

Non-invasive conservation genetics of the critically endangered golden-crowned sifaka (*Propithecus tattersalli*): high diversity and significant genetic differentiation over a small range

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Abstract The genetic structure of the golden-crowned sifaka (*Propithecus tattersalli*) was evaluated in three forest types in the Daraina region between the Loky and Manambato Rivers in northeastern Madagascar. This critically endangered lemur species, only found within this restricted region ($\sim 1,300 \text{ km}^2$), comprises populations which survive in forest fragments of varying size. While this remote region is already affected by human deforestation, the scheduled paving of the national road can potentially bring significant changes in the near future. In 2006, sifakas feces from 82 putative individuals were collected immediately after defecation. DNA samples were genotyped at 13 microsatellite loci to characterize patterns of genetic diversity within and among three sets of forest fragments representing different ecological types. The recent 2CTAB/PCI extraction method and the ‘comparative’ genotype validation approach allowed us to significantly improve the efficiency of amplification procedures. We found that golden-crowned sifaka genetic diversity was surprisingly high despite its narrow distribution and the current level of habitat fragmentation. We

used both spatial and non-spatial Bayesian clustering methods to determine if forest edges correlated with cryptic genetic clusters and found that it was overall the case. However, the results also indicate that migrations are possible between two of the three studied forest complexes despite the presence of the national road. This is probably due to remaining riparian and small forest patches between these two forest complexes. The third forest complex is more differentiated although some migrations/connections may still exist through non-sampled fragments. This strongly suggests that it is necessary to maintain corridors and small patches, and also to explore the genetic diversity of the fragments outside the protected area.

Keywords Conservation genetics · Habitat fragmentation · *Propithecus tattersalli* · Lemurs · Non-invasive sampling · Faeces

Introduction

Madagascar is well known for its extremely diverse and mostly endemic fauna and flora (Goodman and Benstead 2003). However, the island has suffered from drastic environmental changes in last millennia, leading to the extinction of many species (Burney 1999; Dewar 2003). More than 90% of the original forest cover has disappeared and previously connected forest habitats are now fragmented (Smith 1997). Lemurs are forest-dwelling animals and are thus particularly impacted by these changes. Three of the eight families of Malagasy lemurs present at the time of human arrival have disappeared and two additional families have lost their largest-bodied members (Godfrey and Irwin 2007). The exact causes of these extinctions and of the corresponding environmental changes are not fully

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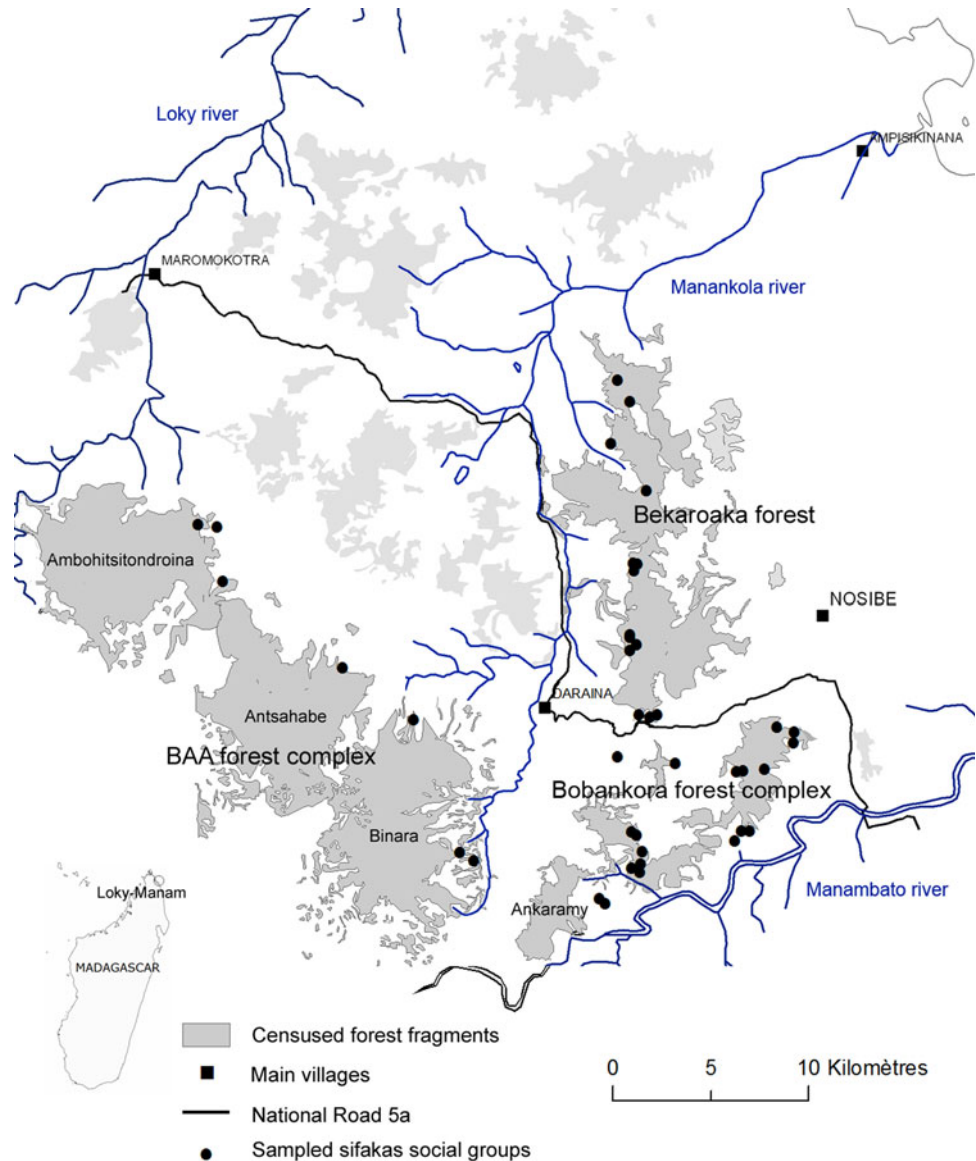
clear. In particular, the relative importance of natural and anthropogenic factors is likely to have varied from one region to another and from a group of species to another (Burney et al. 2004). Even considering human impact only, the debate continues over the nature of these changes (hunting/direct vs. indirect human deforestation) and whether they occurred during the early stages of the human colonization or in the twentieth century. Habitat destruction and fragmentation are clearly among the most serious threats facing lemurs and most species in Madagascar (Mittermeier et al. 2005). Additionally, a number of studies have shown the negative impact of forest fragmentation on species richness (Ganzhorn et al. 2003; Scott et al. 2006; Dunham et al. 2008). However, the consequences of these disturbances on the genetic structure of fragmented populations have not been studied for many species and are still poorly understood. Yet, population genetics studies will be crucial for the effective conservation of many species, now restricted to isolated forest fragments, to develop conservation priorities and to define or create corridors between isolated forest fragments (e.g., Banks et al. 2005; Goossens et al. 2005, 2006; Riley et al. 2006; Radespiel et al. 2008).

The golden-crowned or Tattersall's sifaka (*Propithecus tattersalli*) (Simons 1988) is a critically endangered species of lemurs only found in the Daraina region of northeastern Madagascar (within the subregion of Vohemar, Fig. 1). This is one of the smallest ranges of any lemur (Mittermeier et al. 2005). This large species of the *Indriidae* family was noted for the first time in the 1970s, and remains poorly studied due to its very limited distribution in a relatively remote area (Meyers 1993; Vargas et al. 2002). Its habitat is currently restricted to about fifty small forest fragments (cover no greater than 44,000 ha, often smaller than 1,000 ha) within a mosaic of gallery forests, dry scrub, agricultural zones, and zebu cattle grazing savannas between the Loky and Manambato Rivers. According to Meyers (1993), who performed the only behavioural study published on *P. tattersalli*, golden-crowned sifakas live in social groups of three to 10 individuals that occupy territories of 9–12 ha. Population size estimates range from 6,100 to 10,000 individuals with a potential number of effective breeders between 2,400 and 4,000 (Vargas et al. 2002). Females are thought to be philopatric whereas it is suspected that the subdominant male(s) migrates during the mating season (January) in the middle of the rainy season (Meyers 1993). While the Daraina region is geographically limited, it displays a surprisingly large number of diverse habitat types. Indeed, golden-crowned sifaka can be found in lowland dry deciduous, sub-montane semi-humid and wet evergreen mountain forests. Similarly, the taxonomic composition of

its diet appears to be very flexible, and it has been shown that even over small spatial scales, the diet composition can vary widely between the drier and wetter sites (Meyers 1993; Irwin 2006). In addition to *P. tattersalli*, the eight other sifaka species (genus *Propithecus*) can be found throughout the island in a variety of environments, including both wet and dry forests. In a recent review Irwin (2006), noted that dry forest sifakas, to which *P. tattersalli* belongs, are believed to have a better demographic resiliency in fragmented habitats than wet forest species. Data on the north-eastern species are still lacking, and it is unclear whether golden-crowned sifakas males are able to easily cross open areas and anthropogenic barriers between fragments and mate with individuals from other forest patches. Individuals crossing the savannas have been observed by local people in October and November at the end of the dry season when feeding resources become scarce, hence outside the mating season (Meyers 1993). The Daraina region is expected to be submitted to large and significant disturbances in the near future. Gold mining, already active in some of the forest fragments, may grow fast with the pending tarring of the national road crossing the area (Fig. 1) and with the interest of gold mining companies. It is thus becoming crucial to evaluate the genetic health of this species before major environmental changes take place to develop management strategies in collaboration with local communities, including NGOs.

In this study, we provide the first population genetic data on the golden-crowned sifaka combining non-invasive material sampling and microsatellite genotyping. The advantage of increased sampling efficiency using non-invasive sampling methods, may be counterbalanced by the challenges inherent in low quality/quantity DNA genotyping (Broquet and Petit 2004; Taberlet et al. 1999). The first step of this study was to improve the efficiency of the amplification procedures to obtain reliable consensus genotypes. Altogether we genotyped 82 samples at 13 microsatellites sampled in the three types of forest formations present in the “Station Forestière à Usages Multiples” (S.F.U.M. “Multiple Usage Forest Station”) Loky-Manambato taking account of their ecological variations. The aims of this genetic study were (a) to identify genetically differentiated populations using individual-based analysis without requiring a priori population delineations because little is known on the sifakas capacity to migrate across open areas, (b) to determine the diversity of these *P. tattersalli* inferred populations and their genetic structure, (c) to discuss the resiliency of golden-crowned sifakas to forest fragmentation and the role of riparian corridors between studied forest patches, (d) to identify specific conservation measures for inferred populations.

Fig. 1 Map of the Daraina region, this map represents the three prospected forest complexes and the sampled social groups (*black circles*). The main villages are represented by *black squares*. The national road is represented by a *black line* and the main rivers by *blue lines*



Materials and methods

Non-invasive sample collection, DNA extraction and genotype validation

In order to limit physical contact and possible disturbance of the sifakas, all samples analysed in the present study were collected from faecal material sampled between June and August 2006 (i.e., the dry season) in the “*Station Forestière à usages multiples*” (S.F.U.M) Loky-Manambato (see Fig. 1). Faecal material was obtained from a total of 82 putative individuals belonging to 41 social groups in forest fragments presenting different ecological environments. These fragments were also selected because they were previously chosen by Meyers (1993) for the feeding behaviour and by Vargas et al. (2002) who performed the

first density estimation on *P. tattersalli*. The samples thus came from the dry lowland forest of Bekaraoka (15 social groups, 35 samples), the semi-humid forest complex of Bobankora (19 social groups, 31 samples) and a large forest complex which corresponds to two mountainous evergreen forests (Binara and Antsahabe) and a lowland but hilly dry forest (Ambohitsitondroina) (7 social groups, 16 samples). For simplicity, we shall refer to this third sampling area as BAA (for Binara-Antsahabe-Ambohitsitondroina) (Fig. 1). These fragmented forests include several valleys that were more or less connected to each other, and we tried as much as possible to sample all valleys. All sampling locations were recorded by GPS and mapped using ArcGis 9.1 (Fig. 1). There were however, five individuals from two social groups sampled in Ambohitsitondroina (northern part of BAA) for which the

exact location is thought to be significantly different from the GPS coordinates recorded. Assignment tests suggest that they were most likely sampled in the BAA forest complex. For 21 other samples, the GPS coordinates may differ by a few tens or hundreds of meters but were in the same fragment or valley. We kept these locations for the spatial analyses but we keep in mind that a margin of error is possible for these samples. Given that *P. tattersalli* are probably moving within the same forest the error made is unlikely to affect the general conclusions. Altogether, the sampling locations are thus accurate for 56 samples, very close for 21 samples and likely to be imprecise for 5 samples. In order to reduce the number of samples taken from the same individual but also to use good quality samples, faecal pellets were collected immediately after defecation. For each sample, three to four pellets were stored with 70% ethanol. DNA was extracted using ca. 50 mg from the outer layer of dried faecal pellets using a standard CTAB extraction protocol (Lathuilière et al. 2001) in a Class II microbiological hood. When this method did not provide a good quality extract, we did a second extraction using the 2CTAB/PCI protocol (Vallet et al. 2008). These authors showed that this extraction method improves the ability to obtain high-quality DNA for primate species living in dry or humid habitats with higher amplification success than standard CTAB protocol or QiaAmp DNA Stool Mini Kit (Qiagen).

DNA amplification and microsatellite genotyping

A total of thirteen microsatellite loci were used in this study. Eight loci were selected among the 20 specific polymorphic dinucleotides isolated for *P. tattersalli* (Razafindrakoto et al. 2008) and five among the loci originally designed for *P. coquereli* (Rakotoarisoa et al. 2006), the most closely related sifaka species (Mayor et al. 2004). The loci were selected on the basis of their size (<220 bp) and were thus most likely to amplify non-invasive samples (Taberlet et al. 1999). The microsatellite loci were amplified in a 10 µl reaction consisting of 5 µl of 10× Qiagen Multiplex Buffer, 200 nM of each primer with the fluorescent label (FAM or HEX) on the forward primer, 1 µl of DNA template, and 3.4–3.8 µl ultra-pure H₂O. PCR reactions were conducted in a Eppendorf Mastercycler under the following conditions: initial denaturation of 15 min at 94°C, 40 cycles of 30 s at 94°C, 90 s at 55–60°C, 60 s at 72°C, and a final extension step for 30 min at 72°C. Allelic sizes were determined by separating the amplification products on a DNA sequencing genotyping gel and run on an ABI 3730 (Applied Biosystems). Results were analyzed using the software Peak Scanner (Applied Biosystems). The genotypes were validated using the approach of Frantz et al. (2003).

A number of difficulties can be met with faeces that contain a significant amount of plant remnants, as is the case for folivorous species like the golden-crowned sifaka. Amplification inhibitors may influence genotyping and amplification success although this may often be a result of inadequate DNA extraction methods (Broquet et al. 2007). In order to test the quality of our extracts, evaluate genotyping errors and validate the genotypes generated from our faecal samples, we first randomly selected 12 individuals genotyped at six microsatellites (i.e., randomly selected in the list below) using two methods that are usually used to provide reliable consensus genotypes. The first is the widely used ‘multi-tubes’ approach (Taberlet et al. 1996) and the second is the more recent ‘comparative’ approach of Frantz et al. (2003). In both methods, an allele is recorded only if it was amplified unambiguously at least twice in distinct replicates for heterozygous genotypes and at least three times for homozygous genotypes. However, the conservative ‘multi-tubes’ procedure requires three initial PCR replicates to which four additional replicates are added if no consensus is reached, whereas the ‘comparative’ approach requires only two initial replicates to which new amplifications are added in a stepwise manner. This last approach is expected to require less amplification but might also produce more genotyping errors. For the six tested loci and the 12 individuals, the genotype reconstructed using eight replicates was considered to be the real or consensus one. To evaluate the efficiency of the two approaches, they were both applied to the same individuals and loci by sampling the required number of replicates at random among the eight PCRs. The average minimum number of PCRs required to obtain this reliable genotype was then computed for the two approaches and compared. We considered three widely used indices to evaluate genotyping success for the six tested loci from the eight replicates: (a) the allele dropout (ADO) rate corresponding to the number of observed dropouts (false homozygote genotypes) divided by the total number of successful amplifications of heterozygous genotypes (Broquet and Petit 2004), (b) the false allele (FA) rate computed as the number of observed genotypes with a false allele divided by the total number of positive amplifications and (c) the mean quality index for each locus across individuals (QI) (Miquel et al. 2006) where a score of 1 was given to correct genotypes, and a score of 0 for incorrect genotypes.

Genetic diversity, Hardy–Weinberg equilibrium and isolation by distance

Genetic diversity was first measured for the whole dataset as the mean number of alleles per locus (n_A), the observed (H_o) and expected (H_e) heterozygosities under

Hardy–Weinberg (HW) equilibrium (Nei 1978). Linkage disequilibrium (LD) was estimated across all pairs of loci using the correlation coefficient of Weir (1979). A permutation approach was used to determine which LD values were significant. Wright’s F -statistics were estimated according to Weir and Cockerham (1984). Departure from Hardy–Weinberg equilibrium (HWE) was measured with F_{is} and tested using permutations. All these analyses were performed using the GENETIX 4.0.5 software (Belkhir et al. 1996/2004). The *ar* Inter-individual genetic distance (Rousset 2000) were computed using the program SPAGeDI (Hardy and Vekemans 2002) and a Mantel test (Mantel 1967) was performed to determine if the patterns of differentiation follow isolation-of-distance (Slatkin 1993).

Genetic clustering analysis

In order to investigate the genetic structure and determine the correspondence between the spatial location of genetic discontinuities and the forest patches boundaries, we employed three Bayesian model-based clustering analyses and compared the consistency of their results.

STRUCTURE analysis

The first one is the most widely used approach implemented in STRUCTURE 2.2 (Pritchard and Wen 2004; Falush et al. 2003). To infer the number K of genetically differentiated clusters, we ran the program for a range of K values between one and seven using 20 independent runs of 60,000 iterations (plus a burn-in of 20,000 iterations) for each K value. Runs were performed with the “admixture model” and the “correlated allele frequency” model without prior information and with an initial alpha value set to 1.0. The maximum K value was chosen as the number of sampled forest fragments plus three, as suggested by Evanno et al. (2005). The most probable K value was determined by applying the Evanno et al. (2005) ad hoc summary statistic ΔK , which is based on the rate of change of the “estimated likelihood” between successive K values. Each individual was then assigned to the group for which the posterior probability was the highest, provided that this value was higher than 0.6 as in Coulon et al. (2008). Individuals, whose maximum assigned probability was less than 0.6, were not assigned to any group. Evanno et al. (2005) have shown that their method detects the highest hierarchical structure level. We thus repeated the analysis for each of the clusters identified in the previous step until no substructure was observed ($K = 1$). We plotted the results of the individuals’ assignment on the distribution map. We then used minimum convex polygons (MCP) to spatially delimitate the different clusters. The

MCPs were generated with the Hawth’s analysis tools extension for ARCGIS (Beyer 2004).

For each genetic cluster, we estimated genetic diversity calculating allelic richness per cluster using the software FSTAT (Goudet 2001), H_o and H_e using GENETIX 4.05. Departure from HW equilibrium was measured using Wright’s F_{is} and tested using permutations. Pairwise F_{st} between clusters and average F_{st} values were estimated according to Weir and Cockerham (1984) and tested for departure from the null hypothesis (no genetic differentiation) with 1,000 permutations using GENETIX 4.05.

GENELAND analysis

The second approach incorporates spatial information and is implemented in GENELAND 2.0 (Guillot et al. 2005a, b). All the parameters (including K) are processed simultaneously. However, as suggested in Guillot et al. (2005a), we first performed a series of 10 runs of 100,000 MCMC iterations to infer the most probable number of genetics groups (K) in our samples where K was allowed to vary. K was inferred as the modal number of genetic groups. Then, we performed a second series of 100 independent MCMC runs of 100,000 iterations each with K fixed to the value identified in the first step. The average logarithm of the posterior probability for each of the 100 runs was computed and the 10 runs with the highest mean posterior probability values were kept for inference. As a quick test, we also visually checked the posterior probability for the other runs and found that this probability first decreased abruptly after the twelfth run and then kept decreasing but more gradually (not shown). For both steps, we used the following input parameters: the uncertainty associated with the spatial coordinates was set to 200 m, the mean radius for an inferred territory of 12 ha (Meyers 1993). The maximum rate of the Poisson process was fixed to 75 (the number of individuals) and the maximum number of nuclei in the Poisson–Voronoi tessellation was fixed to 225 (three times the number of individuals as suggested in the manual). We set the number of pixels to 75 along the axis X and 68 along the axis Y . This corresponds to a cell size of ~ 400 m per side which is the estimated diameter of a sifaka territory (Meyers 1993). We then computed the posterior probability of population membership for each pixel of the spatial domain (using a burn-in of 10,000 iterations). We finally checked the consistency of the results across the 10 runs.

TESS analysis

The third approach implemented in the software TESS (Chen et al. 2007) determines the number of genetic units in a similar way than STRUCTURE but takes into account

the spatial organization of individuals and incorporates a regularization procedure with facilitate the decision regarding the number of K (Chen et al. 2007; François et al. 2008). We used TESS in addition to STRUCTURE and GENELAND because its algorithm is expected to be less influenced than STRUCTURE and GENELAND by Isolation-By-Distance (François et al. 2008). The optimal number of genetic units was inferred by sequentially increasing the maximum number of clusters as recommended by François et al. (2008). For each value of K , five runs of 50,000 simulations with a burn-in of 10,000 were performed using the parameter values suggested by the software manual (no F model, no admixture and a spatial interaction value of 0.6). Runs with the smallest value of Deviance Information Criterion (DIC) were selected to choose the optimal value of K .

Results

DNA reliability and genotyping

Out of the 72 genotypes (i.e., 12 individuals \times 6 loci), we could reconstruct a consensus for all except 2 genotypes with the original eight replicates leading to a total of 70 genotypes to which the genotype validation test could be applied. Our results show that the “comparative” and “multi-tubes” approaches were both able to reconstruct the consensus genotype in all 70 cases with less than eight PCRs. However, the main difference between these two approaches was that the ‘comparative’ methods were significantly more efficient (Fig. 2) since it needed $\sim 22\%$

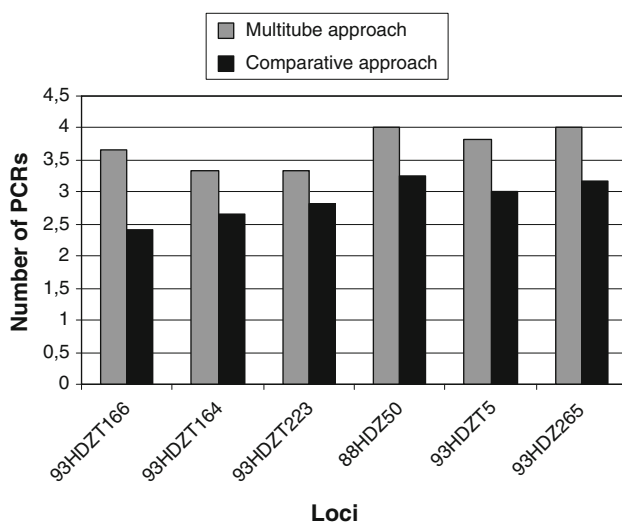


Fig. 2 Comparison between the “multi-tube” and “comparative” approaches. The histogram represents the average number of PCRs required to reach the consensus genotype for each locus for the two methods

Table 1 Genotyping errors and quality indices: allele dropout (ADO) rate, false allele (FA) rate and quality index (QI)

Locus	ADO	FA	QI
93HDZ166	0.20	0.02	0.76
93HDZ164	0.05	0.01	0.88
93HDZ223	0.25	0.02	0.79
88HDZ50	0.33	0.09	0.65
93HDZ5	0.27	0.07	0.69
93HDZ265	0.39	0.03	0.63

less PCR amplifications to obtain the same results (Wilcoxon rank test $W = 36$, P value = 0.0025). Table 1 shows the proportion of allelic dropout (ADO), false alleles (FA) and the quality index (QI), for each tested locus, using the eight original replicates.

We were able to reliably amplify DNA from 77 out of the 82 samples (94%). Interestingly, the five non-amplified samples came from the two wettest forests (Bobankora and BAA). Among the 77 samples, only 45 (58%) could be amplified directly from the DNA extracted using the basic CTAB protocol. The other 32 samples did not produce reliable results until the second extraction method was utilized (i.e., 2CTAB/PCI). In two cases, two samples collected in the same social group and assumed to correspond to two different individuals, had the same multi-locus genotype at all 13 loci. This suggests that they came from the same individual, probably due to sampling errors in the field since defecation of all the members of a sifaka group often happens in a relatively short period of time, with some individuals defecating several times after moving. Thus, out of the 82 sampled putative individuals, 75 individuals were considered, corresponding to a total of 971 genotypes instead of 975 since four genotypes (0.41%) were considered non-reliable using the “conservative” approach, and therefore, were coded as missing genotypes.

Overall genetic diversity

All 13 loci used were polymorphic, with an average of 6.3 alleles ($SD = 1.43$) per locus across all samples (Table 2). The mean H_e value was relatively high (0.72; $SD = 0.09$) (Table 2). Permutation tests for LD were significant in 67 of the 234 pairwise comparisons at the 0.05 level instead of the 12 significant tests that would be expected by chance (0.05×234). However, we could see no trend indicating that some loci were more often involved in significant pairs. The only apparent signal comes from the fact that most of the tests ($42/67 = 67\%$) were observed for individuals sampled in Bekaraoka (Binomial test, P value < 0.001). This could be due to population structure within this set of samples since population structure is known to generate spurious LD between unlinked loci. A significant

Table 2 Summary statistics for the three clusters uncovered with STRUCTURE analysis

Locus name	Whole dataset Na	Cluster 1 (n = 57)				Cluster 2a (n = 13)				Cluster 2b (n = 5)			
		Allelic richness	H _o	H _e n.b.	F _{is}	Allelic richness	H _o	H _e n.b.	F _{is}	Allelic richness	H _o	H _e n.b.	F _{is}
93HDZ5	7	4.47	0.74	0.78	0.052	3.50	0.80	0.60	-0.239	3.00	0.80	0.60	-0.391
88HDZ9	9	5.20	0.91	0.84	-0.091	4.79	0.80	0.53	0.048	2.00	0.80	0.53	-0.600
93HDZ257	7	4.42	0.67	0.71	0.057	3.36	0.40	0.38	0.139	3.00	0.40	0.38	-0.067
93HDZ179	6	3.79	0.82	0.74	-0.121	3.00	0.00	0.00	-0.097	1.00	0.00	0.00	
88HDZ127	7	4.23	0.70	0.74	0.054	4.55	1.00	0.64	0.150	3.00	1.00	0.64	-0.667
88HDZ10	8	5.15	0.89	0.82	-0.087	4.22	0.60	0.47	0.103	2.00	0.60	0.47	-0.333
88HDZ81	7	4.13	0.77	0.75	-0.036	4.38	1.00	0.51	-0.091	3.00	1.00	0.51	-0.200
88HDZ50	5	2.77	0.51	0.47	-0.088	2.76	1.00	0.73	0.158	4.00	1.00	0.73	-0.429
93HDZ265	4	2.51	0.51	0.54	0.060	2.92	0.40	0.53	0.269	2.00	0.40	0.53	0.273
93HDZ164	7	4.59	0.86	0.75	-0.139*	2.93	1.00	0.73	-0.419*	4.00	1.00	0.73	-0.429
93HDZ223	5	3.98	0.67	0.72	0.077	3.35	0.60	0.64	-0.326*	3.00	0.60	0.64	0.077
93HDZ261	5	3.32	0.56	0.57	0.017	2.88	0.80	0.53	0.164	2.00	0.80	0.53	-0.600
93HDZ166	5	3.32	0.61	0.68	0.103	2.00	0.80	0.71	-0.063	3.00	0.80	0.71	-0.143
Average	6.31	3.99	0.71	0.70	-0.01	3.43	0.66	0.66	-0.01	2.69	0.68	0.54	-0.294**

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

H_o observed heterozygosity, H_e n.b. unbiased expected heterozygosity

departure from Hardy–Weinberg was indeed observed with an average F_{is} of 0.039 (P value <0.01) and is probably the result of a Wahlund effect.

Isolation by distance and genetic clustering analysis

We observed a significant correlation between Euclidean geographical distances and genetic inter-individuals distances (Mantel test, Pearson correlation coefficient $R = 0.449$, P value <0.001, 1,000 permutations) suggesting the patterns of differentiation of sampled individuals follow isolation-by-distance.

The STRUCTURE analysis suggested the existence of two main clusters, as this provided the modal value of ΔK (not shown). Cluster 1 consisted mostly of Bobankora and Bekaraoka individuals while cluster 2 contained most of the individuals from BAA with the exception of a social group located in the southeastern edge (Fig. 3a). Five individuals belonging to three different social groups from the very north of Bekaraoka were surprisingly assigned to cluster 2, despite the large geographical distance separating them from BAA. However, when we repeated the analysis for each cluster independently, cluster 2 was clearly subdivided into two groups: the first (2a) including only individuals from BAA and the second (2b) grouping the 5 individuals from the north of Bekaraoka. Cluster 1 did not show further substructure. Altogether it thus appears that three clusters could be identified. Also, we found that there were no individuals who had an estimated membership coefficient lower than 0.6. In other words, all 75

individuals could be assigned to one of the three groups with relatively high confidence as in Coulon et al. (2008). The spatial limits of the three genetic clusters are shown in Fig. 3b and their allelic richness, H_o, H_e and F_{is} are given in Table 2. A high significant average F_{st} was found between the clusters (F_{st} = 0.147, P value <0.001). All pairwise F_{st} between clusters were also highly significant (P value <0.01) ranging between 0.136 (between cluster 1 and 2b) and 0.16 (between cluster 1 and 2a).

GENELAND analysis. In the 10 runs performed to estimate K, the posterior density and the log-likelihood levels reached a plateau well before the end of the MCMC runs, indicating that they have reached convergence. In all the runs, the model number of genetic groups (K) was 4 with nearly no support for other K values (not shown). Therefore, subsequent runs were performed with K fixed to 4. The 10 runs selected on the basis of their posterior probability reached also convergence (not shown). In all cases, the 75 individuals were assigned to 4 spatially defined populations. In 9 out of the 10 runs, the identified clusters were consistent with those obtained with STRUCTURE (see Fig. 4): the main difference is that GENELAND creates a new cluster in the central part of Bekaraoka. When we repeated the Geneland analysis, excluding individuals sampled in BAA in order to have a more homogeneous spatial sampling, the results were consistent since we obtained 3 clusters which corresponded to the 3 first clusters uncovered with the whole sampling. The tenth run (with the lowest posterior probability) presented a slight change in the definition of the clusters: the

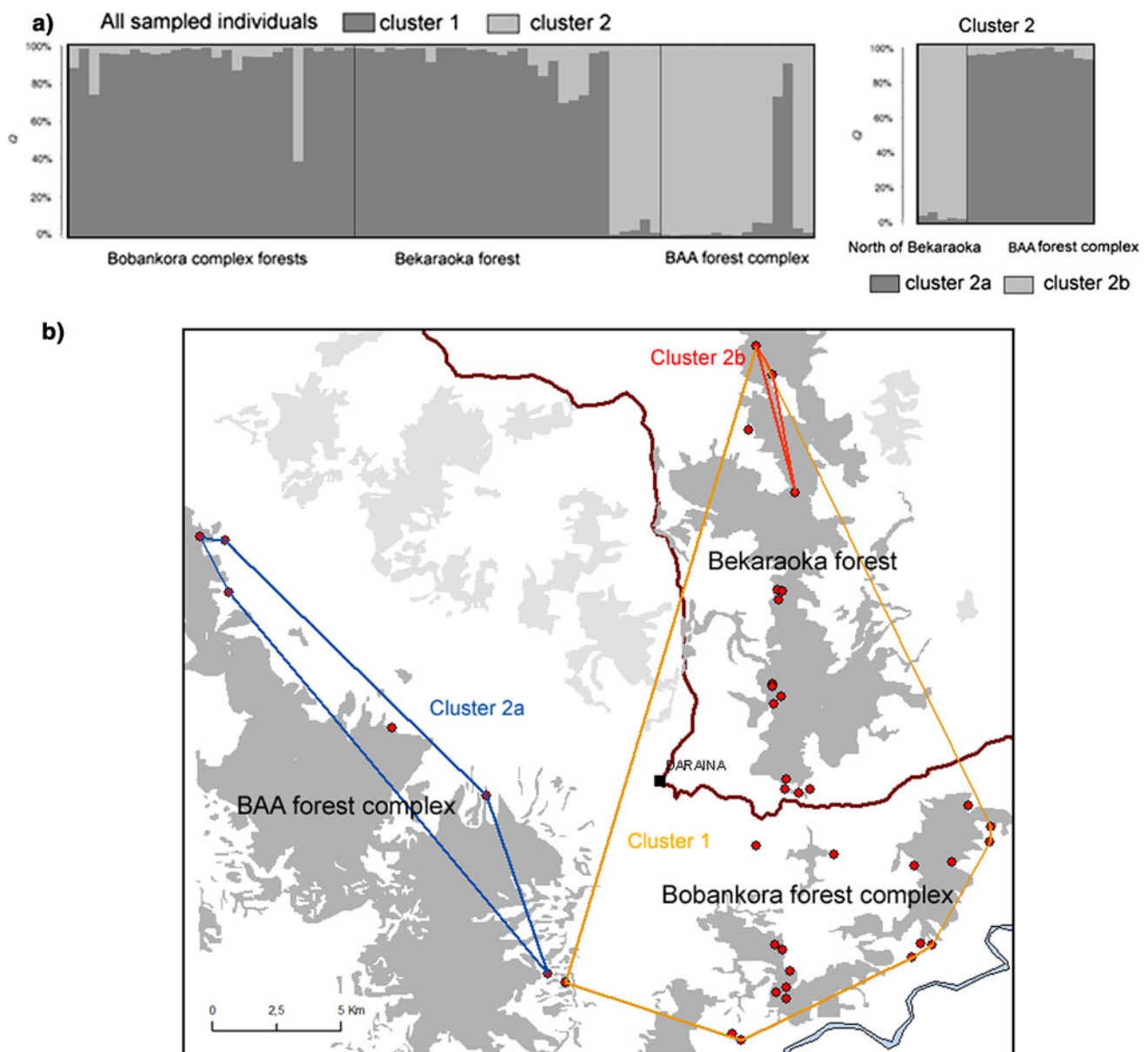


Fig. 3 Genetic clustering analysis with STRUCTURE. **a** Proportional membership (Q) of each sifaka in the clusters identified by STRUCTURE. The *left part* corresponds to the first analysis performed on all individuals and that identified two main clusters (cluster 1 and cluster 2). The *right part* corresponds to the analysis

performed on individuals assigned to cluster 2. Each individual is represented by a single *vertical bar* with its locality of origin below. **b** Minimum convex polygons containing individuals from the same genetic cluster

southernmost samples from Bekaraoka formed a cluster on their own instead of being grouped with Bobankora in the south. Also, the northernmost samples of Bekaraoka were grouped together with the groups from Bekaraoka's central part, instead of forming an independent group. Thus, most changes in the tenth run occur within Bekaraoka.

TESS analysis. Despite the presence of isolation-by-distance, the assignment results obtained for $k = 2$ and $k = 3$ with TESS were very similar with those obtained

with the two previous approaches (data not shown). For $K > 3$, the DIC continues to decrease but the results were not consistent among runs and uncovered genetics units corresponding to related individuals belonging to the same or neighbouring social groups in Bekaraoka. This is also what we observed for $K > 3$ with STRUCTURE. These subdivisions may thus reflect the family-induced structure rather than real population structure (Bergl and Vigilant 2007; Anderson and Dunham 2008).

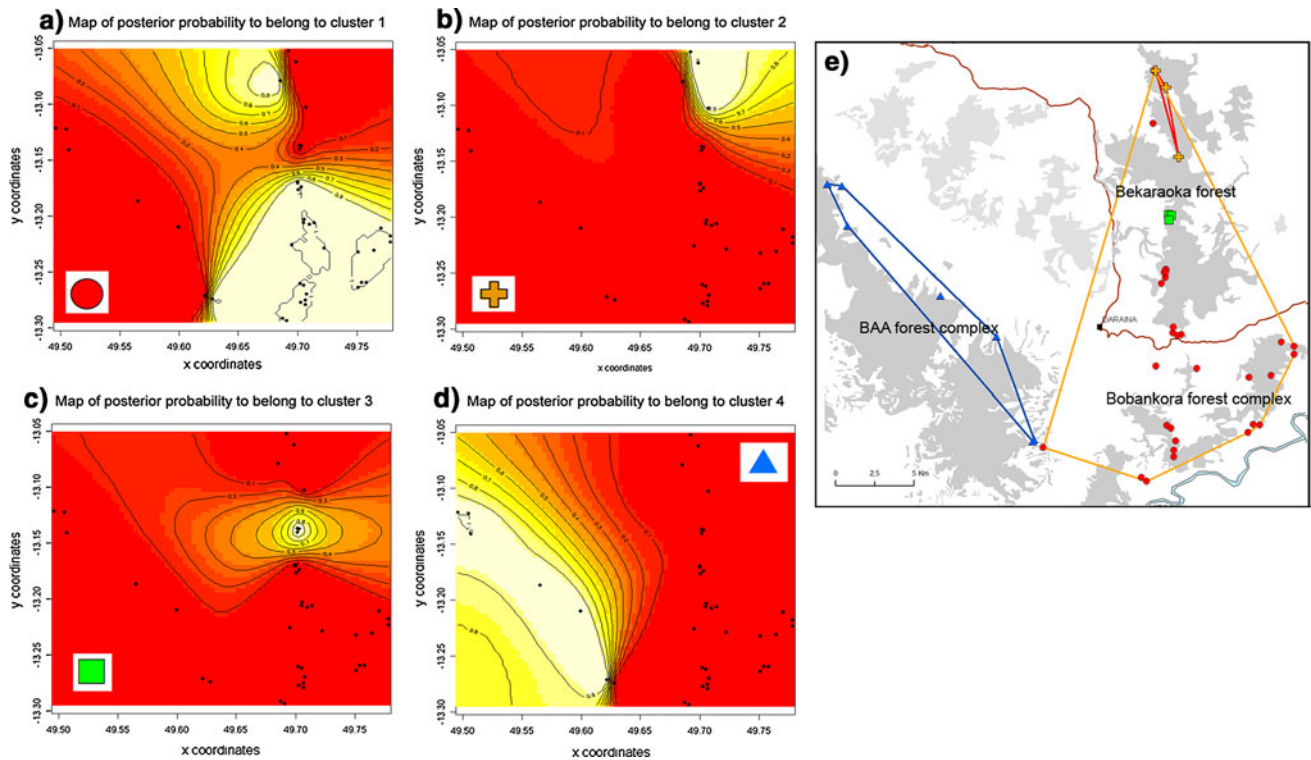


Fig. 4 Spatial genetic clustering analysis with GENELAND. The contour maps show the posterior probability to belong to **a** cluster 1, **b** cluster 2, **c** cluster 3 and **d** cluster 4 computed with GENELAND for

the best run (the 8 following runs were highly similar). Assignment results corresponding to these four clusters were plotted on **e** the map of the sampling area together with the minimum convex polygons

Discussion

Sampling strategy

For the first time, a population genetic study based on a non-invasive sampling strategy was carried out on a lemur species. This allowed us to gather a large amount of genetic material in a relatively short period without any physical contact with individuals. A major difference between our study and most of the studies using this material (e.g., Bellemain et al. 2007 for bears; Bergl and Vigilant 2007 for gorillas; He et al. 2008 for pandas) is that we were able to sample faecal pellets while we were observing animals (but see Lathuillière et al. 2001 for Barbary macaques Goossens et al. 2005, 2006 and Jalil et al. 2008 for orang-utans). Our sampling was made easier by the fact that golden-crowned sifakas groups did not run away when we approached since they are still protected by local taboos (no hunting or other direct pressures). Another possible factor is the fact that *P. tattersalli* appear to have a fast and ineffective plant digestion (Irwin 2006). As a consequence individuals defecated relatively frequently and assuming that the groups were visited in the morning, we did not need to wait for a long time to obtain samples (often less than 2 h). Moreover, sampling fresh material limited DNA degradation via bacteria and fungi, which also contributed to our high success

rates, with 94% of the DNA extracts providing positive PCRs. As a comparison, several recent non-invasive studies obtained lower success rates, e.g., 57% in gorilla (Bergl and Vigilant 2007) and 38% for the Giant Panda He et al. (2008). However, a great part of this success may be due to the recent 2CTAB/PCI extraction method (Vallet et al. 2008) since our success rate was only of 58% before using it. Moreover, the efficiency of this new method is confirmed by the high global amplification success obtained by these authors for *Gorilla* and *Macaca* species (85%) although they did not sample the fecal material just after defecation. Finally, this sampling strategy enabled us to minimize the proportion of individuals sampled multiple times since we were usually able to identify the defecating animals. The fact that social groups were usually small made it relatively easy to identify defecating individuals and hence find their faeces on the ground. As we showed, we only found two cases where the same individual was involuntarily sampled twice. Similarly Goossens et al. (2005, 2006), with a similar sampling strategy, sampled only one individual twice among the 201 genotyped orang-utan individuals. In recent studies, with samples obtained without seeing the individuals, the proportion of samples identified as unique individuals is often much lower. For instance Bergl and Vigilant (2007), estimated that only 38.6% (71 out of 184) of gorilla faecal samples came from unique individuals.

DNA genotype reliability

Our results indicate that the number of PCRs per locus can be minimized without reducing the reliability of the genotypes by following the comparative genotyping protocol proposed by Frantz et al. (2003). For all the tested samples, the same consensus genotypes were reached with fewer PCRs (22% less) than the multi-tubes approach of Taberlet et al. (1996). These results are consistent with those obtained by Hansen et al. (2008) who compared both methods using otter's faeces. These authors showed that, on average, 4.5 (± 0.5) positive PCRs were needed with the comparative approach compared to an average of 6.8 (± 0.6) for the multi-tubes protocols (corresponding thus to a decrease of $\sim 33\%$). Our error rates (overall 5–39% for the dropout allele) were comparable to those of other studies using faeces (Morin et al. 2001; Hansen et al. 2008). Some loci provided much lower error rates than others (e.g., 93HDZT164 compared to 93HDZT265), and a wider-scale analysis would be required to determine the factors responsible for these differences (Taberlet et al. 1999; Hansen et al. 2008). Altogether our results suggest that it should be possible to significantly reduce the cost of faecal DNA studies without reducing the quality of the genotypes. This conclusion still needs to be tested on a wider range of organisms.

Genetic diversity

Golden-crowned sifakas, sampled in the three main forested areas included in the S.F.U.M. appear to maintain a high level of genetic variability ($MNA = 6.3$, $SD = 1.43$ and mean $H_e = 0.72$) despite the large fragmentation level of their environment and an estimated number of individuals ranging between 6,100 and 10,000 individuals for the whole species (Vargas et al. 2002, but see Quéméré et al. 2009 for higher estimates). These diversity values are on the same order than those observed in the few studies available in other sifaka species (Rakotoarisoa et al. 2006 on *P. coquereli* over 20 loci Lawler et al. (2001) on *P. verreauxi* over 16 loci). The golden-crowned sifaka variability levels are also higher than those observed for three mouse lemur species (genus *Microcebus*) by Olivieri et al. (2008) although the latter are expected to have a larger effective size. However, these comparisons should be taken cautiously because of differences in the (a) nature of the markers used (specifically designed vs. heterologous primers) and (b) sampling design in term of extent and resolution of the data (Storfer et al. 2007). For instance, the comparison with other sifaka species is complicated by the fact that both *P. coquereli* and *P. verreauxi* have wider distributions but were sampled in relatively isolated forest

fragments. Until wider sampling is carried out for all these species, the comparisons should be thus be made with care.

Interestingly, we observed that the high H_e values in our samples were also associated with a small number of alleles. This is typically observed in previously large populations subjected to a demographic bottleneck (Nei et al. 1975; Cornuet and Luikart 1996) and this has been linked to habitat fragmentation in other primates (Goossens et al. 2006 in orang-utans; Olivieri et al. 2008 in *Microcebus* species; Craul et al. 2009 in *Lepilemur* species). Preliminary tests performed on the data presented here suggest that this is also the case for *P. tattersalli* (i.e., there is a bottleneck signal) but a full analysis of this question would be beyond the scope of the present study.

Population structure

The analysis of the population structure showed a relatively high and significant level of genetic differentiation between the uncovered genetic clusters (average $F_{st} \sim 0.147$). While we found some evidence of a separation between eastern and western samples (Individuals from BAA appeared to be differentiated from those sampled in the two others forests), the unpaved national road did not appear as a clear barrier to gene flow. Indeed, this road also goes between Bekaraoka and Bobankora but individuals sampled in Bobankora were grouped with the southernmost individuals from Bekaraoka forest using STRUCTURE, GENELAND (Figs. 3, 4) and TESS (not shown). This result is in agreement with field observations: several groups were observed in the network of riparian corridors and small forest patches between these two forest complexes (personal observations by Erwan Quéméré 2006). It should be noted that the shortest Euclidean geographic distances between Bekaraoka/Bobankora and the forests that comprise the BAA, although less than 10 km, correspond to land highly impacted by local residents. Indeed, a large area of agricultural and zebu cattle grazing savanna surrounds Daraina, the main village of the Loky-Manambato region which is located southwest of Bekaraoka and east of BAA, and it seems unlikely to us that sifakas cross this anthropized area. In the southernmost part of the distribution a forest fragment, called Ankaramy and located between Bobankora and BAA (Fig. 1), appeared to be highly impacted by slash-and-burn agriculture (called "Tavy") and no individual was observed in 2006 despite several census missions suggesting again that it is not the road that seems to represent a major barrier to gene flow between the east and the west, at this level. One possible explanation for the patterns observed could actually be the presence of the Manankolana River (Fig. 1). Although it is currently almost dry for much of the year, it may be a significant barrier for sifakas dispersion during the mating season which takes

place during the rainy season. At this stage the lack of data between Bekaraoka and BAA makes it difficult to test this hypothesis. This hypothesis is tempting given the role played by rivers to shape genetic diversity (and speciation) in many lemur species (Wilmé et al. 2006; Olivieri et al. 2008) and in others primates (Eriksson et al. 2004; Goossens et al. 2005; Jalil et al. 2008). Also, the fact that *P. tattersalli*'s range is to a large extent limited by two main rivers further supports the hypothesis. However, at this stage it is not fully supported by the individuals belonging to three different social groups sampled in a valley in the northern part of Bekaraoka. These individuals were first grouped with individuals sampled in BAA using STRUCTURE (Fig. 3a). The genetic proximity of these individuals with those of BAA may reflect connections between the northeastern parts of BAA and Bekaraoka via the unsampled forest fragments to the west of Bekaraoka/northeast of BAA. However, when we repeated this analysis within this cluster, these same individuals were clearly separated from individuals from BAA. The GENELAND analysis confirmed this separation since individuals from the northern part of Bekaraoka correspond to one of the four uncovered clusters. Therefore, in order to better understand the possible links between the groups sampled in the S.F.U.M. it would be important to obtain data from the sifaka groups living in the fragments outside the protected area. In any case, our study should be considered as preliminary, and none of the groups uncovered should be taken at face value. We agree with Anderson and Dunham (2008) who see these methods as exploratory methods, that would be used to increase our understanding of the biology of endangered species (see also Chikhi and Bruford 2005).

To conclude on population structure, *P. tattersalli* seems to be a surprisingly genetically variable species despite a very small distribution area. It would thus be important to perform a fine-scale social and reproductive structure study to determine the exact relationships among individuals from several social groups across the different forest types and across the whole region including the smallest fragments before they disappear.

Conservation implications

Altogether, our results suggest that the level of genetic diversity is relatively high and that there is a strong genetic differentiation despite a rather small distribution area. The first point is relatively good news for the conservation of this species, while the second suggests that unique diversity might be found outside the S.F.U.M. in non-studied forest fragments. The significant diversity maintained in *P. tattersalli* suggests a rather important resiliency to habitat fragmentation combined with an important behavioral

plasticity as revealed by its ability to live in several forest types. The current level of human disturbances may not be as important as in other regions of Madagascar, where other *Propithecus* species are hunted. Also, despite a high level of habitat fragmentation, the genetic diversity may be maintained by the preservation of relatively large forest patches and connections between some of them. Until now, the relatively low human density and strong local taboos have limited the human pressures on their environment. Taboos forbid, for instance, golden-crowned sifakas hunting but also the wood cutting in riparian forests which may be crucial for their migrations. However, causes for concern do exist as the Loky-Manambato region is expected to suffer from significant disturbances in the near future (most likely less than 5 years). The tarring of the national road linking Diego Suarez (Antsiaranana) in the north and the economically important vanilla region on the eastern coast combined with the arrival of immigrant populations and gold mining companies could have significant and negative effects for the biodiversity of the whole region. Our results suggest that the S.F.U.M. should probably be enlarged to capture the unique diversity of this critically endangered species, which is likely to suffer from new anthropogenic pressures in the near future.

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