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

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* 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.

111 A genetic assessment of the littoral forest sub-populations has been never conducted
112 and information on gene flow within these sub-populations and/or between them and
113 the lemurs occurring in the inland humid forest are lacking. The collared brown lemurs
114 seem to have a good ecological tolerance to fragmented habitats and they have also
115 been observed to cross short distances of grassland (Donati et al., 2007b, Ganzhorn et
116 al., 2007). However, the small size of the littoral forest patches left in the area (all less
117 than 300 ha), the unknown dispersal distances, and the presence of rivers and roads
118 between fragments raises questions as to whether these sub-populations have been
119 able to maintain viable levels of genetic diversity.
120 Here, we investigated genetic diversity at eight autosomal short tandem repeats (STRs)
121 and the mitochondrial D-loop region in DNA extracted from fecal samples of three
122 subpopulations living in the littoral forest fragments and two sub-populations from the
123 nearby lowland humid forest of the Tsitongambarika Protected Area (Fort Dauphin
124 region). We aimed at estimating: i) the apportionment of genetic variance between
125 habitats and among sub-populations, including the occurrence of natural or
126 anthropogenic barriers to gene exchange; ii) the correlation between eco-geographic
127 and genetic factors; iii) clues of the historical demography of the species within the
128 region.
129
130 **2. MATERIALS AND METHODS**
131
132 *2.1 Study area*

133 Our research was conducted in the Anosy region on the southeastern coast of
134 Madagascar (Fig. 1) (Ramanamanjato et al., 2002). The largest populations of collared
135 brown lemurs occur in the continuous block of lowland and mid-altitude humid forests
136 growing along the Anosy and Vohimena mountain chains (Andriaholinirina et al., 2014).
137 The most eastern of these chains is today included in the Tsitongambarika Protected
138 Area (hereafter TGK), created in 2008 and covering an area of over 60,000 hectares
139 (Birdlife International, 2011; Schwitzer et al., 2013).
140 Three relict sub-populations of collared brown lemurs occur in littoral forest fragments
141 lying on the sandy coast east of TGK (Fig. 1). One of these fragments is a partially
142 degraded block of around 220 ha in the Mandena Conservation Area (hereafter MND),
143 around 11 km north of Fort Dauphin (Ganzhorn et al., 2007). The other two areas
144 (hereafter S9 and S17) represent more intact blocks of littoral forest (S9: 290ha and
145 S17: 220ha) in the Ste Luce Conservation Zone (hereafter STL), around 30 km north of
146 Fort Dauphin. The area between MND and STL, around 18 km between the nearest
147 points, includes degraded fragments of littoral forest, grasslands, and small rivers (Fig.
148 1). The MND fragment is separated from the nearest edge of TGK by approximately 3
149 km of grassland and eucalyptus plantations, while around 8 km of grassland exist
150 between TGK and the two STL fragments. These last, S9 and S17, are approximately 1
151 km apart at their nearest points but separated by a stretch of lagoon.
152
153 *2.2 Study species*
154

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

199 LEMUR_L_REV (5'-ATGGGCGTAGAGCAAGAAGA-3')] from Wyner et al., (2002). PCR
200 products were purified with the GenElute^{TM} PCR Clean-Up Kit (Sigma, USA).
201 Sequencing reactions were performed for each strand with the ABI PRISM BigDye
202 Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA)
203 according to the manufacturer's recommendations. Lengths of the purified PCR
204 products were measured by the ABI 310 Genetic Analyzer (Applied Biosystems, Foster
205 City, CA). CHROMAS 2.01 (http://chromas-lite.software.informer.com/2.0/) software
206 was used to read ABI electropherograms, whereas DNA Aligment 1.2.0.0
207 (http://www.fluxus-engineering.com/align.htm) and BioEdit 7.1.3
208 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) were used to align the sequences to
209 the Reference Sequence of *Eulemur collaris* (Wyner et al., 2002; Genbank ID:
210 AF257980) and to assign haplotypes.
211 All the sequences have been deposited in GenBank (Accession number: KU196680-
212 KU196722). Haplotype distribution across sites is reported in Supplementary material
213 Table S3.
214
215 *2.6 Quality controls*
216
217 Reliability of microsatellite genotypes was ensured by a stepwise system following
218 Frantz et al. (2003). Briefly, two PCR amplifications per locus were initially performed on
219 each DNA extract and a heterozygous state was called if its alleles were scored at least
220 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 (θ_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).

265 To test whether the inland and coastal collared brown lemurs have experienced genetic
266 bottlenecks we first used the M-ratio approach (Garza and Williamson, 2001). Its
267 rationale is based on the fact that during size declines the recovery in the number of
268 alleles is slower than the range in allele size. In each sample, the k/r ratio (M) averaged
269 across loci, where k is the number of observed alleles and r the range in allele size
270 (maximum size - minimum size +1), was compared to the 95% critical value of M (Mc)
271 obtained by 10,000 simulations under a mutation-drift equilibrium using the program
272 Critical_M (NOAA Fisheries, La Jolla, USA). Mutation reference parameters of a two-
273 phase mutation model were used following Parga et al. (2012) and Peery et al. (2012).
274 Secondly, we used the method implemented in the software Bottleneck v.1.2 (Cornuet
275 and Luikart 1996). It tests the occurrence of a transient excess in the level of
276 heterozygosity compared to that expected under a mutation-drift equilibrium. A
277 Wilcoxon signed-rank test was used to check microsatellite loci showing heterozygosity
278 excess given different proportions of multistep mutations in a two-phase model.
279 FSTAT 2.9.3.2 (www2.unil.ch/popgen/software/fstat.htm) was used to estimate the
280 relatedness (*r*) in lowland and littoral samples following Queller and Goodnight (1989).
281 The Time since the Most Recent Common Ancestor (TMRCA) of mitochondrial lineages
282 was estimated by the Walsh's formula (Walsh 2001) implemented in the online TMRCA
283 calculator (http://clan-donald-usa.org/index.php/tmrca-calculator) using human
284 pedigree-based mutation rate for HVR ($7x10^{-5}$ mut/site/gen, Madrigal et al., 2012) and 8
285 years as averaged generation time (Andriaholinirina et al., 2014). The lower 95%

286 confidence value of the distribution was considered as the minimum time that elapsed
287 since the two haplotypes diverged.
288
289 **3. RESULTS**
290
291 *3.1 Quality controls*
292
293 Microsatellite analyses showed that 5 out of 54 DNA samples (9%) gave a call rate
294 lower than 0.25. They were removed from the STR analyses.
295 Amplification success rates varied from 77% to 93% across the eight loci (mean: 87%),
296 neither false nor null alleles were inferred and no evidence of LD was found
297 (Supplementary material Table S5). Estimated dropout rates varied from 4% to 39%
298 (mean 16%) and three loci (EFR8, 104HDZ127, 104HDZ9) showed significant
299 departures from Hardy–Weinberg equilibrium due to a deficiency in heterozygotes
300 (Supplementary material Table S4). However, deviations were observed in a single
301 population (S9), suggesting that this result may have been caused by the genetic
302 characteristics of the groups under study rather than by genotyping errors.
303 After removing either the S9 sample or the deviating loci from the analyses, HWE was
304 respected for all samples and all loci and the overall pattern of genetic structure and
305 relatedness among groups (lowland and littoral population samples) did not change
306 (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 heterogeneous distribution of diversity, other genetic estimators suggested
323 a common history for lowland and littoral humid forests. In fact, the population diversity
324 parameter theta (Table 1), which is a mutation-scaled measure of effective population
325 size inversely proportional to the amount of drift experienced by the population, showed
326 low and very similar values across samples. This makes it plausible to speculate either
327 a single ancestral population with few breeding animals or synchronous size

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

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

388 **4. DISCUSSION**

389

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

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

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

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

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

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

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

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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.