



The genetic structure of a mouse lemur living in a fragmented habitat in Northern Madagascar

Isa Aleixo-Pais^{1,4,5} · Jordi Salmona^{1,2,3} · Gabriele Maria Sgarlata¹ · Ando Rakotonanahary⁷ · Ana Priscilla Sousa¹ · Bárbara Parreira¹ · Célia Kun-Rodrigues¹ · Tantely Ralantoharijaona⁷ · Fabien Jan¹ · Emmanuel Rasolondraibe⁷ · Tânia Minhós^{1,5,6} · John Rigobert Zaonarivelo⁸ · Nicole Volasoa Andriaholinirina² · Lounès Chikhi^{1,2,3}

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Abstract

Habitat loss and fragmentation are major threats to biodiversity worldwide. Madagascar is among the top biodiversity hotspots and in the past 100 years several species became endangered on the island as a consequence of anthropogenic activities. In this study, we assessed the levels of genetic diversity and variation of a population of mouse lemurs (*Microcebus tavaratra*) inhabiting the degraded forests of the Loky-Manambato region (Northern Madagascar). We used a panel of 15 microsatellite markers to genotype 149 individuals. Our aim was to understand if the elements contributing to the heterogeneity of the landscape, such as forest fragmentation, roads, rivers and open habitat, influence the genetic structure of this population. The results showed that geographic distance along with open habitat, vegetation type and, to some extent, the Manankolana River, seem to be the main factors responsible for *M. tavaratra* population structure in this region. We found that this species still maintains substantial levels of genetic diversity within each forest patch and at the overall population, with low genetic differentiation observed between patches. This seems to suggest that the still existing riparian forest network connecting the different forest patches in this region, facilitates dispersal and maintains high levels of gene flow. We highlight that special efforts targeting riparian forest maintenance and reforestation might be a good strategy to reduce the effect of habitat fragmentation on the genetic diversity of extant *M. tavaratra* populations.

Keywords *Microcebus tavaratra* · Microsatellites · Population genetics · Habitat fragmentation

Isa Aleixo-Pais and Jordi Salmona shared first co-authors.

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✉ Isa Aleixo-Pais
isa.aleixopais@gmail.com

✉ Jordi Salmona
jordi.salmona@gmail.com

✉ Lounès Chikhi
chikhi@igc.gulbenkian.pt

¹ Instituto Gulbenkian de Ciência, 2780-156 Oeiras, Portugal

² Laboratoire Évolution & Diversité Biologique (EDB UMR 5174), Université de Toulouse Midi-Pyrénées, CNRS, IRD, UPS, 31062 Toulouse, France

³ Université Toulouse 3 Paul Sabatier, CNRS; UMR 5174 EDB, 31062 Toulouse, France

Introduction

Habitat loss and fragmentation are major threats to biodiversity worldwide (Barlow et al. 2016; Fahrig 2003; Schipper et al. 2008). The degradation of natural landscapes has been shown to have negative consequences for the genetic

⁴ Organisms and Environment Division, Cardiff School of Biosciences, Cardiff University, Cardiff CF10 3AX, UK

⁵ Centre for Research in Anthropology (CIA-FCSH/NOVA), 1069-061 Lisbon, Portugal

⁶ Department of Anthropology, School of Social Sciences and Humanities, Universidade Nova de Lisboa, 1069-061 Lisbon, Portugal

⁷ Faculté des Sciences, Université de Mahajanga, BP 652, 401 Mahajanga, Madagascar

⁸ Département des Sciences de la Nature et de l'Environnement, Université d'Antsiranana, 201 Antsiranana, Madagascar

diversity of many species, contributing to the decline, endangerment and ultimately, extinction of populations (Lindenmayer and Fischer 2006). The International Union for Conservation of Nature (IUCN) recognises that many forest-dwelling species are threatened to extinction by major forest loss, specifically primates (Vitousek 1997; Lewis et al. 2015; Crooks et al. 2017; Estrada et al. 2017). Moreover, the ability of species to overcome rapid environmental changes relies on genetic variation, which enhances a species' resilience to ecological degradation and retains the capacity of species to adapt (e.g. Keller and Waller 2002; Johansson et al. 2007). Thus, understanding the genetic consequences of HL&F is key for the conservation of many populations living in impacted areas, particularly for biodiversity hotspots.

Madagascar is among the top biodiversity hotspots (Myers et al. 2000; Ganzhorn et al. 2001). This is due to the island's great levels of species richness and endemism, together with the fact that it is one of the most ecologically disturbed countries worldwide (Green and Sussman 1990; Harper et al. 2007). Over the last millennia Madagascar has experienced drastic environmental changes. Several pronounced droughts during the Holocene have replaced humid continuous woodland forests by discontinued mosaic of grasslands and forest patches (Virah-Sawmy et al. 2010; Dewar et al. 2013). During the last century, forests have constantly been lost and altered due to anthropogenic activities, namely fire for slash and burn cultivation, cattle raising, logging, mining activities, introduction of invasive species and hunting (Myers et al. 2000; Harper et al. 2007; Schwitzer et al. 2014). The loss of forest habitats and their increasing degree of fragmentation have been responsible for the rearrangement of Madagascar's biodiversity in terms of number of species, geographic distributions, overall population sizes and genetic diversity (Burney 1999; Quéméré et al. 2012; Salmona et al. 2017). This is particularly true for lemurs which are increasingly confined to reduced and isolated forest patches, and rather small geographic distributions. Lemurs are only found in Madagascar and most species rely exclusively on forests and woodlands (Dufils 2003; Goodman and Benstead 2005). Currently the forest-dwelling lemurs of Madagascar are considered "the most threatened mammal group on Earth" (Schwitzer et al. 2014), with about 94% of all assessed species classified as either vulnerable, endangered, or critically endangered.

A large number of conservation genetics studies focus on identifying barriers, such as roads and agricultural lands, affecting the genetic diversity and differentiation of populations. However, present-day patterns of genetic diversity may be a consequence of several processes, recent or ancient. In the past few years, a major question in landscape genetics has focused on the relative roles of recent human-induced and ancient climate changes on present landscapes (Lorenzen et al. 2011). In Madagascar, this is for instance

the case for the golden-crowned sifaka (*Propithecus tattersalli*), a forest-dwelling large-bodied lemur, whose populations decreased as a consequence of natural and possibly anthropogenic effects in the (Loky-Manambato) region of interest (see below) (Quéméré et al. 2012; Salmona et al. 2017). Madagascar holds a large number of lemur species with very diverse ecological requirements (Mittermeier et al. 2010), but despite the amount of genetic studies on lemur populations (e.g. Craul et al. 2009; Radespiel et al. 2008; Olivieri et al. 2008; Schneider et al. 2010; Lawler 2011; Holmes et al. 2013; Scheel et al. 2015; Nunziata et al. 2016), the impact of HL&F on the genetic diversity of many lemur species is still unknown. Comparative studies across co-distributed species are fundamental for determining the importance of geographical boundaries (Keller et al. 2015) and may also contribute to the debate surrounding the relative roles of climate and human-induced environmental changes. In particular, species with different socio-ecological traits could be useful to that aim. For instance, it is expected that species exhibiting short generation times should be appropriate to detect the genetic signature of habitat fragmentation over short evolutionary periods.

In this study we investigate the northern rufous mouse lemur *Microcebus tavaratra*, in the Loky-Manambato (LM) region (Fig. 1). Mouse lemurs have currently more than 20 recognised species (see Hotaling et al. 2016 for a recent assessment). They represent an interesting evolutionary model as the different species mostly occupy very restricted ranges, occurring in sympatry or over neighbouring areas. The LM is a region exhibiting a well characterised pattern of forest fragmentation, and combines several features that makes it a region of particular interest: (i) it harbours several species of lemurs with distinct biological characteristics; (ii) it is composed of forest patches of different topology, e.g. vegetation, elevation; (iii) it is exposed to anthropogenic pressures and (iv) it is crossed by a main road and a major river (Manankolana). Landscape features of this region have been recognised to influence the genetic structure of sympatric forest dwelling species. The Manankolana river influences the genetic structure of the golden crowned sifaka (*P. tattersalli*) and limits gene flow in the tuff-tailed rat (*Eliurus carletoni*); similarly, the matrix separating forest patches impacts, to some extent, gene flow in both these species (Rakotoarisoa et al. 2013; Quéméré et al. 2010a, b). The genetic diversity of *M. tavaratra* in the LM region has been recently studied using mitochondrial DNA, and the Manankolana river and matrix separating forests were suggested to be major factors determining the genetic structure of this species (Sgarlata et al. 2018). Mouse lemurs are forest-dwelling species, thus they are expected to be affected by forest fragmentation. Here we use microsatellite markers to study the genetic patterns of diversity and differentiation of this species. We investigate the effect of geographic

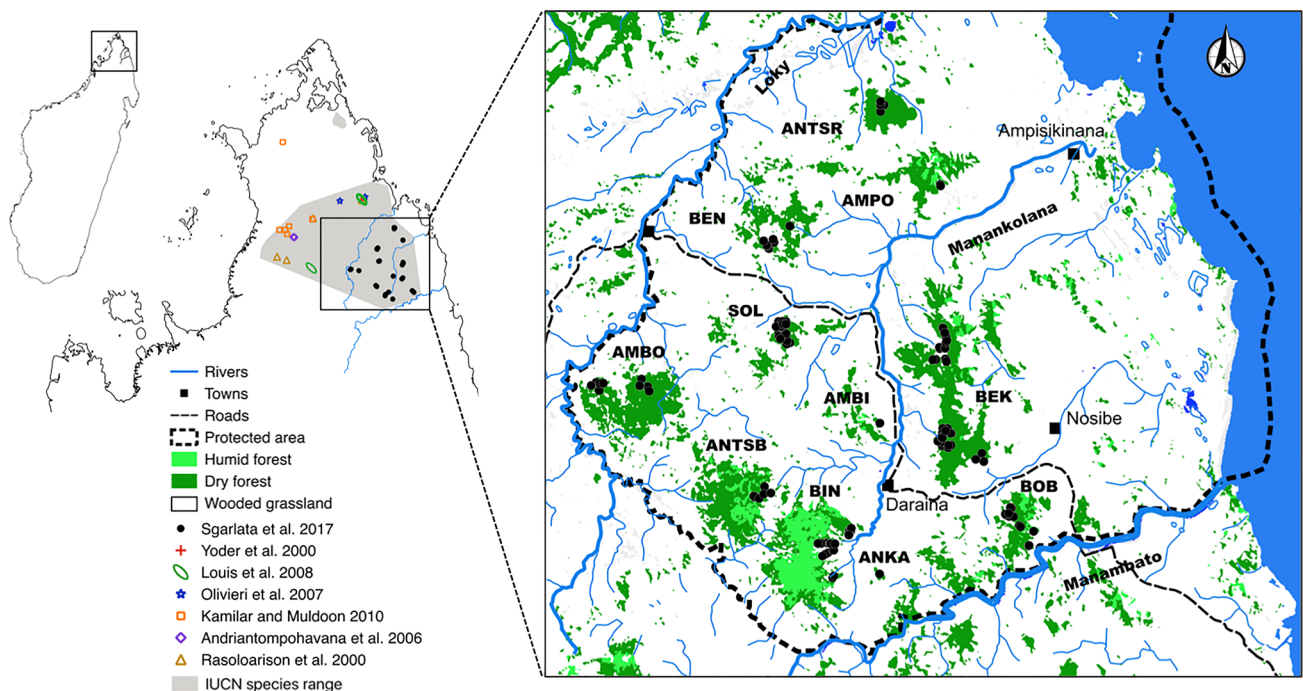


Fig. 1 Geographic range and sampling distribution of *M. tavaratra* across Northern Madagascar Map on the left: distribution range of *M. tavaratra* based on IUCN data and samplings of different studies; Map on the right: present study sampling sites across the Loky-Man-

ambato (LM) region. The black dots correspond to the samples used in this study and the forest patch names are abbreviated (see Table 1 for correspondence to full forest name)

distance and of natural and anthropogenic barriers on the genetic diversity and structure of *M. tavaratra* across part of its distribution range (Mittermeier et al. 2010), and we discuss the implication of our findings for the conservation of this species.

Methods

Study species

The northern rufous mouse lemur (*M. tavaratra*) is a small arboreal lemur (head–body length: 12–14 cm), belonging to the Cheirogaleidae family (Rasoloarison et al. 2000; Mittermeier et al. 2010). It is currently estimated to occur in an area < 5600 km² in north Madagascar (Andriaholinirina et al. 2014) (Fig. 1). Little ecological data are available for *M. tavaratra*, but it is known that *Microcebus* are nocturnal animals with solitarily foraging behaviour during the night and forming sleeping groups during the day (Martin 1972; Radespiel et al. 1998; Radespiel 2000). Although the mating structure of most *Microcebus* species is poorly known, studies on *M. murinus* (a closely related species) have found that females begin to reproduce during their first year (Kappeler and Rasoloarison 2003; Zimmermann et al. 2016). According to the latest IUCN red list assessment, *M. tavaratra*

is classified as “Vulnerable” and is mainly threatened by habitat loss resulting from slash and burn agriculture, charcoal production, uncontrolled bushfires, illegal logging, and mining activities (Andriaholinirina et al. 2014). This species is endemic to Northern Madagascar and can only be found across lowland dry deciduous and transition forests (Fig. 1; Mittermeier et al. 2010; Sgarlata et al. 2018). Previous studies have estimated relatively high population densities (Meyler et al. 2012; Salmona et al. 2014) in the Loky-Manambato region (Table 1; Fig. 1) and have found that most forest fragments comprise relatively large populations (~5000 individuals).

Study area

The LM region is located in the south east of *M. tavaratra* distribution range, in Northern Madagascar, and in the most current literature, no record of *Microcebus* sympatry has been described for this area (Hotaling et al. 2016; Mittermeier et al. 2010). It is a biogeographical transition zone between dry deciduous and humid forests (Goodman and Wilmé 2006), delimited by the Loky and Manambato Rivers. This region is crossed by a relatively shallow river, the Manankolana River, and by a national dirt road connecting two main villages (Fig. 1). It occupies an area of ~2580 km² (Ranirison 2006, unpubl. data) comprising a total forest

Table 1 Northern rufous mouse lemur's genetic diversity

| (a) | | | | | | | | | | | |
|--------------------|-------|---------|-------------------------|-----------------|----------|----------------|----------------------|----------------------|----------------------|----------------------|-----------------------|
| Forest | Abbv. | Cluster | Area (km ²) | Pop. size (ind) | <i>N</i> | <i>n</i> (f/m) | <i>N_A</i> | <i>A_r</i> | <i>H_o</i> | <i>H_e</i> | <i>F_{IS}</i> |
| Ambilondambo | AMBI | N | 8.30 | 1636 | 1 | 1/0 | – | – | – | – | – |
| Ambohitsitondroina | AMBO | E | 38.32 | 6582 | 10 | 10/0 | 4.80 | 4.06 | 0.607 | 0.594 | –0.023 |
| Ampondrabe | AMPO | N | 22.76 | 5448 | 2 | 0/2 | – | – | – | – | – |
| Ankaramy | ANKA | E | 6.39 | 1097 | 1 | 0/1 | – | – | – | – | – |
| Antsahabe | ANTSB | E | 36.94 | 4039 | 10 | 6/4 | 5.27 | 4.35 | 0.632 | 0.610 | –0.037 |
| Antsaharaingy | ANTSR | N | 13.66 | 391 | 7 | 4/3 | 4.07 | 3.92 | 0.629 | 0.611 | –0.032 |
| Benanofy | BEN | N | 25.17 | 4603 | 8 | 6/2 | 5.07 | 4.47 | 0.583 | 0.608 | 0.043 |
| Bekaraoka | BEK | SW | 62.48 | 6637 | 42 | 17/25 | 8.07 | 4.81 | 0.650 | 0.655 | 0.008 |
| Binara | BIN | E | 45.64 | 5439 | 24 | 20/4 | 6.07 | 4.10 | 0.565 | 0.577 | 0.021 |
| Bobankora | BOB | SW | 16.04 | 5217 | 15 | 12/3 | 5.67 | 4.35 | 0.631 | 0.656 | 0.040 |
| Solaniampilana | SOL | N | 22.23 | 5257 | 29 | 19/10 | 6.87 | 4.41 | 0.590 | 0.603 | 0.022 |

| (b) | | | | | | | | | | | |
|--------------------|---------------------|-----------------|----------|----------------|----------------------|----------------------|----------------------|----------------------|-----------------------|--|--|
| Level of hierarchy | | Pop. size (ind) | <i>N</i> | <i>n</i> (f/m) | <i>N_A</i> | <i>A_r</i> | <i>H_o</i> | <i>H_e</i> | <i>F_{IS}</i> | | |
| All samples | Loky-Manambato (LM) | 57,502 | 149 | 95/54 | 10.13 | 10.11 | 0.611 | 0.654 | 0.065*** | | |
| Cluster | Eastern (E) | | 57 | 29/28 | 8.93 | 8.65 | 0.645 | 0.666 | 0.032 | | |
| | Northern (N) | | 47 | 30/17 | 8.13 | 8.04 | 0.594 | 0.626 | 0.052* | | |
| | Southwestern (SW) | | 45 | 36/9 | 7.73 | 7.69 | 0.587 | 0.605 | 0.030 | | |

Genetic diversity estimated across 149 individuals genotyped for 15 microsatellite loci (a) at each forest; and (b) at the overall population (LM region) and at the three major clusters identified in the STRUCTURE and TESS programs. Pop. size (ind): population sizes estimated as the number of individuals within each forest from Salmona et al. (2014); *N*: sample size; *n* (f/m): number of females and males; *N_A*: mean number of alleles; *A_r*: allelic richness; *H_o*: observed heterozygosity; *H_e*: unbiased expected heterozygosity; *F_{IS}*: Wright's *F_{IS}* calculated according to Weir and Cockerham (1984), the significance of the *p*-value was calculated using 10,000 permutations and is represented as follows: **p* < 0.05; ****p* < 0.001

cover area of approximately 440 km² (Vargas et al. 2002), fragmented into eleven major forest patches surrounded by human-altered grasslands, dry scrub and agricultural land (Meyers and Wright 1993; Randrianarisoa et al. 1999). Nine of the forests (AMBI, AMBO, AMPO, ANKA, ANTSR, BEK, BEN, BOB and SOL; refer to Table 1 for full name of the forests) are situated at low to mid elevations and are mostly covered by dry deciduous vegetation (Gautier et al. 2006). In contrast, two high elevation mountain forests (BIN and ANTSB) are covered by a gradient of dry deciduous, transition, humid and ericoid vegetation (Gautier et al. 2006). The preservation of the LM region is managed by the NGO Fanamby under a New Protected Area status (NAP Loky-Manambato; FANAMBY 2010).

Sampling, DNA extraction and microsatellite genotyping

We collected ear biopsies from individuals captured with Sherman traps (H.B. Sherman Traps®) during the dry seasons of 2010 and 2011, according to Rakotondravony and Radespiel (2009) field procedures. At the campsite, morphometric measures were recorded for each mouse lemur captured and compared with previous descriptions of the

species (Rasoloarison et al. 2000). We ensured DNA preservation in field conditions by storing the samples in Queen's lysis buffer (Seutin et al. 1991) and at –20 °C once in the laboratory.

We extracted total genomic DNA from 152 ear biopsies using a DNeasy® blood and tissue kit (Qiagen®). Tissues were incubated overnight at 56 °C in 300 µL of ATL digestion buffer (Qiagen®) including proteinase K (*C_f* = 1 mg/mL), and 20 µL of dithiothreitol (1 M) which proved to be crucial to break down the disulphide bonds between the proteins present in cartilaginous tissue and improve DNA yield (Chen et al. 2003). These samples were firstly barcoded using three mitochondrial DNA markers for the study of Sgarlata et al. (2018) and all were identified as *M. tavaratra*. We amplified by PCR a total of 22 dinucleotide microsatellite loci, previously developed on *M. murinus* (Hapke et al. 2003; Radespiel et al. 2001; Wimmer et al. 2002), in six multiplexes of two to four microsatellite primer pairs (forward primer labelled with biomers.net fluorescent dyes; Table S1). We performed each reaction in a final volume of 10 µL, containing 5 µL of My Taq HS Mix (Bioline), 0.4–1.5 µL of primer mix (initial concentration of 10 µM; see Table S1 for details), and ≈ 20–50 ng of high quality DNA. The PCR initiated with a denaturation step of 94 °C

for 15 s, followed by 36 cycles at 94 °C for 30 s, 48–58 °C for 60 s and 72 °C for 60 s, and a final extension at 72 °C for 10 min. We separated amplification products using capillary electrophoresis (ABI 3130 DNA Analyser; Applied Biosystems®) and determined fragment sizes using the GeneMapper® software (Applied Biosystems®).

Data quality control

We ensured genotype accuracy with two to three PCR replicates and two independent observers scoring genotypes. We discarded seven loci with technical problems or ambiguous peak interpretation: two markers exhibited amplification problems (Mm09n, Mm53), two other showed challenging peak interpretation (Mm06, Mm10) and in three we detected null allele at high frequencies (Mm02, Mm21 and Mm22). We also tested the remaining 15 loci (Table S1) for the presence of null alleles, large allele dropout and scoring errors due to stutter peaks using MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). Out of the 152 initial samples, three did not have enough quality (< 13 validated genotypes) and were discarded, thus 149 samples (98%) constituted the final dataset. These individuals were genotyped across 15 loci, including two monomorphic, which were kept for analyses, except when specified otherwise.

In order to check for correlation among loci we tested for linkage disequilibrium (LD) with the correlation coefficient of Weir (1979) in the GENEPOP web version 4.2 (Raymond and Rousset 2004) and using 10,000 iterations for the test of significance. We corrected LD values for multiple tests using a Bonferroni correction (Rice 1989). Out of 105 pairwise locus comparisons one showed evidence of LD (Mm26b/Mm39, values not shown).

Analysis of genetic diversity

Spatial patterns of genetic diversity were inferred by computing diversity and differentiation measures at distinct geographic scales: within each of the 11 forest fragments, within each cluster as identified by the STRUCTURE, TESS and DAPC clustering methods (see below), and for the whole LM region.

We estimated genetic diversity as the mean number of alleles (N_A), observed heterozygosity (H_o) and unbiased expected heterozygosity (H_e) according to Nei (1978), and we quantified departure from Hardy–Weinberg equilibrium (HWE) (Wright's F_{IS}), using the GENETIX software (Belkhir et al. 1996–2004). We have also calculated the allelic richness (A_r) using the program HP-RARE 1.1 (Kalinowski 2005). Heterozygosities within forest (both H_o and H_e) were tested for correlation with sample size (N), forest area (km²) and census population size using Pearson correlation test. These analyses were performed for all forest

patches, including the ones with small N , because the equation derived by Nei (1978) corrects for small sample sizes.

Analysis of population structure

In order to analyse genetic patterns of population structure we used three distinct approaches: F -statistics, AMOVA and three different clustering methods. F -statistics were estimated using the GENETIX 4.05.2 software and its significance was tested using 10,000 permutations. Deviations from random mating were estimated using F_{IS} and the degree of genetic differentiation within the LM region was estimated by the pairwise F_{ST} , calculated according to Weir and Cockerham (1984). When measuring F -statistics at the level of the forest patch, only forest fragments where $N \geq 7$ were considered. In order to test hypotheses about putative factors shaping population differentiation across the LM region, we used a hierarchical analysis of molecular variance (AMOVA) using 10,000 permutations in the Arlequin software v3.5.1.3 (Excoffier and Lischer 2010). According to the landscape features that occur in the LM region and that may eventually act as a barrier to *M. tavaratra* (see Fig. 1), we carried out two independent AMOVA analyses, which allowed us to study the effect of (i) the Manankolana River; (ii) the national Road, (iii) the forest fragmentation and (iv) vegetation type (humid vs. dry forests). Therefore, we considered two levels of hierarchical population subdivision: we distinguished the east and west coasts of the Manankolana River and, within each coast, we differentiated each forest patch. Similarly, we distinguished two regions located at each side of the National Road that crosses the LM region. Finally, vegetation type was tested by distinguishing BIN and ANTSB forests from the other dry forests within the west river bank.

Clustering methods were used in order to identify genetic groups within the LM region: we used two widely-used Bayesian model-based approaches implemented in the programs STRUCTURE 2.3.4 (Pritchard et al. 2000) and TESS, and also a non-model based method, the Discriminant Analysis of Principal Components (DAPC) implemented in the R package ADEGENET 2.0.1 (Jombart and Ahmed 2011). A brief description of each of these methods is given below.

This program implements a Bayesian model-based approach that does not assume pre-defined groups, assigning individuals to K -clusters with minimum deviations from Hardy–Weinberg proportions and linkage disequilibrium. We used all 149 individuals and 13 loci (excluding the monomorphic loci, see Table S1), and we ran the program for a range of K values from 1 up to 14, assuming an admixture model and correlated allele frequencies. For each K value we performed 20 independent runs of 1,000,000 iterations and a burn-in period of 100,000 steps. We visually checked the convergence of the obtained likelihood of

K for each run. To infer the optimal K , we used the ΔK summary statistic (Evanno et al. 2005) as implemented in Structure harvester software (Earl and vonHoldt 2012). We derived the final assignment of individuals to the K -clusters by averaging the individuals membership coefficients from the 20 independent runs using CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007).

TESS

We used a spatially explicit Bayesian-model approach as implemented in TESS 2.3.1 (Chen et al. 2007). This method detects significant geographic discontinuities in allelic frequencies by implementing a statistical model that accounts for space. The analysis is based on the premise that individuals are more likely to belong to the same cluster when they live geographically close than when they live apart from each other. As in STRUCTURE, TESS also assigns individuals to a pre-defined cluster by estimating the proportion of the individual's genome that originates from each cluster. We varied K between 2 and 13, and for each value of K we performed 20 independent runs of 1,000,000 iterations preceded by a 100,000 burn-in period. We used a spatial iteration value of 0.6, a degree of trend [linear (1)] and the conditional autoregressive (CAR) admixture linear model (Durand et al. 2009). The optimisation of the number of K 's relied on the deviance information criterion (DIC). To infer the optimal K , we averaged the 10 lowest DIC values out of the 20 replicates for each K . The membership coefficients obtained for the optimal K s are represented by pie-charts. Each pie-chart shows the assignment of either one individual or the averaged individuals belonging to one location, to the different clusters. The different colours indicate the different clusters inferred by TESS.

Discriminant analysis of principal components

To assess the genetic variation among forests, we conducted a discriminant analysis of principal components (DAPC). This method consists on a multivariate analysis of principal components that summarises the original number of variables (alleles) into a linear combination of few alleles (the discriminant functions) that maximise the variation between groups and minimise it within groups. Because only some variables maximise variation between groups while minimising it within groups, the DAPC uses a procedure to search for the optimal number of PC's to retain. The DAPC requires prior information on the number of groups, thus, we first ran the K -means clustering method in order to identify the best number of clusters. For this analysis we used the dataset of 13 loci. We also performed an additional DAPC analysis by using $K = 8$ as the pre-defined number of clusters, which

corresponded to the total number of sampled forests in the LM region (excluding forests with $N < 7$).

Isolation by distance and autocorrelation analysis

Limited dispersal leads to a correlation between genetic and geographic distance, typically known as isolation-by-distance (IBD) (Wright 1943; Malecot 1948). As a consequence, individuals that live in close proximity are expected to be more genetically similar than individuals living further apart. To understand if genetic patterns at the LM region could be the product of IBD, we used two approaches.

First, we searched for a statistical correlation between geographic distance and genetic differentiation (among forests or among individuals). Using the R software (R Core team 2017) we fitted a linear regression between the pairwise geographic distance and the genetic distance, measured, respectively, as the *log* euclidian distance and F_{ST} among pairs of forests or Rousset's genetic distance among individuals (Rousset 1997). The significance of the linear regression was calculated using Mantel test (Mantel 1967). The genetic dissimilarity between pairs of individuals was measured by estimating Rousset's genetic distance in the SPAGeDi 1.4 program (Hardy and Vekemans 2002); this estimator is a measure of inter-individual genetic distance analogous to $F_{ST}/(1 - F_{ST})$ and was measured between all pairs of individuals located in the LM region.

Second, in order to investigate patterns of genetic structure over finer geographic scales, we used spatial autocorrelation analysis. Spatial autocorrelation analysis uses the individual as the unit of analysis and thus can be used to investigate biological processes at smaller spatial-scale, such as sex-biased dispersal and dispersal range (Epperson 2005; Aguillon et al. 2017). This analysis investigates genetic similarity (r) between groups of individuals located at different geographical distance classes. The r coefficient varies between -1 and 1 , with positive values representing high levels of relatedness over an area. Spatial autocorrelation was measured in GenALEX 6.5 (Peakall and Smouse 2006, 2012) and classes were defined in order to have the best possible even distribution of sample sizes among classes. By pooling all individuals across the LM region, this resulted in 23 distance classes, differing in length from 1 to 4 km (for a total of 34 km) where the number of dyads (pairwise comparisons among individuals) spanned from 263 to 880. We also calculated r values among individuals located within the same forest fragment using the "Multi pops option", which combines the correlogram obtained from each forest in order to extract common conclusions on the species dispersal process. For each forest fragment, r was estimated for 19 distance classes of 100 m each, with sample sizes varying between 19 and 90 inds. We calculated the 95% confidence

intervals for the observed mean r value by doing 10,000 random permutations.

Results

Geographical patterns of genetic diversity

Among the 15 microsatellite loci used in this study, genetic diversity measured as H_e ranged from 0.32 (Mm26b) to 0.91 (Mm42); two loci (Mm26band Mm51) showed little genetic diversity compared to the other loci and two loci were monomorphic (Mm60 and MmF6; Table S1). Across the LM region the mean number of alleles per locus (and allelic richness) ranged from 4.07 (3.92; ANTSR) to 8.07 (4.8; BEK; Table 1a). Genetic diversity measured by H_e ranged from 0.577 (BIN) to 0.656 (BEK), while H_o ranged from 0.565 (BIN) to 0.650 (BEK; see Table 1a). The highest value for all genetic diversity indexes was observed in the largest forest fragment—BEK ($N=42$), while the lowest value in terms of mean number of alleles (A_r and N_A) was observed in the smallest fragment (ANTSR, $N=7$). BIN, a relatively large forest, showed the lowest diversity values in terms of heterozygosity, despite showing one of the largest number of alleles.

Most of the forest fragments showed non-significant positive F_{IS} values, ranging from -0.037 to 0.043 , suggesting that no significant departures from HWE could be detected at the fragment scale (Table 1a). Contrastingly, within the three groups identified by the clustering methods and at the LM scale (see below and Table 1b), H_e values were generally higher than H_o , resulting in some cases in significant

departures from HWE, which is most likely explained by the Wahlund-effect (Wahlund 1928).

Population structure

Pairwise F_{ST} , IBD and spatial autocorrelation

Levels of genetic differentiation, measured by pairwise F_{ST} , were low but significant among all forest fragments (Table 2). The lowest values (0.028 and 0.032) were found between the neighbouring forests BIN—ANTSB and ANTSB—AMBO in the south-western part of the LM region (Fig. 1), which were connected in the recent past. The highest F_{ST} value (0.093) was found between the ANTSR and AMBO fragments, situated 33 km apart in the northern and south-eastern part of the region, respectively.

The observed pattern of genetic differentiation among forest fragments showed a significant positive signal of IBD, revealing that 55% of genetic differentiation between forest fragments is explained by geographic distance (Mantel test p -value = 0.004, Fig. 2a). However, the level of differentiation is likely affected by environmental factors, such as rivers and matrix separating patches; as some forests in close proximity show relatively high F_{ST} values. For instance, BIN and BOB forests, which are located 15 km apart from each other, show one of the highest pairwise F_{ST} values ($F_{ST}=0.089$), a value comparable with forests located ~ 30 km apart (ANTSR and AMBO; $F_{ST}=0.093$). Although differentiation among groups identified by the clustering methods was not higher than differentiation among forests (Table 2), on average, forests located in the same cluster had lower differentiation values than forests located in different clusters (ranges 0.028–0.070 and 0.045–0.093, respectively). Differentiation

Table 2 Genetic differentiation between forests and clusters

| | SW | | | N | | | E | |
|-------|--------------|--------------|-------|-------|-------|-------|-------|-------|
| | AMBO | ANTSB | BIN | SOL | BEN | ANTSR | BEK | BOB |
| SW | | | | | | | | |
| AMBO | | | | | | 0.034 | 0.053 | |
| ANTSB | 0.032 | | | | | | | |
| BIN | 0.069 | 0.028 | | | | | | |
| N | | | | | | | | |
| SOL | 0.061 | 0.053 | 0.056 | | | | | 0.036 |
| BEN | 0.086 | 0.064 | 0.073 | 0.044 | | | | |
| ANTSR | 0.093 | 0.051 | 0.075 | 0.070 | 0.063 | | | |
| E | | | | | | | | |
| BEK | 0.061 | 0.067 | 0.070 | 0.045 | 0.068 | 0.068 | | |
| BOB | 0.086 | 0.086 | 0.089 | 0.067 | 0.078 | 0.076 | 0.038 | |

Pairwise F_{ST} estimated between forests with $N \geq 7$ (below diagonal, see Fig. 1 for a correspondence between names and geographic localities), and between clusters as identified in the programs STRUCTURE and TESS (above diagonal). Lowest and highest F_{ST} values are highlighted in bold. All pairwise F_{ST} values among forest fragments and among clusters are significant, $p < 0.05$

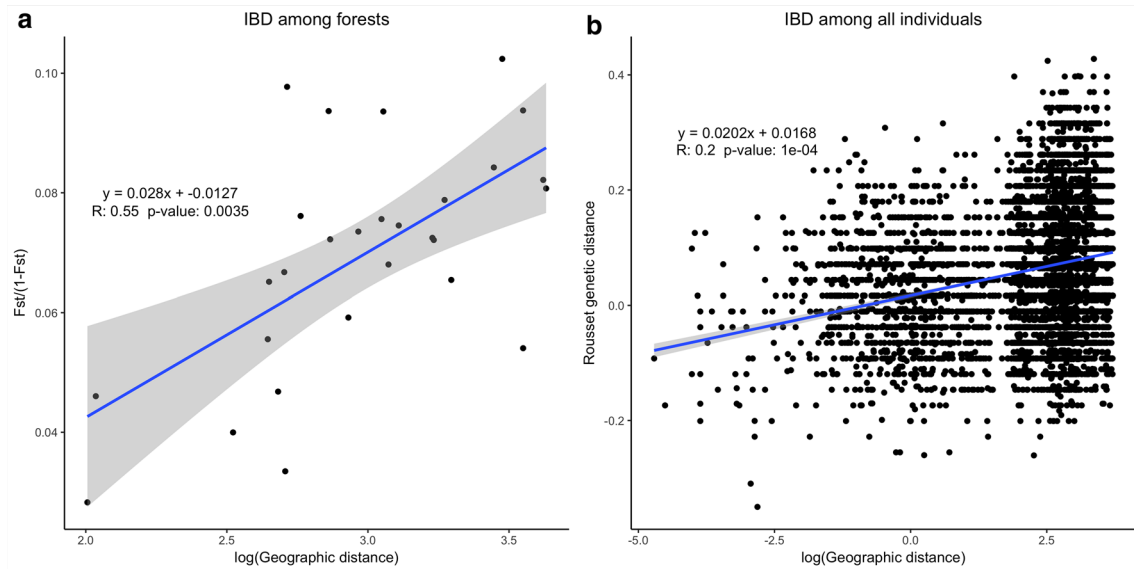


Fig. 2 Pattern of IBD in *M. tavaratra* from the LM region pattern of IBD represented as the relationship between logarithm of Euclidean geographic (in UTM) and genetic distances. **a** Pairwise F_{ST} between all pairs of forests. **b** Rousset's genetic distance between all pairs of individuals

among clusters was higher between the eastern and south-western clusters (0.053), which are separated by the Manankolana River. Also, among forest fragments, the largest pairwise F_{ST} values were observed between eastern and south-western forests (pairwise F_{ST} range 0.061–0.089, Table 2).

Results of the AMOVA underlined the relatively weak genetic differentiation between the “regions” delimited by the two most apparent barriers: the Manankolana River and the national road (Table 3). Most of the variation occurred within forests (~93%), with little variation between the

west and east Manankolana river banks (1.61%) and none among the forests located north and south of the national road (−0.54%). However, the among-forests genetic variation was higher (6.5%), confirming the significant F_{ST} among forest patches and suggesting that the open habitat between forests influences the genetic structure of this species. A significant level of genetic differentiation (3.01%) was found between the dry forests at the north, and the humid transition forest complex at the south-west limit of the species' geographic distribution (ANTSB–BIN),

Table 3 Effect of major landscape features in genetic variance of *M. tavaratra* (AMOVA)

| Source of variation | df | Sum of squares | Variance components | % var. | Fixation index (P-value) |
|---|-----|----------------|---------------------|--------|--------------------------|
| Among river banks | 1 | 30.312 | 0.7931 | 1.61 | $F_{CT}=0.016$ (0.0242) |
| Among forests (within river banks) | 9 | 90.042 | 0.2555 | 5.20 | $F_{SC}=0.053$ (0.0000) |
| Within forests | 287 | 1313.452 | 4.5765 | 93.18 | $F_{ST}=0.068$ (0.0000) |
| Among road sides | 1 | 18.104 | −0.0264 | −0.54 | $F_{CT}=−0.005$ (0.6842) |
| Among forests (within road sides) | 9 | 102.249 | 0.3159 | 6.49 | $F_{SC}=0.065$ (0.0000) |
| Within forests | 287 | 1313.452 | 4.5764 | 94.05 | $F_{ST}=0.0595$ (0.0000) |
| Among river banks | 1 | 30.312 | 0.0675 | 1.37 | $F_{CT}=0.014$ (0.3347) |
| Among forest types (within west river bank) | 1 | 17.297 | 0.1479 | 3.01 | $F_{SC}=0.030$ (0.0000) |
| Within types of forest | 295 | 1386.196 | 4.6990 | 95.62 | $F_{ST}=0.044$ (0.0000) |

The table shows the results of the analysis of molecular variance (AMOVA) across three landscape features in the LM region: (i) The Manankolana River banks—forest groups: [AMBI, AMBO, AMPO, ANTSB, ANTSR, BEN, BIN, SOL] [ANKA, BEK, BOB]; (ii) the two sides of the National Road—forest groups: [AMBI, AMBO, ANKA, ANTSB, BIN, BOB, SOL] [AMPO, ANTSR, BEN, BEK]; and (iii) the type of forest (dry vs. transition/humid) within the West River bank—forest groups: [West River bank (dry forests: AMBI, AMBO, AMPO, ANKA, ANTSR, BEN, SOL) (humid forests: ANTSB, BIN)] [East River bank: (dry forests: BEK, BOB)]. df: degrees of freedom; % variation: the percentage of variation attributed to each hierarchical level for each landscape feature. All values were estimated on 10,000 permutations in Arlequin

suggesting that the type of vegetation may have some effect on the genetic structure of this species (Fig. 1; Table 3).

Spatial autocorrelation values calculated on the whole dataset were positive and significant for the first six distance classes (up to 11 km; Fig. S1a). The highest values were exhibited in the first three distance classes (up to 5 km, $r \sim 0.10$), with r decreasing in the following classes until stabilisation around slightly negative significant values by the seventh distance class (significant genetic dissimilarity for neighbours > 13 km). Because most forests are more than 11 km apart, the negative correlation observed above 13 km suggests that individuals located within the same forest are, on average, more related than individuals located in different forests. This is in agreement with the AMOVA and F_{ST} results supporting the effect of habitat fragmentation on the pattern of genetic differentiation in *M. tavaratra*. At a finer geographic scale (within forests), spatial autocorrelation values were positive and significant only at the first distance class (0–100 m), with values decreasing by the second class (200 m) and varying around zero until 2000 m (Fig. S1b).

Clustering methods and DAPC

The three methods used to infer the best number of genetic clusters (STRUCTURE, TESS and DAPC) generally provided coherent results.

In STRUCTURE, ΔK showed its largest values at $K=3$ and $K=4$, with the highest peak at $K=3$, therefore suggesting the presence of three genetically differentiated groups (Fig. S2a). In TESS we found that a large decrease in DIC occurs from $K=2$ to $K=3$ and from $K=3$ to $K=4$. Although DIC continuously decreases for K values larger than 4, this decrease is less pronounced (Fig. S2b). Thus, TESS seems to indicate $K=3/4$ as the best numbers of clusters. In the DAPC analysis $K=2$ and 3 were considered optimal to describe the data (Fig. S2c), as they showed the lowest BIC values. We decided to consider three clusters as this seemed the simplest accurate solution across all clustering methods. The three clusters roughly correspond to geographic limits of the LM region at the (i) eastern cluster (E), which groups individuals from the BEK and BOB forests; the (ii) southwestern cluster (SW) that assigns individuals from forests located at the southwestern limit of the LM region (AMBO, ANTSB, BIN and the individual sampled at ANKA); and the (iii) northern cluster (N), which assigns individuals from the northern ANTSR, AMPO and BEN forests, and individuals from the two more central forests, AMBI and SOL (Fig. 3a, Fig. S3). The fourth cluster found by STRUCTURE and TESS detects some substructure within the eastern cluster, differentiating individuals from the BEK and BOB forest fragments. Interestingly, the assignment results obtained for $K=3$ and $K=4$ were very similar in both methods (Fig. S3). Finally, although in both methods cluster membership assignment of individuals was generally high, substantial admixture was

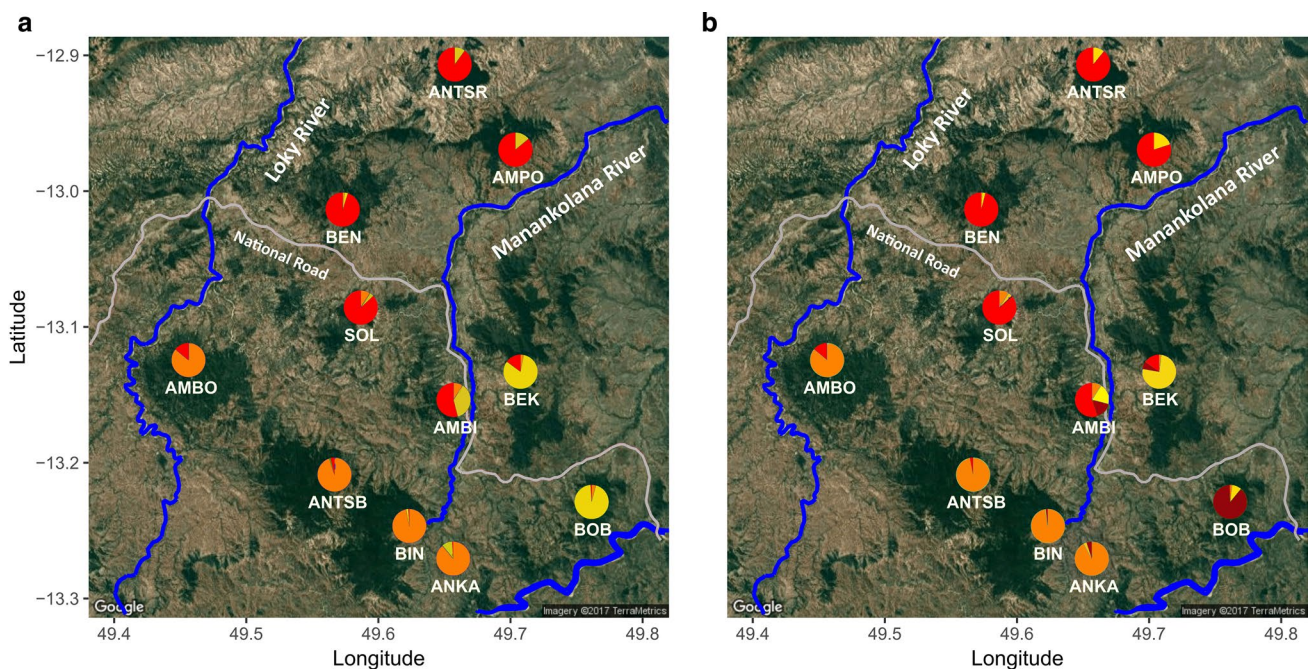


Fig. 3 Bayesian clustering of *M. tavaratra* individuals across the LM region geographical representation of TESS cluster membership coefficient posteriors (pie charts) for values of $K=3$ (a) and $K=4$ (b).

Each colour indicates a cluster. Pie chart size is not representative of the sample size. Rivers are in blue colour and the national dirt road is in grey. (Color figure online)

observed. This agrees with the low F_{ST} values found between clusters. The DAPC analysis requires groups (genetic clusters) to be achieved. Although three clusters were considered optimal to describe the data, we also performed the DAPC by considering eight genetic clusters which correspond to the forests sampled. The distribution of the eight groups along the axis revealed a pattern of differentiation consistent with the real geographical location of the forests in the LM region (Fig. 4 and see Fig. 1), thus supporting the role of geographic distance on the genetic structure detected in *M. tavaratra* populations. Interestingly, BIN forest showed some deviation from its relative real geographic location, by being bottom-shifted relative to the other forests, hence suggesting a greater differentiation or uniqueness.

Discussion

Genetic diversity and differentiation of the northern rufous mouse lemur

Overall, Northern rufous mouse lemurs of the LM region show reasonable levels of genetic diversity, with both observed and expected heterozygosity values above 0.56 in all forests (Table 1). The levels of diversity found in the present study are within the range of those found for other mouse lemur species, for which levels of heterozygosity

were as high as 0.79 and 0.83 for H_e (*M. griseorufus*) and H_o (*M. murinus*), respectively (Table S2). We note, however, that such comparison should be taken with caution since, sampling size, sampling design and marker identity differ across studies. Furthermore, all loci used in this study have been developed in *M. murinus* and not in *M. tavaratra*, and they may thus underestimate the genetic diversity of this species (e.g. Bailey and McLain 2016). For instance, most loci revealed H_e values between 0.78 and 0.94, while two loci showed much more limited diversity, on the order of 0.6 and two other loci diversities below 0.35.

The *M. tavaratra* population inhabiting northern Madagascar has been previously studied, but most studies consist of phylogenetic and morphological analyses. One single study has focused on the genetic structure based on the mitochondrial DNA and has found high levels of diversity in the mitochondrial DNA in most forest fragments, except for the BIN population which showed an extremely low diversity (with absence of diversity at the *D-loop* and *cox2* and levels around 0.15 at *cytb*, Sgarlata et al. 2018).

Despite large discrepancies in forest cover area (6.39–62.48 km²), vegetation, and census population size estimates (~400–4600 ind; Meyler et al. 2012; Salmons et al. 2014), the genetic diversity (A_r , H_o and H_e) was reasonably high and showed little variance across forests of the LM region (var=0.0007, Table 1), with no correlation between any of these factors (Fig. S4). A lack of correlation among these factors was also obtained in the previous work on mitochondrial DNA diversity (Sgarlata et al. 2018).

In a fragmented area such as the LM region, the high levels of genetic diversity observed for *M. tavaratra* can be explained by two possible scenarios: (i) “ancient” and/or (ii) relatively recent habitat loss and fragmentation. In comparison to other lemur species, population sizes are relatively high and may be large enough to maintain relatively high levels of genetic diversity (Table 1 and Table S2). To test so, we compared the observed H_e with expectations from classical population genetic models (one single random-mating population). We used the Ohta and Kimura (1973) equation that estimates H_e at equilibrium as a function of N_e based on microsatellite data. Therefore, we computed the expected H_e within each forest assuming a N_e equal to the estimated census population size. The analytical expectation from classical population genetic models is that H_e is expected to be very similar between populations (variance ~0.0003), which is in agreement with what we observed (variance=0.0007). However, according to theoretical expectations, we should also observe some correlation between genetic diversity and population size. A possible interpretation is that deforestation occurred relatively recently and therefore not enough time passed in order to observe a correlation between population size and genetic diversity.

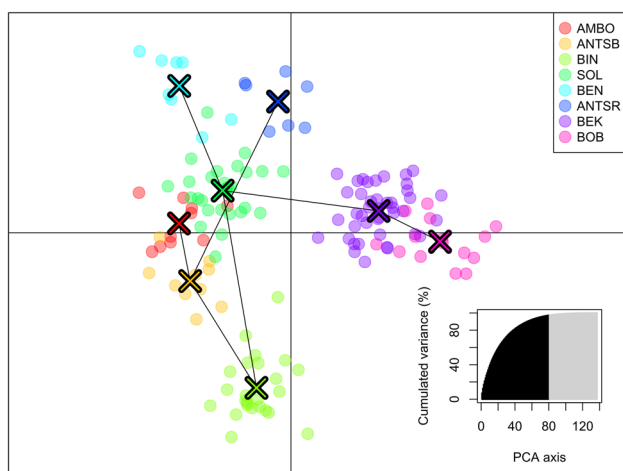


Fig. 4 DAPC analysis on *M. tavaratra* individuals sampled across the LM region Scatterplot of the discriminant analysis of principal component results (DAPC) for $K=8$ (the number of forest patches with $N < 7$). We show results obtained with 80 PCs retained, which explain ~95% of the total variance among groups. The coloured dots represent the position of each sample on the two first discriminant axes. The lines connecting each cross represent the minimum spanning tree based on the squared distances between populations, showing the actual genetic proximities among forests. DAPC shows a close correspondence between genetic structure and the geographical location of the sampled forests

The high levels of genetic diversity together with low but significant levels of differentiation between populations (pairwise $F_{ST} < 0.093$, Table 2), is best explained as a result of relatively recent habitat fragmentation in the LM region. The timing and nature of habitat fragmentation of many regions of Madagascar are still controversial and have been highly debated (Green and Sussman 1990; Burney 1999; Harper et al. 2007). Recent deforestation in this region has been much more limited than in other regions of Madagascar. Quéméré et al. (2012) have suggested that in the last 60 years forest cover in the LM region has changed by 2%. Deforestation has occurred only locally, in the north-western and south-western parts (AMBO and ANTSB forest fragments) of the LM region. This agrees with the clustering methods results, that evidenced the existence of three clusters; these differentiate individuals sampled at east (BEK and BOB—cluster E), from individuals sampled at south-western (AMBO, ANTSB, BIN and ANKA—cluster SW) and north-western forests (ANTSR, AMPO, BEN, SOL, AMBI—cluster N) of the LM region. Such clusters might represent the history of changes in connectivity among the forest fragments of the LM region, including in the same group of forests that have been disconnected more recently. Among the three clusters identified, the SW-cluster (AMBO, ANTSB and BIN) grouped the individuals with the highest membership probabilities (Fig. S3).

Although we could speculate that sub-structuring would likely be detected due to differences in vegetation between the two mountain forests (BIN and ANTSB) and the dry forest (AMBO) (Gautier et al. 2006), as showed by the mitochondrial DNA results (Sgarlata et al. 2018), an additional STRUCTURE analysis showed a lack of sub-structure within the SW-cluster (the most probable number of clusters was $K = 1$; results not shown). The SW cluster was also one of the two genetic clusters detected for the large body sized lemur golden-crowned sifaka by Quéméré et al. (2010a). Forests at the south-western part of the LM region used to form a large forest complex (BAA: BIN, ANTSB and AMBO, Quéméré et al. 2012) and the apparent genetic homogeneity of these forests probably denotes the forest connectivity recorded for the BAA complex since the 1950s (Blasco 1965; Quéméré et al. 2012) with forest loss mainly occurring recently between AMBO and ANTSB. Currently forest corridors are mostly present in the south-western part of the LM region, particularly among ANTSB and BIN patches (Quéméré et al. 2012). Such connectivity may have facilitated dispersal and it may explain why, in addition, we observed the smallest levels of differentiation among these three patches ($F_{ST} = 0.028$ and 0.032 , Table 2).

Open habitat and ecological barriers to gene flow

Most forest-dwelling vertebrates are affected by forest fragmentation. In the LM region this is the case for instance, of the *E. carletoni*, an endemic Malagasy rodent that lives in sympatry with the northern rufous mouse lemur (Rakotoarisoa et al. 2013), and, to some extent, the *P. tattersalli*, a large-body sized lemur species with long dispersal distances (Quéméré et al. 2010b). Although the connectivity among forests through small corridors and riparian forests may have an influence in some patches, our results suggest that *M. tavaratra* genetic structure is influenced by the matrix separating forest patches. This is evidenced by the bi-dimensional discrimination of forest diversity (DAPC), the relatively high percentage of variance among forest patches (AMOVA), and the spatial autocorrelation pattern. The effect of the open habitat may be better observed outside the LM region, where forest fragmentation and, consequently isolation, is more prominent.

The AMOVA results show that most of the genetic variance occurs within forest patches (> 93%), and between 5 and 6.5% occurs among the patches (Table 2): the matrix separating forest patches has a much larger effect than other possible barriers to gene flow—the Manankolana river, the type of forest cover vegetation and the road—whose effects explain a small or no significant percentage of the genetic variance in this species (1.61%, 3% and -0.54% respectively). Our results suggest that ANTSB and BIN humid forests are genetically divergent from the other dry forests (variance $\sim 3\%$, see Table 3), however this population structure may instead be driven by a higher connectivity and much more recent fragmentation, other than vegetation type (humid vs. dry habitat). The AMOVA is known to poorly detect between-group structure when there are few populations per group (< 6, Fitzpatrick 2009) and thus, our results may underestimate the impact of these barriers on the genetic structure of *M. tavaratra* in the LM region. A previous study on mitochondrial genetic diversity of *M. tavaratra* have found a partial effect of the Manankolana river to the genetic structure of this species (Sgarlata et al. 2018). Rivers in Madagascar have a long documented role as drivers of genetic diversification, and the influence of the Manankolana River as a natural barrier to gene flow was clearly demonstrated for the larger bodied golden-crowned sifaka (Quéméré et al. 2010b) and partially for the smaller rodent *E. carletoni* (Rakotoarisoa et al. 2013). Nevertheless, the effects of the Manankolana River are not trivial to determine, in part because of its characteristics: the water flow fluctuates throughout the year as well as the width, and in some sections the river flows underground during the driest periods; its margins harbour significant human settlements (villages) and activities (agriculture) that can potentially limit diurnal and susceptible species dispersal,

but not necessarily species with nocturnal habits, such as mouse lemurs; also there is a dense network of riparian forests present along permanent and seasonal watercourses that may be used as means of dispersal, especially by smaller organisms. We may thus suggest that although the Manankolana River plays a role in structuring *M. tavaratra* populations, it appears not to be the major barrier to dispersal for the LM population. However, it would be important to perform a wider study covering most of the species distribution range to understand the effect of the large Loky River on the species genetic variation. This and other natural and/or anthropogenic features may pose a barrier to gene flow between populations in the LM region and in other areas.

Isolation by distance

IBD was detected across forests, a pattern expected because dispersal in *M. tavaratra* is most likely smaller than the scale at which the study was conducted (Slatkin 1993), as suggested by the spatial autocorrelation analysis (Fig. 2 and Fig. S1). We expect that IBD would not have been detected if forests had been separated, and thus populations drifted independently, a long time ago. Also, we detected spatial autocorrelation among individuals at the first six distance classes (< 11 km) which mostly includes individuals located within the same forest patch (Fig. S1a). Indeed, only two forests are located < 12 km apart (at ~7 km), suggesting that most dispersal events probably occur within forests. Therefore, the negative and significant values of spatial autocorrelation observed at geographical distances larger than 13 km may support the impact of forest fragmentation on the observed pattern of genetic differentiation. Beyond that, analysis at smaller scales (i.e. within fragments) showed that individuals are highly correlated within the first distance classes (0–200 m, Fig. S1). Although little is known about the social behaviour of this species, previous studies on the closely related species *M. murinus* showed that most individuals interact with their neighbours, and dispersal events occurs on a range between 0 and 1000 m, with a median of 250 m mainly due to male dispersal (Radespiel et al. 2003; Schliehe-Diecks et al. 2012). This suggests that most *M. tavaratra* dispersal events are likely limited at short geographical distance, probably occurring within the forest patch to the close neighbourhood of an individual's socio-ecological range.

Also, the DAPC analysis that we performed by using as grouping prior “forest location”, suggests that geographic distance plays a major role on the genetic structure of *M. tavaratra*, as the forest patches distribution on the two discriminant axis of the DAPC is consistent with their relative geographical locations in LM region (Figs. 1 and 4). However, it shows some genetic uniqueness for the BIN forest, whose individuals are bottom-shifted in the DAPC plot. It

is presently difficult to draw a clear conclusion, although we note that genetic diversity (nuclear and mitochondrial) also suggested some unusual genetic pattern in the BIN forest compared to the other forests of the LM region.

Conservation implications

Estimates of population size of *M. tavaratra* point to about 44,000 to 75,000 individuals inhabiting the LM region (Salmona et al. 2014). This number is likely to be three to four times larger across the species' distribution range (Fig. 1; Mittermeier et al. 2010). The species is therefore not facing imminent extinction risk and is indeed classified as “vulnerable” (Andriaholinirina et al. 2014). Moreover, our findings suggest that *M. tavaratra*'s population harbours substantial levels of genetic diversity across the LM region. Particularly, the SW forests seem to have been inhabited by a single large population. The fact that genetic differentiation is affected by geographical distance, open habitat and to some extent by the Manankolana River, demonstrates the importance of forest connectivity across the LM region for the genetic diversity of this species. We suggest that special efforts targeting riparian forests maintenance and reforestation might be a good strategy to reduce the effect of fragmentation on the genetic diversity of extant populations. Large efforts to maintain forest cover are already being conducted in the protected areas of the region, but conservation actions would benefit from more financial support, law enforcement, and national political stability. In addition, a larger sampling encompassing the complete distribution range of *M. tavaratra* and a detailed description of the species behaviour, coupled with landscape genetic modelling approaches (Jaquière et al. 2011; Lowe and Allendorf 2010; Wang et al. 2009) and demographic analyses could contribute greatly to understand the relative effect of current connectivity and past fragmentation on the genetic patterns of this mouse lemur.

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Data availability The complete microsatellite data set of all sampled individuals are available in electronic supplementary material (Online Resource 1).

Compliance with ethical standards

Conflict of interest The authors also declare that they have no conflict of interest.

Ethical approval We conducted this study in agreement with the laws of the International Primatological Society Code of Best Practices for Field Primatology, and with the countries of Madagascar, Portugal and France. The research authorisations [218/10][220/10][113/011][118/011]/MEF/SG/DCB.SAP/SCB were obtained in Madagascar and, the export and import CITES permits 485C-EA09/MG10 and 593C-EA10/MG11 were requested from both Malagasy and Portuguese authorities.

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