# Stuck in fragments: population genetics of the Endangered collared brown lemur *Eulemur collaris* in the Malagasy littoral forest.

Bertoncini S.[1], D'Ercole J.[1,2], Brisighelli F.[3,4], Ramanamanjato J-B.[5], Capelli C.[4], Tofanelli S.[1]* Donati G.[6]*

[1] Dipartimento di Biologia, Università di Pisa, Via Ghini 13, 56126 Pisa, Italy.

[2] Centre for Biodiversity Genomics, University of Guelph, Guelph, N1G 2W1, Ontario, Canada

[3] Sezione di Medicina Legale-Istituto di Sanità Pubblica, Università Cattolica del Sacro Cuore, Roma, Italia

[4] Department of Zoology, University of Oxford, South Parks Road, OX1 3PS Oxford, UK.

[5] QIT Madagascar Minerals, Fort-Dauphin, Madagascar

[6] Department of Social Sciences, Oxford Brookes University, Oxford, UK

Number of text pages: 41; Number of figures: 3; Number of tables: 2

Running headline: Population genetics of *Eulemur collaris*

Key words: *Eulemur collaris*, Littoral Forest, Madagascar, gene flow, fragmentation

* Corresponding authors and co-senior authorship:

Giuseppe Donati

Department of Social Sciences

Oxford Brookes University, UK

gdonati@brookes.ac.uk

Sergio Tofanelli

Department of Biology

University of Pisa, Italy

sergio.tofanelli@unipi.it

## ABSTRACT

### Objectives

The Endangered collared brown lemur (*Eulemur collaris*) is the largest primate living in the littoral forest of southeastern Madagascar, a top priority habitat for biodiversity conservation on the island. Since this lemur is a key seed-disperser, an evaluation of the structure and connectivity of the populations surviving in the forest fragments is urgently needed to guide conservation plans.

### Materials and Methods

Genetic variability at autosomal microsatellites and mitochondrial DNA was investigated in a total of 49 collared brown lemurs sampled by non-invasive methods in three littoral forest fragments and in the nearby lowland humid forest.

### Results

The overall genetic diversity of *E. collaris* in the southeastern coastal region of Madagascar was lower than in other populations, as well as in other lemur species. The population appears highly structured, with less variable and more inbred groups inhabiting the littoral forest fragments compared to the inland area. Major barriers to gene flow were identified isolating littoral forest fragments from each other and from the inland lowland humid forest.

### Discussion

Medium to long-term drift and scarce gene flow is the scenario that best explains the current genetic distribution. Habitat discontinuities such as rivers and grassland between forest fragments played a major role in structuring the population. A common history of size contraction is pointed out by several genetic estimators, indicating a possible ecological crisis triggered around 1300 years ago. The adoption of strategies aimed at facilitating gene flow and population growth appears crucial to delay further loss of genetic diversity.

## 1. INTRODUCTION

Madagascar is known for its unmatched levels of endemic fauna and flora (Goodman and Benstead, 2003). However, the arrival of humans at least 2,500 years ago coincided with the extinction of many species, including 17 taxa of large lemurs (Dewar, 2014). The most recent IUCN reassessment found that 94% of living lemur species are currently threatened, which makes these primates the most endangered group of large vertebrates on earth (Andriaholinirina et al., 2014; Schwitzer et al., 2014a). The main threat to lemur survival is habitat loss and fragmentation, with 52% of forest loss occurring between 1950 and 2010 (Schwitzer et al., 2014b). With this situation continuing, rapid assessments of structure and connectivity of the remnant populations are crucial to define conservation units and to identify priority areas for conservation.

The littoral forest of southeastern Madagascar, a type of lowland humid forest growing on sandy soil, hosts an exceptional level of biodiversity within Madagascar (Dumetz, 1999; Ganzhorn, 2001; Rabenantoandro et al., 2007). Today only small pockets of forest surrounded by grassland are left (Ganzhorn et al., 2007) and the area is severely threatened by intensive human exploitation, such as slash-and-burn cultivation and charcoal production (Bollen and Donati, 2006; Consiglio et al., 2006; Ingram and Dawson, 2006). Additionally, the largest mining project in the country is currently ongoing in the region with an expected further reduction of habitat (Vincelette et al., 2003). Recent paleo-ecological analyses from sedimentary sequences indicate that fluctuations in sea level and rainfall triggered several ecological switches from forest to grassland and vice-versa during the late Holocene (Virah-Sawmy et al., 2009a). Thus,

90 the littoral forest fragments may have also played the role of critical refugia for the local
91 fauna and flora in the extremes of climatic variability on the island.
92 The conservation status of the collared brown lemur (*Eulemur collaris*), the largest
93 lemur species living in the southeastern littoral forest, has been recently updated from
94 the IUCN category of Vulnerable to Endangered for its rapid population decline due to
95 hunting, habitat loss, and fragmentation (Bollen and Donati, 2006; Andriaholinirina et al.,
96 2014). The extirpation of this lemur from its habitat is likely to have a cascade effect on
97 forest regeneration (Ganzhorn et al., 1999; Federman et al., 2016), given its important
98 role as seed disperser for the littoral forest ecosystem (Bollen et al., 2005; Donati et al.,
99 2007a). Hence, there is an urgent need to manage the remaining sub-populations
100 before further environmental or anthropogenic changes take place.
101 The collared brown lemur ranges from Tolagnaro (Fort Dauphin) in the south, to the
102 Mananara River in the north, to the Mandrare River in the west (Andriaholinirina et al.,
103 2014). The largest habitat for this species is currently the inland lowland and semi-
104 montane humid forest, a frayed but continuous habitat separated from the littoral forest
105 fragments by kilometers of grass stretches and wetlands (Mittermeier et al., 2010). An
106 assessment of the overall genetic diversity for this species has been conducted within
107 three protected areas of continuous lowland and semi-montane humid forest
108 (Ranaivoarisoa et al., 2010). The survey revealed a population with an overall
109 appreciable degree of genetic variation and potential disruption of gene flow between
110 northern and southern areas.

A genetic assessment of the littoral forest sub-populations has been never conducted and information on gene flow within these sub-populations and/or between them and the lemurs occurring in the inland humid forest are lacking. The collared brown lemurs seem to have a good ecological tolerance to fragmented habitats and they have also been observed to cross short distances of grassland (Donati et al., 2007b, Ganzhorn et al., 2007). However, the small size of the littoral forest patches left in the area (all less than 300 ha), the unknown dispersal distances, and the presence of rivers and roads between fragments raises questions as to whether these sub-populations have been able to maintain viable levels of genetic diversity.

Here, we investigated genetic diversity at eight autosomal short tandem repeats (STRs) and the mitochondrial D-loop region in DNA extracted from fecal samples of three subpopulations living in the littoral forest fragments and two sub-populations from the nearby lowland humid forest of the Tsitongambarika Protected Area (Fort Dauphin region). We aimed at estimating: i) the apportionment of genetic variance between habitats and among sub-populations, including the occurrence of natural or anthropogenic barriers to gene exchange; ii) the correlation between eco-geographic and genetic factors; iii) clues of the historical demography of the species within the region.

## 2. MATERIALS AND METHODS

### *2.1 Study area*

Our research was conducted in the Anosy region on the southeastern coast of Madagascar (Fig. 1) (Ramanamanjato et al., 2002). The largest populations of collared brown lemurs occur in the continuous block of lowland and mid-altitude humid forests growing along the Anosy and Vohimena mountain chains (Andriaholinirina et al., 2014). The most eastern of these chains is today included in the Tsitongambarika Protected Area (hereafter TGK), created in 2008 and covering an area of over 60,000 hectares (Birdlife International, 2011; Schwitzer et al., 2013).

Three relict sub-populations of collared brown lemurs occur in littoral forest fragments lying on the sandy coast east of TGK (Fig. 1). One of these fragments is a partially degraded block of around 220 ha in the Mandena Conservation Area (hereafter MND), around 11 km north of Fort Dauphin (Ganzhorn et al., 2007). The other two areas (hereafter S9 and S17) represent more intact blocks of littoral forest (S9: 290ha and S17: 220ha) in the Ste Luce Conservation Zone (hereafter STL), around 30 km north of Fort Dauphin. The area between MND and STL, around 18 km between the nearest points, includes degraded fragments of littoral forest, grasslands, and small rivers (Fig. 1). The MND fragment is separated from the nearest edge of TGK by approximately 3 km of grassland and eucalyptus plantations, while around 8 km of grassland exist between TGK and the two STL fragments. These last, S9 and S17, are approximately 1 km apart at their nearest points but separated by a stretch of lagoon.

*2.2 Study species*

155 Collared brown lemurs are cat-sized arboreal strepsirrhines living in multi-male, multi-
156 female groups (Donati et al., 2007a). Average group size is larger in STL (median: 7,
157 range: 2–17, n = 13 groups) than in MND (median: 3, range: 1–6; n = 11 groups)
158 (Donati et al., 2011a). In the lowland humid forest of TGK average group size is 5
159 (range: 2–7; n = 11 groups) in TGK1 (Norscia et al., 2006) and 5 in TGK3 (range: 3-18;
160 n = 32) (Nguyen et al., 2013; Campera et al., unpublished).
161
162 *2.3 Sampling*
163
164 The study protocols were authorized by the Commission Tripartite of the Direction des
165 Eaux et Forêts de Madagascar (Autorisation de recherche
166 n.29/11/MEF/SG/DGF/DCB.SAP/SCB du 20/01/11).
167 A total of 54 fecal samples of collared brown lemurs were collected with a non-invasive
168 method from 2011 to 2013 in the study area. Stool samples in MND (13), S9 (20) and
169 S17 (5) were gathered from habituated lemur groups during behavioral observations
170 (Balestri et al., 2014). TGK1 (7) and TGK3 (9) samples were gathered according to
171 Nguyen et al. (2013) from non-habituated groups while walking line transects. In the
172 latter case, each area was walked only once to avoid sampling the same group twice.
173 Samples were collected from different animals immediately after defecation. Site, group,
174 date, time, and identity of the donor were recorded. Fecal samples were preserved in
175 96% ethanol while in the field and stored at 4°C before further processing for DNA
176 extraction (Balestri et al., 2014).

177

178 *2.4 Microsatellite genotyping*

179

180 DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany)
181 following the manufacturer's instructions. Eight autosomal STR loci (Table 1 and S1)
182 were selected based on the Polymorphic Information Content (PIC mean value: 0,7)
183 and the number of alleles (k mean value: 6) after a careful survey of the available
184 literature (Jekielek and Strobeck, 1998; Tokiniana et al., 2009; Ranaivoarisoa et al.,
185 2010). PCR primers were redesigned using "Primer 3" v. 4.0.0 (Koressaar et al., 2007;
186 Untergasser et al., 2012) (Supplementary material Table S1) to get shorter amplicons
187 (Frantzen et al., 1998). Evidence of null alleles was evaluated with MICRO-CHECKER
188 (van Oosterhout et al., 2004).
189 Amplification products of singleplex reactions were separated using capillary
190 electrophoresis (ABI 310 Genetic Analyzer, Applied Biosystems, Foster City, CA). Allele
191 lengths were called using an internal size standard (ROX-500) and the Gene Mapper
192 software v. 4.0 (Applied Biosystems, Foster City, CA). Individual STR data are shown in
193 Supplementary material Table S2.

194

195 *2.5 Mitochondrial DNA haplotyping*

196

197 Mitochondrial DNA was sequenced at 320 bp of the mtDNA Hyper-Variable Region
198 (HVR) using modified primers [LEMUR_L_FW (5'-TCGTGCATTATGTGCCTTTC-3') and

LEMUR_L_REV (5'-ATGGGCGTAGAGCAAGAAGA-3')] from Wyner et al., (2002). PCR products were purified with the GenElute™ PCR Clean-Up Kit (Sigma, USA). Sequencing reactions were performed for each strand with the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. Lengths of the purified PCR products were measured by the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). CHROMAS 2.01 (http://chromas-lite.software.informer.com/2.0/) software was used to read ABI electropherograms, whereas DNA Aligment 1.2.0.0 (http://www.fluxus-engineering.com/align.htm) and BioEdit 7.1.3 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) were used to align the sequences to the Reference Sequence of *Eulemur collaris* (Wyner et al., 2002; Genbank ID: AF257980) and to assign haplotypes.

All the sequences have been deposited in GenBank (Accession number: KU196680-KU196722). Haplotype distribution across sites is reported in Supplementary material Table S3.

*2.6 Quality controls*

Reliability of microsatellite genotypes was ensured by a stepwise system following Frantz et al. (2003). Briefly, two PCR amplifications per locus were initially performed on each DNA extract and a heterozygous state was called if its alleles were scored at least twice. Amplifications were replicated up to 5 times until an allele state was confirmed

221 twice for heterozygous genotypes and three times for homozygous genotypes. Three
222 blank controls were used in every PCR reaction to detect cross contaminations. The
223 probability of genotyping errors, namely alleles that occurred only once (drop-ins) and
224 PCR failures of one heterozygous allele (dropouts), was evaluated by GIMLET 1.3.3
225 (Valière, 2002). A two-tailed exact test was performed with the GENEPOP v.3.4
226 software (Raymond and Rousset, 1995) to test deviations from Hardy-Weinberg
227 Equilibrium (HWE) and Linkage Disequilibrium across loci (LD) (Supplementary material
228 Table S4 and Table S5).
229 Reliability of mtDNA sequencing was ensured by replicates performed on a sub-total of
230 26 samples, those containing a sufficient amount of DNA after STR analyses and the
231 first round of mitochondrial amplifications.
232
233 *2.7 Statistical analyses*
234
235 The population diversity parameter theta estimated from expected homozygosity under
236 a stepwise mutation model ($\theta_H$, Ohta and Kimura 1973), unbiased diversity index ($h$, Nei
237 1987), pairwise Fst distances (Weir and Cockerham 1984) were calculated using
238 Arlequin, v.3.5.1.2. (Excoffier and Lischer 2010).
239 HP-Rare v. 1.1 (Kalinowski et al., 2005) was utilized to calculate rarefied allelic richness
240 ($A_r$). Detailed investigation of the genetic structure was performed by adopting the
241 software SAMOVA 1.0 (Dupanloup et al., 2002), that explores the grouping criteria
242 maximising the genetic differentiation among sub-populations.

MANTEL 3.0 (John Relethford's Software Page http://employees.oneonta.edu/ relethjh/programs/) was performed to evaluate the correlation between Fst pairwise distances and linear distances between fragments. The final P-value has been calculated upon 1000 permutations.

Using GENEPOP v.3.4 we ran a two-tailed Markov Chain-based test (Guo and Thompson, 1992) for HWE, and estimated the number of effective migrants per generation (Nm) using the private alleles' method of Barton and Slatkin (1986), and the observed ($H_o$) and unbiased expected heterozygosity ($H_e$) under the HWE (Raymond and Rousset, 1995). The Bonferroni correction for multiple tests was applied when necessary (adjusted P-value = observed P-value x n individual tests).

To identify the main genetic barriers between sites, the Monmonier's (1973) maximum difference algorithm was applied to the pairwise Fst matrix on a Delaunay triangulation network (Brassel and Reif 1973), using Barrier v2.2 software (Manni et al., 2004). Briefly, given P sampling points in a two-dimensional Euclidean space a set of triangles is obtained connecting the points as to maximize the minimum angle of all the possible angles of the triangles. Next, an algorithm is applied to identify the edges where pairwise Fst distances between sampling points are the largest. Barriers of first, second and third rank were computed.

Non-parametric Spearman correlations ($r_s$) were used to test the potential association of $A_r$, $H_o$ and $h$ with area size. Although TGK is a continuous block of forest, the two sampling localities (TGK1 and TGK3) were analyzed separately because they are linearly separated by about 30 km (Holmes et al., 2013).

To test whether the inland and coastal collared brown lemurs have experienced genetic bottlenecks we first used the M-ratio approach (Garza and Williamson, 2001). Its rationale is based on the fact that during size declines the recovery in the number of alleles is slower than the range in allele size. In each sample, the k/r ratio (M) averaged across loci, where k is the number of observed alleles and r the range in allele size (maximum size - minimum size +1), was compared to the 95% critical value of M (Mc) obtained by 10,000 simulations under a mutation-drift equilibrium using the program Critical_M (NOAA Fisheries, La Jolla, USA). Mutation reference parameters of a two-phase mutation model were used following Parga et al. (2012) and Peery et al. (2012).

Secondly, we used the method implemented in the software Bottleneck v.1.2 (Cornuet and Luikart 1996). It tests the occurrence of a transient excess in the level of heterozygosity compared to that expected under a mutation-drift equilibrium. A Wilcoxon signed-rank test was used to check microsatellite loci showing heterozygosity excess given different proportions of multistep mutations in a two-phase model.

FSTAT 2.9.3.2 (www2.unil.ch/popgen/software/fstat.htm) was used to estimate the relatedness (*r*) in lowland and littoral samples following Queller and Goodnight (1989).

The Time since the Most Recent Common Ancestor (TMRCA) of mitochondrial lineages was estimated by the Walsh's formula (Walsh 2001) implemented in the online TMRCA calculator (http://clan-donald-usa.org/index.php/tmrca-calculator) using human pedigree-based mutation rate for HVR ($7x10^{-5}$ mut/site/gen, Madrigal et al., 2012) and 8 years as averaged generation time (Andriaholinirina et al., 2014). The lower 95%

confidence value of the distribution was considered as the minimum time that elapsed since the two haplotypes diverged.

## 3. RESULTS

### *3.1 Quality controls*

Microsatellite analyses showed that 5 out of 54 DNA samples (9%) gave a call rate lower than 0.25. They were removed from the STR analyses.

Amplification success rates varied from 77% to 93% across the eight loci (mean: 87%), neither false nor null alleles were inferred and no evidence of LD was found (Supplementary material Table S5). Estimated dropout rates varied from 4% to 39% (mean 16%) and three loci (EFR8, 104HDZ127, 104HDZ9) showed significant departures from Hardy–Weinberg equilibrium due to a deficiency in heterozygotes (Supplementary material Table S4). However, deviations were observed in a single population (S9), suggesting that this result may have been caused by the genetic characteristics of the groups under study rather than by genotyping errors.

After removing either the S9 sample or the deviating loci from the analyses, HWE was respected for all samples and all loci and the overall pattern of genetic structure and relatedness among groups (lowland and littoral population samples) did not change (Supplementary Table S6).

307 Reliability of the sampling methods and informativeness of the chosen STR panel are
308 supported by the fact that all animals showed different STR profiles.
309 MtDNA analysis showed that 11 out of 54 DNA samples (20%), among which the 5
310 samples already excluded by STR analyses, did not yield reliable products. They were
311 removed from sequencing analyses. Replicated sequencing assays of mitochondrial
312 DNA always matched previous results.
313
314 *3.2 Genetic diversity and structure*
315
316 As a whole, the spatial analyses based on both STR (*h, Ar,* $H_o$*,* $H_e$ values) and mtDNA
317 (*h* values), showed that the population samples inhabiting the inland forest of TGK
318 display higher genetic diversity (t-test: P = 0.025 upon STR *h*; P = 0.000 upon mtDNA *h*)
319 than those inhabiting the littoral forests (Table 1). In particular, the samples from MND
320 always exhibited the lowest diversity and the samples from TGK3 (STR *h* excepting) the
321 highest.
322 Despite the current heterogeneous distribution of diversity, other genetic estimators
323 suggested a common demographic history for lowland and littoral humid forests. In fact,
324 the population diversity parameter theta (Table 1), which is a mutation-scaled measure
325 of effective population size inversely proportional to the amount of drift experienced by
326 the population, showed low and very similar values across samples. This makes it
327 plausible to speculate either a single ancestral population with few breeding animals or
328 synchronous size contractions in multiple groups, both followed by independent

329 evolution. A prolonged limitation to gene flow among forest patches was indicated
330 (Table 2) by:
331 i) the high differentiation among sub-populations (mean Fst: 0.236±0.068, adjusted P-
332 value <0.001); ii) the high rate of private alleles and its apportionment within single
333 groups (35.9% of the total alleles; range across groups 0-20%, mean 12.8%) from
334 which a uniform low number of migrants per generation under a migration-drift model
335 was inferred (0.229 ± 0.098 SD; range 0.13-0.50).
336 Significant departures from the Hardy-Weinberg equilibrium were due to heterozygosity
337 deficiency at three loci from the same group (S9). Observed heterozygosity was lower
338 than expected at all loci and in all groups (Table 1 and Table S4), suggesting a
339 moderate-to-high level of inbreeding within groups. A higher level of relatedness was
340 observed between individuals living in littoral ($r$ = 0.345) than in lowland forests ($r$ =
341 0.179).
342 The total study area size showed a positive correlation with allelic richness ($r_s$ = 0.98, P
343 = 0.005) and strong albeit not significant trends with both, Nei's genetic diversity ($r_s$ =
344 0.87, P = 0.054) and observed heterozygosity ($r_s$ = 0.82, P = 0.089). However,
345 geographical distance was not a good predictor of genetic distance (Mantel test: $R^2$ =
346 0.030, P = 0.689). In fact, no substantial differentiation between TGK populations (Fst:
347 0.080, P = 0.046) has been observed despite being separated by about 30 km, whereas
348 an abrupt genetic transition (Fst: 0.268, P = 0.000) was found between the neighboring
349 S9 and S17 fragments. The lack of isolation-by-distance (IBD) is depicted in Figure 2,

where geographic distance was plotted against a normalized measure of Fst varying from zero to infinity ($R^2$ = 0.016, P = 0.725).

The SAMOVA analysis revealed that genetic variance was apportioned according to a two level structure: a higher level separating MND, S17 and S9 littoral fragments in that order; a secondary level clustering the collared lemurs in lowland and littoral forest areas. Accordingly, the optimal number of groups that maximized the among-group variance (Fct, see Table S7) is four. The top-rank barrier (I, Fig. 3) calculated by the Monmonier's algorithm separated the MND sample from TGK1 and S17 samples, while the second- and third-rank barriers (II and III, Fig. 3) further isolated S17 and S9 samples. The boundary formed by combining the three barrier lines crossed the savannah-like ecotone, which separates littoral from lowland forests.

*3.3 Demographic inference from genetic analysis*

Signatures of size contractions were detected (Supplementary Table S8) by means of the M-ratio test for all the sub-samples using an estimation of theta obtained from the observed homozygosity under a stepwise mutation model ($\theta_H$, Ohta and Kimura 1973). A generalized size reduction held also when M was calculated choosing values of theta both lower (θ=0.1) and higher (θ=4) than $\theta_H$, under a more realistic two-phase mutation model with varying multi-step mutations proportions (Pg = 0.10 and 0.22). Only for higher values of theta (10) and/or Pg (0.40), did M fall above the critical ''threshold''

371 value for a mutation/drift equilibrium (Mc), which indicates substantial population
372 stability (Supplementary Table S9).
373 Further support to size reduction is also given by the absolute values of M (range: 0.53-
374 0.67), all of which are below 0.68, commonly considered as critical in bottlenecked
375 vertebrate species (Garza and Williamson 2001) and far lower than those obtained in
376 the Endangered wild populations of *Lemur catta* from South-West Madagascar under
377 equal parameters (range: 0.66-0.71, Parga et al., 2012).
378 Reductions in population size were also identified using the approach based on
379 heterozygote excess (Piry et al., 1999) but only for TGK1 and S9, and only for particular
380 combinations of model parameters (Supplementary Table S10).
381 Mitochondrial variability was remarkably low: only two different haplotypes (HT1 and
382 HT2, Supplementary material Table S3) were found. All fragments were monomorphic
383 for the HT1 haplotype with the exception of TGK3, where four animals (50%) also
384 showed the HT2 haplotype. The two haplotypes differ by seven mutations. Using
385 mutation rates calculated for the human HVRI this difference provides a minimal
386 divergence time between mitochondrial lineages of 704 years and a median of 1352
387 years.
388

389 **4. DISCUSSION**

390

391 Genetically, the collared brown lemurs existing in the Fort Dauphin region showed a
392 highly structured population and low diversity within subpopulations. As expected,

393 diversity loss appears more significant in littoral forest fragments than in the frayed but
394 continuous block of lowland humid forest of the TGK Protected Area (Fig. 1). This
395 pattern is supported by the overall association between genetic diversity and patch size,
396 which, in turn, is a good proxy of population size (Knaepkens et al., 2004; Arroyo-
397 Rodrıguez and Dias, 2010; Holmes et al., 2013). A similar trend towards low genetic
398 diversity in fragmented populations was recently observed in one congeneric species
399 (*Eulemur cinereiceps*, Brenneman et al., 2012) as well as in other genera of the family
400 Lemuridae (*Varecia variegata*, Holmes et al., 2013; *Lemur catta*, Clarke et al., 2015).
401 However, the genetic diversity of the collared brown lemurs from Fort Dauphin's littoral
402 forest appears even lower than that observed in other lemur populations.
403 A previous genetic assessment of four populations of *E. collaris* in three continuous
404 forests located in the central and northern part of the species range revealed higher
405 mean $H_e$, 0.58 (10 loci; Ranaivoarisoa et al., 2010) than that observed in our
406 populations, 0.45 (8 loci). The mean $H_e$ of littoral *E. collaris* is also low when compared
407 to the critically endangered, congeneric *E. cinereiceps*, 0.53 (26 loci), that also has part
408 of its range occurring in littoral forest fragments (Brenneman et al., 2012). Such a
409 pattern holds even when compared with other lemurids living in fragmented forests
410 (*Varecia variegata:* 0.57 [10 loci; Baden et al., 2014] and *Lemur catta:* 0.80 [8 loci;
411 Parga et al., 2012]), and other lemur families (*Propithecus coquereli:* 0.77 [20 loci;
412 Rakotoarisoa et al., 2006]; *Microcebus revelobensis:* 0.60 [8 loci; Olivieri et al., 2008]*;*
413 *Propithecus tattersalli:* 0.72 [13 loci; Quéméré et al., 2010]; *Propithecus perrieri:* 0.64
414 [24 loci; Salmona et al., 2015]). The above comparison should be viewed cautiously due

415 to differences in number and type of loci used, the potential for allelic drop-out, and
416 differences in sample size. Bearing this caveat in mind, the above comparison does
417 indicate a considerable loss of genetic diversity for the sub-populations of *E. collaris* in
418 the littoral forest.
419 Structure analyses showed a great genetic differentiation (Hartl and Clark 1997)
420 between sub-populations, with a mean Fst (0.24) that is to our knowledge the highest
421 observed in any lemur study to date (Baden et al., 2014). The estimated migration rate
422 across sites, about one individual every four generations, suggests that the intervening
423 matrix is difficult for individuals to traverse. Thus, while *E. collaris* is ecologically and
424 behaviorally flexible (Donati et al. 2011a; Campera et al. 2014), the inability for
425 individuals to migrate between sites may hinder gene flow, resulting in inbreeding.
426 Two pairs of samples strongly deviate from the predictions of the isolation-by-distance
427 model: the two sub-populations of TGK were physically distant but not genetically
428 differentiated, while the two sub-populations in the littoral forests of Ste Luce were
429 physically close (around 1 km) but genetically different (Fig. 1). This suggests that
430 habitat discontinuities such as rivers and grassland between forest fragments play a
431 larger role than linear distance in structuring these lemurs. Our analysis of genetic
432 barriers using Monmonier's algorithm showed that littoral forest fragments were quite
433 isolated from each other. The 3-8 km matrix of grassland that separates the littoral
434 forest from the TGK forest is likely to represent one of the primary causes of uneven
435 gene flow for *E. collaris* in the whole area. However, the unexpected presence of a

436 barrier between the very close sites of S9 and S17 at Ste Luce invokes rivers/lagoons
437 as another putative main cause (Fig. 1).
438 The effects of an inhospitable matrix for migration has been demonstrated for other
439 primate (Gossens et al., 2005; Bergl and Vigilant, 2007; Olivieri et al., 2008; Radespiel
440 et al., 2008) and non-primate species (Stangel et al., 1992; Proctor et al., 2005). For
441 example, human-induced savannahs and roads have been shown to restrict gene flow
442 between populations of golden-brown mouse lemurs, *Microcebus ravelobensis*
443 (Radespiel et al., 2008), resulting in low genetic diversity among isolated populations
444 inhabiting forest fragments (Guschanski et al., 2007). In contrast, other species appear
445 less affected by fragmentation as is the case of golden-crowned sifakas, *Propithecus*
446 *tattersalli*, in the north of the island (Quéméré et al., 2010). The low permeability of the
447 matrix in the Fort Dauphin area may be somewhat unexpected because the collared
448 brown lemurs have been reported to use terrestrial locomotion to cross grasslands
449 (Donati et al., 2007b, 2011a; Ganzhorn et al., 2007). However, migration events across
450 open areas are likely to be associated with high costs for the lemurs due to the possible
451 increased risk of predation, hunting, and potential thermoregulatory stress for a species
452 adapted to closed canopy forests (Andriaholinirina et al., 2014; Donati et al., 2011b). It
453 is reasonable to hypothesize that such costs may only be risked in unusual situations,
454 as in the reported case of homing behavior after the relocation of several collared lemur
455 groups in MND (Donati et al., 2007b).
456 Although inferring the underlying demographic history of the collared brown lemur
457 population in the Fort Dauphin region is beyond the scope of this paper, a number of

458 genetic estimators provides support to a history of size contraction and isolation in the
459 area. In this respect, the weaker support for bottlenecks found using the heterozygosity-
460 based approach may rely on its lower sensitivity, especially for severe and ancient
461 population declines (Piry et al., 1999, Cristescu et al., 2010, Peery et al., 2012).
462 However, it's worth to note that the heterozygosity test for a bottleneck is more powerful
463 when sample sizes are large (Cornuet and Luikart 1996), so it is possible that our
464 analysis was underpowered. Moreover, several studies have indicated that the genetic
465 signal of a population contraction can be also produced by sampling from a structured
466 population (Chikhi et al. 2010), thus we can only tentatively conclude that our analysis
467 constitutes a "true" bottleneck.
468
469 A scenario considering medium to long-term isolation and size reduction seems to
470 reconcile the broad excess of homozygotes with the high rate of divergence and the
471 departure from an isolation-by-distance model. Even exercising caution when applying
472 mutation rates calculated for the human HVRI, we obtained a median estimate for the
473 divergence between mitochondrial lineages (1352 years BP) that roughly approaches
474 paleo-ecological evidence of habitat shifts in the region. It has been recently shown that
475 the coastal area of Fort Dauphin has been heavily affected by Late-Holocene climate
476 changes with peaks of aridity in the interval from 950 to 600 years BP, coinciding with
477 large-scale faunal extinction (between 1400 and 500 cal. yr BP) and drought/marine
478 surges (between 1200 and 700 cal. yr BP) over the whole island (Virah-Sawmy et al.,
479 2009a,b; 2010). Since collared brown lemurs are arboreal species, the relatively rapid

480 transition from closed woodland forest to an open habitat dominated by ericoid
481 grassland and *Myrica* bushland (Virah-Sawmy et al., 2009b; 2010) may have caused a
482 significant contraction of *Eulemur* populations.
483 Since archaeological evidence indicates the presence of human settlements in the
484 south-east around 1150 years BP, differentiating between natural and anthropogenic
485 drivers of change remains problematic (Burney et al., 2004; Rakotoarisoa 1997).
486 However, the island-wide phylogeography of five species of *Microcebus* (Yoder et al.,
487 2016) as well as the genetics of the fragmented populations of golden-crowned sifaka
488 (*P. tattersalli*) in the North (Quéméré et al., 2012) have recently supported previous
489 studies (Bond et al., 2008) suggesting that large areas of the island consisted of a
490 mosaic of grassland, humid and dry forest types. It is plausible that it may also apply to
491 the littoral forest region in the south-east with some areas of grassland that have existed
492 for a few millennia while other areas rapidly shifting between grassland, dry forest, and
493 humid forest due to paleo-climatic perturbations, e.g. severe climatic desiccations
494 (Virah-Sawmy et al., 2009a,b; 2010). The question will remain unresolved until a larger
495 number of samples and molecular markers will allow us to better date potential
496 population bottlenecks (Gossens et al., 2005), and more locations are surveyed in TGK
497 to determine the natural levels of sub-structuring in non-fragmented populations
498 (Quemere' et al., 2010).
499 Our results have important implications for *E. collaris* conservation policies. Although
500 the genetic evidence would support a scenario of long-term population tolerance to
501 habitat change it is unclear whether the species may cope with the dramatic forest loss

502 that has rapidly accelerated over the last decades due to human exploitation of natural
503 resources (Ganzhorn, 2001; Bollen and Donati, 2006; Consiglio, 2006; Ingram and
504 Dawson, 2006). A severe loss of genetic diversity and high inbreeding due to small
505 population size is likely to lead to extinction in the medium-long term (Frankham, 1995;
506 Saccheri et al., 1998). Thus, restoration of gene flow and re-stocking of current
507 populations appear as urgent actions to impede further loss of genetic diversity. Despite
508 its reported ability to cross short distances of grassland, our data strongly indicate that
509 the forest-dwelling collared brown lemurs are unable to maintain adequate levels of
510 gene flow in the current landscape. Additionally, recent studies on habitat requirements
511 in littoral forests indicate that these frugivorous lemurs necessitate large ranging areas
512 (Campera et al., 2014). This suggests that current littoral forest populations can only be
513 viable if their current habitat is maintained or extended. Considering the structuring of
514 the sub-populations, animal movements between littoral forest sites should be favored.
515 This could be done, for instance, by setting up forest corridors between S17 and S9 that
516 could allow the lemurs to cross the river. However, since the poor soil fertility that
517 characterizes littoral forests only allows for slow tree growth (Vincelette et al., 2007), the
518 use of translocation for population restocking should also be considered (Britt et al.,
519 2004; Day et al., 2009; IUCN, 2002). The collared brown lemurs appear relatively
520 tolerant to relocations, as indicated by the successful attempt conducted in MND
521 (Donati et al., 2007b), or by the establishment of a population outside of the species
522 range (Jolly et al., 2006; Donati et al. 2009). It is also imperative to reduce hunting
523 pressure and forest loss that are now threatening at an alarming rate the largest

reservoir of the species in the Fort Dauphin region, i.e. the TGK Protected Area (BirdLife International, 2011; Nguyen et al., 2013). Finally, our analyses and recommendations are based on relatively small sample sizes, thus it is always possible that some of our results might change with the addition of larger samples. This latter point underscores the urgent need for more sampling of rapidly declining populations so that biologists can make robust inferences and conservation recommendations pertaining to endangered species.

**Acknowledgements**

We thank the Ministère des Eaux et Forets for their permission to work in Madagascar. We thank for their collaboration the Department of Animal Biology of the University of Antananarivo, the Madagascar Institute for the Conservation of Tropical Environments, and QIT Madagascar Minerals (QMM). We sincerely acknowledge Michela Balestri, Marta Barresi, Marco Campera, Timothy Eppley, Trang Nguyen, Murielle Ravaolahy, Valentina Serra for their help with sample collection, Luca Taglioli, Maddalena Giannì, and Monica Guerrini for their help with data, lab analysis. We thank Filippo Barbanera for technical advice and contribution to an early version of the manuscript. We acknowledge the QMM biodiversity staff, especially Johny Rabenantoandro Laza Andriamandimbiarisoa, Faly Randriatafika, and the field assistants at Ampasy (TGK3), Mandena, and Ste Luce. GD was assisted by a Faculty Grant from Oxford Brookes

University. ST is recipient of a grant from the University of Pisa (ex60%). All authors of this article have no conflict of interest to declare.

**Literature cited**

Andriaholinirina N, Baden A, Blanco M, Chikhi L, Cooke A, Davies N, Dolch R, Donati G, Ganzhorn J, Golden C, Groeneveld LF, Hapke A, Irwin M, Johnson S, Kappeler P, King T, Lewis R, Louis EE, Markolf M, Mass V, Mittermeier RA, Nichols R, Patel E, Rabarivola CJ, Raharivololona B, Rajaobelina S, Rakotoarisoa G, Rakotomanga B, Rakotonanahary J, Rakotondrainibe H, Rakotondratsimba G, Rakotondratsimba M, Rakotonirina L, Ralainasolo FB, Ralison J, Ramahaleo T, Ranaivoarisoa JF, Randrianahaleo SI, Randrianambinina B, Randrianarimanana L, Randrianasolo H, Randriatahina G, Rasamimananana H, Rasolofoharivelo T, Rasoloharijaona S, Ratelolahy F, Ratsimbazafy J, Ratsimbazafy N, Razafindraibe H, Razafindramanana J, Rowe N, Salmona J, Seiler M, Volampeno S, Wright P, Youssouf J, Zaonarivelo J, Zaramody A. 2014. *Eulemur collaris*. The IUCN Red List of Threatened Species 2014.

Arroyo-Rodrıguez V, Dias PAD. 2010. Effects of habitat fragmentation and disturbance on howler monkeys: a review. *Am J Primatol,* 72:1–16.

Baden AL, Holmes SM, Johnson SE, Engberg SE, Louis EE, Bradley BJ. 2014. Species-level view of population structure and gene flow for a critically Endangered primate (*Varecia variegata*). *Ecol Evol,* 4:2675–2692.

566 Balestri M, Barresi M, Campera M, Serra V, Ramanamanjato JB, Heistermann M,
567 Donati, G. 2014. Habitat degradation and seasonality affect physiological stress
568 levels of *Eulemur collaris* in littoral forest fragments. *PLoS One,* 9:e107698.
569 Barton NH, Slatkin M. 1986. A quasi-equilibrium theory of the distribution of rare alleles
570 in a subdivided population. *Heredity,* 56:409–415.
571 Bergl RA, Vigilant. L, 2007. Genetic analysis reveals population structure and recent
572 migration within the highly fragmented range of the Cross River gorilla (*Gorilla*
573 *gorilla diehli*). *Mol Ecol,* 16:501–516.
574 BirdLife International. 2011. Tsitongambarika Forest, Madagascar: biological and socio-
575 economic surveys, with conservation recommendations. Cambridge: Birdlife
576 International.
577 Bollen A, Donati G, Fietz J, Schwab D, Ramanamanjato JB, Randrihasipara L, Van
578 Elsacker L, Ganzhorn JU. 2005. Fruit Characteristics in a Dry Deciduous and a
579 Humid Littoral Forest of Madagascar: Evidence for Selection Pressure through
580 Abiotic Constraints rather than Seed Dispersers. In: Dew L, Boubli JP, editors.
581 Tropical Fruits and Frugivores: The Search for Strong Interactors. Springer
582 Netherlands. p 92–118.
583 Bollen A, Donati G. 2006. Conservation status of the littoral forest of south-eastern
584 Madagascar: a review. *Oryx,* 40:57–66.
585 Bond WJ, Silander JA, Ranaivonasy J, Ratsirarson J. 2008. The antiquity of
586 Madagascar's grasslands and the rise of the C4 grassy biomes. *J Biogeogr,*
587 35:1743–1758.

588 Brassel KE, Reif D. 1979. A procedure to generate Thiessen polygons. *Geogr Anal,*
589 325:31-36.
590 Brenneman RA, Johnson SE, Bailey CA, Ingraldi C, Delmore KE, Wyman TM, Louis
591 EE. 2012. Population genetics and abundance of the Endangered grey-headed
592 lemur *Eulemur cinereiceps* in south-east Madagascar: assessing risks for
593 fragmented and continuous populations. *Oryx,* 46:298–307.
594 Britt A, Welch C, Katz A. 2004. Can small, isolated primate populations be effectively
595 reinforced through the release of individuals from a captive population? *Biol Cons,*
596 115:319-327.
597 Burney DA, Burney LP, Godfrey LR, Jungers WL, Goodman SM, Wright HT, Jull AJT.
598 2004. A chronology for late prehistoric Madagascar. *J Hum Evol,*: 47:25–63.
599 Campera M, Serra V, Balestri M, Barresi M, Ravaolahy M, Randriatafika F, Donati G.
600 2014. Effects of Habitat Quality and Seasonality on Ranging Patterns of Collared
601 Brown Lemur (*Eulemur collaris*) in Littoral Forest Fragments. *Int J Primatol,* 35:957–
602 975.
603 Chikhi L, Sousa VC, Luisi P, Goossens B, Beaumont MA. 2010. The confounding
604 effects of population structure, genetic diversity and the sampling scheme on the
605 detection and quantification of population size changes. *Genetics*, 186:983-95.
606 Clarke TA, Gray O, Gould L, Burrell AS. 2015. Genetic diversity of the ring-tailed lemur
607 (*Lemur catta*) in south-central Madagascar. *Folia Primatol,* 86:76–84.

608 Consiglio T, Schatz GE, McPherson G, Lowry PP II, Rabenantoandro J, Rogers ZS,
609 Rabevohitra R, Rabehevitra D. 2006. Deforestation and plant diversity of
610 Madagascar's littoral forests. *Conserv Biol,* 20:1799–1803.
611 Cornuet JM, Luikart G. 1996. Description and power analysis of two tests for detecting
612 recent population bottlenecks from allele frequency data. *Genetics*, 144:2001-14.
613 Cristescu, R., Sherwin, W.B., Handasyde, K., Cahill, V., Cooper, D.W., 2010. Detecting
614 bottlenecks using BOTTLENECK 1.2.02 in wild populations: the importance of the
615 microsatellite structure. *Conserv Genet,* 11:1043–1049.
616 Day SR, Ramarokoto R, Sitzmann BD, Randriamboahanginjatovo R, Ramanankirija R,
617 Randrianindrina V, Ravololonarivo G, Louis EE. 2009. Re-introduction of diademed
618 sifaka (*Propithecus diadema*) and black and white ruffed lemurs (*Varecia variegata*
619 *editorum*) at Analamazaotra Special Reserve, eastern Madagascar. *Lemur News,*
620 14:32-37
621 Dewar RE. 2014. Early human settlers and their impact on Madagascar's landscapes
622 In: Scales IR, editor. Conservation and Environmental Management in Madagascar.
623 Abingdon Oxford: Routledge. p 44–64.
624 Donati G, Bollen A, Borgognini Tarli SM, Ganzhorn JU. 2007a. Feeding over the 24-h
625 cycle: dietary flexibility of cathemeral collared lemurs (*Eulemur collaris*). *Behav Ecol*
626 *Sociobiol,* 61:1237–1251.
627 Donati G, Ramanamanjato JB, Ravoahangy AM, Vincelette M. 2007b. Traslocation as a
628 conservation measure for a threatened species: the case of *Eulemur collaris* in the
629 Mandena littoral forest, south-eastern Madagascar. In: Ganzhorn JU, Goodman SM,

630 Vincelette M, editors. Biodiversity, Ecology and Conservation of the Littoral
631 Ecosystems of South-eastern Madagascar. Washington: Smithsonian Institution
632 Press. p. 237–246.
633 Donati G, Baldi N, Morelli V, Ganzhorn JU, Borgognini-Tarli SM. 2009. Proximate and
634 ultimate determinants of cathemeral activity in brown lemurs. *Anim Behav*, *77*:317-
635 325.
636 Donati G, Kesch K, Ndremifidy K, Schmidt SL, Ramanamanjato JB, Borgognini Tarli
637 SM, Ganzhorn JU. 2011a. Better few than hungry: flexible feeding ecology of
638 collared lemurs *Eulemur collaris* in littoral forest fragments. *PLoS One,* 6:e19807.
639 Donati G, Ricci E, Baldi N, Morelli V, Borgognini Tarli SM. 2011b. Behavioral
640 thermoregulation in a gregarious lemur, *Eulemur collaris*: Effects of climatic and
641 dietary-related factors. *Am J Phys Anthropol,* 144:355–364.
642 Dumetz N. 1999. High plant diversity of lowland rainforest vestiges in eastern
643 Madagascar. *Biol Conserv,* 8:273–315.
644 Dupanloup I, Schneider S, Excoffier L. 2002. A simulated annealing approach to define
645 the genetic structure of populations. *Mol Ecol,* 1:2571–2581.
646 Excoffier L, Lischer L.. 2010. Arlequin suite ver 3.5: A new series of programs to
647 perform population genetics analyses under Linux and Windows. *Mol Ecol Resour*,
648 10: 564-567.
649 Federman S, Dornburg A, Daly DC, Downie A, Perry GH, Yoder AD, Sargis EJ, Richard
650 AF, Donoghue MJ, Baden AL. 2016. Implications of lemuriform extinctions for the
651 Malagasy flora. *Proc Natl Acad Sci USA,* 113: 5041-5046.

652 Frankham R. 1995. Inbreeding and extinction: a threshold effect. *Conserv Biol,* 9:792–
653 799.
654 Frantz AC, Pope LC, Carpenter PJ, Roper TJ, Wilson GJ, Delahay RJ, Burke T. 2003.
655 Reliable microsatellite genotyping of the Eurasian badger (*Meles meles*) using fecal
656 DNA. *Mol Ecol,* 12:1649–1661.
657 Frantzen MA, Silk JB, Ferguson JW, Wayne RK, Kohn MH, 1998. Empirical evaluation
658 of preservation methods for fecal DNA. *Mol Ecol,* 7:1423–1428.
659 Ganzhorn JU, Fietz J, Rakotovao E, Schwab D, Zinner D. 1999. Lemurs and the
660 regeneration of dry deciduous forest in Madagascar. *Conserv Biol,* 13:1–11.
661 Ganzhorn JU, Lowry PP II, Schatz G, Sommer S. 2001. The biodiversity of
662 Madagascar: one of the world's hottest hotspots on its way out. *Oryx,* 35:346–348.
663 Ganzhorn JU, Andrianasolo T, Andrianjazalahatra T, Donati G, Fietz J, Norscia I,
664 Rakotondranary J, Rakotondratsima BM, Ralison J, Ramarokoto R, Randriamanga
665 S, Rasarimanana S, Rakotosamimanana B, Ramanamanjato JB, Randria G,
666 Rasolofoharivelo T, Razanahoera-Rakotomalala M, Schmid J, Sommer S. 2007.
667 Lemurs in evergreen littoral forest fragments of different size and degrees of
668 degradation. In: Ganzhorn JU, Goodman SM, Vincelette M, editors. Biodiversity,
669 Ecology and Conservation of the Littoral Ecosystems of South-eastern Madagascar.
670 Washington: Smithsonian Institution Press. p 223–236.
671 Garza JC, Williamson EG. 2001. Detection of reduction in population size using data
672 from microsatellite loci. *Mol Ecol,* 10:305–318

673 Goodman SM, Benstead JP. 2003. Natural history of Madagascar. Chicago: University
674 of Chicago Press.
675 Gossens B, Chikhi L, Jalil MF, Ancrenaz M, Lackman-Ancrenaz I, Mohamed M, Andau
676 P, Bruford MW. 2005. Patterns of genetic diversity and migration in increasingly
677 fragmented and declining orang-utan (*Pongo pygmaeus*) populations from Sabah,
678 Malaysia. *Mol Ecol,* 14:441–456.
679 Guo SW, Thompson EA. 1992. Performing the exact test of Hardy-Weinberg proportion
680 for multiple alleles. *Biometrics,* 48:361–372.
681 Guschanski K, Olivieri G, Funk SM, Radespiel U. 2007. MtDNA reveals strong genetic
682 differentiation among geographically isolated populations of the golden brown
683 mouse lemur, *Microcebus ravelobensis*. *Conserv Genet,* 8:809–821.
684 Hartl DL, Clark AG. 1997. Principles of Population Genetics, 3nd edn. Sinauer
685 Associates, Inc, Sunderland, MA
686 Holmes SM, Baden AL, Brenneman RA, Engberg SE, Louis EE Jr, Johnson SE. 2013.
687 Patch size and isolation influence genetic patterns in black-and-white ruffed lemur
688 (*Varecia variegata*) populations. *Conserv Genet,* 14:615–624.
689 Ingram JC, Dawson TP. 2006. Forest cover, condition, and ecology in human-impacted
690 forests, south-eastern Madagascar. *Conserv Soc, 4*(2), 194.
691 IUCN/SSC Re-introduction Specialist Group. 2002. Guidelines for nonhuman primate
692 re-introductions. *Re-introduction News,* 21: 1-32.

693 Jekielek J, Strobeck C. 1998. Characterization of polymorphic brown lemur (*Eulemur*
694 *fulvus*) microsatellite loci and amplification in the family Lemuridae. *Mol Ecol,*
695 8:895–906.
696 Jolly A, Koyama N, Rasamimanana H, Crowley H, Williams G. 2006. Berenty Reserve:
697 a research site in southern Madagascar. In: Jolly A, Sussman RW, Koyama N,
698 Rasamimanana H. editors. Ringtailed Lemur Biology. Springer US. p. 32-42.
699 Kalinowski ST. 2005. HP-Rare: a computer program for performing rarefaction on
700 measures of allelic diversity. *Mol Ecol Notes,* 5:187–189.
701 Knaepkens G, Bervoets L, Verheyen E, Eens M. 2004. Relationship between population
702 size and genetic diversity in Endangered populations of the European bullhead
703 (*Cottus gobio*): implications for conservation. *Biol Cons,* 115:403–410.
704 Koressaar T, Remm M. 2007. Enhancements and modifications of primer design
705 program Primer3. *Bioinformatics,* 23:1289–1291.
706 Madrigal L, Posthumously LC, Melendez-Obando M, Villegas-Palma R, Barrantes R,
707 Raventos H, Pereira R, Luiselli D, Pettener D, Barbujani G. 2012. High
708 mitochondrial mutation rates estimated from deep-rooting Costa Rican pedigrees.
709 *Am J Phys Anthropol,* 148:327-33.
710 Manni F, Guérard E, Heyer E. 2004. Geographic patterns of (genetic, morphologic,
711 linguistic) variation: how barriers can be detected by using Monmonier's algorithm.
712 *Hum Biol,* 76:173-90.
713 Mittermeier RA, Louis EE, Richardson M, Schwitzer C, Langrand O, Rylands AB,
714 Hawkins F, Rajaobelina S, Ratsimbazafy J, Rasoloarison R, Roos C, Kappeler PM,

MacKinnon J. 2010. Lemurs of Madagascar. Washington: Conservation International.

Monmonier M. 1973. Maximum-difference barriers: an alternative numerical regionalization method. *Geogr Anal,* 3:245–261.

Nei M. 1987. Molecular Evolutionary Genetics. New York: Columbia University Press.

Nguyen T, Eppley TE, Donati G. 2013. Rapid assessment of lemur abundance in the lowland rainforest of Ampasy, Tsitongambarika, south-east Madagascar. *Lemur News,* 17:39–43.

Norscia I, Rahanitriniaina OG, Jolly A, Donati G. 2006. Preliminary survey of lemur density in the semimontane rainforest of Anka, Fort-Dauphin region. *Lemur News,* 11:14–17.

Ohta T, Kimura M. 1973. A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. *Genet Res,* 22:201-4.

Olivieri GL, Sousa V, Chikhi L, Radespiel U. 2008. From genetic diversity and structure to conservation: genetic signature of recent population declines in three mouse lemur species (*Microcebus* spp.). *Biol Conserv,* 141:1257–1271.

Parga JA, Sauther ML, Cuozzo FP, Jacky IAY, Lawler RR. 2012. Evaluating ring-tailed lemurs (*Lemur catta*) from southwestern Madagascar for a genetic population bottleneck. *Am J Phys Anthropol*,*147*, 21-29.

Peery MZ, Kirby R, Reid BN, Stoelting R, Doucet-Bëer E, Robinson S, Vásquez-Carrillo C, Pauli JN, Palsbøll PJ. 2012. Reliability of genetic bottleneck tests for detecting recent population declines. *Mol Ecol*, 21:3403-18.

737 Piry S, Luikart G, Cournet J-M. 1999. Bottleneck: a computer pro-gram for detecting
738 recent reductions in the effective populationsize using allele frequency data. *J*
739 *Hered*, 90:502–503.
740 Proctor MF, McLellan BN, Strobeck C, Barclay RM. 2005. Genetic analysis reveals
741 demographic fragmentation of grizzly bears yielding vulnerably small populations.
742 *Proc R Soc Lond B Biol Sci,* 272:2409–2416.
743 Queller DC, Keith F, Goodnight FK. 1989. In Evolution. Estimating Relatedness Using
744 Genetic Markers. Vol. 43, No. 2, pp. 258-275
745 Quéméré E, Louis EE Jr, Ribéron A, Chikhi L, Crouau-Roy B. 2010. Non-invasive
746 conservation genetics of the critically Endangered golden-crowned sifaka
747 (*Propithecus tattersalli*): high diversity and significant genetic differentiation over a
748 small range. *Conserv Genet,* 11:675–687.
749 Quéméré E, Amelot X, Pierson J, Crouau-Roy B, Chikhi L. 2012. Genetic data suggest
750 a natural prehuman origin of open habitats in northern Madagascar and question
751 the deforestation narrative in this region. *Proc Natl Acad Sci USA*,109:13028-33.
752 Rabenantoandro J, Randriatafika F, Lowry PP II. 2007. Floristic and structural
753 characteristics of remnant littoral forest sites in the Tolagnaro area. In: Ganzhorn
754 JU, Goodman SM, Vincelette M, editors. Biodiversity, Ecology and Conservation of
755 the Littoral Ecosystems of South-eastern Madagascar. Washington: Smithsonian
756 Institution Press. p. 65–77.
757 Radespiel U, Rakotondravony R, Chikhi L. 2008. Natural and anthropogenic
758 determinants of genetic structure in the largest remaining population of the

759 Endangered golden-brown mouse lemur, *Microcebus ravelobensis*. *Am J Primatol,*
760 70:860–870.
761 Rakotoarisoa G, Shore G, McGuire S, Engberg S, Louis E, Brenneman R. 2006.
762 Characterization of 20 microsatellite marker loci in Coquerel's sifaka (*Propithecus*
763 *coquereli*). *Mol Ecol Notes,* 6:1119–1121.
764 Rakotoarisoa J. 1997. A cultural history of Madagascar. Evolution and interpretation of
765 the archeological record. In: Goodman SK, Patterson BD, editors. Natural Change
766 and Human Impact in Madagascar. Washington, DC: Smithsonian Institution Press.
767 pp. 331–341.
768 Ramanamanjato JB, Mcintyre PB, Nussbaum RA. 2002. Reptile, amphibian, and lemur
769 diversity of the Malahelo Forest, a biogeographical transition zone in southeastern
770 Madagascar. *Biol Cons,* 11:1791–1807.
771 Ranaivoarisoa JF, Brenneman RA, McGuire SM, Lei R, Ravelonjanahary SS, Engberg
772 SE, Bailey CA, Kimmel LM, Razafimananjato T, Rakotonomenjanahary R, Louis EE
773 Jr. 2010. Population Genetic Study of the Red-Collared Brown Lemur (*Eulemur*
774 *collaris* É. Geoffroy) in Southeastern Madagascar. *TOCONSBJ* 4:1–8.
775 Raymond M, Rousset F. 1995. GENEPOP (version 1.2): population genetics software
776 for exact tests and ecumenicism. *J Hered,* 86:248–249.
777 Saccheri I, Kuussaari M, Kankare M, Vikman P, Fortelius W, Hanski I. 1998. Inbreeding
778 and extinction in a butterfly metapopulation. *Nature,* 392:491–494.
779 Salmona J, Teixeira H, Rasolondraibe E, Aleixo-Pais I, Kun-Rodrigues C,
780 Rakotonanahary AN, Jan F, Rabarivola CJ, Zaonarivelo JR, Volasoa NA, Chikhi L.

781 2015. Genetic Diversity, Population Size, and Conservation of the Critically
782 Endangered Perrier's Sifaka (*Propithecus perrieri*). *Int J Primatol,* 36:1132–1153.
783 Schwitzer C, Mittermeier RA, Davies N, Johnson S, Ratsimbazafy J, Razafindramanana
784 J, Louis EE, Rajaobelina S. 2013. Lemurs of Madagascar: a strategy for their
785 conservation 2013–2016. Bristol: IUCN SSC Primate Specialist Group, Bristol
786 Conservation and Science Foundation, and Conservation International.
787 Schwitzer C, Mittermeier RA, Johnson SE, Donati G, Irwin M, Peacock H, Ratsimbazafy
788 J, Razafindramanana J, Louis EE Jr, Chikhi L, Colquhoun IC, Tinsman J, Dolch R,
789 LaFleur M, Nash S, Patel E, Randrianambinina B, Rasolofoharivelo T, Wright PC.
790 2014a. Conservation. Averting lemur extinctions amid Madagascar's political crisis.
791 *Science,* 343:842–843.
792 Schwitzer C, Chikhi L, Donati G, Irwin M, Johnson SE, Mittermeier RA, Peacock H,
793 Ratsimbazafy J, Razafindramanana J, Louis EE Jr, Colquhoun IC, Tinsman J,
794 Dolch R, LaFleur M, Nash S, Patel E, Randrianambinina B, Rasolofoharivelo T,
795 Wright PC. 2014b. Protecting lemurs-response. *Science,* 344:358.
796 Stangel PW, Lennartz MR, Smith MH. 1992. Genetic variation and population structure
797 of red-cockaded woodpeckers. *Conserv Biol,* 6:283–292.
798 Tokiniana H, Bailey CA, Shore GD, Delmore KE, Johnson SE, Louis EE, Brenneman
799 RA. 2009. Characterization of 18 microsatellite marker loci in the white-collared
800 lemur (Eulemur cinereiceps). *Conserv Genet,* 10:1459.
801 Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG.
802 2012. Primer3--new capabilities and interfaces. *Nucleic Acids Res,* 40:e115.

803 Valière N. 2002. GIMLET: a computer program for analysing genetic individual
804 identification data. *Mol Ecol Notes,* 2:377–379.
805 Van Oosterhout C, Van Heuven MK, Brakefield PM. 2004. On the neutrality of
806 molecular genetic markers: pedigree analysis of genetic variation in fragmented
807 populations. *Mol Ecol,* 13:1025–1034.
808 Vincelette M, Randrihasipara L, Ramanamanjato JB, Lowry PP II, Ganzhorn JU. 2003.
809 Mining and environmental conservation: the case of QIT Madagascar Minerals in
810 the southeast In: Goodman SM, Benstead JP, editors. The Natural History of
811 Madagascar. Chicago: University of Chicago Press. p 1535–1537.
827 Vincelette M, Rabenantoandro J, Randrihasipara L, Randriatafika F, Ganzhorn JU.
828 2007. Results from ten years of restoration experiments in the southeastern littoral
829 forests of Madagascar. Biodiversity, Ecology and Conservation of Littoral Forest
830 Ecosystems in Southeastern Madagascar, Tolagnaro (Fort Dauphin). In: Ganzhorn
831 JU, Goodman SM, Vincelette M, editors. Biodiversity, Ecology and Conservation of
832 the Littoral Ecosystems of South-eastern Madagascar. Washington: Smithsonian
833 Institution Press. p 337–354.
834 Virah-Sawmy M, Gillson L, Willis KJ. 2009a. How does spatial heterogeneity influence
835 resilience to climatic changes? Ecological dynamics in southeast Madagascar. *Ecol*
836 *Monogr*,*79*: 557-574.
837 Virah-Sawmy M, Katherine J, Gillson W, Gillson L. 2009b. Threshold response of
838 Madagascar's littoral forest to sea-level rise. *Global Ecol Biogeogr,* 18:98–110.

839 Virah-Sawmy M, Willis KJ, Gillson L. 2010. Evidence for drought and forest declines
840 during the recent megafaunal extinctions in Madagascar.*J Biogeography*, *37*:506-
841 519.
842 Walsh B. 2001. Estimating the Time to the Most Recent Common Ancestor for the Y
843 chromosome or Mitochondrial DNA for a Pair of Individuals. *Genetics,* 158:897-912
844 Weir BS, Cockerham CC. 1984. Estimating F-Statistics for the Analysis of Population
845 Structure. *Evolution,* 6:1358–1370.
846 Wyner YM, Johnson SE, Stumpf RM, Desalle R. 2002. Genetic assessment of a white-
847 collared x red-fronted lemur hybrid zone at Andringitra, Madagascar. *Am J Primatol,*
848 57:51–66.
849 Yoder AD, Campbell CR, Blanco MB, Dos Reis M, Ganzhorn JU, Goodman SM,
850 Hunnicutt KE, Larsen PA, Kappeler PM, Rasoloarison RM, Ralison JM, Swofford
851 DL, Weisrock DW. 2016. Geogenetic patterns in mouse lemurs (genus *Microcebus*)
852 reveal the ghosts of Madagascar's forests past. *Proc Natl Acad Sci USA,* 113:8049-
853 56.
854
855

## FIGURE CAPTIONS

**Figure 1.** Location of the study sites.

**Figure 2.** An analysis of isolation-by-distance, showing the regression between a normalized measure of genetic distance [(Fst/(1-Fst)] and the geographic distance in linear kilometers for all pairs of population samples.

**Figure 3.** An analysis of genetic barriers using Monmonier's algorithm applied to five vertices and employing Delaunay's triangulation. Edges are associated with Fst pairwise distance measures. I, II, III: respectively first, second and third rank genetic barriers.