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Genetic Differentiation and Demographic History of the Northern Rufous Mouse Lemur (*Microcebus tavaratra*) Across a Fragmented Landscape in Northern Madagascar

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Abstract Phylogeographic barriers, together with habitat loss and fragmentation, contribute to the evolution of a species' genetic diversity by limiting gene flow and increasing genetic differentiation among populations. Changes in connectivity can thus

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affect the genetic diversity of populations, which may influence the evolutionary potential of species and the survival of populations in the long term. We studied the genetic diversity of the little known Northern rufous mouse lemur (*Microcebus tavaratra*), endemic to Northern Madagascar. We focused on the population of *M. tavaratra* in the Loky–Manambato region, Northern Madagascar, a region delimited by two permanent rivers and characterized by a mosaic of fragmented forests. We genotyped 148 individuals at three mitochondrial loci (*D-loop*, *cytb*, and *cox2*) in all the major forests of the study region. Our analyses suggest that *M. tavaratra* holds average genetic diversity when compared to other mouse lemur species, and we identified two to four genetic clusters in the study region, a pattern similar to that observed in another lemur endemic to the region (*Propithecus tattersalli*). The main cluster involved samples from the two mountain forests in the study region, which were connected until recently. However, the river crossing the study region does not appear to be a strict barrier to gene flow in *M. tavaratra*. Finally, the inferred demographic history of *M. tavaratra* suggests no detectable departure from stationarity over the last millennia. Comparisons with codistributed species (*P. tattersalli* and two endemic rodents, *Eliurus* spp.) suggest both differences and similarities in the genetic clusters identified (i.e., barriers to species dispersal) and in the inferred demographic history. These comparisons suggest that studies of codistributed species are important to understand the effects of landscape features on species and to reconstruct the history of habitat changes in a region.

Keywords Genetic structure · Habitat fragmentation · Isolation by distance · Population genetics · Small mammals

Introduction

Destruction and degradation of natural ecosystems is causing widespread biodiversity loss (Schipper *et al.* 2008). Habitat loss leads to fragmentation, the breaking up of habitat into smaller and more isolated fragments separated by a matrix of unfavorable habitat, which itself can reduce genetic diversity and increase inbreeding (Frankham 2005). Worldwide, most remaining forests are subject to fragmentation (Haddad *et al.* 2015). Therefore, it is essential to understand the extent to which habitat size and putative barriers to gene flow (e.g., roads, rivers, and unfavorable habitat) affect species' genetic diversity and structure. For many species, particularly small or nocturnal species, it is difficult and very time consuming to use direct observation or tracking methods to investigate the landscape features that disrupt dispersal (Tesson and Edelaar 2013). However, the combination of population genetics, landscape ecology, and spatial analysis may facilitate the detection of potential barriers to gene flow that are undetectable using nonspatial methods (reviewed in Storfer *et al.* 2006). Genetic data can also be used to reconstruct a species' history, and identify

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factors that have affected ecosystems in the past. By quantifying the effect of such factors on species distribution and diversity, biologists can inform conservation management (Christie and Knowles 2015; Hanski 1994, 1999; Tewksbury *et al.* 2002). Conservation genetics, together with a wide array of disciplines, helps preserve species and their ability to respond to environmental changes, for instance, by identifying the genetic consequences of demographic changes and inbreeding (Frankham *et al.* 2002; Shafer *et al.* 2015; Soulé 1985).

Around 25% of mammalian species are thought to be globally threatened with extinction (Schipper *et al.* 2008), and habitat degradation is the primary threat (Crooks *et al.* 2017), especially in the tropics (Schipper *et al.* 2008). For instance, Madagascar lost almost 40% of its forest cover from 1950 to 2000 (Harper *et al.* 2007), causing the estimated loss of 9% of Malagasy plant and invertebrates (Allnutt *et al.* 2008). Madagascar is one of the world's richest biodiversity hotspots, with an outstanding degree of endemism, containing 3.2% of the world's plant species and 2.8% of vertebrate species as endemics (Myers *et al.* 2000). The diversity of primate fauna (i.e., lemurs) is comparable to that of Brazil, the world's richest country for primates (Schwitzer *et al.* 2013, 2014), which has a land area ca. 14 times larger than Madagascar. Anthropogenic deforestation, forest fragmentation, and hunting are the most serious threats faced by lemurs (Schwitzer *et al.* 2013, 2014). These threats are more severe for regionally endemic species, since localized deforestation is more threatening to these species than it is to those with a larger geographic distribution (Yoder *et al.* 2000).

The genus *Microcebus* (mouse lemur) is, together with the genus *Lepilemur*, one of the two most diverse genera of Malagasy primates, with >20 species (Andriantompohavana *et al.* 2006; Hotaling *et al.* 2016; Louis *et al.* 2006, 2008; Olivieri *et al.* 2007; Radespiel *et al.* 2008a, 2012; Rasoloarison *et al.* 2013). Most of these species have very small distributions and there are almost no biological data on them. The mechanisms and conditions that led to this large radiation are still unclear. Several studies have suggested that large rivers could have acted as barriers to gene flow during the evolution of mouse lemurs (Martin 1995; Olivieri *et al.* 2007; Radespiel *et al.* 2008a; Schneider *et al.* 2010; Wilmé *et al.* 2006), contributing to species divergence. A detailed investigation of the intraspecific genetic diversity may help to understand the major factors that may be involved in the diversification of the genus *Microcebus* (Yoder *et al.* 2000).

We focus on *Microcebus tavaratra*, a nocturnal mouse lemur endemic to Northern Madagascar (Rasoloarison *et al.* 2000). The population size across its entire distribution (Fig. 1) is unknown, but the population in the southernmost area of its distribution, the Loky–Manambato region, numbers ca. 57,000 (Salmona *et al.* 2014). This area is covered by fragmented forests and it is unclear to what extent human activities or natural climate have contributed to the present landscape in this region. Landscape satellite imagery suggests that forest cover remained remarkably stable over the past 60 yr. (Quéméré *et al.* 2012). In contrast, palynological records across Madagascar suggest that forest habitats have been subject to periods of contraction and extension at different periods of the Holocene (Burney *et al.* 2004; Goodman and Rakotozafy 1997; Matsumoto and Burney 1994).

To our knowledge, six genetic studies have focused on the vertebrates of the Loky–Manambato region (Quéméré *et al.* 2010a, b, 2012; Rakotoarisoa *et al.* 2013a, b; Salmona *et al.* 2017), four of which focused on one lemur species (*Propithecus tattersalli*) and the other two on two endemic rodents. We studied the phylogeography and population genetics of *Micocebus tavaratra* across the Loky–Manambato region, using three mitochondrial DNA markers (*cytochrome-b*, *D-loop*, and *cox2*).

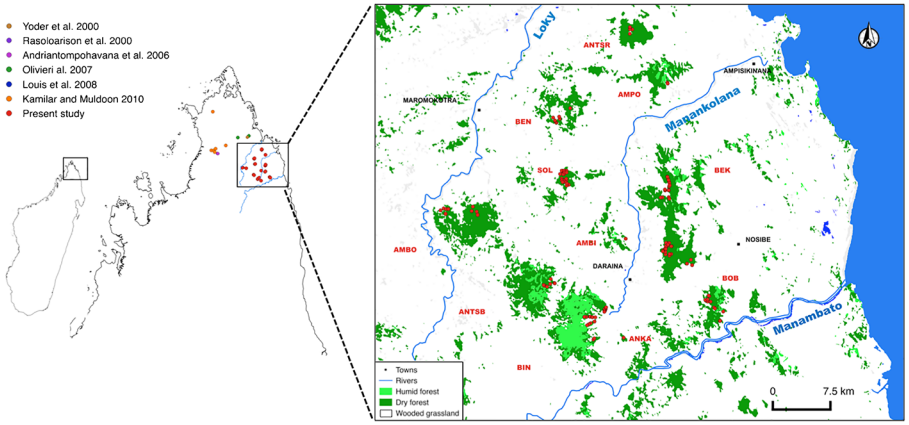

Fig. 1 Sampling distribution of *Microcebus tavaratra* in the Loky–Manambato region, Madagascar. (**Left**) Available presence data for *M. tavaratra*. **(Right)** Forest fragments we sampled in 2010: 11 in the Loky–Manambato region. Red labels indicate fragments we sampled; red dots indicate samples locations. AMBI = Ambilondambo; AMBO = Ambohitsitondroina; AMPO = Ampondrabe; ANKA = Ankaramy; ANTS = Antsahabe; ANTSR = Antsaharaingy; BEK = Bekaraoka; BEN = Benanofy; BIN = Binara; BOB = Bobankora; SOL = Solaniampilana.

Morphological diversity, population density and ecological niche data are available for *M. tavaratra* (Kamilar *et al.* 2016; Meyler *et al.* 2012; Rasoloarison *et al.* 2000; Salmona *et al.* 2014) but other aspects of its ecology, behavior, genetic structure, and demographic history remain to be studied. We aimed to 1) assess the level of genetic diversity in the study area; 2) quantify genetic differentiation among forests and putative subpopulations in relation to open habitats and rivers; and 3) infer putative population size changes. If fragmentation is ancient, then we predict that genetic differentiation should be significant, likely due to limited gene flow across open habitat. Given that the Manankolana River represents a significant barrier for other codistributed species (Quéméré *et al.* 2010a; Rakotoarisoa *et al.* 2013b), we also predict that it should shape genetic differentiation in *M. tavaratra*. Furthermore, we predict that we will identify genetic evidence of recent connectivity between the two mountain forests of the region, likely associated with a common history of forest-cover changes during the late Quaternary vegetational shift (Rakotoarisoa *et al.* 2013a).

Methods

Study Species

Microcebus tavaratra is a small (head–body length: 12–14 cm), omnivorous, nocturnal lemur belonging to the Cheirogaleidae family. It mainly inhabits Northern Madagascar's dry deciduous forests, at altitudes ranging from 20 to 250 m (Fig. 1; Mittermeier *et al.* 2010). *M. tavaratra* is classified as Vulnerable and threatened mainly by hunting and anthropogenic habitat loss and fragmentation resulting from slash-and-burn agriculture, charcoal production, uncontrolled bushfires, illegal logging, and mining activities (Andriaholinirina *et al.* 2014). No life history data are available for *M. tavaratra*, but studies of a closely related species, *M. murinus*, show that females

can begin to reproduce during their first year (Kappeler and Rasoloarison 2003; Zimmermann *et al.* 2016). Therefore, several studies have used 1 yr. as generation time value. However, a recent study used data from wild and captive populations of *Microcebus* and estimated a generation time of 3.0–4.5 yr. (Yoder *et al.* 2016). We consider generation times of 1, 3, and 4.5 yr. in the following analyses.

Study Area

The study region (2580 km^2; Ranirison *unpubl. data*) is bounded by the Loky and Manambato Rivers and crossed by the shallow Manankolana River (Fig. 1). It is a biogeographical transition zone between dry deciduous and humid forests (Goodman and Wilmé 2006), covered by gallery, littoral, dry deciduous, and humid forests (over approximately 440 km^2; Vargas *et al.* 2002) and surrounded by human-altered grasslands, dry scrub and agricultural land (Meyers and Wright 1993; Randrianarisoa *et al.* 1999). The region comprises nine major forest fragments situated at low to mid altitudes (126–728 m) and mostly covered by dry deciduous forests: Ambilondambo, Ambohitsitondroina, Ampondrabe, Ankaramy, Antsaharaingy, Bekaraoka, Benanofy, Bobankora, Solaniampilana (Fig. 1 and Table I; Gautier *et al.* 2006; Goodman and Wilmé 2006). In contrast two high altitude mountain fragments (Binara 1171 m, Antsahabe 1099 m) are covered by a gradient of dry deciduous (up to 500 m), transition (500–600 m), humid and ericoid forests (from 600 m; Gautier *et al.* 2006; Goodman and Wilmé 2006). The region's conservation is managed by the NGO Fanamby under a New Protected Area (NAP Loky–Manambato) status (FANAMBY 2010).

Sampling and Laboratory Procedures

We collected ear biopsies from individuals captured with Sherman traps (H. B. Sherman Traps®), during the dry season in 2010 and 2011, according to established field procedures (Rakotondravony and Radespiel 2009). We set the traps between 18:00 and 20:00 h along trails and checked them the following day starting at 07:00 h. We brought the captured mouse lemurs to the campsite and marked, weighed, and measured them. We released captured individuals at the site of capture later the same day. We recorded the GPS point at the site of capture and extracted the altitude information from a digital elevation model (DEM) database using GPS Visualizer (http://www.gpsvisualizer.com/elevation). We stored the biopsies in Queens Lysis Buffer (Seutin *et al.* 1991) at ambient temperature in the field and subsequently at -20 °C. We extracted total genomic DNA from 148 samples, using a mammalian DNA isolation protocol (Laird *et al.* 1991). We modified this protocol to increase the amount of extracted genomic DNA by incubating each biopsy overnight at 37 °C in 300 μL of digestion buffer (100 mM EDTA, 100 mM NaCl, 50 mM Tris pH 8 and 1% sodium dodecyl sulfate) and 30 μL of Proteinase K at 10 mg/mL (Promega no. V3021). We amplified and sequenced three mitochondrial (mtDNA) loci using published primers: cytochrome *b* (*cytb*; 1110 bp) (Irwin *et al.* 1991), *D-loop* (514 bp) (Gerloff *et al.* 1999), and the cytochrome *c* oxidase subunit II (*cox2*; 684 bp) (Adkins and Honeycutt 1994). We carried out amplifications in a 10-μL reaction with final concentrations of 1.0 μM for each primer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1× NH_4 reaction buffer, and 0.05 unit of GoTaqFlexi DNA polymerase (Promega no. M8305). Thermocycling consisted

Table I Genetic diversity of *Microcebus tavaratra* in the Loky–Manambato region of Madagascar, 2010–2011

Forest name	Abbreviation	*N*	*D-loop*					*cytb*					*cox2*					All					Altitude (m)	Forest type	Area (km^2)
			N hap	*S*	*h*	π*	priv. (%)	*N* hap	*S*	*h*	π*	priv (%).	*N* hap	*S*	*h*	π*	priv. (%)	*N* hap	*S*	*h*	π*	priv. (%)			
Ambilondambo	AMBI	1	1	/	/	/	0	1	/	/	/	0	1	/	/	/	0	1	/	/	/	0	253	D	8.3
Ambohitsitondroina	AMBO	9	4	10	0.75	5.92	50	4	3	0.75	0.93	50	2	3	0.22	0.97	0	6	16	0.89	2.03	83	89–231	D	38.32
Ampondrabe	AMPO	2	1	0	0	0	100	1	0	0	0	0	1	0	0	0	0	1	0	0	0	100	108–133	D–T	22.76
Ankaramy	ANKA	1	1	/	/	/	0	1	/	/	/	0	1	/	/	/	0	1	/	/	/	0	529	D	/
Antsahabe	ANTSB	10	4	23	0.73	10.11	75	3	15	0.38	2.79	67	3	3	0.38	0.88	33	6	41	0.84	3.8	83	374–678	T–H	36.94
Antsaharaingy	ANTSR	8	5	22	0.86	21.37	80	4	5	0.82	2.03	25	2	3	0.54	2.35	0	6	30	0.93	6.33	83	53–84	D	13.66
Bekaraoka	BEK	41	14	43	0.85	22.82	71	12	22	0.81	3.94	83	7	9	0.73	3.83	14	17	74	0.89	8.01	53	91–245	D–T	62.48
Benanofy	BEN	8	4	25	0.64	20.49	100	5	4	0.86	1.2	80	2	3	0.57	2.51	0	8	32	1	5.79	100	201–432	D	25.17
Binara	BIN	24	1	0	0	0	0	2	1	0.16	0.15	50	1	0	0	0	0	2	1	0.16	0.07	50	163–521	T–H	45.64
Bobankora	BOB	15	7	28	0.86	23.12	71	6	7	0.8	2.25	67	4	5	0.77	3.73	0	8	40	0.87	7.23	88	138–393	T–H	16.04
Solaniampilana	SOL	29	5	12	0.51	7.62	60	6	5	0.57	0.67	50	3	2	0.14	0.2	67	9	19	0.61	2.04	78	88–137	D	22.23
Total	Tot	148	38	58	0.92	24.42	/	33	40	0.91	3.53	/	10	12	0.71	3.38	/	59	110	0.95	8.01	/	53–678	/	334.78
Loky–Manambato 23 inds.	/	23	16	47	0.96	27.13	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	53–678	/	/
Northernmost forests	/	23	12	42	0.92	28.02	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	48–510	/	/

N = number of individuals; *N* hap. = number of haplotypes; *S* = number of variable nucleotides; *h* = haplotype diversity; π = nucleotide diversity; priv. = percentage of private haplotypes; * = values multiplied by 10^3 ; D = dry forest; T = transition forest; H = humid forest; / = information not available

of an initial denaturing temperature of 94 °C for 2 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 45–55 °C and 1.15 min at 72 °C, and a final extension of 10 min at 72 °C (Guschanski *et al.* 2006; Horvath *et al.* 2008). The annealing temperature of *D-loop*, *cytb*, and *cox2* was set respectively to 55 °C, 50 °C, and 45 °C. We sequenced the polymerase chain reaction (PCR) products in an ABI 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, CA) and edited the sequences using BioEdit v7.2.5 (Hall 1999).

For comparative purposes, we retrieved additional sequences from Genbank, specifically 23 *D-loop*, 11 *cytb*, and 8 *cox2* sequences of *Microcebus tavaratra* from forests north of the study region (Electronic Supplementary Material [ESM] Fig. S1 and Table SI). We also downloaded *D-loop*, *cytb*, and *cox2* sequences of 18 other mouse lemur species (ESM Table SII). The forests north of the study region correspond to the Analamerana (ANALM), Andrafiamena (ANDF), Andavakoera (AND), and Ankarana (ANKR) forests. We aligned the sequences using the Clustal W method (Thompson *et al.* 1994), in BioEdit (Hall 1999). We translated protein-coding sequences (*cytb* and *cox2*) to confirm the absence of stop codons. To obtain an artefactual indel-free sequence alignment, we aligned the protein-coding sequences according to the translated protein sequence alignment.

We performed the following analyses: 1) using each mtDNA locus separately; 2) concatenating all the loci, as this can increase the resolution of the genetic analyses; and 3) concatenating *cytb* and *cox2* sequences, as they are thought to have similar mutation rate (Blair *et al.* 2014).

Genetic Diversity and Differentiation

We computed haplotype (h) and nucleotide diversity (π) using DnaSP 5.0 (Librado and Rozas 2009). We used jModelTest v2.1.5 (Darriba *et al.* 2012) to select the best-fitting model of nucleotide substitution, choosing the most represented substitution model among those suggested using several selection-model methods (AIC, BIC, DT, LhRT, LdRT). The selected nucleotide substitution models were Hasegawa–Kishino–Yano evolution model (HKY; Hasegawa *et al.* 1985) plus rate variation among sites (G) for the *D-loop*; HKY plus proportion of invariable sites (I) for *cytb*; HKY for *cox2*; and HKY plus I for *cytb* + *cox2* + *D-loop*. We tested whether genetic diversity (h, π, no. of haplotypes and no. of private haplotypes) correlated with census population size (estimated in Salmona *et al.* 2014), forest area, and sample size for the eight populations with $N > 7$. We used Pearson correlation tests when data were normally distributed (tested using Shapiro–Wilky test) and Spearman correlation otherwise. We performed all correlation analyses in R v3.2.3 (R Core Team 2015). We also compared the genetic diversity of *Microcebus tavaratra* between two neighboring regions divided by the perennial Loky River: the study region and northernmost forests (ESM Fig. S1). To overcome the difference in sample size between the two regions, we randomly subsampled 23 individuals from the study region and computed the genetic diversity indexes.

We investigated genetic structure using several approaches: pairwise Φ_{ST}, isolation by distance, spatial autocorrelation, spatial analysis of molecular variance (SAMOVA), barrier identification using Monmonier's algorithm on a Delaunay triangulation (BARRIERS), and haplotype network. We excluded forests with less than eight individuals

(AMPO, ANKA, AMBI), with a final dataset of 144 individuals belonging to the remaining eight forests.

The pairwise Φ_{ST} is an estimator of genetic differentiation among populations that considers genetic distance and haplotype frequency (Excoffier *et al.* 1992). We computed pairwise Φ_{ST} values among forests with ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010), using the dataset with the three concatenated loci, TN93 as substitution model (Tamura and Nei 1993), and 10^4 random permutations.

We performed a Mantel test between genetic (Φ_{ST}) and geographic distances to detect patterns of isolation by distance in GenAlex (Peakall and Smouse 2006, 2012). The configuration and topology of suitable habitat patches can affect the type of isolation by distance pattern that emerges (Hutchison and Templeton 1999; van Strien *et al.* 2015). Some landscape configurations result in null or negative correlations between genetic and geographic distance, and in positive correlations when only close populations are considered. Thus, we should consider only neighboring deme pairs when investigating landscape effects in gene flow (van Strien *et al.* 2015). These pairs can be defined by Delaunay triangulation (Goldberg and Waits 2010). We computed the Delaunay triangulation using BARRIERS v2.2 (Manni *et al.* 2004). We assessed the linear relationship between genetic and geographic distances for neighboring deme pairs using a Pearson correlation test with 10^3 random permutations (in R v3.2.3; R Core Team 2015). This test has greater power than the Mantel test when using data vectors instead of distance matrices (Legendre and Fortin 2010). We also performed a spatial autocorrelation analysis using the three concatenated loci to investigate the spatial scale of variation in the dispersal process (ESM).

We used two complementary approaches to identify genetic barriers in our study population. First, we performed a spatial analysis of molecular variance (SAMOVA) using SAMOVA v2.0 (Dupanloup *et al.* 2002) to identify groups of forests that are genetically homogeneous or maximally differentiated from each other. We tested for two to seven groups (K) using forests with more than seven samples ($N = 8$). To select the optimal number of groups, we considered the maximum value of genetic variance resulting from differences among groups (FCT estimator), excluding configurations with single-population groups (Magri *et al.* 2006). Second, we investigated the presence of up to three barriers to gene flow using the Monmonier algorithm (Monmonier 1973) in BARRIERS v2.2 (Manni *et al.* 2004). This approach uses a geometrical map of the surveyed populations (Delaunay triangulation) and a genetic distance matrix (pairwise Φ_{ST}) to identify genetic barriers where differences between pairs of populations are largest. To assess barriers robustness we computed pairwise Φ_{ST} matrix from 100 bootstrapped DNA sequences datasets (per forest) generated with SEQBOOT in PHYLIP v3.69 (Felsenstein 1989). The algorithm used in BARRIERS has a higher detection efficiency than SAMOVA, but BARRIERS may identify spurious genetic barriers, owing to the nature of the algorithm (Dupanloup *et al.* 2002).

Lastly, to reveal the evolutionary relationship between the sampled populations we constructed the 95% most likely haplotype networks (Clement *et al.* 2000) in PopArt v1.7 (Leigh and Bryant 2015). We used the three datasets (148 ind.; *cytb_cox2*; *D-loop*; *cytb_cox2_D-loop*) to compute three distinct networks, including the northernmost sequence data for *M. tavaratra* (ESM Fig. S1 and Table SI).

Demographic Analyses

We used three different approaches to determine whether the population departs from mutation-drift equilibrium expectations and to infer its demographic history: 1) we computed three summary statistics: Tajima's D, Ramos R_2, and Fu's F_S (Fu 1997; Ramos-Onsins and Rozas 2002; Tajima 1989); 2) we estimated the mtDNA effective population size over time using the Extended Bayesian Skyline Plot (EBSP) in BEAST v1.8.1 (Drummond *et al.* 2012; Heled and Drummond 2008); and 3) we constructed the sequence mismatch distribution. Exploring different sampling strategies can help to overcome the confounding "structure effect" in demographic history inferences (Chikhi *et al.* 2010; Heller *et al.* 2013; Mazet *et al.* 2015, 2016; Städler *et al.* 2009). Therefore, we used four sampling strategies: 1) all samples from a single forest or identified spatial cluster (*local sampling*); b) two, three, or four randomly picked samples from each forest, excluding forests with only one, two, or three individuals, respectively (*pooled sampling*); 3) one randomly picked sample from each forest (*scattered sampling*); and 4) all samples from the surveyed area (*structured sampling*). We used 10 repetitions for *pooled* and *scattered* sampling.

We used ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010) to calculate Tajima's D, Ramos R_2, and Fu's F_S statistics and compared them to 10^3 coalescent-based simulations to determine their departure from the expected value under a population at equilibrium. Under the assumption of neutrality, positive Tajima's D and F_S values suggest population contraction, whereas negative values suggest a population expansion. Tajima's D is considered more powerful at detecting old and stronger population decline, whereas F_S exhibits more power with recent and soft contraction (Depaulis *et al.* 2003). Negative values of Ramos's R_2 support signatures of population contraction, while positive values support expansion.

We used EBSP implemented in BEAST v1.8.1 to infer the demographic history of the study population. We used unlinked loci substitution and clock models, but linked tree models because they belong to the same nonrecombinant DNA molecule (mtDNA), and specified a linear model of population size. We applied a strict clock model for all loci. We sampled the substitution rate (u) from a normal distribution: 1) truncated to an upper limit of 1×10^{-4}, with a mean of 7.2×10^{-8} and standard deviation (SD) of 7.25×10^{-7} for *D-loop* (Vigilant *et al.* 1991); and 2) with mean of 1.32×10^{-8} and SD of 4.0×10^{-9} for *cytb* and *cox2* (Blair *et al.* 2014). For *D-loop* we used a mutation rate range of 11.5–17.3% (substitutions/site) per 10^{-6} years, as estimated from the human–chimpanzee split (Vigilant *et al.* 1991) and similar to the value used in a study of the closely related species *Microcebus murinus* (Schneider *et al.* 2010). For *cytb* and *cox2*, we used the value of mutation rate estimated for two closely related species, *M. murinus* and *M. griseorufus* (Blair *et al.* 2014). We set a lognormal distribution for the prior of κ (transversion/transition) with initial value of 1) 1.356 for *D-loop*, 2) 0.8825 for *cytb*, and 3) 1.033 for *cox2*, as estimated in jModeltest v2.1.5. We set a $1/X$ prior distribution to estimate the demographic population size and a Poisson prior distribution to estimate demographic population size changes. We used a Markov Chain Monte Carlo (MCMC) of 2×10^8 generations with 25% burn-in to allow optimal acceptance rates of 10–70%. Following the recommendations of BEAST v1.8.2 tutorial (http://beast.bio.ed.ac.uk/tutorials), we adjusted the weights of EBSP operators to improve MCMC mixing. We carried out the analyses with the HKY

substitution model selected with jModeltest (see earlier): 1) plus gamma correction (G) for *D-loop*, sampled from an exponential prior distribution (initial value: 0.10); 2) plus invariant sites (I) sampled from a uniform prior distribution (initial value: 0.88) for *cytb*; and 3) without any other parameter for *cox2*. We checked for convergence of effective sample size (ESS >200) of all parameters in Tracer v1.6 (Rambaut *et al.* 2014).

We also estimated the mitochondrial DNA effective population size (mtDNA N_e), using a Bayesian coalescent-based constant-size model, implemented in BEAST v1.8.1 (Drummond *et al.* 2012), using the foregoing settings. The software estimates the value of population size as N_e multiplied by τ, where τ is the generation time in years. To obtain an estimate of the mtDNA N_e, we divided the estimated value by τ, testing different generation time values (i.e., 1, 3, or 4.5 yr.; Yoder *et al.* 2016).

We constructed sequence mismatch distributions for forests with $N > 7$ individuals. A sequence mismatch distribution is the distribution of the number of nucleotide mismatches between all pairs of DNA sequences belonging to a population sample (Rogers and Harpending 1992; Slatkin and Hudson 1991). The distribution is sensitive to changes in population size. It is usually bell shaped in populations that have increased demographically in the past as a single population with no subdivisions (Rogers and Harpending 1992), and shows one or two modes in structured populations that have passed through a range expansion, depending on the population density and the number of migrants exchanged between neighboring populations (Ray *et al.* 2003). We investigated mismatch distributions under demographic or spatial expansion models in ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010). To test the goodness-of-fit of the observed mismatch distribution to that expected under demographic or spatial population expansion model, we computed the sum of squared deviations (*ssd*) and the raggedness index (*rg*) with 10^3 replicates. We performed this analysis on two datasets: *D-loop* ($u = 7.2 \times 10^{-8}$) and *cytb_cox2* ($u = 1.32 \times 10^{-8}$).

Data Availability The datasets generated during the current study are available in the NCBI repository, including the GPS coordinates of the sampled individuals (MF141095–MF141538).

Ethical Note We conducted this study in agreement with the laws of the countries of Portugal, France, and Madagascar (CITES; permit ID 593C–EA10/MG11) and with the International Primatological Society Code of Best Practices for Field Primatology. The authors declare that they have no conflict of interest.

Results

Genetic Diversity

We analyzed three mtDNA loci from 148 *Microcebus tavaratra* individuals collected at altitudes between 53 and 678 m (Table I), thus mainly in dry (up to 500 m) and transition (500–600 m) habitats. Overall, the study population showed a haplotype diversity of 0.95 and a nucleotide diversity of 8.01×10^{-3} (Table I). We found haplotype diversity >0.84 in most of the forests, except for SOL ($h = 0.61$) and BIN ($h = 0.16$), the latter showing only two haplotypes despite a sample size of 24 individuals. Three forests

(ANTSR, BEK, and BOB) had the highest values of both haplotype and nucleotide diversity. Total haplotype diversity was ≈ 0.9 for *D-loop* and *cytb*, and ≈ 0.7 for *cox2*, while nucleotide diversity was $\approx 24 \times 10^{-3}$ for *D-loop*, $\approx 3 \times 10^{-3}$ for *cytb* and *cox2*. We found no significant correlation between genetic diversity and forest area, census population size, or sample size (ESM Tables SIII and SIV).

The *D-loop* genetic diversity in the study region was similar to that in the northernmost forests (Table I). Comparing genetic diversity across *Microcebus* species, *Microcebus tavaratra* shows high (*D-loop* and *cytb*) to average (*cox-2*) haplotype diversity (Table II, Fig. 2, ESM Table SV). *M. tavaratra* have average (*D-loop* and *cox-2*) and low (*cytb*) levels of nucleotide diversity, with *M. griseorufus*, *M. murinus*, and *M. myoxinus* showing the highest *D-loop* nucleotide diversity. However, the available sample size varies among species and is likely to affect the results.

Population Structure

Pairwise Φ_{ST} values ranged from 0.07 to 0.94, most were higher than 0.2, and all were significant, except that between BOB and BEN (Table III). Mantel and Pearson correlation tests revealed weak and nonsignificant correlations between pairwise genetic and geographic distances (Mantel statistics, $R < 0.001$, $P = 0.45$; Pearson $\rho = -0.089$, $P = 0.76$, ESM Fig. S2), suggesting the absence of simple isolation

Table II Sample sizes for published genetic data retrieved from NCBI for mouse lemur species

Species	*D-loop* *N*	*cytb* *N*	*cox2* *N*
M. griseorufus	145	102	38
M. murinus	498	44	50
M. lehilahytsara	2	17	17
M. myoxinus	8	30	28
M. tavaratra	148	148	148
M. berthae	4	8	4
M. bongolavensis	24	2	2
M. danfossi	6	7	7
M. jollyae	3	–	7
M. macarthurii	2	2	2
M. mittermeieri	6	19	31
M. ravelobensis	85	15	18
M. sambiranensis	6	12	10
M. simmonsi	13	36	45
M. gerpi	6	6	6
M. mamiratra	4	–	–
M. marohita	–	3	3
M. tanosi	–	10	10

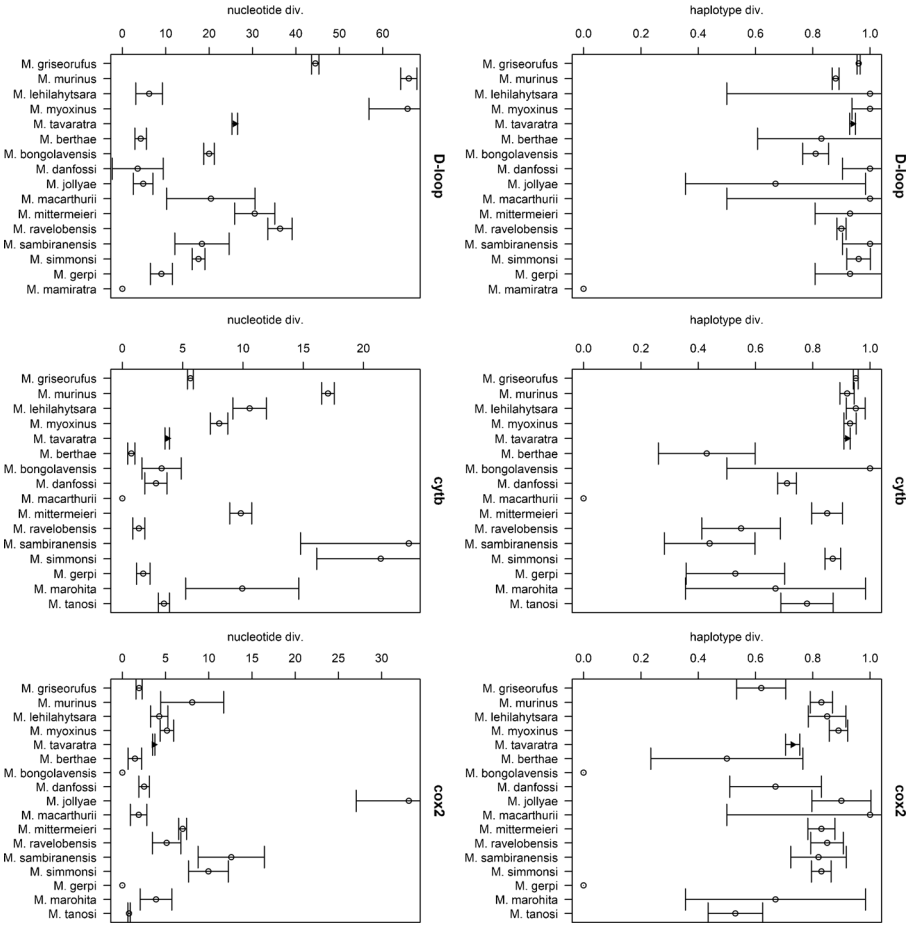

Fig. 2 Comparative genetic diversity in mouse lemurs. Haplotype **(right)** and nucleotide **(left)** diversity for three mitochondrial loci (*D-loop*, *cytb*, *cox2*). Nucleotide diversity values are multiplied by 10^3. Points show the mean values. Bars around points show the standard deviation. Triangles highlight data for *Microcebus tavaratra*.

by distance patterns in mtDNA loci in our study region. The autocorrelation analysis showed near null and nonsignificant values (ESM Fig. S3), suggesting that, within forests, geographically closer individuals are not more similar or dissimilar to each other than expected by chance. Using SAMOVA, we identified four forest clusters ($K = 4$; highest FCT value and no single-forest clusters) with similar molecular variance within forests (48.80%) and among forests clusters (45.36%): 1) ANTSB–BIN; 2) AMBO–SOL; 3) BEK–BOB; 4) ANTSR–BEN (ESM Table SVI). ANTSB and BIN was the only cluster present in all tested configurations ($K = 2$ to $K = 7$; Table SVI), showing the strong similarity of the two humid mountain forests as well as their clear differentiation from the other forests of the region. The Monmonier's maximum difference algorithm confirmed the four clusters identified by SAMOVA and further emphasized the geographical concordance between barriers and the Manankolana River (Fig. 3). The haplotype

Table III Pairwise comparisons of genetic differentiation in *Microcebus tavaratra* among eight forests in the Loky–Manambato region of Madagascar surveyed in 2010–2011 and with more than seven genotyped individuals

	AMBO	ANTSB	ANTSR	BEK	BEN	BIN	BOB	SOL
AMBO								
ANTSB	0.74 (**0.000**)							
ANTSR	0.50 (**0.002**)	0.54 (**0.000**)						
BEK	0.19 (**0.001**)	0.33 (**0.000**)	0.23 (**0.001**)					
BEN	0.53 (**0.001**)	0.53 (**0.000**)	0.21 (**0.035**)	0.22 (**0.001**)				
BIN	0.94 (**0.000**)	0.13 (**0.000**)	0.83 (**0.000**)	0.48 (**0.000**)	0.83 (**0.000**)			
BOB	0.27 (**0.004**)	0.46 (**0.000**)	0.19 (**0.026**)	0.07 (**0.034**)	0.15 (0.061)	0.70 (**0.000**)		
SOL	0.14 (**0.026**)	0.79 (**0.000**)	0.62 (**0.000**)	0.30 (**0.000**)	0.64 (**0.000**)	0.90 (**0.000**)	0.42 (**0.000**)	

P-values are in parentheses and values in bold represent a *P*-value <0.05, based on 10^4 random permutations

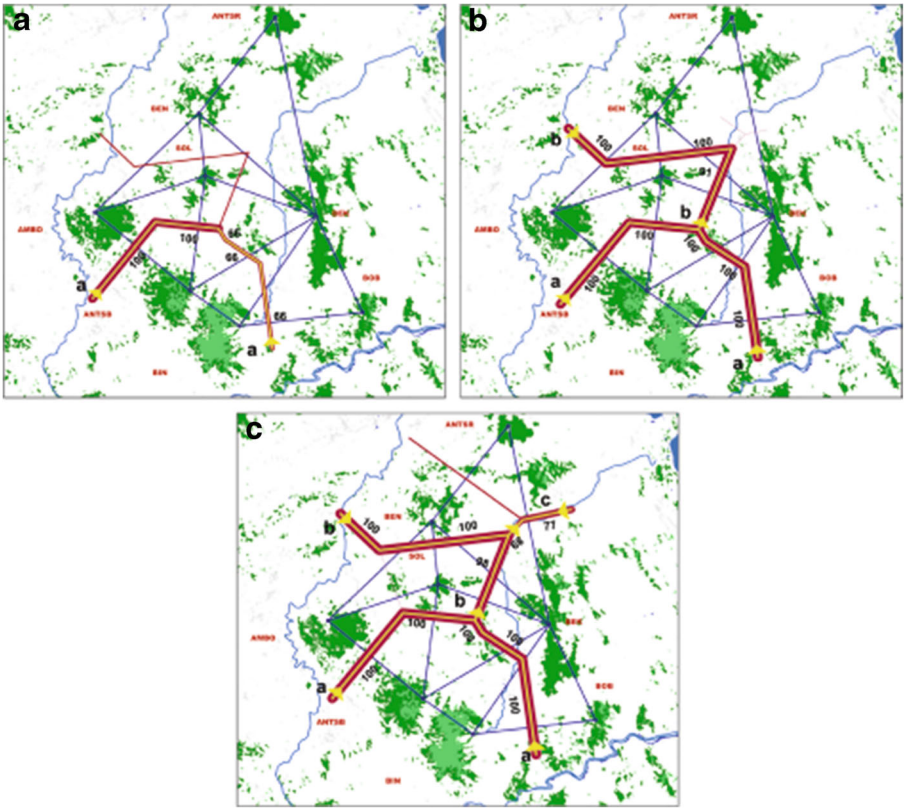

Fig. 3 Identification of genetic barriers among subpopulations of *Microcebus tavaratra* in the Loky–Manambato region of Madagascar in 2010–2011. Numbers are bootstrap support values for each segment. Blue lines represent the Delaunay triangulation (see Methods). Red segments constitute barriers computed from 100 bootstrapped matrices. Yellow lines represent the genetic barriers estimated from the Φ_{ST} matrix. **(a)** Detection of the first barrier, labeled as *a*. **(b)** Detection of the first (*a*) and second (*b*) barriers. **(c)** Detection of the first (*a*), second (*b*), and third (*c*) barriers.

networks (Fig. 4 and ESM Fig. S4) revealed a high proportion of private haplotypes and genealogical diversity in the study region; the latter describes the number of evolutionary steps between haplotypes. Apart from the ANTSB and BIN forests, which appear to be closely related in all networks, we found no easily interpretable phylogeographic pattern in the Loky–Manambato region. We could not visually recover the clusters identified with SAMOVA and BARRIERS in the haplotype network. At a larger geographical scale, the *D-loop* network did not show a clear distinction between the haplotypes from the study region and those from northern-most forests.

Demography

Nearly all Tajima's *D*, Fu's F_s, and Ramos R_2 computed for each sampling strategies and identified clusters were nonsignificant (ESM Table SVII). The exceptions were *cytb_cox2*_SOL, *cytb_cox2*_AMBO_SOL, and *cytb*, for which we found positive R_2

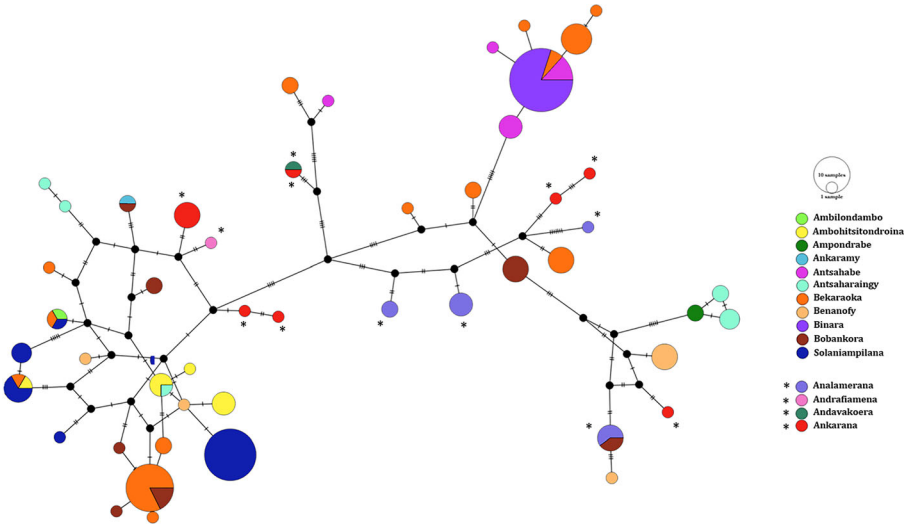

Fig. 4 Unrooted haplotype network of haplotypes of *Microcebus tavaratra*. Network constructed using samples collected in the Loky–Manambato region of Madagascar between 2010 and 2011 and published data from the northernmost forests. Parsimony mtDNA haplotype network obtained from *D-loop* by using a 95% parsimony connection limit. Sizes correspond to the relative frequencies and colors to the geographical location of the different haplotypes. Black circles on the interlinking branches represent missing haplotypes and sticks correspond to mutational steps between haplotypes. Asterisk marks haplotypes from the northernmost forests.

and negative Tajima's D and F_s, all suggesting a past expansion under the assumption of no spatial structure. We did not test *D-loop*_BIN because of its lack of polymorphisms.

We could not reject the null hypothesis of demographic and/or spatial expansion for most mismatch distribution analyses (Fig. 5, ESM Figs. S5–S8). The demographic expansion assumption was rejected for *D-loop*_BEK, *D-loop*_BOB, *D-loop*_BEK_BOB, *cytb_cox2*_BOB, and *cytb_cox2*_ANTSB but the spatial expansion assumption was not. When we grouped all the samples together (Fig. 5), mismatch distributions showed a bimodal pattern, typical of a spatial expansion (Ray *et al.* 2003). We observed no differences in the outcome of the mismatch analyses for the three sampling strategies (*local*, *scattered*, and *pooled*), all supporting the hypothesis of past population expansion event(s) (ESM Table SVII, Figs. S5–S8). We could not compute the mismatch distributions of BIN (both *D-loop* and *cytb_cox2* datasets) and ANTSB–BIN cluster (*cytb_cox2* dataset), likely because of the low mtDNA diversity of these populations (Table I).

For most sampling strategies the EBSP analysis suggested that the effective population size (mtDNA N_e) did not change over time (Fig. 6, ESM Fig. S9). Its narrow confidence interval shows that it converged reliably to a unique optimum (Fig. 6). Only the *scattered* sampling scheme showed a slight recent increase in N_e, bounded by a large confidence interval (ESM Fig. S9a). Most forests showed comparable mtDNA N_e estimates, but BEK showed estimates one order of magnitude larger than other forests (ESM Fig. S9e). Under the constant-size coalescent model, we estimated median mtDNA N_e for the study region, testing different generation time (GT) values: 315,100 individuals (GT = 1 yr), 105,033 individuals (GT = 3), and 70,022 individuals

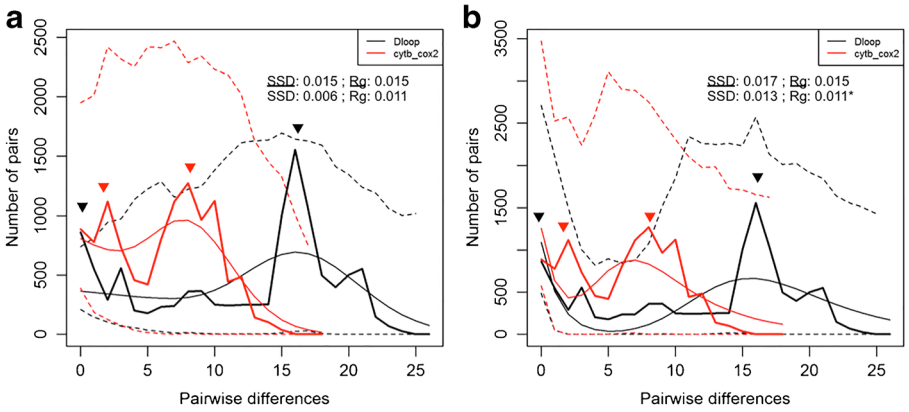

Fig. 5 Mismatch distribution under the assumption of expansion based on samples of *Microcebus tavaratra* obtained in the Loky–Manambato region of Madagascar. Mismatch distributions were calculated from 148 individuals collected in 2010–2011. SSD represents the sum of the squared deviation and Rg the raggedness index of the *D-loop* dataset; SSD and Rg relate to the *cytb_cox2* dataset. Continuous lines show the expected distribution; thick continuous lines show the observed distribution; dashed lines show the 95% confidence interval. $*P < 0.05$. Triangles show the two modes identified in the distributions (black: *D-loop* and red: *cytb_cox2*). **(a)** Demographic expansion. **(b)** Spatial expansion.

(GT = 4.5) (ESM Table SVIII). Based on population genetics theory, this suggests a very large nuclear N_e of between 280,000 (GT = 4.5) and 1.2×10^6 (GT = 1). However, the model used assumes a panmictic population and population structure will likely affect these values (Wakeley 2001).

Discussion

We collected *Microcebus tavaratra* individuals at altitudes higher (53–673 m) than it was previously thought to occur (25–250 m; Mittermeier *et al.* 2010). This suggests that *M. tavaratra* can be found in transition habitats (above 500 m), and thus may not be restricted to lowland dry forests. Our results show that genetic diversity of *M. tavaratra* is average among mouse lemurs. We also found significant population structure. This structure is partly influenced by rivers, but they do not appear to be clear and strong barriers to dispersal for this species. We identified strong genetic similarity between samples from the two mountain forests of the study region, which were connected until recently. Finally, we found no departure from stationary demographic history in the recent past.

Genetic Diversity and Effective Population Size

We found variable levels of genetic diversity among forests in the study region, which were not associated with either the estimated census population sizes or the forest areas. We found very little genetic diversity in the mountain forest BIN compared to the other forests, which could suggest a much smaller population size. However, in another study we found that the BIN forest holds levels of genetic diversity similar to those of the other forests for microsatellite markers, unlike mtDNA (Aleixo-Pais *et al. unpubl. data*). One

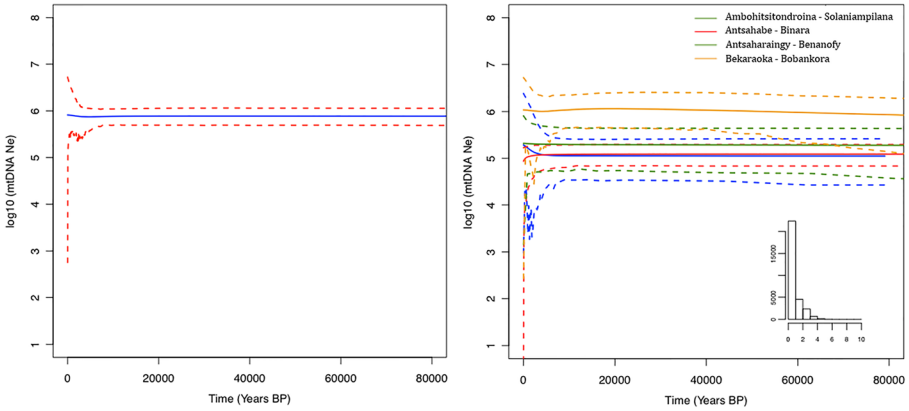

Fig. 6 Demographic history (estimated effective population size) of *Microcebus tavaratra* from the Loky–Manambato region of Madagascar, 2010–2011. **(Left)** EBSP analysis of all samples together (*structured* sampling strategy). **(Right)** EBSP analysis of groups identified by SAMOVA and BARRIERS. The solid line represents the median of the mtDNA effective population size over time. Dashed lines above and below the median values bound the 95%-HPD. The right panel includes a histogram of posterior size change values.

possible interpretation of these patterns is that *Microcebus tavaratra* colonized BIN relatively recently, and that very few females contributed to the population. These results suggest that the variable levels of genetic diversity in the study region may be linked to factors other than population size and forest area. At a larger geographical scale, we found that *M. tavaratra* harbors similar values of diversity in the forests north of the Loky River and in the study region.

Compared to other *Microcebus* species, haplotype diversity values are among the highest for *cytb* and high or average for the *D-loop* and *cox-2*. Nucleotide diversity estimates show average (*D-loop* and *cox-2*) and low (*cytb*) levels of diversity for *Microcebus tavaratra*, and a particularly high level of *D-loop* nucleotide diversity for *M. griseorufus*, *M. murinus*, and *M. myoxinus*, the mtDNA marker mostly used to assess intraspecific genetic diversity. These three mouse lemur species have a larger geographic range than *M. tavaratra* (Mittermeier *et al.* 2010), which may explain why they have higher nucleotide diversity than *M. tavaratra*. However, differences in sample size and area surveyed in the published data are likely to affect the estimated levels of diversity, so we should treat these comparisons with caution.

We found a large effective mtDNA population size in our study region, which varied (70,022–315,100) depending on the assumed generation time. Based on population genetics theory, this suggests a nuclear effective population size (N_e) between 280,000 and 1.2×10^6. We interpret this calculation with care because it is influenced by population structure, and thus by gene flow. The effective population size of a structured population typically increases as gene flow decreases (Mazet *et al.* 2016; Nei and Takahata 1993; Wakeley 2001). In addition, although we used genetic samples from the study region for our calculation, the estimated effective population size is likely to be influenced by the rest of the species' distribution and therefore does not represent an accurate estimate of the true N_e for the study region. The overestimation of the N_e for *M. tavaratra* in the study region might explain why we found an N_e much higher than the census population size (ca. 57,000 individuals; Meyler *et al.* 2012; Salmona *et al.* 2014), when in natural populations is generally the opposite (Frankham 2007; Palstra and

Ruzzante 2008). An additional explanation is that the census population size of *M. tavaratra* may be underestimated because part of the population may be undergoing extended torpor (Schülke and Ostner 2007). A comparison with census estimates (N_c) is thus unavoidably difficult and should probably not be overinterpreted, although it is important in elucidating the ecological factors that may affect population diversity and persistence (Kalinowski and Waples 2002; Palstra and Fraser 2012). Given that the distribution limits of *M. tavaratra* have not yet been fully investigated and likely extend both south and north (Kamilar *et al.* 2016) of the currently accepted distribution, both N_e and N_c should be considered as tentative at this stage. Altogether, the high genetic diversity of *M. tavaratra* is consistent with its potentially large distribution area and population size.

Population Structure

Based on a variety of complementary approaches, our results suggest that the population of *Microcebus tavaratra* is structured. At the study region level, our analyses suggest that the clearest genetic cluster corresponds to the Binara and Antsahabe mountainous forests, which were connected in the recent past and are the most humid forests in the region. Genetic evidence from a forest-dependent rodent associated with humid habitats (*Eliurus tanala*) also suggests that the populations in these two massifs are not very differentiated. In contrast, genetic data from the closely related rodent species associated with dry habitat (*E. carletoni*) did not support the same conclusion (Rakotoarisoa *et al.* 2013a, b). It is difficult at this stage to draw strong conclusions but additional studies of other codistributed species will help determine whether ecology (humid vs. dry) or some other factor explains these partly varied patterns.

Our analyses further suggested that open habitat likely played a significant role in the observed genetic structure of *Microcebus tavaratra*, as previously reported for other mouse lemur species (Guschanski *et al.* 2006; Olivieri *et al.* 2008; Radespiel *et al.* 2008b; Schneider *et al.* 2010) and for *Propithecus tattersalli*, endemic to the study region (Quéméré et al. 2010a). Although we identified some spatial genetic structure in *M. tavaratra*, we found no pattern of isolation by distance. We cannot directly determine which factors explain the absence of isolation by distance, but a similar lack of isolation by distance was described for *Eliurus carletoni* (Rakotoarisoa *et al.* 2013b), living in the same region and sharing a similar ecological niche (Carleton 2003).

Finally, our analyses suggest that the Manankolana River represents a potential barrier to gene flow, in line with previous studies of *Propithecus tattersalli* (Quéméré *et al.* 2010a, b) and *Eliurus carletoni* (Rakotoarisoa *et al.* 2013b). However, as for *E. carletoni*, this river does not represent a strict barrier to gene flow, in agreement with the absence of a clear haplotype network clustering of the forests on the two sides of the river. Similarly, at a larger geographical scale the haplotype networks did not reveal any divergence between populations north and south of the Loky River (i.e., between the study region and the northernmost forests); however, the sampling outside the study region is too limited at this stage to derive strong conclusions.

Demographic History

Most demographic history analyses revealed either no major change in population size or, in the case of the mismatch distributions, possible demographic and spatial

expansion. These results agree with previous findings for populations of *Eliurus tanala* located in the two mountain forests BIN and ANTSB (Rakotoarisoa *et al.* 2013a). In contrast, two other mammals living in the dry habitat of the study region, *Eliurus carletoni* and *Propithecus tattersalli* harbor genetic signatures of a population decline likely to have occurred since the Last Glacial Maximum (Quéméré *et al.* 2012; Rakotoarisoa *et al.* 2013a; Salmona *et al.* 2017). A genetic signature of population decline and habitat loss and fragmentation was also detected in *P. perrieri* in the neighboring Analamerana and Andrafiamena forests (Salmona *et al.* 2017), north of the Loky River and where the northernmost samples of *Microcebus tavaratra* come from. It is unclear whether the absence of clear demographic signal in *M. tavaratra* is due to limited information in the mtDNA data or to a real constant population size in the recent past, because EBSP analysis may not recover the correct demographic history (Grant 2015; Grant *et al.* 2012). Further investigation with more informative markers (microsatellite and RAD-seq data) will likely provide more insights into the demographic history of this species.

Conclusions

We detected some level of population structure but no clear signal of population size change in *Microcebus tavaratra* in the Loky–Manambato region of Madagascar. Comparisons with codistributed species suggest that some landscape features may play a role in some species but not in others, even though the species are associated with the same habitat. For instance, populations of *M. tavaratra* (associated with dry habitat) living in the two mountain forests Binara and Antsahabe were not very genetically differentiated, as also shown for *Eliurus tanala* (associated with humid habitat), but in contrast with results for *E. carletoni* (associated with dry habitat). This suggests that if we want to understand the history of species, and thus the history of habitat changes in a region, studies incorporating codistributed species may be particularly important.

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