Received: 2 July 2019 | Revised: 20 October 2019 | Accepted: 31 October 2019

DOI: 10.1002/ajp.23070

AMERICAN JOURNAL OF PRIMATOLOGY WILEY

RESEARCH ARTICLE

Genetic and morphological diversity of mouse lemurs (*Microcebus* spp.) in northern Madagascar: The discovery of a putative new species?

Gabriele Maria Sgarlata[1*] | Jordi Salmona[1,2*] | Barbara Le Pors[1] | Emmanuel Rasolondraibe[3] | Fabien Jan[1] | Tantely Ralantoharijaona[3] | Ando Rakotonanahary[3] | Jacquis Randriamaroson[3] | Adam Joseph Marques[1] | Isa Aleixo-Pais[1,4,5] | Tiago de Zoeten[1] | Dhurham Said Ali Ousseni[3] | Simon Benjamin Knoop[1] | Helena Teixeira[1,6] | Vivien Gabillaud[1] | Alex Miller[1,7] | Mohamed Thani Ibouroi[3,8,9] | Solofonirina Rasoloharijaona[3] | John Rigobert Zaonarivelo[8] | Nicole Volasoa Andriaholinirina[3] | Lounès Chikhi[1,2]

[1]Instituto Gulbenkian de Ciênca, Oeiras, Portugal

[2]Laboratoire Évolution & Diversité Biologique (EDB UMR 5174), IRD, CNRS, UPS, Université de Toulouse Midi-Pyrénées, Toulouse, France

[3]Département de Biologie Animale et Ecologie, Faculté des Sciences, Université de Mahajanga, Mahajanga, Madagascar

[4]Organisms and Environment Division, Cardiff School of Biosciences, Cardiff University, Cardiff, UK

[5]Centre for Research in Anthropology (CRIA-FCSH/NOVA), Lisbon, Portugal

[6]Institute of Zoology, University of Veterinary Medicine Hanover, Hanover, Germany

[7]School of Human Sciences, The University of Western Australia, Crawley, Australia

[8]Département des Sciences de la Nature et de l'Environnement, Université d'Antsiranana, Antsiranana, Madagascar

[9]Institut des Sciences et Industrie du Vivant et de l'environnement, AgroParisTech, Paris, France

Correspondence
Gabriele Maria Sgarlata, Instituto Gulbenkian de Ciênca, Rua da Quinta Grande, 6, P-2780-156 Oeiras, Portugal.
Email: gabriele.sgarlata@gmail.com

Lounès Chikhi, Laboratoire Évolution & Diversité Biologique (EDB UMR 5174), IRD, CNRS, UPS, Université de Toulouse Midi-Pyrénées, 118 route de Narbonne, Bât. 4R1, 31062 Toulouse cedex 9, France.
Email: chikhi@igc.gulbenkian.pt; lounes.chikhi@univ-tlse3.fr

Funding information
Fundação para a Ciência e a Tecnologia, Grant/Award Numbers: PD/BD/114343/2016, PTDC-BIA-EVL/30815/2017, PTDC/BIA-BEC/100176/2008, PTDC/BIA-BIC/4476/2012, SFRH/BD/64875/2009; 2015–2016 BiodivERsA, Grant/Award Numbers: 01LC1617A, ANR-16-EBI3–0014, Biodiversa/0003/2015;

Abstract

Tropical forests harbor extremely high levels of biological diversity and are quickly disappearing. Despite the increasingly recognized high rate of habitat loss, it is expected that new species will be discovered as more effort is put to document tropical biodiversity. Exploring under-studied regions is particularly urgent if we consider the rapid changes in habitat due to anthropogenic activities. Madagascar is known for its extraordinary biological diversity and endemicity. It is also threatened by habitat loss and fragmentation. It holds more than 100 endemic primate species (lemurs). Among these, *Microcebus* (mouse lemurs) is one of the more diverse genera. We sampled mouse lemurs from several sites across northern Madagascar, including forests never sampled before. We obtained morphological data from 99 *Microcebus* individuals; we extracted DNA from tissue samples of 42 individuals and amplified two mitochondrial loci (cytb and cox2) commonly used for species identification. Our findings update the distribution of three species (*Microcebus tavaratra*, *Microcebus arnholdi*, and *Microcebus mamiratra*), including a major increase in the distribution

*Gabriele Maria Sgarlata and Jordi Salmona are shared first co-authors.

Investissement d'Avenir grant of Agence Nationale de la Recherche, Grant/Award Number: ANR-10-LABX-25-01; Rufford Small Grant Foundation, Grant/Award Numbers: 10941-1, 12973-1; LIA BEEG-B (Laboratoire International Associé – Bioinformatics, Ecology, Evolution, Genomics and Behaviour, CNRS); Labex - TULIP, Grant/Award Numbers: ANR-10LABX-41, ANR-11-IDEX-0002-02

area of *M. arnholdi*. We also report the discovery of a new *Microcebus* lineage genetically related to *M. arnholdi*. Several complementary approaches suggest that the newly identified *Microcebus* lineage might correspond to a new putative species, to be confirmed or rejected with additional data. In addition, morphological analyses showed (a) clear phenotypic differences between *M. tavaratra* and *M. arnholdi*, but no clear differences between the new *Microcebus* lineage and the sister species *M. arnholdi*; and (b) a significant correlation between climatic variables and morphology, suggesting a possible relationship between species identity, morphology, and environment. By integrating morphological, climatic, genetic, and spatial data of two northern *Microcebus* species, we show that the spatial distribution of forest-dwelling species may be used as a proxy to reconstruct the past spatial changes in forest cover and vegetation type.

KEYWORDS
connectivity, cryptic species, phylogeny, species delimitation, sympatry

1 | INTRODUCTION

Tropical forests hold an extraordinary amount of biological diversity and new species and taxa continue to be discovered at a high rate (Burgin, Colella, Kahn, & Upham, 2018). Nevertheless, tropical ecosystems are currently undergoing rapid environmental changes, mainly due to human activities (Venter et al., 2016), which pose a serious and concrete risk to the persistence of world biodiversity.

Madagascar is a recognized biodiversity hotspot (Myers, Mittermeier, Mittermeier, Fonseca, & Kent, 2000), whose forest-dwelling species are increasingly threatened by extensive and ongoing habitat loss and fragmentation (Harper, Steininger, Tucker, Juhn, & Hawkins, 2007; Schwitzer et al., 2014; Waeber, Schuurman, & Wilmé, 2018). This threat is especially serious for the species that are distributed in restricted ranges (i.e., microendemics), a category that includes a high percentage of Malagasy taxa (Wilmé, Goodman, & Ganzhorn, 2006).

In the present study, we focus on mouse lemurs (genus *Microcebus*), a small-sized nocturnal primate group that occurs in a wide variety of habitat types across Madagascar (Kappeler & Rasoloarison, 2003). The number of recognized *Microcebus* species has greatly increased from two species, more than 20 years ago, to more than 20 putative species today (Ross and Kappeler, 2006; Andriantompohavana et al., 2006; Hotaling et al., 2016; Louis et al., 2006, 2008; Olivieri et al., 2007; Radespiel et al., 2008, 2012; Rasoloarison, Weisrock, Yoder, Rakotondravony, & Kappeler, 2013). Although earlier studies applied morphological data as criteria to identify new *Microcebus* species (e.g., Rasoloarison, Goodman, & Ganzhorn, 2000; Zimmermann, Cepok, Rakotoarison, Zietemann, & Radespiel, 1998), more recently the use of genetic data has played a major role in the discovery of new *Microcebus* taxa in Madagascar. Genetic data, 15–20 years ago, in particularmitochondrial DNA (mtDNA), appeared to supersede morphological data, as they were not always sufficient to discriminate *Microcebus* species (Yoder, Burns, & Génin, 2002; Yoder et al., 2005). More recently, Weisrock et al. (2010) used mtDNA and four nuclear loci to provide a more robust estimate of *Microcebus* species delimitations. Although the effective population size of mtDNA is smaller than that of nuclear DNA, Weisrock et al. (2010) observed a noticeable concordance between mtDNA lineages and patterns of nuclear genetic structure, hence suggesting that mtDNA was often sufficient to identify different species. In the most recent comprehensive study on *Microcebus* phylogenetics, Hotaling et al. (2016) used a multilocus genetic dataset (mtDNA and nuclear loci) and applied a Bayesian approach based on the multispecies coalescent (MSC) model (Rannala & Yang, 2003; Yang, 2002) to assess the validity of species delimitation. However, Sukumaran and Knowles (2017) have shown that the MSC model cannot always separate species divergence from intraspecies population structure, and they concluded that a rigorous approach able to distinguish between population- and species-level structure still needs to be developed. Altogether, this suggests that the identification of species should not rely only on genetic or genomic data but should integrate other types of data, including morphological or behavioral data, when available.

In this work, we present the results of surveys across northern Madagascar, where five *Microcebus* species have been described: *Microcebus arnholdi*, *Microcebus tavaratra*, *Microcebus mamiratra*, *Microcebus margotmarshae*, and *Microcebus sambiranensis*. We collected *Microcebus* samples from several forest fragments for which nearly no information is currently available. We obtained morphological data from 99 new *Microcebus* individuals, and we genotyped 42 individuals for two mtDNA loci (cytb and cox2) commonly used for species identification and representing currently the main reference for species identification in the *Microcebus* genus (see however, Louis & Lei, 2016 for a recent use of mitogenome for 23 species). Mitochondrial genetic data were used to construct phylogenetic trees using both maximum-likelihood (ML) and Bayesian approaches. We used the MSC model to test species delimitation hypotheses and compare our results with *Microcebus* species previously defined or

confirmed using this approach (see Hotaling et al., 2016). Considering the limitations of the MSC model in distinguishing population structure from species divergence (Sukumaran & Knowles, 2017), we also used an *ad hoc* analysis that compares phylogenetic and geographic distances within and between sister-groups.

Most *Microcebus* species have been described based on the phylogenetic species concept, which considers genetic monophyly as evidence for species delimitation (Cracraft, 1983). However, additional data, such as morphology and climate, can provide further lines of evidence to support species delimitation (De Queiroz, 2005, 2007) by identifying clear morphological differences and habitat preferences among the hypothesized species. Therefore, we used georeferenced climatic and morphological data of the sampled individuals (a) to assess whether habitat preferences and phenotypic differences could be detected among the four *Microcebus* taxa studied here; and (b) to quantify the contribution of the climatic factors measured on morphological variability. According to the "recent divergence" hypothesis, we expect that taxa genetically more divergent will show stronger morphological and habitat differences compared to taxa showing lower genetic divergence (see, for instance, Struck et al., 2018; Chenuil et al., 2019).

Finally, we consider (a) the "niche conservatism" hypothesis, defined as the tendency of species to retain aspects of their fundamental niche over time (Wiens et al., 2010); and (b) the fact that *Microcebus* spp. are mostly forest-dependent species, to qualitatively reconstruct the forest cover changes that occurred in northern Madagascar during the late Quaternary. In particular, we integrate information on habitat preferences, morphological differences, and geographical location of two northern mouse lemur's species typically associated with either dry (*M. tavaratra*) or humid (*M. arnholdi*) habitat and discuss the biogeographical changes that could have led to their present fragmented distribution.

2 | METHODS

2.1 | Study species

In the present study, we focus on three of the five *Microcebus* species occurring in northern Madagascar (Figure 1): *M. arnholdi*, *M. tavaratra* and *M. mamiratra*.

M. arnholdi (head-body length: 10–12.6 cm) has been described using two mtDNA loci from a population sampled at Montagne d'Ambre (AMBRE), northern Madagascar (Figure 1; Louis et al., 2008). Little is known about this species, except that it occurs in humid habitats of northern Madagascar (Mittermeier et al., 2010). It was thought to be exclusively present in AMBRE until Weisrock et al. (2010) found that an individual sampled at Ambanja, more than 150 km south of AMBRE (Figure 1), was genetically clustering with individuals from AMBRE. *M. arnholdi* is currently classified as Endangered (Andriaholinirina et al., 2014).

M. tavaratra (head-body length: 12–14 cm) inhabits dry deciduous and transition forests of northern Madagascar, between 50–670 m of altitude (Mittermeier et al., 2010; Rasoloarison et al., 2000;

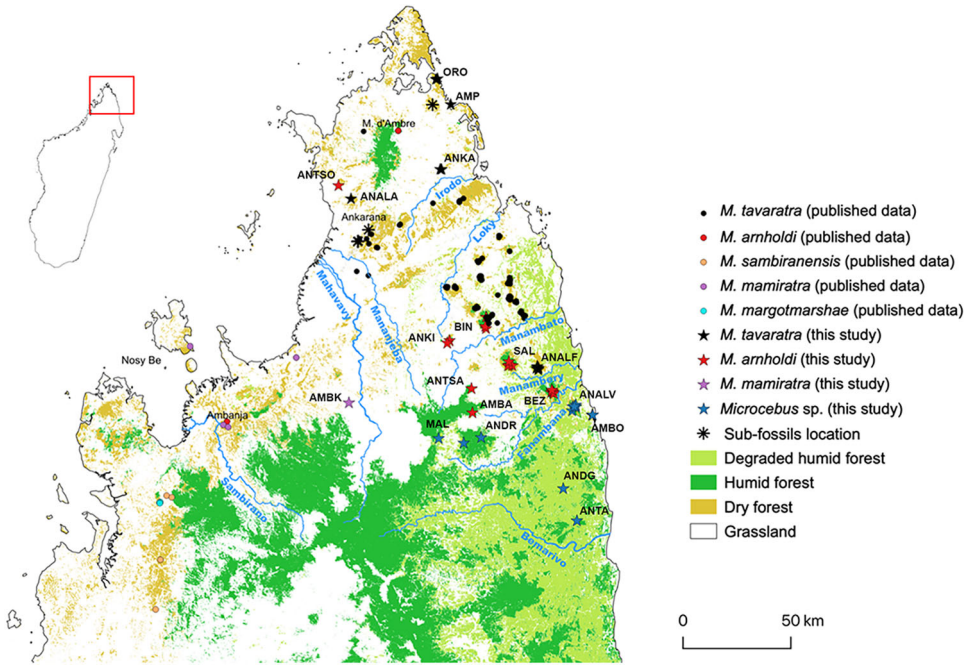

FIGURE 1 Sampling distribution of *Microcebus* spp. across northern Madagascar. Black labels represent forest fragments surveyed between 2010 and 2017. AMBA, Ambaliha; AMBK, Ambakirano; AMBO, Ambohitrandrina; AMP, Ampiho; ANALA, Analabe; ANALF, Analafiana; ANALV, Analalava; ANDG, Andrangotra; ANDR, Andravory; ANKA, Ankarongana; ANKI, Ankinjanala; ANTA, Antananivo; ANTSA, Antsahavary; ANTSO, Antsoroby; BEZ, Bezavona; BIN, Binara; MAL, Maladialina; ORO, Oronjia; SAL, Salafaina. Stars indicate the individuals sampled in the present study. Circles indicate published data. Asterisks indicate subfossil localities for *Indri indri*, *Prolemur simus* and *Propithecus cf. diadema*

Sgarlata et al., 2018). Originally described from morphological and genetic data by Rasoloarison et al. (2000) and Yoder et al. (2000), it is currently known to occur in the regions of the Ankarana, Analamerana, Andavakoera, Andrafiamena, and Loky-Manambato, and probably in the lowland forests of AMBRE (Figure 1). *M. tavaratra* is classified as Vulnerable (Andriaholinirina et al., 2014).

M. mamiratra (head-body length: 10.5–12.7 cm) occurs in the humid primary and secondary forests of the small Lokobe Strict Nature Reserve of Nosy Be Island (Andriantompohavana et al., 2006; Olivieri et al., 2007). It was first described by Andriantompohavana et al. (2006) and later independently reported synonymous with *Microcebus lokobensis* by Olivieri et al., 2007 before being recognized as one single species. It is classified as Critically Endangered (Andriaholinirina et al., 2014).

2.2 | Study sites

Nineteen sites were visited between 2010 and 2017 (Figure 1 and Table 1). Fieldwork varied across years but was typically conducted between May and October to avoid the rainy season. We conducted our sampling in several dry deciduous forest fragments of the far north: the Oronjia (or Orangéa, abbreviated as ORO) new protected area (NAP for Nouvelle Aire Protégée), the Ampiho (AMP) forest part of the Ambodivahibe NAP, the Ankarongana forest (ANKA), the Analabe (ANALA), and Antsoroby forests (ANTSO). We collected samples from one dry forest located south of the Manambato River, Analafiana (ANALF), and a littoral forest south of Fanambana River, Ambohitrandrina (AMBO; Figure 1). We also surveyed several humid forest fragments: the Binara (BIN) mountain forest located within the Loky-Manambato NAP; the Salafaina and Bezavona-Ankirendrina forests, located between the Manambato and Fanambana Rivers; the Ankinjanala forest located at the headwater of the Loky River; the Antsahavary and Ambaliha (AMBA) forests within the Corridor of Marojejy-Anjanaharibe Sud-Tsaratanana (COMATSA) NAP; Analalava (ANALV) forest, south of the Fanambana River; the Andravory humid massif between north COMATSA and Fanambana River; the Andrangotra (ANDG) forest, a small fragment close to Antsirabe-Nord village, and the Antananivo (ANTA) forest, part of the Makirovana-Tsihomanaomby forest complex NAP, located south of the Fanambana River. We also visited a small forest fragment close to the Ambakirano village, at the east of the Mahavavy River (AMBK).

2.3 | Sampling and laboratory procedures

We captured mouse lemur individuals at night (between 18:00 and 07:00) using Sherman traps (H. B. Sherman Traps®), following

TABLE 1 Collecting sites and genetic data of the *Microcebus* individuals sampled across northern Madagascar

Forest	Abbreviation	Year	N	cytb	cox2	Morpho (N)	Altitude (m)	Latitude	Longitude	Forest type	Clade
Oronjia	ORO	2015	2	1	1	2	15, 82	−12.255	49.378	D	*Microcebus tavaratra*
Ampiho	AMP	2017	1	1	–	1	9	−12.361	49.440	D	*M. tavaratra*
Ankarongana	ANKA	2013	2	2	2	2	194, 254	−12.634	49.403	D	*M. tavaratra*
Analabe	ANALA	2017	1	1	–	10	101	−12.763	49.021	D	*M. tavaratra*
Analafiana	ANALF	2013	5	5	5	5	41–90	−13.456	49.831	D	*M. tavaratra*
Antsoroby	ANTSO	2017	1	1	–	1	56	−12.709	48.966	D	*Microcebus arnholdi*
Binara	BIN	2010	3	3	3	27	526–781	−13.278	49.606	T-H	*M. arnholdi*
Salafaina	SAL	2013	4	4	4	4	309–653	−13.444	49.711	T-H	*M. arnholdi*
Bezavona	BEZ	2013	5	5	5	5	231–600	−13.555	49.897	T-H	*M. arnholdi*
Ankinjanala	ANKI	2015	2	2	2	1	714–993	−13.355	49.447	T-H	*M. arnholdi*
Antsahavary	ANTSA	2015	1	1	–	1	951	−13.548	49.550	H	*M. arnholdi*
Ambaliha	AMBA	2015	1	1	1	1	972	−13.649	49.556	H	*M. arnholdi*
Analalava	ANALV	2013	5	5	5	5	63–899	−13.617	49.991	T-H	*Microcebus* sp.
Ambohitrandrina	AMBO	2013	3	3	3	3	15–17	−13.649	50.069	D	*Microcebus* sp.
Andrangotra	ANDG	2015	1	1	1	1	252	−13.959	49.951	H	*Microcebus* sp.
Maladialina	MAL	2014	1	1	1	1	986	−13.759	49.413	H	*Microcebus* sp.
Andravory	ANDR	2014	2	2	2	2	899, 954	−13.764	49.558	H	*Microcebus* sp.
Antananivo	ANTA	2014	1	1	1	1	259	−14.093	50.012	H	*Microcebus* sp.
Ambakirano	AMBK	2015	1	–	1	–	253	−13.616	49.026	H	*Microcebus mamiratra*
Total	–	–	42	40	37	73	–	–	–	–	–

Note: N = n° of individuals; cytb = n° of individuals genotyped for cytb locus; cox2 = n° of individuals genotyped for cox2 locus; Morpho (N) = sample size for morphological data; Altitude = altitude of the capture site; D = dry; H = humid; T-H = transition-humid; Clade: the mtDNA clade to which the genotyped individuals cluster. "–" = information not available.
Abbreviation: mtDNA, mitochondrial DNA.

established field procedures (Rakotondravony & Radespiel, 2009). We collected ear biopsies and 12 morphological measurements (Olivieri et al., 2007; see Supporting Information B), and released individuals at their capture site later the same day. Our handling protocol for biopsy sampling and morphological measurements does not require anesthetization of the captured individuals. We recorded the global positioning system (GPS) coordinates of the capture site and acquired the altitude information from a digital elevation model database using GPS Visualizer (http://www.gpsvisualizer.com/elevation). We stored the biopsies in Queen's lysis buffer (Seutin, White, & Boag, 1991) at ambient temperature in the field (between 2 and 6 months, depending on when the individual was caught) and at −20°C without changing buffer solution, once the samples are shipped to our laboratory. We extracted total genomic DNA from 42 new samples using a Qiagen modified protocol described in Sgarlata et al. (2018) and Aleixo-Pais et al. (2018). We amplified and sequenced two mitochondrial (mtDNA) loci using published primers: cytochrome b (cytb; 1,140 bp; Irwin, Kocher, & Wilson, 1991) and the cytochrome c oxidase subunit II (cox2; 684 bp; Adkins & Honeycutt, 1994). Details on the polymerase chain reaction (PCR) amplification can be found in Sgarlata et al. (2018). We sequenced the PCR products in an ABI 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, CA) and edited the sequences using GENEIOUS PRO v10.2.3 (http://www.geneious.com, Kearse et al., 2012). Data for both mtDNA loci were available for 36 out of 42 new *Microcebus* samples.

We retrieved additional mtDNA sequences from Weisrock et al. (2010) and Hotaling et al. (2016). The complete dataset does not include data for *M. margotmarshae*, given that sequences on the same mtDNA loci were not available. The complete datasets consisted of (a) cytb sequences for 313 individuals, representing 22 described *Microcebus* species; and (b) cox2 sequences for 335 individuals, representing 24 described *Microcebus* species. We built an additional dataset by combining cytb and cox2 sequences. We aligned and visually checked the sequences using the Clustal Omega method (Sievers et al., 2011). We performed a format conversion of DNA alignments using ALTER (Glez-Peña, Gómez-Blanco, Reboiro-Jato, Fdez-Riverola, & Posada, 2010).

2.4 | Phylogenetic analyses

We used two statistical methods for phylogenetic analysis: a Bayesian Markov Chain Monte Carlo (MCMC) approach implemented in MRBAYES v3.2.1 (Ronquist et al., 2012) and an ML approach implemented in RAXML v8.2.X (Stamatakis, 2014; see Appendix for more details).

2.5 | Species validation

Phylogenetic analyses showed the presence of a monophyletic clade never reported before, closely related to *M. sambiranensis* and *M. arnholdi* (Figure 2). For the sake of simplicity, we will refer to the new mtDNA clade as "*Microcebus* sp." in the rest of the manuscript. We tested the species delimitation hypothesis between the three sister-groups, *M. sambiranensis*, *M. arnholdi* and the new *Microcebus* sp. clade, using the BPP v3.4 software (Yang, 2015), a Bayesian MCMC program for analyzing DNA sequence alignments under the MSC model (Rannala & Yang, 2003; Yang, 2002). While the method is usually used with multilocus data, it has also been shown that such a model can accurately assign species identity with even one locus (Yang & Rannala, 2017; see Appendix for further details).

Methods based on the MSC model, as BPP software, under some conditions, cannot efficiently distinguish species and population genetic structure, thus leading to the identification of spurious species (Sukumaran & Knowles, 2017). Intraspecific genetic structure arises when there is reduced gene-flow among populations, which can be due, for instance, to (a) geographical distance (i.e., isolation by distance; Wright, 1943); and/or (b) geographical isolation, as a consequence of the presence of a partial barrier between populations (Avise, 2000). To assess whether *M. arnholdi* and the new *Microcebus* sp. clade represent intraspecific lineages isolated due to geographical distance, we compared phylogenetic and geographical distance among individuals (within and between clades). This is very similar to the commonly performed test of "isolation by distance," and the assumption we make is that individuals belonging to the same species should show a similar level of genetic differentiation when separated by similar geographic distances. If the two mtDNA lineages relate to intraspecific population structure, it would be more likely to observe, for the same geographic distance, similar values of phylogenetic distance between interclades and intraclade comparisons. Of course, this cannot be an absolute test but a reasonable one at this stage. We performed this analysis with the cytb, cox2 and combined cytb+cox2 dataset in R (R Core Team, 2018). We estimated phylogenetic distances between individuals with the adephylo R package (Jombart, Balloux, & Dray, 2010), using the patristic distance, that is the sum of branch lengths on the path between individuals. We used pairwise.wilcox.test function in R to assess significant differences in the distribution of phylogenetic distance values between intraclade and interclades groups. Last, we compared the proportion of nucleotide differences of recognized *Microcebus* species pairs with that of *Microcebus* sp.–*M. arnholdi* clade pair. We computed the proportion of nucleotide differences on the cytb and cox2 datasets using the software MEGA v7 (Kumar, Stecher, & Tamura, 2016).

2.6 | Morphological analyses

We used 12 morphological measurements to assess whether morphological differences could be detected between *M. arnholdi*, *M. tavaratra*, *Microcebus* sp., and *M. mamiratra* clades. We performed the analysis on four groups of individuals: (a) *M. tavaratra*–*M. arnholdi*; (b) *M. tavaratra*–*M. arnholdi* in BIN forest; and (c) *M. arnholdi*–*Microcebus* sp. clade; (d) *M. arnholdi*–*Microcebus* sp. clade-*M. mamiratra*

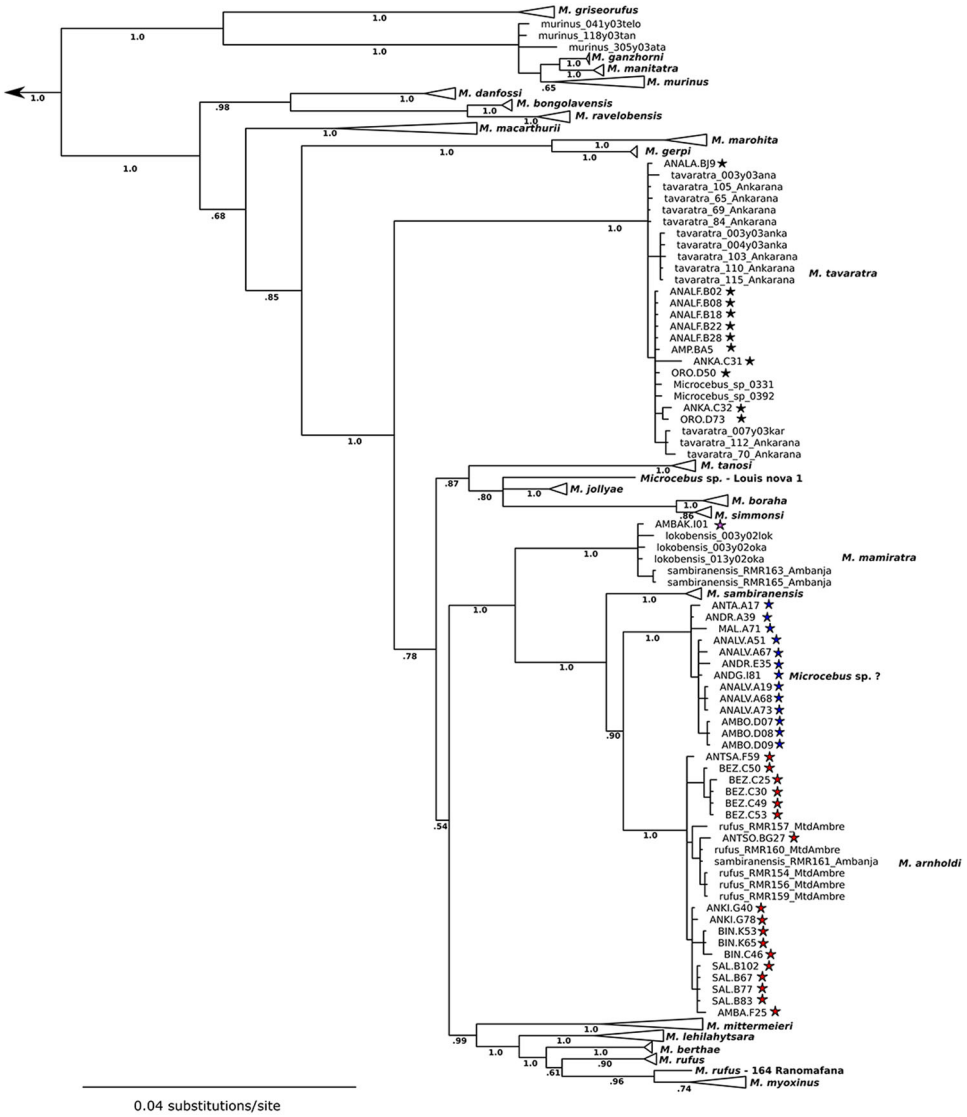

FIGURE 2 Bayesian phylogenetic tree of the *Microcebus* genus. We analyzed the combined mtDNA dataset (cytb + cox2) using the Bayesian Markov Chain Monte Carlo approach implemented in MRBAYES v3.2.1. Stars show the 42 new sampled individuals. Data from other *Microcebus* species were retrieved from Weisrock et al. (2010) and Hotaling et al. (2016). The numbers below branches are posterior probabilities. The sample ID does not refer necessarily to species identity, but does correspond to the sample ID present in the NCBI database

(Table S3). For comparison purposes, we also used morphological data of individuals from AMBRE (Station de Roussettes; $N = 18$) and Ankarana (ANKAR; $N = 7$), for which no genetic data are presented here, and BIN ($N = 24$; genetically identified as *M. tavaratra* in Sgarlata et al., 2018). AMBRE is inhabited by *M. arnholdi* (Louis et al., 2008), while Ankarana and BIN by *M. tavaratra* (Andriantompohavana et al., 2006); this is why data from these sites were used as reference for species morphological assignment. In addition, we conducted the same analysis on *Microcebus bongolavensis–Microcebus ravelobensis*, well-known *Microcebus* sister species for which similar morphological measures were available (Olivieri et al., 2007). We used the K-means clustering algorithm to identify the most likely number of divergent groups based on morphological information (see Appendix for more details). We also used the Discriminant Analysis of Principal Component (DAPC) to identify which morphological variables contribute most significantly to the morphological structure. DAPC analysis was performed using the dapc function in adegenet R package (Jombart, 2008; Jombart & Ahmed, 2011).

Finally, we examined whether a certain percentage of individual morphological variability could be explained by the effect of abiotic factors. We used the individual coordinates of the first or second dimensions of the K-means clustering analysis and 19 bioclimatic variables extracted from the WorldClim v2 dataset (Fick & Hijmans, 2017). Bioclimatic information for the georeferenced locations of the sampled individuals was obtained using the raster and sp R packages (Bivand, Pebesma, & Gómez-Rubio, 2013; Hijmans, 2017; Pebesma & Bivand, 2010). To disentangle the effects of either geography (as proxy for genetic relatedness) or climate on morphological variability, we used variance-partitioning analysis for estimating the proportions of variation explained by (a) climate controlling for geography

(Climate | Geography); (b) geography controlling for climate (Geography | Climate); and (c) the joint contribution of geography and climate (Geography AND Climate). We performed the same analyses using as a response variable "mtDNA identity", instead of "morphology". We then selected, among the 19 bioclimatic variables, those that showed low levels of collinearity, measured as Pearson correlation ($R < 0.2$) and assessed differences of those variables on the distribution of the *Microcebus* species studied here. Species pairwise comparisons were assessed for significance using the nonparametric Wilcoxon test.

3 | RESULTS

From an extensive survey effort including forests never sampled before, we present new mitochondrial genetic data for 42 *Microcebus* individuals and new morphological data from 99 individuals. We used these data to clarify the taxonomy and phylogeography of mouse lemurs in northern Madagascar. Using ML and Bayesian coalescent-based methods to reconstruct *Microcebus* phylogeny, we propose to update the distribution of three species (*M. tavaratra*, *M. arnholdi*, and *M. mamiratra*). Moreover, our phylogenetic analyses reveal a novel mtDNA lineage which, based on several complementary approaches, is supported to be a possible new *Microcebus* species.

3.1 | Phylogenetic analyses

The phylogenetic analyses performed with the combined mtDNA loci (i.e., cox2 + cytb) were consistent with previously published *Microcebus* phylogenies (Figure 2; Hotaling et al., 2016; Louis & Lei, 2016; Weisrock et al., 2010). Both ML (RAXML) and Bayesian (MRBAYES) methods produced similar trees, with few differences in clade topology (Figure 2; Figures S1 and S2). In particular, the cytb + cox2 RAXML phylogenetic tree showed discordance for dichotomies between *Microcebus tanosi* and *M. tavaratra*, inconsistency on the sister-species of *Microcebus rufus* and lack of monophyly for *Microcebus gerpi* (Figure S2c). In addition, we noticed that, in both RAXML and MRBAYES trees, three *Microcebus murinus* individuals (top of Figure 2 and Figure S2c) could not be associated unambiguously to the *M. murinus* clade, likely due to the shorter mtDNA sequences of these individuals compared to the whole dataset. All previously described *Microcebus* species ($N = 24$) showed monophyly (Figure 2). Besides the three *M. murinus* individuals, the only exception is a divergent individual of *M. rufus*, which was also reported in Hotaling et al. (2016).

Among the 42 newly collected samples, 11 clustered with the *M. tavaratra* clade, therefore, extending its distribution range to forests north of the Irodo River (ORO, AMP, and ANKA), south-west of the M. d'Ambre (ANALA) and south of the Manambato River (ANALF; Figure 1). One additional individual clustered with the *M. mamiratra* clade broadening its distribution to the gallery forest neighboring the Ambakirano village, along the Mahavavy River in northwestern Madagascar. Moreover, our phylogenetic analyses showed that one individual from the ANTSO forest, south-west of AMBRE, and 16 individuals from humid forests south of the Loky River were genetically associated with the *M. arnholdi* clade, greatly extending its distribution. One of the most remarkable results was that the remaining 13 samples, collected south of the Manambato River, formed a new monophyletic clade (*Microcebus* sp.), sister to *M. arnholdi*. The monophyly of the new clade was well supported in all analyses except when using the cox2 dataset alone. In the following sections, we assess whether the new mtDNA clade would represent a new *Microcebus* species.

3.2 | Species validation analyses

The mean proportion of pairwise differences (p-distance) of cytb among all recognized *Microcebus* species ranged from 0.9% and 15.3% (Figure S3a). The p-distance between *M. arnholdi* and the new *Microcebus* sp. clade (1.5%) appeared greater than the p-distance between several currently recognized species pairs ($N = 4$), namely *Microcebus simmonsi–Microcebus boraha* (0.9%), *Microcebus manitatra–Microcebus ganzhorni* (1%), *M. ganzhorni–M. murinus* (1.2%) and *M. ravelobensis–M. bongolavensis* (1.2%). For cox2 we found that *Microcebus* species diverged between 0.7% and 16.3% (Figure S3b). Again, several recognized species pairs ($N = 3$) exhibited p-distances smaller than *M. arnholdi*-new *Microcebus* sp. clade pair (1.3%): *M. simmonsi–M. boraha* (0.7%), *M. manitatra–M. ganzhorni* (1%), *Microcebus* sp.–*M. sambiranensis* (1.2%).

When we used the BPP species delimitation method to test whether *M. arnholdi*, *M. sambiranensis*, and *Microcebus* sp. represented distinct species, we found strong support of species divergence (Table 2), robust to all combinations of priors (minimum posterior probability = 0.9997). The results of the randomized datasets showed (as expected) zero support for species divergence among the three clades. Finally, we performed BPP tests on the species pairs that showed lower p-distance than *Microcebus* sp.–*M. arnholdi* (Table S4). Overall, we found the support of species delimitation also for these species' pairs, although for some prior combinations posterior probability values were very low (Table S4a).

We further performed an *ad hoc* test that compares phylogenetic and geographical distance among individuals (within and between taxa), to assess whether *Microcebus* sp. would correspond to a new *Microcebus* species or to intraspecific genetic structure within *M. arnholdi*. For all mtDNA datasets (Figure 3 and Figure S4), we found, for similar values of geographical distance, overall higher inter than intra clades phylogenetic distances (Wilcoxon signed-ranks test: $\chi^2 = 278.43$; $df = 2$; $p < .001$). We performed the same analysis on *M. bongolavensis–M. ravelobensis*, one of the species pairs with lower p-distance than *Microcebus* sp.–*M. arnholdi* (Figure 3b). Again, we observed overall higher inter than intraclades phylogenetic distances (Wilcoxon signed-ranks test: $\chi^2 = 143.78$; $df = 2$; $p < .001$), although at a lower extent than *Microcebus* sp.–*M. arnholdi* comparison (Figure 3; Figures S4 and S5).

TABLE 2 Posterior probabilities from the species delimitation test for *Microcebus* sp., *Microcebus arnholdi*, and *Microcebus sambiranensis*

	alg0	alg0-random	alg1	alg1-random
Small pop. & deep div.	1	0	1	0
Small pop. & recent div.	1	0	1	0
Medium pop. & deep div.	1	0	1	0
Medium pop. & recent div.	1	0	1	0
Large pop. & deep div.	0.9998	0	1	0
Large pop. & recent div.	0.9998	0	0.9997	0

Note: alg0 and alg1: results using either algorithm0 or algorithm1; alg0-random and alg1-random present the results for the randomized dataset. We performed the BPP analyses on the combined mtDNA dataset (cytb + cox2). The first column describes the combination of priors (effective population size and time of divergence) used in the analyses. The species delimitation test suggests with high probability that the three clades correspond to distinct species.
Abbreviation: mtDNA, mitochondrial DNA.

3.3 | Morphological analyses

We used 12 morphological variables across all captured *Microcebus* species. Summary statistics for each species are presented in Table 3. To assess whether species differ morphologically we performed K-means clustering analyses on the morphological data (Table S3 and Figure 4a–c). The analyses carried out with *M. arnholdi* and *M. tavaratra* (Groups I and II; Table S3), identified $K = 2$ as the most likely number of clusters and allowed discriminating both species (Figure S6a,b and Table S5a,b), with clusters partitioned along the dimension explaining most variation (36.4% for Group I and 55.1% for Group II). The individual from ANTSO forest clustered with AMBRE samples, wherein *M. arnholdi* is known to occur (Louis et al., 2008). Most BIN individuals clustered with *M. tavaratra*, except three (BIN.C46, BIN.K53, and BIN.K65) that were associated with *M. arnholdi* (Figure 4b and Table S5b). The analysis carried out with *M. arnholdi* and *Microcebus* sp. showed $K = 3$ as the most likely number of clusters (Figure S6c). However, one of the three clusters included only one individual (Figure 4c). The other individuals were scattered in two clusters that were not representing species/clade identity. Similarly, the K-means analysis performed on the three genetically close clades (*M. arnholdi*, *Microcebus* sp., and *M. mamiratra*; Table S3, Group IV) did not identify morphological differences (data not shown). For comparison, we also assessed whether morphological differences would be detectable in another well-known group of sister species (viz., *M. bongolavensis*–*M. ravelobensis*; Olivieri et al., 2007). The K-means analysis determined that $K = 2$ was the best number of clusters (Figure S7); however, *M. ravelobensis* individuals were distributed across the two clusters, not showing a clear morphological divergence between the two species.

We first performed a DAPC analysis on the species comparison that showed clear morphological differences, that is, *M. arnholdi*–*M. tavaratra* (Group I dataset; Table S3). We used species identity as prior groups, to identify the morphological variables that significantly contribute to the morphological structure between the two species (Figure S8c). Our results show that "Tail length" is the morphological variable that has the largest effect on phenotypic structure, being on average longer in *M. tavaratra* than in *M. arnholdi* (Figure S8d). We also performed DAPC analysis on the "cryptic" sister-taxa *M. arnholdi*–*Microcebus* sp. and *M. bongolavensis*–*M. ravelobensis*, considering that morphological differences might be constrained to

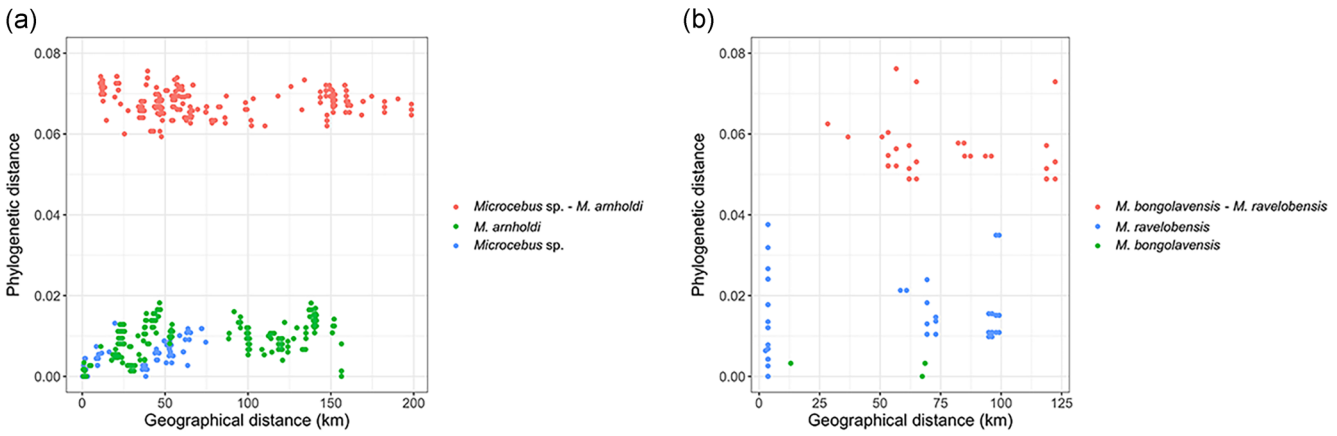

FIGURE 3 Relationship between phylogenetic and geographic distance. These results refer to the analyses performed with the combined mtDNA dataset (cytb + cox2). Phylogenetic distance is measured as patristic distance, which is the sum of branch lengths on the path between individuals. (a) *Microcebus* sp.–*Microcebus arnholdi*, and (b) *Microcebus bongolavensis* –*Microcebus ravelobensis* comparisons showed, for the same geographic distance, higher interclade than intraclade phylogenetic distance. We expect higher values of phylogenetic distance with respect to geography for individuals belonging to different species than for those of the same species. Abbreviation: mtDNA, mitochondrial DNA

TABLE 3 Mean and standard deviation of the morphometric variables for each *Microcebus* taxon

	Microcebus arnholdi (*N* = 34)	*Microcebus tavaratra* (*N* = 51)	*Microcebus* sp. (*N* = 13)	*Microcebus mamiratra* (*N* = 1)
Ear length	20.5 ± 2.7	21.4 ± 2.5	17.8 ± 2.8	21.6
Ear width	12.6 ± 1.5	13.3 ± 1.2	12 ± 1.9	11.7
Head length	33.8 ± 2.2	34.1 ± 2	33.5 ± 3.1	30.7
Head width	20.5 ± 1.4	20.6 ± 1.4	19.9 ± 1.7	22.2
Snout length	7.9 ± 1.3	7.9 ± 1.2	7.9 ± 1.9	11.4
Interorbital dist.	21.5 ± 1.5	21.5 ± 1.3	20.9 ± 1.7	25.0
Intraorbital dist.	7 ± 1.4	7.1 ± 1.5	6.5 ± 0.8	6.8
Lower leg length	38.2 ± 4	39.8 ± 2.9	34.8 ± 4.8	40.1
Hind foot length	21.2 ± 1.9	21.9 ± 1.7	19.7 ± 2.4	21.6
Body length	79.7 ± 11.4	81.8 ± 10.2	76.3 ± 18.2	96.0
Tail length	145.5 ± 19.7	153.3 ± 18.8	134.3 ± 19.6	149.0
Tail circumference	19.7 ± 2.4	19.9 ± 2.2	19.8 ± 3.3	19.0

Note: All variables are expressed in mm.

a few variables instead of the whole organism. For *M. arnholdi*–*Microcebus* sp., DAPC analysis showed that "Ear length," "Ear width," and "Tail length" were the morphological variables that have the largest effect on phenotypic structure between *M. arnholdi* and *Microcebus* sp.; however, differences for each variable were not significant (Figure S9a–c). Whereas, *M. bongolavensis*–*M. ravelobensis* showed "Intraorbital distance" and "Hind foot length" as the significant discriminant morphological variables, being, for both variables, longer in *M. bongolavensis* than in *M. ravelobensis* (Figure S9d–f).

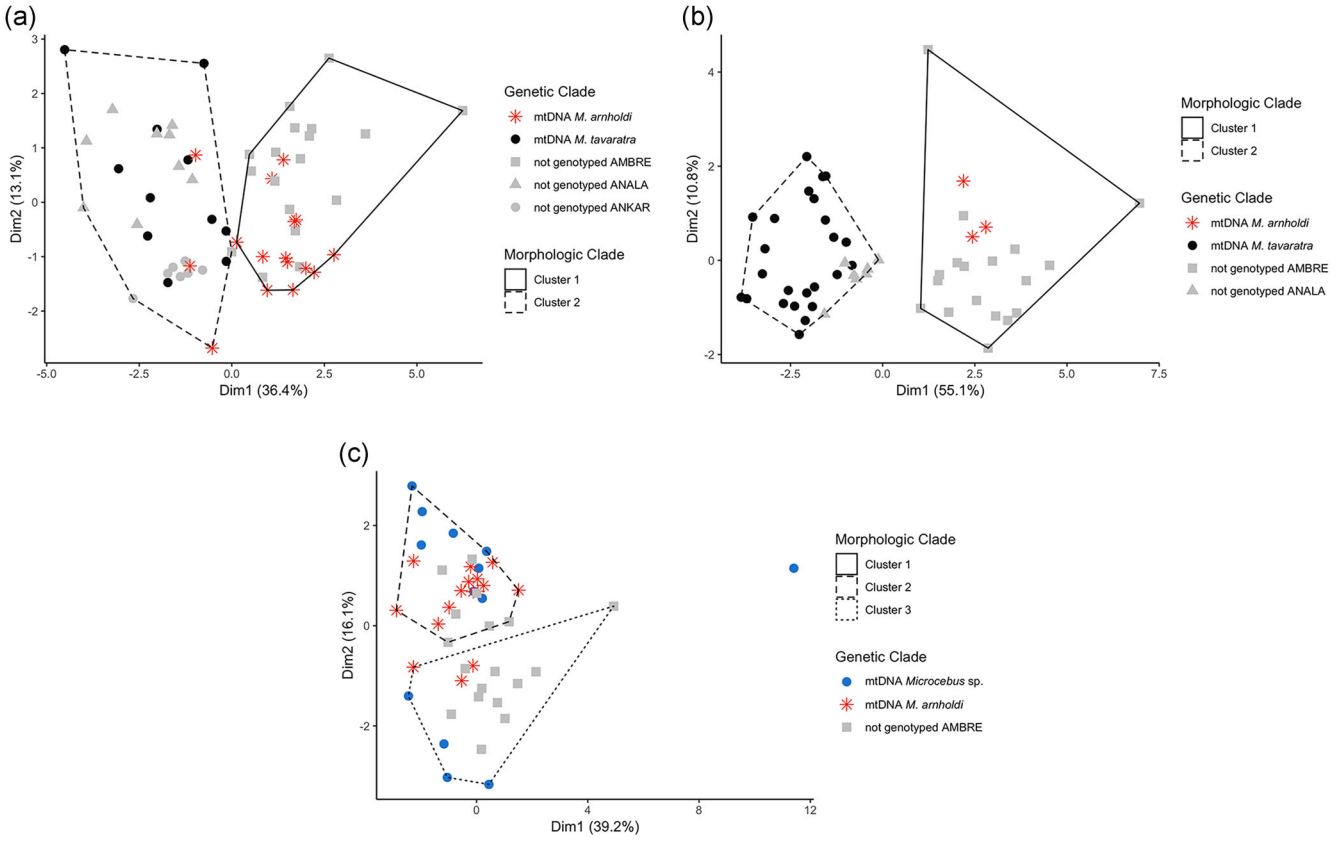

FIGURE 4 K-means analyses on *Microcebus tavaratra*, *M. arnholdi*, and *Microcebus* sp. morphological data. The K-means analyses show morphological differences between *M. tavaratra* and *M. arnholdi*, but none between *M. arnholdi* and *Microcebus* sp. (a) Group I: *M. arnholdi* (Cluster 1) versus *M. tavaratra* (Cluster 1) from Northern Madagascar; (b) Group II: *M. arnholdi* (Cluster 1) versus *M. tavaratra* (Cluster 2) from Binara forest; (c) Group III: *M. arnholdi* versus *Microcebus* sp. from northern Madagascar. AMBRE, Montagne d'Ambre; ANALA, Analabe; ANKAR, Ankarana

TABLE 4 Partial redundancy analyses

a)				
Morphology		**Adj.R^2**	**% Variance**	***p* Value**
Group I	Climate | Geography	0.56	48.92	.001
	Climate AND Geography	0.01	51.03	NA
	Geography | Climate	0.00	0.05	.65
Group II (only BIN)	Climate | Geography	0.00	4.29	.420
	Climate AND Geography	0.32	55.99	NA
	Geography | Climate	0.06	39.72	.386
Group III	Climate | Geography	0.00	0.46	.75
	Climate AND Geography	0.41	73.33	NA
	Geography | Climate	0.21	26.21	.00
b)				
Clade Identity		**Adj.R^2**	**% Variance**	***p* Value**
Group I	Climate | Geography	0.63	53.92	.001
	Climate AND Geography	0.14	38.25	NA
	Geography | Climate	0.05	7.83	.001
Group II (only BIN)	Climate | Geography	0	0.55	.883
	Climate AND Geography	0.4	61.77	NA
	Geography | Climate	0	37.68	.52
	Climate | Geography	0.04	4.36	.024
Group III	Climate AND Geography	0.47	70.21	NA
	Geography | Climate	0.22	25.43	.001

Note: Group I: *M. arnholdi* versus *M. tavaratra* in northern Madagascar; Group II: *M. arnholdi* versus *M. tavaratra* in BIN forest; Group III: *Microcebus* sp. versus *M. arnholdi*. Climate | Geography: effect of climate controlling for spatial autocorrelation; Climate AND Geography: joint effect of climate and geography; Geography | Climate: effect of geography controlling for climate; Adj.R^2: adjusted R^2, a measure of the predictive power; % Variance: the proportion of the variance. NA: significance cannot be tested. We assessed the effect of climate and geography on (a) morphological variability and (b) mtDNA clade identity of the studied mouse lemur species. The results suggest a significant relationship between morphology, clade identity, and climate for *M. tavaratra* and *M. arnholdi* (Group I).
Abbreviations: BIN, Binara forest; mtDNA, mitochondrial DNA.

We investigated the effects of climate and geography on morphology by conducting a partial redundancy analysis, first on the continuous morphological coordinates, and then on the discrete morphological clusters identified with the K-means approach. For *M. tavaratra* and *M. arnholdi* (Group I; Table S3), we detected a significant association between morphology and climate (adjusted $R^2 = 0.56$; Table 4a). Accordingly, partial redundancy analyses on clade identity suggest a significant relationship between species identity and climate (adjusted $R^2 = 0.63$; Table 4b). However, when we performed the same analyses only on the *M. tavaratra* and *M. arnholdi* individuals occurring in BIN (Group II, removing AMBRE and ANKAR; Table S3), we could not detect any significant relationship (Table 4a,b). We assessed which variable was contributing to climatic divergence in *M. tavaratra* and *M. arnholdi* distributions and we found that "Maximum temperature of the warmest month" and "Precipitation of Coldest Quarter" were the two climatic variables showing significant differences between the two species (Figure 5).

For the *M. arnholdi*–*Microcebus* sp. comparison (Group III in Table 4a), we detected a positive association with geography (adjusted $R^2 = 0.21$; $p < .001$) and the joint contribution of geography and climate (adjusted $R^2 = 0.41$; p = not testable). Also, the partial redundancy analysis performed on cluster identity did not show correspondence between clusters and abiotic factors (Group III in Figure 4c and Table 4b).

4 | DISCUSSION

4.1 | Update of species distributions

M. tavaratra was known to occur between forests north of the Irodo River and the Manambato River, including also the Ankarana Special Reserve (Figure 1). Our phylogenetic analyses not only confirm that *M. tavaratra* occurs in forests located north of its previous putative distribution but is also present south of the Manambato River, in the ANALF forest (Figure 1). These results lead to a total increase of the species-area distribution of 6.6% (Figure 6) and include locations that were previously predicted by ecological niche modeling (Kamilar, Blanco, & Muldoon, 2016). Overall, we observed that current

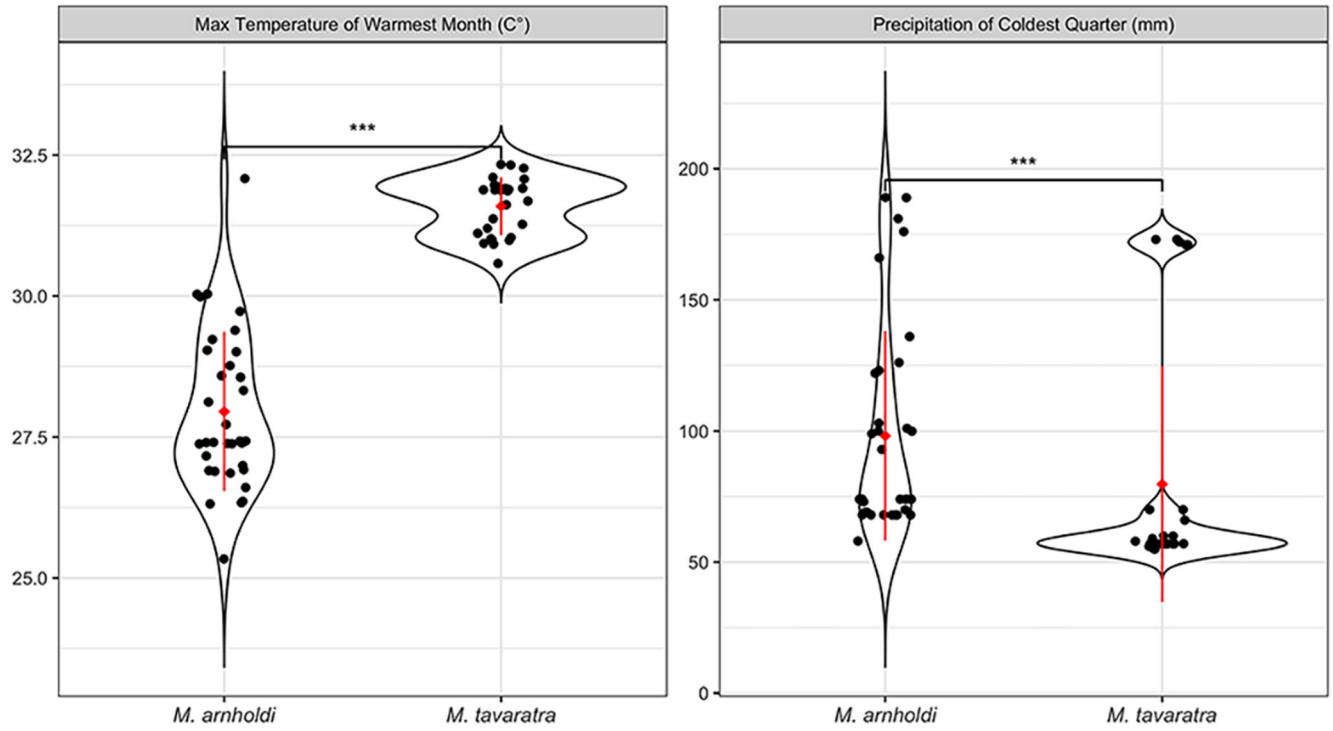

FIGURE 5 Bioclimatic differences between *M. tavaratra* and *M. arnholdi*. Among the 19 bioclimatic variables tested in the presented study, we selected those that showed a level of colinearity (measured as correlation) <0.2. Each dot represents the climatic value extracted from the site in which each *Microcebus* individual was sampled. We observed significant climatic differences between *M. tavaratra* and *M. arnholdi* sampling sites. ***$p < .01$

M. tavaratra populations are scattered across the region in forests that are separated by nonforested habitat (grasslands, agricultural land, etc.), suggesting past connectivity between present-day dry forest fragments.

M. mamiratra was first reported in the Nosy Be Island and later along the north-west coast (Figure 1; Andriantompohavana et al., 2006; Olivieri et al., 2007). Our results provide evidence of its presence in the riparian forest neighboring Ambakirano, along the

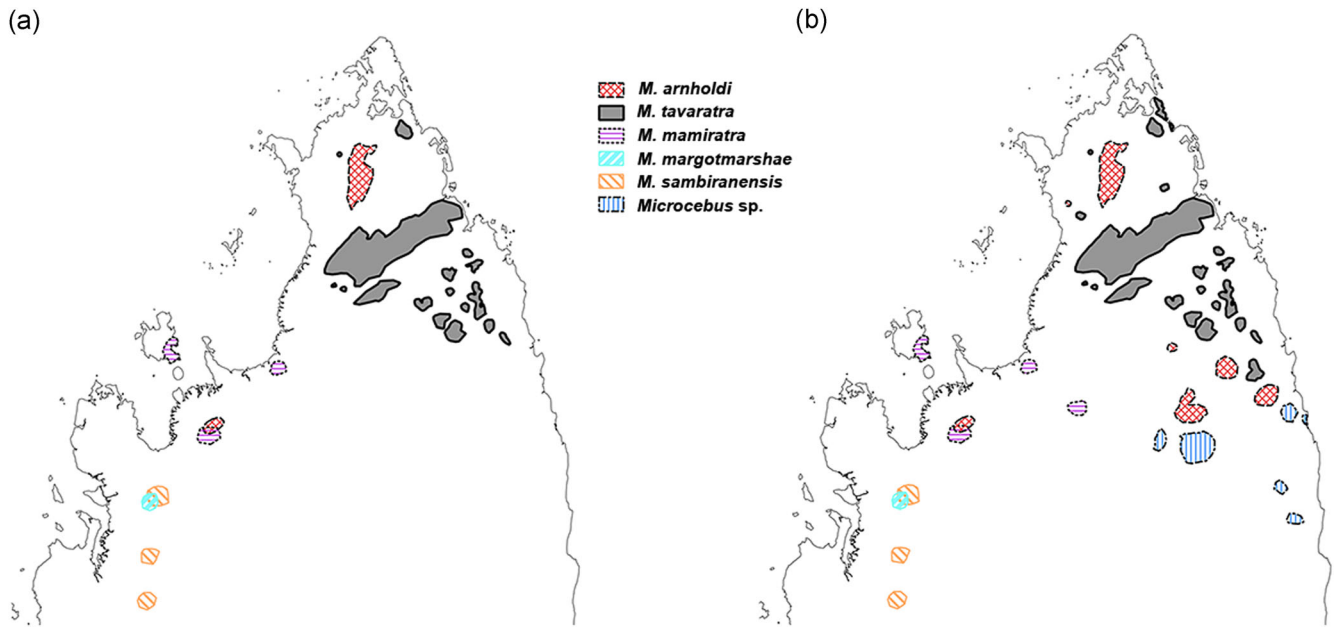

FIGURE 6 Species distribution of northern *Microcebus*. (a) Species distributions before the present study; (b) species distributions after the present study. Our results support an increase of the area of occurrence for *M. tavaratra* (6.6%), *M. arnholdi* (114%), and *Microcebus mamiratra* (29%)

Mahavavy River (AMBK, Figure 1), therefore, widening its distribution of 29% to the east and suggesting ancient forest connectivity among these three localities (Figure 6).

Our results also greatly extend *M. arnholdi's* distribution area (114%). The species was thought to be mostly limited to the mountainous forest of Montagne d'Ambre until it was described in Ambanja, 130 km south-west of Montagne d'Ambre, by Weisrock et al. (2010; Figure 6). Our results suggest that the distribution of *M. arnholdi* also extends south-east but appears to be restricted to the humid forest patches of northern Madagascar (Figure 1). As for the other species, this scattered distribution suggests that these forests may represent relict habitats of an ancient continuous humid forest. The scattered distribution of *M. arnholdi* in humid forests is surprisingly located within a well-recognized dry biogeographic zone (BIN and SAL; Figure S11), and its presence in the small humid area within the dry ANTSO forest (Figure 1; Figures S10 and S11), points to humid vegetation as a key factor for *M. arnholdi* resilience. For BIN and SAL forests, altitude likely has a major role in maintaining a humid microclimate, as it has been demonstrated for other tropical regions (e.g., Anderson et al., 2018). The case of the ANTSO forest is different as this forest is located at a low altitude and mainly composed of dry vegetation. Interestingly, we sampled one *M. arnholdi* individual in the only part of that fragment that exhibited moist vegetation. Physical characteristics of the soil (e.g., geomorphology) are a known factor affecting the tree access to water, and, therefore, the type of vegetation at the land surface (Chase, Johnson, & Martin, 2012). Thus, we hypothesize that particular geomorphological characteristics can contribute to the persistence of humid microclimate suitable to the survival of *M. arnholdi*, even at a small spatial scale such as that of the ANTSO forest.

4.2 | A new location of *Microcebus* sympatry

Importantly, we discovered the presence of *M. arnholdi* in the BIN mountainous forest, previously thought to be only inhabited by *M. tavaratra*. Our results represent the first demonstration of co-occurrence of *M. tavaratra* and *M. arnholdi* (see BIN in Figure 1). Several *Microcebus* species pairs occur in sympatry in western Madagascar, which generally include the widely distributed grey mouse lemur (*M. murinus*). *M. murinus* has a very wide distribution, likely due to a recent expansion across western Madagascar (Schneider, Chikhi, Currat, & Radespiel, 2010). As a consequence, it is co-distributed with three locally restricted congeneric species: *M. ravelobensis*; *Microcebus berthae*; *Microcebus myoxinus* (Rasoloarison et al., 2000; Schmid & Kappeler, 1994; Zimmermann et al., 1998). The cooccurrence we describe may also be the result of a recent expansion of one of the two species (*M. arnholdi* or *M. tavaratra*). Our result raises to four the number of published co-occurrences of unrelated *Microcebus* species (not including contact zones between sister species). Further genetic analyses are required to determine whether interbreeding occurs between *M. tavaratra* and *M. arnholdi* in BIN.

4.3 | Insights on recent forest changes from forest-dwelling species distributions

Our findings confirm that *M. tavaratra* and *M. arnholdi* are distributed in areas characterized by divergent climatic conditions (Groups I and II in Table 4a), which can be represented by the two bioclimatic variables showing low levels of collinearity (Figure 5). Unlike *M. arnholdi*, *M. tavaratra* seems to be more adapted to dry habitats (Figure 5 and Table 4b). This explains its broad presence in the mostly dry northern Madagascar (Figure 1). Differences in climatic preferences are also associated with clear differences in morphology between *M. tavaratra* and *M. arnholdi* individuals (Figure 4a,b), supported by the significant contribution of climate on morphology (Groups I and II in Table 4a). Interestingly, we found that "Tail length" is the morphological variable that contributes the most to the phenotypic divergence between *M. tavaratra* and *M. arnholdi* (Figure S8c), being shorter in the *Microcebus* species commonly associated to colder habitat (*M. arnholdi*; Figure S8d and Figure 5). This is consistent with previous studies showing that mice raised at warm temperatures have a significantly longer tail than those raised in cold temperatures (Serrat, 2014). The mechanism behind this phenotype needs to be explored, but according to Allen's rule (Allen, 1877) this could represent an adaptive trait that, under cold temperatures, reduces heat loss through body extremities (e.g., tail).

Our results suggest a possible link between taxonomy, morphological variability, and environmental factors for these two species. Thus, assuming that *M. tavaratra* and *M. arnholdi* have retained the ecological traits related to their current niche (viz., "niche conservatism"; Wiens et al., 2010) during the late Quaternary, we can use present distribution of the dry-associated (*M. tavaratra*) and humid-associated (*M. arnholdi*) mouse lemurs to reconstruct the recent history of dry and humid forests in northern Madagascar. Looking at the large distribution and presence of *M. tavaratra* in northern dry forests and the broad scattered distribution of *M. arnholdi* in humid forests, we speculate that current species distributions are the result of a recent shift from humid to dry forest cover in northern Madagascar, with consequent range expansion of *M. tavaratra* and contraction of *M. arnholdi*. In fact, the finding in the currently dry forests of the Ankarana and Montagne des Français of *Propithecus cf. diadema*, *Indri indri*, and *Prolemur simus* subfossils, is particularly notable. Indeed, these larger body-size lemurs currently occur only in the humid forests of eastern Madagascar, which suggests that during early-mid Holocene, northern Madagascar was more humid than today (Godfrey, Jungers, Simons, Chatrath, & Rakotosamimanana, 1999; Jungers, Godfrey, Simons, & Chatrath, 1995; Simons et al., 1995; Figure 1). Therefore, we propose that when humid habitats were more continuous than today, *M. arnholdi* would have had a larger and continuous distribution than *M. tavaratra*. Then, the increase of dry conditions during the Holocene may have caused the contraction and disconnection of humid forests and an increase of dry habitats (Gasse & Van Campo, 1998; Virah-Sawmy, Willis, Gillson, & Williams, 2010), confining *M. arnholdi* populations in the remaining humid

forest fragments, while *M. tavaratra* populations would have been able to increase their range in the expanding dry forests. Subsequent dry events and possible human activities may have further fragmented dry forests to the extent that we observe today. Similar scenarios have been proposed to explain the genetic diversity, demographic history, and distribution of northern Madagascar's tuft-tailed rats (*Eliurus carletoni* and *Eliurus tanala*; Rakotoarisoa, Raheriarisena, & Goodman, 2013a, 2013b), sifaka (*Propithecus tattersalli*; Quéméré, Amelot, Pierson, Crouau-Roy, & Chikhi, 2012; Salmona, Heller, Quéméré, & Chikhi, 2017) and the leaf chameleons (e.g., *Brookesia ebenaui* and *Brookesia minima*; Raxworthy & Nussbaum, 1995). More genetic data coupled with population divergence time estimates and/or spatial simulations would help infer which humid to dry climatic oscillation likely led to the current distribution of mouse lemurs in northern Madagascar.

4.4 | New mtDNA lineage

Our phylogenetic analyses identified a novel mtDNA clade (*Microcebus* sp., Figure 2) closely related to the *M. arnholdi* clade. The proportion of pairwise differences between *M. arnholdi* and *Microcebus* sp. was larger than the differences observed between well accepted pairs of species ($N = 3$ or 4; Figure S3a,b). Using a Bayesian coalescent-based species delimitation test (BPP software) we found support to consider *M. sambiranensis*, *M. arnholdi* and *Microcebus* sp. as distinct species (Table 2). The BPP results for *M. ganzhorni*, *M manitatra*, and *M. murinus* and those for *Microcebus danfossi*, *M. bongolavensis* (Table S4), were much weaker under several parameter combinations. Although our analyses are limited to the use of two mitochondrial loci, our BPP results for *M. arnholdi* and *M. sambiranensis* are in agreement with those of Hotaling et al. (2016), which used a combined dataset of two mtDNA and four nuclear loci.

The BPP software uses an implementation of the MSC model, and recent work (Sukumaran & Knowles, 2017) suggested that, under certain conditions, BPP results can be considered reliable for species delimitation only if other types of data are used (e.g., ecological, ethological). Here we have used a simple approach, which may help to distinguish intra and interspecies genetic structure. We compared the individual phylogenetic and geographic distances within and among clades (Figure 3; Figures S4 and S5). Overall, we observed that phylogenetic distances were larger between *M. arnholdi* and *Microcebus* sp. (red dots in Figure 3a) than within each clade, whichever geographic distance we considered (blue and green dots in Figure 3a). This suggests that geographic distance may not be the factor limiting gene flow among the populations of these two clades. Furthermore, we may exclude that *M. arnholdi* and the new monophyletic clade are the results of present physical barriers to gene-flow, given that *M. arnholdi* individuals sampled in forests separated by large open habitat and several permanent rivers still cluster within the same clade (Figure 1). Conversely, the MAL and AMBA samples are located within the same continuous forest but belong to different clusters.

Despite the genetic evidence of species delimitation for *M. arnholdi* and *Microcebus* sp., morphological analysis with K-means approach did not show clear differences between these two taxa (Figure 4c), similarly to another group of sister species (*M. bongolavensis*–*M. ravelobensis*) for which similar morphological variables were available (Figure S7). When we used DAPC analysis on morphological data, however, both *M. arnholdi*–*Microcebus* sp. and *M. bongolavensis*–*M. ravelobensis* comparisons identified three morphological variables that most differentiate each taxon from its pair. But only *M. bongolavensis*–*M. ravelobensis* comparison showed significant differences between the distribution of two of the three identified morphological variables (Figure S9). Following these results, we attempted to assess whether the morphological clusters identified in *M. arnholdi*–*Microcebus* sp. comparison would reflect differences in abiotic factors rather than species identity. We did not find support for this hypothesis; however, we found evidence for the contribution of geography on morphological variability (Group III in Table 4a). Early studies reported that morphological traits could be effective for species recognition in the *Microcebus* genus (Yoder et al., 2000); however, this is not always the case (Rasoloarison et al., 2013; Yoder et al., 2005). Hence, the lack of morphological differences between *M. arnholdi* and the individuals of the new mtDNA clade does not necessarily reject the species hypothesis for the new mtDNA lineage. Moreover, according to the "recent divergence hypothesis", it is expected that more genetically divergent taxa (*M.* tavaratra–*M. arnholdi*) would show stronger phenotypic differences than less genetically divergent one (*Microcebus* sp.–*M. arnholdi*).

Altogether, these results support the hypothesis that the new mtDNA clade may represent a distinct species. However, several *Microcebus* species are female philopatric (Radespiel, Lutermann, Schmelting, Bruford, & Zimmermann, 2003), resulting in strong mitochondrial genetic structure which may not necessarily relate to species delimitation. Therefore, we propose that the new mtDNA clade is considered at best a tentative hypothesis of distinct species, to be confirmed or rejected through the application of nuclear genomic data and/or other ecological information.

4.5 | Conservation implications

These results have major implications for the conservation of northern Madagascar *Microcebus* species. Based on the preliminary results of this study, most distribution updates of the species studied here were considered in the recent IUCN Red List assessments conducted in May 2018, Antananarivo, Madagascar. However, we have to highlight that deforestation rates in northern Madagascar have been rising in the last decade, sustained by strong demands for charcoal and hardwood by neighboring major towns, and international demand for precious woods and vanilla (e.g., Vieilledent et al., 2018, Waeber, Wilmé, Mercier, Camara & Lowry, 2016). Therefore, there is a real danger that, for instance, the populations in lowland forests of the new *Microcebus* sp. (i.e., ANALV, AMBO, ANDG, and ANTA) will not last long. Indeed, the two unprotected forests surveyed in 2013 (ANALV, AMBO) had almost entirely disappeared by 2018 (JS, BLP, ER pers. obs.). This points out the urgency to

further extend the Malagasy protected area system to remote locations, to save the yet unrevealed diversity it hosts.

ACKNOWLEDGMENTS

We thank the Direction Générale du Ministère de l'Environnement et des Forêts de Madagascar, Direction Régionale de l'Environnement, Ecologie et des Forêts (DREEF DIANA and SAVA), Madagascar's Ad Hoc Committee for Fauna and Flora and Organizational Committee for Environmental Research (CAFF/CORE), Conservation International, WWF, Missouri Botanical Garden and the Fanamby NGO. This study benefited from the continuous support of the Department of Animal Biology and Ecology, University of Mahajanga, the Department of Animal Biology, University of Antananarivo, Department of Natural and envornmental Sciences at the University of Antsiranana and from the participation of Malagasy MSc students, field assistants, volunteers, and many great local guides and cooks which we warmly thank for their help in the field and for sharing their incomparable knowledge of the forest, misaotra anareo jiaby. This research was funded through the 2015–2016 BiodivERsA COFUND call for research proposals, with the national funders ANR (grant number ANR-16-EBI3–0014), FCT (grant number Biodiversa/0003/2015) and PT-DLR (grant number 01LC1617A). It was also partly funded by the FCT (grant numbers PTDC/BIA-BEC/100176/2008, PTDC/BIA-BIC/4476/2012, and PTDC-BIA-EVL/30815/2017 to L.C., SFRH/BD/64875/2009 to J.S., PD/BD/114343/2016 to G.S), the LabEx entitled TULIP (grant numbers ANR-10LABX-41, ANR-11-IDEX-0002-02), the LIA BEEG-B (Laboratoire International Associé-Bioinformatics, Ecology, Evolution, Genomics and Behaviour, CNRS) between the CNRS and IGC, the Investissement d'Avenir grant of the Agence Nationale de la Recherche (CEBA: ANR-10-LABX-25-01), the Rufford Small Grant Foundation (grant number 10941-1 to JS and 12973-1 to MTI) and the Groupement de Recherche International (GDRI) Biodiversité et developpement durable-Madagascar. In addition, we thank the ITI facility of Instituto Gulbenkian de Ciencia, in particular, João Garcia, for its logistical support in the computational analyses.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The generated genetic datasets have been deposited in the NCBI repository, including the GPS coordinates of the sampled individuals (MN699484 - MN699560). Morphological data are provided in Supporting Information B.

ETHICS STATEMENT

We conducted this study in agreement with the laws of the countries of Portugal, France, and Madagascar (CITES; permit IDs 485C-EA09/MG10, 559C-EA10/MG13, 782C-EA12/MG14, 1035C-EA12/MG15, 994C-EA11/MG17). Our research complied with the ASP Principles for the Ethical Treatment of Nonhuman Primates and the International Primatological Society Code of Best Practices for Field Primatology.

ORCID

Gabriele Maria Sgarlata http://orcid.org/0000-0002-4115-6453
Jordi Salmona http://orcid.org/0000-0002-1950-5805
Barbara Le Pors http://orcid.org/0000-0001-8326-3794
Adam Joseph Marques http://orcid.org/0000-0002-6070-0506
Isa Aleixo-Pais http://orcid.org/0000-0003-2730-3688
Tiago de Zoeten http://orcid.org/0000-0001-8619-2573
Alex Miller http://orcid.org/0000-0001-6439-1010
Mohamed Thani Ibouroi http://orcid.org/0000-0003-2934-6622
Lounès Chikhi http://orcid.org/0000-0002-1140-0718

REFERENCES

Adkins, R. M., & Honeycutt, R. L. (1994). Evolution of the primate cytochrome c oxidase subunit II gene. *Journal of Molecular Evolution*. *38*, 215–231. https://doi.org/10.1007/BF00176084

Aleixo-Pais, I., Salmona, J., Sgarlata, G. M., Rakotonanahary, A., Sousa, A. P., Parreira, B., … Chikhi, L. (2018). The genetic structure of a mouse lemur living in a fragmented habitat in Northern Madagascar. *Conservation Genetics*. *20*, 229–243. https://doi.org/10.1007/s10592-018-1126-z

Allen, J. A. (1877). The influence of physical conditions in the genesis of species. *Radical Review*, *1*, 107–140.

Anderson, J., Keppel, G., Thomson, S., Randell, A., Raituva, J., Koroi, I., … Kleindorfer, S. (2018). Changes in climate and vegetation with altitude on Mount Batilamu, Viti Levu, Fiji. *Journal of Tropical Ecology*, *34*(5), 316–325. https://doi.org/10.1017/S0266467418000299

Andriaholinirina, N., Baden, A., Blanco, M., Chikhi, L., Cooke, A., Davies, N., & Zaramody, A. (2014). Microcebus tavaratraMicrocebus tavaratra. The IUCN Red List of Threatened Species 2014: e.T41571A16113861. https://doi.org/10.2305/IUCN.UK.2014-1.RLTS.T41571A16113861.en.

Andriantompohavana, R., Zaonarivelo, J. R., Engberg, S. E., Randriamampionona, R., Mcguire, S. M., Shore, G. D., … Louis, E. E. (2006). Mouse lemurs of northwestern Madagascar with a description of a new species at Lokobe Special Reserve. *Occasional papers/Museum of Texas Tech University*. *259*, 1–24.

Avise, J. C. (2000). *Phylogeography: The History and Formation of Species*. Cambridge, MA: Harvard University Press.

Bivand, R. S., Pebesma, E., & Gómez-Rubio, V. (2013). *Applied Spatial Data Analysis with R*. New York: Springer-Verlag.

Borcard, D., & Legendre, P. (2002). All-scale spatial analysis of ecological data by means of principal coordinates of neighbour matrices. *Ecological Modelling*, *153*, 51–68. https://doi.org/10.1016/S0304-3800(01)00501-4

Borcard, D., & Legendre, P. (1994). Environmental control and spatial structure in ecological communities: An example using oribatid mites (Acari, Oribatei). *Environmental and Ecological Statistics*, *1*, 37–61. https://doi.org/10.1007/BF00714196

Borcard, D., Legendre, P., & Drapeau, P. (1992). Partialling out the spatial component of ecological variation. *Ecology*, *73*, 1045–1055. https://doi.org/10.2307/1940179

Burgin, C. J., Colella, J. P., Kahn, P. L., & Upham, N. S. (2018). How many species of mammals are there? *Journal of Mammalogy*. *9*, 1–14. https://doi.org/10.1093/jmammal/gyx147

Charrad, M., Ghazzali, N., Boiteau, V., & Niknafs, A. (2014). NbClust: An R Package for determining the relevant number of clusters in a dataset. *Journal of Statistical Software*. *61*, 10828.

Chase, M. N., Johnson, E. A., & Martin, Y. E. (2012). The influence of geomorphic processes on plant distribution and abundance as reflected in plant tolerance curves. *Ecological Monographs*, *82*, 429–447. https://doi.org/10.1890/11-2145.1

Chenuil, A., Cahill, A. E., Délémontey, N., Du Salliant Du Luc, E., & Fanton, A. (2019). Problems and questions posed by cryptic species. A framework to guide future studies. In E. Casetta, J. Marques da Silva & D. Vecchi (Eds.), *From Assessing to Conserving Biodiversity*. History, Philosophy and Theory of the Life Sciences (24, pp. 77–106). Switzerland: Springer Nature. https://doi.org/10.1007/978-3-030-10991-2_4

Cracraft, J. (1983). Species concepts and speciation analysis. *Current Ornithology*. *1*, 159–187.

Dray, S., Legendre, P., & Peres-Neto, P. R. (2006). Spatial modelling: A comprehensive framework for principal coordinate analysis of neighbour matrices (PCNM). *Ecological Modelling*, *196*, 483–493. https://doi.org/10.1016/j.ecolmodel.2006.02.015

Fick, S. E., & Hijmans, R. J. (2017). WorldClim 2: New 1-km spatial resolution climate surfaces for global land areas. *International Journal of Climatology*. *37*, 4302–4315. https://doi.org/10.1002/joc.5086

Gasse, F., & Van Campo, E. (1998). A 40,000-yr pollen and diatom record from Lake Tritrivakely, Madagascar, in the Southern Tropics. *Quaternary Research*. *49*, 299–311. https://doi.org/10.1006/qres.1998.1967

Glez-Peña, D., Gómez-Blanco, D., Reboiro-Jato, M., Fdez-Riverola, F., & Posada, D. (2010). ALTER: Program-oriented conversion of DNA and protein alignments. *Nucleic Acids Research*. *38*, W14–W18. https://doi.org/10.1093/nar/gkq321

Godfrey, L. R., Jungers, W. L., Simons, E. L., Chatrath, P. S., & Rakotosamimanana, B. (1999). *Past and Present Distributions of Lemurs in Madagascar* (pp. 19–53). New York: Kluwer Academic/Plenum.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology*. *59*, 307–321.

Harper, G. J., Steininger, M. K., Tucker, C. J., Juhn, D., & Hawkins, F. (2007). Fifty years of deforestation and forest fragmentation in Madagascar. *Environment Conservation Journal*. *34*, 325–333. https://doi.org/10.1017/S0376892907004262

Hijmans, R. J. (2017). raster: Geographic data analysis and modeling. *R package version*, *2*, 6–7.

Hotaling, S., Foley, M. E., Lawrence, N. M., Bocanegra, J., Blanco, M. B., Rasoloarison, R., & Weisrock, D. W. (2016). Species discovery and validation in a cryptic radiation of endangered primates: Coalescent-based species delimitation in Madagascar's mouse lemurs. *Molecular Ecology*. *25*, 2029–2045. https://doi.org/10.1111/mec.13604

Irwin, D. M., Kocher, T. D., & Wilson, A. C. (1991). Evolution of the cytochrome b gene of mammals. *Journal of Molecular Evolution*. *32*, 128–144. https://doi.org/10.1007/BF02515385

Jombart, T. (2008). adegenet: A R package for the multivariate analysis of genetic markers. *Bioinformatics*, *24*, 1403–1405. https://doi.org/10.1093/bioinformatics/btn129

Jombart, T., & Ahmed, I. (2011). Adegenet 1.3-1: New tools for the analysis of genome-wide SNP data. *Bioinformatics*. *27*, 3070–3071. https://doi.org/10.1093/bioinformatics/btr521

Jombart, T., Balloux, F., & Dray, S. (2010). Adephylo: New tools for investigating the phylogenetic signal in biological traits. *Bioinformatics*. *26*, 1907–1909. https://doi.org/10.1093/bioinformatics/btq292

Jungers, W. L., Godfrey, L. R., Simons, E. L., & Chatrath, P. S. (1995). Subfossil *Indri indri* from the Ankarana Massif of northern Madagascar. *American Journal of Physical Anthropology*. *97*, 357–366. https://doi.org/10.1002/ajpa.1330970403

Kamilar, J. M., Blanco, M. B., & Muldoon, K. M. (2016). Ecological niche modeling of mouse lemurs (Microcebus sp.) and its implications for their species diversity and biogeography. In S. M. Lehman, U. Radespiel & E. Zimmermann (Eds.), *The Dwarf and Mouse Lemurs of Madagascar: Biology, Behavior, and Conservation Biogeography of the Cheirogaleidae* (pp. 449–618). Cambridge, United Kingdom: Cambridge University Press.

Kappeler, P. M., & Rasoloarison, R. M. (2003). Microcebus, mouse lemurs, Tsidy. In S. M. Goodman & J. P. Benstead (Eds.), *The Natural History of Madagascar* (pp. 1310–1315). Chicago, USA: University of Chicago Press.

Kassambara, A., & Mundt, F. (2017). factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R package version 1.0.5.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., & Drummond, A. (2012). Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for Bigger datasets. *Molecular Biology and Evolution*. *33*, 1870–1874. https://doi.org/10.1093/molbev/msw054

Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., & Calcott, B. (2017). PartitionFinder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*. *34*, 772–773.

Louis, E. E., Coles, M. S., Andriantompohavana, R., Sommer, J. A., Engberg, S. E., Zaonarivelo, J. R., & Brenneman, R. A. (2006). Revision of the mouse lemurs (*Microcebus*) of eastern Madagascar. *International Journal of Primatology*. *27*, 347–389. https://doi.org/10.1007/s10764-006-9036-1

Louis, E. E., Engberg, S. E., Mcguire, S. M., Mccormick, M. J., Randriamampionona, R., Ranaivoarisoa, J. F., & Lei, R. (2008). Revision of the mouse lemurs, *Microcebus* (primates, Lemuriformes), of Northern and Northwestern Madagascar with descriptions of two new species at Montagne d'Ambre National Park and Antafondro Classified Forest. *Primate Conservation*, *23*, 19–38. https://doi.org/10.1896/052.023.0103

Louis, E. E., & Lei, R. (2016). Mitogenomics of the family Cheirogaleidae and relationships to taxonomy and biogeography in Madagascar. In U. Radespiel, E. Zimmermann & S. M. Lehman (Eds.), *Dwarf and mouse lemurs of Madagascar: biology, behavior, and conservation biogeography of the Cheirogaleidae*. Cambridge (UK): Cambridge Universtiy Press.

Mittermeier, R.A., Hawkins, F., Kappeler, P.M., Langrand, O., Louis, E.E., Mackinnon, J., & Mackinnon, J. (2010). Lemurs of Madagascar (Conservation International).

Myers, N., Mittermeier, R. A., Mittermeier, C. G., Fonseca, G. A. B. da, & Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature*, *403*, 853–858. https://doi.org/10.1038/35002501

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., & Wagner, H. (2018) vegan: Community Ecology PackageR package version 2.5-2https://CRAN.R-project.org/package=vegan

Olivieri, G., Zimmermann, E., Randrianambinina, B., Rasoloharijaona, S., Rakotondravony, D., Guschanski, K., & Radespiel, U. (2007). The ever-increasing diversity in mouse lemurs: Three new species in north and northwestern Madagascar. *Molecular Phylogenetics and Evolution*, *43*, 309–327. https://doi.org/10.1016/j.ympev.2006.10.026

Pebesma, E. J., & Bivand, R. S. (2010). Using rainfall radar data to improve interpolated maps of dose rate in the Netherlands. *The Science of the total environment*, *409*(2), 123–133.

De Queiroz, K. (2005). A unified species concept and its consequences for the future of taxonomy. *Proceedings of the California Academy*

of Sciences, *56*(June), 196–215. https://doi.org/10.1073/pnas.0502030102

De Queiroz, K. (2007). Species concepts and species delimitation. *Systematic Biology*, *56*(6), 879–886. https://doi.org/10.1080/10635150701701083

Quéméré, E., Amelot, X., Pierson, J., Crouau-Roy, B., & Chikhi, L. (2012). Genetic data suggest a natural prehuman origin of open habitats in northern Madagascar and question the deforestation narrative in this region. *Proceedings of the National Academy of Sciences*, *109*, 13028–13033. https://doi.org/10.1073/pnas.1200153109

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Radespiel, U., Lutermann, H., Schmelting, B., Bruford, M. W., & Zimmermann, E. (2003). Patterns and dynamics of sex-biased dispersal in a nocturnal primate, the grey mouse lemur, *Microcebus murinus*. *Animal Behaviour*, *65*, 709–719. https://doi.org/10.1006/anbe.2003.2121

Radespiel, U., Olivieri, G., Rasolofoson, D. W., Rakotondratsimba, G., Rakotonirainy, O., Rasoloharijaona, S., & Randrianarison, R. M. (2008). Exceptional diversity of mouse lemurs (*Microcebus* spp.) in the Makira region with the description of one new species. *American Journal of Primatology*. *70*, 1033–1046. https://doi.org/10.1002/ajp.20592

Radespiel, U., Ratsimbazafy, J. H., Rasoloharijaona, S., Raveloson, H., Andriaholinirina, N., Rakotondravony, R., & Randrianambinina, B. (2012). First indications of a highland specialist among mouse lemurs (*Microcebus* spp.) and evidence for a new mouse lemur species from eastern Madagascar. *Primates*, *53*, 157–170. https://doi.org/10.1007/s10329-011-0290-2

Rakotoarisoa, J.-E., Raheriarisena, M., & Goodman, S. M. (2013a). Late quaternary climatic vegetational shifts in an ecological transition zone of northern Madagascar: Insights from genetic analyses of two endemic rodent species. *Journal of Evolutionary Biology*, *26*(5), 1019–1034. https://doi.org/10.1111/jeb.12116

Rakotoarisoa, J.-E., Raheriarisena, M., & Goodman, S. M. (2013b). A phylogeographic study of the endemic rodent Eliurus carletoni (Rodentia: Nesomyinae) in an ecological transition zone of northern Madagascar. *Journal of Heredity*, *104*(1), 23–35. https://doi.org/10.1093/jhered/ess083

Rakotondravony, R., & Radespiel, U. (2009). Varying patterns of coexistence of two mouse lemur species (*Microcebus ravelobensis* and *M. murinus*) in a heterogeneous landscape. *American Journal of Primatology*. *71*, 928–938. https://doi.org/10.1002/ajp.20732

Rambaut, A., Drummond, A. J., & Suchard, M. (2014). Tracer, v1.6.

Rannala, B., & Yang, Z. (2003). Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. *Genetics*, *164*, 1645–1656.

Rannala, B., & Yang, Z. (2013). Improved reversible jump algorithms for Bayesian species delimitation. *Genetics*, *194*, 245–253.

Rasoloarison, R. M., Goodman, S. M., & Ganzhorn, J. U. (2000). Taxonomic revision of mouse lemurs (*Microcebus*) in the western portions of Madagascar. *International Journal of Primatology*. *21*, 963–1019. https://doi.org/10.1023/A:1005511129475

Rasoloarison, R. M., Weisrock, D. W., Yoder, A. D., Rakotondravony, D., & Kappeler, P. M. (2013). Two new species of mouse lemurs (Cheirogaleidae: *Microcebus*) from eastern Madagascar. *International Journal of Primatology*. *34*, 455–469. https://doi.org/10.1007/s10764-013-9672-1

Raxworthy, C. J., & Nussbaum, R. A. (1995). Systematics, speciation and biogeography of the dwarf chameleons (Brookesia; Reptilia, Squamata, Chamaeleontidae) of northern Madagascar. *Journal of Zoology*. *235*, 525–558.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*. *61*, 539–542. https://doi.org/10.1093/sysbio/sys029

Salmona, J., Heller, R., Quéméré, E., & Chikhi, L. (2017). Climate change and human colonization triggered habitat loss and fragmentation in Madagascar. *Molecular Ecology*, *26*(19), 5203–5222. https://doi.org/10.1111/mec.14173

Schmid, J., & Kappeler, P. M. (1994). Sympatric mouse lemurs (*Microcebus* spp.) in western Madagascar. *Folia Primatol*. *63*, 162–170. https://doi.org/10.1159/000156812

Schneider, N., Chikhi, L., Currat, M., & Radespiel, U. (2010). Signals of recent spatial expansions in the grey mouse lemur (*Microcebus murinus*). *BMC Evolutionary Biology*. *10*, 105. https://doi.org/10.1186/1471-2148-10-105

Schwitzer, C., Mittermeier, R., Johnson, S., Donati, G., Irwin, M., Peacock, H., … Chikhi, L. (2014). Averting lemur extinctions amid Madagascar's political crisis. *Science*, *343*, 842–843. https://doi.org/10.1126/science.1245783

Serrat, M. A. (2014). Environmental temperature impact on bone and cartilage growth. *Comprehensive Physiology*. *4*, 621–655. https://doi.org/10.1002/cphy.c130023

Seutin, G., White, B. N., & Boag, P. T. (1991). Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology*. *69*, 82–90. https://doi.org/10.1139/z91-013

Sgarlata, G. M., Salmona, J., Aleixo-Pais, I., Rakotonanahary, A., Sousa, A. P., Kun-Rodrigues, C., … Chikhi, L. (2018). Genetic differentiation and demographic history of the northern rufous mouse lemur (*Microcebus tavaratra*) across a fragmented landscape in northern Madagascar. *International Journal of Primatology*. 1–25. https://doi.org/10.1007/s10764-018-0015-0

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., & Li, W. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology.7*, 539. https://doi.org/10.1038/msb.2011.75

Simons, E. L., Burney, D. A., Chatrath, P. S., Godfrey, L. R., Jungers, W. L., & Rakotosamimanana, B. (1995). AMS 14C dates for extinct lemurs from caves in the Ankarana Massif, northern Madagascar. *Quaternary Research*. *43*, 249–254. https://doi.org/10.1006/qres.1995.1025

Stamatakis, A. (2006). RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*. *22*, 2688–2690.

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and postanalysis of large phylogenies. *Bioinformatics*. *30*, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Struck, T. H., Feder, J. L., Bendiksby, M., Birkeland, S., Cerca, J., Gusarov, V. I., … Dimitrov, D. (2018). Finding Evolutionary Processes Hidden in Cryptic Species. *Trends in Ecology & Evolution*, *33*(3), 153–163. https://doi.org/10.1016/j.tree.2017.11.007

Sukumaran, J., & Knowles, L. L. (2017). Multispecies coalescent delimits structure, not species. *Proceedings of the National Academy of Sciences*. *114*, 1607–1612. https://doi.org/10.1073/pnas.1607921114

Venter, O., Sanderson, E. W., Magrach, A., Allan, J. R., Beher, J., Jones, K. R., & Watson, J. E. M. (2016). Sixteen years of change in the global terrestrial human footprint and implications for biodiversity conservation. *Nature Communications*. *7*, 12558. https://doi.org/10.1038/ncomms12558

Vieilledent, G., Grinand, C., Rakotomalala, F. A., Ranaivosoa, R., Rakotoarijaona, J.-R., Allnutt, T. F., & Achard, F. (2018). Combining global tree cover loss data with historical national forest-cover maps to look at six decades of deforestation and forest fragmentation in Madagascar. *Biological Conservation*, *222*, 189–197. https://doi.org/10.1016/j.biocon.2018.04.008

Virah-Sawmy, M., Willis, K. J., Gillson, L., & Williams, J. (2010). Evidence for drought and forest declines during the recent megafaunal extinctions in Madagascar. *Journal of Biogeography*. *37*, 506–519. https://doi.org/10.1111/j.1365-2699.2009.02203.x

Waeber, P.O., Schuurman, D., & Wilmé, L. (2018). Madagascar's rosewood (Dalbergia sp.) stocks as a political challenge (PeerJ Inc.). https://doi.org/10.7287/peerj.preprints.27062v1

Waeber, P. O., Wilmé, L., Mercier, J.-R., Camara, C., & Lowry, P. P., II (2016). How effective have thirty years of internationally driven conservation and development efforts been in Madagascar? *PLOS ONE*, *11*(8):e0161115. https://doi.org/10.1371/journal.pone.0161115

Weisrock, D. W., Rasoloarison, R. M., Fiorentino, I., Ralison, J. M., Goodman, S. M., Kappeler, P. M., & Yoder, A. D. (2010). Delimiting species without nuclear monophyly in Madagascar's mouse lemurs. *PLOS ONE*, *5*):e9883. https://doi.org/10.1371/journal.pone.0009883

Wiens, J. J., Ackerly, D. D., Allen, A. P., Anacker, B. L., Buckley, L. B., Cornell, H. V., … Stephens, P. R. (2010). Niche conservatism as an emerging principle in ecology and conserva- tion biology. *Ecology Letters*, *13*(10), 1310–1324. https://doi.org/10.1111/j.1461-0248.2010.01515.x

Wilmé, L., Goodman, S. M., & Ganzhorn, J. U. (2006). Biogeographic evolution of Madagascar's Microendemic Biota. *Science*, *312*, 1063–1065. https://doi.org/10.1126/science.1122806

Wright, S. (1943). Isolation by distance. *Genetics*, *28*, 114–138.

Yang, Z. (2002). Likelihood and Bayes estimation of ancestral population sizes in hominoids using data from multiple loci. *Genetics*, *162*, 1811–1823.

Yang, Z. (2015). The BPP program for species tree estimation and species delimitation. *Current Zoology*. *61*, 854–865.

Yang, Z., & Rannala, B. (2010). Bayesian species delimitation using multilocus sequence data. *Proceedings of the National Academy of Sciences*, *107*, 9264–9269.

Yang, Z., & Rannala, B. (2017). Bayesian species identification under the multispecies coalescent provides significant improvements to DNA barcoding analyses. *Molecular Ecology*. *26*, 3028–3036. https://doi.org/10.1111/mec.14093

Yoder, A. D., Burns, M. M., & Génin, F. (2002). Molecular evidence of reproductive isolation in sympatric sibling species of mouse lemurs. *International Journal of Primatology*. *23*, 1335–1343. https://doi.org/10.1023/A:1021187106641

Yoder, A. D., Olson, L. E., Hanley, C., Heckman, K. L., Rasoloarison, R., Russell, A. L., … Goodman, S. M. (2005). A multidimensional approach for detecting species patterns in Malagasy vertebrates. *Proceedings of the National Academy of Sciences*, *102*, 6587–6594. https://doi.org/10.1073/pnas.0502092102

Yoder, A. D., Rasoloarison, R. M., Goodman, S. M., Irwin, J. A., Atsalis, S., Ravosa, M. J., & Ganzhorn, J. U. (2000). Remarkable species diversity in Malagasy mouse lemurs (primates, Microcebus). *Proceedings of the National Academy of Sciences*, *97*, 11325–11330. https://doi.org/10.1073/pnas.200121897

Zimmermann, E., Cepok, S., Rakotoarison, N., Zietemann, V., & Radespiel, U. (1998). Sympatric mouse lemurs in north-west Madagascar: A new rufous mouse lemur species (*Microcebus ravelobensis*). *Folia Primatologica*, *69*, 106–114. https://doi.org/10.1159/000021571

SUPPORTING INFORMATION

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

How to cite this article: Sgarlata GM, Salmona J, Pors BL, et al. Genetic and morphological diversity of mouse lemurs (*Microcebus* spp.) in northern Madagascar: The discovery of a putative new species? *Am J Primatol*. 2019;0:e23070. https://doi.org/10.1002/ajp.23070

APPENDIX: DETAILED METHODS

Phylogenetic analyses

In MRBAYES v3.2.1, we used four MCMC chains, each at the default temperature and running for 15,000,000 generations. Trees were sampled every 15,000 generations and 25% of total sampled trees were discarded as the "burn-in." To assess MCMC convergence, we checked for stationarity of the log probability of the data over time, we verified that the potential scale reduction factor was close to 1 and we used Tracer v1.6 (Rambaut, Drummond, & Suchard, 2014) to confirm that all estimated sample size values were >200. In RAXML v8.2.X, we used the rapid Bootstrap option with automatic detection of the sufficient number of bootstrap replicates (option: "autoMR"). In both approaches, we estimated the phylogeny for each mtDNA dataset (cox2, cytb, and cox2_cytb). We used sequences of *Cheirogaleus major* (EU825335), *Cheirogaleus medius* (EU825333), *Cheirogaleus crossleyi* (EU825354) and *Mirza coquereli* (U53571) as outgroups (retrieved from GeneBank). Retained posterior distributions of trees are summarized in MRBAYES to build a consensus tree, while in RaxML majority-rule consensus tree is estimated from the Bootstrap replicates. Phylogenetic trees were visualized in FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and modified in Inkscape (https://inkscape.org/).

We estimated the best partitioning scheme and best-fit model of evolution for each mtDNA dataset in PARTITIONFINDER v2.1.1 (Guindon et al., 2010; Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017; Stamatakis, 2006) using the greedy algorithm and selecting the best model according to AICc criteria. We performed a separate analysis for each phylogenetic method, given that MRBAYES and RAXML accept only some of the models of evolution that can be estimated in PARTITIONFINDER. Details on the selected model of evolution for each mtDNA dataset and phylogenetic method are presented in Supporting Information A (Table S1).

Species validation

We carried out the species delimitation analysis using a user-specified guide tree (option "A10"; Rannala & Yang, 2013; Yang & Rannala, 2010) that defines the *M. sambiranensis*, *M. arnholdi* and *Microcebus* sp. clades as different species. The BPP v3.4 software requires prior information on ancestral population size (θ) and the age of the root of the species tree (τ), and as in Hotaling et al. (2016) we tested a wide range of prior values for a total of six combinations of θ and τ prior values (Supporting Information A, Table S2). We ran BPP on the combined mtDNA dataset (cytb + cox2), with four repetitions for each combination of prior values, using alternatively rjMCMC algorithm0 or algorithm1, a burn-in of 100,000 generations and a total of 10,000 MCMC thinning samples. We assess BPP robustness by performing the

same analyses on datasets in which individuals were randomly assigned to one of the three clades.

Morphological analysis

We used K-means clustering, an approach that uses an unsupervised machine learning algorithm for partitioning individuals in a set of predefined K-groups by minimizing differences within-group and maximizing differences between groups. We estimated the optimal number of predefined K-groups using NbClust function in the NbClust R package (Charrad, Ghazzali, Boiteau, & Niknafs, 2014). This function computes 26 indices, each proposing the best number of K-groups, and the K value with higher support among the 26 indices is selected as the best clustering scheme. After performing the K-means clustering analysis using the K-means function in R, we validated the quality of the clustering using the average silhouette approach, implemented in the factoextra R package (Kassambara & Mundt, 2017).

We performed partial redundancy analyses to test the partial contribution of geography (as a proxy for genetic relatedness) and climate on morphological variability (Borcard & Legendre, 1994; Borcard, Legendre, & Drapeau, 1992). Significance was assessed using 1,000 permutations (analysis of variance function in R). In this analysis, we reduced the 19 bioclimatic variables to their most relevant and significant components by performing PCA analyses. Geographic distances were transformed in principal coordinates of neighbor matrices to account for positive spatial autocorrelation among individuals (Borcard & Legendre, 2002; Dray, Legendre, & Peres-Neto, 2006). Partial redundancy analyses and tests for significance were performed in R using the vegan package (Oksanen et al., 2018).