51
FST-ED (PC1) ^a	3.94	0.013	11.80	FST-MD (PC1) ^d	8.02	0.005	50.06
FST-ED (PC2) ^b	12.33	0.001	33.99				
Partial dbRDA							
Variables/ controlled	F	p	% of variables	Variables/ controlled	F	p	% of variables
FST-GD/ED	1.23	0.268	2.65	FST-MD/ED	1.77	0.159	5.42
FST-ED/GD	6.10	0.001	3.41	FST-ED/MD	2.12	0.09	7.86

Note: In marginal tests of dbRDA, contribution of each explanatory variable (GD, ED, MD) to genetic differentiation was tested separately, while in partial (conditional) tests, the pure contribution of each factor was obtained after removing the covariate effects of the other factors. For the effects of ED and MD, only the PC axes that contributed significantly to genetic differentiation were represented. Explanatory variables: ^aPC1 = BIO6, BIO4, BIO9, BIO16, BIO13, BIO7, BIO1, BIO3, BIO18; ^bPC2 = BIO8, ELV, pH; ^cPC1 = BIO8, BIO10, BIO5, ELV, pH, SILT; ^dPC1 = TFW, PL, RLSSL, ASL, OW, OWE, CL, AL.

Where species distribution overlap (Allorge-Boiteau, 2013; Cribb & Hermans, 2009; Portères, 1954) (Figure 1), a combination of stratified populations and admixed individuals was detected, mainly in the northwest (CMK, ATS, BBL, MSL, AKF) and southwest (AKL, MLT) populations. These results, therefore, confirm possible sympatry and suggest that genetic exchanges are possible between sympatric species. Hybridization is a common phenomenon among *Vanilla* species (Bory et al., 2010; Divakaran et al., 2006; Gigant et al., 2011; Nielsen & Siegmund, 1999), with an absence of interspecific genetic incompatibility, particularly between closely related species (Bory et al., 2010).

4.3 | Genetic discontinuities and speciation: forest and montane refugia, and riverine barriers

As genetic groups potentially corresponded to different species, diversification processes of leafless *Vanilla* populations in Madagascar could be interpreted as speciation mechanisms. Five mechanisms of speciation are recognized to be a pattern of diversification common

for many taxa in Madagascar: ecological constraint influenced by bioclimatic disparities, western rainforest refugia, montane refugial diversification, riverine barriers, and watersheds (Vences et al., 2009). Interestingly, a genetic diversity center was revealed in the populations from west of Madagascar (highest H_e and A_r values, Table 1). Diversification could have resulted from a postglacial colonization from a western refuge area (Vences et al., 2009), to the north and the south, if considering the cline in diversity as the direction of spread (Hewitt, 1996). A similar scenario was suggested on the basis of paleoclimatic data for palm trees in the northeast of Madagascar (Rakotoarinivo et al., 2013), that have then recolonized the eastern corridor. The general pattern of genetic diversity showed a weak but significant latitudinal shift in the genetic diversity distribution ($r_{\text{He-Latitude}} = 0.47, p < .04$) along the western coast of Madagascar. In addition to the probable effect of climatic oscillation explained previously, the lowest genetic diversity detected in southern populations might be partially linked to population size reductions (bottlenecks), as small and isolated populations are characterized by a low genetic diversity and linkage disequilibrium among loci (Frankham et al., 2010; Ouborg et al., 2006). Indeed, populations in the south of

Madagascar are often exploited for medicinal use (Randrianarivony et al., 2017) and reduced by deforestation, as the two southwestern populations (ANJ and SAG) are found in unprotected areas close to villages. Low genetic diversity in fragmented habitats was also observed for some tropical tree populations in Africa (Andrianoelina et al., 2006, 2009; Farwig et al., 2007). Also, conjugated effects of deforestation and climate crisis, particularly emphasized in the south, could impact on seedlings recruitment (Moles & Westoby, 2004; Rasmussen, 2002).

Significant regions of genetic discontinuities were revealed with BARRIER analysis, between adjacent populations belonging to different genetic groups/species and indicating limited gene flow between these groups (Figure 4b). The most significant genetic barrier ($0.23 < F_{ST} < 0.6$) was observed between the genetic group C7 (AND), encountered in high altitude (up to 800 m) (Figure 4a, b) in a 49 ha conserved forest (Gould & Gabriel, 2015), and the others localized in low altitude, confirming probable differentiation by isolation. AND population could be the result of a “montane refugia speciation” supposing that population may have survived in high altitude during the climate shift, has adapted over time and remained isolated from the others (Fjeldsaå & Lovett, 1997; Vences et al., 2009). Moreover, the AND population showed a high clonality (73%) which probably explains the significant heterozygote excess ($FIS < 0$) detected (Halkett et al., 2005).

Rivers can constitute permanent geographic barriers that separated the continuous distribution range of populations leading to vicariant divergence (Vences et al., 2009). Five genetic boundaries identified by BARRIER analysis matched river separations. Indeed, population KRD and KRM belonging to different genetic groups are geographically separated by two small rivers in the west: Morondava and Maharivo (barrier g) (Figure 4a, b). The genetic barrier numbered d traces the path of the great Mangoky river separating the mid-West population (KRM) from southwest populations (Figure 4a, b). Barriers h, i, and k can be linked to rivers in the north (Figure 4a, b). To what extent gene flow can be impeded by riverine barriers will have to be further assessed when better knowledge will be obtained regarding leafless *Vanilla* species pollinators and dispersers. Nevertheless, those riverine barriers might explain part of, but not all the genetic differentiation observed between populations/species. The occurrence of IBD, IBE, and IBA was therefore also assessed.

4.4 | Combined effects of IBA and IBE as drivers of genetic divergence

Considering the geographic distribution of the genetic groups identified (Figure 4), some of them seem to be confined to well-defined biomes (e.g., C5, C6 and C7) and AMOVA showed a weak but significant (6%) genetic variation between ecoregions. Tested separately on 23 populations, IBD and IBE played a significant role ($R^2_{adj} = 45.42\%$ and $R^2_{adj} = 45.79\%$, respectively) on genetic differentiation. However, partial dbRDA showed a relatively weak ($R^2_{adj} = 3.4\%$), but significant influence of environmental factors on genetic differentiation and no contribution of IBD. The combined effect of geographic

and environmental distance (IBD \cap IBE) was the most explanatory ($R^2_{adj} = 42.8\%$). Indeed, spatial autocorrelation was detected between geographic and environmental distance (Figure 6c). This can be explained by the presence of clear ecoregions determined by the presence of a central mountain range and bioclimatic differences between west/east and north/south, which can lead to eco-geographic constraint speciation (Vences et al., 2009). The analysis of the reduced dataset (eight populations) revealed no IBD and no correlation between IBD and IBE. The suppression of this correlation allowed the pure contribution of these processes to be determined (Wang & Bradburd, 2014). Results from this reduced dataset confirmed the significant occurrence of IBE ($R^2_{adj} = 52.51\%$) (with most discriminant variables being related to temperature, elevation, and soil pH) (Table 3). Moreover, significant IBA ($R^2_{adj} = 50.06$) (concerning flower size-related measures) was also revealed in this dataset. But, there was a strong correlation between environment and morphological variables, making it difficult to disentangle their relative contribution to the genetic differentiation. Two evolutionary patterns can possibly explain this situation: Either IBA of floral traits could indicate pollinator-mediated ecological selection, or ecological isolation could have led to drift-induced morphological variations in flower size (Waser & Campbell, 2004). Pollinator-mediated divergent ecological selection acts on flower traits (size, color, shape, odor...), therefore promoting isolation by adaptation (IBA) via floral isolation (Van der Niet et al., 2014). In orchids, pollinator specialization is common (Darwin, 1859; Peter & Johnson, 2014; Tremblay, 1992) and requires association between floral characteristics and insect traits (Micheneau et al., 2009; Petersson, 2015; Tremblay, 1992). For example, an association between spur length of some Angraecoid species and proboscis length of pollinators has been observed in Madagascar (Micheneau et al., 2009). Flower-size variations in *Vanilla* species could be indicative of a pollinator specialization. Indeed, as noticed in Costa Rica, given the major size differences observed between *V. planifolia* and a newly described species *V. sotoarenasii*, it is unlikely that the same bee species can perform pollination efficiently in both species (Azofeifa-Bolaños et al., 2017). Similarly, in Peru, small size bees (*Melipona* sp., *Euglossa* sp.) were shown to be unable to remove pollen from the large flowers of *V. grandiflora*, as opposed to *Eulaema meriana* (Householder et al., 2010; Lubinsky et al., 2006). Therefore, the eastern populations, with much larger flowers (as shown for AND), might be characterized by different pollinators than the western ones, and southwestern populations of *V. decaryana* (C5), with the smallest flowers, could be concerned as well by a certain level of pollinator specificity. However, various arguments are more in favor of the fact that most *Vanilla* species probably share the same pollinators in Madagascar. For *V. bosseri* in the Kirindy forest (KRD), Petersson (2015) identified three visiting bee species as possible pollinators: *Macrogalea ellioti*, *Liotrigona madecassa*, and *Liotrigona mahafalya*. These three bees have a north-west-south distribution (Pauly et al., 2001) matching the combined distributions of western *Vanilla* species. Another potential bee pollinator was also indicated based on floral morphology traits, *Lithurgus pullatus* which has a disjunct southwestern and northeastern distribution (Pauly

et al., 2001). Moreover, in Mayotte, South Africa and Madagascar, leafless *Vanilla* species (*V. humblotii*, *V. roscheri*, *V. bosseri*, respectively) all have potential pollinator species from the Allodapini group (Gigant et al., 2014; Gigant, De Bruyn, et al., 2016; Petersson, 2015). In addition to bee species, the sunbird *Nectarinia coquerelli* was also identified as visitor and putative pollinator of *V. humblotii* in Mayotte (Gigant, De Bruyn, et al., 2016). At least one to two species belonging to the same genus, *Nectarinia notata* and *Nectarinia souimanga*, were observed in 31 dry forest sites in Madagascar (Raherilalao & Wilmé, 2008). High interannual variation in precipitation was observed in the west of Madagascar, which is associated with unpredictable patterns of flowering and fruiting (Dewar & Richard, 2007). This is consistent with the generalist hypothesis, at least for the western species: Some pollinators would compensate for the absence of the others to ensure species reproduction. Our results showing the existence of possible hybrids between some sympatric species in the west of Madagascar reinforces this hypothesis, as also demonstrated for two sympatric leafless *Vanilla* species in Puerto Rico (*V. barbellata* and *V. claviculata*) (Nielsen, 2000) for which the occurrence of hybrids suggested the same pollinator. In the genus *Vanilla*, in America, the known pollinators of wild species of the *V. planifolia* and *V. pompona* groups are similar, and they are all orchid-bee species from the genera *Euglossa* and *Eulaema* (Andriamihaja et al., 2020).

In plants, ecological isolation, apart from pollinator-mediated divergent selection, is mainly based on the selection against migrants (Lowry et al., 2008; Nosil et al., 2005). Our results showed that discriminant environmental variables were related to temperature, altitude, and soil (pH). Altitude influence was previously discussed in the “montane refugia speciation” possibly involved for the high-altitude population AND. Soil pH can affect mycorrhizal fungi composition (van Aarle et al., 2002; Porter et al., 1987). For orchids, establishment and growth is highly dependent on suitable mycorrhizal fungi for seed germination in addition to suitable pollinator populations for reproduction (Ackerman et al., 1996; Rasmussen, 2002; Rasmussen et al., 2015). In Puerto Rico, Cuba, and Costa Rica, different mycorrhizal fungi were detected in different sites associated with different *Vanilla* species, and fungal specificity for *Vanilla* seed germination and growth was shown (Porrás-Alfaro & Bayman, 2007). Characterizing the mycorrhizal fungi in each of the studied populations could allow to verify this, as done on orchids in the Central Highlands of Madagascar (Yokoya et al., 2015). Species differentiation may also be related to the different phenology of the species, influenced by climatic and ecological conditions such as temperature and altitude (Petrauski et al., 2019; Sobel et al., 2009). Indeed, *V. humblotii* and *V. perrieri* have overlapping phenologies (November–April and November–December, respectively) (Allorge-Boiteau, 2005; Portères, 1954), but *V. bosseri* and *V. madagascariensis* flower earlier (September–October and June–October, respectively) (Allorge-Boiteau, 2005, 2013; Portères, 1954). *V. decaryana* is reported to flower in December–January (Allorge-Boiteau, 2013; Portères, 1954). Although the leafless *Vanilla* species *V. claviculata* and *V. barbellata* were shown to have overlapping phenologies in Puerto Rico, facilitating hybridization (Nielsen, 2000), differences in

flowering periods with another sympatric species (*V. dilloniana*) have also been reported (Nielsen & Siegismund, 1999).

All results combined, the present study suggested a pattern of speciation possibly driven by the existence of riverine barriers and forest and montane refuges, in combination with isolation by ecology, as a result of adaptation to local habitats, possibly linked to local diversity in mycorrhizal fungi, and differences in flowering phenology, although pollinator-mediated divergent selection in these *Vanilla* species could not be ruled out and might depend on the species concerned. Pollinator specificity could also be a consequence of floral differentiation consecutive to ecological speciation. Our results also showed the efficiency of a population genetics approach using highly variable microsatellite markers and the importance of large sample sizes for reliable estimates of the genetic relationship between recently diverged species, as shown for *Carappa* spp. (Duminil et al., 2012) and *Coffea* spp. (Razafinarivo et al., 2013).

This study is the first to provide an overview on the patterns of population genetic and morphological differentiation and to address issues related to the evolutionary mechanisms of several natural leafless *Vanilla* populations from Madagascar. It is nevertheless necessary to point out that this work has certain limitations, including the limited number of polymorphic microsatellite markers used, the small sample sizes of some populations (MAN, SAG, ZVB) and the absence of morphological data of one identified genetic group. The presence of new and significantly different genetic and morphological groups in the east, not yet botanically described, is interesting and will require further sampling and genetic studies in the eastern region, which is not specifically described as harboring leafless *Vanilla* species (Allorge-Boiteau, 2005; Cribb & Hermans, 2009; Portères, 1954). Further studies on the Madagascar species reproduction biology (incompatibility, phenology, pollinators, seed dispersion, mycorrhizal fungi...) are needed. The Madagascar leafless *Vanilla* species will also need to be compared to sister species from the SWIO area to gain a broader phylogeographical view of their evolution.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION

Cathucia F. Andriamihaja: Data curation (lead); Formal analysis (lead); Investigation (equal); Methodology (lead); Writing-original draft (lead); Writing-review & editing (equal). **Aro V. Ramarosandrana:** Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Supervision (equal); Validation (equal); Writing-review & editing (equal). **Michel Grisoni:** Conceptualization (equal); Funding acquisition (equal); Supervision (equal); Validation (equal); Writing-review & editing (equal). **Vololoniaina H. Jeannoda:** Conceptualization (equal); Supervision (equal); Validation (supporting); Writing-review & editing (equal). **Pascale Besse:** Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Supervision (equal); Validation (equal); Writing-original draft (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

Morphological measurement data and microsatellite data used to produce Figure 4, Figure 5, and Table 2 are accessible on UMR PVBMT Cirad dataverse (<https://dataverse.cirad.fr/dataverse/pvbmt>; <https://dataverse.cirad.fr/dataset.xhtml?persistentId=doi:10.18167/DVN1/GWXWCB>).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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CHAPITRE 3

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Systematique des vanilliers aphylls endémiques du Sud-Ouest de l'océan Indien

« De Madagascar, je peux dire aux naturalistes que c'est vraiment leur terre promise. La nature semble s'y être retirée dans un sanctuaire privé pour travailler sur des modèles différents de ceux qu'elle a créés ailleurs. À chaque pas, on rencontre les formes les plus étranges et les plus merveilleuses »
Philibert Commerson

3.1. Présentation et résumé du chapitre

La délimitation du complexe d'espèces aphylls du genre *Vanilla* de la région du sud-ouest de l'océan Indien (SWIO) a longtemps été un défi taxonomique, en raison de la différenciation morphologique limitée et de l'absence de variation dans les régions chloroplastiques. Ce complexe regroupe sept espèces botaniques connues : *V. madagascariensis*, *V. bosseri*, *V. decaryana* et *V. perrieri* endémiques de Madagascar, *V. humblotii* présumée endémique de l'archipel des Comores, mais également présente à Madagascar, *V. roscheri* de la côte Est africaine et *V. phalaenopsis* endémique des Seychelles (Chapitre 1). Dans le Chapitre 2, nous avons pu démontrer, à partir des marqueurs microsatellites, l'existence de deux clusters génétiques malgaches localisés à l'Est de Madagascar, en plus des cinq taxons reconnus dans la littérature. Une taxonomie intégrative a donc été menée dans ce Chapitre 3. Nous avons enrichi les données microsatellites et morphologiques utilisées dans l'étude précédente avec de nouvelles données, et réalisé un séquençage ITS pour valider le niveau taxonomique de ces clusters génétiques malgaches, et démêler les relations phylogénétiques entre les espèces du sud-ouest de l'océan Indien. Sur la base des analyses phylogénétiques, génotypiques et morphologiques, neuf espèces ont été identifiées dans les îles du sud-ouest de l'océan Indien, dont sept sont présentes à Madagascar et les deux autres sont réparties respectivement aux Seychelles et à l'Est du continent Africain. D'après l'analyse phylogénétique, le groupe des vanilliers aphylls est originaire de Madagascar, d'où elle s'est diversifiée, à partir d'un ancêtre folié africain. Ensuite, trois événements ultérieurs de colonisation indépendants de Madagascar vers les autres territoires de la région sud-ouest de l'océan Indien se sont produits au sein des deux principaux clades : espèces à fleurs blanches versus espèces à fleurs jaunes. D'autre part, trois espèces botaniques connues, *V. madagascariensis*, *V. bosseri* et *V. phalaenopsis*, sont nettement différenciées sur l'analyse de regroupement génétique à partir des microsatellites bien qu'elles soient conspécifiques sur l'arbre ITS, ce qui suggère une récente spéciation. Les deux nouvelles espèces malgaches sont décrites et une nouvelle clé d'identification de ces sept espèces aphylls du genre *Vanilla* endémiques de Madagascar est proposée dans ce chapitre.

3.2. Integrative taxonomy and phylogeny of leafless *Vanilla* orchids from the South West Indian ocean region reveal two new Malagasy species

Running title: Two new *Vanilla* species from Madagascar.

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Abstract: The leafless *Vanilla* species complex from the Southwestern Indian ocean (SWIO) region has long been a taxonomic challenge, due to limited patterns of morphological differentiation and an absence of variation within chloroplast sequences. This complex regroups seven known morphospecies: *V. madagascariensis*, *V. bosseri*, *V. decaryana* and *V. perrieri* endemics to Madagascar, *V. humblotii* presumed as endemic to Comoros Archipelago but also present in Madagascar, *V. roscheri* from the East African coast and *V. phalaenopsis* endemic to Seychelles. A previous population genetics study using microsatellite markers allowed to distinguish, in addition to the five recognized Malagasy taxon, two other genetic clusters present in the East of the island. An integrative taxonomy approach was therefore conducted by combining microsatellite and morphological data used in the previous study with new datasets, and by adding ITS sequencing data, to validate the taxonomic level of these Malagasy genetic clusters and unravel phylogenetic relationships between SWIO species. As a result, based on phylogenetic, genotypic and morphological evidences, nine species were discriminated in the SWIO region, including seven in Madagascar with two new Eastern species. The leafless *Vanilla* group originated and diversified in Madagascar, from an ancestor of African descent, with three subsequent independent colonization events from Madagascar to the other territories of SWIO within the two main lineages (white versus yellow flower species). Three known morphospecies, *V. madagascariensis*, *V. bosseri* and *V. phalaenopsis*, are conspecific but nevertheless differentiated albeit recently. The new Malagasy species are described and a new identification key is proposed.

Keywords: microsatellite, ITS, morphology, systematics, Madagascar, species delimitation, phylogeography

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3.2.1. Introduction

All biological scientists and conservationists strongly need to properly define the taxonomic units they work on. For many disciplines, such as botany, species are the fundamental units on which studies will be based. Species is denoted on the basis of many criteria of which resemblance between species members is the most widely accepted (Aldhebiani, 2018). These criteria used to define species boundaries have changed over time due essentially to the rapid development of new technologies and the birth of evolutionary concepts (Rouhan & Gaudeul, 2021). Linnaeus was one of the first systematist to propose a species definition. According to him, members of a species have relatively constant characters and tend to breed (Larson, 1968). Years later, De Candolle proposed another definition which integrated, in addition to the criteria of resemblance and reproduction, a condition of fertile offspring of species members (De Candolle, 1813). In the 19th century, Darwin's theory of evolution revolutionized species concepts. According to the book "On the origin of species", Darwin species concept can be formulated as follows: a fundamental unit of evolution that is constituted by populations of organisms united by common descent and that have been differentiated from the other species after natural selection by adaptation to environmental conditions (Darwin, 1859; Stamos, 2013). On the basis of these earlier theories, many recent species concepts have been proposed from the 20th century (De Queiroz, 2005, 2007; Aldhebiani, 2018; Rouhan & Gaudeul, 2021) among which the most widely held are the following: biological (Mayr, 1942), evolutionary (Simpson, 1951; Wiley, 1978), ecological (Van Valen, 1976), phenetic (Sokal, 1986) and phylogenetic species concepts (De Queiroz, 2005, 2007; Aldhebiani, 2018).

The biological species concept (BSC) considers the total reproductive isolation as an indication of species status (Mayr, 1942; Aldhebiani, 2018). This concept has been widely discussed, as strict reproductive isolation is rarely achieved, for example in the case of closely related species living in sympatry. This situation would imply the recognition of fewer species taxon than the number currently admitted (Dayrat, 2005; De Queiroz, 2007; Aldhebiani, 2018). The evolutionary species concept (EvSC) was first proposed by Simpson (1951) and revised by Wiley (1978). It considers species as a "single lineage of ancestral descendent populations of organisms which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate" (Wiley, 1978). In addition to his definition, Wiley (1978) stated four corollaries following the definition, among which the reproductive isolation requirement. He also argued that the morphological or phenetic differences between two evolutionary species is not mandatory (Wiley, 1978). But, the impossibility or difficulty to test if species have a different evolutionary trajectory and the reproductive isolation requirement make this concept not easy to apply. Van Valen introduced the ecological species concept (EcSC) in defining species as a set of organisms occurring in a distinct adaptive zone, called a niche (Van Valen, 1976). EcSC is not effective when different species occupy the same habitat. In the phenetic species concept (PheSC), species is defined as a taxon whose members are on average more similar to each other and distinct to members of other taxon based on as many phenetic traits as possible and using clustering and distance methods (Sokal, 1986; Aldhebiani,

2018). Phenetic traits should be quantifiable and can be issued from different sources of data like cytology, phytochemistry, anatomy, embryology and frequently morphology. Morphological features are sometimes subject to phenotypic plasticity (variation according to the environment), which makes phenetic classification doubtful, especially when the number of characters used for species delimitation is low. Still in this perspective of clustering methods, Mallet (1995) suggested another approach using genotypic data (e.g. allozymes, microsatellites, SNP or single nucleotide polymorphism, ... (Henry, 2001)) and morphological data. According to the genotypic species concept (GSC), a species is a genotypic cluster that can hybridize but forms few or no intermediates with other genotypic clusters (Mallet, 1995). The maintenance of independent evolving units in the case of sympatry, despite the continuity of gene flow between them, is the result of various processes like ecological adaptation that selects disruptively against intermediates or hybrids (Mallet, 1995). With the genotypic species concept, the difference between species and subspecies is small (Mallet, 1995). According to Hausdorf (2011), genotypic clusters represent the initial stages of speciation, thus cannot yet be recognized as species and should be regrouped with the ancestral species. Finally, the phylogenetic species concept (PhySC) defines species as a set of organisms that have the same ancestor (monophyly requirement) (De Queiroz, 2005, 2007; Aldhebiani, 2018). As no clear guidelines exist for identifying the node where the speciation event occurs (Mallet, 1995; Aldhebiani, 2018), this may lead to an oversubdivision (Mallet, 1995). PhySC is not practical for direct field descriptions. Also, it is crucial to choose the adequate molecular marker in phylogenetic methods depending on the taxonomic level one wants to delimit (Besse, 2014a).

All these species concepts are complementary but also have some limitations. The problem of species delimitation is amplified when species have recently diverged or when they are cryptic or closely related (De Queiroz, 2005; Barley et al., 2013; Pinheiro, Dantas-Queiroz, & Palma-Silva, 2018). Indeed, characters resulting from a complete speciation are not all observable in a recently diverged species (De Queiroz, 2005). For cryptic and closely related species, the problem is that morphological traits cannot distinguish them even if they form distinct genetic groups (Bickford et al., 2007). There are also some case (e.g. adaptive radiation) where genetical divergence between species is low although morphological differentiation is high (Barley et al., 2013). Today, it is recognized that the best way to circumvent species is to use several methods (morphological analyses, phylogeny, genetic structuring...) and different species concepts (Rouhan & Gaudeul, 2021). This principle of taxonomy, called integrative taxonomy (Dayrat, 2005), is based on the idea that species delimitation using different data sources and methods is more rigorous than when using a single type of data (Dayrat, 2005; Schlick-Steiner et al., 2010). By combining different approaches, integrative taxonomy tends to be more standard (Dayrat, 2005) because the limitations of each approach are offset by the advantages of the others. Therefore, integrative taxonomy has been shown as the most efficient for the delimitation of recently diverged, cryptic or closely related species (e.g. Dayrat, 2005; Duminil et al., 2012; Pansarin & Ferreira, 2019; Pessoa et al., 2012).

In this paper, we applied this strategy to resolve the taxonomic puzzle associated with a recent group of leafless *Vanilla* Plum. ex Mill. species (Orchidaceae) from the South West Indian ocean region (SWIO) (Madagascar, Comoros archipelago, Seychelles, Eastern coast of Africa) (Andriamihaja et al., 2020). Seven leafless species are described botanically in this region, but their floral morphology is very similar. Our recent population genetics work suggested that there might be more species than the five initially described in Madagascar (Andriamihaja et al., 2020, 2021), and nothing is known about the phylogenetic relationships between the Malagasy species and sister species from the SWIO region. Species can be allopatric (Madagascar vs Seychelles vs Comoros vs East of Africa, or West vs East of Madagascar), but cases of sympatry are frequent for *Vanilla* species from Western Madagascar (Andriamihaja et al., 2020, 2021). Hybrid individuals were detected in areas where at least two species were sympatric (Andriamihaja et al., 2021). As interspecific hybridization has also already been observed between several *Vanilla* species (either naturally or through breeding), even between the most genetically distant (Nielsen, 2000; Divakaran et al., 2006; Minoo et al., 2008), adopting BSC would call into question many species so far described in the genus. In addition, the fertility of interspecific hybrids was demonstrated, as in the case of the species *V. × tahitensis* J. W. Moore resulting from the hybridization of *V. planifolia* Andrews and *V. odorata* C. Presl (Lubinsky et al., 2008). The EcSC is not applicable to *Vanilla* species because they are often sympatric (Nielsen, 2000; Soto Arenas & Dressler, 2010; Andriamihaja et al., 2020), therefore occupy the same ecological niches and need the same resources. Since it is impossible or difficult to test if Malagasy leafless *Vanilla* species have a different evolutionary trajectory given that they are sympatric and can hybridize, the EvSC concept is also difficult to apply.

Therefore, only the Phylogenetic, Genotypic and Phenetic concepts might be applicable for the *Vanilla* species under survey, particularly those from Madagascar. SWIO leafless species form a recent (4.4 Mya) monophyletic group as shown using chloroplast DNA markers (*rbcL*, *psaB*, *psbB*, *psbC*) (Bouetard et al., 2010). These cpDNA markers were however unable to resolve species under PhySC within this recent group because of the lack of sequence variation. As demonstrated in barcoding experiments for *Vanilla* species (Besse et al., 2021), the sequencing of the nuclear Internal Transcribed Spacer (ITS) region of the ribosomal DNA is necessary to reach a sufficient level of discrimination, and will therefore be used to apply the PhySC to this recent group of species and resolve the phylogenetic relationships within this clade. Moreover, a previous thorough microsatellite markers (SSR or Simple Sequence Repeats) study at the population level combined with morphological evidences allowed to resolve some of the Malagasy species (seven genetic groups were revealed for five described botanical species) (Andriamihaja et al., 2021), demonstrating the power of combined GSC and PheSC analyses, that will therefore be applied in complement to the PhySC to further resolve species delimitation. According to Mallet (1995), the GSC approach is powerful in orchids where interspecific hybridization is frequently observed.

We therefore initiated this study to answer major questions that were raised by our previous SSR study of Madagascar population differentiation (Andriamihaja et al., 2021). We studied

new accessions from leafless species from the SWIO group (Seychelles, East African coast, Comoros archipelago) in addition to Madagascar accessions, using ITS sequencing and SSR genotyping (outgroups consisting of leafy African and Malagasy species were also added in the ITS analysis). We also performed new field prospections that allowed the morphological characterization of the genetic group from Manompana (Madagascar) that was not studied earlier. This allowed us to unravel the phylogenetic relationships and biogeography of leafless *Vanilla* species distributed in the SWIO as well as to resolve, using an integrative taxonomy approach, the number of species present in Madagascar, with the discovery and description of two new leafless *Vanilla* species in the East of Madagascar.

3.2.2. Materials and Methods

a. Sampling design

This study included 38 leafless *Vanilla* samples collected during field surveys carried out in Madagascar (2017-2019), as well as eight samples from the South West Indian ocean region (Mayotte, Seychelles, Zanzibar and South Africa) and three leafless samples coming from botanical gardens and maintained in the Biological Resources Center (BRC) Vatel collection in Reunion island (Table 1, Table S1). Samples were chosen to cover all distribution areas of the seven leafless *Vanilla* species indicated in the literature (Portères, 1954; Cribb & Hermans, 2009; Allorge-Boiteau, 2013; Andriamihaja et al., 2020) and to represent all genetic groups from Madagascar revealed in a previous study (Andriamihaja et al., 2021) (Figure 1). Fieldwork and sampling in Madagascar were conducted according to permit numbers 201/17/MEEF/SG/DGF/DSAP/SCB.Re and 205/17/MEEF/SG/DGF/DSAP/SCB.Re delivered by the Ministry of environment and sustainable development. In addition, 14 leafy specimens from Africa and Madagascar and one outgroup from America were also studied to complete the dataset. A total of 64 samples were used, including 45 samples used in previous studies (Andriamihaja et al., 2021; Besse et al., 2021) and 19 samples specific to this study (Table S1). They are all registered and stored in BRC Vatel.

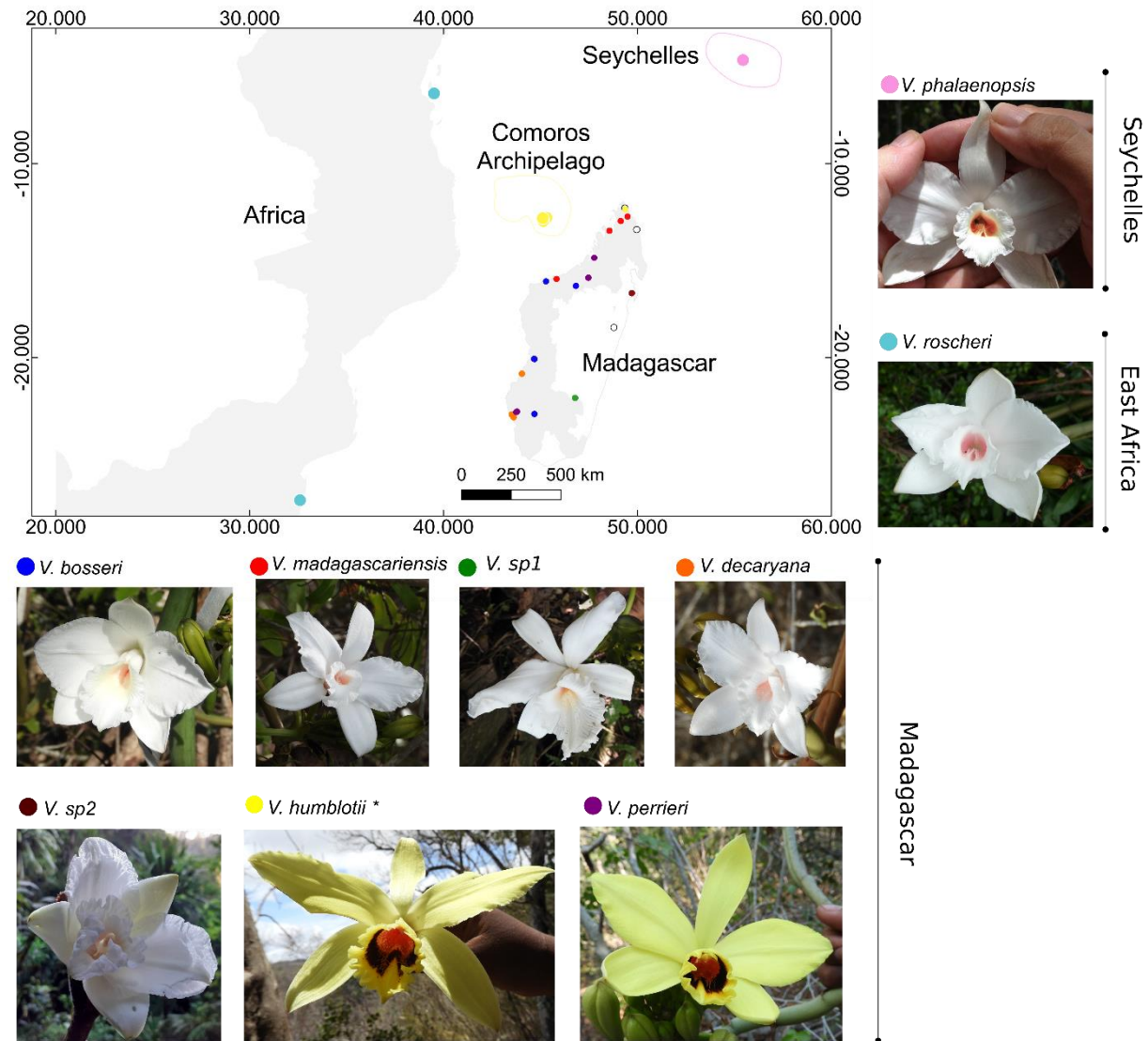


Figure 1. Sampling map

Circles indicate sample locations. Species names were refined according to a previous study on genetic structuring (Andriamihaja et al., 2021), botanical description and geographical distribution (Portères, 1954; Cribb & Hermans, 2009; Soto-Arenas & Cribb, 2010; Allorge-Boiteau, 2013). White circles surrounded by black lines correspond to unspecified samples. *V. sp1* and *V. sp2* represent samples that could not be matched to known species on the basis of their morphological characteristics and genetic grouping (Andriamihaja et al., 2021). Distribution of leafless *Vanilla* species in the South West Indian ocean region are highlighted in grey. * : *V. humblotii* is both present in Comoros Archipelago and Madagascar (Portères, 1954; Cribb & Hermans, 2009). (Photo *V. bosseri*; *V. madagascariensis*; *V. sp1*; *V. decaryana*: Cathucia F. Andriamihaja; *V. perrieri*: Hoby N. Nomenjanahary, *V. sp2*: Michel Grisoni, *V. humblotii*: Johnson G. Andrianantenaina, *V. roscheri*: Rodolphe Gigant, *V. phalaenopsis*: Pascale Besse).

b. Phylogenetic analyses and divergence estimates

The first analysis carried out consisted in building a phylogenetic tree. DNA was extracted using the Dneasy plant Mini kit (Qiagen Hilden Germany). Because preliminary assessments showed that chloroplast genes (*rbcl*, *psaB*, *psbB*, *psbC*, and *matK*) were not variable enough to

discriminate this group of leafless *Vanilla* species (Bouetard et al., 2010, and data not shown), we used the nuclear ITS region. As in many plant species (China Plant BOL Group et al., 2011), the ITS region is among the mostly used and efficient marker in *Vanilla* phylogeny (Lubinsky et al., 2008; Soto Arenas & Dressler, 2010; Azofeifa-Bolaños et al., 2017; Villanueva-Viramontes et al., 2017), as it shows more variations at several taxonomic levels including species and subspecies and can be especially helpful for closely related species delimitation (Besse, 2014a; Besse et al., 2021). The ITS region was amplified via Polymerase Chain Reaction (PCR) using the primers *ITS 17SE* and *ITS 26SE* (Besse, 2014b). Amplification reactions included 20 µl of Gotaq master Mix 2X (Promega, Madison, Wisconsin, USA), 0.8 µl each of forward and reverse primers (10uM), 14.4 µl of H₂O and 4µl of DNA (10 ng/µl). The PCR cycling profile consisted in 3 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60 °C, 45 s at 72°C and final elongation of 10 min at 72°C. The amplification products were sent to Genoscreen (Lille, France) for sequencing. Amplicons were sequenced in both directions (reverse/forward). The 54 sequence data obtained have been submitted to the GenBank database under accession numbers MW879465- MW879518, 10 others are issued from previous work (Besse et al., 2021) (Table S1). Nucleotide sequences were aligned using the MEGAX software (Kumar et al., 2018) and checked manually to avoid possible errors. Analyses were performed using four phylogenetic methods: Neighbor-joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Analysis. The first three were implemented with MEGAX software (Kumar et al., 2018) and the last one was done under BEAST 1.10.4 (Suchard et al., 2018). The nucleotide substitution model by Motoo Kimura + gamma distribution (K80+G) was selected, as it was identified to be the best model according to the Bayesian Information Criterion (BIC) and decision-theoretic performance-based approach (DT) calculated with jModelTest 2.1.10 software (Posada, 2008). For Bayesian method phylogeny reconstruction, two simultaneous independent runs of Bayesian Monte Carlo Markov Chain (MCMC) was run for 20,000,000 generations, with a tree sampled every 2000 generations. The convergence of the two independent runs was tested with Tracer 1.7.1. According to the test of molecular clocks using the Maximum Likelihood method in MEGAX, the null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level ($P= 6.220E-0.75$). Therefore, times of divergences between leafless *Vanilla* species from the SWIO region were estimated using the relaxed molecular clock approach implemented in BEAST 1.10.4. Strict and uncorrelated (exponential and lognormal) relaxed clock models were tested with K80+G substitution model and calibrated Yule of speciation (Heled & Drummond, 2012). In addition, comparison between marginal likelihood of the three analyses calculated (under Tracer 1.7.1, (Rambaut et al., 2018)) via path sampling approach in BEAST indicated that uncorrelated exponential relaxed clock was the most appropriate model. For the calibration, we used the divergence date of the SWIO leafless *Vanilla* group inferred by Bouetard et al. (2010). This calibration point was set at a normal prior age of 4.4 Mya with a standard deviation of 2.3 Mya (95% HPD: 0.6-8.2) close to the estimated dates from Bouetard et al. (2010). TreeAnnotator 1.10.4 was used to summarize the sample of post-burning trees as a single tree after discarding the first 10% as burn-in. The summarized tree was finally viewed

and constructed using Figtree 1.4.4 (Rambaut, <http://tree.bio.ed.ac.uk/software/figtree/>) and Inkscape (<https://inkscape.org/fr/>).

c. Taxonomic diagnosis using a population genetics approach

Clades obtained from phylogenetic analysis were further diagnosed using a population genetics approach. This method is often recommended for closely related species delimitation (Duminil & Di Michele, 2009; Wang et al., 2019) as they can be considered to be highly differentiated populations (Queloz et al., 2010).

Table 1. List of specimens used in this study, with classification and geographical origin
Species names were defined according to genetic structuring analyses in Andriamihaja et al. (2021), morphological description and geographical location in the literature (Portères, 1954; Cribb & Hermans, 2009; Allorge-Boiteau, 2013). Accession voucher numbers are detailed in Table S1.

Classification ^a	Species	Geographical origin	Wild population or collection	N
Xanata Tethya				
/ Aphyllae	<i>V.sp1</i> ^a	Madagascar	AND	4
	<i>V.sp2</i> ^a	Madagascar	MAN	5
	<i>V. perrieri</i> Schltr. ^a	Madagascar	AKL, BFD, BTM	5
	<i>V. bosseri</i> L.Allorge ^a	Madagascar	AKL, BBL, KR D, ZVB	6
	<i>V. madagascariensis</i> Rolfe ^a	Madagascar	AMB, ANK, ANM, CMK	4
	<i>V. decaryana</i> H. Perrier ^a	Madagascar	ANJ, ATD, KRM	4
	<i>V. humblotii</i> Rchb.f. ^a	Madagascar	MDF	4
	Unknown ^a	Madagascar	EST	1
	Unknown ^a	Madagascar	Diego (trad. Doctor)	2
	Unknown ^a	Madagascar	Tulear (Antsokay arboretum)	1
	Unknown ^a	Madagascar	Vohemar (Floribis collection)	2
	<i>V. humblotii</i> Rchb.f. ^a	Mayotte	Moya, Sohoa, Choungi	3
	<i>V. phalaenopsis</i> Rchb.f. ^a	Seychelles	Mahé, JB Lyon	3
	<i>V. roscheri</i> Rchb.f. ^a	East Africa	South Africa, Zanzibar	3
Unknown ^a	SWOI unspecified	MNHN Cherbourg, RBG Kew	2	
Xanata Tethya				
/ Foliosae	<i>V. africana</i> Lindl. ^b	Africa	MNHN Cherbourg	1
	<i>V. crenulata</i> Rolfe ^b	Africa	MNHN Cherbourg	3

Classification ^a	Species	Geographical origin	Wild population or collection	N
	<i>V. françoisii</i> H. Perrier ^b	Madagascar	MAN	7
	<i>V. polylepis</i> Summerh. ^b	Africa	RBG Kew	1
	<i>V. imperialis</i> Kraenzl. ^b	Africa	MNHN Cherbourg, Denmark	2
Xanata Xanata / Foliosae	<i>V. palmarum</i> Salzm. ex Lindl. ^b	America	Brazil	1
			total	64

^a Sub genus & section according to Soto-Arenas & Cribb (2010)/ section according to Portères (1954). ^a leafless species, ^b leafy species.

For that, microsatellite genotyping data of the 49 leafless *Vanilla* samples were collected: a subset of the data was issued from previous studies (Gigant et al., 2014, 2016; Andriamihaja et al., 2021) and 14 new samples were studied (Table S1) at seven microsatellite loci (*mVroCIR04*, *mVroCIR05*, *mVhuCIR03*, *mVhuCIR04*, *mVhuCIR06*, *mVhuCIR07*, *mVhuCIR08*) designed by Gigant et al. (2012). PCR amplifications and microsatellite genotyping were performed as described previously (Andriamihaja et al., 2021). Assignment of individuals to genetic clusters on the basis of multi-locus genotypes was performed using Bayesian clustering analyses implemented in the STRUCTURE software (Pritchard et al., 2000). The model with no admixture and correlated alleles frequencies was chosen. The number of genetic clusters (K) was set from 1 to 11 with 10 replicate runs for each K-value, 100,000 burn-in and 1,000,000 Markov chain Monte Carlo (MCMC) replications. The most probable number of K was determined by plotting the ad hoc criteria Ln P(X|K) (Pritchard et al., 2000) and the summary likelihood statistics ΔK (Evanno et al., 2005). The best K corresponds normally to the highest value of mean Ln P(X|K) and ΔK (Pritchard et al., 2000; Evanno et al., 2005). Bar plot of assignment test obtained from STRUCTURE was generated via POPHELPER package in R (Francis, 2017).

d. Morphological measures in Madagascar species

In *Vanilla* species, the morphological differences are mainly based on floral traits (Soto-Arenas & Cribb, 2010; Soto Arenas & Dressler, 2010). So, floral characters of all Malagasy genetic entities identified from molecular analyses were compared. For that, the results of a previous work on eight populations representing six genetic groups in Madagascar were reexamined (Andriamihaja et al., 2021). In addition, a fieldwork was carried out in Manompana (North East of Madagascar, *V. sp2*) in December 2021 to complete the floral dataset. The 10 collected flowers were measured and scanned in accordance to the previous methods (Andriamihaja et al., 2021), so as to make the comparison possible. Consequently, 17 floral traits and nine color variables were assessed (Andriamihaja et al., 2021). The floral measurements were obtained

either directly *in situ* using a scale and a caliper, or after processing the images under IMAGE J software (Abràmoff et al., 2004). Color variables were extracted after transforming scanned floral images into CIE (L*a*b*) color space under R 3.5.1 software (R Core Team, 2019) according to the script of Kendal et al. (2013). The 26 morphological measurements obtained were added to those used in the previous study (Andriamihaja et al., 2021) to reperform a Principal Component Analysis (PCA) using FACTOMINER (Lê et al., 2008). Means and standard deviation of the ten most explanatory variables from PCA were calculated. Multivariate analysis of variance (MANOVA) was conducted using R software to test the statistical significance of floral distinctiveness based on the priori groupings of genetic structuring result. Differences between genetic groups for these floral characters were determined using a Bonferroni corrected pairwise t-test under R software. The stems of the different genetic groups were also observed in a general way in order to detect possible differences between them.

3.2.3. Results

a. Phylogenetic ITS reconstruction

The ITS region sequences compared were 762 bp long (including part of the *ITS1*, the *5.8s RNA* gene, and part of the *ITS2* regions). The topology of the trees obtained with the different phylogenetic analysis methods was generally similar. For this reason, only the result issued from Bayesian analyses with BEAST is presented in this paper (Figure 2). As also demonstrated in previous study on *Vanilla* genus using chloroplast genes (Bouetard et al., 2010), leafless *Vanilla* species from the SWIO region were monophyletic (Figure 2).

Contrary to what was obtained by Bouetard et al. (2010), our results showed that the leafless group is positioned closer to *V. crenulata* Rolfe and *V. africana* Lindl. than to *V. imperialis* Kraenzl. and *V. polylepis* Summerh. (Figure 2, Figure S1). Nevertheless, the phylogenetic relationships of these African as well as Malagasy (*V. francoisii* H. Perrier) leafy species with the leafless group were poorly resolved (Bayesian node support probability < 50%), which precludes any further insight on the precise origin of the leafless species group before conducting a more thorough sampling of African leafy species.

ITS sequences were however very powerful to resolve species within the leafless monophyletic group, the main focus of our study. Our results showed, with strong support (100%), a clear division of the leafless *Vanilla* group into two evolutionary lineages based on flower color rather than geographical origin: one clade (100%, 1.92 Mya) grouping all white flower species from Seychelles, East African coast and Madagascar, and one more recent clade (100%, 0.87 Mya) comprising yellow flower species from the Comoros archipelago and Madagascar (Figure 2).

Within the white flower leafless group, two main clades were revealed from BEAST analysis: one comprising species from West of Madagascar (*V. madagascariensis* Rolfe, *V. bosseri* L. Allorge, *V. decaryana* H. Perrier), Seychelles (*V. phalaenopsis* Rchb.f.) and the East African coast (*V. roscheri* Rchb.f.), the second with accessions from East of Madagascar (*V. sp1*, *V.*

sp2) (Figure 2). The first one was further divided into three well defined clades: *V. roscheri* (East African coast) (100%), *V. decaryana* (Madagascar) (100%) and a more ancient monophyletic group (100%) comprising accessions from species *V. madagascariensis*, *V. bosseri* and *V. phalaenopsis*. Based on BEAST results, *V. roscheri* and *V. decaryana* formed a monophyletic group, but with a very low Bayesian node support probability (50%), which did not hold in ML analysis (Figure S1). The second clade within the white flower species was an early diverging monophyletic group (Bayesian node support probability = 76%) comprising two leafless populations from East of Madagascar, with group from the East coast (*V. sp2*, Bayesian node support probability = 100%) being older (0.87 Mya) than the one from the South East in the Malagasy highlands' domain (*V. sp1*, Bayesian node support probability = 100%, 0.44 Mya) (Figure 2). In accordance with the relatively low Bayesian node support probability (76%) revealed in the Bayesian method, the ML method indicated that *V. sp1* and *V. sp2* did not form a monophyletic group (Figure S1).

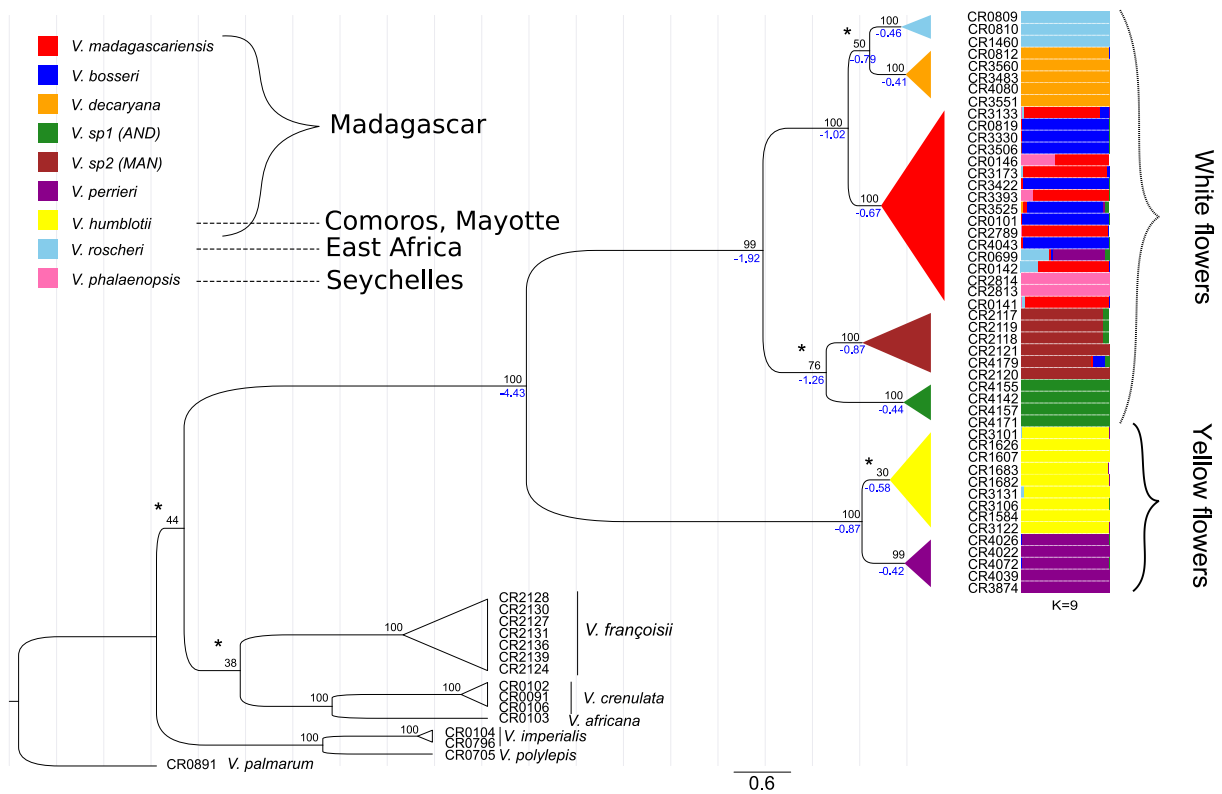


Figure 2. Bayesian phylogenetic tree (BEAST)

Based on ITS region and genetic clustering analysis (STRUCTURE) using seven microsatellite loci (K=9) of leafless *Vanilla* species from the SWIO region. Bayesian node support probabilities (black) as well as internal node time of divergence (blue) are displayed on the BEAST tree. Unreliable nodes (as confirmed by ML in Figure S1) with low Bayesian node support probabilities are indicated by stars. In the Structure plot, each individual is represented by a horizontal bar with color proportional to their membership to the nine genetic groups computed from the Bayesian analysis. Species names (and geographical location) corresponding to the nine clusters are indicated.

Within the yellow flower group, *V. perrieri* Schltr. from Madagascar formed a well-individualized group (100%) from the poorly defined (30%) *V. humblotii* Rchb.f. group (from

Madagascar and the Comoros archipelago) (Figure 2). The Maximum likelihood tree (Figure S1) clearly showed that *V. perrieri* differentiated recently from *V. humblotii*.

b. Microsatellite genetic assignement

On the basis of the Ln P(X|K) criteria (Pritchard et al., 2000), the most probable number of genetic clusters was K=9. But considering the result of Evanno's method, the best K was estimated at 4, with also a small peak at nine (Figure S2). Thus, we considered that the best K was nine combining the two methods. Among the K=9 clusters estimated by both Evanno and Pritchard methods, seven matched the previously defined Malagasy genetic groups in Andriamihaja et al. (2021): four corresponded to already described species endemics to Madagascar (*V. madagascariensis*, *V. bosseri*, *V. decaryana*, *V. perrieri*), two represented the two populations from the East of Madagascar (*V. sp1*, *V. sp2*) and one corresponded to a species supposedly endemics to Comoros archipelago but that was confirmed to be also present in Madagascar (*V. humblotii*). This was further confirmed here by showing that *V. humblotii* accessions from Comoros archipelago and Madagascar were grouped in the same genetic cluster. In addition, two new genetic clusters were revealed in the present study, corresponding to the newly studied species from the SWIO (*V. phalaenopsis* from Seychelles and *V. roscheri* from East Africa) (Figure 2). Moreover, microsatellite genetic assignment allowed to separate the three species (*V. madagascariensis*, *V. bosseri* and *V. phalaenopsis*) that were unresolved within a monophyletic group revealed by ITS phylogenetic analysis (Figure 2). Assignment probabilities were generally higher for almost all specimens, including unknown accessions, suggesting a clear clustering. Only two accessions, issued from botanical garden collections, did not match clearly to a species cluster, CR0146 and CR0699, and could be interspecific hybrid species. As for ITS analysis, the two Malagasy Eastern groups (*V. sp1* and *V. sp2*) were well-differentiated genetically based on microsatellite analyses.

c. Morphological identification of Malagasy species

Morphological analyses were performed to compare the Eastern *V. sp2* population with other previously studied Malagasy species (Andriamihaja et al., 2021). The two principal axes of the PCA, representing 70.7% of the total variation, indicated a clear separation of four major non-overlapping floral groups, three of which were already described previously (Andriamihaja et al., 2021): i) yellow flower group (*V. humblotii* and *V. perrieri*), ii) Western Malagasy species (*V. decaryana*, *V. bosseri*, *V. madagascariensis*), iii) population from the South East of Madagascar living in high altitude (*V. sp1*) and iv) a new group corresponding to the population from Manompana in the North East of Madagascar (*V. sp2*) (Figure 3).

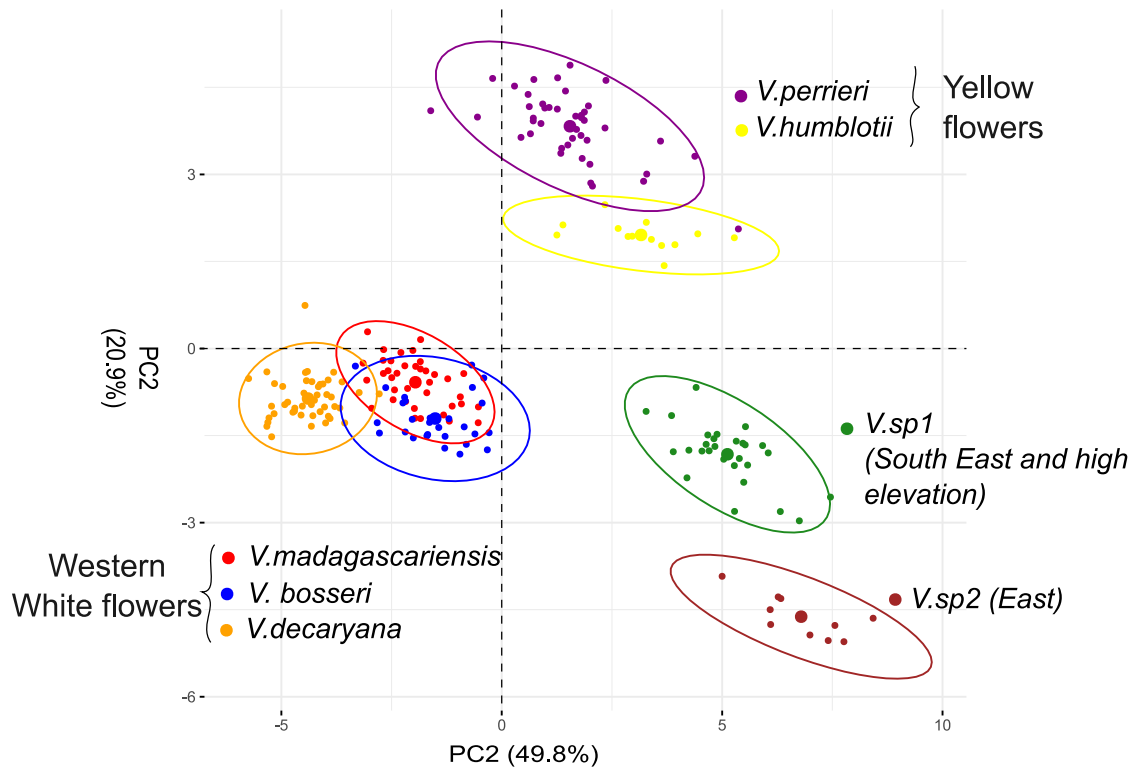


Figure 3. PCA using 26 floral traits of the seven Malagasy genetic groups

In general, the different genetic groups overlapped in the space of the first two principal components (PCs), with the exception of the two Eastern groups, which showed a clear discontinuity (Figure 3). The first PCA component explained 49.8 % of the total floral variations and was associated with the size of the flower. PC1 allowed to clearly separate the three Western white flower species (*V. decaryana*, *V. madagascariensis*, *V. bosseri*) from the four others (two yellow flowers species and two white flower genetic groups from the East). The color of the petal (a^*b^* of the inside face), the color of the sepal (b^* of the outside face) and the color of the labellum ($L^*a^*b^*$ of the inside face) contributed most strongly to the PC2, which explained 20.9% of the total floral variations. PC2 mainly differentiated white flower species from yellow flower species.

As already demonstrated by our previous study, six of the genetic groups showed specific morphological features (Andriamihaja et al., 2021) (Table 2). Indeed, MANOVA demonstrated a significant floral differentiation of grouping ($P < 0.001$). Reusing the morphological datasets of our previous study (Andriamihaja et al., 2021), we found the same discriminating features, but we were here able to determine that accessions from the 7th genetic group (*V. sp2*) formed a distinct morphological group from all other previously studied species: i) the amount of shine and yellow of the inside face of petals that allowed to distinguish the two yellow flower species, *V. humblotii* and *V. perrieri*; ii) the small size of flowers (petals < 5 cm long), characteristic of *V. decaryana*; iii) the absence of hair on the labellum allowing to discriminate *V. bosseri* from *V. madagascariensis* and iv) the large size of flowers (petals > 7 cm) enabling to discriminate *V. sp1* (AND), located in the South East, from the Western species with white flowers. The

newly studied Eastern genetic group (*V. sp2*, MAN) had the largest floral parts, the heaviest flowers with an average flower weight of 13.1g (Figure 4, Table 2) and floral parts with more rounded apex, a result specific to this study. The 10 most discriminating floral traits are presented in Table 2, with 9 characters already raised in Andriamihaja et al. (2021) and one new (Column length).

Table 2. Bonferroni corrected pairwise t-test of ten floral parameters (mean ± sd) among seven Structure genetic groups.

Means followed by the same letter in the same row are not significantly different (p-value > 0.05). Morphological data of *V. humblotii*, *V. madagascariensis* Rolfe, *V. perrieri* Schltr., *V. bosseri* L. Allorge, *V. decaryana* H. Perrier and *V. sp1* were taken from Andriamihaja et al. (2021).

Floral Traits	<i>V. humblotii</i>	<i>V. madagascariensis</i>	<i>V. perrieri</i>	<i>V. bosseri</i>	<i>V. decaryana</i>	<i>V. sp1</i>	<i>V. sp2</i>
LS	16.6±2.3 ^a	7.2±0.8 ^b	13.7±1.9 ^c	9.4±1.2 ^d	4.8±0.4 ^e	21.4±2.5 ^f	20.5±3.4 ^f
TFW	6.1±1.1 ^a	2.9±0.4 ^b	4.2±0.9 ^c	3.3±0.5 ^b	1.8±0.5 ^d	7.9±1.0 ^e	13.1±1.5 ^f
BIFP	41.4±5.9 ^a	-2.6±0.7 ^b	58.5±8.8 ^c	-2.2±0.8 ^b	-2.4±0.7 ^b	-1.8±0.6 ^b	0.5±0.03 ^b
AIFP	-8.1±1.1 ^a	1.8±0.4 ^b	-10.3±1.7 ^c	2.3±0.4 ^b	1.8±0.7 ^b	1.8±0.5 ^b	-1.5±0.1 ^d
OW	4.9±0.5 ^a	3.6±0.3 ^b	4.1±0.5 ^c	3.8±0.3 ^b	3.3±0.3 ^d	5.9±0.6 ^e	7±1.2 ^f
PL	7.5±0.5 ^a	5.8±0.5 ^b	6.5±0.6 ^c	5.9±0.6 ^b	4.2±0.3 ^d	8.8±0.5 ^e	7.5±0.4 ^a
BIL	40.5±1.9 ^a	2.5±0.9 ^b	39.8±6.6 ^a	2.2±1.3 ^b	-0.8±0.5 ^c	0.8±0.6 ^{b,c}	0.6±0.04 ^{b,c}
CL	2.5±0.2 ^a	1.8±0.1 ^b	2.4±0.2 ^a	2.2±0.2 ^c	1.6±0.2 ^d	3.3±0.1 ^e	3.1±0.1 ^f
ASL	7.1±0.6 ^a	5.9±0.6 ^b	6.6±0.7 ^a	5.9±0.5 ^b	4.2±0.3 ^c	8.5±0.5 ^d	7.2±0.4 ^a
OWE	1.2±0.4 ^a	0.6±0.1 ^b	0.8±0.2 ^c	0.6±0.1 ^b	0.5±0.1 ^b	1.7±0.3 ^d	2.8±0.8 ^e

LS: labellum surface (cm²), TFW: total floral weight (g), BIFP: b* of inside face of petal, AIFP: a* of inside face of petal, OW: ovary width (mm), PL: petal length (cm), BIL: b* of inside of labellum, CL: column length (cm), ASL: adaxial sepal length (cm), OWE: Ovary weight (g).

3.2.4. Discussion

a. Phylogenetic relationships and phylogeography of Malagasy leafless species and sister species from the South West Indian ocean region

Based on the Internal Transcribed Spacers (ITS) region, which is the most used sequence in plant classification and in orchid phylogeny (Tsai et al., 2005; Soto Arenas & Dressler, 2010; Sharma et al., 2012; Azofeifa-Bolaños et al., 2017), this study provides further support for the monophyly of the leafless *Vanilla* group from the SWIO region, as already found by Bouetard et al. (2010) using chloroplast DNA.

As opposed to the chloroplast DNA (Bouetard et al., 2010), the ITS region clearly resolved the phylogeny of this leafless *Vanilla* group with high Bayesian node support probabilities values and the separation of many known species into several monophyletic groups (Figure 2).

Although the precise relationships between African leafy species and the leafless *Vanilla* group from the SWIO region was poorly resolved in our study, the African related origin of the common ancestor of the group and its migration to Madagascar was clearly demonstrated by Bouetard et al. (2010). Similarly, several Malagasy plants originated from historical migrations from Africa (Forrest & Hollingsworth, 2003; Micheneau et al., 2008; Wikström et al., 2010; Anthony et al., 2010; Callmander et al., 2011; Strijk et al., 2012). Ali and Huber (2010) demonstrated that, rather than land bridges (such as the Davie Ridge in the Mozambique channel), strong winds and current flowing from the East coast of the African continent eastward towards Madagascar during the Paleogene period (about 66 to 23 Mya (Luterbacher et al., 2004)) may have facilitated colonization events from Africa to Madagascar. Our phylogenetic reconstruction based on different methods strongly supports an initial split of the leafless group into two main lineages: a clade of yellow flower species and a clade of white flower species. Both leafless groups have differentiated in Madagascar, from where they have then colonized nearby islands (Seychelles, Comoros archipelago) and backwards to the Eastern African coast by transoceanic migration, as will be discussed below. Long-distance colonization events have already occurred several times in the evolution of the genus *Vanilla* and specifically at the origin of leafless species (Bouetard et al., 2010).

The clade of species with white flowers was possibly formed earlier than the one with yellow flower species based on the relaxed molecular clock analysis. It was divided into two main groups showing a clear East/West Madagascar biogeographic disjunction, with the most ancestral lineage (estimated about 1.26 Mya) represented by the two Eastern Malagasy populations of which the Northeastern Manompana population (*V. sp2*) was the most ancient (0.87 Mya) (Figure 2). The second monophyletic group (100 %) within the clade of white flower species was divided into three clades: i) a strongly supported clade consisting of accessions from Madagascar and Seychelles (*V. madagascariensis*, *V. bosseri*, *V. phalaenopsis*), appeared about 0.67 Mya, ii) *V. roscheri* group from East African coast (100 %) and iii) the most recent clade (about 0.41 Mya) formed by *V. decaryana* from South of Madagascar (100 %). This structuration in the white flower clade suggests a Malagasy origin. The common ancestor of this clade differentiated in Madagascar, most probably first in the East, followed by migration and differentiation in the West by a non-adaptive mechanism as inferred in the “western forest refugia” and “Montane refugia” scenarios (Vences et al., 2009), as suggested in our previous study (Andriamihaja et al., 2021). However given the uncertainty of the divergence dates estimates (Table S2), it is also possible that a leafless ancestor could have been widespread in Eastern and Western Madagascar and then has adapted to humid versus dry conditions, giving rise to two phylogenetic lineages, under the “ecogeographic constrain” scenario (Vences et al., 2009). Cases of diversification along West/East environmental gradients have already been addressed several times in Madagascan animals (e.g. Glaw et al., 2009; Vences et al., 2009; Vences & Glaw, 2003; Yoder & Heckman, 2006; Younger et al., 2019). In Malagasy plants, analogous cases of differentiation between Western versus Eastern species have been also reported within *Canarium* L. genus (Federman et al., 2018) and several genus of the Sarcocaulaceae (Randrianasolo & Miller, 1999; Aubriot et al., 2016). Nevertheless, it is

noteworthy that the group of Western Malagasy species has then given rise, by transoceanic migration, to *V. roscheri* in the Southeastern coast of Africa and to *V. phalaenopsis* in Seychelles. Cameron (2011) suggested that *V. roscheri*, *V. phalaenopsis* and *V. madagascariensis* were probably morphological variations of the same species. The ITS phylogeny clearly showed that this is not the case for *V. roscheri*. On the other hand, *V. phalaenopsis* from Seychelles and *V. madagascariensis* (and *V. bosseri*) from Madagascar appeared as conspecific species on the ITS tree. They nevertheless represent recently diverged distinct species as demonstrated using microsatellite genetic structuring (Figure 2).

The leafless *Vanilla* group with yellow flowers has given rise to two known botanical species, *V. humblotii* and *V. perrieri* (Portères, 1954). Despite ancient records of *V. humblotii* as endemics to the Comoros archipelago (Portères, 1954), the present phylogenetic analysis confirmed its presence also in Madagascar, a fact that was already noticed by Cribb & Hermans (2009) and previously suggested on the basis of population genetics approaches (Andriamihaja et al., 2021). Indeed, we demonstrate here that *V. humblotii* accessions from Madagascar and the Comoros archipelago belong to the same species.

According to Soto-Arenas & Cribb (2010), *V. perrieri* may well be conspecific to the Malagasy leafy species (*V. françoisii*). But based on our results, *V. perrieri* has differentiated from *V. humblotii* present in northern Madagascar, most probably as a consequence of isolation by ecology (Andriamihaja et al., 2021). Indeed, if *V. humblotii* was only observed in the North of Madagascar, *V. perrieri* has extended along the West coast from the North to the South, towards much more dryer ecological niches (Allorge-Boiteau, 2005; Cribb & Hermans, 2009). In fact, the Western coast of Madagascar is made up of four ecoregions ranging from the deciduous forests of the North with an average annual rainfall between 1000 and 1500 mm to the spiny thickets in the arid zones of the extreme South with a maximum annual rainfall of 500 mm (Burgess et al., 2004; Andriamihaja et al., 2020). The existence of geographically separated populations of *V. humblotii* raises major questions on the origin and biogeographic history of this species in the SWIO that will require further genetic diversity studies in the different islands to be fully resolved. Nevertheless, as in the case of white flower species, the ITS tree topology suggests a Malagasy origin for yellow flower species, therefore implying that *V. humblotii* has colonized the Comoros archipelago from Madagascar, adding another transoceanic migration event to those already demonstrated for the white flower group from Madagascar to Seychelles and the Southeastern African coast. Based on paleogeographic reconstructions and paleoceanographic modeling, it was demonstrated that since the Miocene (about 23 to 6 Mya (Sluiter et al., 2016)), currents and winds have been, like in the present times, oriented from Madagascar to Africa (Ali & Huber, 2010), thus the Malagasy origin of *V. roscheri* (East Africa) ancestor is possible. Likewise, an ancestry of *V. humblotii* from Madagascar is therefore more plausible than from the Comoros. On the basis of molecular, geological and bathymetric analyses, Warren et al. (2010) showed that the low sea level in the past (about 0.5 Mya) allowed the enlargement of currently small islands such as Seychelles, Maldives and Chagos Archipelago. Also, additional islands appeared between the Seychelles

and the Mascarene Islands (Warren et al., 2010), probably facilitating the recent dispersal of the ancestor of *V. phalaenopsis* from Madagascar. Inter-islands migrations from Madagascar to the other islands of the SWIO region have also been reported many times in plants including orchids (Yuan et al., 2005; Micheneau et al., 2008; Wikström et al., 2010; Jaros et al., 2016; Kainulainen et al., 2017; Le Péchon et al., 2020). In *Vanilla*, these transoceanic dispersal events may have occurred through floating vegetation mats moved by a sea currents and winds (Renner, 2004; Zhang et al., 2007), or by frugivorous migratory animals (zoochory) of which birds and bats have been suspected to be the main dispersal vectors for *Vanilla* species in the SWIO region and elsewhere (Lubinsky, Van Dam, & Van Dam, 2006; Bory et al., 2008, 2010; Bouetard et al., 2010; Gigant et al., 2011), facilitated here by the short distance between the different territories (<2000 km).

b. Leafless *Vanilla*: how many species in Madagascar?

Morphological taxonomy was the first method used in plant classification (Mallet, 1995; De Queiroz, 2005, 2007; Householder et al., 2010; Aldhebiani, 2018; Rouhan & Gaudeul, 2021). From the description of the first species in the *Vanilla* genus (Rolfe, 1896) to the recent new discoveries (Pansarin, 2010; Pansarin & Miranda, 2016; Flanagan & Mosquera-Espinosa, 2016; Fraga et al., 2017; Parizaca, 2019), floral traits have been the basis of *Vanilla* taxonomy. *Vanilla* is one taxonomically complex plant group (Gigant et al., 2011; Andriamihaja et al., 2020), with a number of species increasing at a significant rate, which has led to frequent taxonomic revisions (Rolfe, 1896; Portères, 1954; Soto-Arenas & Cribb, 2010; Soto Arenas & Dressler, 2010; Karremans et al., 2020). Multivariate grouping of morphological traits allowed us to distinguish four non-overlapping groups of leafless *Vanilla* species from Madagascar, with flower size and color being the discriminating features (Figure 3, Table 2). Although the characteristics of the flowers are the most discriminating in *Vanilla* genus, visual comparisons we have made between stems of the different genetic groups as well as an ongoing study showed differences in the abundance and forms of warts on the stems (J.G. Andrianantenaina, pers com.). However, *Vanilla* species may show phenotypic plasticity and intraspecific variation (Bory et al., 2010; Soto-Arenas & Cribb, 2010; Soto Arenas & Dressler, 2010) and sometimes different species may have similar morphological traits (Portères, 1954; Soto-Arenas & Cribb, 2010) requiring specialist expertise to distinguish species. We therefore used a complementary approach within an integrative taxonomic framework combining PheSC, PhySC and GSC. The use of several sources of data has already proven its effectiveness in the definition of *Vanilla* species in Costa Rica and in the revision of the genus (Soto-Arenas & Cribb, 2010; Soto Arenas & Dressler, 2010; Azofeifa-Bolaños et al., 2017).

The combination of phylogeny using ITS, morphological analysis and ISSR clustering methods have already been used in the *Vanilla* systematic analysis and allowed to delimit *V. planifolia* and its relatives in the Mexican Yucantan peninsula (Villanueva-Viramontes et al., 2017). In our study, the genotypic method approach based on microsatellite data, combined with floral morphological data, identified all five species previously described botanically in Madagascar (*V. decaryana*, *V. bosseri*, *V. madagascariensis*, *V. humblotii*, *V. perrieri*) (Portères, 1954;

Allorge-Boiteau, 2005; Cribb & Hermans, 2009). In addition, two new genotypic clusters were detected in the East of Madagascar (Figures 2, 4, Table 2). The seven Malagasy genetic clusters identified matched those previously revealed by Andriamihaja et al. (2021), showing the power of a population assignment analysis despite the small intraspecific sampling used here. Although sometimes with overlap, each morphological group matched one genotypic cluster (Figure 3). The use of morphological and genotypic data was effective to discriminate plant species in several genera such as *Ancistrocladus* Wall. (Ancistrocladaceae) (Turini et al., 2014), *Phoenix* L. (Arecaceae) (Pintaud et al., 2010), *Carapa* Aubl. (Meliaceae) (Duminil et al., 2006) and *Epidendrum* L. (Orchidaceae) (Pessoa et al., 2012).

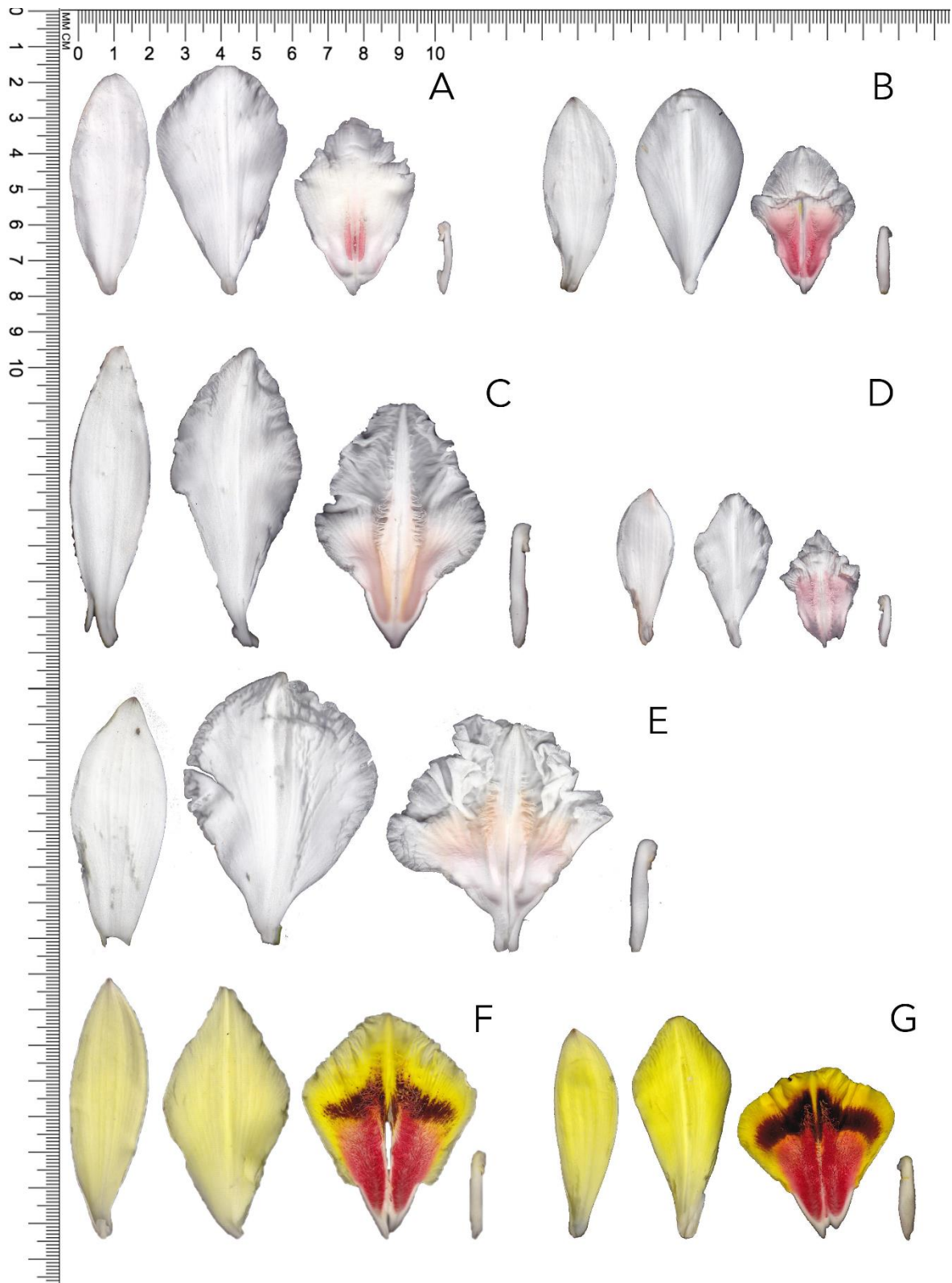


Figure 4. Scanned flower parts of different leafless *Vanilla* species

Scanned flower parts (sepal, petal, labellum, column) of the different leafless *Vanilla* species from Madagascar: A: *V. bosseri*, B: *V. madagascariensis*, C: *V. sp1*, D: *V. decaryana*, E: *V. sp2*, F: *V. humblotii*, G: *V. perrieri*.

When considering the PhySC approach using ITS, the results matched those from the PheSC and GSC for three leafless *Vanilla* species identified in Madagascar (*V. decaryana*, *V. humblotii*, *V. perrieri*) and for the two new species from East of Madagascar (*V. sp1*, *V. sp2*). However, although ITS analysis revealed that *V. madagascariensis* and *V. bosseri* were conspecific species (Figure 2), microsatellites revealed that these two species are clearly distinct and have recently diverged in Madagascar. They also show a clear morphological differential feature (no hair on the labellum) of *V. bosseri* (Figure 4).

In conclusion, this integrative taxonomy approach therefore identifies seven leafless species native to Madagascar (*V. decaryana*, *V. bosseri*, *V. madagascariensis*, *V. perrieri*, *V. humblotii*, *V. sp1*, *V. sp2*) that have distinct morphological and genotypic traits. All species form distinct monophyletic groups, apart from *V. bosseri* and *V. madagascariensis* that are conspecific and recently diverged from each other.

The present study is the first comprehensive contribution to understanding the relationships among some leafless species within the genus *Vanilla*. This study provides also a clear taxonomic placement of Malagasy *Vanilla* species using integrative taxonomy and multi-territorial samples. However, the low resolution of the relationships between leafless species from the SWIO region and their leafy sister species from Africa and Madagascar, as well as the lack of comparative flower samples from Seychelles, East Africa and Comoros archipelago are limitations and require further sampling and study. Nevertheless, our study demonstrates, according to various complementary evidences (morphological, phylogenetic, genotypic), a major result with the discovery of two new leafless *Vanilla* species endemic to Madagascar, and distributed in the East of the island, outside the generally admitted distribution range of leafless *Vanilla* species (Portères, 1954; Cribb & Hermans, 2009; Allorge-Boiteau, 2013) (Figure 1). These two species are further described and referred to as *V. allorgii* sp. nov. (*V. sp1*) and *V. atsinananensis* sp. nov. (*V. sp2*).

c. Taxonomic treatment

i. Description of new species

Vanilla allorgii C. F. Andriamihaja, sp. nov. (figures 4,5,6)

Type: Madagascar, province Fianarantsoa, district Ambalavao, Tsaranoro, Lon: 46°46'37.93" E, Lat: 22°05'4.77" S. Collectors: C.F. Andriamihaja, N.H. Nambinintsoa, A. Botomanga. Holotype (Holotype : ACA n°7) deposited at the Herbarium of Parc Botanique et Zoologique de Tsimbazaza (TAN!) , Antananarivo. Dec 2018.

Diagnosis—Similar to *Vanilla madagascariensis* Rolfe. (paratypes, ACA n°2, deposited at Herbarium du jardin botanique Tsimbazaza d'Antananarivo (TAN!)), but differs in the larger flowers (petals: $8.8 \pm 0.5 \times 3.3 \pm 0.6$ cm versus $5.8 \pm 0.5 \times 3 \pm 0.3$ cm in *V. madagascariensis*; sepals: $8.6 \pm 0.4 \times 2.2 \pm 0.3$ cm versus $5.8 \pm 0.5 \times 2.1 \pm 0.2$ cm in *V. madagascariensis*, lip surface: 21.4 ± 2.5 cm² vs 7.3 ± 0.8 cm²), a lip with orange peach throat (vs red throat in *V. madagascariensis*) and fleshy lumps on the upper surface with trichomes on the lateral margins (vs. abundant red trichomes from the base to the middle of the lobe).

Description—Hemi-epiphytic vine, more than 5 m long. Aerial roots brown, pubescent and ramified for nutrition. Roots whitish to brown, glabrous and non ramified for fixation. Stems, 8-10 mm diam, green, smooth when old and sometimes verrucose when young, cylindrical, fleshy, flexuous, with two longitudinal grooves. Internodes 3-7 cm long. Inflorescence racemose, 20-25 cm long, ca 15-35 flowered, sometimes terminal and sometimes located on axillary branches. Leaves green, ca 6-8 cm long, lanceolate, acuminate, rudimentary at the apex of young stems, aborted when stems age. Floral bracts ovate and apically acute. Flowers white, ephemeral with one flower opening per day and 2-days anthesis process (each floral piece opens successively); pedicel and ovary green, 50-80 × 4.8-7.5 mm, terete, smooth, curved; buds green at the base and dark green at the apex, apex acute. Sepals white in the adaxial surface, brownish green with white border in the abaxial surface, fused with petals, lanceolate, nerved, smooth border, inrolled, apex acute, 7-10 cm long. Petals fused with labellum, nerved, white, with a green and conspicuous longitudinally line on the abaxial surface, ovate, inrolled, 7-10 cm long, apex acute. Labellum trilobed, attached to the dorsal side of column, veined, with orange peach throat from the base to the middle of the labellum and white in the middle of the labellum to the apex, margin undulate, 6-8 × 4-6 cm; midlobe with two canals in front of the column, two row of glabrous callus at base, callus tufted in the central part. Column subterete, 30-37 × 4-6 mm, white, flattened at the ventral part; stigma transversely broadly oblong. Rostelum quadrangular, 2-4 × 3-6 mm. Anther basifixed and oblong.

Distribution—This species is known from Tsaranoro valley forest (Lon: 46°46'15.45"E, Lat: 22° 5'10.97"S) fragment near Andringitra National Park in the South East and central highlands' domain of Madagascar, district of Ambalavao. According to flower pictures on Tropicos (Charles Rakotovo, n°7236!), it is also present in forests in district of Ivohibe, region of Ihorombe (South East).

Ecology and Habitat—Tsaranoro valley is accessed via the National Road 7 (RN7) south of Ambalavao by taking the road to Vohitsaoka and then to the village of Morarano. The place is located at an altitude of 850-945 m in the Western valleys of Andringitra mountains. This natural barrier retains the humidity of blowing winds from the Eastern coast, therefore Tsaranoro has a warmer and dryer climate. The average annual temperature is 19.4°C and the annual rainfall is 1071 mm (<https://fr.climate-data.org/>). Population of *V. allorgii* was discovered in a sacred grove, a burial place for the local communities. Vegetation is a remnant of highland humid forest surrounded by wooded grassland mosaic (Moat & Smith, 2007). Forest cover ranged from 20 to 60%. The most common species are *Dombeya* Lam. species (Sterculiaceae), *Euphorbia tetrapera* Baker (Euphorbiaceae) and *Tragia tiverneana* Leandri (Euphorbiaceae). The forest canopy is at 8 m height. *V. allorgii* is a root-climber, found clinging either to trees or to rocks for light interception.

Phenology— Initiation of flowering has been observed in the month of October 2018, corresponding to the beginning of rainy season in Madagascar. Flowering has continued until December 2018.

Etymology—This species is dedicated to Dr Lucile Allorge, a botanist who has been working for a long time on the plants of Madagascar, especially on Malagasy endemic *Vanilla*.

Proposed conservation status—The Tsaranoro Valley Forest is remnant of sacred forests of less than 60 ha, managed by a local organization. Very few fruits were observed during our fieldwork at Tsaranoro indicating a loss of interaction with pollinators. Based on a previous genetic analysis (Andriamihaja et al., 2021), *Vanilla* population in Tsaranoro has a high clonality rate, thus a very low genetic diversity that may reduce the adaptive capacity of the population to climate change and threaten the species in the long terme. Given these threats explained above and the restricted ditribution of the species, although the forests are protected, it seems appropriate to include *V. allorgii* as endangered [EN]. However, additional studies of others populations are needed to validate this conservation status. Then a formal assessment should be carried out according to the IUCN Red list criteria to confirm the status.

Additional specimens examined—MADAGASCAR. 1—District Ivohibe. Commune rurale Ivohibe. Fokontany Longoza. Forêt de moyenne altitude à gros arbres élevés et savane herbeuse de Vohibory. Rivière Efanôla. Lon: 22°35'15"S and Lat: 46°41'03"E, 733 m, 07 December 2016, Charles Rakotovao, 7236 [flower photographs on Tropicos !] (MO, P, TAN). 2— District Ivohibe à Ihosy. Grande liane crassulescente, 8-10 m recouvrant des Euphorbes coralliformes. October 1992, L. Allorge, P01801828 (MNHN !). 3—Bassin du Mananara, vallée de la Menarahaka, restes de forêt xérophile près d'Analavoka. Alt 700-800m, October 1924, H. Humbert, P00102694, (MNHN !).

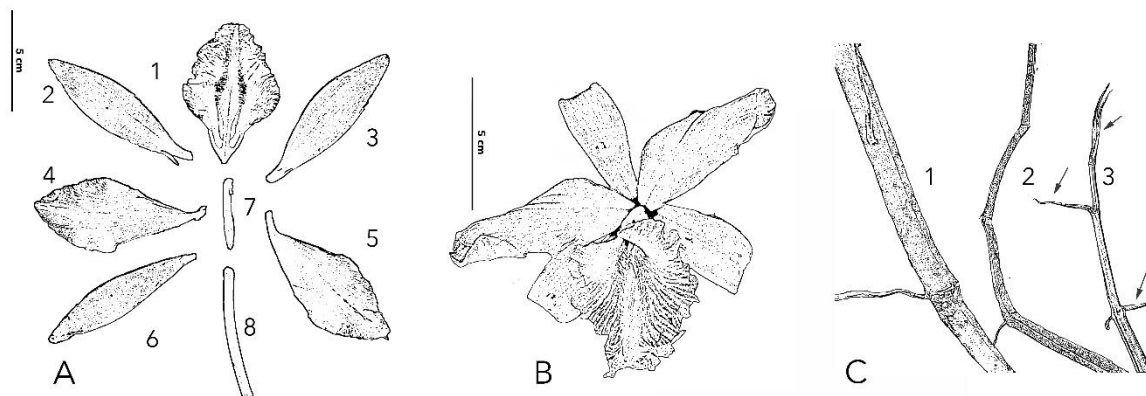


Figure 5. *Vanilla allorgii* C.F. Andriamihaja

A— Dissected perianth: labellum (1), left lateral sepal (2) right lateral sepal (3), left petal (4), right petal (5), adaxial sepal (6), column (7), pedicel and ovary (8). B— Flowers. C—Portion of stem (1), rudimentary leaves (2,3) and root. (Image by P. Besse).

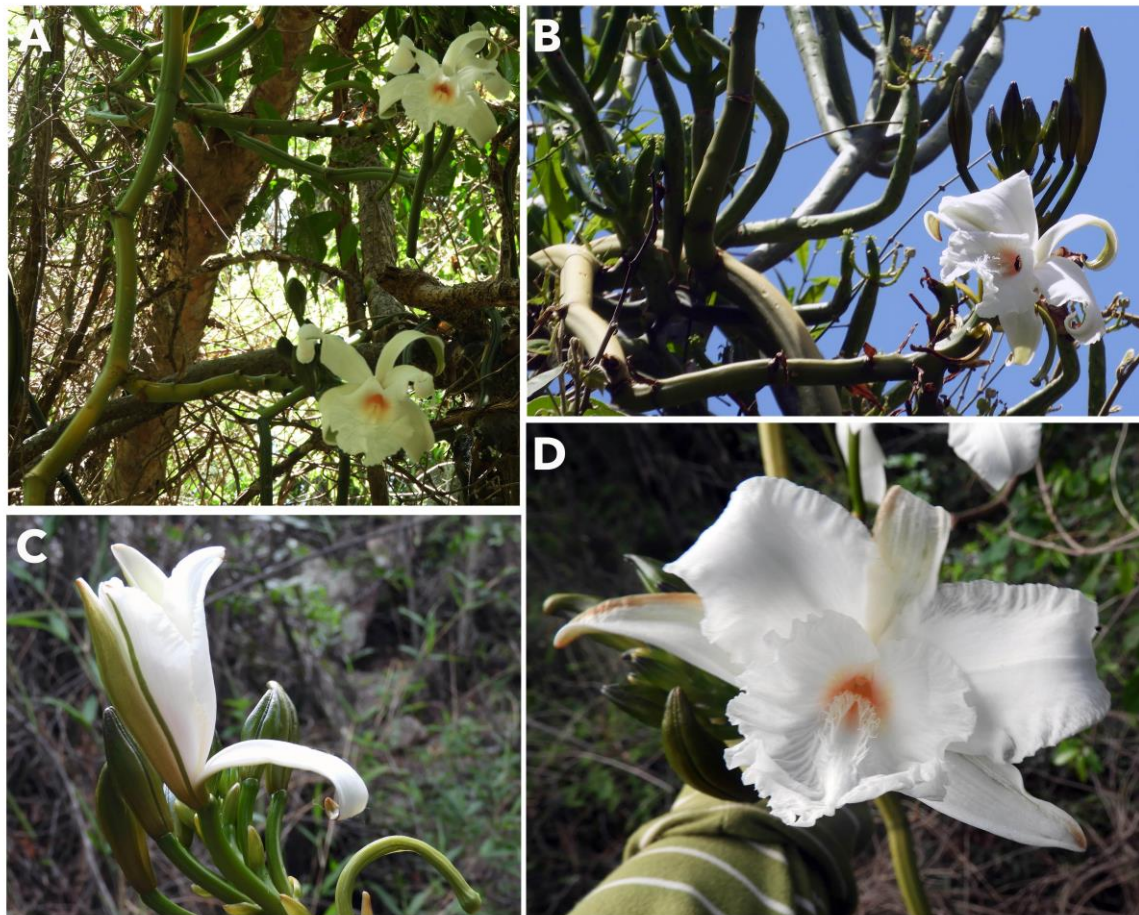


Figure 6. *Vanilla allorgii* C.F. Andriamihaja

A, B—vegetative habit of plant. C—inflorescence with opening flower showing abaxial surface of sepals and petals. D—fully opened flower with front view of labellum. Photographs: A and B by N. H. Nambinintsoa, C and D by C.F. Andriamihaja.

Vanilla atsinananensis C. F. Andriamihaja, sp. nov. (figures 4,7,8)

Type: Madagascar, province Toamasina, region Analanjirofo, district Soanierana Ivongo, commune Manompana, Ambodiriana protected forest, Lon: 49°41'46.7" E, Lat: 16°40'33.0" S. Collectors: M. Grisoni, N.H Nambinintsoa, A. Botomanga. Holotype (Holo, n° ACA 8) deposited at Herbarium of Parc Botanique et Zoologique de Tsimbazaza (TAN!), Antananarivo. Jan 2021.

Diagnosis— Similar to *Vanilla allorgii* C.F. Andriamihaja., but petals are obovate (vs ovate in *V. allorgii*) and larger (5 ± 0.6 cm versus 3.3 ± 0.6 cm in *V. allorgii*), lip is flabellate with orange peach throat only in the middle of the labellum (vs from the base of the labellum to the middle part in *V. allorgii*), flowers are weighty, stems are verrucose in old parts as well as in young parts.

Description—Hemi-epiphytic vine climbing along tree trunks reaching more than 7m in length. Terrestrial roots brown, verrucose stuck to tree trunks up to 10 m long and exploring soil for nutrition. Aerial roots whitish to brown, glabrous and non ramified for fixation. Stems, 5-15 mm diam, green, verrucose, cylindric, fleshy, flexuous, with two longitudinal grooves.

Internodes 5-18 cm long. Inflorescence racemose, ca 10-30 flowered, sometimes terminal and sometimes located on axillary branches. Floral bracts ovate and apically acute. Leaves green, ca 2-4 cm long, lanceolate, acuminate, rudimentary at the apex of young stems, aborted when stems age. Flowers white, ephemeral; pedicel and ovary green, 67-81 × 6-8 mm, terete, smooth; buds green, apex obtuse. Sepals white in the adaxial surface, green with white border in the abaxial surface, fused with petals, ovate, nerved, smooth border, apex acute, 5-8 cm long. Petals fused with labellum, nerved, white, with a green and conspicuous longitudinal line on the abaxial surface, obovate, 6-8 cm long, apex obtuse. Labellum trilobed, attached to the dorsal side of column, veined, with orange peach throat in the middle of the labellum and white in the other parts of the labellum, margin very undulate, 5-7 × 4-7 cm; midlobe with two canals in front of the column, two row of glabrous callus at base, callus tufted in the central part. Column subterete, 29-32 × 5-6 mm, white, flattened at the ventral part; stigma transversely broadly oblong. Rostelum quadrangular, 4-6 × 3-4 mm. Anther basifixed and oblong.

Phenology— Flowers have been collected in December.

Distribution—The species has been firstly reported in Ambodiriana reserve in the district of Soanierana Ivongo (North East of Madagascar) managed by ONG ADAFAM delegated site manager. One population was also observed in the South of Toamasina (Lon: 48°46'27.07" E, Lat: 18°26'47.50" S), Madagascar. The species was also seen in 2017 in the forest flap of Ambatoharagnana, commune of Sahambala, district of Toamasina II according to the photos of Benjamina Ralajjaona, Giovanni A. Rakotonirina, Anthony R. Syde, Harisandy M. Rasoanindriana & P. Antilahimena on Tropicos (photo n°51!).

Ecology and Habitat— The Ambodiriana forest (240 ha) is located two hours hike from the town of Manompana, North of Toamasina (Eastern Madagascar). The Ambodiriana forest is located in the East coast at an altitude of 100-250 m. It is exposed to the Alizé, with 2937 mm of annual rainfall and 23.1°C of average annual temperature (<https://fr.climate-data.org>). Vegetation is a lowland humid forest (Moat & Smith, 2007) with trees reaching more than 30 meters in height such as *Canarium madagascariensis* Engl. (Burseraceae), *Anthostema madagascariensis* Baill. (Euphorbiaceae) and *Uapaca ferruginea* Baill. (Euphorbiaceae). Forest cover ranged from 40 to 80%. Ambodiriana is home to about hundred orchid species (<https://www.adafam.org/>) including *V. atsinananensis* which grows as hemiepiphyte on supporting trees.

Etymology— The specific epithet (*atsinananensis*) comes from the word “atsinanana” that means “East” in the Malagasy language. It makes reference to the East coast distribution of the species.

Proposed conservation status— Given the low number of individuals in the Ambodiriana forest, *V. atsinananensis* might be considered as endangered (EN) or vulnerable (VU). A formal assessment according to IUCN red list criteria (IUCN, 2001) should however be done to confirm this status.

Additional specimens examined—MADAGASCAR. 1—District Toamasina II, Commune Rurale Sahambala, Fokontany Sahavongo, village le plus proche Sahavongo, lambeau de forêt

d'Ambatoharagnana, Ambinanin'ny Bikôka. Lon: 18°01'28"S and Lat: 49°05'25"E, 515 m, 01 February 2017, Benjamina Ralajjaona, Giovanni A. Rakotonirina, Anthony R. Syde, Harisandy M. Rasoanindriana & P. Antilahimena, 51 [flower photographs on Tropicos !] (MO, TAN). 2—Route de Fort-Carnot (5km de la bifurcation), Janvier 1964, J. Bosser. 19049, P00334809 (MNHN!).

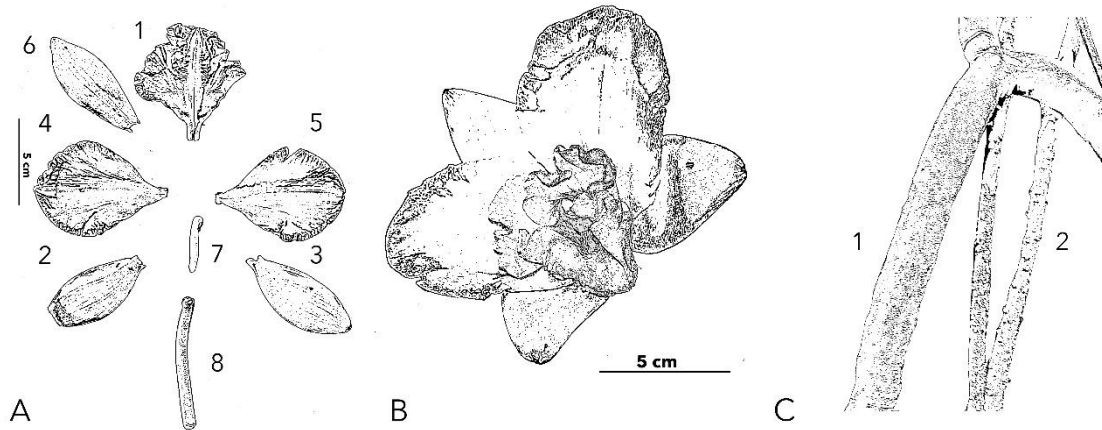


Figure 7. *Vanilla atsinananensis* C.F. Andriamihaja. A—Dissected perianth: labellum (1), left lateral sepal (2) right lateral sepal (3), left petal (4), right petal (5), adaxial sepal (6), column (7), pedicel and ovary (8). B—Flowers. C—Parts of stem (1) and root (2). (Image by P. Besse).

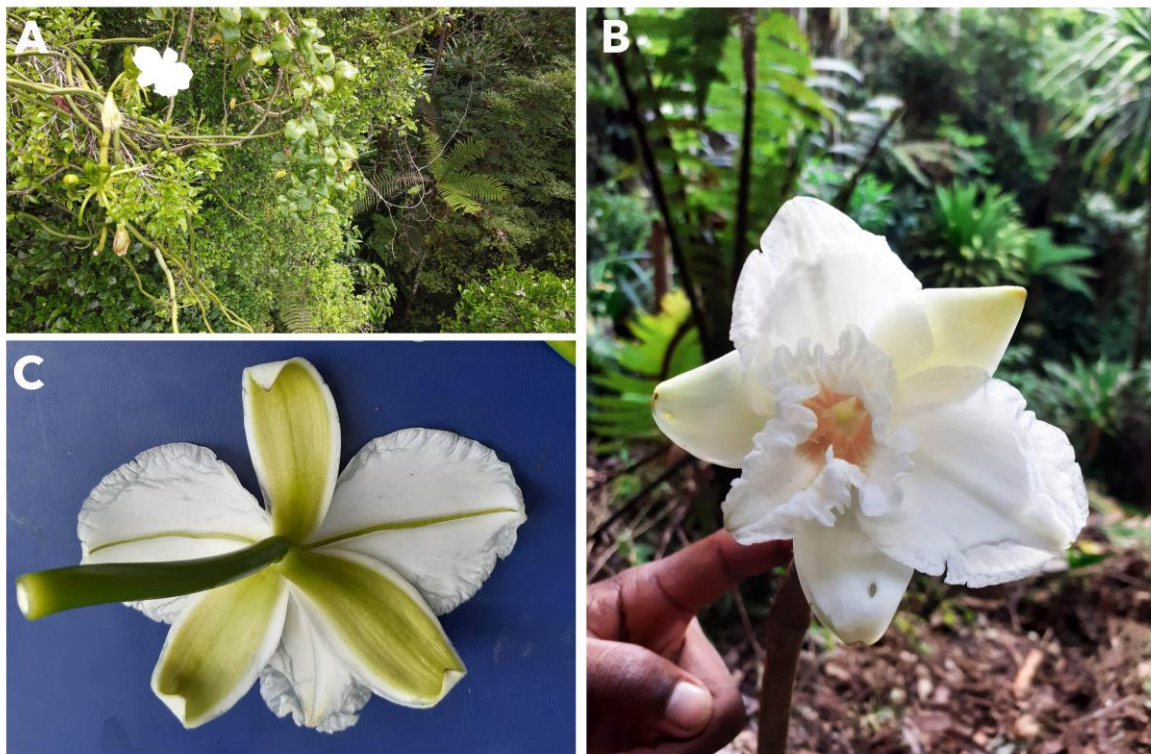


Figure 8. *Vanilla atsinananensis* C.F. Andriamihaja. (A) vegetative habit of plant, (B) back view of flower showing abaxial surface of sepals and petals, (D) fully opened flower with front view of labellum. Photographs: M. Grisoni.

ii. Identification key to leafless Vanilla species from Madagascar

In addition to including *V. bosseri* described by Allorge and the two new species discovered from our analyzes (*V. allorgii* and *V. atsinananensis*), we also provide a new revised key to the leafless *Vanilla* species from Madagascar based on Soto-Arenas & Cribb (2010).

Key to leafless Vanilla species from Madagascar

- 1. White flowers.....2
- 1. Yellow flowers..... 6
- 2. Petals 70-98 mm long. Flowers 7-15 g. Lip undulate with peach orange throat: species from East of Madagascar..... 3
- 2. Petals 30-70 mm long. Flowers 1-4 g. Lip with pinkish, reddish or yellowish throat: species from West of Madagascar.....4
- 3. Buds green at the base and dark green at the apex. Sepals brownish green in the abaxial surface, lanceolate, inrolled and measuring 7-10 cm long. Petals ovate, inrolled with apex acute. Labellum with orange peach throat from the base to the middle of the labellum and white in the middle of the labellum to the apex *V. allorgii*.
- 3. Buds green with apex obtuse. Sepals green in the abaxial surface, ovate, and measuring 5 – 8 cm long. Petals obovate with apex obtuse. Labellum with orange peach throat in the middle of the labellum and white in the other parts of the labellum. *V. atsinananensis*.
- 4. Column of 9-19 mm long. Sepals 25-49 mm long, lip with pinkish color *V. decaryana*.
- 4. Column 14-30 mm long. Sepals 48-73 mm long.....5
- 5. Lip obovate, obtuse, the margins not undulate, with two lines of long papillae or fleshy hair and pink-reddish throat..... *V. madagascariensis*.
- 5. Lip with yellow-pink throat, without fleshy hair and with red protrusion between two crests..... *V. bosseri*.
- 6. Flowers 5-8 g. Petals and sepals with clear yellow, 65-85 mm long. Lip papillose. Column 20-28 mm long..... *V. humblotii*.
- 6. Flowers 2-7 g. Petals and sepals with intense yellow color. Petals 51-79 mm long. Lip not papillose. Column 18-30 mm..... *V. perrieri*.

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CHAPITRE 4

CHAPITRE 4

Diversité génétique et conservation des vanilliers aphyllés à Madagascar

« Si le biote, au cours des âges, a construit quelque chose que nous aimons mais ne comprenons pas, alors qui, à part un fou, se débarrasserait des pièces apparemment inutiles ? Conserver chaque roue et chaque rouage est la première précaution d'un bricolage intelligent. »
Aldo Leopold

4.1. Présentation et résumé du chapitre

Selon la dernière évaluation des orchidées, environ 4342 espèces sont potentiellement menacées (en danger critique d'extinction [CR], en danger [EN], ou vulnérable [VU]). Non seulement réputée pour sa biodiversité exceptionnelle et sa position de premier producteur mondial de *Vanilla planifolia*, Madagascar est également la région qui compte le plus d'espèces d'orchidées potentiellement menacées. Ce point chaud de la biodiversité abrite un grand nombre d'espèces aphyllés du genre *Vanilla*, dont deux nouvelles espèces identifiées et décrites dans le Chapitre 3. Ces espèces représentent des ressources précieuses à la fois sur le plan scientifique, culturel, et économique (Chapitre 1), grâce à leur capacité d'adaptation à la sécheresse, à leur résistance aux maladies causées par les champignons et à leur utilisation dans la médecine traditionnelle. Malheureusement, elles sont menacées par la destruction massive des habitats naturels à Madagascar. Dans cette étude, nous avons fourni des informations sur la variabilité génétique, la biologie de la reproduction et la distribution géographique des sept espèces aphyllés malgaches pour contribuer à la planification de leur conservation. D'après les résultats obtenus, les deux nouvelles espèces décrites sont fortement menacées, soit par la petite taille de la population pour le cas de *V. atsinananensis*, soit par la perte d'interaction avec les pollinisateurs pour le cas de *V. allorgii*. Malgré son importante fructification naturelle, *V. decaryana* montre une faible variabilité génétique et une faible taille effective des populations, ayant entraîné un taux de consanguinité élevé pour cette espèce. *V. humblotii* présente, d'après nos résultats, une grande variabilité génétique, comparé aux trois espèces précédentes. Cependant, la petite taille effective de la population de *V. humblotii* peut entraver sa survie à court et à long terme. En accord avec leur large répartition, *V. perrieri* et *V. bosseri* ont la plus grande diversité génétique et des tailles effectives de populations atteignant le minimum requis pour le maintien de la diversité à court terme. Bien qu'ayant une distribution potentielle

limitée, *V. madagascariensis* possède une diversité génétique importante et une taille effective de population suffisante pour le maintien à court terme de son potentiel évolutif et de sa valeur adaptative. Cependant, la perte probable des habitats appropriés de *V. madagascariensis* dans le Nord pourrait rendre ses populations vulnérables. La modélisation de la niche écologique réalisée a démontré que les espèces de vanilliers aphyllés malgaches ont été, en général, capables de surmonter les oscillations climatiques passées, les contraintes anthropiques actuelles et pourraient supporter le réchauffement climatique futur. Ce chapitre a permis de mettre à jour les informations sur ces sept espèces, fournissant la base pour développer de nouvelles prospections de terrain et recherches qui seront essentielles pour leur conservation et leur valorisation durable.

4.2. Present and future status of native leafless *Vanilla* species in Madagascar

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Abstract: According to the last assessment on orchids, about 4342 species are possibly threatened (critically endangered [CR], endangered [EN], or vulnerable [VU]). Not only renowned for its exceptional biodiversity and its position as the leading producer of the first commercial orchid (*Vanilla planifolia*), Madagascar is also the region with the most potentially endangered orchid species. This biodiversity hotspot has the highest number of leafless *Vanilla* species including two recently discovered species. They represent valuable agricultural resources as they offer interesting traits such as adaptation to drought, resistance to *Fusarium* and medicinal properties. Unfortunately, they are threatened by the massive destruction of natural habitats in Madagascar. In this study, we provided information on the genetic variability, reproductive biology and geographic distribution of the seven Malagasy leafless *Vanilla* species for planification of effective conservation. Our results supported that the two novel species are highly endangered, either by small population size (*V. atsinananensis*) or by loss of interaction with pollinators (*V. allorgii*). Despite the high natural fruit set, *V. decaryana* showed a low genetic variability and low effective population size, accelerating inbreeding. *V. humblotii* displayed a greater genetic variability, but its small effective population size may hamper its short- and long-term survival. In line with their wide distribution, *V. perrieri* and *V. bosseri* have the highest genetic diversity and effective population sizes reaching the minimum required for maintaining diversity in the short term. Although limited to the North, *V. madagascariensis* has considerable genetic diversity and sufficient effective population size for short term maintenance of its evolutionary potential and fitness, but could be vulnerable due to the loss of suitable habitat. The ecological niche modelling performed demonstrated

that Malagasy leafless *Vanilla* species have been, in general, able to overcome past climatic oscillations, current anthropogenic constraints and could sustain climate warming. This study allowed to update information on these seven species, a basis for further surveys and studies that will be essential for their conservation.

Keywords: Conservation, orchid, genetic diversity, ecological niche modelling, microsatellite, reproductive biology

4.2.1. Introduction

Biodiversity conservation represents one of the 17 key challenges listed in the 2030 Agenda for Sustainable Development (United Nations, 2015). There is now unequivocal evidence that biodiversity, the sum of genetic, phenetic, species, communities and ecosystem variations (Dirzo and Raven, 2003; Rands et al., 2010; Swingland, 2001), is the guarantee of food security, long-term environmental stability and contributes to public health (Arnold et al., 2021; Kilpatrick et al., 2017; Loreau and de, 2013). Unfortunately, the number of wild fauna and plants has been rapidly declining in the last years due to anthropogenic processes including habitat destruction, over-exploitation, species introductions, pollution and climate change (Dirzo and Raven, 2003; Ewers and Didham, 2005; Gurevitch and Padilla, 2004; Kuussaari et al., 2009; Thomas et al., 2004). In addition, Shaffer (1981) identified also four stochastic events that may lead to population extinction: demographic, environmental and genetic stochasticity, and natural disasters. Managing biodiversity requires the availability of sufficient and accurate information to make effective intervention decisions. According to the International Union for Conservation of Nature and Natural Resources (IUCN), biodiversity conservation needs to be addressed at the three different levels: genetic, species and ecosystem (IUCN, 1980; McNeely et al., 1990; WRI et al., 1992). In the 1970s, Frankel introduced the concept of conservation genetics (Frankel, 1974, 1970), which is an approach using the different genetic tools and methods to preserve species (Frankham et al., 2010). Although often overshadowed by other scientific disciplines in conservation strategies, genetics plays a significant role in conservation actions as it helps to clarify species taxonomy, identify population structure, define ESUs (Evolutionary Significant Units), detect inbreeding and loss of diversity, calculate effective population size and estimate gene flow (Fay, 2018; Frankham, 2010; Schwartz et al., 2007). Species viability depends on the population size and on genetic variation required for populations to adapt to environmental change (Frankham et al., 2010; Hedrick and Miller, 1992). To predict the rate of inbreeding and loss of genetic diversity, a key genetic parameter called “Effective population size (N_e)” was defined (Frankham, 1995; Frankham et al., 2014; Franklin, 1980; Wright, 1931). N_e refers to the size of an idealized population with the same variance of gene frequency, rate of inbreeding and rate of loss of genetic diversity as the actual population under study (Frankham, 1995; Husemann et al., 2016). Closely linked to genetics, the reproductive biology of species may also provide important information for designing conservation programs (Carrió et al., 2009; Weller, 1994). For example, the dependence level of plant species on pollinators to produce seeds determines their reproductive success and may reduce genetic diversity, consequently threatening small population survival in the case of

pollinator scarcity (Rymer et al., 2005; Weller, 1994). Nevertheless, self-pollinating species or pollination-dependent species from small populations, despite having high natural fruit set, may also be affected by inbreeding depression (Frankham et al., 2010; Weller, 1994). Knowledge about the distribution patterns of species over time and space can also greatly improve the understanding and monitoring of biodiversity (Liu et al., 2013; Morales et al., 2015; Wang et al., 2015). Species distribution modelling allows to predict the potential habitat outside the known range of occurrence, to identify priority conservation areas, to understand the biogeographical history of the species and to model the potential impacts of climate change in the future (Liu et al., 2013; Scheldeman and Zonneveld, 2010).

Because of their small size, limiting the population distribution areas, islands are the first victims of massive extinction of biodiversity (Dirzo and Raven, 2003; Purvis et al., 2000; Simberloff, 2000). Madagascar is listed among the top ten “hyper-threatened” biodiversity hotspots on the basis of exceptional species richness, concentration of endemic species and habitat loss (Brooks et al., 2002; Ganzhorn et al., 2001). Almost half of the known plants in Madagascar are threatened or extinct (Brooks et al., 2002) and more than 3,000 endemic species would be vulnerable to climate change (Bellard et al., 2014). From these, most are distributed in the Eastern littoral forests, the Western dry deciduous forests and the woody formations in the Central highlands, habitats identified as the most fragmented and degraded (Brown et al., 2015; Ganzhorn et al., 2001). These three endangered ecosystems are the main refuges of orchid species in Madagascar (Cribb and Hermans, 2009, 2007). With more than 800 orchid species (90 % endemic) (Cribb and Hermans, 2009, 2007) of which 646 threatened species, Madagascar is part of the priority areas for orchid conservation (Zizka et al., 2020). Malagasy orchids, like sister species in the other regions, are subject to ecological constraints (soil, climate, mycorrhizal fungi, pollinators, host trees..) to develop in a given environment (Fay, 2018; McCormick and Jacquemyn, 2014; Yokoya et al., 2015), making their conservation a complex challenge, especially in the context of global environment change (Fay and Chase, 2009; Gale et al., 2018). Due to the difficulty of taxonomic identification and illegal trade problems, all orchids are listed in the appendices of the Convention on International Trade in Endangered Species (CITES) (Hinsley et al., 2017). Among the 57 orchids genus on this continental island, *Vanilla* is the most emblematic with one economically important introduced species (*V. planifolia*), two leafy endemic species and seven leafless endemic species (Cribb and Hermans, 2009; Portères, 1954) including two recently described (Andriamihaja et al., in prep). Adapted to drought, resistant to diseases and used in traditional medicine in Madagascar (Rakotoarivelo et al., 2019; Randriamiharisoa et al., 2015; Randrianarivony et al., 2017; Raveloarison et al., 2017), the leafless *Vanilla* species present interesting agronomic and pharmacological traits for future sustainable use. Like all plants, *Vanilla* species are highly threatened by rapid deforestation due to land use change and fire in Madagascar, which resulted in a forest cover loss of about 44% over a 60-years period (Vieilledent et al., 2018). In addition, the two new species were only so far observed in two isolated populations of the East coast and Central highland of Madagascar (Andriamihaja et al., 2021, Andriamihaja et al., in prep). Therefore, there is an urgent need to identify the most endangered species and define the necessary

conservation actions. The aim of this study was to determine the status of leafless *Vanilla* species using a multidisciplinary approach: genetic monitoring based on microsatellite data and reproductive biology assessment, to evaluate the genetic and biological status of the species; and ecological niche modelling over time (past, present, future) to verify if climate variation influences species distributions. Such information will provide precious guidelines for the conservation of these *Vanilla* species in Madagascar.

4.2.2. Materials and Methods

a. Study area and sampling

The study area extends along the entire range of leafless *Vanilla* species in Madagascar defined in the literature (Allorge-Boiteau, 2013, 2005; Andriamihaja et al., 2021, 2020; Cribb and Hermans, 2009; Portères, 1954) and indicated in online databases (Tropicos, iNaturalist, MNHN) (Figure 1).

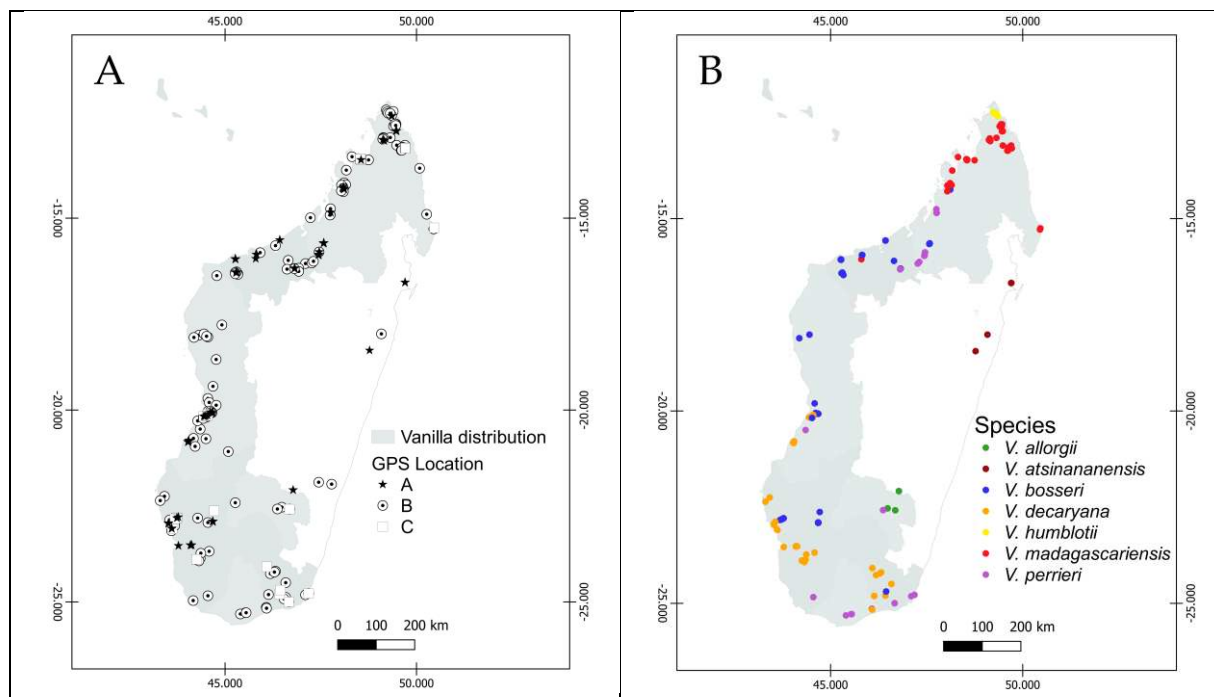


Figure 1. Sampling map

A—Leafless *Vanilla* species area distribution (in grey) according to Allorge-Boiteau (2005, 2013); Cribb & Hermans (2009) and Portères (1954) and individuals location points from different sources of data. A: GPS coordinates from Andriamihaja et al. (2021) and Andriamihaja et al. (in prep), B: GPS locations from online databases (Tropicos, iNaturalist and MNHN), C: GPS locations from Collection of University of Antananarivo. B— Distribution of the different species: only specimens verified on the basis of the results of the genetic clustering analysis and morphological evaluation of photos and herbaria in online databases (Tropicos, iNaturalist, MNHN) are indicated and were included in the species distribution modelling analysis.

Seven leafless *Vanilla* species are observed in different biogeographical regions, six are endemic to Madagascar and one to Madagascar and the Comoros archipelago (Andriamihaja et al., 2020, 2021, in prep.). The drier and warmer forests in the West coast (dry deciduous and succulent woodland forests) and the arid lands in the South (spiny thickets) constitute the main zones where five among the seven species (*V. bosseri*, *V. decaryana*, *V. madagascariensis*,

V. humblotii, *V. perrieri*) were observed until now due to their adaptation to drought. Cribb & Hermans (2009) also indicated an additional wetter range area of *V. madagascariensis* in the Northeastern coast (lowland forests). Although rare, a few individuals have been recently observed and newly described in the East (*V. atsinananensis*) and in a cooler and elevated zone near the Andringitra Mountain (subhumid forest) (*V. allorgii*) (Andriamihaja et al., 2021, Andriamihaja et al., in prep). Our study used genetic data already available in the database of the Biological Resources Center (BRC) Vatel in Reunion island and used in previous studies (Andriamihaja et al., 2021, Andriamihaja et al., in prep), GPS coordinates from previous studies (Andriamihaja et al., 2021, Andriamihaja et al., in prep), and GPS locations from online databases. In addition, we also collected and dried on silica gel until DNA extractions new stem samples from the *Vanilla* collection at the University of Antananarivo. These new specimens collected were vouchered and deposited in the collections of the BRC Vatel in Reunion island.

b. Genetic analysis

Of the 712 samples used, 614 were classified as species based on our previous studies (Andriamihaja et al., 2021, Andriamihaja et al., in prep) and 98 corresponded to new samples from the *Vanilla* collection of the University of Antananarivo. In order to identify the species the new specimens belong to, a genetic clustering analysis was performed. For that, DNAs of the 98 samples were extracted using DNEASY plant kit from Qiagen, then amplified with seven microsatellites primer pairs produced by Gigant et al. (2012). The PCR amplifications were as described in Andriamihaja et al. (2021). Fragments were separated by capillary electrophoresis with an automated sequencer 3130XL ABI Genetic Analyzer (Applied Biosystems). Peaks were detected and allele sizes were scored manually using GENEIOUS v.7.0 (Olsen et al., 2014). The STRUCTURE software based on Bayesian method was run to assign the new specimens to the different known species (Pritchard et al., 2000). To do this, we used a dataset consisting of microsatellite data (from six or seven loci) of the 98 new samples as well as 151 individuals (among the 613 previously studied) representing the seven-known species. Structure analysis were performed following the protocol in Andriamihaja et al. (in prep).

The genetic analysis was conducted by species after classifying each individual into the seven different species based on Structure clustering and after removing individuals appearing as hybrids (probability of assignment less than 0.80). After identifying clonal individuals using GenAlEx software v. 6.503 (Peakall and Smouse, 2006), we calculated the clonality rate of each species by dividing the number of unique genotypes or “genets” (G) by the total number of individuals or “ramets” (N) (Halkett et al., 2005). Then, genetic analyses were conducted on the “genets” dataset. For each species, the estimated parameters of genetic diversity were: the total number of alleles for all loci (Na), the mean observed number of alleles per loci (Al), the total number of private alleles (Pa), the allelic richness corrected for sample size (Ar) according to Mousadik & Petit (1996), observed heterozygosity (Ho), expected heterozygosity (He), estimator of inbreeding coefficient (Fis). Na, Al, Ar, Pa, Ho, He and Fis were calculated from GenAlEx and Ar was estimated from FSTAT 2.9.4 (Goudet, 1995). We used the online version

GENEPOP (Raymond, 1995) to test for deviation from Hardy-Weinberg equilibrium and to detect probable linkage disequilibrium between all pairs of loci in each species with a Holm's sequential Bonferroni correction to adjust the p-values of multiple hypothesis test (Abdi, 2010). Null allele frequencies at each locus for each species were calculated via INEst v.2.2. using a Bayesian approach (IIM) (Chybicki and Burczyk, 2009).

Effective population size (N_e) of each species was calculated using the Linkage Disequilibrium (LD) method assuming random mating, achievable from NeEstimator software v2 (Do et al., 2014). For each N_e estimated, NeEstimator calculated 95% confidence interval (CI) with JackKnife procedure. Following the recommendation in Waples & Do (2010), the allelic frequencies of 0.02 and 0.05 were chosen as conservative thresholds to minimize the effect of the presence of rare alleles (Do et al., 2014).

c. Reproductive biology

i. Flowering characterization

At least, one site per species was selected for flowering characterization. Along a transect ranging from 200 m to 4,700 m long, we counted the total number of individuals (N) and the number of flowering individuals (N_f) in each site (Table S1). The number of individuals per km of transect was then calculated from these data in order to compare the different observation sites. One individual was defined as a stem rooting in the soil and climbing along the base of a plant support, one meter apart from the others and not sharing the support with them. The number of inflorescences of each flowering individual was recorded as well as the number of flowers buds (B), the number of opened flowers (OF), the number of closed flowers (CF), the number of pods (P), the number of floral scars (FS), and the number of ovaries (O) remaining on each inflorescence (Figure 2A). The total number of inflorescences (It) and the average number of opened flowers per inflorescence per day ($OFme$) were calculated on the basis on these collected data. The average number of flowers produced per inflorescence (Ftm) was calculated as follows:

$$Ftm = \frac{\sum_1^{It} OF + B + CF + P + FS + O}{It}$$

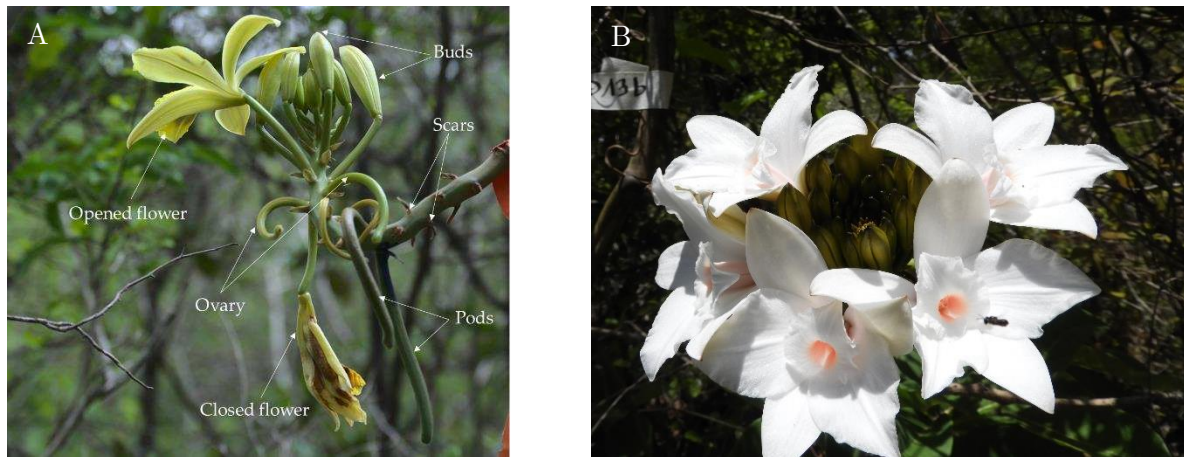


Figure 2. *Vanilla*

A— *Vanilla perrieri* inflorescence in the Befandrama (BFD) site, showing the different stages of floral and fruit development. B— *Vanilla madagascariensis* inflorescence in the Maromandia (MMD) site, showing five simultaneously opened flowers. (Photos A: Johnson G. Andrianantenaina, B: Cathucia F. Andriamihaja).

ii. Natural fruit set

The mean natural fruit set was estimated by dividing the total number of pods (P) recorded by the total number of flowers produced (Ft) (measured by adding the number of floral scars and pods on an inflorescence if the observation was done at the end of the flowering period). The incoming flowers i.e. buds as well as opened flowers, closed flowers, and ovaries were not taken into account if the observation was done during the early or mid-blooming period. The number of inflorescences used for the estimation varied from six in Manompana population (*V. atsinananensis*) to 105 in Tsaranoro (*V. allorgii*) (Table S2).

iii. Compatibility systems

The compatibility systems of three species (*V. madagascariensis*, *V. bosseri*, *V. perrieri*) were assessed by carrying out pollination experiments in three sites in Madagascar (BFD, MND, MSL). For this purpose, three treatments were performed on identified and marked individuals: i) no treatment on flowers (spontaneous self-fertilization), ii) hand self-pollination of single flowers (manual self-fertilization), iii) hand self-pollination between the flowers of two individuals of the same species at least 10 m apart (cross-pollination) (Table S3).

We also performed reciprocal interspecific crossing tests in two sites where two different species were in sympatry (*V. madagascariensis* \times *V. bosseri* in MSL, *V. perrieri* \times *V. madagascariensis* in BFD) (Table S3). Each crossing test was made very early in the morning after checking for the absence of pollen movement. Unlike intraspecific crossing tests, the number of flowers and individuals available for interspecific tests at the sites during the field period did not allow for multiple replications (Table S3). Tested flowers were carefully labeled, marked with the treatment performed and the parents. The inflorescences were bagged after treatments were performed for isolating the flowers from animal visits. The bags were removed the day after treatment. Crossing tests were verified 15 to 28 days after the pollination experiments.

Unsuccessful pollination can be recognized a few days after treatment by the fall of the faded flower and the absence of swelling of the ovary (Gigant et al., 2016; Shadakshari et al., 2003).

d. Ecological niche modelling

There are several methods and software for species distribution modelling (SDM) (Gobeyn et al., 2019), but all these approaches use in general presence/absence and environmental input data (Gobeyn et al., 2019). Since the absence data were not available due to the large size of Madagascar and the wide range of leafless *Vanilla* species, we opted for the MaxEnt algorithm which has the efficiency of requiring only presence data (Phillips et al., 2006) to generate a global map of the potential habitats of species.

Occurrence data of the seven Malagasy *Vanilla* species were generated in two different ways. First, we conducted genetic clustering analyses using dataset from Andriamihaja et al. (2021; in prep) and the additional data from the collection of Antananarivo University. This first step allowed us to classify the samples issued from University of Antananarivo collection into the seven known species and to determine the spatial distribution of individuals belonging to these seven species in Madagascar (Figure 2B). We also collected other location data available online in Tropicos (<https://www.tropicos.org/>), iNaturalist (<https://www.inaturalist.org/>) and MNHN (<https://science.mnhn.fr/>) in order to add additional points for niche modelling. For the dataset collected on the internet, the classification of the recorded points by known species was done by analyzing the available photos and herbarium records and based on the identification criteria proposed in Andriamihaja et al. (in prep) (Figure 2B).

Three categories of modeling were performed: i) past, ii) current and iii) future projections of species range. The historical environmental data consisted of 19 bioclimatic variables from the Last Glacial Maximum (LGM), between 26.5 and 19,000 years before present (Clark et al., 2009), available from worldclim (<http://www.worldclim.com/past>).

For the current environmental data, we used the 14 weakly correlated variables (11 current bioclimatic variables, pH, Elevation, NDVI_{max}) identified previously as those that contributed most to the differentiation of Malagasy leafless *Vanilla* (Andriamihaja et al., 2021), as well as eight additional ecological variables (geology type, vegetation type, human population density, digital elevation model, vegetation production, human influence index, aspect and slope) were taken account in order to refine environmental characteristics (see Rakotoarinivo et al., 2013) (Table S4). An initial analysis using all 22 variables was first run to determine the relative contribution of each variable to the model. Then, only the variables that contributed the most to the model (>2%) were retained for producing the final model of the species habitat niche.

Three future climate projection models were tested for modeling the future leafless *Vanilla* distribution: i) the Canadian Center for Climate Modeling and Analysis (CCCMA), ii) the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and iii) the Hadley Center for Coupled Model version 3 (HadCM3). They are each composed of nine climatic

variables: minimum annual rainfall (realmat), mean annual rainfall (realmar), minimum temperature of coldest month (mintemp), maximum temperature of hottest month (maxtemp), minimum precipitation of the driest month (minprec), maximum precipitation of the wettest month (maxprec), evapotranspiration total of 12 month added together (etptotal), water balance for the year (wbyear = realmar-etptotal), number of months with a positive water balance (wbpos). For each of these three models, projections for 2050 and 2080 were used under two emission scenario types A2 and B2 based on different economic and demographic growth assumptions expressed in terms of greenhouse gas emissions. Future climate layers were sourced from IPCC (Intergovernmental Panel on Climate Change) database (http://apps.ipcc-data.org/cgi-bin/ddc_nav/dataset=tar_gcm).

Past, present and future potential ecological niches of the seven leafless Malagasy *Vanilla* species were inferred by using the default settings of MaxEnt software version 3.4.4, as described by Scheldeman & Zonneveld (2010). MaxEnt calculates the probability of occurrence of a species in any raster cell (30" arc, ~ 1 km x 1 km size (Fick and Hijmans, 2017)) by comparing the similarity of that cell with cells in the raster where the species was actually observed using a maximum entropy algorithm (Phillips et al., 2006). To assess the fit of each model to the data, MaxEnt calculates the area under the receiver operating characteristic or AUC (Scheldeman and Zonneveld, 2010). The AUC values range from 0 to 1, with a value higher than 0.7 indicating an acceptable fit of the model to the data and 0.5 indicating a poor fit no better than random (Araujo et al., 2005; Scheldeman and Zonneveld, 2010). However, as the use of AUC in model evaluation is often debated (Lobo et al., 2008; Scheldeman and Zonneveld, 2010; Synes and Osborne, 2011), MaxEnt outputs were also checked and compared to presence records and known ecological information on species. The species models produced from MaxEnt were imported in QGIS 3.18.1 (<https://www.qgis.org>) to construct the distribution maps and to calculate the total surface of the ecological niches of each species in Madagascar. For that, the cumulative probabilities of occurrence were transformed from continuous to binary forms (0 or 1) using the 10-percentile training presence as cut-off. The 10-percentile training presence is the probability that 90% of the presence points fall within the potential distribution area, the remaining 10% are those that have an unusual environment and do not fall within the boundaries of the effective niche (Radosavljevic and Anderson, 2014; Scheldeman and Zonneveld, 2010). In other words, sites with a probability above the threshold have suitable environmental conditions for the species while sites below the threshold correspond to areas where the species could not occur. The proportion of suitable habitat (P) for each species was estimated as follows:

$$P = \frac{\text{Total surface of ecological niches}}{\text{Total surface of Madagascar}} \times 100$$

The potential global distribution of leafless *Vanilla* in Madagascar, all species combined, was determined by adding the individual distributions models of the seven species in QGIS. The proportion of each zone corresponding to different amounts of co-occurring species was

calculated via the previous formula (P). The total percentage of the distribution area of all leafless *Vanilla* species (Pt) was estimated by adding the different proportions (P) of areas potentially occupied by one or different species.

4.2.3. Results

a. Genetic analysis

Among the 712 accessions available, after exclusion of accessions with missing data (23 samples from University of Antananarivo) and of hybrids (n=31), a total of 658 accessions were studied ("ramets"). After clone exclusion, a total of 604 unique genotypes ("genets") were found and used in genetic analysis, including 529 dataset from previous studies (Andriamihaja et al., 2021, in prep) and 75 new data (Table S5). Indices of allelic variation and genetic diversity are given in Table 1, as well as information on deviations from the HW equilibrium, Fis values and estimations of effective population sizes Ne.

Of the seven species studied, only *V. allorgii*, whose individuals were collected in a small forest, showed a high clonality rate (genotypic diversity = 27%). A total of 258 alleles were found with an average number of 12 alleles/locus/species. Number of alleles ranged between 20 (*V. allorgii*) to 163 (*V. bosseri*), with mean number of alleles per locus (Al) ranging from 2.86 to 23.29 in the same species, respectively. As expected, allelic richness (Ar) was less strongly influenced by sample size and varied from 2.86 to 8.14. In total, 121 private alleles (P) were recorded, the lowest number was attributed to *V. allorgii* (P = 1) and the highest number (P= 42) was found in *V. bosseri*. The observed heterozygosity (Ho), the expected heterozygosity (He) and allelic richness of *V. bosseri* (Ho= 0.65, He = 0.82, Ar= 8.14) and *V. perrieri* (Ho= 0.62, He = 0.78, Ar= 6.66) were high, whereas the species *V. allorgii* showed the lowest genetic diversity (Ho=0.59, He= 0.40, Ar=2.86). Averaged over all loci, deviations from HW equilibrium ($p < 0.001$) were found in all species, with a heterozygote deficit (FIS > 0) for six species (maximum in *V. decaryana* with 0.43) and a strong heterozygote excess for *V. allorgii* (FIS < 0). None of the 21 loci combinations tested were significantly in LD after SB correction. All species, except *V. allorgii*, have a relatively high frequency of null alleles (> 0.1) at two (*V. humblotii*), three (*V. atsinananensis*, *V. bosseri*), four (*V. madagascariensis*), five (*V. decaryana*) and seven (*V. perrieri*) microsatellite loci.

Table 1. Summary of genetic analysis per species at seven microsatellite loci

	<i>V. allorgii</i>	<i>V. atsinananensis</i>	<i>V. bosseri</i>	<i>V. decaryana</i>	<i>V. humblotii</i>	<i>V. madagascariensis</i>	<i>V. perrieri</i>
N	30	12	203	139	30	167	77
G	8	10	195	123	30	162	76
G/N (%)	27	83	96	88	100	97	99
Na	20	35	163	85	60	118	108
Al	2.86 ± 0.74	5.00 ± 0.69	23.29 ± 4.13	12.14 ± 2.57	8.57 ± 1.54	16.86 ± 2.37	15.43 ± 1.62
Ar	2.86 ± 1.95	4.55 ± 1.45	8.14 ± 3.11	4.37 ± 2.36	5.57 ± 2.45	5.86 ± 2.35	6.66 ± 1.42
Pa	1	5	42	18	7	24	24
Ho	0.59 ± 0.18	0.49 ± 0.09	0.65 ± 0.04	0.36 ± 0.08	0.58 ± 0.08	0.54 ± 0.09	0.62 ± 0.08
He	0.40 ± 0.10	0.59 ± 0.06	0.82 ± 0.06	0.55 ± 0.10	0.69 ± 0.08	0.67 ± 0.10	0.78 ± 0.03
FIS	-0.36 ± 0.23	0.14 ± 0.15	0.20 ± 0.05	0.43 ± 0.10	0.14 ± 0.09	0.21 ± 0.05	0.20 ± 0.10
HWE	***	**	***	***	***	***	***
Ne1 (0.02) [95%CI]	infinite	24 [3-infinite]	263 [157-597]	39 [25-65]	35 [16-203]	103 [67-176]	69 [44-126]
Ne2 (0.05) [95%CI]	infinite	24 [3-infinite]	204 [73-infinite]	19 [9-37]	38 [11-infinite]	70 [42-130]	46 [19-220]

N: number of samples per species (ramet); G: number of unique genotypes (genet); G/N: genotypic diversity; Na: total number of alleles for all loci; Al: mean number of alleles per locus ± SD; Ar: mean allelic richness per locus ± SD (Mousadik and Petit, 1996); Pa: total number of private allele; Ho: observed heterozygosity over all loci; He: expected heterozygosity over all loci; FIS: fixation index; HWE: results of test for departures from Hardy-Weinberg equilibrium (NS $p > 0.05$, * $p < .05$, ** $p < .01$, *** $p < .001$); and, Ne: effective population with 95% confidence interval (CI) for allelic frequencies thresholds of 0.02 and 0.05 obtained from JackKnife method.

As for *V. allorgii* for which Ne estimation failed, the small size of the *V. atsinananensis* population did not allow to precisely estimate the effective size Ne (very large confidence intervals and Ne slightly above N), their results must therefore be taken with caution. For the remaining species, lower or approximately equal values of Ne compared to N were detected, therefore estimated values can be accepted as accurate as suggested by England et al. (2006). *V. bosseri* had the highest Ne with estimates around 263 [95% CI: 157-597] and 204 [95% CI:

73-infinite] at the 0.02 and 0.05 thresholds, respectively (Table 1). In contrast, *V. decaryana* and *V. humblotii* had the lowest Ne values with estimates around 39 [95% CI: 25-65] and 19 [95% CI: 9-37], and 35 [95% CI: 16-203] and 38 [95% CI: 11- infinite] at the cut-off of 0.02 and 0.05, respectively.

b. Reproductive biology

i. Flowering characteristics

Eight to 59 individuals were found along the selected transects (Table 2), with a mean of 51 individuals / transect / site.

Table 2. Summary of floral characteristics of the seven leafless *Vanilla* species from Madagascar

Species	Sites codes	T (km)	N	N/km	Nf	Nf/N (%)	Ima	Ime	Ftm	OFma	OFme
<i>V. allorgii</i>	AND	0.3	59	197	31	52	12	3.8 ± 3.2	25 ± 8	1	1.11 ± 0.57
<i>V. atsinananensis</i>	MAN	1.5	8	5	1	13	6	-	20 ± 7	1	1
<i>V. bosseri</i>	MSL	2.3	50	22	30	60	8	2 ± 1.6	29 ± 9	2	0.84 ± 0.59
<i>V. decaryana</i>	AJA	0.6	56	90	6	9	10	4.4 ± 3.4	29 ± 7	5	1.13 ± 1.08
	ATA	0.2	26	163	9	35	8	4.4 ± 2.6	24 ± 7	2	0.92 ± 0.74
<i>V. madagascariensis</i>	MMD	4.7	52	11	24	46	11	3.1 ± 2.9	41 ± 20	5	1,30 ± 0,98
<i>V. humblotii</i>	MDF	1.8	84	47	3	7	2	1.1 ± 0.3	24 ± 6	2	0,88 ± 0,83
<i>V. perrieri</i>	BFD	1.1	75	71	23	31	5	1.9 ± 1.3	31 ± 8	3	0,68 ± 0,82

T: Transect, N: total number of individuals along the transect, N/km: number of individuals per km calculated, Nf: number of flowering individuals along the transect, Nf/N (%): percentage of flowering individuals along the transect, Ima: the maximum number of inflorescences on a flowering individual, Ime: the average number of inflorescences on a flowering individual, Ftm: The average number of flowers produced per inflorescence, OFma: the maximum number of opened flowers per inflorescence per day, OFme: the average number of opened flowers per inflorescence per day.

The populations of *V. allorgii* (AND) and *V. decaryana* (ATA) were the densest with approximatively 197 and 163 individuals per kilometer of transect, respectively. In opposite, the number of individuals constituting the population of *V. atsinananensis* in the Ambodiriana (MAN) forest and *V. madagascariensis* in Maromandia (MMD) was very low with about five and eleven individuals per kilometer of transect, respectively.

If the great majority of individuals (> 65%) were in vegetative state in five sites (MAN, AJA, ATA, MDF, BFD) where four species (*V. atsinananensis*, *V. decaryana*, *V. humblotii*, *V. perrieri*) can be found, about half of the individuals observed along the transect at the MMD (*V. madagascariensis*), AND (*V. allorgii*) and MSL (*V. bosseri*) sites had produced inflorescence during the study period. Populations of the two yellow flower species produced the fewest inflorescences per flowering individual (Ima = 5 for *V. perrieri*; Ima = 2 for *V. humblotii*), and the maximum value Ima = 12 was observed for *V. allorgii* (Table 2). Except

for *V. madagascariensis*, which showed a mean number of flowers produced per inflorescence of 41, each inflorescence yielded twenty to thirty flowers on average (Table 2). Flower opening was successive with zero to five opened flowers per inflorescence per day. If for *V. allorgii* and *V. atsinananensis* the maximum number of simultaneously opened flowers per day per inflorescence was strictly limited to one, it reached a maximum of two in *V. humblotii* and *V. bosseri*, three in *V. perrieri* and could go up to five in *V. decaryana* and *V. madagascariensis* (Table 2, Figure 2B).

ii. Natural fruit set

The natural fruit sets of the leafless *Vanilla* populations were relatively low, ranging from 0.61% in the AND site (*V. allorgii*) to 11.88% in the MLT site (*V. decaryana*), both at mid-blooming (Table). On the basis of the six inflorescences of a single flowering individual observed in the MAN population, the natural fruit set of *V. atsinananensis* was 4.13 %. The natural fruit set of *V. decaryana* varied among populations, it ranged from 1.79 % in ATA population to 11.88% in the MLT population. The natural fruit set of *V. madagascariensis* was around 2.69% (measured at mid-flowering) and 5.66% (measured at late flowering). Despite the low flowering rate of the MDF population (*V. humblotii*), its naturel fruit set (9.80%) was important compared to other species. Whether measured at mid or end blooming period, the natural fruit set of *V. perrieri* hovered around 4%. Measured during mid-flowering, the natural fruit set of *V. bosseri* (4.71%) was close to that of *V. perrieri* (Table).

Table 3. Summary of the natural fruit sets estimated for the seven leafless *Vanilla* species from nine sites in Madagascar

Species	Sites codes	Flowering period	Number of inflorescences	P	Ft	Natural fruit set (%)
<i>V. allorgii</i>	AND	Middle	105	10	1652	0.61
<i>V. atsinananensis</i>	MAN	End	6	5	121	4.13
<i>V. bosseri</i>	MSL	Middle	49	37	785	4.71
<i>V. decaryana</i>	AJA	Beginning	22	0	2	0
	AJA*	End*	20	12	570	2.11
	ATA	Beginning	38	1	56	1.79
	MLT	Middle	35	19	160	11.88
<i>V. madagascariensis</i>	MMD	Middle	33	17	633	2.69
	MMD	End	82	210	3711	5.66
<i>V. humblotii</i>	MDF	Middle	8	5	51	9.80
<i>V. perrieri</i>	BFD	End	53	63	1320	4.77
	BFD	Middle	15	13	294	4.42

*: inflorescences of previous year, P: pods, Ft: total number of flowers produced

iii. Compatibility systems

All spontaneous self-fertilization tests conducted were unsuccessful (Table 4).

Table 4. Results of pollination experiment tests

Treatment	Species	Site codes	Number of treatments	Number of success (%)
Spontaneous self-fertilization	<i>V. bosseri</i>	MSL	11	0 (0)
	<i>V. madagascariensis</i>	MMD	15	0 (0)
	<i>V. perrieri</i>	BFD	18	0 (0)
Manual self-fertilization	<i>V. bosseri</i>	MSL	11	9 (81.8)
	<i>V. madagascariensis</i>	MMD	16	14 (87.5)
	<i>V. perrieri</i>	BFD	23	20 (86.9)
Cross-pollination between two individuals of the same species	<i>V. bosseri</i>	MSL	11	8 (72.7)
	<i>V. madagascariensis</i>	MMD	16	15 (93.7)
	<i>V. perrieri</i>	BFD	15	13 (86.7)
Cross-pollination between two individuals of different species	<i>V. madagascariensis</i> x <i>V. bosseri</i>	MSL	1	1 (100)
	<i>V. bosseri</i> x <i>V. madagascariensis</i>	MSL	1	0 (0)
	<i>V. madagascariensis</i> x <i>V. perrieri</i>	BFD	3	1 (33.33)
	<i>V. perrieri</i> x <i>V. madagascariensis</i>	BFD	3	3 (100)

However, self-compatibility was verified for all the three studied species with high rates of success (81.8 to 87.5 %) in hand self-fertilization of a single flower. Hand-crossing between two different individuals of the same species, distant at least 10m apart, also showed high success rates (72.7 to 93.7%) (Table 4). Of the six hand-crossing tests conducted between individuals of *V. perrieri* (yellow flower species) and *V. madagascariensis* (white flower species) in the BFD site, four resulted in 15 days-old fruits. Also, one of the two interspecific hand-crossing tests between *V. madagascariensis* (white flower species) and *V. bosseri* (white flower species) produced a 28 days-old pod in the MSL site.

c. Species distribution models

A total of 746 data points has been identified, classified by species and used to construct the species distribution models (Figure 1B). From these, 65 points were collected from online databases (MNHN, Tropicos, iNaturalist), 583 points correspond to the locations of non-hybrid individuals from previous studies (Andriamihaja et al., 2021; in prep), and 98 points were obtained from records of the collection of the University of Antananarivo after clustering analysis with STRUCTURE using six to seven microsatellite markers (assignment probability > 80%) (data not shown).

For all species, the distribution range during the last glacial maximum appears to be wider than the current distribution area (Figure 3). Among the 19 bioclimatic variables in the LGM, contributing variables appear different for all species. But, the precipitation of warmest quarter, isothermality (Mean diurnal range/Temperature annual range) and the precipitation of coldest month are the ones that are common to all species, and often the most contributing. The produced single model of the past distribution of leafless *Vanilla* species, those combining the individual predictions for the seven species, suggests that the genus was present in the great majority of Madagascar's surface (Percent of total Madagascar surface occupancy Pt = 84%, AUC= 0.904-0.993) during LGM, with the Northwest and Southwest being the main areas of sympatry. The areas of sympatry (N= two to five species recorded in the same site) represented about 50% of the total area of Madagascar against 34% of area where a single species occurs (Figure 4).

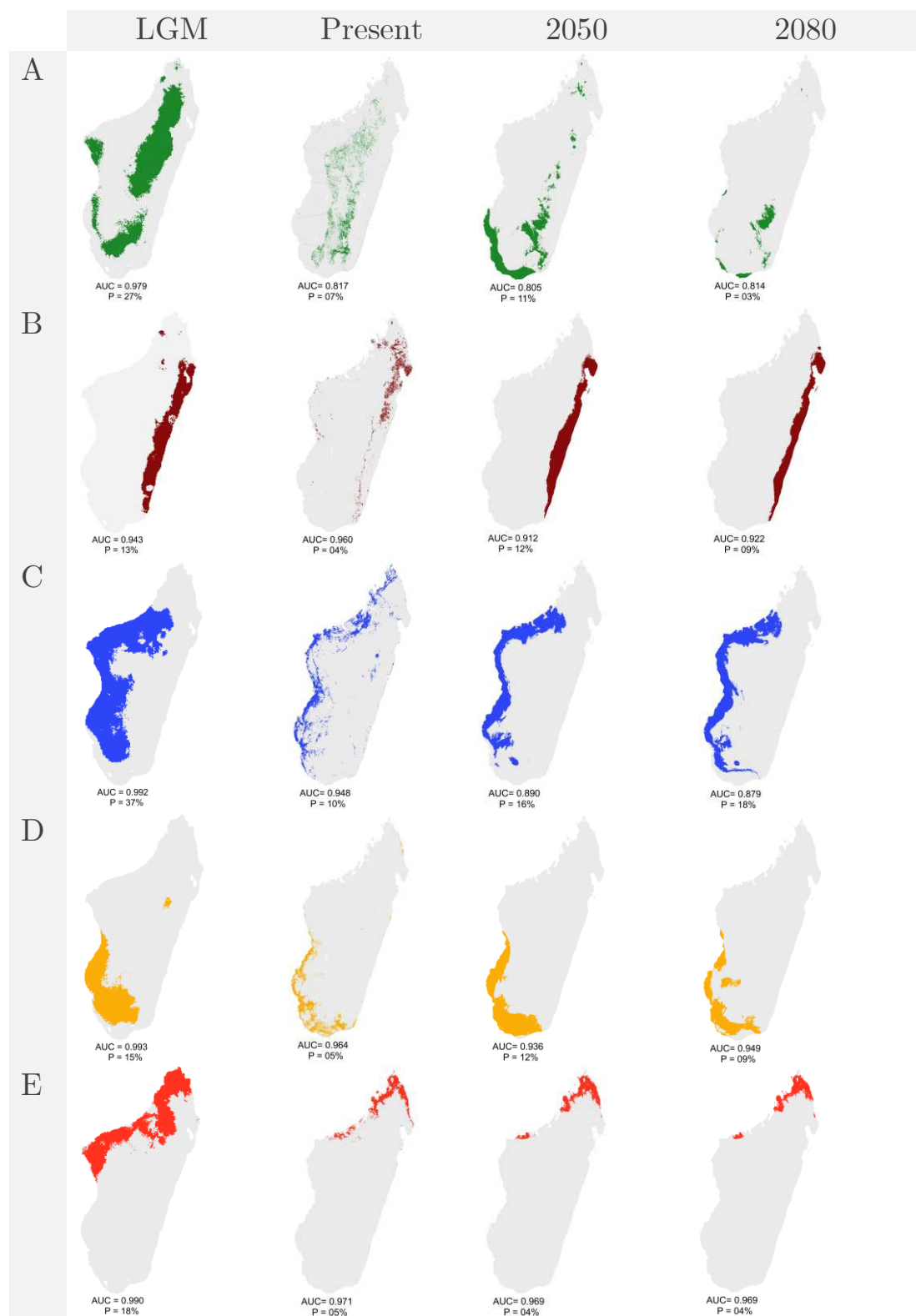
Among the 22 variables used in initial present distribution analysis, 19 were retained for the final analysis. The most explanatory variables of leafless *Vanilla* habitat distribution (explaining more than 2% of the predicted distribution) differ from one species to another (Table 5).

Table 5. List of variables that contributed to the present ecological niche modelling of each species

Species	Variables
<i>V. allorgii</i>	Vegetation type, geology type, NDVI max, vegetation production, pH
<i>V. atsinananensis</i>	Vegetation production, vegetation type, altitude
<i>V. bosseri</i>	Geology type, precipitation seasonality, vegetation production, NDVI _{max} , vegetation type, digital elevation model, human influence index, isothermality (mean diurnal range/ temperature annual range), slope
<i>V. decaryana</i>	Precipitation of wettest quarter, vegetation type, digital elevation model, geology type, human influence index, vegetation production, temperature seasonality
<i>V. humblotii</i>	Vegetation type, geology type, digital elevation model
<i>V. madagascariensis</i>	Minimum temperature of coldest month, temperature seasonality, human influence index, precipitation seasonality, NDVI _{max} , mean temperature of driest quarter, vegetation type, altitude
<i>V. perrieri</i>	Vegetation type, geology type, human population density, digital elevation model, precipitation seasonality, habitat aspect, mean temperature of wettest quarter, temperature seasonality, mean temperature of wettest quarter, isothermality

Nevertheless, vegetation type, geology type, human index influence and the digital elevation model or altitude, were the most relevant variables for the majority of species. Based on the obtained AUC values (> 0.8), and on the comparison between presence data points; distribution models and the species ecology information, the model predicting performance can be considered as good (Figure 3).

Although reduced compared to the past (LGM), the current overall distribution of leafless *Vanilla* species remains broad in Madagascar (Pt = 31%, AUC = 0.817-0.971), with sympatry areas (Pt = 10%) where two or even three species can cohabit in the North West and South West (Figure 4). Consistent with their distribution in the past, *V. perrieri* and *V. bosseri* are visibly the most widely distributed species, today found along the entire Western coast covering an area of P= 14% (AUC = 0.914) and 10% (AUC = 0.948) in Madagascar, respectively (Figure 3). The five other species are probably restricted to the Northwest (*V. madagascariensis*, *V. humblotii*), Southwest (*V. decaryana*), East (*V. atsinananensis*), and Central highlands (*V. allorgii*) regions, each covering less than 10% (AUC = 0.817-0.871) of Madagascar's land area (Figure 3).



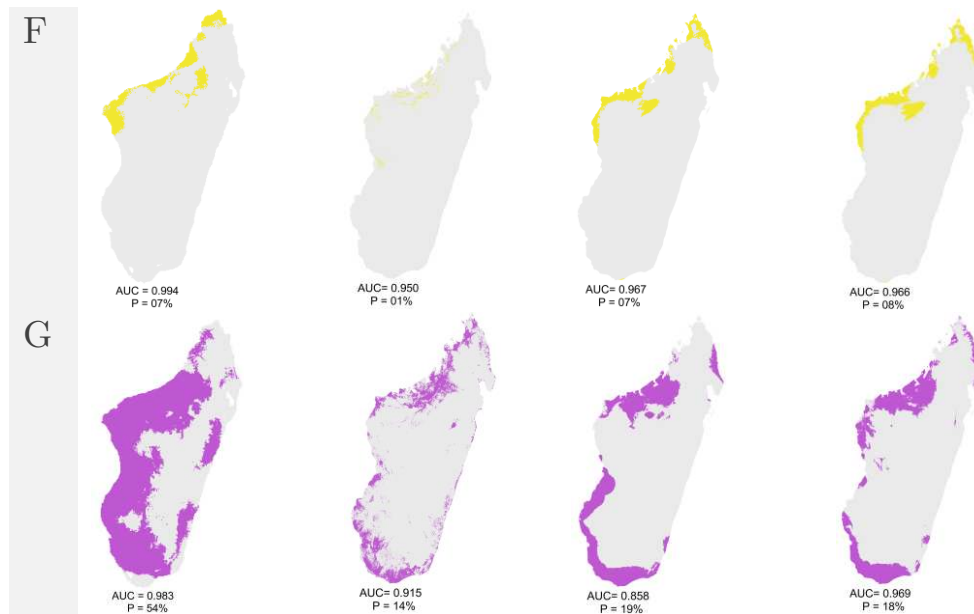


Figure 3. Potential past (Last Glacial Maximum), present and future (2050 and 2080 with the CSIRO climate change model and A2 scenarios) occurrence areas of the seven leafless *Vanilla* species from Madagascar. À: *V. allorgii*, B: *V. atsinananensis*, C: *V. bosseri*, D: *V. decaryana*, E: *V. madagascariensis*, F: *V. humblotii*, G: *V. perrieri*. AUC>0.90: excellent; 0.80<AUC<0.90: good; 0.70<AUC<0.80: acceptable, 0.60<AUC<0.70: poor, 0.50<AUC<0.60: invalid (Araujo et al., 2005). Proportion (P) of suitable habitats are indicated.

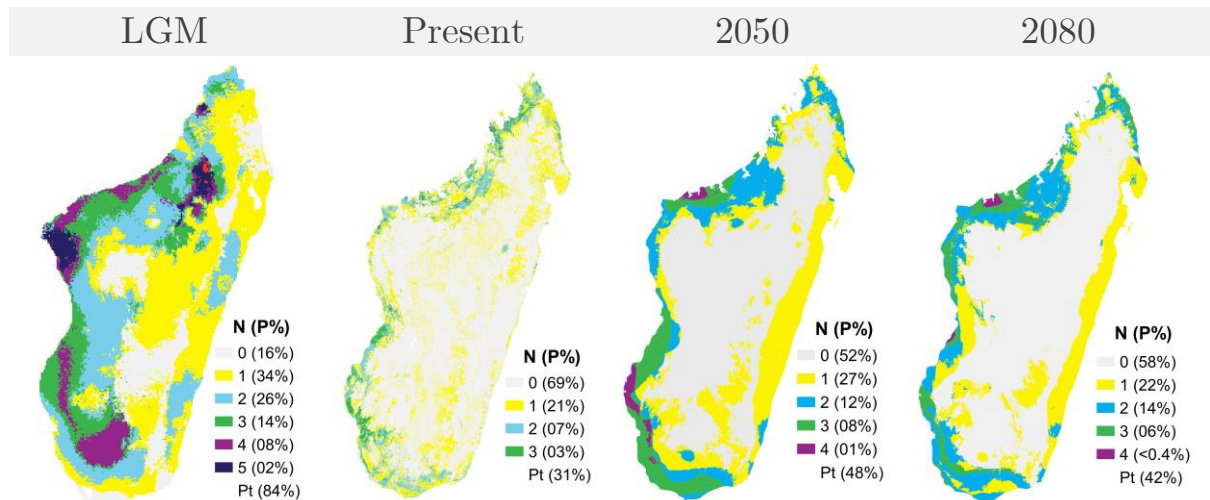


Figure 4. Potential past (Last Glacial Maximum), present and future (2050 and 2080 with the CSIRO climate change model and A2 scenarios) distribution areas of leafless *Vanilla* species from Madagascar (all species combined). The different colors represent different amounts of co-occurring species. P%: proportion of the different occurrence areas. Pt: total percentage of ecological niche of leafless *Vanilla* species.

Among the three future climate projection models tested (CSIRO, CCCM, HadCM3) and the two emission scenario types (A2, B2), the CSIRO model and the scenario A2 gave highest AUC and matched the most to the presence data points, so they are the climate scenario models that were considered for the final modelling. Precipitation is the predominant variable in the future potential distribution of *V. allorgii*, *V. atsinananensis* and *V. bosseri* while it is rather the minimum temperature for the two Northern species (*V. humblotii* and *V. madagascariensis*). The future distribution is mainly a function of water balance for the year

for *V. decaryana* and precipitation and evapotranspiration for *V. perrieri*. Under the climate change scenarios, most species (except *V. madagascariensis*) may possibly maintain, even expand their area of occurrence in 2050 (AUC = 0.805-0.969) (Figure 3). *V. allorgii*, which is currently observed in the Central highlands, is likely to be more concentrated in the Southern part of the island in the 2050 climate projection (AUC = 0.814) (Figure 3A). *V. atsinananensis* could expand its habitat along the entire East coast (AUC = 0.922) (Figure 3B). The Western range of *V. bosseri* and *V. perrieri* (Figure 3C, 3G), the Northern distribution of *V. madagascariensis* (Figure 3E) and *V. humblotii* (Figure 3F), as well as the Southern distribution of *V. decaryana* (Figure 3D) should be maintained in the future (Figure 3C, 3G). The potential ecological niche of *V. madagascariensis* could slightly decrease from P= 5% at present (AUC = 0.969) to P= 4% in 2050 (AUC = 0.969), as areas of distribution may completely disappear. For *V. bosseri*, suitable habitat in the extreme North and South of the island may be lost but could be compensated by its expansion along the West coast (AUC = 0.890) (Figure 3C). Habitat losses in the far North may also occur for *V. perrieri* (AUC = 0.858). In 2050, the North-West and the South-West should remain the main areas where species richness (up to four species) is the highest. The suitable habitats of four species (*V. allorgii*, *V. atsinananensis*, *V. decaryana*, *V. perrieri*) should then probably reduce in the face of higher change under the 2080 climate projection and A2 scenarios (AUC = 0.814-0.969) while *V. bosseri* and *V. humblotii* should continue their expansion (AUC = 0.879 and 0.966, respectively) (Figure 3). In summary, global potential suitable habitats of leafless *Vanilla* species and sympatric areas should globally increase between present and future although a slight reduction might be observed between 2050 and 2080 (Figure 3, Figure 4).

4.2.4. Discussion

Leafless *Vanilla* species are commonly known as “Vahinamalona” in Madagascar (Rakotonandrasana et al., 2017; Randriamiharisoa et al., 2015). They are collected in different Western regions (especially in the South) to treat various diseases (Randriamiharisoa et al., 2015; Randrianarivony et al., 2017). Conservation and sustainable use of medicinal plants require the consideration of many factors including habitat specificity, distribution range, population size, species diversity, growth rate and reproductive system (Chen et al., 2016). Here, we will address the genetic diversity, the reproductive biology and the ecological niches evolution of the seven species in Madagascar over time (past, present, future) to evaluate short and long-term survival. Then, we discuss the implications for the conservation of these endemic species.

a. *Vanilla* leafless species in Madagascar: reproductive and genetic status

Population densities of the Malagasy leafless *Vanilla* species varied from five (*V. atsinananensis*) to 197 (*V. allorgii*) individuals per kilometer. Population density is a demographic parameter strongly related to the conservation status of the habitat. Studies conducted in several forests around the world including in Madagascar indicated that sacred

forests are the most strongly conserved (Bhagwat and Rutte, 2006; Rath et al., 2020; Wadley and Colfer, 2004). A sacred forest is, in general, a well-preserved place whose access and management are governed by local communities following traditional rules (Bhagwat and Rutte, 2006). In many cases, part or all of these landscapes may never be subjected to resource use due to their cultural, traditional and spiritual significance (Bhagwat and Rutte, 2006; Rath et al., 2020). In this study, the highest abundance (> 150 individuals/km) was attributed to populations of two species (population AND of *V. allorgii*, population ATA of *V. decaryana*) found in small sacred forests, while *V. madagascariensis* (N= 11 individuals/ km) population (MMD), with one of the lowest densities, is located near a road within a secondary forest. The AJA population of *V. decaryana* (N= 90 individuals/km) and the BFD population of *V. perrieri* (N= 71 individuals/km) are found in forests without formal protection, but which are managed by the nearby villages. The forest in which *V. humblotii* is found (MDF, N=47 individuals/km) is part of the new protected areas of Madagascar created after 2015. The MSL population of *V. bosseri* (N= 22 individuals/km) is located in the new protected area of Bongolava Maintso that is still subject to frequent illegal logging, fire for charcoal despite its status (Miladera, 2008). Although located in a protected area, the population of *V. atsinananensis* in Manompana (MAN) has a very small size (N= 5 individuals/km). In the genus *Vanilla*, the extreme rarity of individuals in their natural habitats is a fact that has also been reported for other species (Barona-Colmenares, 2018; Flanagan et al., 2018; Fraga et al., 2017; Karremans and Lehmann, 2018). If in many cases the rarity is due to the destruction of unprotected habitats (Barona-Colmenares, 2018; Karremans and Lehmann, 2018), it may also possibly be the consequence of the low colonization opportunities of the species due to the natural disjunction of suitable environmental conditions within habitat (Phillips et al., 2011). In other words, species requiring special conditions (e.g. specialization to mycorrhiza, association with specific tutors as the case of *V. palmarum* (Barberena et al., 2019), association to specific edaphic conditions, etc.) may be restricted to areas where these conditions are satisfied. As leafless *Vanilla* species are heliophilous, they generally form disjunct patches of populations confined in open (light) areas within dense forests with very large trees (like Ambodiriana humid forest (MAN)). Thus, the locations of the transect chosen for the inventory can influence the density of individuals.

Flowering characteristics of Malagasy leafless *Vanilla* species were similar to the other *Vanilla* species in other regions. In most cases, *Vanilla* flowers are ephemeral with an average of one to three opened flowers per inflorescence per day (Gigant et al., 2016; Soto Arenas and Dressler, 2010), as observed for the Malagasy species *V. allorgii*, *V. atsinananensis*, *V. bosseri*, *V. humblotii* and *V. perrieri*. But we observed the highest recorded values so far in the *Vanilla* genus with up to five simultaneous flowers opened for *V. decaryana* and *V. madagascariensis* (Figure 2B). The average number of flowers produced per inflorescence varied from 20 (*V. atsinananensis*) to 41 (*V. madagascariensis*) (Table 2). It was about 29 ± 7 for *V. decaryana* according to our results, a number included in the range reported by Portères (1954) for this species (20-40 flowers). In contrast to our results, Portères (1954) indicated a lower maximum number of 20 flowers (instead of 41 ± 12) per inflorescence for *V. madagascariensis*. Portères

(1954) mentioned a June-October flowering season for *V. madagascariensis*, but we observed that it could last until January (pers.obs), which could explain the higher flowering rate recorded. Portères (1954) also reported a higher range of 40 to 50 flowers (instead of 24 ± 6) for *V. humblotii*. However, the study of *V. humblotii* in Mayotte indicated a number of 34 ± 12 flowers per inflorescence (Gigant et al., 2016) which is close to our estimation. All Malagasy leafless *Vanilla* species showed higher numbers of flowers per inflorescence than the sister species *V. roscheri* (14 ± 8 flowers per inflorescence) in South Africa (Gigant et al., 2014). Seven (*V. humblotii*) to 60 % (*V. bosseri*) of observed individuals were in flowering stage. This low flowering rate of *V. humblotii* has also been reported in Mayotte (M'sa, 2014). However, in the Kirindy (KRD), less than 5% of *V. bosseri* individuals inventoried had produced an inflorescence in 2014 (Petersson, 2015). Several factors may affect flowering parameters estimation. First, the number of samples used could greatly impact the estimation. In this study, flowering characterization of *V. humblotii* was based on the few inflorescences (<10) observed. Also, if the majority of the population is constituted by juvenile individuals, the rate of flowering will be low because plants should attain a certain biomass and age to bloom (Lopez and Runkle, 2005). The climatic and ecological differences between observation sites and climatic variations between years could also affect flower production, pollination, anthesis timing and flowering period (Lopez and Runkle, 2005; Scaven and Rafferty, 2013).

Malagasy leafless *Vanilla* species, as many other orchids (Micheneau et al., 2009; Tremblay, 1992), are exclusively pollinator-dependent with hermaphrodite flowers and a rostellum membrane preventing spontaneous self-pollination. Indeed, spontaneous pollination tests conducted did not result in fruits, confirming strict dependence on pollinators for fruit set. The crossbreeding tests carried out confirmed however the self-compatibility of all species (Table 4). The dependence in pollinators and the self-compatibility of leafless *Vanilla* species was also demonstrated in *V. bosseri* in the Kirindy Forest (Petersson, 2015), in *V. humblotii* in Mayotte (Gigant et al., 2016) and in *V. roscheri* in East Africa (Gigant et al., 2014). For Malagasy leafless *Vanilla* species, natural fruit set was low, ranging from 0.61 % (*V. allorgii*) to 11.88% (*V. decaryana*), in accordance with published fruit sets in allogamous *Vanilla* and other deceptive rewardless orchid species (Ackerman et al., 1996; Gigant et al., 2016, 2014; Householder et al., 2010; Macedo, 2000; Pansarin et al., 2014; Tremblay, 1992; Tremblay et al., 2005). These rates varied also depending on the site for the same species (e.g. *V. decaryana*: 1.79 % in ATA, 0-2.11% in AJA and 11.88 % in MLT). In deceptive rewardless orchids, natural fruit set is limited by pollinator availability rather than being resource dependent (Tremblay et al., 2005). As the fragmentation or degradation of the habitat could influence the availability of pollinator populations (Rathcke and Jules, 1993), it could also impact plant natural fruit set. In this study, populations of *V. decaryana* with low natural fruit set are located in degraded (AJA) and small sacred forest (ATA) surrounded by agricultural land and villages. Conversely, the forest in which MLT is located is part of a larger forest continuum surrounding the Onilahy River. If *V. humblotii* natural fruit set in Madagascar was estimated around 9.80% in this study, the rate ranged from 0% to 10.02 % in five sites in the Comoros archipelago islands (M'sa, 2014) but it was only about 0.62% and 1.2% in Mayotte during two field surveys

conducted (Gigant et al., 2016), probably depending on the degree of habitat degradation or fragmentation in the various sites. In the Kirindy protected forest (KRD), the natural fruit set of *V. bosseri* was estimated at 3.96 %, a value close to the one obtained from this study for the Marosely protected forest (MSL) population (4.71%). Among all leafless *Vanilla* species studied so far, the sister species from East Africa (*V. roscheri*) and the distantly related American species (*V. barbellata*) had the highest rate of natural fruit set, with values of 26.3% and 18.2%, respectively (Gigant et al., 2014; Tremblay et al., 2005).

In areas of sympatry (Figure 4), natural hybridization between species is possible and could affect the evolution and conservation of the involved species. Indeed, hybridization could lead to speciation, play a role in the transfer of adaptive traits and increase genetic diversity (Anderson, 1948; Arnold, 1997; Rieseberg and Carney, 1998), or may lead to the extinction of rare taxa as a result of outbreeding depression and genetic assimilation (Ellstrand et al., 1999; Levin et al., 1996; Wolf et al., 2001). Hybridization is a possible phenomenon in *Vanilla* genus (Divakaran et al., 2006; Lubinsky et al., 2006; Minoo et al., 2008; Nielsen, 2000). Indeed, the crossing tests between *V. madagascariensis* and *V. bosseri* and between *V. perrieri* and *V. madagascariensis* carried out in this study were successful (Table 4). Furthermore, our previous study using microsatellite markers identified possible hybrid individuals in Madagascar (Andriamihaja et al., 2021).

Like most *Vanilla* species, the leafless species from Madagascar can also multiply by asexual or vegetative reproduction. Using microsatellite data, the clonality rate varied from 1% to 17% for six species, a value relatively within the same range of the one observed for *V. humblotii* in Mayotte (12% (Gigant et al., 2016)) as well as for the Puerto Rican leafless species, *V. claviculata* (6%) and *V. barbellata* (5%) (Nielsen, 2000). Among the seven Malagasy leafless species, *V. allorgii* is the only one with a high clonality rate (73%) resulting in Hardy-Weinberg disequilibrium ($FIS < 0$) due to heterozygous excess, a common feature of clonal populations (Balloux et al., 2003; Halkett et al., 2005; Meloni et al., 2013). The Hardy-Weinberg exact test conducted on the six other species indicated rather a disequilibrium with heterozygote deficit ($FIS > 0$) probably due to null alleles or to consanguineous mating through geitonogamy (also observed in Mayotte for *V. humblotii* (Gigant et al., 2016)) made possible by the occurrence of simultaneous flowers on the same individual (Table 2). After verifying, for each species, the correlation between the mean squared correlation of allele frequencies at different gene locus (r^2) and the product of two fixation index (FST) for pairs of loci as explained in Waples (2015) (data not shown), the probability that the heterozygote deficiency was the result of species sub-structuring into different populations (Wahlund effect) was low. The genetic diversity is influenced by several factors including natural selection, gene flow and mutation rates (Frankham et al., 2010). Current genetic variation may also reflect past events of the species, such as range expansion or decline, founder events or population bottlenecks and habitat fragmentation (Frankham et al., 2010; Hewitt, 2004). As expected (Nybom, 2004), species with narrow distribution (e.g. *V. allorgii*, *V. atsinananensis*, *V. decaryana*) had lower genetic diversity than those widely distributed (*V. bosseri* and *V. perrieri*). For *V. roscheri* located in

range edge distribution on the banks of the Lake Sibaya (South Africa), a drastic reduction of diversity was observed with a single homozygous genotype, despite the high fruit set (Gigant et al., 2014). The reproductive system was also demonstrated to be a determinant factor of genetic variation in plants (Hamrick and Godt, 1996; Nybom, 2004). In general, the genetic diversity of Malagasy leafless *Vanilla* species ($H_o = 0.49-0.65$) was relatively high and matched the values reported for long-lived perennials or outcrossing species ($H_o = 0.63$ (Nybom, 2004)). Furthermore, in accordance with their clonality rates which may promote geitonogamy (Halkett et al., 2005), *V. humblotii* from Sohoa population (Mayotte) ($G/N = 88\%$) had a lower genetic diversity ($H_e = 0.450$) (Gigant et al., 2016) than population of *V. humblotii* in Madagascar ($G/N = 100\%$, $H_e = 0.69$). Probably for the same reason, the *V. allorgii* population with high clonality ($G/N = 27\%$) showed the lowest diversity ($H_e = 0.40$, $Ar = 2.86$) of the Malagasy species.

The effective population size (N_e) is an essential parameter for conservation of species, as it can influence the allelic frequencies, the inbreeding rate and the genetic diversity of a species (Frankham et al., 2010). To avoid inbreeding depression and maintain genetic diversity, a N_e greater than 50 is essential in the short-term, and greater than 500 is required in the long-term (Frankham et al., 2014, 2010). If the population size is larger than the estimated N_e ($N > N_e$), the value of N_e is generally unbiased (England et al., 2006). In opposite, if $N < N_e$, then N_e may be underestimated (England et al., 2006). In this study, it was difficult, or even impossible, to estimate the N_e of *V. atsinananensis* and *V. allorgii*, respectively, because of the small population sizes. The species with the highest genetic diversity, *V. bosseri*, showed also the highest N_e ($N_e = 204-263$), a value which may be underestimated as $N < N_e$. The effective population sizes of *V. madagascariensis* ($N > N_e$, $N_e = 103$) and *V. perrieri* ($N > N_e$, $N_e = 69$) exceeded the minimum N_e value of 50 for short-term survival of species at 0.02 cut-off. *V. decaryana* and *V. humblotii*, in contrast, showed the lowest N_e ($N_e < 50$ at 0.02 and 0.05 cut-offs), values that did not reach the minimum requirement of 50. This result is consistent with the finding of Schoen & Brown (1991) that in general N_e is correlated with species diversity and inbreeding coefficient, species with lowest diversity and highest FIS having the lowest N_e (the case of *V. decaryana*) (Schoen and Brown, 1991). According to Waples & Do (2010), the estimates of N_e obtained should be reliable when the N_e is small (100-200 or less) and the size of population used for estimation is about 50 individuals. When N_e is less than 50, a population of 25 to 30 individuals should also provide useful N_e estimation (Waples and Do, 2010). Nevertheless, the N_e estimated in this study are indicative and should be considered with caution since the Linkage Disequilibrium method should be conducted on isolated populations, with discrete generations, and under random mating (Wang et al., 2016; Waples et al., 2014), which might not be the case of most Malagasy leafless *Vanilla* species (long-lived due to vegetative multiplication and $FIS > 0$).

b. Past, current and future suitable habitats

Based on our previous study, differentiation of the seven Malagasy leafless *Vanilla* species resulted from isolation by environmental factors (Andriamihaja et al., 2021). Thus, the suitable habitats of some species are probably different although they are all adapted to dry conditions. In Madagascar, the Last Glacial Maximum period was marked by an intense drought and a temperature decrease of 4°C compared to the present times (Burney, 2004). Since ecological niche modeling indicated that the potential ranges of all species were primarily a function of precipitation and isothermality, the 4°C temperature drop does not appear to be a true limiting factor in the suitable habitats of these species. Leafless *Vanilla* species would likely have occupied all dry locations without any real change of seasons (isothermality) as they are adapted to drought.

During LGM, the Central highlands were dominated by ericoid bushes (Burney, 2004). Tropical montane forests were located in lower elevations than today, along the East coast, the Northwestern and the Central parts of the island (Burney, 2008, 2004; Goodman and Jungers, 2014). According to Burney (2004), today's species isolated in high regions would have probably been distributed over a much larger area in the past. This is probably the case for *V. allorgii*, currently located in a high-altitude forest (800-1000 m), but which, according to the modeling, probably had a wider ecological niche distributed in the central part of Madagascar, in the west and the south during LGM. In opposite, the current Western vegetation and the Western late Pleistocene vegetation could have been not very different from today during the LGM (Burney, 2004). That could explain the similar past and present distribution configuration of the Western species. Indeed, as in the present time, four species may have had an exclusively Western distribution (*V. bosseri*, *V. decaryana*, *V. madagascariensis*, *V. humblotii*), one species was predominantly distributed along the East coast (*V. atsinananensis*), one species probably skirted the Central highlands and the West (*V. allorgii*), and one species potentially occurred in the West and slightly in the East (*V. perrieri*). However, there is some bias to the range distribution predicted by the models because it does not consider some ecological variables on which *Vanilla* species are highly dependent such as plant tutors, pollinators, mycorrhizal fungi (McCormick et al., 2012; McCormick and Jacquemyn, 2014; Porrás-Alfaro and Bayman, 2007). The integration of those variables, for example “vegetation” variable, would much improve our past predictions.

In any case, the potential habitat areas of the different species have been considerably reduced in the present time, essentially due to deforestation resulting from burning for charcoal (Burney, 2004) and human activities increasing pre-existing natural fire (e.g. in Central highlands) or spreading fire to environment where it is naturally scarce (e.g. East, Southwest, North) (Burney, 2004; Heled and Drummond, 2012). Indeed, three interdependent variables were identified by the models as contributing most to the current species distributions (Table 5): i) the vegetation type, ii) the vegetation production and iii) the human influence index based on human population density, land transformation, human access and infrastructure (Sanderson et al., 2002). Human arrived and settled in Madagascar about two millennia ago (Burney,

2004). The fire ecology has been progressively changed since the first human settlement, appearing firstly in the Southwest, then spreading along the West coast to wetter forests, until reaching the highlands about 1400 years before the present (Burney, 2004). Eastern forests were the last to be affected by human activities (Burney, 2004). According to different studies, forest cover of Madagascar has been decreased from 20,000 Kha in 1895 (Dufils, 2008) to 8,925 Kha in 2014 (Vieilledent et al., 2018), a loss of approximatively 11,000 Kha in a 119-year interval. The present niche modeling matches with the known distribution of species but allows identifying new areas for prospections, particularly for the recent discovered species *V. allorgii* and *V. atsinananensis*. As in the past, *V. bosseri* and *V. perrieri* are most widely distributed along the West Coast (*V. perrieri* extending more to the South), *V. madagascariensis* and *V. humblotii* are restricted to the Northwest, *V. decaryana* is limited to the Southwest and the two species, *V. allorgii* and *V. atsinananensis*, are specific to Central highlands and the East coast, respectively. This implies two new facts: i) the distribution of the leafless species is expected in a larger proportion in the East, an area of distribution never described before (Cribb and Hermans, 2009; Portères, 1954), ii) *V. madagascariensis* has got a much narrower distribution than published in the literature (Cribb and Hermans, 2009), probably because of taxonomic confusion with three other species (*V. bosseri*, *V. atsinananensis*, *V. allorgii*).

Based on the climate change scenario, results of MaxEnt analysis indicated in general the maintenance or the expansion of potential suitable habitats of leafless *Vanilla* species in Madagascar towards 2050 compared to the current environmental conditions (Figure 3, Figure 4), an expected result as leafless *Vanilla* species are adapted to drought conditions. Indeed, climate changes expected cause an increase in temperature seasonality and mean annual temperature, as well as an increase precipitation during the rainy season and a decrease in rainfall during the dry season in Madagascar (Tadross et al., 2008; Vieilledent et al., 2016). The same trend, i.e. stability or increase in geographical distribution, could also occur for some deciduous perennial and aridity-adapted species in Mongolia, China and Ethiopia (Li et al., 2020; Noulèkoun et al., 2017; Qin et al., 2020) and for baobab species adapted to very dry and hot areas (*Adansonia grandidieri*) in Madagascar (Vieilledent et al., 2013; Wan et al., 2021). However, despite this expansion of ecological niches, some habitats may disappear in the North (e.g. *V. bosseri*, *V. perrieri*, *V. madagascariensis*), probably due to the increase of humidity in this area. According to Tadross et al. (2008), indeed, it is possible that the Northern region would become progressively wetter, and temperature increases would be moderated by moisture, clouds and rainfall in this region. Due to climate change, some of the extended distribution areas in 2050 (e.g. *V. allorgii*, *V. atsinananensis*, *V. decaryana* and *V. perrieri*) could disappear towards 2080 (Figure 3, 4). Indeed, the latest study on climate trends and future climate change in Madagascar has shown that, depending on the projections used, rainfall will probably increase between 2050 and 2080 (Raholijao et al., 2019). As in the case of three baobab species adapted to the dry climate of Southern Madagascar (*Adansonia grandidieri*, *Adansonia rubrostipa*, *Adansonia za*) (Wan et al., 2021), the majority of Malagasy leafless *Vanilla* species are therefore precipitation sensitive. This precipitation increase between 2050 and 2080 should be about 0.5% in the Northwest, 0.4% in the East coast, 0.4% in the

Central highlands and probably no increase in the Southwest (Raholijao et al., 2019). A study on 74 endemic plants of Madagascar indicated significant changes in suitable habitats by 2080 for many species, with 25 species that could expand their geographical distribution and 42 species exhibiting habitat reduction (Schatz et al., 2008). They demonstrated that distribution areas of species found in the humid littoral forests, the Central highlands sub humid forests, and the far Northern dry forests would probably exhibit dramatic reduction by 2080 (Schatz et al., 2008). They also indicated that all species located in the Western and Southwestern dry to arid forest would probably maintain or increase their range in 2080 (Schatz et al., 2008). Nevertheless, the distribution of orchid populations depends, in addition to climate factors, on different biological and ecological parameters including availability of mycorrhizal fungi for seed germination and availability of pollinators for seed production (Ackerman et al., 1996; McCormick and Jacquemyn, 2014; Rasmussen et al., 2015, 2015). Studies have demonstrated that *Vanilla* species are associated with soil fungi (Porrás-Alfaro and Bayman, 2007). Yet, soil biomes as well as the distribution, structure and abundance of insect communities may be impacted by climate change (Jansson and Hofmockel, 2020; Nooten et al., 2014). As with past distribution, future projections do not account for human influences, vegetation or other biotic factors influencing orchid species distribution (McCormick and Jacquemyn, 2014). So, the distribution modelling of the seven species could be further improved by considering human influence and vegetation as well as other biotic factors (pollinators, disperses, mycorrhiza distribution), when knowledge becomes available, as performed for *V. planifolia* on the American continent (Ellestad et al., 2021).

c. Conservation implications for each species

To determine forest threats and define conservation plans, conservationists typically look at several parameters including the overall conservation status of plant and animal species. Based on a solid foundation of research and monitoring, the International Union for Conservation of Nature (IUCN) is widely recognized as the most reliable reference tool for assessing species status. The IUCN Red List uses a set of specific criteria based on population size, distribution area and number of mature individuals to assign each species or subspecies to one of nine categories (IUCN, 2001). The present work does not yet allow defining the IUCN categories of the seven Malagasy species. However, it provides a set of key information (Ne, biological reproduction, suitable habitats) that may be useful in determining the threats to these species. On the basis of effective population size criteria and biological reproduction, *V. allorgii* could be considered as critically endangered. Indeed, the only known *V. allorgii* population to date, located in a sacred forest with limited area, showed the highest density (197 plants/km) and had a high flowering rate (52%) but the lowest natural fruit set (fruit set = 0.61%), most likely due to the loss of interaction with pollinators. As a consequence, a strong clonality rate (73%) was detected resulting in an excess of heterozygotes. This population of *V. allorgii* will be, in the long term, threatened by the loss of genetic diversity as it already shows the lowest allelic richness (2.86) and He (0.40). Our modeling results are essential to identify suitable areas to urgently survey to verify if other populations exist. Even if the distribution predictions showed

that its suitable niche should increase in 2050, a clear decrease in this distribution area could occur around 2080 if no specific measures are taken. Genetic and reproductive status of the other identified populations should be determined and conservation plans should be established in all populations identified in order to limit the future impacts of anthropogenic activities that could further reduce the distribution of the species.

V. atsinananensis has been, until now, only observed in two Eastern forests of Madagascar. In the Manompana (MAN) population, the number of individuals was low ($N=11$, unique genotype = 9) over a distance of 1.5 km and the flowering rate was among the lowest (13%). Small populations, like *V. atsinananensis*, are more prone to extinction as they are more vulnerable to genetic drift (Ellstrand and Elam, 2021) and inbreeding (at a faster rate than large population), and can therefore suffer from genetic constraints (reduced capacity of adaptation) (Frankham et al., 2010). Given the few available information on this species, the minimum effective population size rule and the population density, *V. atsinananensis* could be considered as critically endangered. Like for *V. allorgii*, a major effort is urgently needed to prospect suitable habitats identified in the present study. According to the present modelling, this species seemed to have the smallest ecological niche compared to other species. Nevertheless, its range could still extend along the Eastern coast of Madagascar. Moreover, a MNHN herbarium plate (Andriamihaja et al., in prep), which we did not use in our analyses, indicates a location that agrees with our current modeling results.

V. decaryana is also among the most endangered of the seven species. Indeed, this species showed the lowest effective population size ($N_e=19-39$), the highest inbreeding coefficient ($FIS=0.43$) and the lowest number of private alleles among the Western white flower species. Although its natural fruit set can be high, showing a good interaction with pollinators in some populations ($MLT=11.88\%$), the flowering rate is variable (9 to 35%). As *V. decaryana* is adapted to arid conditions in the South, the ecological niches should increase in 2050 compared to the current distribution. However, the modelling predicted a decrease between 2050 and 2080. Here, four populations of *V. decaryana* among the seven studied were found in unprotected forest (AJA, SAG, MLT, ITB), one in sacred forest (ATD) and two in a national park and a new protected area (KRM, MFD). According to Frankham (2003), genetic diversity is in general high when gene flow between several populations of the species occur in a region. But, despite the fact that identified populations of *V. decaryana* are geographically close, their genetic diversity was low probably resulting from low gene flow between populations. Indeed, previous study demonstrated the existence of barriers to gene exchange between the *V. decaryana* populations studied (Andriamihaja et al., 2021), suggesting reproductive isolation of populations. The massive collection for medicinal use of the species in the South (Rakotoarivelo et al., 2019; Randriamiharisoa et al., 2015; Randrianarivony et al., 2017) combined with this low effective population size and low genetic diversity are extremely worrying and could threaten the short and long time survival of *V. decaryana*.

V. humblotii has been classified as vulnerable and considered as a protected species in Mayotte according to the IUCN France assessment (IUCN et al., 2014). Despite the high natural fruit

set and the high level of genetic diversity, the only known population of *V. humblotii* in Madagascar has a very low flowering rate (7%). At the same time, its effective population size is low ($N_e = 35$ and 38 at cut off of 0.02 and 0.05) and the modelled current distribution appears to be very limited unlike the other species. Therefore, overall, the species appears to face a significant danger within our study site in Madagascar. Nevertheless, N_e of *V. humblotii* may be underestimated as $N < N_e$ and predicted future scenarios show considerable increase in the amounts of suitable habitats.

V. madagascariensis is the white flower species with the most limited distribution area (only in the North) (Figure 3E). Its genetic diversity and natural fruit set was relatively good despite the heterozygote deficit ($FIS > 0$). Based on effective population size and considering the adaptation to drought, *V. madagascariensis* seems viable in the short term ($N_e \geq 50$) but should be considered as vulnerable given the N_e value that does not meet the threshold required for long-term survival. Besides, potential distributions of *V. madagascariensis* in the North may be declining in the future, requiring further studies of the soil and symbiotic associations to help prevent this reduction.

V. bosseri and *V. perrieri* are the species with the highest genetic diversity in terms of allelic richness and observed heterozygosity (Table). These species are distributed along the whole West coast of Madagascar, the widest distribution (Figure 3). The flowering rate reached the value of 30% (*V. perrieri*) and 60% (*V. bosseri*) and the natural fruit sets were relatively good (4%) compared to what is expected in wild *Vanilla* populations. For *V. bosseri*, the effective population size of 204-263, may be underestimated as $N < N_e$ (England et al., 2006), and appears sufficient for short-time survival and not far from the value defined by Frankham et al. (2010) for long-term survival. For *V. perrieri*, N_e is smaller ($N_e = 49-69$) and barely reaches the values defined for short-term survival. Considering the reproductive status, the current and future widest distribution and the rule of Frankham, *V. bosseri* and *V. perrieri* do not seem to be threatened in the short term, despite the observed inbreeding ($FIS > 0$). However, species distribution modelling predicts a future (2050, 2080) loss of some *V. bosseri* and *V. perrieri* habitats in the far North and in some regions in the West.

By combining the current distribution areas of the seven species, potential areas of sympatry were identified. Among these regions, special attention should be given to the districts of Ambanja (AMB), Mitsinjo (in Complexe Mahavavy Kinkony reserve, CMK), Soalala, Marovay (in Ankarafantsika National Park, AKF), Analalava, Besalampy, Ambilobe in the North and the district of Morombe, Morondava, Betioky Atsimo, Toliara II, Belo sur Tsiribihina in the South, where up to three species could currently cohabit together (Figure 4). Four districts (Mitsinjo, Soalala, Morombe, Toliara II) among those previously mentioned as potential current sympatric areas would also probably host up to four species towards 2050. In view of their specific richness, these regions should be further surveyed for the conservation of leafless *Vanilla* species in Madagascar. Indeed, maintaining the evolutionary processes that have generated taxonomic biodiversity is the most suitable approach for the conservation of taxonomically complex groups (Ennos et al., 2005) like the genus *Vanilla* (Andriamihaja et al., 2020; Bory et

al., 2010). Thus, sympatric zones must be regarded as key areas when developing strategies for conservation as they provide a step for evolutionary processes.

4.2.5. Conclusion

It is now globally recognized that biodiversity faces an unprecedented decline since many years. This situation is more worrying in developing countries where natural ecosystems are rapidly converted to agricultural land and urban area causing climate and biotic changes. These changes could impact severely the conservation of some groups of orchids due to their life cycle depending on many parameters such as the availability of tutors (for epiphytes), the abundance of pollinators, the symbiotic association with soil mycorrhiza and abiotic factors. Thus, the conservation of orchids requires knowledge of several biological parameters including population genetics, reproductive biology, demography and distribution, to be effective. Among the 1770 orchids listed in the global online database of the IUCN (<https://www.iucnredlist.org/>), only 13 *Vanilla* species have been classified within the nine categories and none of the leafless *Vanilla* species located in Madagascar is present in this list. This study is a first step towards a conservation plan of the Malagasy leafless *Vanilla* species. It provided information to further evaluate the conservation status of these seven Malagasy leafless *Vanilla* native species. It also allowed to identify new potential areas of distribution of different species in Madagascar. As most allogamous *Vanilla* species, they can exhibit sexual as well as asexual reproduction, they have low natural fruit set (<30%), they are self-compatible and could hybridize with each other. Genetic diversity is generally high for all species except for the Central highland species *V. allorgii* which also exhibits the lowest rate of fruit set and the highest rate of clonality. Distribution of the seven species is currently quite wide except for *V. humblotii* which is present on only 1% of the surface of Madagascar. But, thanks to their capacity to adapt to drought, these species could expand their ecological niches in the future. In any cases, the results of our past, present and future modeling indicate that the distribution pattern of leafless *Vanilla* species is sensitive to moisture, vegetation and human influence. Our data do not yet allow to classify each species in the IUCN categories, because we lack a wider assessment of population sizes of the species at the scale of Madagascar. Also, future distribution modelling is not adapted to IUCN criteria because temporal scale is different and the assumption of a relationship between range distribution area and population size is not verified (Akçakaya et al., 2006). However, based on reproductive strategy, genetic analysis and species distribution area, we obtained informations suggesting that two species (*V. allorgii* and *V. atsinananensis*) may be critically endangered, one species (*V. decaryana*) could be endangered, two may be vulnerable (*V. humblotii* and *V. madagascariensis*) and two could be considered as near threatened or least concern (*V. perrieri* and *V. bosseri*). If considering the revised recommendations for 50/500 rules to 100/1000 (Frankham et al., 2014), then the long term evolutionary potential of all species is threatened and most species (except *V. bosseri* and *V. madagascariensis*) might suffer inbreeding depression in short time. Nevertheless, studies on pollination and mycorrhizal associations as well new prospections of other populations have to be made to confirm our results regarding genetic diversity and reproductive biology of the seven leafless species and to

improve the delimitation of their ecological niches and to measure potential sizes in Madagascar.

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CHAPITRE 5

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Conclusion générale et perspectives

« Nous n'atteindrons jamais l'harmonie avec la terre, pas plus que nous n'atteindrons la justice ou la liberté absolues pour les personnes. Dans ces aspirations supérieures, l'important n'est pas d'atteindre, mais de s'efforcer. »

Aldo Leopold

Ce travail a été initié avec la volonté de résoudre la question générale de la taxonomie du complexe d'espèces aphyllés appartenant au genre *Vanilla* à Madagascar face à l'insuffisance d'informations sur les populations de ces espèces, afin de disposer des éléments nécessaires à la définition d'un schéma de conservation. D'après Olivieri et al. (2016), la biologie de la conservation devrait également promouvoir les processus évolutifs à tous les niveaux de la biodiversité en plus de veiller à la conservation des habitats, de la richesse spécifique ou des espèces.

Dans cette perspective, la problématique de cette thèse a été abordée en essayant tout d'abord de comprendre les mécanismes d'évolution en jeu dans la divergence des espèces de vanilliers aphyllés à Madagascar. Les résultats du Chapitre 2 ont indiqué un effet combiné de plusieurs facteurs incluant les barrières géographiques (rivières) responsables d'une différenciation entre populations géographiquement proches. Par ailleurs, des effets combinés de l'IBD/IBE et de l'IBE/IBA ont été détectés, suggérant une spéciation liée aux conditions écologiques, elles-mêmes fortement structurées géographiquement à Madagascar. Les facteurs abiotiques (climat, sol) comme biotiques (phénologie, mycorhizes) sont probablement responsables de cet isolement par les facteurs environnementaux (IBE). L'isolement par adaptation (IBA) détecté sur les caractères floraux est, quant à lui, soit issu de la sélection divergente liée à une spécificité de pollinisateurs, soit une conséquence probable de l'IBE, selon les espèces considérées. Néanmoins, des études supplémentaires sur les facteurs biotiques seront nécessaires pour compléter ces informations.

Ensuite, les questions liées à la taxonomie de ces espèces ont été traitées dans le Chapitre 3. La combinaison de plusieurs outils (génétiques et morphologiques) a permis d'identifier neuf espèces de vanilliers aphyllés dans la région Sud-Ouest de l'Océan Indien (SOOI) dont sept espèces déjà recensées dans la littérature et deux nouvelles espèces décrites au cours de cette thèse. Bien que morphologiquement très similaires, les espèces de Madagascar ont finalement chacune des traits floraux qui permettent de les distinguer allant de la forme des fleurs (taille des fleurs, texture du labelle) à leur couleur (couleur de la fleur et/ou du labelle) (Chapitres 2 et 3). Ainsi, la difficulté de l'identification taxonomique de ces espèces est bien liée à leur origine récente et à leur capacité de s'hybrider, mais l'utilisation de marqueurs génétiques appropriés via une approche intégrative a permis de résoudre ce puzzle taxonomique. Par ailleurs, les analyses phylogénétiques ont permis de montrer que, via une origine africaine, l'ancêtre commun de toutes ces espèces aphyllés de la zone Sud-Ouest de l'Océan Indien était

d'abord présent à Madagascar et a conduit à la diversification de deux grands clades respectivement d'espèces à fleurs blanches et à fleurs jaunes. La colonisation des autres îles du SOOI (et un retour vers l'Afrique) s'est faite ultérieurement au sein de ces clades à partir de Madagascar. Par ailleurs, nos résultats suggèrent également que l'espèce foliée (*V. francoisi*) endémique de Madagascar aurait une origine phylogénétique différente de celle des espèces de vanilliers aphylls.

Enfin, certaines informations importantes pour la conservation de ces espèces ont été apportées dans le Chapitre 4. Sur la base des tailles effectives estimées des populations, des taux de fructification et de floraison et de la modélisation des distributions écologiques spatiales et temporelles, deux espèces apparaissent comme en danger critique, une semble en danger, deux sont probablement vulnérables, et les deux dernières seraient probablement soit quasi menacées, soit à préoccupation mineure à Madagascar.

Ainsi, ce travail de thèse permet de développer trois conclusions principales :

- Madagascar constitue un modèle intéressant pour l'étude de la spéciation des orchidées ;
- Des études complémentaires sont nécessaires afin d'approfondir nos connaissances sur les mécanismes d'évolution de ces espèces ;
- Une stratégie de conservation doit être mise en place pour les espèces menacées.

Ces trois points constituent les principaux éléments de discussion dans ce dernier chapitre avant de proposer des applications possibles des résultats issus de ce travail.

5.1. Madagascar : un modèle d'étude pour l'évolution des orchidées

Représentant environ 6 à 11% des espèces à graine, la famille des Orchidaceae fait partie des groupes de plantes les plus diversifiés (Pillon and Chase, 2007). Après la Nouvelle-Guinée, Madagascar se classe en deuxième position parmi les îles continentales ayant la plus grande proportion d'orchidées endémiques, avec près de 1000 espèces dont environ 83% sont endémiques (Cribb and Hermans, 2009; Taylor et al., 2019), faisant d'elle l'un des sites d'études les plus importants sur les orchidées et leur conservation. Appelée île-continent en référence à sa grande superficie, Madagascar présente sept écorégions (Chapitre 1) ayant certainement joué un rôle majeur dans la diversification des orchidées en permettant la radiation des espèces colonisatrices en plusieurs espèces distinctes dans des environnements différents (spéciation écologique), un mécanisme de « contrainte éco-géographique » (Vences et al., 2009). Chacune de ces écorégions a une structure distincte de communautés végétales qui reflète la diversité des types de sols, des substrats rocheux, de l'altitude et du climat (Goodman, 2008). Chez les vanilliers aphylls, nous avons vu que *V. allorgii* et *V. atsinananensis*, réparties respectivement dans les forêts subhumides (jusqu'à 900-1000 m) et humides de l'Est, ont des caractéristiques morphologiques et génétiques assez éloignées des cinq autres espèces de l'Ouest de Madagascar localisées dans les forêts denses sèches ou arides. Des cas similaires d'espèces appartenant à des niches écologiques différentes sont fréquents dans d'autres genres de la famille des Orchidaceae

à Madagascar (Cribb and Hermans, 2009). Par exemple, les espèces *Brachycorythis disoides* et *Brachycorythis pleistophylla* qui sont toutes les deux endémiques de Madagascar sont respectivement réparties dans les hautes terres centrales (Antananarivo, Fianarantsoa) et dans les zones côtières (Antsiranana, Majunga, Toamasina) (Cribb and Hermans, 2009). Le genre *Grammangis* est constitué de deux espèces endémiques dont l'une (*G. ellisii*) est distribuée dans les écorégions humides et subhumides du Nord et de l'Est jusqu'à une altitude de 1300 m, tandis que la deuxième (*G. spectabilis*), est uniquement présente dans l'écorégion sèche et aride du Sud-Ouest à une altitude entre 300 et 800 m (Cribb and Hermans, 2009).

Les mécanismes ayant favorisé l'importante diversification des orchidées constituent un sujet très exploré depuis l'époque de Darwin (Cozzolino and Widmer, 2005; Darwin, 1859; Givnish et al., 2015; Johnson et al., 2018; Schiestl and Schlüter, 2009; Scopece et al., 2008). Une variété de traits adaptatifs a pu faciliter la divergence écologique des orchidées incluant la photosynthèse CAM, l'épiphytisme, l'association à des mycorhizes, l'évolution de la pollinie et la pollinisation par déception (Ayasse et al., 2011; Givnish et al., 2015; Otero et al., 2007; Schiestl and Schlüter, 2009).

D'après Givnish et al. (2015), la photosynthèse CAM (Crassulacean Acid Metabolism) peut probablement accélérer la spéciation et la diversification des orchidées en permettant aux espèces de coloniser des sites plus exposés et plus secs que leurs milieux d'origines. Les travaux réalisés par Silvera et al. (2009) sur plusieurs espèces d'orchidées américaines ont montré que la photosynthèse en C3 est l'état ancestral et la CAM a évolué plusieurs fois indépendamment avec plusieurs inversions dans la grande famille des Orchidaceae. À Madagascar et en Afrique, les travaux de Bone et al. (2015) sur des espèces appartenant à la sous-tribu Eulophiinae (Orchidaceae) ont démontré que la photosynthèse CAM est associée au changement environnemental (climat et sol). En effet, le mécanisme CAM a évolué dans quatre lignées d'orchidées terrestres dont les ancêtres communs sont originaires d'habitats humides à Madagascar (Bone et al., 2015). Ces quatre lignées ont colonisé des niches écologiques présentant un gradient différent de sécheresse (Bone et al., 2015). Pour les lignées CAM ayant les richesses spécifiques les plus importantes, d'autres traits supplémentaires sont également apparus (hétérophyllie et panachure des fleurs) (Bone et al., 2015). L'épiphytisme est étroitement lié à la photosynthèse CAM (Silvera et al., 2009) et permet également d'envahir une nouvelle zone écologique (Givnish et al., 2015). D'après les travaux de Silvera et al. (2009), les épiphytes de basses altitudes ont une plus forte prévalence en voie photosynthétique CAM et les épiphytes dans les forêts nuageuses humides de haute altitude ont une tendance à se différencier rapidement. Cependant, Bone et al. (2015) ont démontré chez les espèces appartenant à la sous-tribu Eulophiinae, qu'au contraire, le passage du mode épiphyte des lignées ancestrales au mode terrestre des lignées actuelles semble coïncider avec l'aridification du climat et l'expansion des habitats plus ouverts à Madagascar. Ainsi, le processus de spéciation liée à la photosynthèse CAM est encore difficile à cerner et est sûrement associé à d'autres facteurs importants de la spéciation. En majeure partie héli-épiphytes, parfois épiphytes (Cameron, 2011; Portères, 1954; Soto Arenas and Cameron, 2003; Soto Arenas and

Dressler, 2010), les espèces appartenant au genre *Vanilla* sont connues pour avoir une stratégie de photosynthèse CAM (Gehrig et al., 1998; Reinert et al., 1997; Silvera et al., 2009; Ting, 1989). Compte tenu du fait que la stratégie CAM est présente aussi bien chez des espèces foliées (ex *V. planifolia* (Gehrig et al., 1998; Reinert et al., 1997; Ting, 1989), *V. chamissonis* (Reinert et al., 1997), *V. inodora* (Silvera et al., 2005)) adaptées aux milieux humides que chez les espèces aphyllées (ex *V. barbellata* (Ting, 1989)) adaptées aux milieux secs, il est peu probable que la photosynthèse CAM soit impliquée spécifiquement comme mécanisme d'adaptation aux milieux arides pour les vanilliers aphyllés du SOOI, mais peut expliquer la grande variété d'habitats dans lesquels les espèces du genre ont pu s'adapter.

L'étude de Bone et al. (2015) a également révélé l'évolution de la mycohétérotrophie (association plantes-champignons) chez certaines espèces ayant colonisé les zones sèches du Sud de l'Afrique (Bone et al., 2015). L'association avec des mycorhizes permet aux plantes de se priver d'un appareil photosynthétique (feuilles chlorophylliennes) (Bidartondo et al., 2004), et par conséquent de limiter l'évapotranspiration et aussi de s'adapter aux environnements secs (Bone et al., 2015). Toutes les orchidées entretiennent une relation symbiotique avec des champignons pour leur germination (McCormick et al., 2012; McCormick and Jacquemyn, 2014). Si pour certaines cette association se passe durant le premier stade de développement de la plante (germination, protocorme), pour d'autres espèces l'association est nécessaire pendant toute la durée de la vie (McCormick et al., 2012; McCormick and Jacquemyn, 2014) et peut parfois aboutir à une spécificité aux mycorhizes (Otero et al., 2007). Une étude réalisée dans une forêt tropicale du Mozambique a démontré une forte relation entre le type d'habitat (prairies, savanes, forêt de *Faidherbia*, forêt d'*Acacia*, savane boisée Miombo) et les communautés mycorhiziennes du sol (Rodríguez-Echeverría et al., 2017). Les analyses réalisées en Amérique sur *V. planifolia*, *V. poitaei*, *V. phaenantha* et *V. aphylla* ont montré que ces différentes espèces, qui sont localisées dans différents sites, sont associées à différents champignons mycorhiziens (Porrás-Alfaro and Bayman, 2007). Cependant, ces résultats différents entre les quatre espèces de vanilliers pourraient également s'expliquer par la différence du milieu (climat, sol, etc.) dans lesquelles elles ont été échantillonnées (Porrás-Alfaro and Bayman, 2007). À Madagascar, la diversité de champignons mycorhiziens associés aux orchidées varie avec la saison (climat), le type de sol et le type de forêts (Yokoya et al., 2021, 2015). Comme nos résultats (Chapitre 2) ont indiqué un rôle probable des facteurs abiotiques (pH du sol, température et précipitation) dans la différenciation des vanilliers aphyllés, l'analyse de leur association symbiotique avec les champignons du sol constitue aussi une piste intéressante à explorer. Une étude en cours dans le cadre de la thèse de Botomanga Alemao à l'Université d'Antananarivo traite actuellement ces questions liées aux mycorhizes. L'étude consiste, entre autres, à estimer le taux de germination en milieu naturel et tester le taux de germination par graine en condition artificielle. Les premiers résultats indiquent un taux quasi nul de la régénération naturelle dans plusieurs sites (Ramarosandratana A.V., pers. com.), ce qui suggère une incapacité des semis à atteindre le stade adulte pour différentes raisons possibles dont l'appauvrissement du sol en matières organiques et/ou l'absence de

champignons mycorhiziens symbiotiques pourtant cruciaux pour la germination des orchidées (McCormick et al., 2012; Porras-Alfaro and Bayman, 2007).

L'évolution de la pollinie, en conduisant au placement précis des pollens sur différentes parties du corps d'un pollinisateur, tout comme la pollinisation par déception (mimétisme des partenaires potentiels ou de la nourriture), ont été montrés comme étant des facteurs importants de l'isolement reproductif (induit par une spécificité aux pollinisateurs) chez les orchidées (Givnish et al., 2015). En effet, les variations évolutives des tailles d'éperon chez certaines orchidées se produisent même en l'absence de récompense (nectar) (Givnish et al., 2015). Les insectes sont connus pour être les principaux pollinisateurs chez les orchidées (Dressler, 1967; Jersáková et al., 2006; Schiestl and Schlüter, 2009), ayant parfois conduit à des cas de spécificité aux pollinisateurs (Darwin, 1859; Householder et al., 2010; Lubinsky et al., 2006; Micheneau et al., 2009; Peter and Johnson, 2014; Tremblay, 1992). À Madagascar, les insectes sont très diversifiés. Par exemple, le pays présente un taux d'endémisme très élevé en espèces d'abeilles (90% d'espèces endémiques et cinq genres endémiques) (Eardley et al., 2009). Des cas d'interactions spécifiques entre des insectes et des orchidées ont été reportés à plusieurs reprises dans la grande île (Nilsson et al., 1992, 1986; Petersson, 2015; Wasserthal, 1997). Comme les insectes ont un schéma de distribution varié à Madagascar allant d'une très large répartition (ex : *Halictus jucundus*) à une seule localité (ex : *Nubenomia luridipes*) (Pauly et al., 2001), cela aurait probablement joué un rôle dans la spéciation écologique entre les espèces d'orchidées appartenant à des écorégions très différentes de l'île. Pour le cas des vanilliers aphyllés de la zone SOOI, seules deux études ont permis, à ce jour, de déterminer les pollinisateurs potentiels (Gigant, 2012; Gigant et al., 2016; Petersson, 2015). À Madagascar, dans la population KRD de *V. bosseri*, quatre espèces d'abeilles ont été identifiées comme étant des candidates potentielles pour la pollinisation de l'espèce (Petersson, 2015) incluant trois espèces largement réparties du Nord au Sud de la côte Ouest (*Macrogalea ellioti*, *Liotrigona madecassa*, *Liotrigona mahafalya*) (Pauly et al., 2001) et une espèce présente uniquement dans le Sud-Ouest et au Nord-Est de l'île (*Lithurgus pullatus*) (Pauly et al., 2001). Les pollinisateurs potentiels sont donc probablement similaires pour les espèces de Nord et de l'Ouest (*V. madagascariensis*, *V. bosseri*, *V. decaryana*, *V. humblotii*, *V. perrieri*), mais pourraient être différents pour les espèces de l'Est (*V. atsinananensis* et *V. allorgii*), notamment considérant les différences importantes au niveau de la taille des fleurs. Tenant compte du profil de distribution des pollinisateurs et la présence de quelques individus hybrides révélés par nos résultats (Chapitre 2), nous suspectons plutôt un système généraliste de pollinisation, du moins pour les vanilliers aphyllés du Nord et de l'Ouest. Ainsi, l'identification des pollinisateurs associés à chaque espèce, voire des disperseurs potentiels, serait un atout considérable pour approfondir nos connaissances sur les mécanismes de spéciation écologique en jeu et la biologie de la reproduction, mais aussi pour affiner les cartes de distribution des espèces du genre *Vanilla* à Madagascar, comme ce qui a été réalisé pour *V. planifolia* (Ellestad et al., 2021).

Madagascar fait également partie d'un système insulaire comprenant plusieurs îles océaniques également à forte endémicité. La Réunion, par exemple, est l'île océanique la plus riches en

orchidées (Taylor et al., 2019). Mise à part leur richesse spécifique, l'isolement géographique des îles (frontières bien délimitées), leur petite superficie combinée à leurs milieux très hétérogènes ont été depuis longtemps reconnus comme très utiles pour comprendre les mécanismes évolutifs et écologiques (Warren et al., 2015). Les îles ont été les modèles choisis lors de l'élaboration de plusieurs théories dont celle de la sélection naturelle (Darwin, 1859) ou celle de l'équilibre dynamique (MacArthur, 1963; Wilson and MacArthur, 1967). Ce système insulaire de la région SOOI présente un fort intérêt dans les études de phénomènes de migration inter-îles. Chez les orchidées, et en particulier chez les vanilliers, la dispersion à longue distance est un phénomène fréquent (Bouetard et al., 2010; Givnish et al., 2015). À l'issue de leurs analyses phylogénétiques, Bouetard et al. (2010) ont, en effet, conclu que trois événements de migration transocéanique à partir du continent africain ont probablement entraîné la diversification des espèces aphyllées du genre *Vanilla* : i) la migration vers les îles du Sud-Ouest de l'Océan Indien pour les espèces du groupe « *V. phalaenopsis* », ii) vers les îles des Caraïbes pour les espèces du groupe « *V. barbellata* » et iii) vers l'Asie pour les espèces du groupe « *V. aphylla* » (Figure 1a,b,c). Les résultats du Chapitre 3 suggèrent, pour le cas des vanilliers aphyllés de la zone SOOI, trois événements supplémentaires de migration depuis Madagascar à partir des clades des espèces à fleurs blanches et des espèces à fleurs jaunes (Figure 1d,e,f). Ces événements de migration auraient probablement été, dans le cas de *V. roscheri* et *V. humblotii*, favorisés par les vents et les courants marins de Madagascar en direction de l'Afrique à partir du Miocène (Ali and Huber, 2010) combinés aux fronts cycloniques connus pour souffler principalement d'Est en Ouest dans la partie occidentale de l'Océan Indien (Wells, 2008). De plus, d'autres éléments génétiques viennent conforter la présence de *V. humblotii* à Madagascar avant de migrer vers l'Archipel des Comores : la diversité génétique est plus élevée pour la population de *V. humblotii* à Madagascar ($He = 0,69$) (Chapitre 4) comparée à celles des Comores et de Mayotte ($He_{global} = 0,21$) (Gigant, 2012; Gigant et al., 2016; M'sa, 2014). En effet, le déclin de diversité génétique, par les effets fondateurs successifs, indique généralement le sens de la colonisation (Hewitt, 1996; Prugnolle et al., 2005). Ces données génétiques sont actuellement en cours de ré-analyse, île par île, à l'échelle de l'espèce *V. humblotii* afin d'obtenir un degré élevé de précision. En ce qui concerne *V. phalaenopsis* des Seychelles, sa récente différenciation via un ancêtre d'origine malgache peut résulter d'une migration transocéanique qui a pu être facilitée par l'apparition de nombreuses petites îles aux alentours des Seychelles et de Madagascar il y a environ 0.5 Ma résultant de la baisse du niveau de la mer (Warren et al., 2010). Dans la région SOOI, la présence de plusieurs populations d'orchidées appartenant à la même espèce ou de plusieurs espèces appartenant au même genre dans les différentes îles reflète ces phénomènes migratoires inter-îles (Andriananjamanantsoa, 2015; Cribb and Hermans, 2009; Jaros et al., 2016; Micheneau et al., 2008; Pailler et al., 2018). La migration transocéanique d'une espèce végétale est rendue possible grâce à l'intervention de multiples vecteurs de dispersion, y compris le vent, les courants marins (Renner, 2004; Zhang et al., 2007) et les animaux migrateurs (oiseaux et mammifères) (Nathan, 2006). Parmi la faune capable de migrer à longues distances, les chauves-souris frugivores sont connues pour être d'importants disperseurs de pollen et de graines (Shilton et al., 1999).

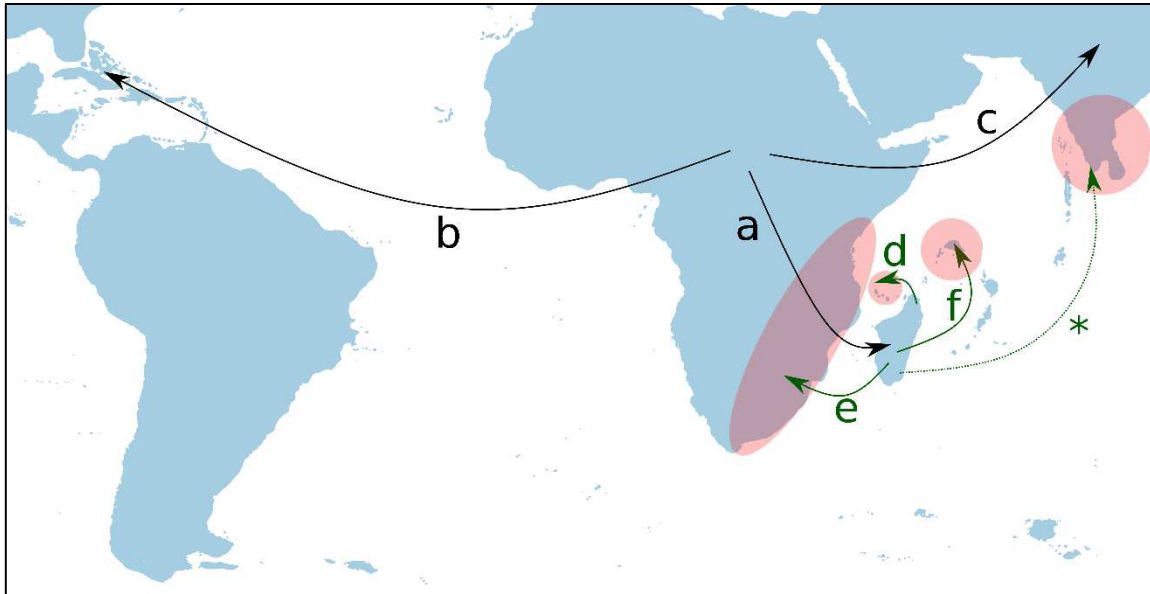


Figure 1: Événements supposés de dispersion dans l'histoire des vanilliers aphyllés

Figure réalisée à partir d'une carte du monde datant de l'âge de la dernière glaciation (~21 000 Ma), montrant les différentes îles qui sont apparues dans la région SOOI (Source : <http://www.worldclim.com/past>). a—Migration de l'Afrique vers Madagascar (Bouetard et al., 2010) (groupe « *V. phalaenopsis* » 4,4 Ma). b— Migration de l'Afrique vers les Caraïbes (Bouetard et al., 2010) (groupe « *V. barbellata* » 3,53Ma). c—Migration de l'Afrique vers l'Asie il y a 4,60 Ma (Bouetard et al., 2010) (groupe « *V. aphylla* ». d—Migration de Madagascar vers l'Archipel des Comores < 0,58 Ma (*V. humblotii*). e—Migration de Madagascar vers l'Afrique de l'Est (*V. roscheri* 0,46 Ma). f—Migration de Madagascar vers les Seychelles (*V. phalaenopsis* < 0,67 Ma). *—Migration de Madagascar vers l'Inde et le Sri Lanka (*V. walkeriae*).

Dans la région SOOI, une analyse phylogénétique réalisée sur des populations de *Miniopterus* suggère qu'au moins deux ou trois événements historiques de colonisation depuis Madagascar vers l'Archipel de Comores ont eu lieu, facilités par le sens du vent en direction de l'Ouest (Weyeneth et al., 2008). Il est donc possible que ces espèces de chauve-souris soient impliquées dans la dispersion des espèces d'orchidées, plus particulièrement celles des vanilliers aphyllés du SOOI. En effet, les chauves-souris sont décrites comme disperseurs possibles de *V. insignis* et vérifiés de *V. pompona* en Amérique tropicale (Soto Arenas and Dressler, 2010). Quoiqu'il en soit, le transport de propagules peut aussi simplement avoir été réalisé via des radeaux de végétations flottants (Bouetard et al., 2010).

Enfin, Madagascar est potentiellement un lieu privilégié pour étudier les phénomènes d'hybridation et/ou d'isolement reproducteur chez les orchidées grâce aux nombreuses zones de sympatrie où cohabitent plusieurs espèces génétiquement et morphologiquement proches (Cribb and Hermans, 2009). L'hybridation naturelle des orchidées de Madagascar est très peu documentée. Le seul cas d'hybridation recensé dans les publications, à notre connaissance, concerne celui de deux espèces sympatriques du genre *Cynorkis* endémiques des hautes terres malgaches (Hermans and Rajaovelona, 2019). Dans le cas des vanilliers aphyllés de Madagascar, la présence d'individus hybrides dans certains sites (ex : CMK, ATS, BBL, MSL, AKL, MLT) (Chapitre 2—Figure 4) ainsi que l'obtention de gousses âgées de 15 et 28 jours à partir des tests de pollinisation manuels effectués entre deux espèces à fleurs blanches (*V.*

madagascariensis et *V. bosseri*) et deux espèces ayant des fleurs de différentes couleurs (*V. madagascariensis* et *V. perrieri*), permettent de démontrer que l'isolement reproducteur ne s'applique pas à ce complexe d'espèces récentes (Figure 2).



Figure 2: Distribution spatiale des individus de *V. madagascariensis* (rouge), de *V. bosseri* (bleu) et des individus hybrides entre les deux espèces (blanc)

Figure représentant la population de la forêt du Complexe Mahavavy Kinkony (CMK).

5.2. L'évolution des vanilliers aphyllés du Sud-Ouest de l'océan Indien : une histoire encore lacunaire

Les analyses moléculaires de génétique des populations et phylogénétiques ainsi que les nombreux travaux de description morphologique ont contribué à une bien meilleure compréhension de la systématique des vanilliers aphyllés de la zone Sud-Ouest de l'Océan Indien. De grands progrès ont été réalisés au cours de cette thèse dans la délimitation de ce complexe d'espèces récentes. En additionnant principalement deux concepts d'espèces (géotypique et morphologique), les statuts taxonomiques des cinq vanilliers aphyllés malgaches décrits dans la littérature (*V. bosseri*, *V. decaryana*, *V. humblotii*, *V. madagascariensis*, et *V. perrieri*) ont été vérifiés et deux nouvelles espèces ont été décrites (*V. allorgii* et *V. atsinananensis*).

Si l'on tient compte uniquement du concept phylogénétique, la condition de monophylie exigée est remplie pour *V. allorgii*, *V. atsinananensis*, *V. decaryana* et l'espèce de l'Afrique de l'Est *V. roscheri*.

Par contre, si *V. humblotii* et *V. perrieri* forment bien deux groupes monophylétiques sur l'arbre BEAST (Chapitre 3—Figure 2), le clade *V. humblotii* est peu résolu, voire plutôt paraphylétique sur l'arbre ML (Chapitre 3—Figure S1). Ceci pose donc, selon le concept d'espèce phylogénétique, la question de les considérer comme une même espèce. D'après le concept unifié de spéciation de De Queiroz (2007), il suffit que les lignées évoluent séparément pour pouvoir les classer en espèce. Comme aucun hybride entre *V. humblotii* et *V. perrieri* n'a été identifié par les analyses de la structuration génétique et compte tenu de la différenciation

génétique à partir des microsatellites et de la différenciation morphologique, il serait logique d'accepter le statut de ces deux espèces sur la base des recommandations de De Queiroz.

Aussi, les espèces *V. bosseri*, *V. madagascariensis* et *V. phalaenopsis* sont indissociables au sein d'un même groupe monophylétique et devraient être considérées comme une même espèce selon le concept d'espèce phylogénétique. *V. phalaenopsis* des Seychelles est séparée géographiquement des deux espèces malgaches (*V. bosseri* et *V. madagascariensis*). Compte tenu de cette séparation géographique et de la différenciation génétique sur la base de marqueurs microsatellites, *V. phalaenopsis* peut être considérée comme une unité taxonomique qui évolue certainement indépendamment de *V. bosseri* et *V. madagascariensis*. Néanmoins, une comparaison morphologique de *V. phalaenopsis* avec les deux espèces malgaches (*V. bosseri* et *V. madagascariensis*) sera nécessaire pour valider le concept d'espèce morphologique. Contrairement aux résultats de l'analyse phylogénétique, l'analyse de la structuration génétique sur la base des microsatellites indique que *V. bosseri* et *V. madagascariensis* forment deux unités évolutives clairement différentes et ce malgré leur situation sympatrique dans plusieurs zones de Madagascar (ex CMK, BBL, ATS, MSL). Les analyses morphologiques révèlent aussi des différences nettes d'ornementation du labelle entre ces deux espèces. Des études complémentaires sur le phénomène d'hybridation en milieu naturel seraient toutefois utiles afin de comprendre le mécanisme permettant le maintien de la différenciation entre ces deux unités taxonomiques. En effet, les tests de croisement effectués manuellement entre les deux espèces n'ont été suivis que jusqu'aux stades de gousses et ont été pratiqués sur un nombre limité d'individus. Comme l'hybridation n'est efficace que si les individus hybrides survivent et sont capables de se reproduire (Baack et al., 2015), des tests supplémentaires de la valeur sélective et de fertilité des individus hybrides permettraient de vérifier l'isolement post-zygotique des deux espèces en sympatrie. Toutefois, les individus hybrides entre ces espèces détectés par l'analyse microsatellite semblent montrer que de tels hybrides sont bel et bien viables (Chapitre 2). Un échantillonnage populationnel plus conséquent associé à une analyse spatiale fine de la structuration génétique dans les zones de sympatrie où des individus hybrides ont été détectés (Figure 2) pourrait permettre de vérifier l'introgession. C'est, par exemple, ce qui a été fait pour étudier l'occurrence de l'hybridation et la structure de la population hybride entre *Ligularia cymbulifera* et *Ligularia tongolensis* dans quatre zones d'hybridation en Asie (L. Hu et al., 2021). Les hybrides de différentes classes (génération F1, génération F2) peuvent être identifiés à partir des méthodes statistiques bayésiennes (Anderson and Thompson, 2002; L. Hu et al., 2021; Pritchard et al., 2000). L'analyse de la structuration génétique à petite échelle (analyse de la distribution non aléatoire des génotypes) permet d'estimer plusieurs paramètres dont l'isolement par la distance, le flux génétique et le niveau de consanguinité (Vekemans and Hardy, 2004). Cette analyse permettrait de déterminer : i) la distribution des espèces sympatriques dans le site, ii) si les hybrides sont oui ou non distribués de façon aléatoire dans la forêt, iii) l'intensité du flux génétique intraspécifique et interspécifique, iii) l'abondance des hybrides par rapport aux parents. Pour le concept d'espèce génotypique, Mallet (1995) considère que si les hybrides sont absents ou rares dans les zones de sympatrie, les deux clusters génotypiques peuvent être considérés comme des espèces. En

d'autres termes, un flux génétique élevé entre les espèces sympatriques indiquerait une évolution collective, remettant en cause tous les concepts d'espèce et l'identité taxonomique des espèces concernées. En ce sens, une étude poussée des zones de sympatrie entre espèces des deux clades majeurs (espèces à fleurs jaunes versus fleurs blanches), telles que AKL (avec *V. bosseri* et *V. perrieri*) ou MLT (avec *V. decaryana* et *V. perrieri*) dans lesquelles des hybrides sont détectés par analyse microsatellite (Chapitre 2) ou biologiquement possibles par croisement jusqu'au stade gousses tout du moins (ex BFD avec *V. madagascariensis* et *V. perrieri*) (Chapitre 4), voire observés (une fleur hybride jaune pâle observée à BFD) (Chapitre 1), revêt un intérêt scientifique majeur.

De nos jours, il existe de plus en plus d'études taxonomiques utilisant des outils récents de séquençage nouvelle génération, NGS ou « Next Generation Sequencing ». NGS est le terme générique qui désigne l'ensemble des méthodes permettant l'analyse rapide et peu coûteuse d'un grand nombre d'acides nucléiques (ADN, ARN) simultanément pour de nombreux individus (Bateman, 2020). Ces techniques sont utilisées pour produire, assembler et lire les séquences d'un génome complet (lecture longue) ou se limiter à des zones d'intérêt spécifiques ou à un petit nombre de gènes individuels dans le génome (lecture courte) (Behjati and Tarpey, 2013; T. Hu et al., 2021). Les NGS offrent plusieurs approches capables de produire ou génotyper des milliers de nucléotides variables ou SNP (Single Nucleotide Polymorphism) à l'échelle du génome. Parmi ces techniques, le RAD-seq (Restriction-site Associated DNA) et le GBS (Genotyping By Sequencing) sont actuellement parmi les plus utilisées pour générer des marqueurs SNPs et clarifier la taxonomie et les relations phylogénétiques des groupes d'espèces relativement proches (Ahrens et al., 2017; Bateman, 2020; Bateman et al., 2018; Favre et al., 2021). Dans notre cas, l'utilisation des marqueurs microsatellites a été privilégiée au détriment des marqueurs SNPs compte tenu du fait que les populations ont été échantillonnées à travers Madagascar au cours de différentes missions de terrain s'étalant sur plusieurs années. Ainsi, les données de génétique moléculaire ont été générées progressivement, en ajoutant de plus en plus d'accessions, ce qui n'aurait pas pu être fait en utilisant par exemple la méthode GBS, qui serait la plus appropriée pour générer des SNPs dans de telles populations (Favre et al., 2021). Étant donné que les populations des différents groupes génétiques sont maintenant identifiées, une approche en utilisant des SNPs pourrait être envisagée dans le futur afin d'affiner les relations phylogénétiques entre ces vanilliers aphyllés malgaches et les espèces sœurs des Seychelles et de l'Afrique de l'Est. L'utilisation d'un grand nombre de locus tels que les SNPs apporterait aussi des informations plus précises sur les processus liés à la conservation tels que la consanguinité, la dépression hybride, les paramètres démographiques (migration, taille effective des populations, goulot d'étranglement..), l'adaptation, la diversité et les flux génétiques (Allendorf et al., 2010; Mostert-O'Neill et al., 2021; Ouborg et al., 2010). En utilisant des SNPs, par exemple, il serait possible de détecter les événements passés de changements démographiques qui pourraient être mis en relation avec la distribution passée et présente des espèces (Garot et al., 2019; Mays et al., 2018; Ren et al., 2017). De telles analyses à grande échelle du génome permettraient aussi d'aborder l'évolution des populations via une approche de génomique des populations (Luikart et al., 2003; Ouborg et al., 2010). À partir de ces

analyses génomiques, la détermination, par la méthode des locus outliers, des régions du génome sous sélection et donc ayant été impliquées potentiellement, par exemple, dans l'adaptation ou la spéciation écologique serait également réalisable (Chapman et al., 2016; Prunier et al., 2011). Sachant que la séquence annotée du génome de *Vanilla planifolia* est maintenant disponible (Hasing et al., 2020; et projet VaniSeq en cours à l'UMR PVBMT), il serait même possible d'identifier à quels gènes ces régions correspondent.

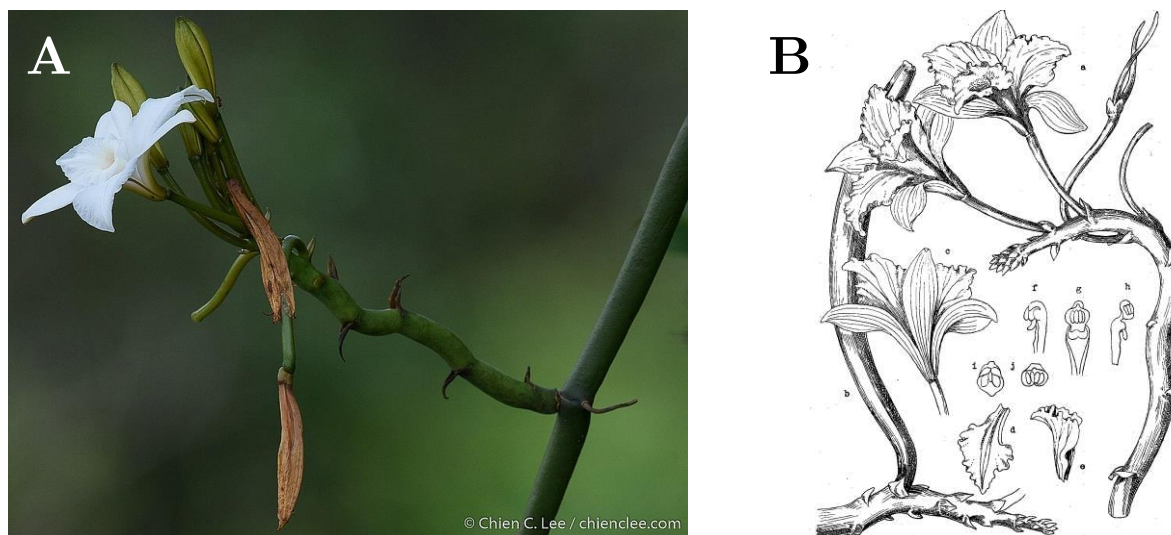


Figure 3. *Vanilla walkeriae*

A—Photo de *V. walkeriae* (© Chien C. Lee) provenant de la base de données en ligne inaturalist (www.inaturalist.org). D'après l'auteur, la photo a été prise au Sri Lanka, dans le district d'Anuradhapura, dans la division de Nochchiyagama. Lon : 80.011043 E et Lat : 8.420743 N. B— *V. walkeriae* Wight (Portères, 1954): a) tige et inflorescence; b) infrutescence et fruit; c) fleur épanouie; d) labelle, vu par sa face supérieure; e) labelle, vu de profil, sommet du gynostème; f) vue latérale; g) vue antérieure; h) anthère soulevée; i) et j) anthère.

Enfin, la relation entre l'espèce aphyllé *V. walkeriae* du Sri Lanka et de l'Inde et les espèces du SOOI soulève une dernière question. Soto-Arenas and Cribb (2010), dans sa révision taxonomique du vanillier, classe cette espèce parmi le groupe « *V. phalaenopsis* » du SOOI. D'après sa description, *V. walkeriae* possède des fleurs similaires à *V. madagascariensis* et *V. phalaenopsis*, mais avec une gorge jaune (Soto-Arenas and Cribb, 2010). Dans son ouvrage, Portères (1954) indique également une grande affinité entre *V. walkeriae* et *V. perrieri* (Figure 3. *Vanilla walkeriae*). Les rares photos disponibles dans les bases de données en ligne montrent une très forte similitude entre cette espèce et les espèces à fleurs blanches du SOOI (Figure 3). Vu cette ressemblance frappante qui existe entre *V. walkeriae* et les espèces malgaches, il est fort probable que l'ancêtre de *V. walkeriae* soit originaire de Madagascar et ait migré en Inde et au Sri Lanka (Figure 1). Bien que la distance entre Madagascar et l'Asie soit très grande (environ 3800 km), le flux migratoire entre ces deux régions aurait été, d'après les travaux de Warren et al. (2010), facilité dans le passé par la direction du courant marin et du vent et/ou l'apparition de petites îles ainsi que l'élargissement de la superficie des îles existantes actuelles (ex : Seychelles, Maldives, archipel des Chagos) résultant de la diminution du niveau de la mer (Figure 1). Ces chaînes d'îles entre Madagascar et l'Asie auraient donc probablement servi

de pont facilitant la dispersion aussi bien d'animaux que de plantes entre ces deux zones grâce à la réduction de la longueur de la traversée océanique (Warren et al., 2010). C'est le cas, par exemple, des espèces du genre *Exacum* (Gentianaceae) endémiques de plusieurs régions de l'Asie y compris le Sri Lanka et l'Inde dont l'ancêtre commun serait originaire de Madagascar (Yuan et al., 2005).

5.3. Recommandations sur la conservation des vanilliers aphyllés à Madagascar

À ce jour, l'un des documents officiels les plus importants dans les politiques de conservation de la biodiversité demeure le texte signé lors de la convention sur la diversité biologique (Convention on Biological Diversity) (United Nations, 1992). Dans ce document, deux principaux modes de conservation sont définis : la conservation « *ex situ* » et la conservation « *in situ* ».

La conservation « *ex-situ* » est « *la conservation des composantes de la diversité biologique en dehors de leurs habitats naturels* » (United Nations, 1992) en vue de maintenir le pool génétique (Pullin, 2002). D'après Pullin (2002), la mise en place d'une stratégie de conservation *ex-situ* doit se faire normalement dès que le nombre d'individus d'une espèce dans son milieu naturel devient relativement faible. De telles recommandations vers une conservation *ex-situ* ont été faites pour le cas de *V. roscheri* (Gigant et al., 2014) en Afrique du Sud afin d'assurer la préservation du génotype unique et fixé présent dans les populations sur les rives du Lac Sibaya. De la même façon, le CBNM Mayotte a mis en place une collection *ex-situ* représentative de la diversité de *V. humblotii* à Mayotte (M'sa et al., 2013), notamment pour les populations à très faibles effectifs, selon les recommandations proposées par Gigant et al. (2016). Dans le cas des vanilliers aphyllés malgaches, la densité des populations de *V. atsinananensis* est très faible, la population de *V. allorgii* est à dominance clonale, les répartitions géographiques et les tailles effectives estimées des populations de chaque espèce indiquent pour certain une menace pour la survie à court terme (*V. decaryana*, *V. humblotii*) ou pour d'autres une difficulté pour la survie à long terme (*V. madagascariensis*, *V. perrieri* et *V. bosseri*) (Tableau 1). Par conséquent, les sept espèces de vanilliers aphyllés de Madagascar doivent faire l'objet d'une conservation « *ex-situ* ».

Actuellement, l'Université d'Antananarivo procède à la mise en place d'une collection vivante des individus de chaque groupe génétique ou espèce, ces individus sont plantés sous les conditions appropriées dans des serres. Un doublon représentatif (quelques individus par groupe génétique/espèce) est envisagé dans le Centre de Recherche Biologique (CRB) Vatel de La Réunion. Une collaboration avec le jardin botanique de Tsimbazaza d'Antananarivo est également en cours pour la mise en place d'une collection vivante de quelques individus appartenant aux deux nouvelles espèces décrites dans le cadre de cette thèse (*V. atsinananensis* et *V. allorgii*). L'un des principaux enjeux de la conservation *ex-situ* est de pouvoir représenter le schéma de la diversité génétique des espèces dans leur milieu naturel (Pullin, 2002). C'est

pourquoi, pour le cas de l'Université d'Antananarivo, chaque espèce est représentée, dans la collection, par des individus appartenant aux différents sites dans lesquels nous avons travaillé.

Tableau 1: Résumé récapitulatif des menaces et des statuts UICN proposés pour chaque espèce ainsi que les recommandations pour leur conservation

Espèces	Menaces	Statut UICN provisoire	Recommandations
<i>V. allorgii</i>	Perte de diversité génétique Perte d'interactions avec les pollinisateurs Taux de clonalité élevé	CR	Conservation <i>ex-situ</i> Prospection d'autres populations Pollinisation manuelle Identification des pollinisateurs Repeuplement de sites appropriés
<i>V. atsinananensis</i>	Petite taille de la population	CR	Conservation <i>ex-situ</i> Prospection d'autres populations Repeuplement de sites appropriés
<i>V. bosseri</i>	Destruction des habitats (feux) dans certains sites	LC	Conservation <i>ex-situ</i> Atténuation des pressions sur les habitats
<i>V. decaryana</i>	Consanguinité Fragmentation des habitats Collecte des tiges Ne <50	EN	Conservation <i>ex-situ</i> Mise en place de corridors entre habitats fragmentés Translocation d'individus Plantations pour réduire les collectes en milieu naturel
<i>V. humblotii</i>	Faible taux de floraison Ne <50	VU	Conservation <i>ex-situ</i> Prospection d'autres populations Mise en place d'une aire protégée Repeuplement de sites appropriés
<i>V. madagascariensis</i>	Perte d'habitats dans le futur	VU	Conservation <i>ex-situ</i>

Espèces	Menaces	Statut UICN provisoire	Recommandations
	Destruction des habitats		Prospection d'autres populations Atténuation des pressions Mise en place d'une aire protégée
<i>V. perrieri</i>	Destruction des habitats (feux) Ne <100	NT	Conservation <i>ex-situ</i> Atténuation des pressions

CR : en danger critique, EN : en danger, VU : vulnérable, NT : quasi menacée, LC : à préoccupation mineure

D'autres techniques alternatives de conservation *ex-situ* pourraient également être appliquées dont la conservation au froid des graines, des gousses, des pollens et des tissus (Divakaran et al., 2006a; Flanagan and Mosquera-Espinosa, 2016; Pullin, 2002). Ces techniques modernes ont l'avantage de maximiser la conservation de la diversité génétique (Flanagan and Mosquera-Espinosa, 2016). La conservation *ex-situ* pourrait permettre de procéder, si besoin, à la réintroduction des espèces dans leur milieu naturel. Pour le succès de la réintroduction des orchidées à Madagascar, il serait essentiel de s'assurer que les champignons associés aux espèces soient présents dans l'habitat à restaurer (Yokoya et al., 2021).

Le grand problème de la conservation « *ex-situ* » est que les espèces sont isolées des interactions écologiques qu'elles entretenaient dans leur milieu naturel (Pullin, 2002). D'où la nécessité de « *la conservation des écosystèmes et des habitats naturels ainsi que le maintien et la reconstitution des populations viables d'espèces dans leur milieu naturel et, dans le cas des espèces domestiquées ou cultivées, dans le milieu où elles ont développé leurs propriétés distinctives* », une stratégie appelée conservation « *in situ* » (United Nations, 1992). Les actions à entreprendre pour la conservation « *in situ* » dépendent de la taille des populations et des niveaux de menace sur l'espèce concernée (Frankham et al., 2010; Pullin, 2002). En d'autres termes, conserver une espèce dans son milieu naturel revient à élaborer des stratégies pour augmenter la taille de la population, pour limiter la consanguinité dans les petites populations et pour gérer les populations fragmentées (Frankham et al., 2010). Pour cela, la première étape consiste normalement à identifier les unités à conserver (Crandall et al., 2000; de Guia and Saitoh, 2007; Moritz, 1994). Les unités de conservation sont des groupes de populations d'une espèce contenant la diversité nécessaire à la survie de l'espèce à court et long terme. Afin de préserver le maximum de diversité génétique dans le minimum de populations possible, plusieurs unités de conservation ont été définies au fil du temps : les ESUs (Evolutionary Significant Units) (Moritz, 1994; Ryder, 1986), les MUs (Management Units) (Moritz, 1999, 1994), les OCUs (Operational Conservation Units) (Doadrio et al., 1996), les FGEUs (Fundamental Geographic and Evolutionary Units) (Riddle and Hafner, 1999), les FCUs

(Functional Conservation Units) (Maes et al., 2004) et les RGUC (Relevant Genetic Units for Conservation) (Pérez-Collazos et al., 2008). Bien que peu recommandées dans certaines situations (Crandall et al., 2000; Kizirian and Donnelly, 2004), les unités de conservations définies par Moritz sont parmi les plus utilisées actuellement (de Guia and Saitoh, 2007). Moritz (1994) a évoqué la nécessité de déterminer les ESUs et les MUs pour la conservation des espèces menacées. Les unités évolutives significatives (ESUs) sont des populations géographiquement séparées et génétiquement très distinctes, par conséquent ayant des trajectoires évolutives indépendantes (Moritz, 1994) et/ou des caractères morphologiques adaptatifs différents (Crandall et al., 2000; de Guia and Saitoh, 2007). En d'autres termes, une ESU représente une unité infraspécifique présentant un flux génétique très restreint avec les autres unités (ESUs) de l'espèce (Fraser and Bernatchez, 2001), nécessitant ainsi une gestion séparée. La conservation des ESUs permet le maintien du patrimoine génétique et du potentiel évolutif d'une espèce sur le long terme (Moritz, 1994). Les ESUs correspondent souvent à des ensembles de populations présentant une monophylie réciproque (Moritz, 1994). Dans la pratique, elles sont généralement identifiées en créant un arbre phylogénétique à partir de données de séquençage et/ou un arbre Neighbor joining construit sur la base de matrices de distances (génétique, morphologique, écologique, géographique) (Crandall et al., 2000; de Guia and Saitoh, 2007; Diniz-Filho and De Campos Telles, 2002; Hedrick et al., 2001; Morgan et al., 2008; Moritz, 1994). Chaque ESU est composée d'un ensemble de populations structurées récemment appelées unités de gestion (MUs) qui doivent également être surveillées et gérées pour la conservation à court terme (Moritz, 1999, 1994). Contrairement aux ESUs, les MUs ne sont identifiées que par une divergence significative des fréquences alléliques indépendamment des relations phylogénétiques (Moritz, 1994). Le calcul des distances génétiques entre les populations (ex : indices de différenciation, F_{ST}) ainsi que les méthodes bayésiennes de structuration génétique (ex STRUCTURE) permettent de définir les MUs (Hedrick et al., 2001; Moritz, 1994; Palsboll et al., 2007). Pour le cas des vanilliers du SOOI, nos résultats phylogénétiques montrent que chaque espèce serait a priori constituée d'une seule ESU. Une analyse complémentaire des résultats de la structuration (génétique, géographique, écologique, morphologique) et des flux génétiques des populations de chaque espèce constituera une étape utile pour identifier les MUs à considérer dans les stratégies de conservation à court terme.

Pour l'instant, seulement une population a pu être étudiée pour chacune des espèces *V. atsinananensis* et *V. allorgii*. La première action la plus importante pour ces deux espèces serait donc de prospecter d'autres populations en se basant sur les résultats de la modélisation des niches écologiques potentielles (Chapitre 4). Une fois que les populations seront identifiées, des études complémentaires (génétiques et biologiques) devraient être réalisées afin de déterminer si les mêmes menaces liées respectivement à la faible densité et/ou à la forte clonalité subsistent dans ces populations. Le cas échéant, il faudrait identifier les facteurs responsables de ces menaces afin d'optimiser les conditions de reproduction (pollinisateurs, mycorhizes, etc.) (Frankham et al., 2010) et proposer des mesures permettant de réduire ces menaces. Pour *V. allorgii* ($FIS < 0$), par exemple, l'identification des pollinisateurs serait une étape importante dans la stratégie de sa conservation. Si nécessaire, des pollinisations manuelles devront être

faites en collaboration avec les communautés locales. C'est, par exemple, ce qui a été fait pour l'espèce *Angraecum longicalcar*, une orchidée listée comme en danger critique [CR] par l'IUCN, qui est menacée non seulement par la petite taille des populations (12 individus connus, endémicité locale), par le feu et la dégradation des habitats, mais également par la probable extinction de son pollinisateur naturel (Rajaovelona and Gardiner, 2016). En effet, les recherches réalisées sur cette espèce ont montré que les plantes semblent ne produire aucune gousse dans leur milieu naturel (Rajaovelona and Gardiner, 2016). Si malheureusement aucune autre population n'existe, l'établissement de nouvelles populations dans des niches écologiques potentielles permettrait d'augmenter la taille de la population. Dans son ouvrage, Frankham et al. (2010) propose la mise en place d'au moins dix populations dans différents sites pour minimiser le risque de maladie et l'effet des catastrophes naturelles. Les projets de réintroduction d'une espèce dans des habitats naturels sont assez fréquents à Madagascar. Par exemple, plusieurs individus de deux espèces d'orchidées, *Cynorkis purpurea* et *Benthamia cinnabarina*, ont été réintroduits à Itremo (Madagascar) par le Royal Botanical Garden de Kew en 2015 (Yokoya, 2015). Il en est de même pour l'espèce *Angraecum longicalcar* qui a fait l'objet d'une plantation dans un habitat similaire à son habitat naturel (Rajaovelona and Gardiner, 2016). Dans le cas de *V. allorgii* et *V. atsinananensis*, les niches écologiques potentielles sont relativement larges d'après les résultats de la modélisation (Chapitre 4).

Pour les populations ayant une tendance à une faible variabilité génétique (ex : *V. allorgii*, *V. atsinananensis*, *V. decaryana*) ou à une forte consanguinité (ex : *V. decaryana*), l'introduction d'individus issus d'autres populations (translocation) permettrait d'augmenter la diversité génétique (Frankham et al., 2010). Cependant, l'introduction doit se faire en choisissant des populations proches génétiquement pour éviter les problèmes de dépression hybride. Allendorf et al., (2010) propose quatre règles à respecter pour l'introduction ou la translocation entre deux populations : i) la similarité écologique, ii) la similarité phénotypique, iii) la similarité de l'ensemble du génome indiquée par les marqueurs neutres et iv) la similarité génétique au niveau des locus adaptatifs. Dans la pratique, Mortiz suggère que la translocation devrait se faire plutôt entre deux MUs au sein d'une ESUs qu'entre deux MUs appartenant à deux ESUs différents (Moritz, 1999). La mise en place d'un corridor forestier à partir des actions de restauration écologique serait très important pour limiter les impacts de la fragmentation des habitats (cas des populations de *V. decaryana*). En effet, le maintien de la connectivité entre les populations permettrait de faciliter l'expansion de leurs aires de répartition, comme recommandé par Yesuf et al. (2021) à l'issue de leurs études sur quelques espèces rares de Madagascar. Limiter la fragmentation des populations serait aussi bénéfique pour maintenir le flux génétique entre elles (Frankham et al., 2014). Dans certaines localités de Madagascar, plus particulièrement dans le Sud (ex site SAG), les tiges de *V. decaryana* sont fortement exploitées. Les menaces concernent également l'exploitation intensive des arbres forestiers qui sont les principaux tuteurs de ces vanilliers aphyllés. D'après nos observations, les tiges de vanilliers sont arrachées intégralement par les collecteurs, par conséquent les individus se font très rares (seulement dix individus échantillonnés sur un transect d'environ 1 km à SAG). Ainsi, une production agricole via des plantations (comme pour *V. planifolia*)

pourrait être envisagée dans les zones où les vanilliers sont fortement collectés afin de réduire la pression de collecte en milieu naturel (Frankham et al., 2010).

Si plusieurs populations de *V. madagascariensis* majoritairement situées dans des aires protégées ont été identifiées dans cette étude, la seule population de *V. humblotii* observée jusqu'à présent se situe dans la nouvelle aire protégée MDF. Les stratégies de conservation à appliquer pour ces deux espèces considérées comme vulnérables, compte tenu de leur distribution restreinte au Nord et de la taille effective des populations, consistent à maintenir les habitats actuels à travers la mise en place de nouvelles zones de conservation qui peuvent être soit des aires protégées, soit des forêts à gestion communautaire. Des efforts pour rechercher d'autres populations de *V. humblotii* seraient également bénéfiques. Comme pour *V. atsinananensis* et *V. allorgii*, une installation de nouvelles populations dans les niches écologiques potentielles pourrait être envisagée dans le cas où aucune autre population n'est identifiée.

En ce qui concerne les espèces qui sont largement réparties comme *V. bosseri* et *V. perrieri*, limiter la fragmentation et la destruction des habitats permettrait d'assurer le continuum écologique et de favoriser les flux génétiques entre les populations.

Les mesures écologiques et biologiques de conservation doivent s'accompagner de réglementations et législations pour être efficace. Suite au constat des naturalistes soulignant une perte importante des surfaces forestières (Montagne and Ramamonjisoa, 2006), Madagascar fut le premier pays africain à avoir développé un plan d'action environnemental national datant des années 1990 (Goodman, 2008). Depuis cette date, la politique de conservation malgache vise, non seulement à créer de nouvelles zones protégées, mais aussi à appliquer une nouvelle approche de « gestion contractuelle des écosystèmes » faisant des populations riveraines les acteurs principaux de gestion (Montagne and Ramamonjisoa, 2006). Ainsi, en plus des aires protégées déjà mises en place depuis les années 1920, de nouvelles aires protégées ainsi que des forêts à gestion communautaires se sont formées progressivement dans l'île. Les sept espèces aphylls malgaches se trouvent soit dans des zones classées aires protégées, soit dans des forêts dont la gestion a été transférée légalement à des communautés locales, soit dans des forêts sacrées, mais sans protection légale, soit dans des habitats non protégés (Chapitre 1—Tableau 1). À Madagascar, la participation des communautés locales dans la conservation s'est avérée être une approche puissante dans la gestion des forêts (Rajaovelona and Gardiner, 2016; Rasolofoson et al., 2015; Tengö et al., 2007). D'autre part, l'examen de plusieurs sites forestiers à Madagascar a montré que la cogestion entre organismes de conservation et les communautés riveraines semble être la stratégie la plus efficace en termes de stabilisation, voire de croissance, du couvert forestier, et d'équilibre des intérêts entre les utilisateurs (McConnell and Sweeney, 2005). Par conséquent, toutes les zones non protégées qui abritent les espèces de vanilliers aphylls ayant des niches écologiques potentielles restreintes (ex : *V. madagascariensis*, *V. humblotii*, *V. allorgii*, *V. atsinananensis*), plus particulièrement celles où cohabitent plusieurs espèces (ex AKL et MLT) devraient être classées parmi les aires protégées ou du moins parmi les zones à gestion communautaire. Dans les aires protégées qui sont encore victimes de feux

et d'exploitation illicite (ex MSL), les surveillances et la contribution des communautés riveraines doivent être renforcées.

En résumé, pour toutes les espèces, l'optimisation de leurs conditions environnementales à travers les actions comme la gestion des récoltes, la gestion des feux, la restauration écologique et l'amélioration des qualités du sol seraient bénéfiques pour leur sauvegarde. Les zones où la richesse spécifique est la plus élevée comme le cas des différents sites dans la province de Majunga (CMK, ATS, BBL, NMK, AKF, BFD, BTM) et de Tuléar (AKLL) (Chapitre 4) devraient avoir un statut d'aire protégée ou du moins devraient être intégrées dans les plans communaux de développement et de conservation. D'autant que ces zones de sympatrie permettraient de conserver les processus évolutifs (Ennos et al., 2005), les hybrides représentent peut être une solution évolutive d'avenir pour ce complexe d'espèces ?

5.4. Application des résultats

Cette thèse traite à la fois des aspects fondamentaux en biologie évolutive et des applications évidentes dans les programmes de conservation, d'amélioration génétique et de valorisation durable des espèces de vanilliers aphyllés de Madagascar. Elle a contribué, d'une manière générale, à une meilleure compréhension des conditions associées et des mécanismes évolutifs qui sous-tendent la formation d'un complexe d'espèces appartenant à la grande et emblématique famille des Orchidées. La résolution des problèmes d'identification taxonomique permettra de faciliter l'authentification du matériel botanique dans le cadre de futures recherches sur la biologie, l'écologie ou la valorisation de ces espèces. Comme nous l'avons souligné, les vanilliers aphyllés sont utilisés dans la médecine traditionnelle à Madagascar et constituent un réservoir potentiel de molécules actives pour l'industrie cosmétique. Cependant, les recherches effectuées sur ces espèces, par exemple les analyses chimiques des gousses (Ramaroson-Raonizafinimanana, 1988), ont été jusqu'à présent conduites sur des bases taxonomiques encore floues. À partir de nos résultats, les espèces pourront être dorénavant identifiées facilement et une analyse par espèce pourrait être conduite afin de vérifier la variabilité intraspécifique ou la différence interspécifique des composantes chimiques intéressantes. Les vanilliers aphyllés présentent également des caractères de résistance à la pourriture des racines (causée par le champignon *Fusarium*) (Divakaran et al., 2006b; Koyyappurath et al., 2015), mais aussi de tolérance à la sécheresse, un trait important dans le contexte du changement climatique global. Ces espèces représentent, ainsi, des candidats potentiels pour de futurs programmes d'amélioration génétique de la vanille cultivée (*V. planifolia*) (Divakaran et al., 2006b) dont la gousse est mondialement commercialisée. En somme, les résultats obtenus à l'issue de cette thèse pourront constituer un document de référence pour les chercheurs qui travaillent sur un modèle d'étude similaire, pour les futures entreprises industrielles qui voudraient valoriser ces vanilliers aphyllés et pour les centres de recherches en amélioration génétique. En ce sens, il pourrait contribuer au développement scientifique, économique et bio-industriel à Madagascar, soutenant le développement d'une

nouvelle filière innovante via l'utilisation durable de ces espèces aphylls endémiques de Madagascar.

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CHAPITRE 2

Table S1: Geographical location, elevation and number of genotyped individuals (n) for the studied populations.

Sites	Code	Longitude	Latitude	Élévation (m)	n
Montagne des Français	MDF	49.354203	-12.335875	215	29
Analamerana	ANM	49.477491	-12.724982	58	27
Ankarana	ANK	49.12918	-12.952675	117	22
Ambanja	AMB	48.50000	-13.610000	143	27
Maromandia	MND	48.088309	-14.218457	41	41
Befandrama	BFD	47.760048	-14.852435	132	24
Antsianitia	ATS	46.427925	-15.570435	19	27
Marosely	MSL	47.578808	-15.648803	280	41
Complexe Mahavavy-Kinkony	CMK	45.824605	-15.945627	16	26
Betaramahamay	BTM	47.444201	-15.964932	211	34
Baie de Baly	BBL	45.273094	-16.068887	15	26
Ankarafantsika	AKF	46.816657	-16.302281	95	24
Namoroka	NMK	45.302633	-16.402629	107	24
Kirindy Forest	KRD	44.657645	-20.067082	55	33
Kirindy Mitea	KRM	44.045367	-20.820437	24	29
Ankililoaka	AKL	43.777311	-22.791984	274	25
Zombitse Vohibasia	ZVB	44.675825	-22.911742	749	17
Antandroka	ATD	43.518649	-22.946485	31	23
Anja	ANJ	43.605442	-23.085189	11	24
Mahaleotse	MLT	44.095938	-23.518483	89	38
Saint-Augustin	SAG	43.780511	-23.538178	20	9
Manompana	MAN	49.610000	-16.960000	41	11
Andringitra	AND	46.777304	-22.08464	887	30

Table S2: Characteristics of the seven nuclear microsatellites of the genus *Vanilla* developed by Gigant et al. (2011) from *V. humblotii* and *V. roscheri*.

Locus name	Repeat motif	Primer sequence (5'-3')	GenBank
mVroCIR04	(ga) 18	F: AAGGTACAAGATCCCCGTCA R: CCGCGAGCTTTATTCTACCA	JN222574
mVroCIR05	(ga) 15	F: GCTATTTCCCACGAACCCTA R: AACCATTGCCAGAAGCCTAA	JN222575
mVhuCIR03	(ag) 24	F: GTCGCATTACATAGCTTCG R: TCTTCCTACCGCTGTCTGTCT	JN222562
mVhuCIR04	(ag) 13	F: GGATACTTCCGGTGA CTCCA R: GCTCTGGCTCTGTGGTTAGG	JN222563
mVhuCIR06	(tg) 10	F: CTTGGTTGTTCTGCTGGATG R: CCCTAGAACCCATGTCTTGC	JN222564
mVhuCIR07	(ga) 16	F: CCACGTAGATCAAACACAGCA R: AGAAACTGTTAGAAATCCCAAGC	JN222565
mVhuCIR08	(ga) 15	F: TTA ACTCTTCGCCCATTAAGC R: CCTTCGAGCTTTCCGTTC	JN222566

Table S3. Mean values of the environmental characteristics of the 23 leafless *Vanilla* populations in Madagascar. For population codes, see table S1. For variable codes and units, see table S4.

	MDF	ANM	ANK	AMB	MND	BFD	ATS	MSL	CMK	BTM	BBL	AKF	NMK	MAN	KRD	KRM	AND	AKL	ZVB	ATD	ANJ	MLT	SAG
Elv (m)	216	58	117	16	50	132	19	283	16	211	15	96	107	20	55	25	942	274	749	28	11	89	22
NDVIM	0.68	0.67	0.68	0.79	0.62	0.55	0.51	0.61	0.62	0.53	0.69	0.65	0.62	0.84	0.72	0.67	0.58	0.56	0.49	0.63	0.62	0.53	0.56
pH	5.89	6.04	5.63	5.73	5.65	6.22	6.14	5.72	5.84	5.81	6.25	5.63	5.82	5.3	5.86	6.1	5.6	6.38	5.68	7.3	6.73	6.53	6.78
Sand (%)	60	65	63	53	57	69	55	65	69	66	65	59	66	49	67	65	54	61	61	72	64	61	71
Clay (%)	23	21	18	26	24	17	25	19	17	16	19	22	19	28	22	23	27	24	23	18	22	24	20
Silt (%)	17	14	18	21	19	15	20	16	13	15	15	19	15	23	12	12	18	15	16	9	13	16	9
BIO1	25	26	26	26	26	26	26	25	27	26	27	26	26	24	25	25	20	24	22	25	25	24	24
BIO2	9	10	10	10	11	12	11	13	12	14	10	14	12	9	13	12	12	13	14	12	12	14	11
BIO3	74	75	13	70	71	78	70	76	71	76	66	81	73	62	63	60	65	63	67	61	58	62	58
BIO4	117	131	136	145	142	113	147	152	144	167	146	121	136	214	221	250	246	263	264	276	284	310	292
BIO5	31	32	32	33	34	33	34	33	35	35	34	35	34	32	35	35	29	34	33	34	34	35	34
BIO6	19	19	19	19	18	19	18	16	18	16	19	18	18	17	15	15	10	13	11	15	15	13	15
BIO7	12	14	13	14	16	15	16	17	17	18	15	17	16	14	21	20	19	21	21	20	20	22	19
BIO8	26	27	27	27	27	27	28	26	28	27	28	27	27	26	27	28	23	26	25	28	28	28	27
BIO9	24	25	24	24	24	25	26	23	25	24	25	25	24	23	22	22	17	20	19	22	21	21	21
BIO10	26	27	27	27	27	27	28	26	28	28	28	27	27	23	27	28	23	26	25	28	28	28	28
BIO11	24	24	24	24	24	25	24	23	25	24	25	25	24	16	22	22	17	20	19	21	21	20	21
BIO12	1139	1390	1563	2064	1797	1552	1683	1490	1446	1473	1228	1440	1325	3316	919	703	963	664	742	577	575	604	543
BIO13	305	322	403	497	479	452	411	458	418	444	403	421	405	332	276	214	246	167	188	144	146	156	137
BIO14	11	27	14	27	8	2	24	0	12	0	1	1	2	104	2	1	6	3	5	2	3	4	3
BIO15	117	94	114	100	110	124	94	132	117	129	135	125	127	43	127	131	110	112	107	113	112	109	109
BIO16	773	825	1035	1264	1154	1084	993	1096	988	1078	935	1013	958	1277	663	532	626	450	482	399	394	404	365
BIO17	47	97	45	84	29	8	105	2	42	2	6	6	9	370	6	6	28	11	19	11	13	16	14
BIO18	520	825	873	880	764	826	699	838	707	674	572	954	289	1247	663	532	626	450	482	399	350	349	321
BIO19	55	100	46	90	29	13	130	2	42	2	6	6	9	603	6	6	34	13	20	12	14	18	17

Table S4. List of the 25 environmental variables

Code	Variables	Units
Elv (m)	Elevation	meter
NDVIM	Maximum Normalized Difference Vegetation index	
pH	Soil pH in H ₂ O at 5 cm of depth	
Sand	Quantity of sand between 5 and 15 cm of deep	%
Clay	Quantity of clay at 5 cm of depth	%
Silt	Quantity of silt at 5 cm of depth	%
BIO1	Annual Mean Temperature	°C
BIO2	Mean diurnal range (mean of monthly max temp-min temp)	°C
BIO3	Isothermality	BIO2/BIO7 * 100
BIO4	Temperature seasonality	Standard deviation * 100
BIO5	Max temperature of warmest month	°C
BIO6	Min temperature of coldest month	°C
BIO7	Temperature annual range (BIO5-BIO6)	°C
BIO8	Mean temperature of wettest quarter	°C
BIO9	Mean temperature of driest quarter	°C
BIO10	Mean temperature of warmest quarter	°C
BIO11	Mean temperature of coldest quarter	°C
BIO12	Annual precipitation	millimeter
BIO13	Precipitation of wettest month	millimeter
BIO14	Precipitation of driest month	millimeter
BIO15	Precipitation seasonality	Coefficient of variation * 100
BIO16	Precipitation of wettest quarter	millimeter
BIO17	Precipitation of driest quarter	millimeter
BIO18	Precipitation of warmest quarter	millimeter
BIO19	Precipitation of coldest quarter	millimeter

Table S5. Matrix of pairwise F_{ST} values between 23 leafless *Vanilla* population from Madagascar calculated from neutral microsatellite datasets, all values are significant (p-value <0.001). For population code, see Table S1.

Pop	MDF	ANM	ANK	AMB	MND	BFD	ATS	MSL	CMK	BTM	BBL	AKF	NMK	KRD	KRM	AKLL	ZVB	ATD	ANJ	MLT	SAG	MAN	
ANM	0.30																						
ANK	0.30	0.04																					
AMB	0.31	0.05	0.09																				
MND	0.29	0.03	0.08	0.01																			
BFD	0.19	0.17	0.19	0.21	0.17																		
ATS	0.21	0.11	0.14	0.16	0.14	0.12																	
MSL	0.21	0.12	0.14	0.17	0.14	0.11	0.03																
CMK	0.22	0.04	0.07	0.07	0.05	0.11	0.03	0.04															
BTM	0.22	0.21	0.22	0.26	0.22	0.03	0.15	0.14	0.14														
BBL	0.21	0.10	0.12	0.15	0.12	0.09	0.02	0.01	0.02	0.11													
AKF	0.22	0.13	0.16	0.19	0.16	0.11	0.03	0.02	0.04	0.14	0.02												
NMK	0.23	0.13	0.16	0.19	0.15	0.12	0.04	0.01	0.05	0.15	0.02	0.01											
KRD	0.20	0.12	0.15	0.17	0.14	0.12	0.02	0.02	0.04	0.15	0.03	0.01	0.02										
KRM	0.38	0.27	0.28	0.31	0.28	0.27	0.24	0.20	0.22	0.28	0.22	0.22	0.24	0.18									
AKLL	0.19	0.17	0.19	0.22	0.18	0.07	0.09	0.09	0.09	0.10	0.06	0.09	0.11	0.09	0.28								
ZVB	0.24	0.15	0.18	0.22	0.17	0.14	0.06	0.02	0.06	0.16	0.03	0.03	0.02	0.02	0.24	0.09							
ATD	0.38	0.30	0.31	0.34	0.30	0.27	0.25	0.22	0.25	0.29	0.24	0.24	0.24	0.19	0.07	0.29	0.25						
ANJ	0.46	0.38	0.39	0.42	0.37	0.37	0.35	0.30	0.33	0.37	0.32	0.33	0.34	0.28	0.07	0.38	0.35	0.12					
MLT	0.33	0.23	0.24	0.27	0.24	0.21	0.21	0.16	0.19	0.22	0.17	0.17	0.19	0.16	0.12	0.23	0.20	0.17	0.17				
SAG	0.40	0.31	0.33	0.35	0.31	0.28	0.28	0.23	0.26	0.28	0.25	0.24	0.27	0.22	0.16	0.31	0.28	0.20	0.20	0.06			
MAN	0.32	0.29	0.30	0.32	0.28	0.22	0.14	0.17	0.20	0.23	0.19	0.19	0.21	0.19	0.39	0.23	0.24	0.41	0.52	0.33	0.44		
AND	0.37	0.37	0.39	0.41	0.35	0.30	0.25	0.24	0.26	0.30	0.25	0.25	0.27	0.23	0.46	0.28	0.28	0.49	0.60	0.39	0.53	0.41	

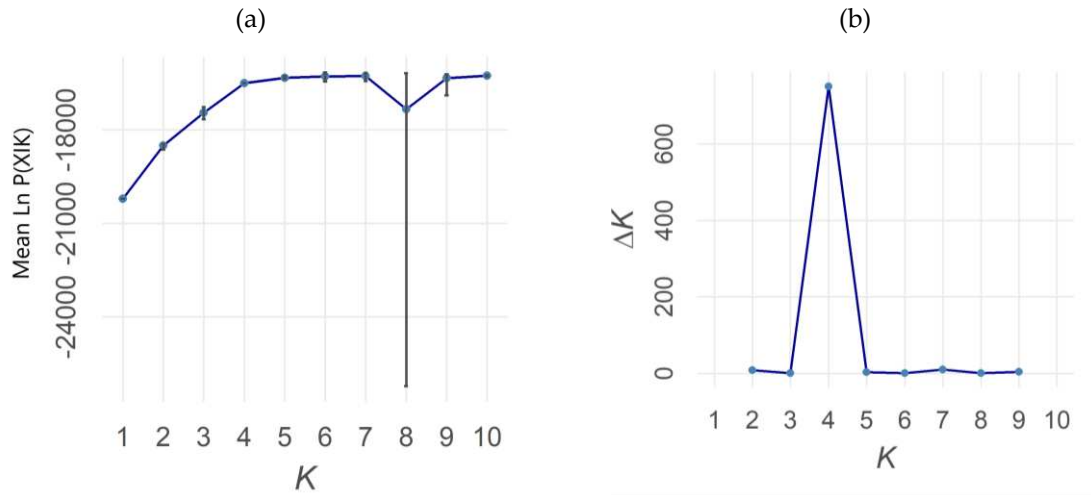


Figure S1. Probability and distribution from STRUCTURE

Plot of (a) the mean posterior probabilities of K, $\text{Ln } P(X|K)$, as implemented in STRUCTURE Software (Pritchard et al., 2000) and (b) the distribution ΔK values from STRUCTURE with $K=4$ based on Evanno method (Evanno et al., 2005).

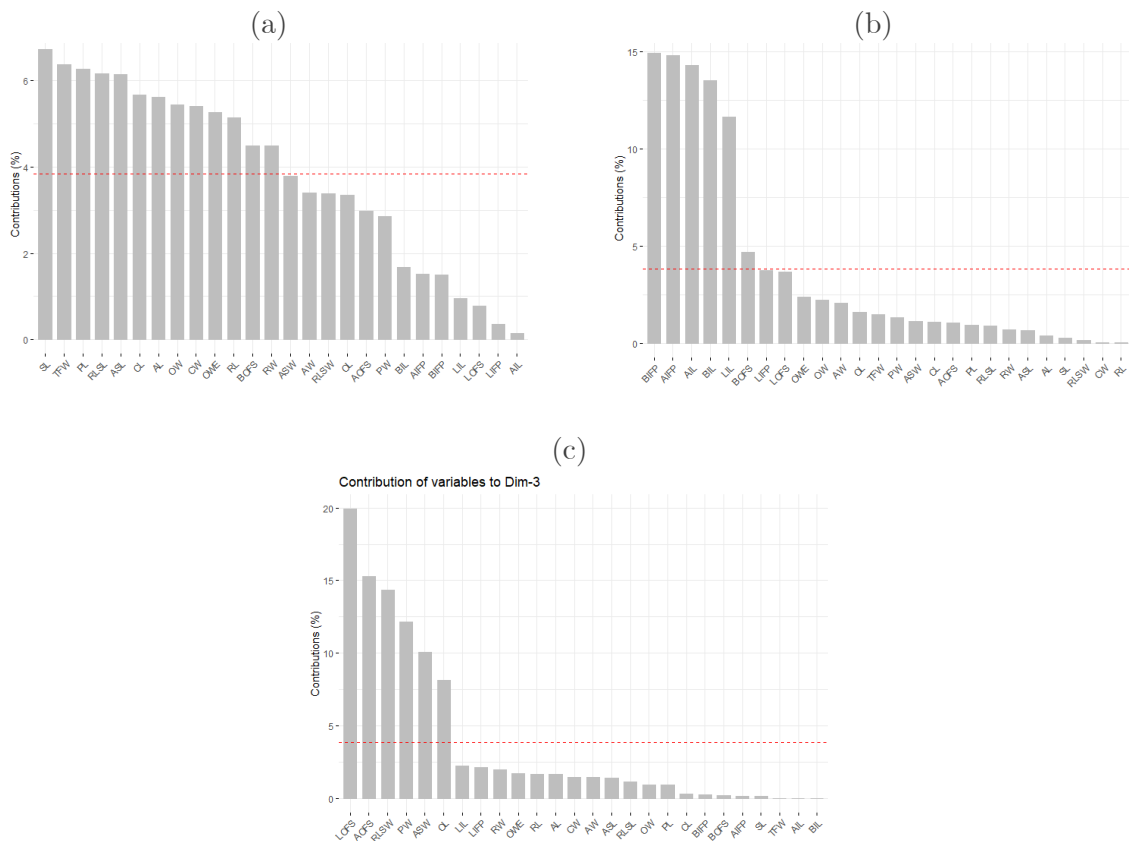


Figure S2. Contribution (%) of the 26 morphological variables to the three principal components of PCA: (a): PC1, (b): PC2, (c): PC3

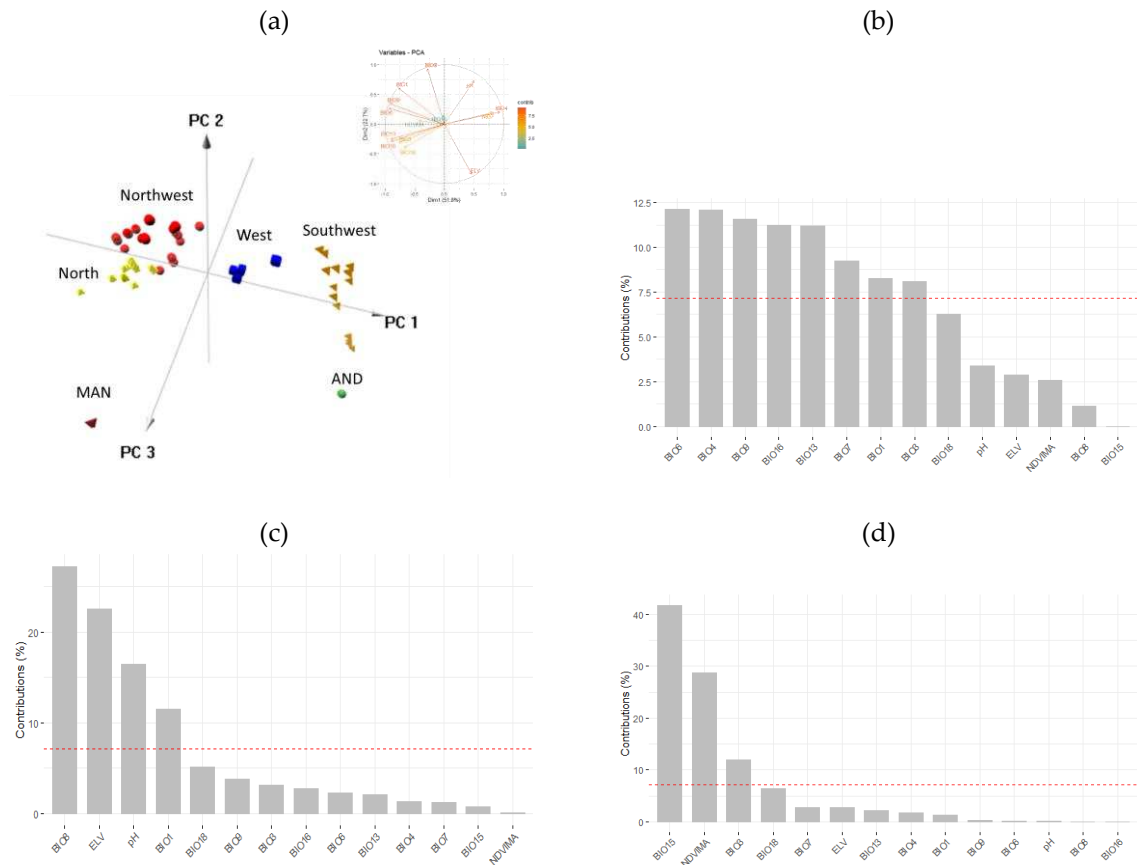


Figure S3. PCA analysis and principal components
3D PCA plot of 14 uncorrelated environmental variables of 23 populations (a) and contribution (%) of the 14 environmental variables to the three principal components of PCA: (a): PC1, (b): PC2, (c): PC3

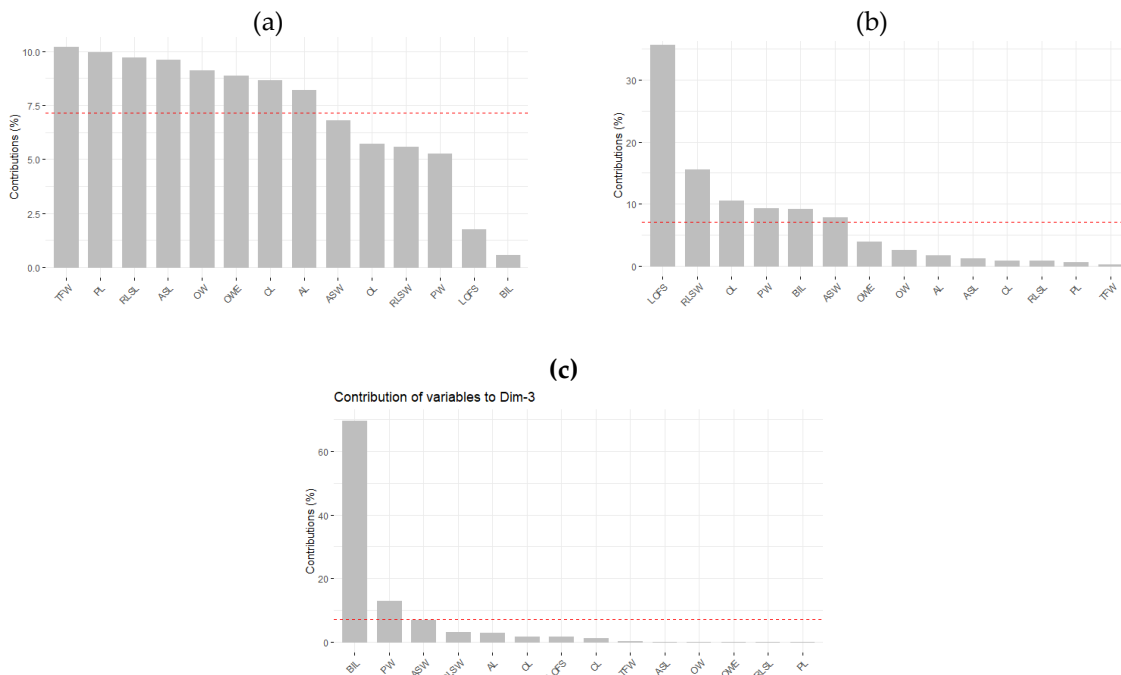


Figure S4. Contribution (%) of the 14 morphological variables to the three principal components of PCA: (a): PC1, (b): PC2, (c): PC3

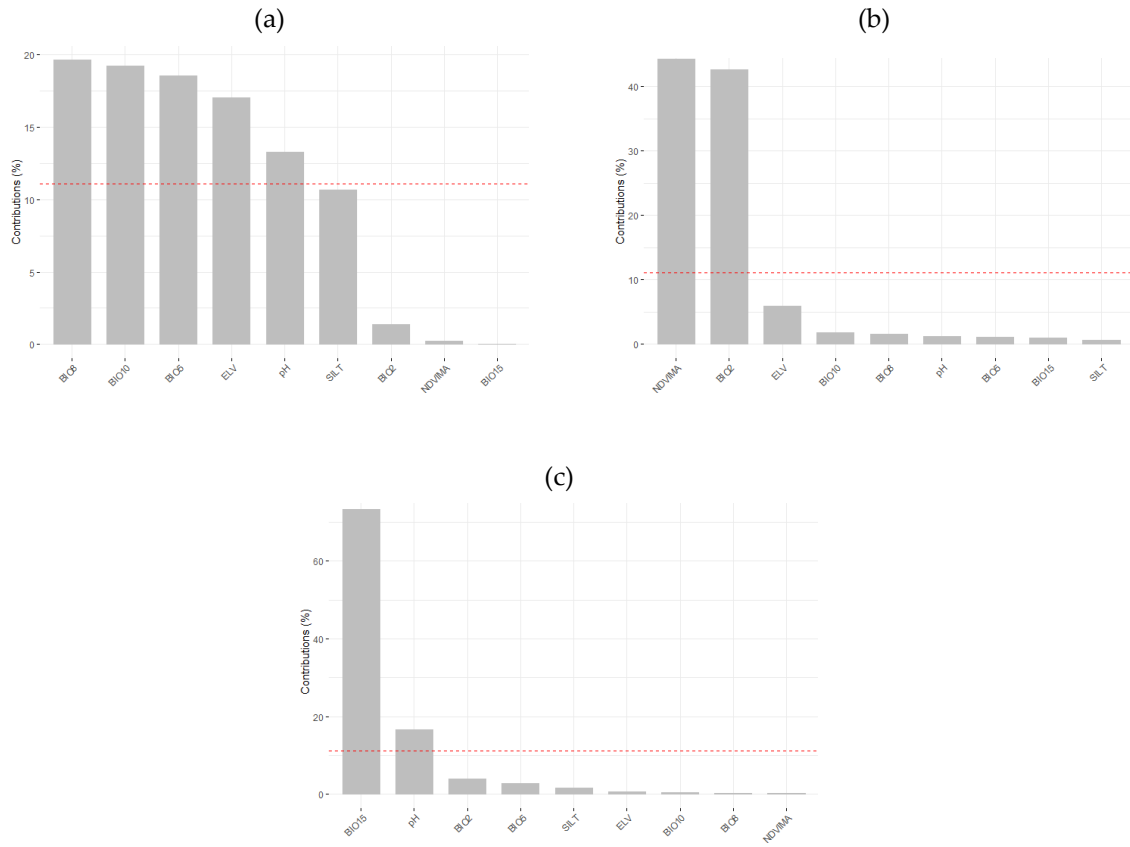


Figure S5. Contribution (%) of the 9 environmental variables to the three principal components of PCA: (a): PC1, (b): PC2, (c): PC3

CHAPITRE 3

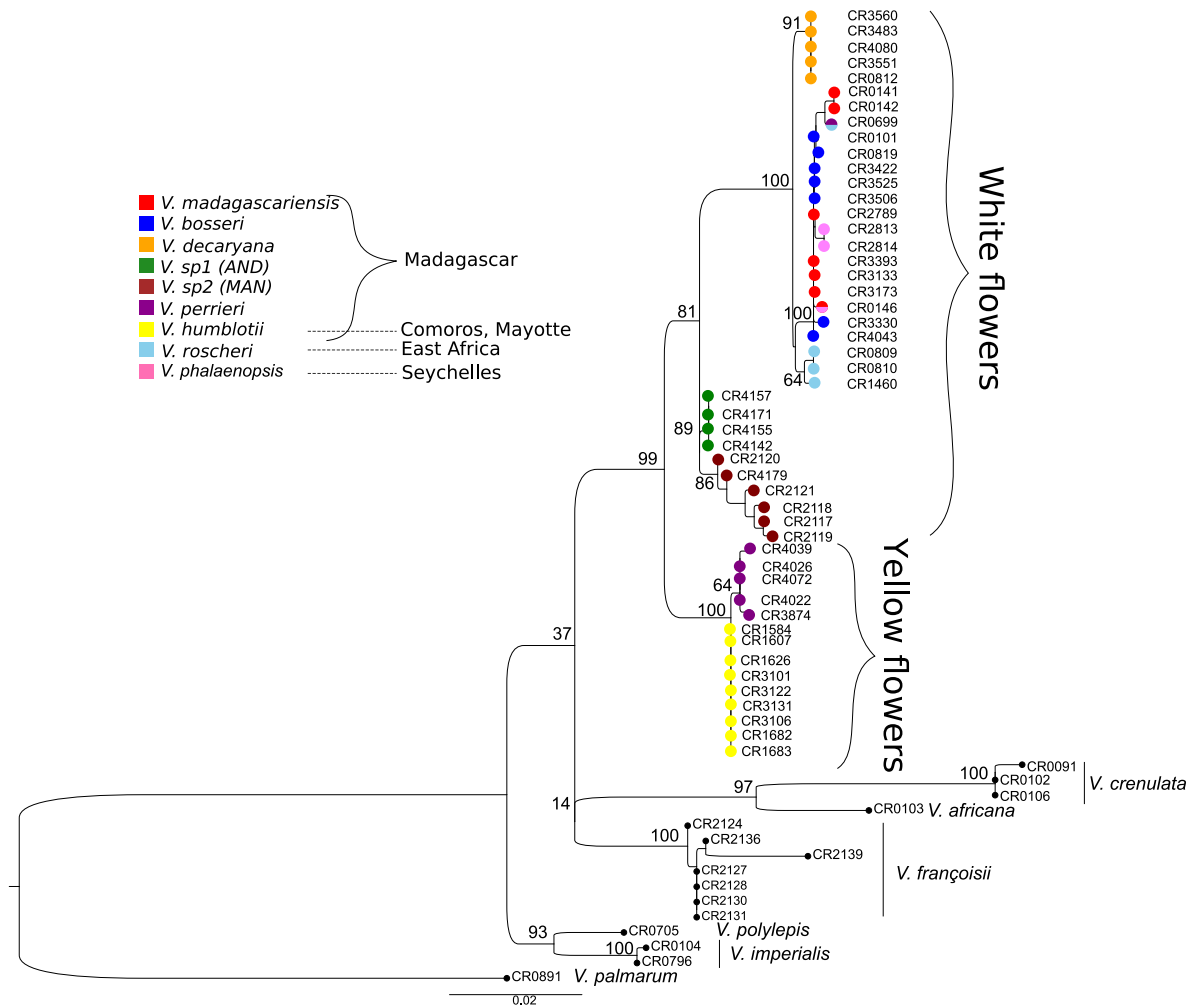


Figure S1. Maximum Likelihood tree of leafless *Vanilla* species from SWIO region based on ITS region. Bootstrap supports (%) (black) are displayed on the tree.

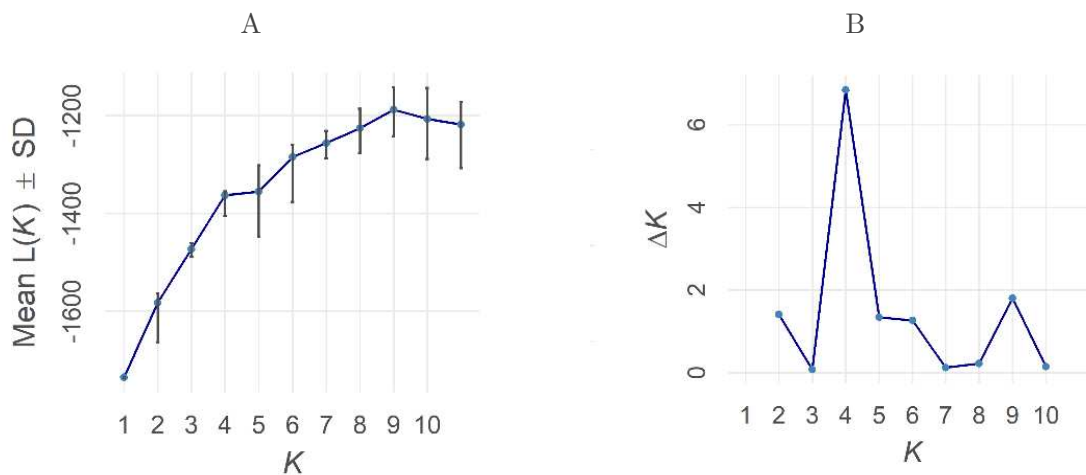


Figure S2. A) Mean posterior probabilities of K, Ln P(X|K), as implemented in STRUCTURE software (Pritchard et al., 2000). B) Distribution of ΔK values from STRUCTURE algorithm based in Evanno's method (Evanno et al., 2005).

Table 1. List of specimens used in this study.

Species names were defined according to genetic structure based on previous study (Andriamihaja et al., 2021), morphological description and geographical location in the literature (Portères, 1954; Cribb & Hermans, 2009; Allorge-Boiteau, 2013). New samples specific to the microsatellite study were indicated by *. The remaining microsatellite data of leafless *Vanilla* species were taken from previous study: Andriamihaja et al. (2021)^a, (Gigant et al., 2016)^b, (Gigant et al., 2014)^c. GenBank codes for new 54 ITS sequences are indicated by **. ITS sequence data of the 10 other samples was obtained from Besse et al. (2021).

Voucher ¹	Species	Source	Longitude	Latitude	GenBank reference
<u>CR0091</u>	<i>V. crenulata</i>	MNHN ²			MW829675
<u>CR0102</u>	<i>V. crenulata</i>	MNHN ²			MW879481**
<u>CR0106</u>	<i>V. crenulata</i>	MNHN ²			MW829676
<u>CR0103</u>	<i>V. africana</i>	MNHN ²			MW829667
<u>CR0104</u>	<i>V. imperialis</i>	MNHN ²			MW829685
<u>CR0705</u>	<i>V. polylepis</i>	Kew ³			MW829699
<u>CR0796</u>	<i>V. imperialis</i>	Denmark ³			MW829686
<u>CR0891</u>	<i>V. palmarum</i>	Brazil			MW829694
<u>CR2124</u>	<i>V. françoisii</i>	Madagascar	49.7017	-16.66974	MW829681
<u>CR2127</u>	<i>V. françoisii</i>	Madagascar	49.70171	-16.66978	MW829682
<u>CR2128</u>	<i>V. françoisii</i>	Madagascar	49.70176	-16.67027	MW879485**
<u>CR2130</u>	<i>V. françoisii</i>	Madagascar	49.7017	-16.66977	MW879486**
<u>CR2131</u>	<i>V. françoisii</i>	Madagascar	49.70171	-16.66979	MW879487**
<u>CR2136</u>	<i>V. françoisii</i>	Madagascar	49.7018	-16.66976	MW879488**
<u>CR2139</u>	<i>V. françoisii</i>	Madagascar	49.70173	-16.66993	MW879489**
CR0101 *	<i>Unknown</i>	MNHN ²			MW879511**
CR0141 *	<i>Unknown</i>	Madagascar ⁴	49,959098	-13,398657	MW879512**
CR0142 *	<i>Unknown</i>	Madagascar ⁴	49,95001	-13,396445	MW879513**
CR0146 *	<i>V. phalaenopsis cf.</i>	Lyon ³			MW879506**
CR0699 *	<i>Unknown</i>	Kew ³			MW879514**
CR0809 ^b	<i>V. roscheri</i>	Zanzibar	39,468706	-6,329453	MW879509**
CR0810 ^b	<i>V. roscheri</i>	Zanzibar	39,499461	-6,371267	MW829708
CR0812 *	<i>Unknown</i>	Madagascar ⁵			MW879515**
CR0819 *	<i>Unknown</i>	Madagascar ⁶	46,816657	-16,302281	MW879474**
CR1460 ^b	<i>V. roscheri</i>	South Africa	32,58867	-27,35962	MW879510**
CR1584 ^c	<i>V. humblotii</i>	Mayotte	45,10562	-12,81273	MW879490**
CR1607 ^c	<i>V. humblotii</i>	Mayotte	45,13435	-12,95688	MW879491**
CR1626 ^c	<i>V. humblotii</i>	Mayotte	45,2984	-12,78302	MW879492**
CR1682 *	<i>Unknown</i>	Madagascar ⁷	49,340018	-12,28228	MW879516**

Voucher ¹	Species	Source	Longitude	Latitude	GenBank reference
CR1683 *	<i>Unknown</i>	Madagascar ⁷	49,340018	-12,28228	MW879517**
CR2117 ^a	<i>V. sp2</i>	Madagascar	49,70351	-16,67454	MW879469**
CR2118 ^a	<i>V. sp2</i>	Madagascar	49,70362	-16,67448	MW879470**
CR2119 ^a	<i>V. sp2</i>	Madagascar	49,70354	-16,67454	MW879471**
CR2120 ^a	<i>V. sp2</i>	Madagascar	49,69861	-16,67587	MW879472**
CR2121 ^a	<i>V. sp2</i>	Madagascar	49,69855	-16,67557	MW879473**
CR2789 ^a	<i>V. madagascariensis</i>	Madagascar	48,543004	-13,455841	MW879497**
CR2813 *	<i>V. phalaenopsis</i>	Mahé (Seychelles)			MW879507**
CR2814 *	<i>V. phalaenopsis</i>	Mahé (Seychelles)			MW879508**
CR3101 ^a	<i>V. humblotii</i>	Madagascar	49,354203	-12,335875	MW879493**
CR3106 ^a	<i>V. humblotii</i>	Madagascar	49,353453	-12,336973	MW879494**
CR3122 ^a	<i>V. humblotii</i>	Madagascar	49,353365	-12,340385	MW879495**
CR3131 ^a	<i>V. humblotii</i>	Madagascar	49,354839	-12,339791	MW879496**
CR3133 ^a	<i>V. madagascariensis</i>	Madagascar	49,477912	-12,724712	MW879498**
CR3173 ^a	<i>V. madagascariensis</i>	Madagascar	49,126024	-12,954172	MW879499**
CR3330 ^a	<i>V. bosseri</i>	Madagascar	45,274021	-16,067842	MW879475**
CR3393 ^a	<i>V. madagascariensis</i>	Madagascar	45,817042	-15,94609	MW879500**
CR3422 ^a	<i>V. bosseri</i>	Madagascar	44,675825	-22,911742	MW879476**
CR3483 ^a	<i>V. decaryana</i>	Madagascar	43,605376	-23,084468	MW879482**
CR3506 ^a	<i>V. bosseri</i>	Madagascar	44,657645	-20,067082	MW879477**
CR3528 ^a	<i>V. bosseri</i>	Madagascar	44,681156	-20,075398	MW879478**
CR3551 ^a	<i>V. decaryana</i>	Madagascar	44,03251	-20,833722	MW879479**
CR3560 ^a	<i>V. decaryana</i>	Madagascar	44,029252	-20,832241	MW879483**
CR3874 ^a	<i>V. perrieri</i>	Madagascar	47,760153	-14,852589	MW879501**
CR4022 ^a	<i>V. perrieri</i>	Madagascar	47,458123	-15,878717	MW879502**
CR4026 ^a	<i>V. perrieri</i>	Madagascar	47,457811	-15,879484	MW879503**
CR4039 ^a	<i>V. perrieri</i>	Madagascar	43,738393	-22,815007	MW879504**
CR4043 ^a	<i>V. bosseri</i>	Madagascar	43,777421	-22,791761	MW879480**
CR4072 ^a	<i>V. perrieri</i>	Madagascar	43,776318	-22,792011	MW879505**
CR4080 ^a	<i>V. decaryana</i>	Madagascar	43,518594	-22,946076	MW879484**
CR4142 ^a	<i>V. sp1</i>	Madagascar	46,777304	-22,08464	MW879465**
CR4155 ^a	<i>V. sp1</i>	Madagascar	46,777979	-22,085406	MW879466**
CR4157 ^a	<i>V. sp1</i>	Madagascar	46,777741	-22,085371	MW879467**
CR4171 ^a	<i>V. sp1</i>	Madagascar	46,777128	-22,084671	MW879468**

Voucher ¹	Species	Source	Longitude	Latitude	GenBank reference
CR4179 [*]	Unknown	Madagascar	48,774186	-18,446528	MW879518**

¹ Voucher names are based on collection code in the BRC Vatel (Reunion), leafy species are underlined, others are leafless species, ² Muséum National d'Histoire Naturelle, Cherbourg collection, ³ Botanical Garden, ⁴ Floribis collection (Vohemar), ⁵ Antsokay Arboretum, ⁶ Ankarafantsika (Madagascar), ⁷ Traditional doctor (Diego).

Table S2. Estimated divergence times from Bayesian relaxed clock analyses based on ITS markers and using uncorrelated exponential relaxed clock.

Mean and 95% HPD of the posterior probability distribution are in Mya. Node date of the common ancestor of the leafless *Vanilla* species from the SWIO region is taken from Bouetard et al. (2010).

Node	Mean [95% HPD]
Common ancestor of yellow flower clade and white flower clade	4.43 [0.44-8.27]
Common ancestor of <i>V. perrieri</i> and <i>V. humblotii</i>	0.87 [0.01-2.74]
<i>V. humblotii</i>	0.58[0.01-1.79]
<i>V. perrieri</i>	0.42[0.01-1.28]
Common ancestor of Madagascar Eastern species (<i>V. sp1</i> and <i>V. sp2</i>) and other white flower species	1.92[0.04-4.98]
Common ancestor of <i>V. sp1</i> and <i>V. sp2</i>	1.26[0.04-3.78]
<i>V. sp1</i>	0.44[0-1.61]
<i>V. sp2</i>	0.87[0.02-2.77]
Common ancestor of <i>V. madagascariensis</i> , <i>V. bosseri</i> , <i>V. phalaenopsis</i> , <i>V. decaryana</i> and <i>V. roscheri</i>	1.02[0.01-2.97]
Common ancestor of <i>V. madagascariensis</i> , <i>V. bosseri</i> and <i>V. phalaenopsis</i>	0.67[0.01-2.01]
<i>V. roscheri</i>	0.46[0-1.47]
<i>V. decaryana</i>	0.41[0-1.26]

CHAPITRE 4

Table S1. List of sites selected for flowering characterization

Species	Site	Code	Conservation status	Longitude	Latitude	Observation date	Transect (km)
<i>V. allorgii</i>	Tsaranoro	AND	SC	44.521769	-20.1412150	9/12/2018	0.3
<i>V. atsinananensis</i>	Ambodiriana	MAN	NPA	49.701394	-16.672233	18/12/2020	1.5
<i>V. bosseri</i>	Marosely	MSL	NPA	47,5750150	-15,6556310	20/10/2019	2.3
<i>V. decaryana</i>	Anja	AJA	UP	43,6044650	-23,0848790	16/12/2018	0.6
	Antandroka	ATA	SC	43,5185360	-22,9459550	21/12/2018	0.2
<i>V. madagascariensis</i>	Maromandia	MMD	UP	48,0878320	-14,2181240	20/11/2018	4.7
<i>V. humblotii</i>	Montagne des Français	MDF	NPA	49,3533360	-12,3370310	05/11/2019	1.8
<i>V. perrieri</i>	Befandrama	BFD	UP	47,7599550	-14,8529650	02/11/2019	1.1

SC: sacred forest, NPA: New Protected Areas, UP: Unprotected areas

Table S2. The sites and the number of inflorescences by species observed for calculating the natural fruit set

Species	Sites	Sites codes	Observation date	Flowering period	Number of inflorescences
<i>V. allorgii</i>	Tsaranoro	AND	9/12/2018	Middle	105
<i>V. atsinananensis</i>	Ambodiriana	MAN	27/01/2021	End	6
<i>V. bosseri</i>	Marosely	MSL	20/10/2019	Middle	49
<i>V. decaryana</i>	Anja	AJA	16/12/2018	Beginning	22
	Anja*	AJA*	16/12/2018	End*	20
	Antandroka	ATA	21/12/2018	Beginning	38
	Mahaleotse	MLT	19/12/2018	Middle	35
<i>V. madagascariensis</i>	Maromandia	MMD	20/11/2018	Middle	33
	Maromandia	MMD	19/01/2019	End	82
<i>V. humblotii</i>	Montagne des Français	MDF	05/11/2019	Middle	8
<i>V. perrieri</i>	Befandrama	BFD	12/01/2019	End	53
	Befandrama	BFD	01/11/2019	Middle	15

*: inflorescences of the previous year

Table S3. Summary of compatibility system tests performed on three species present in three study sites in Madagascar

Treatment	Species	Date of treatment	Site codes	Number of treatments
Spontaneous self-fertilization	<i>V. bosseri</i>	19/10/2019	MSL	11
	<i>V. madagascariensis</i>	25/10/2019	MMD	15
	<i>V. perrieri</i>	30/10/2019	BFD	18
Manual self-fertilization	<i>V. bosseri</i>	19/10/2019	MSL	11
	<i>V. madagascariensis</i>	25/10/2019	MMD	16
	<i>V. perrieri</i>	30/10/2019	BFD	23
Cross-pollination between two individuals of the same species	<i>V. bosseri</i>	19/10/2019	MSL	11
	<i>V. madagascariensis</i>	25/10/2019	MMD	16
	<i>V. perrieri</i>	30/10/2019	BFD	15
Cross-pollination between two individuals of different species	<i>V. madagascariensis</i> x <i>V. bosseri</i>	20/10/2019	MSL	1
	<i>V. bosseri</i> x <i>V. madagascariensis</i>	20/10/2019	MSL	1
	<i>V. madagascariensis</i> x <i>V. perrieri</i>	01/11/2019	BFD	3
	<i>V. perrieri</i> x <i>V. madagascariensis</i>	01/11/2019	BFD	3

Table S4. Variables used in Maxent species distribution modelling. Variables in bold are those used for final predictions.

Variables	Type
Annual mean Temperature (BIO1)	Climate
Isothermality (BIO3)	Climate
Temperature seasonality (BIO4)	Climate
Mean Temperature or Warmest Month (BIO6)	Climate
Temperature Annual Range (BIO7)	Climate
Mean Temperature of Wettest Quarter (BIO8)	Climate
Mean Temperature of Driest Quarter (BIO9)	Climate
Precipitation of Wettest Month (BIO13)	Climate
Precipitation seasonality (BIO15)	Climate
Precipitation of Wettest Quarter (BIO16)	Climate
Precipitation of Warmest Quarter (BIO18)	Climate
Maximum Normalized difference vegetation index (NDVI)	Habitat
pH	Habitat
Geology type	Habitat
Vegetation type	Habitat
Vegetation production	Habitat
Altitude	Habitat
Digital elevation model	Habitat
Aspect	Habitat
slope	Habitat
Human population density	Anthropogenic
Human influence index	Anthropogenic

LES 23 SITES D'ETUDE DANS LE CADRE DE LA THESE



Figure 1 Ankarantsika (AKF)
Espèces rencontrées : *V. madagascariensis*, *V. bosseri*,
V. perrieri



Figure 2. Ankarana (ANK)
Espèce rencontrée : *V. madagascariensis*



Figure 3. Analamerana (ANM)
Espèce rencontrée : *V. madagascariensis*



Figure 4. Antsianitia (ATS)
Espèces rencontrées : *V. madagascariensis*, *V. bosseri*



Figure 5. Befandrama (BFD)
Espèces rencontrées : *V. madagascariensis*, *V. perrieri*



Figure 6. Betaramahamay (BTM)
Espèce rencontrée : *V. perrieri*



Figure 7. Complexe Mahavavy kinkony (CMK)
Espèces rencontrées : *V. madagascariensis*, *V. bosseri*



Figure 8. Montagne des Français (MDF)
Espèce rencontrée : *V. humblotii*



Figure 9. Maromandia (MND)
Espèce rencontrée : *V. madagascariensis*



Figure 10. Marosely (MSL)
Espèces rencontrées : *V. madagascariensis*, *V. bosseri*



Figure 11. Namoroka (NMK)
Espèce rencontrée : *V. bosseri*



Figure 12. Namoroka (NMK)



Figure 13. Ankililoaka (AKL)
Espèces rencontrées : *V. bosseri*, *V. perrieri*

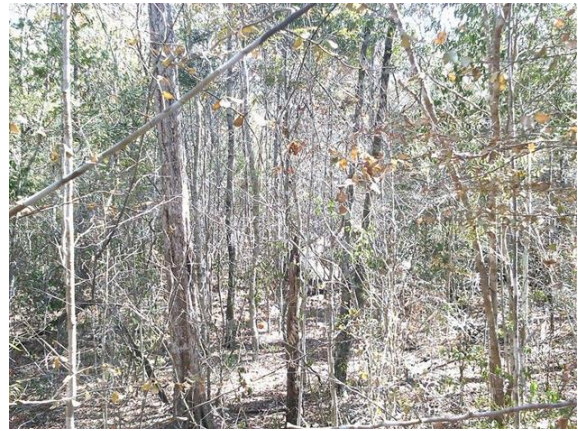


Figure 14. Kirindy (KRD)
Espèce rencontrée : *V. bosseri*



Figure 15. Kirindy Mitea (KRM)
Espèce rencontrée : *V. decaryana*

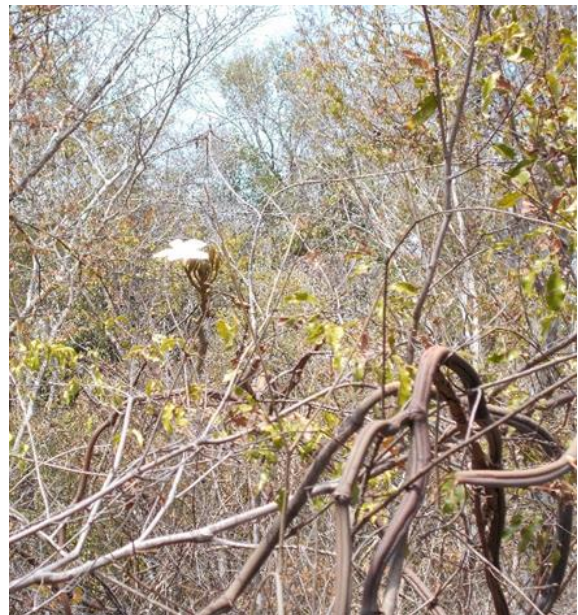


Figure 16. Zombitse (ZVB)
Espèce rencontrée : *V. bosseri*



Figure 17. Anja (ANJ)
Espèce rencontrée: *V. decaryana*



Figure 18. Antandroka (ATD)
Espèce rencontrée: *V. decaryana*



Figure 19. Mahaleotse (MLT)
Espèces rencontrées : *V. decaryana*, *V. perrieri*



Figure 20. Saint Augustin (SAG)
Espèce rencontrée : *V. decaryana*



Figure 21. Ambanja (AMB)
Espèce rencontrée : *V. madagascariensis*



Figure 22. Tsaranoro Andringitra (AND)
Espèce rencontrée : *V. allorgii* sp. nov



Figure 23. Manompana (MAN)
Espèce rencontrée : *V. atsinananensis* sp. nov



Figure 24. Manompana (MAN)

PERIODE DE FLORAISON DES SEPT VANILLIERS APHYLLES DE MADAGASCAR

Espèces	Août	Sept	Oct	Nov	Déc	Janv
<i>V. bosseri</i>	■					
<i>V. madagascariensis</i>		■				
<i>V. perrieri</i>				■		
<i>V. humblotii</i>				■		
<i>V. decaryana</i>					■	
<i>V. allorgii</i>			■			
<i>V. atsinananensis</i>					■	

Caractérisation des espèces aphyllées du genre *Vanilla* endémiques de Madagascar en vue de leur conservation

Résumé

Selon les plus récentes évaluations de la biodiversité, le nombre d'espèces végétales et animales recensé est estimé à près de huit millions. Cette richesse spécifique est le produit d'un long processus évolutif résultant de l'adaptation des organismes aux changements fréquents de leur environnement. La diversité biologique est inégalement répartie sur la planète, étant majoritairement concentrée sur quelques points chauds. Appelées « hotspots », ces zones sont les plus vulnérables aussi bien aux impacts des activités humaines qu'aux changements globaux actuels, causant un déclin important de la biodiversité. C'est le cas de la région Sud-Ouest de l'océan Indien (SOOI), un centre d'endémicité en orchidées. Leader mondial en production de gousses de vanille, cette région abrite également une diversité importante en espèces aphyllées endémiques appartenant au genre *Vanilla* Plum. ex. Miller (Orchidaceae), majoritairement distribuées à Madagascar. Les vanilliers aphyllés, par leur capacité d'adaptation à la sécheresse, leur résistance aux maladies fongiques et leurs propriétés médicinales, présentent un intérêt économique et scientifique majeur. Ces espèces forment un groupe monophylétique différencié récemment (4,4 Ma) à partir d'un ancêtre folié africain. Elles ont des traits morphologiques similaires et sont indissociables à partir de marqueurs chloroplastiques, rendant leur statut taxonomique douteux. A Madagascar, elles sont réparties principalement dans les forêts sèches de la côte Ouest, classées parmi les écosystèmes les plus menacés de l'île. Dans l'objectif de dénouer la taxonomie des vanilliers aphyllés et contribuer à leur conservation, des prospections suivies d'échantillonnages ont été réalisés dans plusieurs localités de Madagascar, couvrant une grande partie de leurs zones de distribution renseignées. Ensuite, une approche intégrative combinant la génétique, la biologie et l'écologie a été réalisée pour résoudre la taxonomie de ces espèces et déterminer leur statut de conservation. D'après les analyses des données microsatellites et morphologiques, la différenciation des sept groupes génétiques identifiés à Madagascar résulterait de plusieurs facteurs dont principalement les barrières géographiques (rivières), l'isolement par les facteurs environnementaux (IBE) (température, élévation, pH du sol) fortement corrélé à la géographie (IBD), et l'isolement par adaptation (IBA) de traits floraux (résultant soit de l'IBE soit d'une sélection divergente par les pollinisateurs). L'étude taxonomique intégrative (phylogénie ITS, analyse populationnelle microsatellite et morphologique) divise les vanilliers du SOOI en deux grands clades : espèces à fleurs blanches versus espèces à fleurs jaunes. Dans le clade des espèces à fleurs blanches, *V. decaryana* (Madagascar) et *V. roscheri* (Afrique de l'Est) forment chacune un groupe monophylétique tandis que *V. madagascariensis* (Madagascar), *V. bosseri* (Madagascar) et *V. phalaenopsis* (Seychelles) apparaissent conspécifiques (ITS), mais récemment différenciées (microsatellites). L'étude révèle également la présence de deux nouvelles espèces, réparties à l'Est, qui ont été nommées *V. allorgii* et *V. atsinananensis* et décrites. Dans le clade des aphyllées à fleurs jaunes, *V. perrieri* (Madagascar) semble s'être différenciée de *V. humblotii* (Madagascar et Comores). La configuration de l'arbre ITS soutient une origine malgache de ces deux grands clades, suivie de trois colonisations indépendantes vers l'Afrique de l'Est, l'Archipel des Comores et les Seychelles. D'après les tests de pollinisations réalisés, les espèces aphyllées sont auto-compatibles mais dépendent de pollinisateurs et peuvent se croiser entre elles. Les analyses de la diversité génétique, de la biologie de la reproduction et la modélisation spatiale et temporelle des niches écologiques des sept espèces malgaches suggèrent que *V. allorgii* et *V. atsinananensis* sont menacées respectivement par la faible densité des populations et une perte d'interaction avec les pollinisateurs. La fragmentation des populations naturelles de *V. decaryana* a entraîné une perte de diversité génétique ainsi qu'une forte consanguinité. *V. madagascariensis* et *V. humblotii* ont des zones de distribution restreintes dans le Nord, pouvant menacer leur survie à long terme. *V. perrieri* et *V. bosseri* semblent être les moins vulnérables car largement réparties le long de la côte Ouest. Des mesures de conservation *ex situ* et *in situ* sont proposées pour optimiser la conservation de ces vanilliers sauvages à Madagascar, un pays abritant une histoire évolutive impressionnante de la grande famille des Orchidaceae.

Mots clés : Orchidaceae, spéciation, diversité génétique, biologie de la reproduction, taxonomie intégrative, structuration génétique, phylogénie, modélisation de la distribution des espèces.

Characterization of leafless *Vanilla* species endemics to Madagascar with a view to their conservation

Abstract

The most recent biodiversity assessments indicate a number of about eight million plant and animal species in the world. This specific richness is the product of a long evolutionary process resulting from the adaptation of organisms to frequent changes in their environment. Biological diversity is unevenly distributed on the planet, being mostly concentrated in a few regions, called "hotspots". These regions are the most vulnerable to both human activities and current global changes, causing a significant decline in biodiversity. This is the case of the South West Indian ocean (SWIO) region, a center of orchid endemism. In addition to being a leader in *Vanilla planifolia* pods production, SWIO also presents an important specific richness in endemic leafless plants belonging to the genus *Vanilla* Plum. ex. Miller (Orchidaceae), mainly distributed in Madagascar. Because of their ability to adapt to drought, their resistance to fungal diseases and their medicinal properties, leafless *Vanilla* species are of major economic and scientific interest. They form a monophyletic group recently (4.4 Ma) diverged from an African leafy ancestor. They cannot be differentiated by morphological traits and chloroplast genes, making their taxonomic status questionable. In Madagascar, leafless *Vanilla* species are distributed mainly in the dry forests along the West coast, classified among the most threatened ecosystems of the island. In order to unravel the taxonomy of Malagasy leafless *Vanilla* species and contribute to their conservation, field surveys followed by sampling were carried out in several localities of Madagascar, covering a large part of their known distribution areas. Then, an integrative approach combining genetics, biology and ecology was carried out to solve the taxonomy of these species and determine their conservation status. According to genetic clustering using microsatellite loci and morphological analyses, the seven genetic groups identified in Madagascar differentiated likely due to several factors, including geographical barriers (rivers), isolation by environmental factors (IBE) (temperature, elevation, soil pH) strongly correlated to geographical isolation (IBD), and isolation by adaptation (IBA) of floral traits (resulting either from IBE or from divergent selection by pollinators). The integrative taxonomic strategy (ITS phylogeny, population-based microsatellite and morphological analyses) divides SOOI leafless *Vanilla* species into two major clades: white-flower versus yellow-flower species. In the white-flower species clade, *V. decaryana* (Madagascar) and *V. roscheri* (East Africa) each forms a monophyletic group, while *V. madagascariensis* (Madagascar), *V. bosseri* (Madagascar) and *V. phalaenopsis* (Seychelles) appear conspecific (ITS) although recently differentiated (microsatellites). The study also reveals the presence of two new species, distributed in the East, named *V. allorgii* and *V. atsinananensis*, and described. Within the yellow-flower leafless species clade, *V. perrieri* (Madagascar) appears to have differentiated from *V. humblotii* (Madagascar and Comoros). The ITS phylogenetic tree structure supports a Malagasy origin of these two major clades, followed by three independent colonization events to East Africa, the Comoros Archipelago and the Seychelles. The pollination tests conducted suggest that all species are self-compatible but pollinator-dependent and can interbreed. Analyses of genetic diversity, reproductive biology, and spatial and temporal modelling of suitable habitats indicated that *V. allorgii* and *V. atsinananensis* are threatened by low population density and loss of interaction with pollinators, respectively. Fragmentation of natural populations of *V. decaryana* has resulted in loss of genetic diversity and high inbreeding. *V. madagascariensis* and *V. humblotii* have restricted distribution areas in the North, which may threaten their long-term survival. *V. perrieri* and *V. bosseri* appear to be the least vulnerable because they are widely distributed along the West coast. *Ex situ* and *in situ* conservation measures are proposed to optimize the protection of these wild *Vanilla* species in Madagascar, a country harboring an impressive evolutionary history of the large Orchidaceae family.

Keywords : Orchidaceae, speciation, genetic diversity, reproductive biology, integrative taxonomy, genetic structuring, phylogeny, species distribution modelling.