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Mitochondrial Genetic Diversity in the Crowned Sifaka (*Propithecus coronatus*) in a Fragmented Landscape

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Abstract: *Propithecus coronatus* is an endangered, diurnal forest-dwelling lemur of northwestern Madagascar. We conducted the first extensive population genetic study for this species. We designed new primers to amplify and sequence the mitochondrial D-loop of 125 individuals from 14 localities in the northern part of the species' distribution. Our aim was to assess the genetic variability and differentiation of this species in a fragmented landscape. Compared to other lemurs, crowned sifakas have a moderate level of haplotype diversity (0.853) and a low nucleotide diversity (1.21%). Despite the considerable forest fragmentation in the region surveyed, the species does not show strong signals of genetic structure as shown by the Φ_{ST} estimates, the network of haplotypes, and the limited correlation between genetic and geographic distance. The current mtDNA estimated effective population size was relatively large (median: 11,262; 95% HPD: 5,107−20,083), in agreement with recent census estimates, suggesting that a large number of individuals is still present across the species range. Using the Extended Bayesian Skyline Plot (EBSP) approach to reconstruct the demographic history of the species, we did not detect any genetic signal of change in population size. Despite the ongoing loss and fragmentation of their habitat, the population still harbors subtantial genetic diversity, likely as a partial consequence of a taboo against hunting the crowned sifaka among the Sakalava ethnic group inhabiting the area.

Key Words: *Propithecus coronatus*, crowned sifaka, Madagascar, mitochondrial DNA, genetic diversity, demographic history, endangered species, conservation

Introduction

Madagascar has an exceptional concentration of endemic species (about 80%). Many of them are restricted to small forest patches; a result of climate change during the Pleistocene and of the fragmentation, degradation, and destruction of Madagascar's forests by humans, particularly since the 1970s. The relative contribution of each is still under debate (Virah-Sawmy *et al.* 2009; Matsumoto and Burney 1994; McConnell and Kull 2014; Scales 2014). Paleoecological data suggest that, at least for some regions, forest fragmentation has been caused by natural events (Matsumoto and Burney 1994; Virah-Sawmy *et al.* 2009; Vorontsova *et al.* 2016) that predate the arrival of humans on the island, now thought to have started around 4000 years ago (Gommery *et al.* 2011; Dewar *et al.* 2013). It is clear, however, that anthropogenic activities—slash-and-burn agriculture, charcoal production, fires for zebu cattle pasture, logging and, to a lesser extent, mining—are regarded as the major recent causes for forest loss and fragmentation. Accordingly, it has been shown that forest cover in Madagscar decreased by 50% over 60 years from 1950 to 2010 (Harper *et al.* 2007; ONE 2013; Schwitzer *et al.* 2014a, 2014b).

The lemurs are prominent among Malagasy endemics, with 110 species and subspecies currently recognized

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(Mittermeier *et al.* 2010; Schwitzer *et al.* 2013)[1], and, as a group, they are among the most threatened vertebrates in the world (Schwitzer *et al*. 2014a, 2014b). Four of the nine species of sifaka (*Propithecus*: Indriiidae) are Critically Endangered (CR)—*P. candidus*, *P. perrieri*, *P. tattersalli*, and *P. diadema*—and the remaining five are Endangered (EN)—*P. coquereli*, *P. coronatus*, *P. deckenii*, *P. edwardsi*, and *P. verreauxi* (IUCN 2014).

Our study focused on the crowned sifaka (*Propithecus coronatus*), a medium-sized (head and body length 39−45 cm), diurnal, arboreal, and folivorous species living in riparian, semi-deciduous, dry forests or in mangroves (Petter and Andriatsarafara 1987; Mittermeier *et al.* 2010). Crowned sifakas live in small social groups of 2 to 8 individuals (Ramanamisata *et al.* 2014; Razanaparany *et al.* 2014). Typically, the dominant female of a group reproduces every two years, and it is usually the males that disperse (C. Pichon and F. Bayart unpubl. data). The European Endangered Species Program (EEP) data on *P. coronatus* show that females can live to at least 20 years, and males to at least 25 years in captivity (Roullet 2014). Based on data from *P. verreauxi*, the generation time could be as high as 19.5 years (Lawler *et al.* 2009; Morris *et al.* 2011). This is a parameter for which there is little available data, however, and authors have used values of 3 to 17.5 years (Quéméré *et al.* 2012).

The geographical distribution of the species extends from the rivers Betsiboka and Mahavavy in the northwest of Madagascar (the area of the present study) to the rivers Tsiribihina and Manambolo in the southwest (Tattersall 1986; Thalmann and Rakotoarison 1994; Rasoloharijaona *et al.* 2005; Wilmé *et al*. 2006; Razafindramanana and Rasamimanana 2010; Rakotonirina *et al.* 2014; Salmona *et al.* 2014). The Betsiboka/Mahavavy river region was once believed to encompass the entire range of *P. coronatus*; their presence to the southeast was documented only recently (Tattersall 1986; Thalmann and Rakotoarison 1994; Thalmann *et al.* 2002; Rasoloharijaona *et al.* 2005; Wilmé *et al.* 2006; Razafindramanana and Rasamimanana 2010; Rakotonirina *et al.* 2014). Its range is characterized by mosaics of forest fragments surrounded by grassland and farmland (Fig. 1). The fragmentation of their habitat is believed to have led to a population decline of about 50% in the last decades, human activities being the most important cause (Mittermeier *et al.* 2010; IUCN 2014).

Surveys by Salmona *et al.* (2014) have indicated between 4,226 and 36,672 individuals in the northern part of the species' range, delimited by the Betsiboka and Mahavavy rivers. By extrapolation they estimated between 130,000 and 220,000 individuals for the species, although this is probably an overestimate because the forests between the rivers Tsiribihina and Manambolo are more fragmented than in the north of their range.

The first protected area for the species, the Nouvelle Aire Protégée of Antrema (NAP-Antrema − category VI; previously SFUM, Antrema), was established in 2001 (Gauthier *et al.* 1999) and is jointly managed by the National Museum of Natural History (MNHN) of Paris (France) and the University of Antananarivo (Madagascar). It is located in the northern coastal part of the species' range. The landscape of the NAP-Antrema includes dry forest, mangroves and grasslands; the first two being typical habitats for *P. coronatus* (Gauthier *et al.* 1999; Salmona *et al.* 2014). This particular area has attracted the interest of conservation biologists because of the remarkably high density of crowned sifakas: about 300 ind/km² in a single forest fragment (Badrala forest; Pichon *et al.* 2010) (Fig. 1).

Two other protected areas exist in the northern area delimited by the Betsiboka and the Mahavavy rivers. The NAP-Mahavavy-Kinkony (category V) was established in 2006 and is managed by the Malagasy NGO Asity (Fig. 1); it includes dry forests, a lake, a saline bay and a riparian delta ecosystem that are the home to several threatened species (Schwitzer *et al*. 2013). The NAP-Bombetoka-Belemboka (category V) was established in 2007 and was managed by the Malagasy NGO Fanamby until 2011 (Fig. 1). The three protected areas were set up as biodiversity conservation projects in collaboration with the local human populations (i.e. the *Sakalava* ethnic group) (Gauthier *et al.* 1999; FANAMBY 2008; Schwitzer *et al.* 2013; FEM 2015). The Sakalavas do not usually hunt sifakas because to do so is taboo (Harpet *et*

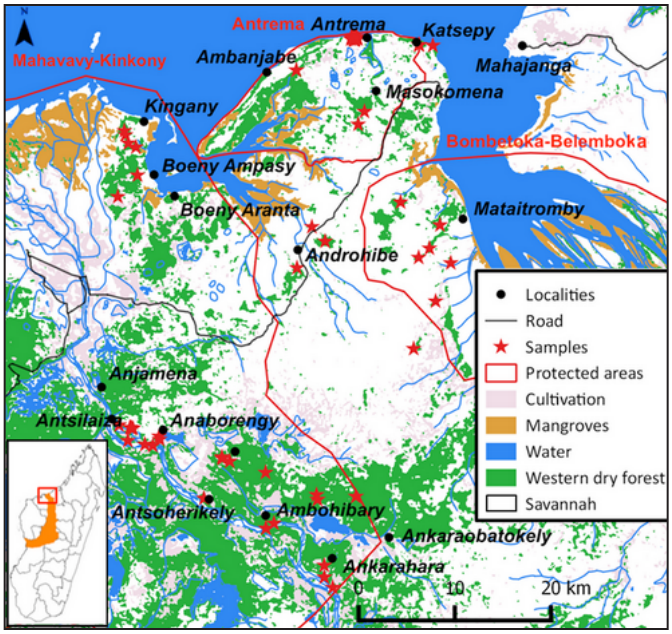

Figure 1. The study area in central western Madagascar. The study was carried out in the north of the range of *P. coronatus* (see Salmona et al. 2014). The villages named are those closest to the sampling sites. Bombetoka-Belemboka, Antrema, and Mahavavy-Kinkony are the protected areas in the study area.

1 The count of 103 taxa by Schwitzer *et al.* (2013) along with species described and reinstated subsequently: *Microcebus tanosi* Rasoloarison *et asl.*, 2013; *Microcebus manitatra* Hotaling *et al.*, 2016; *Microcebus ganzhorni* Hotaling *et al.*, 2016; *Microcebus boraha* Hotaling *et al.* 2016; *Cheirogaleus lavasoensis* Thiele, Razafimahatratra and Hapke, 2013; *Cheirogaleus andysabini* Lei *et al.*, 2015; and *Cheirogaleus thomasi* (Forsyth Major, 1894).

al. 2008). While this traditional taboo is widespread in the northern region, it may be more strictly respected in the NAP-Antrema because the traditional leader of the Sakalava ethnic group, Prince Tsimanendry, lives nearby.

The genetic diversity of endangered species is a useful indicator to establish effective conservation measures to diminish the level of inbreeding and to promote contact between populations that have been isolated because of habitat degradation. This is the issue we address here. To date, there have been no available population genetic studies concerning *P. coronatus*. We non-invasively collected DNA material and, as a first step, sequenced and analyzed a partial DNA sequence of the mitochondrial D-loop region of 125 individuals from the north of the species range. Mitochondria are transmitted along the female line, without recombination, and divergence between mitochondrial sequences results from mutation only.

We first measured mitochondrial diversity in the NAP-Antrema sifakas (79 individuals; AMB, ANT, KAT, MAZ sites), including one of the forests with the high population densities. We subsequently extended our assay to a wider area (~1600 km²), adding 56 samples from other sites. Female *P. verreauxi* (sister species of *P. coronatus*) are very territorial and tend not to migrate for reproduction (Richard *et al.* 1993 and references therein). Crowned sifakas are reluctant to cross grassland, preferring to move through wooded areas, although they have been observed to cross several hundred meters of open grassland (F. Bayart pers. obs.). The same is true for their sister species, *P. tattersalli* and *P. perrieri* (Quéméré *et al.* 2010a; Banks *et al.* 2007, and references therein). The increasing discontinuity in the forest canopy resulting from deforestation (ONE 2013), and direct field observations (Salmona *et al.* 2014) strongly suggest that *P. coronatus* is experiencing a marked demographic decline (IUCN 2014). We aim to contribute to the conservation of this lemur by estimating their genetic diversity, examining any geographical patterns, and inferring putative population size changes.

Methods

Fieldwork and fecal sample collection

Fecal samples were obtained from the northern part of the species range (northwestern Madagascar), the region delimited by the Mahavavy and the Betsiboka rivers (Fig. 1). Vegetation there is characterized by grassland (85%) and dry forest (13%) (data from Salmona *et al.* 2014). Sixty-nine samples came from the Antrema site and 56 were obtained from other locations in the northern part of the species' range. Fresh fecal material was collected during the day in the dry season over three years (2009−2011). Salmona *et al.* (2014) and Salmona *et al.* (2015) provide further details. Feces were picked up directly after observed defecation, leaving little chance that the same individual was sampled twice. They were stored immediately in dry conditions using silica gel (Quéméré *et al.* 2010b). Avoiding duplicate sampling of the same animal was made easy in Antrema where there was an ongoing long-term behavioural ecology project with well-identified individuals and groups. Fecal samples were obtained from 14 locations (Table 1, Fig. 1), named according to the closest village or town or to the name of the forest. DNA was extracted from a subgroup of the available fecal samples.

Laboratory procedures

A total of 125 samples were obtained, including 79 from NAP-Antrema, mostly from the Badrala forest (n = 69, Table 1; the site called "Antrema"). DNA was extracted from the outer layer of dried feces according to the 2CTAB/PCI protocol (Vallet *et al.* 2007). New primers were designed to amplify the mitochondrial Hypervariable Sequence I (HVS-1) of the D-loop. To do so, we aligned the mitochondrial D-loop sequences of 61 individuals of several lemur species (Appendix, Tab. S1) and identified two conserved regions very likely to be conserved also in *P. coronatus*. These conserved regions enabled the design of two primers: PcorL-15978 (5'-CACCTTCAGCACCCAAAGCTG-3'); PcorH-16423 (5'-TGATGGTTTCACGGAGGATGGTAG-3'). DNA was amplified in a total volume of 25 µl, containing 200 µM of each dNTP, Colorless GoTaq® Reaction Buffer 1X (1.5 mM $MgCl_2$), 100 nM of each primer, 1 Unit of GoTaq® DNA Polymerase, 0.4 µg/µl of BSA and 1 µl of DNA samples diluted 10 times. The amplification was performed by a Mastercycler® Eppendorf thermalcycler with a first denaturation at 95°C for 5 minutes, followed by forty cycles of denaturation at 95°C for 1 minute, with an annealing process at 58° for 30 seconds and extension phase at 72°C for 1 minute. The final extension occurred at 72°C for 10 minutes.

DNA double-strand sequencing was performed by Genoscreen on an Applied Biosystems 3730XL sequencer using the Sanger method. Each sequence chromatogram was visually inspected using the software BioEdit v7.2.5 (Hall 1999). To be certain that primers had amplified the target HVS-1 region, all obtained sequences were aligned against Nucleotide collection (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences analyzed in this paper had a length of 395 base-pairs after the deletion of flanking conserved portions (including the primers).

Computation of the genetic diversity

The 125 sequences were aligned using BioEdit v7.2.5 (Hall 1999) with the Clustal W method (Thompson *et al.* 1994). In the general analysis, the number of samples coming from the Antrema site was too high (n = 69) to be compared to the other 13 sampling sites (total n° of individuals = 56). We therefore used a subset of 11 sequences from the Antrema site in those analyses where uneven samples were an issue. The highest probable combination of 11 sequences from a random distribution was obtained by sampling 11 individuals as shown in Figure S1 (Appendix). From these 67 D-loop sequences (56 + 11), we computed haplotype diversity (h) and nucleotide diversity (π) by using DnaSP 5.0 Software (Librado and Rozas 2009). To identify the best model of

nucleotide substitution, we used jModelTest v2.1.5 (Darriba *et al.* 2012) and the Akaike Information Criterion (AIC) (Akaike 1973). By applying the jModelTest we estimated the best-fitting mutation model to be the HKY (Hasegawa *et al.* 1985), with a proportion of invariable sites of 0.86 and a kappa value of 20. The genetic diversity among lemur species was assessed on D-loop sequences retrieved from GenBank (references in Table 2).

Spatial analysis

We investigated the genetic structure using several complementary approaches. We first computed the pairwise estimator of genetic differentiation Φ_{ST} (signficance estimated by 10,000 permutations) among sampling sites (Excoffier *et al.* 1992) with ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010). We note that Φ_{ST} values were computed using the Tamura and Nei (TN93) substitution model (Tamura and Nei 1993) instead of the HKY model because ARLEQUIN v3.5.1.2 does not implement the latter.

The correlation between genetic and geographic distances was assessed by the Mantel test using GenAlex software (Peakall and Smouse 2006, 2012) with a significance established after 10,000 random permutations. Pairwise genetic distances between test-sites were computed from sequences by using the TN93 model (Tamura and Nei 1993) in MEGA v6.0 (Tamura *et al.* 2013).

To test whether grassland and farmland act as barriers to gene flow, we applied the Analysis of Molecular Variance (AMOVA) in ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010) in order to see which combination of samples, once they were put into groups, would have explained the higher fraction of the molecular variance between groups. We first computed the molecular variance (among sites in group and within sites) for all the 14 sampling sites all together, that is in one group. We then computed the same estimators for two groups as there is a large grassland area dividing the northern groups from the southern. Finally we tested the existance of four groups according to the prioximity of the sites and to the presence of grassland separating them (see details in Fig. S2; Appendix). The statistical significance of the fixation indexes was assessed using permutation procedures based on 10,000 runs.

A network of haplotypes was obtained by using TCS v1.21 (Clement *et al.* 2000), with 95% statistical parsimony. This method estimates the evolutionary steps among haplotypes, finally representing a 95% probable set of the most likely networks.

Demographic analysis

To estimate the mitochondrial DNA effective population size (mtDNA *Ne*), we used two Bayesian coalescent-based methods as implemented in the BEAST software v1.8.1 (Drummond *et al.* 2012). The first method assumes a constant population size over time. The second is the Extended Bayesian Skyline Plot (EBSP) analysis that allows population size to vary. The EBSP is a non-parametric Bayesian coalescent-based approach that estimates population-size (that is N_e multiplied by *tau*, where *tau* is the generation time in years) for each coalescent interval, without *a priori* specification of any demographic model (Heled and Drummond 2008). This analysis reconstructs the population size history over time and estimates the number of population change points, which are defined by the "demographic population size change" parameter. In this study, we considered three different values of generation time (3, 6 and 17.5 years) and we estimated mtDNA N_e, dividing the population size parameter by *tau* (i.e. generation time). We used a Markov Chain Monte Carlo (MCMC) of 1×10^8 steps. We removed the first 25% steps (burn-in) and we checked for convergence of all parameters and for acceptable Effective Sample Size values (ESS >200) in Tracer v1.6 (Rambaut *et al.* 2014). The analysis was carried out with the HKY+I evolution model. The prior for invariant sites was set as uniform with an initial value of 0.86. We applied a strict clock model, based on the hypothesis of unvaried mutation rate within species, with the clock rate sampled from a normal distribution with mean of 7.2×10^{-8} (standard deviation = 0.725×10^{-8}). To date, an accurate estimation of the D-loop mutation rate for lemurs is not available. We therefore used the 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). UPGMA trees were used as starting trees of the MCMC analyses. The nucleotide frequencies were estimated from a uniform prior distribution with an initial value of 0.25. A log-normal distribution was set for the prior of kappa (transversion/transition) with an initial value of 20.0. Lastly, we used a 1/X prior distribution for estimating the demographic population size and a Poisson prior distribution for the estimation of the population size changes.

In addition, to determine whether population departed from mutation-drift equilibrium we computed three summary statistics Tajima's D, Ramos R_2, and Fu's F_S (Tajima 1989; Fu 1997; Ramos-Onsins and Rozas 2002) that are sensitive to such departures. We used ARLEQUIN v3.5.1.2 to calculate these statistics. The statistical significance was determined by 10,000 coalescent simulations. Under a Wright-Fisher model without selection and population structure, positive Tajima's D and F_S values suggest population contraction, whereas in the case of a population expansion Tajima's D and F_S are negative. The R_2 statistic exhibits either a negative or a positive value when population experiences, respectively, contraction or expansion.

To overcome the confounding effect of population structure, we followed the approaches suggested by Chikhi *et al.* (2010), Heller *et al.* (2013), and Städler *et al.* (2009). Four sampling strategies were used:

1) *Local sampling,* either 69 samples ("Ant") or 11 samples ("Ant11") or seven samples ("Ant7") from the single site of Antrema;
2) *Pooled sampling*, two (or three) samples randomly picked from each site, excluding the sites with only one individual, were pooled in the same dataset ("pool_2i" or "pool_3i");

3) *Scattered sampling*, a dataset composed by pooling one randomly picked sample from each site ("scat_1i");
4) *Structured sampling*, all samples from the surveyed area plus either 69 or 11 or seven samples from Antrema (respectively "All_Ant", "All_Ant11", "All_Ant7").

Three repetitions were used for *pooled* and *scattered* sampling to test whether the results were robust.

Results

Genetic diversity and structure

We present results for the entire northernmost part of the range of *P. coronatus* shown in Figure 1; not just NAP-Antrema, even though the majority of the 125 sequences obtained came from this single area. As mentioned, we pooled 11 sequences of the 69 of Antrema and added them to the other sites for a total of 11+56 = 67 mitochondrial D-loop HVR1 sequences of a length of 395 base-pairs. The alignment of the 67 sequences showed the presence of 17 variable nucleotide positions: 16 transitions and one transversion (4.3% of the 395 bp).

Two localities of the 14 in the geographic region under investigation showed a haplotype diversity (h) of 0.5, while the diversity of the other 12 sites ranged from 0.6 to 1 (Table 1). Overall, haplotype (h) and nucleotide (π) diversity were 0.85 ± 0.022 and 1.2% ± 0.11%, respectively. The NAP-Antrema (sampling sites AMB, ANT, KAT, MAZ in Table 1) showed average genetic diversity indices; haplotype and nucleotide diversity of 0.75 ± 0.06 and 1.1% ± 0.23%, respectively.

The 67 mitochondrial partial D-loop sequences are displayed in a haplotype network representation (Fig. 2) obtained by applying a 95% statistical parsimony criterion. A total of 11 haplotypes were found and one (H3) was shared among nine sampling sites (AMB, MAT, MAZ, KIN, ANT, ANS, ANO, ANK, AND), four (H8, H9, H10, H11) were detected in one site only (ANO, KAT, ANK) (Fig. 2). From the unrooted network, the maximal number of mutational steps between the most divergent haplotypes (H10; H4) corresponds to 16. The distribution of the different haplotypes among the different test-sites showed no geographic pattern.

The 91 pairwise Φ_{ST} values for the 14 test-sites ranged from -1 to 0.63, with a mean very close to zero (mean Φ_{ST} = -0.017; see Table S2 in the Appendix). The pairwise Φ_{ST} values

Table 1. Sampling sites and measures of genetic diversity of the crowned sifaka. Each site is indicated with its complete name and its abbreviation (Abbr.). Ind. = number of individuals; Hap = number of haplotypes per site; Sites = number of variable sites; π = nucleotide diversity; h = haplotype diversity; sd = standard deviation; GPS NS = latitude; GPS EW = longitude; * = values multiplied by 100; # = decimal degree GPS data in WGS 84 coordinate reference system. Antrema_07 and Antrema_11: estimates for the Antrema sampling site using a sub-sample of 7 or 11 samples, respectively (see Methods section). Total Ant 07 or 11 = estimates for all sampling sites, with either 7 or 11 individuals from Antrema. NAP-Antrema = includes the four sampling sites placed in the protected area (AMB, ANT11, KAT, MAZ).

	Abbr.	Ind.	Hap.	Sites	π*	sd π*	h	sd h	GPS NS(#)	GPS EW(#)
Ambanjabe	AMB	3	2	3	0.51	0.24	0.67	0.31	-15.743	46.069
Ambohibary	AMO	4	2	9	1.14	0.60	0.50	0.27	-16.160	46.070
Androhibe	AND	3	2	4	0.68	0.32	0.67	0.31	-15.910	46.100
Ankarahara	ANK	4	4	5	0.76	0.20	1.00	0.18	-16.200	46.135
Antrema	ANT	69	4	9	0.96	0.04	0.67	0.02	-15.710	46.166
Antrema_07	ANT7	7	3	8	1.04	0.23	0.76	0.12	-15.710	46.166
Antrema_11	ANT11	11	3	8	0.97	0.17	0.71	0.08	-15.710	46.166
Antsilaiza	ANS	11	5	15	1.26	0.26	0.82	0.01	-16.070	45.920
Antsoherikely	ANO	3	3	14	2.36	0.67	1.00	0.27	-16.145	46.015
Boeny Ampasy	BOE	1	1	-	-	-	-	-	-15.840	45.960
Boeny Aranta	BON	1	1	-	-	-	-	-	-15.860	45.925
Katsepy	KAT	5	3	12	1.67	0.42	0.80	0.16	-15.714	46.214
Kingany	KIN	4	2	7	0.89	0.47	0.50	0.27	-15.790	45.950
Mataitromby	MAT	7	3	4	0.56	0.12	0.67	0.16	-15.880	46.260
Masokomena	MAZ	2	2	2	0.51	0.25	1	0.50	-15.760	46.175
Tsiandrarafa	TSI	8	5	14	1.3	0.31	0.89	0.01	-16.100	46.040
Total	/	125	11	17	1.12	0.07	0.80	0.02	/	/
Total Ant 07	/	63	11	17	1.22	0.11	0.86	0.02	/	/
Total Ant 11	/	67	11	17	1.20	0.11	0.85	0.02	/	/
NAP-Antrema	/	21	6	15	1.10	0.23	0.748	0.058	/	/

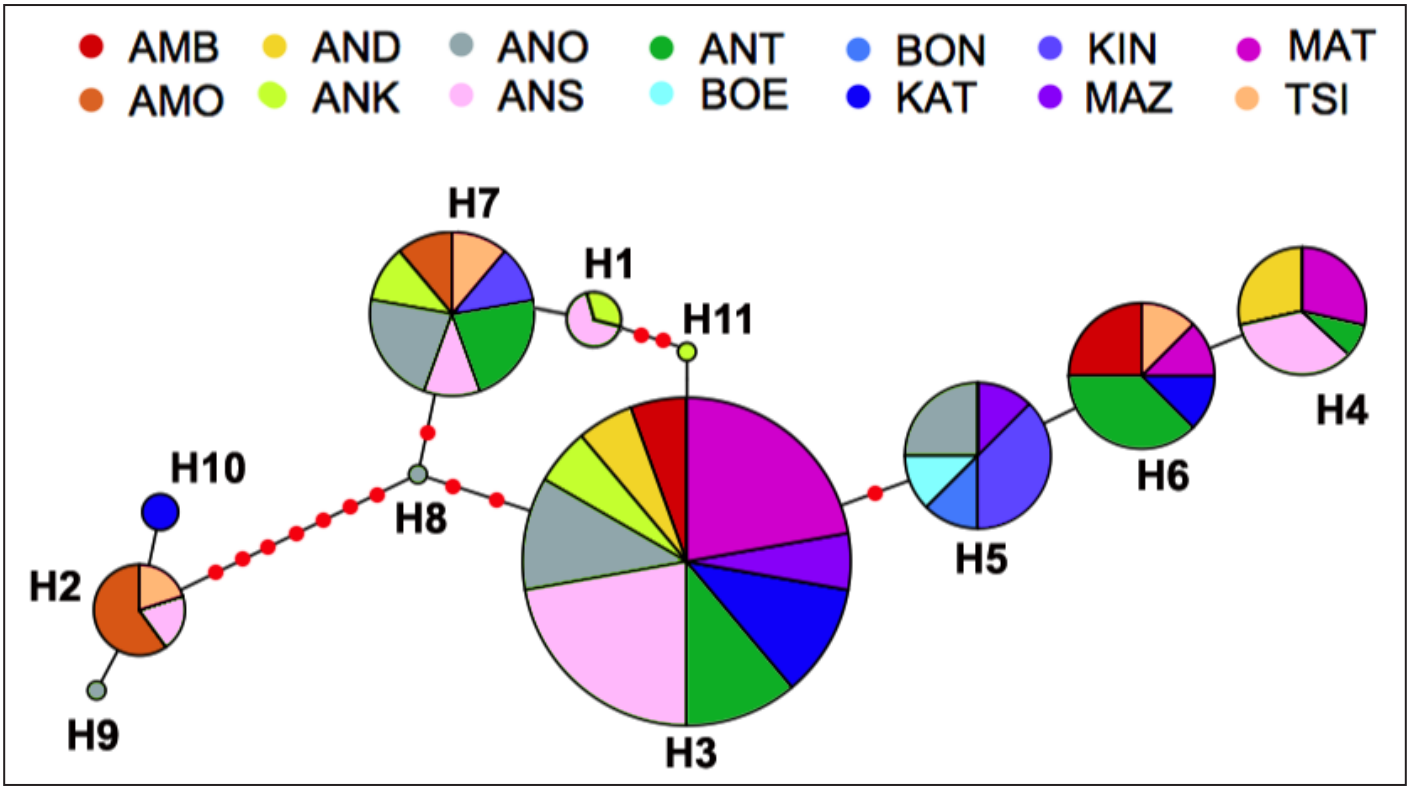

Figure 2. Haplotype network of *P. coronatus* obtained with D-loop sequences from this study. Each circle represents a single haplotype and the size is proportional to its frequency in the dataset. Small red circles represent estimated haplotypes, missing in the dataset. The 14 different colors indicate the sampling sites (see Table 1). Solid lines represent 95% parsimonious connections of single mutation between haplotypes. The loop indicates that different set of single-point mutations can give rise to the same haplotype.

exhibited a very large variance, likely due to the small number of samples per site. Indeed, only some pairwise comparisons, those for the Ambohibary and Mataitromby sites, exhibited a significant genetic differentiation (AMO-ANT; AMO-ANS; AMO-MAT; ANK-MAT) with Φ_{ST} values ranging from 0.386 to 0.659. All other values were not significant, with thirteen Φ_{ST} pairwise comparisons between 0 and 0.1 and forty-seven lower than zero. The Mantel test, comparing the pairwise matrix of genetic differentiation with the corresponding matrix of linear geographic distances, yielded a significant correlation (Rxy = 0.329; P = 0.003; R^2 = 0.11; Fig. 3).

Concerning the AMOVA analysis, it appears that when samples are put together in one single group, the differences among them explain 10.91% of the molecular variance, but this percentage falls to ~4% when we put the samples in 2 or 4 groups. The two-group hypothesis corresponds to the northern and the southern parts of the sampled area that are divided by grasslands (in white in the middle of Fig. 1), whereas the four-group hypothesis corresponds to a north/south/east/west partition of samples around the same grassland zone. The north/south partition into two groups explains more variance (~11%) than the four-group partition, suggesting that, overall, the genetic differentiation of crowned sifakas is explained mainly by the grasslands dividing northern and southern sites (Table 3 and Fig. S2 for more details).

Demographic history

Under a constant-size coalescent model, we obtained a median mtDNA effective population size of 11,883 individuals and a 95% highest posterior-density (HPD, that is the shortest interval that contains 95% of the most probable values) between 5,107 and 20,083 (assuming a generation time of six years, Table 4). Estimations using generation times of three or 17.5 years are shown in Table S3 and suggest that larger generation times will produce smaller effective size estimates, the opposite happens for shorter generiation times. This is a likely consequence of the fact that genetic drift experienced for a particular time may result in a genetic diversity in a species with long generation time and small effective population size similar to that in a species with a short generation time and large effective population size.

Very similar values were obtained for the "All_Ant" (69 samples from Antrema site plus 56 from all other sites) and "All_Ant7" (seven samples from Antrema site plus 56 from all other sites) sampling strategies (see section "Demographic analyses" in methods for details about the alternative sampling).

In nearly all cases, the four sampling strategies (*local*, *scattered*, *pooled* and *structured*) showed that most of the demographic summary statistics were not significant (Table 5), the only exception being the Antrema sample when *local sampling* was used ("Ant" and "Ant_11"; Table 5). In

that case, we found positive and significant values for all the statistics.

The demographic history inference (mtDNA N_e over time), using the EBSP, was a stationary population (median mtDNA N_e: 32,172), with a large confidence interval (95% HPD: 1,471–373,933). The demographic size change estimate was zero (median; 95% HPD between 0 and 2/3), except for Antrema, for which we detected a signal of one past event of population size change (Table 5).

Discussion

Genetic diversity

Using mitochondrial DNA, we have obtained the first estimates of the genetic diversity of *Propithecus coronatus*. This diversity was addressed in geographic terms (the so-called genetic structure) for the northern part of the estimated geographic range of crowned sifaka. The nucleotide diversity detected in *P. coronatus* (π = 1.21%) was within the range of the values observed for other *Propithecus* species for which mtDNA data are available (0.42%–1.53%) (*P. candidus*, *P.*

Table 2. D-loop genetic diversity of sifakas and other lemurs. EN = Endangered; CR = Critically Endangered; Length = number of base pairs; N = number of sequences; Sites = number of variable sites; hap = number of haplotypes; *h* = haplotype diversity; π = nucleotide diversity expressed as a percentage; Tot. census pop. size = census population size; References for demographic data = references of the total population size estimates; /: not available

Species	IUCN status	Length	N	Sites	hap	*h*	π (%)	Tot. census pop. size	References for genetic diversity	Referencs for demographic data
Propithecus coronatus	EN	395 bp	63	17	11	0.86	1.21	130,000 to 200,000	This study	Salmona *et al.* (2014)
Propithecus candidus	CR	395 bp	7	2	2	0.29	1.15	<1000 *c.*250	Andriantompohavana *et al.* (2006) Mayor *et al.* (2002, unpubl.)	IUCN 2014
Propithecus tattersalli	CR	~560 bp	/	/	/	/	/	>10,000	Bailey *et al*. (2016)	Quéméré *et al.* (2010a)
Propithecus coquereli	EN	560 bp	82	17	12	0.76	0.66	>50,000	/	Kun-Rodrigues *et al.* (2014)
Propithecus edwardsi	EN	395 bp	13	13	4	0.71	1.53	*c.*40,000	Andriantompohavana *et al.* (2006) Mayor *et al.* (2002 unpubl.)	Irwin *et al.* (2005)
Propithecus verreauxi	EN	395 bp	7	11	5	0.91	1.35	/	Andriantompohavana *et al.* (2006)	/
Propithecus perrieri	CR	~560 bp	51	0	1	0	0	*c.*917	Bailey *et al*. (2016)	Banks *et al.* (2007)
Propithecus deckeni	EN	/	/	/	/	/	/	/	/	/
Propithecus diadema	CR	395 bp	23	15	4	0.72	0.99	/	Mayor *et al.*(2002 unpubl.) Andriantompohavana *et al.* (2006)	/
Lepilemur edwardsi	EN	496 bp	121	/	23	0.89	0.49	/	Craul *et al.* (2009)	/
Varecia variegata	CR	395 bp	186	44	24	0.84	2.67	/	Louis Jr. *et al.* (2006) Baden *et al.* (2014) Andriantompohavana *et al.* (2006)	/
Microcebus ravelobensis	EN	395 bp	113	113	26	0.94	7.91	/	Guschanski *et al.* (2006) Olivieri *et al.* 2007	/
Microcebus danfossi	EN	477 bp	6	39	6	1.00	3.55	/	Guschanski *et al.* (2006) Olivieri *et al.* (2007)	/
Eulemur cineirescens	CR	452 bp	108	39	11	0.82	2.1	<1000	Delmore *et al.* (2013) Johnson *et al.* (2008)	Vasey (1997)

Table 3. Analysis of molecular variance (AMOVA). From left to right, the three columns give the percentage of molecular variation "Among groups", "Among sites in groups" and "Within sites". The p-value is indicated in parenthesis. Details on the sites grouping are specified in the "Group composition" column (see also Table 1).

	Among groups	Among sites in groups	Within sites	Group composition
4 groups	8.55% (P= 0.053)	4.28 % (P = 0.17)	87.17 % (P = 0,04)	(AMB, ANT, MAZ, KAT) (MAT, AND) (KIN, BOE, BON) (ANS, ANO, AMO, ANK, TSI)
2 groups	11.06% (P = 0.02)	4.28% (P = 0.14)	84.67% (P = 0.03)	(AMB, ANT, MAZ, KAT, MAT, AND, KIN, BOE, BON) (ANS, ANO, AMO, ANK, TSI)
1 group	/	10.91% (P = 0.03)	89.09% /	All 14 sites

Table 4. MtDNA effective population size. Eff. Pop. Size Mean = the mean value of the mtDNA effective population size; Eff. Pop. Size Median = the median value. 95% HPD is the 95% confidence interval of the mean. These values were estimated from three different datasets. All_Ant = 56 samples + 69 from Antrema; All_Ant7 = 56 samples + 7 from Antrema; All_Ant11 = 56 samples + 11 from Antrema.

	Eff. pop. size mean	Eff. pop. size Median	95% HPD
All_ Ant	10,077	9,586	[4,132; 16,708]
All_ Ant7	12,105	11,374	[4,672; 20,640]
All_ Ant11	11,883	11,262	[5,107; 20,083]

coquereli, *P. edwardsi*, *P. verreauxi*, and *P. diadema*) (Tables 1 and 2). *Varecia variegata*, *Microcebus ravelobensis, M. danfossi* and *Eulemur cineirescens* present higher π values compared to *Propithecus*. The lower nucleotide diversity in *Propithecus* compared to the species listed in Table 2 (apart from *Lepilemur edwardsi*), might suggest either lower mutation rates or smaller effective population sizes (Nabholz *et al.* 2008). At this stage, it is difficult to identify the most likely explanation, and inter-specific comparisons will require further research. In particular, the estimation of the mutation rate across species will allow comparison of the levels of interspecific genetic diversity (Leffler *et al.* 2012), with a potential application to the assessment of extinction risks and conservation policies (see below and Akçakaya 2000).

Genetic Structure

The results illustrated in Figure 3 suggest that there is a limited, though significant, correlation between genetic differentiation and geographical distance across the region surveyed, even if the samples from different test-sites did not show a strong signal of genetic differentiation, as the majority of the Φ_{ST} values were not significant (Table S2). This suggests that the sub-populations may still be connected to each other, or have been connected until recent times. If the fragmentation of the habitat is a threat to *P. coronatus*, it seems that ancient fragmentation in this geographical region did not isolate the female populations that are, overall, quite homogeneous; only the Ambohibary and Mataitromby sample sites showed significant Φ_{ST} values (>0.36).

This absence of a clear spatial pattern of diversity is confimed both by the haplotype network (Fig. 2) that failed to reveal any phylogeographic patterns, and by the AMOVA analysis, as 89.09% of molecular variance is found within sampling sites and only 10.91% of it is explained by variance between sampling sites. We are aware, however, that more samples should be typed because in this study we are dealing with a low number of individuals in an area that is relatively large (1,600 km²)

Previous studies on other sifaka species suggest that they may not be among the species most affected by recent habitat fragmentation. Quéméré *et al.* (2010b), for instance, found that genetic differentiation in the golden-crowned sifaka, *P. tattersalli*, was influenced more by a river dividing its range

Table 5. Demographic history. The first column shows the sampling strategies tested in this study. Sampling = indicates the strategy (see Methods); n = number of individuals; hap = number of haplotypes in each strategy; sites = number of variable sites; π = nucleotide diversity; *h* = haplotype diversity; sd = standard deviation; D = Tajima's D; R2 = Ramos-Onsins & Rozas; Fs = Fu's Fs; 95% - bounds = boundaries of the 95% confidence interval; SizeChan = median demographic size changes and 95% confidence interval;° = values multiplied by 100; * = P-value <0.05; ** = P-value<0.01.

	Sampling	n	hap	sites	π °	sd (π) °	*h*	sd (*h*)	D	95% - bounds	R2	95% - bounds	Fs	95% - bounds	SizeChange
scat_1i_01	scattered	14	7	15	0.99	0.26	0.87	0.06	-0.70	[-1.76; 1.72]	0.14	[0.09; 0.22]	-0.16	[-4.48; 4.73]	0; [0. 2]
scat_1i_02	scattered	14	5	9	0.83	0.16	0.79	0.07	0.60	[-1.77; 1.74]	0.18	[0.09; 0.23]	1.37	[-3.68; 4.16]	0; [0. 2]
scat_1i_03	scattered	14	8	16	1.34	0.27	0.92	0.04	0.20	[-1.76; 1.71]	0.16	[0.09; 0.22]	-0.28	[-4.54; 4.44]	0; [0. 2]
pool_2i_01	pooled	24	9	16	1.20	0.18	0.88	0.04	0.38	[-1.69; 1.76]	0.15	[0.07; 0.20]	0.33	[-5.16; 5.0]	0; [0. 2]
pool_2i_02	pooled	24	8	16	1.22	0.19	0.87	0.03	0.43	[-1.73; 1.74]	0.15	[0.07; 0.20]	1.17	[-5.07; 4.99]	0; [0. 2]
pool_2i_03	pooled	24	9	16	1.43	0.17	0.91	0.03	1.13	[-1.73; 1.78]	0.17	[0.07; 0.20]	0.94	[-5.18; 5.02]	0; [0. 2]
pool_3i_01	pooled	30	9	16	1.32	0.15	0.87	0.03	0.99	[-1.70; 1.82]	0.16	[0.07; 0.19]	1.33	[-5.53; 5.32]	0; [0. 2]
pool_3i_02	pooled	33	10	17	1.40	0.15	0.88	0.03	1.06	[-1.71; 1.83]	0.16	[0.06; 0.19]	1.09	[-5.65; 5.24]	0; [0. 2]
pool_3i_03	pooled	33	10	17	1.54	0.12	0.90	0.02	1.51 *	[-1.70; 1.87]	0.18 *	[0.06; 0.19]	1.50	[-5.88; 5.34]	0; [0. 2]
All_Ant	structured	125	11	17	1.12	0.07	0.80	0.02	1.05	[-1.59; 1.93]	0.13	[0.04; 0.16]	2.67	[-7.44; 6.88]	0; [0. 2]
All_Ant7	structured	63	11	17	1.22	0.11	0.86	0.02	1.00	[-1.64; 1.89]	0.14	[0.05; 0.17]	1.56	[-6.68; 6.12]	0; [0. 2]
All_Ant11	structured	67	11	17	1.20	0.11	0.85	0.02	0.99	[-1.68; 1.90]	0.14	[0.05; 0.17]	1.65	[-6.69; 6.34]	0; [0. 2]
Ant	local	69	4	9	0.94	0.05	0.67	0.02	2.62 **	[-1.64; 1.97]	0.21 **	[0.04; 0.18]	7.96 **	[-5.25; 5.18]	1; [0. 3]
Ant7	local	7	3	8	1.04	0.23	0.76	0.12	1.34	[-1.58; 1.64]	0.26	[0.12; 0.31]	2.98	[-2.71; 3.64]	1; [0. 2]
Ant11	local	11	3	8	0.97	0.17	0.71	0.08	1.65 *	[-1.71; 1.76]	0.24 *	[0.1; 0.24]	4.18 *	[-3.62; 4.11]	1; [0. 3]

than by forest fragmentation. Quéméré *et al.* (2010b) also found a significant correlation between genetic differentiation and geographical distance and, in another study, they detected signals of ancient changes in population size related to climatic events rather than to the current levels of habitat fragmentation (Quéméré *et al.* 2012). Similarly, Salmona *et al.* (2015) found isolation by distance but a limited role played by grassland as a barrier to the dispersal of *P. perrieri* (one of the most endangered primates in the world). Other studies (for example, Jimenez and Vargas 2000) support the idea that grassland may not empede gene flow between neighboring forests in *P. coronatus* populations.

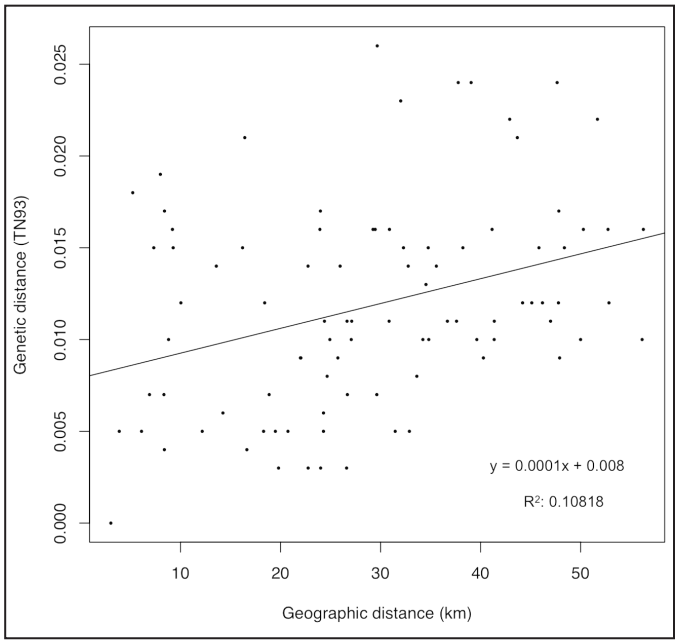

Figure 3. Isolation by distance among sampling sites. Plot of pairwise genetic distances (according to the TN93 model in Tamura and Nei (1993) versus corresponding geographic distances (kilometers). A significant linear regression is shown.

It is important to note, however, that habitat fragmentation is suspected to have significantly increased in recent decades across the known area of occupancy of *P. coronatus* (Rakotonirina *et al.* 2013; Ramanamisata and Razafindraibe, 2013; Razafindramanana *et al.* 2013; Salmona *et al.* 2014), though other authors have suggested an opposite trend (Andriamasimanana and Cameron, 2014). Concerning the area under investigation (see Fig. 1, Table 3), grassland divides the north and the south and this partition is the one making most sense in terms of the AMOVA analysis (11% of the variance is explained by a north/south grouping—see Table 3 and Fig. S2). We can suggest that a circulation of female individuals from the north to the south, and vice versa, is unlikely throughout the grassland itself, but becomes possible from neighboring forest fragments.

Assuming that the barriers to dispersal are the same in the three sister species (*P. coronatus*, *P. tattersalli* and *P. perrieri*), the absence of large rivers in the area investigated could also explain the weak genetic structure. Moreover, paleoenvironmental data revealed that the local landscape was characterized by a mosaic of dry forest and grassland even about 3,500 years BP, and by grassland with fire-adapted trees and ruderal herbs in the last 1000 years (Matsumoto and Burney 1994; Burns *et al.* 2016). These findings suggest that

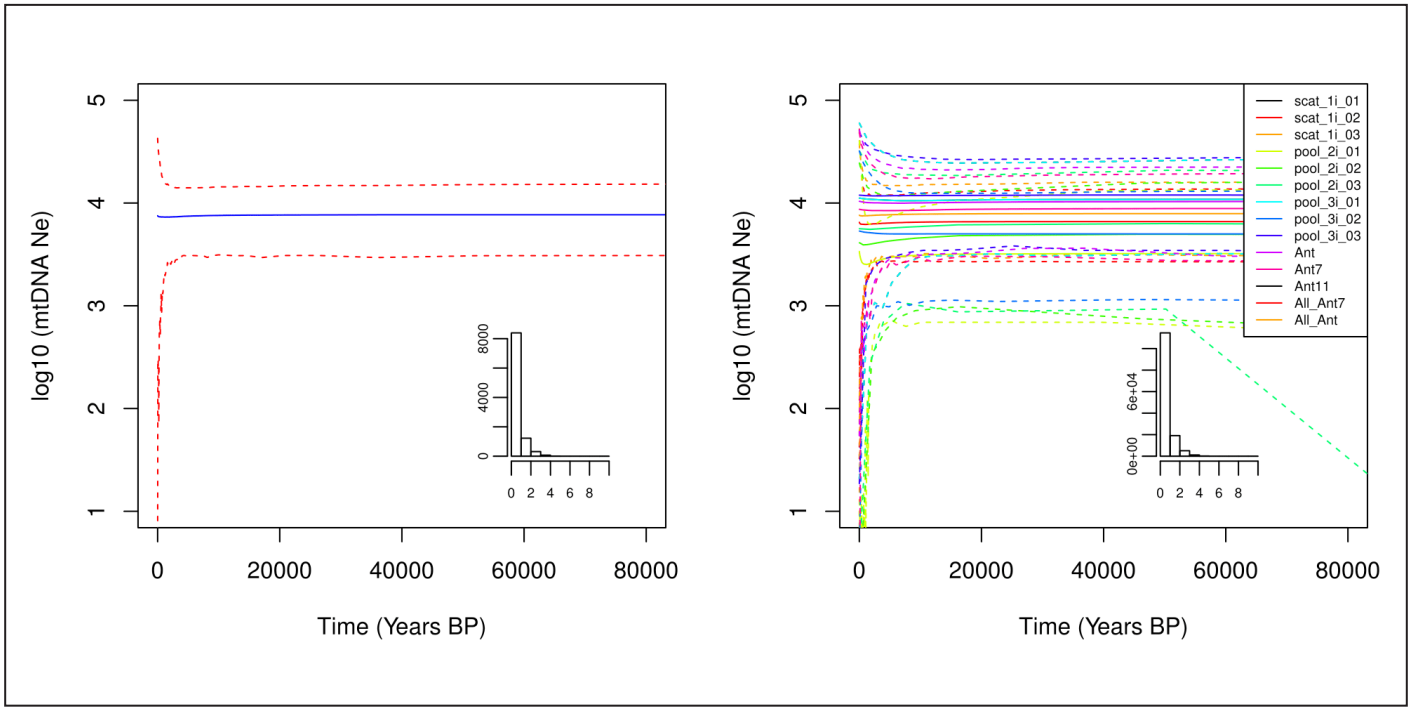

Figure 4. Extended Bayesian skyline plot using four sampling strategies. Plots of the median estimates (solid line) of the mtDNA effective population size over time obtained from D-loop sequences of *P. coronatus*. The dashed lines represent the 95% HPD boundaries. The histograms show the Posterior Size Change (PSC) values. The left graph shows the demographic trend using the"All_Ant11" dataset. The right graph shows the demographic trend of all other datasets. Scat = "scattered"; pool = "pooled."

crowned sifakas have long coped with fragmented landscapes, over at least 3,500 years, with a likely recent increase in the last decades (Harper *et al.* 2007).

The weak genetic differentiation detected may thus reflect the current or recent past abilities of females in traversing grassland and other open habitats to occupy dispersed forest patches. While female philopatry seems to be more predominant amongst *Propithecus*, female dispersal has been shown to occur under certain condition (Morelli *et al.* 2009). Although there is no record of *P. coronatus* crossing wide gaps of open habitat, other sifaka species are able to do so (Meyers and Wright 1993; Richard *et al.* 1993; Mayor and Lehman 1999), and its sister species *P. verreauxi* is known for its ability to walk on the ground (Mittermeier *et al.* 2010).

Demographic history

Most of the methods that we used to reconstruct the demographic history of *P. coronatus* implicitly or explicitly assume that there is no structure and that the samples were obtained from an isolated panmictic population. As demonstrated by several recent studies, population structure can generate spurious signals of population size change (Städler *et al.* 2009; Chikhi *et al.* 2010; Heller *et al.* 2013). To better control for the confounding effect of population structure, Städler *et al.* (2009) suggested the use of a *scattered* sampling strategy (one sample retrieved from each deme) as it gives results comparable, in terms of genealogical structure, to a panmictic population that has experienced the same temporal demographic history (for example, contraction). More recently, Heller *et al.* (2013) suggested the *pooled* sampling strategy as the best one to detect signals of population size expansion and decline. Because of this we used various sampling strategies for our demographic analyses.

Altogether the Extended Bayesian Skyline Plot (EBSP) analyses suggest a constant mtDNA N_e over the time, whichever sampling scheme was adopted. The EBSP showed wide confidence intervals, however, for mtDNA N_e and for the number of population-size-change events (Fig. 4 and Table 4). Since with confidence intervals containing one or more changes it is not possible to retain with certainty a constant population hypothesis, our results should be considered with caution (Table 5; <http://beast.bio.ed.ac.uk/tutorials>) because the lack of signal could be related to a lack of statistical power.

Under the coalescent assumption of constant population size, by using software BEAST we estimated a median mtDNA N_e of 11,883 (95% HPD: 5,107−20,083; generation time of 6 years) that falls within the range of the values obtained by EBSP (median: 32,172; 95% HPD: 1,471–373,933). This agreement was expected and suggests that, at least at the geographical scale surveyed and for mtDNA, a constant population size model is a reasonable choice for *P. coronatus*. These results were confirmed to a large extent by three summary statistics (Tajima's *D*, Fu's *F*s and R_2). With the exception of the Antrema sampling ("Ant" and "Ant11"; Table 5), which showed significant but contradictory signals between the three statistics, most of the results were not suggesting any particular departure from mutation-drift equilibrium.

In short, when using mtDNA our results suggest that there is no genetic signal of contraction or expansion in *P. coronatus* across the northern part of its distribution. It would be important to complement this study with the addition of nuclear markers such as microsatellites. Microsatellites have been developed for this species (Lei *et al.* 2008) and could therefore be used to determine whether the lack of genetic structure can be confirmed and whether recent demographic events not visible with mtDNA can be detected with nuclear markers.

It is important to link the genetic estimates for the population size of crowned sifaka with recent census data concerning the region under investigation. The population was estimated to be between 4,226 and 36,672 individuals, and most probably above 10,000, with some localities harboring high densities (>300 ind/km²; Salmona *et al.* 2014). While these authors did not survey the southern part of the range, they suggested that there could be as many as 130,000 to 220,000 individuals across the entire species' distribution. As the authors themselves admit, these figures are disputable, though they can be regarded as an upper limit. If the current mtDNA N_e is between 5,107 and 20,083 (median: 11,262), this implies by definition (assuming that they are evolving neutrally in a panmictic population with an equal breeding sex ratio) a theoretical nuclear N_e of 20,428 to 80,332 (median: 45,048) as the effective population size of mitochondria is ¼ of nuclear genes. The estimated nuclear N_e is, therefore, rather high when compared to the census sizes computed in the northern area by Salmona *et al.* (2014), thus suggesting that a recent population decline might have occurred despite the fact that mtDNA data do not allow us to detect it with existing methods. To conclude, while mtDNA effective population size estimations (using EBSP and the constant population-size model) depend on the length of the generation time, still debated in *P. coronatus*, we note that the N_e we estimated is in agreement with the extrapolation of Salmona *et al.* (2014). Again, more samples (from the southern distribution of the species) and more genetic data accross all samples are needed to obtain more reliable estimates.

Conservation implications

In this study, we found substantial mitochondrial genetic diversity within the NAP-Antrema and along the Mahavavy riverbanks. Interestingly, these are the areas with high densities of *P. coronatus* (up to 300 ind/km²; Salmona *et al.* 2014). While *P. coronatus* benefits from considerable conservation efforts in the NAP-Antrema, we believe that more efforts are necessary along the Mahavavy riverbanks, inside and outside the Mahavavy-Kinkony protected area.

The high population densities found by Salmona *et al*. (2014) and the substantial genetic diversity found in the region (this study) are likely a partial consequence of a taboo against hunting the crowned sifaka among the Sakalava population inhabiting the area (Harpet *et al.* 2008). Conservation

measures in all areas should reinforce this traditional respect in their conservation and community outreach strategies. The NAP-Antrema community, for instance, benefits from the presence of the traditional leader of this region, Prince Tsimanendry, who lives in Antrema, in his efforts to protect one of the surviving primary coastal dry forests in northwestern Madagascar (Gauthier *et al.* 1999).

Our results reveal limited genetic structure in the northern distribution of *P. coronatus*. This suggests that crowned sifakas were recently and may still be able to move between neighboring forest fragments and, potentially, across grassland. However, since sifakas may have a long generation time and the landscape has been particularly impacted in the last 60 years (Harper *et al.* 2007; ONE 2013; Schwitzer *et al.* 2014a, 2014b), it is difficult to determine when or if the dispersal stopped between forest fragments.

Future conservation efforts should focus on re-establishing connectivity between forest fragments of the region and on maintaining current available habitat. Regular monitoring of *P. coronatus* and its habitat is also needed. Anthropogenic disturbances can be expected to increase in the near future throughout Madagascar (for example, slash-and-burn, illegal logging). Based on current human population growth rates in Madagascar (3.39% per year), Vieilledent *et al.* (2013) estimated that the popuation size may double from 21 million in 2011 to 40 million in 2045. This will undoubtedly increase significantly the intensity of deforestation to values as high as 1.17% per year by 2030 (from 1990 to 2000, it was around 0.9% per year; Harper *et al.* 2007) and may threaten *P. coronatus*' populations viability accordingly.

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Appendix

Table S1. Primer design based on conserved regions. Dloop (a) and tRNA (b) tables show the 61 sequences of lemur species, aligned to identify the conserved sequences in designing the primer. Using MITOMAP human project, the more conserved regions surrounding the mitochondrial Hypervariable Region 1 have been identified (c): (i) tRNA proline gene; and (ii) Conserved Domain which divides HVS-1 from HVS-2 within the Control Region.

(a)

Definition	ID	Definition	ID
Avahi cleesei BEMA8	DQ856036	*Avahi occidentalis* MAR46	DQ856042
Avahi cleesei BEMA9	DQ856037	*Avahi occidentalis* MAR52	DQ856043
Avahi cleesei BEMA13	DQ856039	*Avahi occidentalis* MAR54	DQ856044
Avahi cleesei BEMA14	DQ856040	*Avahi occidentalis* MAR55	DQ856045
Avahi laniger AND14	DQ856018	*Avahi occidentalis* MAR56	DQ856046
Avahi laniger AND19	DQ856019	*Avahi occidentalis* MAR60	DQ856047
Avahi laniger AND33	DQ856020	*Avahi occidentalis* MAR61	DQ856048
Avahi laniger AND34	DQ856021	*Avahi unicolor* ANT5.8	DQ856032
Avahi laniger NARA4.1	DQ856022	*Avahi unicolor* ANT5.9	DQ856033
Avahi laniger NARA4.2	DQ856023	*Avahi unicolor* ANT5.10	DQ856034
Avahi laniger NARA4.10	DQ856024	*Avahi unicolor* ANT5.12	DQ856035
Avahi laniger NARA4.11	DQ856025	*Indri indri* JAR4	DQ855966
Avahi laniger NARA4.13	DQ856026	*Indri indri* MIZA5.3	DQ855967
Avahi laniger NARA4.17	DQ856027	*Homo sapiens*	NC_012920
Avahi laniger NARA4.18	DQ856028	*Pan troglodytes*	NC_001643
Avahi laniger NARA4.23	DQ856029	*Propithecus candidus* JAR14	DQ855969
Avahi laniger NARA4.31	DQ856030	*Propithecus coquereli*	NC_01105
Avahi laniger NARA4.32	DQ856031	*Propithecus coquereli* BOR2	DQ855971
Avahi laniger RANO2.10	DQ855979	*Propithecus coronatus* JAM4.7	DQ855974
*Avahi laniger*RANO66	DQ855975	*Propithecus deckeni* BEMA4	DQ855973
Avahi laniger RANO199	DQ855976	*Propithecus diadema* TAD13	DQ855970
Avahi laniger RANO319B	DQ855977	*Propithecus perrieri* ANAL10	DQ855968
Avahi laniger RANO348	DQ855978	*Propithecus tattersalli* DAR4.2	DQ855972
Avahi occidentalis MAR29	DQ856041		

(b)

Species	ID	Species	ID
Lepilemur hubbardorum	NC_014453	*Eulemur f. fulvus*	AB371086
Lepilemur ruficaudatus	NC_021953	*Eulemur f. mayottensis*	AB371087
Hapalemur griseus	NC_021950	*Avahi laniger*	NC_021940
Eulemur macaco	AB371088	*Propithecus coquereli*	NC_01105
Eulemur rufus	NC_021948	*Varecia variegata*	AB371089
Lemur catta	AJ421451	*Prolemur simus*	NC_021959
Eulemur mongoz	AM905040	*Cheirogaleus medius*	NC_021945

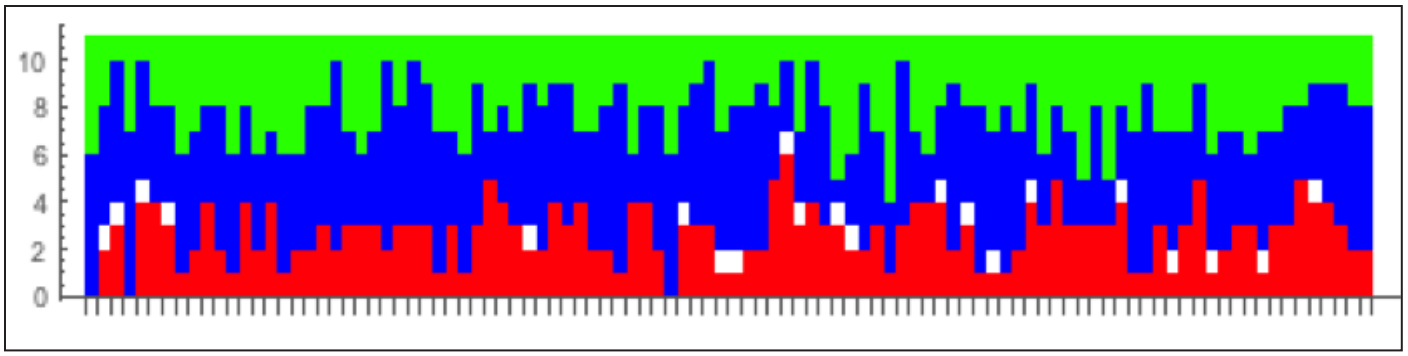

Figure S1. Random sampling of 11 individuals, among the 69 samples from the Badrala Forest. To avoid biased estimation of the genetic diversity and differentiation due to higher sample size from Antrema (n = 69) with respect to the other sampling sites (total n = 56), we built a random distribution of 11 individuals from the 69 individuals of Antrema. The most probable combination was used for further analysis. In order to visualize the probability distribution of 11 random samples, we randomly chose 100 combinations. The probability distribution is a "Multivariate Hypergeometric Distribution" with four variables, one for each haplotype detected among the 69 samples of Antrema (H3: 17; H4: 1; H6: 29; H7: 22). One hundred combinations, randomly chosen, are shown in this graph; each vertical rectangle corresponds to a combination. The colors represent the four haplotypes and the number of sampled individuals for each haplotype is indicated by the height of the colored portion of the rectangle. Red = H3; White = H4; Blue = H6; Green = H7. The most probable combination is **H3:** 3, **H4:** 0, **H6:** 5, **H7:**3, with probability of 0.068187. The probability distribution and the most probable combination were computed using Mathematica (<www.wolfram.com/mathematic>).

Table S2. Pairwise Φ_{ST} values among 14 sites. The table shows the pairwise comparisons of the genetic differentiation among the 14 sites. Almost all pairwise Φ_{ST} are not significant (* = P-value < 0.05; ** = P-value < 0.01).

	AMB	AMO	AND	ANK	ANT	ANS	ANO	BOE	BON	KAT	KIN	MAT	MAZ	TSI
AMB	0													
AMO	0,63	0												
AND	-0,17	0,63	0											
ANK	0,43	0,49	0,45	0										
ANT	-0,07	0,53 **	0,05	0,10	0									
ANS	0,01	0,40 **	0,02	-0,02	-0,04	0								
ANO	0,06	-0,04	0,10	-0,04	0,01	-0,08	0							
BOE	-0,51	0,52	-0,34	0,34	-0,32	-0,28	-0,56	0						
BON	-0,51	0,52	-0,34	0,34	-0,32	-0,28	-0,56	0	0					
KAT	0,13	0,10	0,18	0,19	0,17	0,05	-0,21	-0,22	-0,22	0				
KIN	-0,03	0,54	0,08	0,19	-0,07	-0,03	-0,04	-1,00	-1,00	0,13	0			
MAT	-0,14	0,66 **	-0,11	0,39 *	0,02	0,01	0,21	-0,19	-0,19	0,21	0,09	0		
MAZ	-0,20	0,55	-0,03	0,24	-0,16	-0,18	-0,14	-1,00	-1,00	-0,06	-0,31	-0,24	0	
TSI	0,13	0,30	0,19	-0,05	0,01	-0,05	-0,21	-0,21	0,01	-0,03	0,16	-0,11	-0,18	0

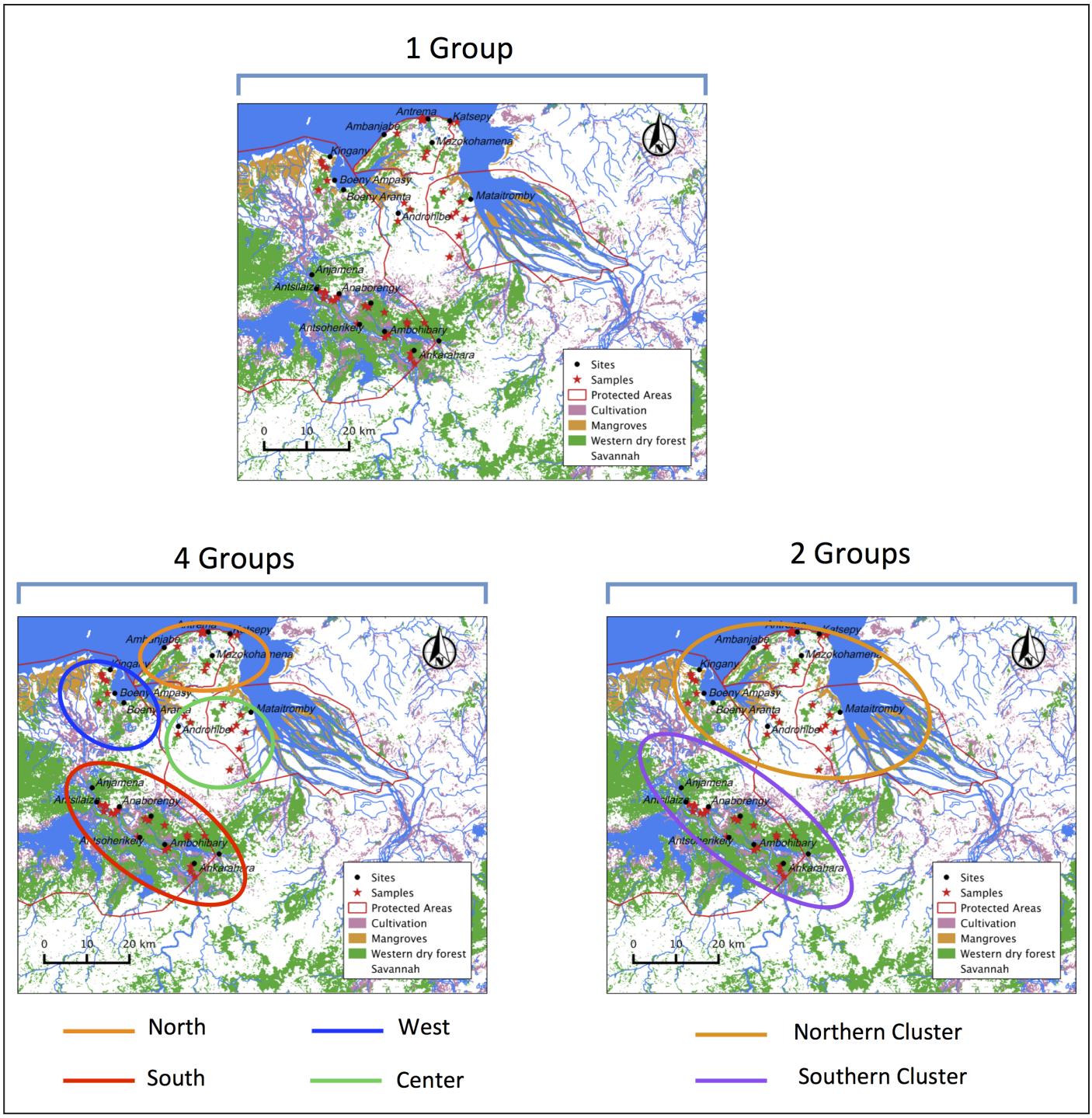

Figure S2. Visual framework of tested groupings in AMOVA analyses. Above − All the 14 sampling sites are included in one group. Below left − Four groups were defined, according to the presence of artificial barriers (e.g., main roads) and natural barriers (e.g., small rivers, savannah and cultivated lands), which would relatively isolate the four groups: North (AMB, ANT, MAZ, KAT), South (ANS, AMO, ANO, ANK, TSI), West (KIN, BOE, BON), Center (MAT, AND). Below right − The two groups were justified by the presence of a relatively large stretch of grasslands between the two groups of sites: Northern (AMB, ANT, MAZ, KAT, KIN, BOE, BON, MAT, AND), Southern (ANS, AMO, ANO, ANK, TSI). Northern and Southern cluster included; 33 and 30 individuals, respectively.

Table S3. mtDNA effective population size. Eff. Pop. Size Mean = the mean value of the mtDNA effective population size; Eff. Pop. Size Median = the median value. 95% HPD is the 95% confidence interval of the mean. These values were estimated from three different datasets. Estimations obtained assuming generation time of a) 3 years and b) 17.5 years.

a)

	Eff. pop. size mean	Eff. pop. size Median	95% HPD
All_ Ant	20,154	19,172	[8,265; 33,417]
All_ Ant7	24,209	22,747	[9,343; 41,280]
All_ Ant11	23,767	22,524	[10,213; 40,167]

b)

	Eff. pop. size mean	Eff. pop. size Median	95% HPD
All_ Ant	3,455	3,287	[1,417; 5,729]
All_ Ant7	4,150	3,899	[1,602; 7,077]
All_ Ant11	4,074	3,861	[1,751; 6,886]