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Evaluating Ring-Tailed Lemurs (*Lemur catta*) From Southwestern Madagascar For a Genetic Population Bottleneck

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ABSTRACT In light of historical and recent anthropogenic influences on Malagasy primate populations, in this study ring-tailed lemur (*Lemur catta*) samples from two sites in southwestern Madagascar, Beza Mahafaly Special Reserve (BMSR) and Tsimanampetsotsa National Park (TNP), were evaluated for the genetic signature of a population bottleneck. A total of 45 individuals (20 from BMSR and 25 from TNP) were genotyped at seven microsatellite loci. Three methods were used to evaluate these populations for evidence of a historical bottleneck: M-ratio, mode-shift, and heterozygosity excess tests. Three mutation models were used for heterozygosity excess tests: the stepwise mutation model (SMM), two-phase model (TPM), and infinite allele model (IAM). M-ratio estimations indicated a potential bottleneck in both populations under some conditions. Although mode-shift tests did not strongly indicate a population bottleneck in the recent historical past when samples from all individuals were included, a female-only analysis indicated a potential bottleneck in TNP. Heterozygosity excess was indicated under two of the three mutation models (IAM and TPM), with TNP showing stronger evidence of heterozygosity excess than BMSR. Taken together, these results suggest that a bottleneck may have occurred among *L. catta* in southwestern Madagascar in the recent past. Given knowledge of how current major stochastic climatic events and human-induced change can negatively impact extant lemur populations, it is reasonable that comparable events in the historical past could have caused a population bottleneck. This evaluation additionally functions to highlight the continuing environmental and anthropogenic challenges faced by lemurs in southwestern Madagascar. Am J Phys Anthropol 147:21–29, 2012.

It is well documented that the endemic fauna and flora of Madagascar have been negatively impacted by historical and recent anthropogenic activities. Historically, the arrival of humans to Madagascar appeared to trigger the extinction of endemic taxa such as birds, mammals, and reptiles (Dewar, 2003). More recently, continuing rates of deforestation due to human population pressure and the extraction of natural resources have destroyed the habitats that harbor extant lemurs and other vertebrate fauna, thereby altering the community and population structure of endemic species (Green and Sussman, 1990; Sussman et al., 1994, 2003; Mittermeier et al., 2006). Also, despite being culturally prohibited (i.e., "fady") among many of the ethnic groups in southwestern Madagascar to hunt lemurs, there is a long history in the regional archaeological record of hunting the now extinct "subfossil" lemurs, as well as extant lemur forms (e.g., *Propithecus*) (Perez et al., 2005; Loudon et al., 2006). Similarly, climate factors such as cyclones and droughts also influence patterns of mortality and persistence of vertebrate communities in Madagascar (Ganzhorn, 1995; Gould et al., 1999; Wright, 1999; Richard et al., 2002; Dewar and Richard, 2007). The demographic disruption of populations, whether due to habitat destruction, hunting, climate, or disease, can alter the genetic composition of a population, and several analytical techniques are available for determining the genetic consequences of

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population disruption (Allendorf and Luikart, 2006). One class of population genetic analyses examines how patterns of genetic diversity deviate from theoretically expected equilibrium conditions and/or a neutral expectation (reviewed in Chikhi et al., 2010).

As part of an on-going study of the behavior, ecology, genetics, health, and life history patterns of ring-tailed lemurs (*Lemur catta*), we present information on the genetic diversity of two *L. catta* populations. Our motivation for this analysis stems from the observation that both climate and anthropogenic factors have negatively influenced wild ring-tailed lemur populations (Gould et al., 1999; Whitelaw et al., 2005; Loudon et al., 2006; Sauther et al., 2006; Fish et al., 2007; Sauther and Cuozzo, 2009). We seek to determine if these disruptions have left a genetic signature in the genotypes of extant *L. catta* populations. Specifically, we test the hypothesis that *L. catta* populations from two particular locations in southwestern Madagascar, Beza Mahafaly Special Reserve (BMSR) and Tsimanampetsotsa National Park (TNP), have experienced a genetic bottleneck.

MATERIALS AND METHODS

Sample collection locations and protocol

Samples were collected from wild *Lemur catta* inhabiting two areas of southwestern Madagascar. Beza Mahafaly Special Reserve (BMSR) (23°30′S, 44°40′E) includes a protected, fenced 80 ha parcel of intact gallery forest that has not been significantly affected by human disturbance for more than 20 years. Although a small "protected" parcel, a number of the BMSR ring-tailed lemur troops regularly move outside of this fenced area (e.g., Sauther and Cuozzo, 2009). Surrounding this parcel is habitat exhibiting various levels of anthropogenic disturbance and various habitat types (e.g., Cuozzo and Sauther, 2004; Sauther et al., 2006; Sauther and Cuozzo, 2009). Tsimanampetsotsa National Park (TNP) (24°06′S 43°50′E), a site located approximately 135 km southwest of Beza Mahafaly (Cuozzo et al., 2008), is a 48,000 hectare reserve located in southwestern Madagascar, 15km east of the Mozambique Channel. The vast limestone plateau of TNP is populated by dense, spiny, succulent, xerophilic vegetation (Razafimanjato et al., 2007). This park contains primarily intact habitat, with few anthropogenic effects or invasive plant foods (Hammer and Ramilijaona, 2009; Youssouf, Sauther and Cuozzo, unpublished data).

Blood samples were collected from these two sites from May through August 2006. At BMSR individuals from six different troops were sampled. At TNP individuals from four different troops (separated by 3 km) were sampled. Lemurs were darted and captured using a Dan-Inject blow dart system (Dan-Inject, North America, Fort Collins, CO) and the drug Telazol (Fort Dodge Laboratories, Fort Dodge, IA). Doses were determined based on protocols that have been developed over 20 years of lemur captures at BMSR (e.g., Sussman, 1991; Cuozzo and Sauther, 2006; Sauther et al., 2006; Miller et al., 2007). Blood samples were collected while lemurs were under general anesthesia. Licensed veterinarians and veterinary students administered the anesthesia. Blood was collected using standard venipuncture techniques, and was replaced by an equal volume of Lactated ringer's solution (Abbot Laboratories, Chicago, IL). No more than 1% body weight (1 mL/100 g; the accepted safe volume) was collected from each individual, and approximately 100–120 μL were placed on Schleicher & Schuell IsoCode® cards (Keene, NH, USA) for genetic analysis. Following collection, samples were allowed to dry and were stored at room temperature. All animal handling was conducted with Institutional Animal Care and Use Committee (IACUC) approval from the University of Colorado and the University of North Dakota.

DNA extraction, amplification, and genotyping

A total of 45 samples were analyzed, 20 from BMSR (9 males and 11 females) and 25 from TNP (15 males and 10 females). A 0.3 mm diameter hole-punch was collected from each sample card using a Harris Uni-Core instrument (Whatman Inc., Clifton, NJ) for DNA extraction. DNA was isolated using an extraction protocol based on IsoCode card manufacturer (Schleicher & Schuell) recommendations. IsoCode cards are impregnated with a proprietary reagent mixture; to extract DNA, each hole-punch sample was heated in 100 μL of water at 100°C for 15min. Ten microliters of Tris-EDTA (1M Tris Base, 0.5M EDTA Solution, pH 8.0) was added to this 100 μL DNA extract, as this was found to enhance PCR (polymerase chain reaction) amplification. PCR was performed in 12.5 μL reactions, using 1–3 μL of DNA extract. The reaction mixture consisted of GeneAmp® 10X PCR Buffer (Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 pM of each primer (forward and reverse), and 0.5 U of AmpliTaq® DNA Polymerase (Applied Biosystems). Forward primer sequences were fluorescently labeled with 6FAM™, NED™, PET™, and VIC® (Applied Biosystems). The 8 microsatellite markers used in this study were chosen because they amplified well and were highly polymorphic (see Supporting Information for a listing of microsatellites used).

PCR reactions were carried out on an ABI 2720 thermal cycler under the following reaction conditions: an initial denaturation for 2 min at 95°C, 32 cycles of denaturing for 30 s at 95°C, 30 s at a primer-specific annealing temperature (Supporting Information), extension at 72°C for 45 s, followed by a final extension at 72°C for 5 min. PCR products were electrophoresed on a 2.0% agarose gel with 1× TAE buffer at 140 V for 30 min, with a positive and negative control and 100 bp DNA ladder (New England Biolabs, Ipswich, MA).

Products were separated using a multicapillary ABI 3730 sequencer with 500LIZ size standard (Applied Biosystems). GeneMapper® software (Applied Biosystems), version 4.0, was used to identify allele fragment lengths. PCR amplification was repeated a minimum of two to three times per marker, per animal, using DNA isolated from separate extractions to verify genotypes.

Testing for a genetic bottleneck

To determine whether the two lemur populations showed evidence of undergoing a genetic bottleneck, we used two approaches. The first was the M-ratio test (Garza and Williamson, 2001), which detects reductions in population size using a ratio of observed number of alleles to the overall range in allele size. Estimated values of M can be compared to critical values of M (M_c), which are calculated based upon three input parameters: θ ($=4N_e u$, where N_e is the effective population size and u is mutation rate), p_s (the proportion of one-step mutations) and Δ_g (the average size of one-step mutations).

Where $M<M_c$, it is inferred that a population has experienced a bottleneck. In these analyses, M and M_c were calculated under the two-phase model using standard parameters (p_s = 0.9, Δ_g = 3.5) suggested by Garza and Williamson (2001). Because the prebottleneck value of theta for these populations was not definitively known, we set theta at a range of values between 0.1 and 10 (e.g. Busch et al., 2007).

The second set of methods we employed used the program BOTTLENECK; this program is capable of evaluating genotypic data with only four polymorphic microsatellite loci and 20–30 individuals, and it assumes that the genetic loci are unlinked and selectively neutral (Piry et al., 1999). Heterozygosity excess can be indicative of a population bottleneck (Cornuet and Luikart, 1996). Using this program, a sign test, standardized differences test, or a Wilcoxon signed rank test (Cornuet and Luikart, 1996; Luikart and Cornuet, 1998) can detect heterozygosity excess relative to what would be expected under mutation-drift equilibrium based on existing allele frequencies. One limitation of this test is that it is only capable of detecting relatively recent bottlenecks—those which have occurred less than $4N_e$ generations ago, where N_e is the effective population size; this test additionally assumes that each sample chosen for analysis is representative of a population without substructure and without immigration (Luikart and Cornuet, 1998).

The BOTTLENECK program is also capable of identifying populations that have undergone historical bottlenecks via the mode-shift test, which detects disruptions in allele frequency distributions (Luikart et al., 1998). In a nonbottlenecked population, many alleles are found at low frequencies while fewer alleles are found at intermediate frequency classes, creating an "L-shaped" distribution. In contrast, bottlenecked populations—due to the loss of rare alleles—show a distortion of this L-shape, such that the greatest numbers of alleles are found in intermediate frequency classes (a "mode shift").

To evaluate our genotypic datasets for the existence of null alleles, we used MICRO-CHECKER, version 2.2.3 (van Oosterhout et al., 2004). Number of alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E) (Nei, 1978) were determined per locus for each population using POPGENE, version 1.31 (Yeh et al., 1999). Tests for Hardy-Weinberg equilibrium within each population were performed in POPGENE. Only loci found to be in Hardy-Weinberg equilibrium were used in analyses. Further details about the genetic structure of these populations will be reported elsewhere (Parga et al., in prep).

BOTTLENECK analyses were conducted on the two *L. catta* populations using samples from males and females. This was followed by a "female-only" analysis for both populations, where samples from males were excluded because males are primarily the dispersing sex in *L. catta* (Sussman, 1991, 1992; Sauther and Cuozzo, pers. obs.). The inclusion of samples from immigrants can obscure the genetic effects of a bottleneck via the introduction of rare alleles (Luikart and Cornuet, 1998; Keller et al., 2001; Busch et al., 2007), prompting us to run analyses with and without males. Three mutation models were used: the infinite allele model (IAM), the stepwise mutation model (SMM) and the two-phase model (TPM) (Di Rienzo et al., 1994). In the TPM, the proportion of mutations greater than one-step was set between 15 and 30% (corresponding to 70–85% of mutations in the SMM). These are commonly used TPM parameters in studies testing for heterozygosity excess using microsatellite markers (Goossens et al., 2006; Bergl et al., 2008). For each model, one thousand iterations were used. The iterations function to generate simulated datasets that can be used to compare levels of observed heterozygosity in the data with that which would be expected under mutation-drift equilibrium. Wilcoxon tests were applied, as this is the most powerful and robust test when using less than 20 polymorphic microsatellite loci (Piry et al., 1999). Significant values indicate higher than expected levels of heterozygosity (heterozygosity excess). One-tailed tests were used, with significance set at $P < 0.05$.

TABLE 1. Number of alleles and heterozygosity for each locus

Population	Locus	No. of alleles	H_O	H_E
Beza Mahafaly	Lc5	7	0.750	0.778
	Lc6	5	0.750	0.734
	Lc7	10	0.900	0.838
	69HDZ267[a]	10	0.800	0.816
	69HDZ299	7	0.700	0.795
	Efr09	8	0.800	0.740
	L-2	11	0.850	0.825
	Em12	11	0.850	0.864
Tsimanampetsotsa	Lc5	7	0.800	0.782
	Lc6	6	0.720	0.708
	Lc7	11	0.800	0.835
	69HDZ267	10	1.000	0.866
	69HDZ299	8	0.760	0.802
	Efr09	7	0.680	0.772
	L-2	10	0.760	0.804
	Em12[a]	14	0.880	0.818

H_O, observed heterozygosity; H_E, Nei's (1978) unbiased estimate of expected heterozygosity.
[a] Showed a significant departure from Hardy-Weinberg equilibrium.

RESULTS

Table 1 shows the number of alleles, observed heterozygosity and Nei's (1978) unbiased estimate of expected heterozygosity for each locus within each population. All loci showed high levels of heterozygosity in both populations. Observed heterozygosity for each marker was comparable with—and in many cases greater than—that reported for these markers in other lemur studies, including those on *Eulemur fulvus* (Jekielek and Strobeck, 1999) and captive and wild *L. catta* (Pastorini et al., 2005; Zaonarivelo et al., 2007). Additionally, all microsatellites were highly polymorphic, with allele number across loci ranging between 5 and 14 (Table 1). MICRO-CHECKER did not find any evidence of null alleles, scoring errors, or large allele dropout. Two loci showed significant deviations from Hardy-Weinberg equilibrium: 69HDZ267 for BMSR and Em12 for TNP. These loci were omitted from the analyses, leaving seven polymorphic microsatellite loci per population that were used in each analysis.

Results from the M-ratio tests were mixed, and largely depended on the value of theta chosen. Results were not significant when using larger values for theta, but for the two lower values of theta (θ = 1.0 and 0.1), average values of M calculated across all loci fell below the critical "threshold" value (M_c) for both populations, indicating a bottleneck (Table 2).

Within the BOTTLENECK program, the mode-shift test did not detect a disruption in allele frequency

distributions for either population when including samples from all available individuals. When the analysis was repeated using only samples from females, TNP revealed a mode shift whereas BMSR did not; however, the number of females in the TNP sample was small ($n = 10$).

The results of the BOTTLENECK analyses for heterozygosity excess using all individuals in each population are shown in Table 3. Values represent the proportion of simulated datasets that exhibited departures from equilibrium that were as large as or larger than those observed in the data. Under the SMM, neither population showed a pattern of heterozygosity excess (Wilcoxon, BMSR: $P < 0.656$; TNP: $P < 0.711$). However, under the IAM, both the BMSR population and the TNP population showed significant heterozygosity excess (Wilcoxon, $P < 0.004$ for both populations). Under the TPM (the intermediate model), both populations continued to show heterozygosity excess when the percentage of mutations in SMM was set at 70% (which is equal to 30% mutations of more than one step) (Wilcoxon, BMSR: $P < 0.04$; TNP: $P < 0.02$). When the percentage of mutations in SMM was set higher, at 75–80%, only TNP was significant for heterozygosity excess ($P < 0.03$; Table 3). The results of the female-only analysis did not differ from the more inclusive analysis, except in one case; under the TPM (at 85% in SMM), the P-value ($P = 0.055$) from Table 3 for the TNP population changed to a significant value: $P < 0.008$.

TABLE 2. Values of theta (θ) used in the M-ratio test with results

Population	Theta	Average M	M_c
Beza Mahafaly	10	0.66	0.57
	4	0.66	0.64
	1	**0.66**	**0.73**
	0.01	**0.66**	**0.83**
Tsimanampetsotsa	10	0.71	0.59
	4	0.71	0.65
	1	**0.71**	**0.74**
	0.01	**0.71**	**0.83**

Average M was calculated across all loci for each population; values indicating a population decline (where $M < M_c$) are in bold.

DISCUSSION

In this study, we tested the hypothesis that *L. catta* populations in southwestern Madagascar would show evidence of a historical bottleneck under the assumption that such an event might be caused by human-induced habitat disturbance and/or climate factors. Taken together, results from the various tests applied were somewhat equivocal, with mixed support for a population decline only under the M-ratio and heterozygosity excess tests, but seem to suggest that *L. catta* in southwestern Madagascar may have undergone a bottleneck in the recent past.

The mode-shift test did not provide convincing evidence for a bottleneck in either population, but because this test is sensitive to sample size and should ideally be used with at least 30 individuals (Luikart et al., 1998), our use of this test likely suffered from low power. A female-only analysis revealed a shift in the allele frequency distribution for TNP, but because the sample size of females was small, additional tests were required to more solidly evaluate the possibility of a bottleneck in this population.

M-ratio tests provided stronger evidence for a population bottleneck. These tests indicate a population decline in the recent past for both TNP and BMSR under the two lower (but not higher) values of theta. However, the interpretation of these results rests on assumed values of theta. If the lower theta values are accurate—for example, if prebottleneck effective population size was small in TNP and BMSR—then a bottleneck is more likely to have occurred among *L. catta* in these locations.

Results from the BOTTLENECK tests for heterozygosity excess were likewise mixed. Our ability to determine whether each population exhibits heterozygosity excess depends on which mutation model best describes the microsatellite loci used in our analysis. Although the IAM, which produces a new allele for every mutation event, is often deemed inappropriate for microsatellites, several empirical studies have shown that this model sometimes better characterizes microsatellite loci than the SMM (Estoup et al., 1995; O'Connell et al., 1997). Microsatellites manifest a pattern of incremental growth of repeat units punctuated by both random large deletions as well as point mutations (Wierdl et al., 1997;

TABLE 3. Results from BOTTLENECK analyses under 3 mutation models across all loci

			Microsatellite Loci								
Population	Model	% in SSM[a]	Lc5	Lc6	Lc7	69HDZ 267	69HDZ 299	Efr09	L-2	Em12	Wilcoxon p
Beza Mahafaly	IAM	–	0.166	0.061	0.214	–	0.085	0.444	0.489	0.105	**0.004**
	TPM	70	0.305	0.137	0.424	–	0.199	0.217	0.221	0.267	**0.039**
	TPM	75	0.330	0.150	0.468	–	0.207	0.194	0.222	0.309	0.188
	TPM	80	0.370	0.134	0.485	–	0.218	0.196	0.212	0.309	0.234
	TPM	85	0.374	0.197	0.482	–	0.244	0.160	0.193	0.328	0.344
	SMM	–	0.425	0.268	0.288	–	0.396	0.053	0.056	0.465	0.656
Tismanampetsosta	IAM	–	0.114	0.254	0.274	0.022	0.126	0.146	0.416	–	**0.004**
	TPM	70	0.221	0.496	0.443	0.067	0.288	0.289	0.319	–	**0.012**
	TPM	75	0.256	0.486	0.433	0.066	0.316	0.349	0.332	–	**0.012**
	TPM	80	0.269	0.512	0.409	0.057	0.349	0.344	0.284	–	**0.027**
	TPM	85	0.296	0.463	0.353	0.088	0.370	0.380	0.246	–	0.055
	SMM	–	0.516	0.26	0.168	0.229	0.425	0.434	0.096	–	0.711

The values represent the proportion of simulated datasets which show a departure from mutation drift equilibrium that is as large as or larger than that observed in the data. The last column represents P-values resulting from Wilcoxon one-tailed tests for heterozygosity excess across all loci for each mutation model within each population; statistically significant values are in bold. Missing values reflect loci that were omitted from bottleneck analyses because they showed significant departures from Hardy-Weinberg equilibrium.
[a] For the two-phase model (TPM), the % of single-step mutations allowed.

Kryglyak et al., 1998; Ellegren, 2004); this results in a distribution of allele sizes that does not always conform to the SMM (Shriver et al., 1993; Deka et al., 1995) and in some cases may be better fit by the IAM (e.g., Estoup et al., 1995; O'Connell et al., 1997). Other studies have found a good fit between the observed distribution of allele sizes and the SMM (e.g., Edwards et al., 1992; Valdes et al., 1993; reviewed in Ellegren, 2004). Most studies indicate that the mutational processes at microsatellite loci are more complex than the simple increase/decrease of single repeat units (Kryglyak et al., 1998; Ellegren, 2004) and the IAM and SMM represent two extremes (Estoup and Cornuet, 1999). In this regard, it is widely thought that the TPM best describes the mutation processes at microsatellite loci (Di Rienzo et al., 1994; Ellegren, 2000; Schlotterer, 2000; Estoup et al., 2002). We therefore assume that the TPM provides the best characterization of the mutational processes at microsatellite loci, and primarily use the results from this model to infer whether these two *L. catta* populations show heterozygosity excess.

Whereas heterozygosity excess was strongly supported for the TNP population under the TPM, the BMSR population showed a much more attenuated signal of heterozygosity excess under this mutation model, as the majority of parameters used for the TPM failed to reveal statistically significant results. In general, statistical power increases in tests for heterozygosity excess when more polymorphic microsatellite loci are used and, to a lesser degree, when sample size increases (Cornuet and Luikart, 1996). Thus, evidence for heterozygosity excess among *L. catta* at TNP was found in this study with only moderate statistical power, as only seven microsatellite markers and a conservative number of samples from each population were used in this evaluation. While we are confident that the TNP population of *L. catta* exhibits heterozygosity excess, it is difficult to identify a single causal factor that might best explain these results. It is also difficult to rectify why dissimilar results were found between the two *L. catta* populations.

It was initially surprising that the TNP *L. catta* population may have experienced a bottleneck in the recent past, and furthermore, that the signal indicating a population bottleneck in tests for heterozygosity excess was actually stronger at this location than at BMSR. TNP has been protected since 1927 due to its unique flora and fauna. It became a national park in the 1960s and a Ramsar site in 1998. In addition, the area is viewed as sacred for the people living in the locality (Hammer and Ramilijaona, 2009). As such, there is little anthropogenic change within this area. Human activities in TNP are limited to the collection of medicinal plants and human burials (although vegetation can be cut down to construct such graves). The area is also too dry and/or rocky for agriculture. Examination of forest cover maps shows that the area surrounding TNP is much more intact than at BMSR (Harper et al., 2007).

There is a possibility that the heterozygosity excess found in this study at TNP (and to a lesser extent, at BMSR) was not caused by a recent demographic bottleneck, but rather, may have been caused by sampling individuals from a structured population (e.g., one that is comprised of several different subpopulations within the larger population) or one in which there has been a recent reduction in gene flow (Wakeley, 1999; Broquet et al., 2010; Chikhi et al., 2010; Peter et al., 2010). We deem this as unlikely, because samples within each *L. catta* population were collected from multiple social groups which could potentially interbreed. There is also no evidence for decreased gene flow within these sites, as male dispersal regularly occurs at BMSR. For example, in December 2009 and again in June/July 2010, male ring-tailed lemurs originally captured and collared in Parcel 1 at BMSR have been observed and followed as far as 8 km from the study population described herein (Sauther and Cuozzo, unpublished data). In addition, it should be noted that surrounding each of the *L. catta* study locations are additional "pockets" of *L. catta* social groups, as has been demonstrated by Axel and Maurer (2011) for BMSR. Also, in an area 20 km north of the TNP study region, in December 2009 we (MLS and FPC) observed ring-tailed lemur troops in highly degraded habitat along the limestone plateau that forms much of TNP. Admittedly, it is unknown how these unsampled individuals fit into the overall metapopulation structure of *L. catta* in southwestern Madagascar.

Despite the above caveats, we maintain that our results indicate the potential for a past bottleneck in these two populations, especially in light of the ecological history of *L. catta* in southwestern Madagascar. First, lemurs in southwestern Madagascar have been subject to significant levels of anthropogenic disturbance, such as habitat alteration via livestock grazing and tree cutting (Whitelaw et al., 2005; Loudon et al., 2006; Sauther et al., 2006; Fish et al., 2007; Sauther and Cuozzo, 2009). Close proximity to human habitation has likely also had a negative effect on genetic diversity of wild *L. catta* populations through its adverse effects on lemur health. For example, *L. catta* in BMSR near areas of human habitation show increased incidence of parasites and other diseases, and a high degree of terrestriality in this area is linked to coprophagy of human and livestock waste (Loudon, 2009; Fish et al., 2007; Gemmill and Gould, 2008).

Secondly, a single large stochastic environmental event in the recent past—if damaging enough—could have potentially caused a genetic bottleneck. Lemur populations in southwestern Madagascar are subjected to cyclones (Wright, 1999) and periods of extremely low rainfall (Gould et al., 1999; Richard et al., 2002; Lawler et al., 2009). Either of these events could have potentially resulted in such a drastic loss of genetic diversity as to cause a genetic bottleneck to occur. Unlike recurrent events that take place with some regularity or predictability (and are therefore poor candidates for causing a bottleneck signal), random stochastic climatic events cannot be ruled out as potential causative factors for a genetic bottleneck in these populations. One recent severe drought that occurred around BMSR during 1991–1992 (Sauther, 1998; Gould et al., 1999) has proven that mortality can be exceedingly high following such an event. Following the 1991–1992 drought, the total numbers of individuals in the BMSR *L. catta* study population declined precipitously by nearly 50% within two to three years, while infant mortality rose to 80% during the drought and immediate post-drought period (Gould et al., 2003). As in BMSR, rainfall in TNP can be highly variable from one year to the next (European Commission Joint Research Centre Global Environment Monitoring Unit http://bioval.jrc.ec.europa.eu/PA/). Demographic data on another strepsirhine in BMSR, Verreaux's sifaka (*Propithecus verreauxi*), similarly shows the negative effects that low rainfall can have on lemur survival and reproduction (Richard et al., 2000, 2002; Lawler et al., 2009; Lawler, 2011).

TABLE 4. Evidence for population bottlenecks in various lemur species

Species	Mutation models showing significance[a]	Other evidence	Potential causal factors implicated	Reference
Red-collared brown lemur (*Eulemur collaris*)	IAM, SMM	–	Anthropogenic	1
Milne-Edwards' sportive lemur (*Lepilemur edwardsi*)	–	Bayesian analysis[b,c]	Anthropogenic	2
Mouse lemur (*Microcebus* spp.)	IAM, TPM	Bayesian analysis[b,c]	Anthropogenic	3
Black and white ruffed lemur (*Varecia variegata*)	IAM, TPM	Mode shift[a]	None given	4
Red ruffed lemur (*Varecia rubra*)	IAM, TPM	Mode shift[a]	Anthropogenic	5
Ring-tailed lemur (*Lemur catta*)	IAM, TPM	Mode-shift[a], M-ratio[d]	Anthropogenic, climatic events	This study

[a]Piry et al., 1999.
[b]Beaumont, 1999.
[c]Storz and Beaumont, 2002.
[d]Garza and Williamson, 2001.
References: (1) Ranaivoarisoa et al., 2010; (2) Craul et al., 2009; (3) Olivieri et al., 2008; (4) Louis et al., 2005; (5) Razakamaharavo et al., 2010.

Another example of a major stochastic perturbation occurred on January 22, 2005, when cyclone Ernest hit southwest Madagascar. At BMSR, the cyclone ripped flowers and leaves off of large trees, toppled many old growth trees and severely flooded the entire eastern portion of Parcel 1, including the research camp (Youssouf, pers. obs.). The cyclone had a dramatic effect on plant phenology in the area. Most notably, it resulted in a nearly complete failure of *Tamarindus indica* fruit, which is an important fall-back resource for *L. catta* at BMSR (Sauther and Cuozzo, 2009; Whitelaw, 2010). Our demographic data indicate that, similar to the effects of drought, the cyclone had a strong effect on mortality patterns. Notably, adult female and subadult mortality was 31% during the cyclone year compared with 7% during a noncyclone, nondrought year and infant mortality was 69% the year of the cyclone as compared to only 22% during a nondrought, noncyclone year (Sauther and Cuozzo, 2008).

Although only a weak signal of heterozygosity excess was detected for BMSR, one would intuit that the severe mortality induced by these stochastic climatic events (Dewar and Richard, 2007; Lawler et al., 2009) would have more strongly registered in the genotypes of the BMSR *L. catta* population. However, it is possible that the genetic effective size of the BMSR population is well below the census size. In such cases, populations can experience rather severe demographic mortality, but such mortality will not unduly influence genetic diversity (Vucetich et al., 1997; Storz et al., 2002). Droughts have a similar effect on the mortality of the sympatric *P. verreauxi* at BMSR, but no significant heterozygosity excess was detected in the *P. verreauxi* BMSR population (Lawler, 2008, 2011).

Several recent studies of other lemur populations have revealed genetic evidence for bottlenecks (Table 4). These investigations have used a variety of methods to assess the loss of genetic diversity, and many include the test for heterozygosity excess used here (Piry et al., 1999). Altogether, a picture is emerging of Madagascar as a location plagued by recent anthropogenic change; in many cases, this is the primary explanation offered by authors interpreting the genetic signals of population decline (Table 4). It is clear that anthropogenic changes are dramatically affecting lemur habitats and lemur biology in southwestern Madagascar (Sauther and Cuozzo, 2009). Following these earlier studies, our data further illustrate the potential role of human actions on the biology of wild lemur populations.

CONCLUSIONS

The evidence presented here suggests that a possible population bottleneck occurred in the historical past among *L. catta* in Madagascar. Although the mode-shift test alone did not provide convincing evidence of population decline, the M-ratio and heterozygosity excess tests provided some support for the hypothesis that TNP (and to a lesser degree, BMSR as well) experienced a population bottleneck in the recent past. Admittedly, unaccounted for population substructure or reduction of gene flow within these areas could have created a false bottleneck signal in the absence of a true demographic population bottleneck (Broquet et al., 2010; Chikhi et al., 2010; Peter et al., 2010). Nevertheless, given what is known about human-induced changes in southwestern Madagascar (Whitelaw et al., 2005; Loudon et al., 2006; Sauther et al., 2006; Fish et al., 2007; Sauther and Cuozzo, 2009) and environmental perturbations that occur stochastically in this part of the world (Richard et al., 2000, 2002; Gould et al., 2003; Sauther and Cuozzo, 2008; Lawler et al., 2009; Lawler, 2011), it is conceivable that a genetic bottleneck could have resulted in the past from one of these causal factors if the event had markedly negative effects on lemur survival and reproduction. Presumably, the evidence presented here which indicates the potential for loss of genetic diversity in these two populations could apply to the species as a whole if the factor(s) causing the purported bottleneck were present throughout the species' range, which is quite extensive (Sussman et al., 2003; Goodman et al., 2006).

Where bottlenecks occur, the detrimental effects caused by loss of genetic diversity can be widespread. The loss of genetic variation accompanying a bottleneck can lead to inbreeding depression as well as the fixation of deleterious alleles in a population, both of which can markedly decrease the survival rates of individuals (Lande, 1994; Mills and Smouse, 1994). Indeed, *L. catta* in captivity that are more inbred show greater incidence of parasites, lower immunocompetence, and are more likely to die of disease earlier than are more outbred individuals (Charpentier et al., 2008).

Molecular assessments such as that performed in the present study are therefore an important tool to use in

the identification of areas and populations most at conservation risk. Additionally, although our study's primary purpose was to investigate the potential for a genetic bottleneck among *L. catta* in Madagascar, evaluations such as this that document existing levels of genetic diversity (Table 1) are useful in assessing the overall genetic "health" of endemic lemur populations.

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