Conserv Genet
DOI 10.1007/s10592-013-0455-1

RESEARCH ARTICLE

Patch size and isolation influence genetic patterns in black-and-white ruffed lemur (*Varecia variegata*) populations

Sheila M. Holmes · Andrea L. Baden · Rick A. Brenneman · Shannon E. Engberg · Edward E. Louis Jr. · Steig E. Johnson

Received: 1 October 2012 / Accepted: 24 January 2013

Abstract Land use in Madagascar has resulted in extensive deforestation and forest fragmentation. Endemic species, such as the black-and-white ruffed lemur (*Varecia variegata*), may be vulnerable to habitat fragmentation due to patchy geographic distributions and sensitivities to forest disturbance. We tested for genetic differentiation among black-and-white ruffed lemur groups in two sites in a large forest patch and three sites in smaller patches. We also investigated the relationship between the genetic diversity of populations and patch configuration (size and isolation), as well as the presence or absence of past genetic bottlenecks. We collected blood ($n = 22$ individuals) or fecal ($n = 33$) samples from lemurs and genotyped the extracted DNA for 16 polymorphic microsatellites. Bayesian cluster analysis and F_{ST} assigned individuals to three populations: Ranomafana (two sites in continuous forest), Kianjavato (two fragments separated by 60 m of non-forest), and Vatovavy (a single fragment, more isolated in time and space). Vatovavy showed significantly lower allelic richness than Ranomafana. Kianjavato also appeared to have lower allelic richness than Ranomafana, though the difference was not significant. Vatovavy was also the only population with a genetic bottleneck indicated under more than one mutation model and a significant F_{IS} value, showing excess heterozygosity. These results indicate that a small geographic separation may not be sufficient for genetic differentiation of black-and-white ruffed lemur populations and that patch size may influence the rapidity with which genetic diversity is lost following patch isolation.

Keywords Forest fragmentation · Genetic differentiation · Diversity · Bottleneck · Madagascar

S. M. Holmes (✉) · S. E. Johnson
Department of Anthropology, University of Calgary, 2500 University Drive NW, Calgary, AB T2N 1N4, Canada
e-mail: holmess@ucalgary.ca

A. L. Baden
Department of Anthropology, Yale University, New Haven, CT, USA

R. A. Brenneman · S. E. Engberg · E. E. Louis Jr.
Grewcock Center for Conservation and Research, Omaha's Henry Doorly Zoo, 3701 South 10th St, Omaha, NE 68107, USA

Introduction

Madagascar, an island country known for its high endemism and species diversity, has undergone major deforestation and forest fragmentation. Less than 10 % of the original primary forests remain (Myers et al. 2000). Furthermore, both the amount of forest cover in isolated patches and the amount close to the forest edge have quadrupled from the 1950 to 2000s (Harper et al. 2007). Land use practices such as logging, mining, and slash and burn agriculture (*tavy*) continue to threaten Madagascar's unique flora and fauna, including several lemur species (Mittermeier et al. 2010).

Habitat fragmentation consists of four main components: habitat loss, increase in number of habitat patches, decreasing size of habitat patches, and increasing isolation of habitat patches (Andrén 1994; Fahrig 2003). These components have a non-linear relationship, with patch configuration increasing in importance as the amount of original habitat remaining decreases (Andrén 1994; Fahrig 2003). Given the increase in the prevalence of isolated forest patches in Madagascar in recent decades, it may be important to examine how the configuration (i.e., size and

Published online: 03 February 2013

isolation) of forest patches influences population dynamics in endangered native fauna. Previous studies of patch characteristics have indicated that patch isolation over time and space tends to be positively related to the genetic differentiation of populations (Grativol et al. 2001; Segelbacher et al. 2003; Yamamoto et al. 2004). Patch size, on the other hand, tends to be correlated with population size (e.g. small mammal species; Pardini et al. 2005; *Alouatta* spp. reviewed in Arroyo-Rodríguez and Dias 2010), and there is evidence that both metrics may be positively correlated with genetic diversity (Stangel et al. 1992; Knaepkens et al. 2004; Drees et al. 2011). Additionally, genetic bottlenecks may impact future diversity by making populations more susceptible to loss of heterozygosity and allelic richness via genetic drift and inbreeding depression (Frankham 1995; Saccheri et al. 1998; Keller and Waller 2002). This loss of genetic diversity can lead to higher risk of local extirpation in these populations (Saccheri et al. 1998; Harper et al. 2003). Therefore, determining how different patch configurations relate to the genetic parameters of lemur populations can indicate how to best manage land-use practices to minimize genetic differentiation and loss of genetic diversity.

In this study, we evaluate the effect of the size and isolation of forest patches on the genetic differentiation, genetic diversity, and presence of recent bottleneck events in and among black-and-white ruffed lemur, *Varecia variegata*, populations at two sites within a large continuous forest and three sites in a network of smaller forest patches. First, we test the hypothesis that there is a positive relationship between forest discontinuity and genetic differentiation. Black-and-white ruffed lemurs tend to avoid human encroachment (see "Study species" section), therefore we predict population boundaries to match those of forest patches, i.e., each patch will encompass a separate population. However, distance between sites may play a role due to the naturally patchy geographic distribution of the black-and-white ruffed lemur (Balko 1998; Andrainarivo et al. 2008). In this case we would see population differentiation among relatively distant sites, even within a continuously forested environment, though not necessarily between close forest fragments. Second, we examine the relationship between patch size, isolation and genetic diversity. Given that population size, often limited by patch size, tends to be correlated with genetic diversity (Frankham 1996), we hypothesize that populations in larger patches will have the highest genetic diversity, and populations in the smallest patches will have the lowest genetic diversity. We therefore predict that smaller patches will have lower allelic richness and will be more likely to show signs of inbreeding. Finally, we investigate whether past demographic events that can decrease genetic diversity can be detected in the study populations. We predict that black-and-white ruffed lemurs in the network of smaller, more isolated patches will have undergone population bottlenecks or founder effects. Assessing the effects of patch size and isolation on genetic differentiation and diversity will assist in evaluation of conservation risks for this critically endangered primate as habitat fragmentation continues to increase in Madagascar.

Materials and methods

Study species

The critically endangered black-and-white ruffed lemur has been directly impacted by habitat loss and human encroachment (IUCN 2011). Though found throughout the eastern rainforests of Madagascar (Baden et al. 2008; Balko 1998), populations are unevenly distributed within this range and are typically found at low densities (Balko 1998; Andrainarivo et al. 2008). This geographic pattern may be partially explained by adaptations to the stochastic climatic events such as cyclones. However, its current distribution pattern is likely exacerbated by anthropogenic disturbance (Vasey 2005) since the black-and-white ruffed lemur is largely absent from heavily logged areas, and is most abundant in undisturbed forest (White et al. 1995). Additionally, while the black-and-white ruffed lemur's reproductive capabilities may be adapted to the cycles of severe environmental disturbance that naturally occur in Madagascar (e.g. cyclones, heavy rains, and windstorms), individuals failed to successfully reproduce for at least 4 years (i.e., equivalent to the period from birth to sexual maturity; Morland 1991) following severe habitat disturbances at Manombo Special Reserve (Ratsimbazafy 2002; Louis et al. 2005) and Ranomafana National Park (Baden et al. in press). These patterns indicate this species' sensitivity to environmental disturbance. Furthermore, the closely related red ruffed lemur (*V. rubra*) has shown signs of a genetic bottleneck in degraded forest (Razakamaharavo et al. 2010); thus the black-and-white ruffed lemur may suffer genetic consequences from forest fragmentation as well.

Black-and-white ruffed lemur social groups are multimale, multi-female with a fission fusion social organization (subgroup composition changes throughout the course of a day) (Morland 1991). We refer to these fission fusion aggregations as social groups in this paper. Both sexes of this species disperse from their natal groups, with some additional targeted eviction of females (Erhart and Overdorff 2008).

Study sites

Sample and data collection took place at five sites in the rainforests of southeastern Madagascar (Fig. 1). Two study sites, Mangevo and Vatoharanana, were located in Ranomafana

National Park, a 41,600 ha protected area that is part of a larger continuous forest that spans much of the eastern escarpment of Madagascar. The other three sites were located in a series of forest fragments of varying size and isolation distance in the Tsitola–Vatovavy area, east of Ranomafana. Examination of topographical maps indicates that this landscape was continuously forested as recently as 1969 (Kianjavato topographic map© Foiben-Taosarintanin' I Madagasikara, Institut National de Géodésie et Cartographie). We sampled black-and-white ruffed lemur individuals in three forest patches: Tsitola (~954 ha), Sangasanga (64 ha) and Vatovavy (644 ha). Forest patches, separated by a mixture of anthropogenic grasslands, agriculture, roads, and settlements (with the exception of Tsitola and Sangasanga, separated by anthropogenic shrub and grasslands), were delineated for patch size and isolation distance measures using Google Earth (Google Inc. 2009) landcover layers by Digital Globe (August 4, 2005) and Spot 5 (April 18, 2011). Distances between sites are listed in Table 1.

Table 1 Distances between sites (km) where black-and-white ruffed lemur samples were collected

	Vatoharanana	Tsitola	Sangasanga	Vatovavy
Mangevo	8.34	25.83	33.12	40.57
Vatoharanana	–	25.83	33.12	40.57
Tsitola		–	0.06	7.51
Sangasanga			–	5.89

Distance between Mangevo and Vatoharanana was measured as the shortest distance between capture locations. All other distances were measured in Google Earth (Google Inc. 2009) as the shortest distance between forest edges defined by landcover layers by Digital Globe (August 4, 2005) and Spot 5 (April 18, 2011) and rounded to the nearest 10 m)

Sample collection

We obtained samples from black-and-white ruffed lemurs at Mangevo ($n = 10$) and Vatoharanana ($n = 12$) in 2005–2007. Field assistants from Omaha's Henry Doorly Zoo and Aquarium (OHDZA) and the Madagascar Biodiversity Partnership (MBP) immobilized study individuals with 10 mg/kg estimated body weight of Telazol® (Fort Dodge Animal Health, IA), administered by Dan-Inject® (Børkop, Denmark) Model JM CO_2-powered projection rifle and 9 mm disposable Pneu-Darts™ (Williamsport, PA). Whole blood (1 ml/kg) samples were collected from the femoral vein and stored in blood storage solution (Longmire et al. 1992) at room temperature until transfer to OHDZA for deposit in a −80 °C freezer.

S. Holmes and MBP field assistants collected fecal samples from ruffed lemurs that had been previously

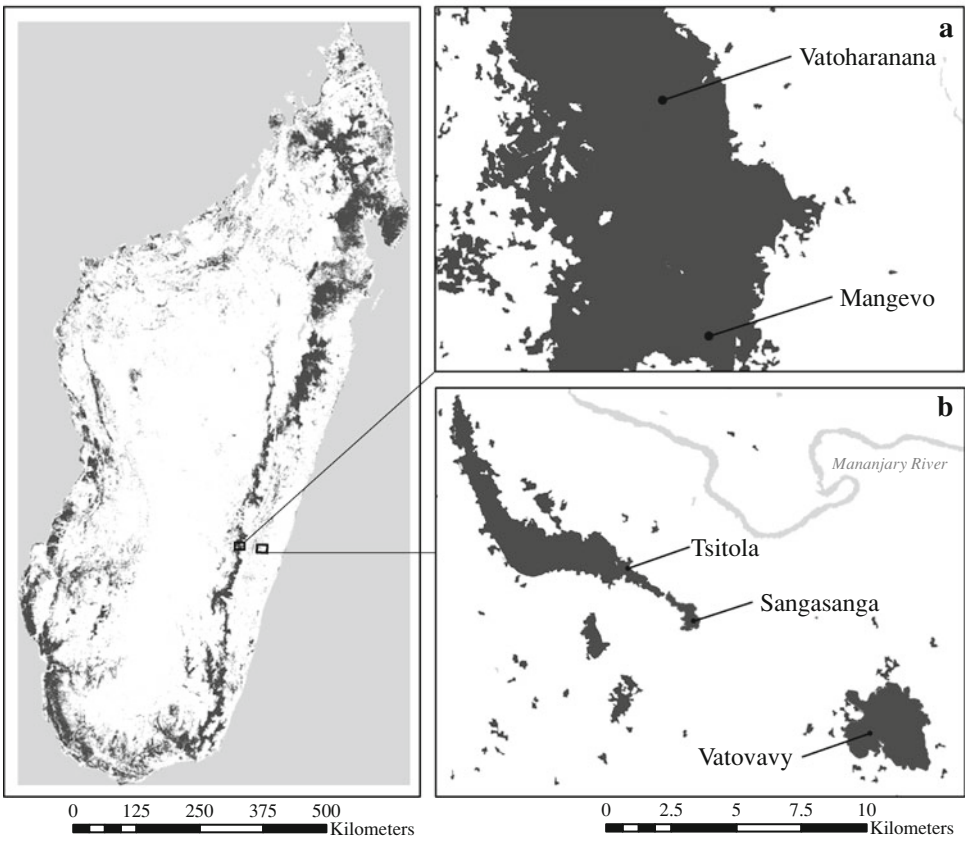

Fig. 1 Map of Madagascar showing forest cover (GIS layers courtesy of Conservation International and Gerber (2010), and modified based on Google Inc. (2009)). *Light grey* areas are water, *white* is non-forest, and *dark grey* is forest. **a** *Inset* of Ranomafana National Park locations where black-and-white ruffed lemur samples were obtained: Vatoharanana and Mangevo. **b** *Inset* of Tsitola–Vatovavy forest fragments. Samples were obtained from lemurs in the Tsitola, Sangasanga, and Vatovavy forest fragments

collared for individual identification in Tsitola ($n = 9$), Sangasanga ($n = 11$), and Vatovavy ($n = 13$) in May–September 2010. Assistants collected 2–5 samples per lemur, and removed seeds from fecal samples prior to preservation in RNA*later*® (Life Technologies, Grand Island, NY) at a ratio of 1 ml feces to 5 ml RNA*later*®. Samples were kept at room temperature for 15–105 days until transported to OHDZA, where they were stored in a −20 °C freezer until DNA extraction. Samples from Sangasanga, and Vatovavy represent a substantial proportion of the adult populations present in these fragments (ca. 50–100 %) while samples from Tsitola encompassed the social group geographically closest to the Sangasanga forest patch. All immobilizations, handling, sample collections, and export/import protocols adhered to and were approved by the OHDZA's Institutional Animal Care and Use Committee (IACUC), Stony Brook University IACUC, University of Calgary Animal Care Committee, Malagasy wildlife authorities, Convention on International Trade in Endangered Species regulations, and US Fish & Wildlife Service.

Genotyping

We extracted nuclear DNA (nucDNA) for microsatellite analysis from blood using proteinase k digestion followed by a standard phenol–chloroform extraction protocol (Sambrook et al. 1989). We extracted DNA from fecal samples using the QIAamp® DNA Stool Mini Kit (QIAGEN, USA) protocol modified according to Wikberg et al. (2012). We used 1.4 ml of the fecal sample solution, centrifuged for 1 min at $1{,}000\times g$ before removing the supernatant and further modified the protocol by allowing the DNA to lyse in ASL buffer for 4–5 h, vortexing every half hour. We performed multiple extractions on more than one fecal sample per individual and genotyped an average of seven replicates to account for the increased likelihood of false alleles and allelic dropout in non-invasively collected DNA (Pompanon et al. 2005).

Eighteen microsatellite loci were selected based on scoring ease of fecal DNA from 25 developed for the black-and-white ruffed lemur (Louis et al. 2005), and were used to genotype all individuals. The loci used were as follows: 51HDZ9, 51HDZ20, 51HDZ25, 51HDZ44, 51HDZ247, 51HDZ436, 51HDZ485, 51HDZ560, 51HDZ598, 51HDZ646, 51HDZ790, 51HDZ816, 51HDZ833, 51HDZ946, 51HDZ948, 51HDZ1020. We carried out PCR amplifications in a 25 µl volume with 20–50 ng nucDNA template, 12.5 pmol unlabelled and fluorescently labelled primers, 1.5 mm $MgCl_2$, 200 µm dNTP, 10 mm Tris (pH 8.3), 50 mm KCl, and 0.5 U *Taq* DNA polymerase (Promega; Madison, WI). We completed amplification in MBA Satellite 0.2G thermal cyclers (Thermo Electron Corp, Waltham, MA) with a thermal profile as follows: 35 cycles of 30 s at 95 °C, 30 s at a primer-specific annealing temperature, and 30 s at 72 °C with a 10 min final extension phase at 72 °C. We determined allele sizes using ABI 3130*xl* Genetic Analyzers (Life Technologies; Grand Island, NY) which separates PCR products using electrophoresis and determined fragment lengths using GeneScan™ 500XL ROX™ size standard in GeneMapper software v. 4.0. The genotyping error rate was 15.19 % due to high rates of allelic dropout and false alleles. False alleles were typically obvious and we used 3–11 replicates to ensure accurate determination of allele sizes in non-invasive samples. If there were no contradictory results, only three PCR products were needed. If at least one replicate did not agree, additional replicates were used until at least a five-replicate majority was achieved. The observed probability of identity ($P_{(ID)}$; Waits et al. 2001) for all markers was 1.60×10^{-13}.

Statistical analyses

We used the software GENEPOP v4.0.10 (Raymond and Rousset 1995; Rousset 2008) to evaluate population and locus conformity or departure from Hardy–Weinberg Equilibrium (HWE) allele frequencies and the software FSTAT v2.9.3 (Goudet 1995, 2001) to investigate linkage disequilibrium. We then performed the following statistical analyses to address each objective. Some statistics are sensitive to sample size [e.g. Bayesian cluster analysis (Pritchard et al. 2000) and allelic richness (Leberg 2002)], however minimum sample size requirements for all statistical tests were surpassed, we used a relatively large number of markers to increase power, and we used multiple statistics where possible to corroborate findings.

First, we examined the relationship between patch isolation and population differentiation. We employed FSTAT to determine Wright's fixation index, F_{ST} (Weir and Cockerham 1984), an estimate of genetic differentiation among populations. F_{ST} generally provides an effective estimation of population differentiation provided this statistic is not translated into gene flow. However, F-statistics are based on several assumptions, including that populations are in migration–drift equilibrium, which may be unrealistic (Whitlock and McCauley 1999). Given that populations may take many generations to reach new equilibria between genetic drift and migration, a violation of this assumption could lead to an F_{ST} value that is more indicative of historical equilibria than of contemporary patterns (Whitlock and McCauley 1999). We also utilized the software STRUCTURE v2.0, which performs Bayesian cluster analysis, to group individuals into the most probable number of populations (Pritchard et al. 2000). Using the test statistic Δk, the second order rate of change of the likelihood of each k value (number of groups), determined through 100,000 independent runs (following a burn-in of 50,000 iterations), we verified the most likely number of genetic clusters (Evanno et al. 2005). Finally, we determined

the exact G-statistic to measure gene diversity among populations, using GENEPOP to take into account the gene diversity within populations (Nei 1973).

Second, to address the connection between genetic diversity and patch size or isolation, we calculated the inbreeding coefficient F_{IS}, as well as allelic richness across populations and loci using FSTAT. Different sample sizes across populations can bias estimates of the average number of alleles per locus due to variable detection of rare alleles (Leberg 2002); therefore, we standardized allelic richness to the smallest sample size for any locus in any population of 11 diploid individuals using rarefaction to correct for this bias.

Third, we compared the presence of genetic bottlenecks in patches of different sizes and isolation distances using the software BOTTLENECK (Cornuet and Luikart 1996; Piry et al. 1999) to calculate the probability of recent declines in effective population size (N_e). We compared the observed heterozygosity (H_O) to the expected heterozygosity at mutation-drift equilibrium (H_{Eeq}), by calculating the number of alleles in the population sample and sample size, under the infinite alleles model (IAM), the stepwise mutation model (SMM), and two-phase mutation model (TPM; Di Rienzo et al. 1994). We also used the mode-shift indicator in BOTTLENECK to generate a qualitative description of distribution of allele frequencies of a population (Piry et al. 1999). Following a genetic bottleneck in a population, there is a disproportionate drop in low frequency alleles, leading to a shift in the mode of the allele frequencies from low to intermediate which is detectable for only a few dozen generations using the mode-shift indicator (Luikart et al. 1998). Immigration among populations can increase the chance of Type II errors in BOTTLENECK statistics and indicators by introducing rare alleles to a population while leaving heterozygosity relatively unaffected (Cornuet and Luikart 1996; Luikart and Cornuet 1998), and heterozygosity excess tests have limited power with small sample sizes (Peery et al. 2012). These factors could prevent detection of bottlenecks in our study populations if there is migration among fragments.

Results

Marker performance

Most of the markers used adhered to HWE frequencies, however locus 51HDZ609 deviated significantly from HWE and had a high presence of null alleles and was therefore dropped from analyses. Locus 51HDZ598 also differed significantly from HWE; however, when populations were examined independently, 51HDZ598 differed from HWE only in one population (Kianjavato) and was thus retained in analyses. With an adjusted p value of 0.000033, none of the pairs of loci were significant for linkage disequilibrium (results not shown). Locus 51HDZ27 was also dropped from analyses due to high monomorphism in the study populations. The mean allelic richness per locus for the remaining 16 loci was 3.48 ± 0.31.

Genetic differentiation

The F_{ST} analysis identified three distinct populations (Table 2). The Ranomafana sites, Mangevo and Vatoharanana, were not significantly differentiated from one another, nor were the two forest patches adjacent to the Kianjavato village, Sangasanga and Tsitola. We validated this result using the analysis STRUCTURE. The most likely number of populations was three, assigning the individuals from Mangevo and Vatoharanana to one population (hereafter referred to as Ranomafana), and grouping the individuals from Sangasanga and Tsitola together (hereafter, Kianjavato). All the individuals from Vatovavy were assigned to one source population, independent of other fragments (see Fig. 2). Due to these results, all following tests were based on the three populations, Ranomafana, Kianjavato, and Vatovavy.

F_{ST} values were re-calculated based on the three distinct populations and all pair-wise comparisons were verified to be significant. Additionally, the results of the G-test applied across all loci using Fisher's method for pair-wise population comparisons were significant ($p < 0.00001$) for each population pair, as well as across all three populations.

Genetic diversity

Table 3 shows the results of HWE conformity, genetic diversity, and bottleneck tests for each population. When results were averaged across loci using Fisher's test, no population was significantly different from the expected HWE frequencies. Vatovavy was the only population with a significant F_{IS} value. Pairwise t-tests of allelic richness with a Bonferroni correction indicated that the Vatovavy population had significantly lower allelic richness than the Ranomafana population ($p = 0.015$). The Kianjavato population did not differ significantly from either of the other two populations (Ranomafana: $p = 0.158$; Vatovavy: $p = 0.109$). The trend showed that Ranomafana population had the highest allelic richness and Vatovavy the lowest.

Bottleneck

Results from BOTTLENECK tests for heterozygosity excess and mode-shift corroborate the findings of the F_{IS} statistics. Vatovavy was the only population for which

Table 2 Genetic differentiation (F_{ST}) between study sites

	Mangevo	Vatoharanana	Sangasanga	Tsitola	Vatovavy
Mangevo	–	0.0052	**0.0002**	**0.0002**	**0.0001**
Vatoharanana	0.0393	–	**0.0002**	**0.0001**	**0.0002**
Sangasanga	0.2435	0.1998	–	0.0064	**0.0001**
Tsitola	0.279	0.2377	0.0573	–	**0.0002**
Vatovavy	0.291	0.2113	0.2107	0.1989	–

F_{ST} values are below the diagonal and *p* values are above. *p* values below the Bonferroni-adjusted 5 % significance level for multiple comparisons (0.0050) are in bold

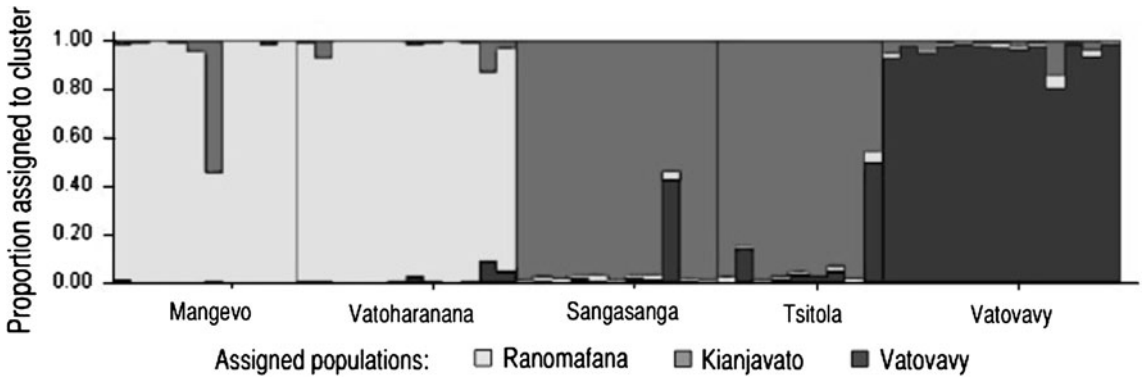

Fig. 2 Results from program STRUCTURE, indicating the proportion of each sampled individual assigned to each of 3 black-and-white ruffed lemur populations of origin

heterozygosity excess was detected under both the IAM and TPM. Vatovavy also displayed a mode-shift: a significant deficit in the abundance of low-frequency alleles with respect to intermediate allele frequency classes.

Discussion

Genetic differentiation

The black-and-white ruffed lemur communities located in continuous forest habitat at Ranomafana (Mangevo and Vatoharanana) were not genetically differentiated from one another, despite a geographic distance of approximately 8.3 km. These results, subject to the limitations of the tests used (e.g. sample size, assumption of migration–drift equilibrium), contrast with patterns found in golden-brown mouse lemurs (*Microcebus ravelobensis*), which exhibited genetic differentiation between sites located within continuous forest (Radespiel et al. 2008). It is perhaps surprising that we did not see similar genetic differentiation at Ranomafana, given the highly patchy distribution of the black-and-white ruffed lemur at this site (White et al. 1995). Sampling of additional sites within large forest patches may be required to detect genetic differentiation. Populations were divergent when found in isolated patches, but only at larger distances. Social groups in Tsitola and Sangasanga (together, Kianjavato) were not genetically differentiated, though the Kianjavato population was genetically differentiated from that of Vatovavy. Similarly, Quéméré et al. (2010) found that golden-crowned sifaka (*Propithecus tattersalli*) population genetic differentiation, while strongly affected by forest patch connectivity on a regional scale, was only weakly related to the connectivity of forest fragments on a larger landscape scale due to the varied isolation of patches. Patterns of genetic differentiation among isolated habitat patches have been recorded both in other lemurs in Madagascar (*M.* spp.; Olivieri et al. 2008; Radespiel et al. 2008) and other mammals elsewhere (*A. caraya*; Oklander et al. 2010; *Rhinopithecus bieti*; Liu et al. 2009; *Pteromys volans*; Lampila et al. 2009).

A caveat to the suggestion that the Kianjavato–Vatovavy divide represents a substantial isolation barrier to migration in the black-and-white ruffed lemur is the unknown historical demography of these populations. It is possible that effective population size and genetic variation at Vatovavy decreased prior to habitat fragmentation, masking current population trends (Jordan et al. 2009). Similar patterns of genetic differentiation may be arrived at through different combinations of gene flow, genetic drift, selection, and a number of other processes. For example, isolation-by-distance and vicariance are often correlated,

Table 3 Genetic diversity in three populations of black-and-white ruffed lemurs

Population	Hardy–Weinberg					Bottleneck			
	χ^2	*p*	F_{IS}	Allelic richness	SE allelic richness	IAM	TPM	SMM	Mode-shift
Ranomafana	40.372	0.0979	0.01 (>0.05, ns)	3.23	0.26	**0.04431**	0.25223	1.00000	Normal
Kianjavato	18.9609	0.9408	0.019 (>0.05, ns)	2.83	0.28	0.52817	0.86026	0.23120	Normal
Vatovavy	28.9609	0.3129	**−0.185 (<0.01)**	**2.47**	0.26	**0.00171**	**0.02148**	0.14648	**Shifted**
Total	88.2937	0.4113	–	3.48	0.31	–	–	–	–

Fisher's test χ^2 and *p* values for departure from Hardy–Weinberg equilibrium are presented for each population, as well as the total for all three populations. F_{IS} values (significance levels based on confidence intervals obtained by bootstrapping over loci (95 % CI = −0.085 to 0.032, 99 % CI = −0.104 to 0.050)) and allelic richness per population and across populations (averaged across loci and based on a population size of 11 diploid individuals) are also presented, along with the standard error (SE) of the mean allelic richness per population and across populations. Heterozygosity excess, under each of three mutation models, and mode-shift tests are presented for the detection of recent genetic bottlenecks. Significant results are in bold

limiting the ability to distinguish between the effects of each on genetic differentiation (Bossart and Prowell 1998). Additionally, if populations have not yet reached migration–drift equilibrium, F_{ST} values will reflect historical population structure rather than current patterns (Whitlock and McCauley 1999). In this case, while it is likely that population genetic differentiation was associated with forest connectivity, the naturally patchy geographic distribution of this species, exacerbated by a decrease in population size due to unknown factors, may have subdivided populations prior to deforestation.

Genetic diversity

We investigated whether the genetically differentiated populations showed evidence of reduced diversity according to patch size or isolation. We found that larger patches had greater allelic richness than smaller patches. Given that Kianjavato formed a single population spanning two adjacent patches, we saw an identical pattern with isolation: the larger the (combined) patch area, the higher the allelic richness (Table 3). A similar general trend towards lower genetic diversity in smaller populations in more fragmented habitats was found in gray-headed lemurs (*Eulemur cinereiceps*; Brenneman et al. 2012). Vatovavy was the only population with a significant F_{IS} value; it was positive, indicating an excess of heterozygosity compared to allelic richness (see "Bottleneck discussion", below). In contrast, if heterozygosity and allelic richness had both decreased to a similar degree, we might see a non-significant F_{IS} value. This was observed in populations of red deer (*Cervus elaphus*), where deer in fragmented habitats demonstrated significantly less allelic richness and heterozygosity than deer in continuous habitats, but no difference in F_{IS} (Dellicour et al. 2011).

The deforestation and forest fragmentation in this system is relatively recent. Based on examination of older topographic maps, we estimate the time since initial discontinuation of forest between the Kianjavato and Vatovavy populations (1969 or more recently) to be 5–6 generations [one generation is equal to 8 years with minimum age at first reproduction for the black-and-white ruffed lemur ~2.5–4 years (Foerg 1982; Brockman et al. 1987; Morland 1991) and maximum age in wild *V.* spp. populations estimated between 13 and 15 years (pers. obs; Vasey, pers. comm)]. It is therefore noteworthy that deforestation among and around the Kianjavato patches is likely to have occurred later than that between Kianjavato and Vatovavy. Within-patch heterozygosity levels take longer to reach equilibrium following subpopulation isolation than do differentiation measures and may reflect past, rather than current, landscape connectivity (Keyghobadi et al. 2005). Therefore, it is possible that over time the Kianjavato population will experience a decrease in genetic diversity.

Bottleneck

As with the genetic diversity results, Vatovavy was the only population with significant heterozygosity excess compared to expected heterozygosity under mutation drift equilibrium under two mutation models. Although the likelihood of a bottleneck in this population was not found to be significant under the SMM, this is the most conservative model and the least likely to identify a heterozygosity excess with respect to mutation drift equilibrium (Luikart and Cornuet 1998). The population at Vatovavy also showed evidence of a mode-shift, which is expected under all three mutation models in the case of a population bottleneck (Luikart et al. 1998). These results, along with the significant positive F_{IS} value (Table 3), suggest the prediction of a past genetic bottleneck in the smaller, more isolated patch. Similar genetic bottlenecks attributed to anthropogenic deforestation within the last few hundred years have been indicated previously in the black-and-white ruffed lemur (Louis et al. 2005), as well as in the closely related red ruffed lemur (*V. rubra*; Razakamaharavo et al. 2010), the Milne-Edwards sportive lemur (*Lepilemur edwardsi*; Craul et al. 2009) and

three northwestern mouse lemurs (*M.* spp.; Olivieri et al. 2008). Thus, marked genetic consequences from a reduction in population size are present across lemur taxa and environments.

It is noteworthy that, despite the recent and considerable deforestation that has occurred in the Tsitola–Vatovavy region over the past 50 years, and the separation from the Ranomafana forest patch before that time, the Kianjavato population did not show signs of a population bottleneck. It is possible that the larger total area of this patch network than Vatovavy may have supported a sufficient population size to maintain allelic richness and heterozygosity (Luikart et al. 1998; Busch et al. 2007). As a caveat to this, however, bottleneck signals may be influenced by immigration, the timing and duration of bottlenecks, sample size, and pre-bottleneck diversity levels (Peery et al. 2012). While immigration among distant fragments is unlikely due to the ecology of this species, it is even less likely to occur in Vatovavy than in Kianjavato. Vatovavy is at the eastern edge of the remaining habitat patches for this species, and therefore could receive no immigration from the east. Therefore, the Kianjavato population is more likely to violate the no-immigration assumption of the bottleneck tests (Luikart and Cornuet 1998) with immigrants masking the signal of a past genetic bottleneck. Also, as noted above, deforestation around the Kianjavato forest patches likely occurred later than deforestation between Kianjavato and Vatovavy, making any potential genetic bottleneck more recent in the former, and therefore more difficult to detect.

Conclusions

In summary, black-and-white ruffed lemur groups showed a lack of genetic differentiation within forest patches and when separated by a small gap between forest patches, yet diverged into separate populations over a larger patch isolation distance. While lemurs in the collectively larger patch cluster of Kianjavato did not undergo a genetic bottleneck, nor a significant deficit in genetic diversity, when compared to those in the largest forest patch, Ranomafana, lemurs in the smaller, more isolated patch had begun to lose genetic diversity in the form of allelic richness, likely due to a demographic bottleneck further isolated by habitat fragmentation. It is important to note, however, that deforestation in the Kianjavato area was likely more recent than habitat loss around Vatovavy; therefore, we may yet see significant reduction in genetic diversity in this patch cluster as time since isolation and/or population decline increases. Prevention of further patch isolation and decrease in patch size will be important in decreasing conservation risks for these populations. The potential for land-use practices to further isolate forest patches and decrease patch size is cause for conservation concern, and must be addressed in proactive management plans.

Acknowledgments We would like to thank all of the local and foreign research assistants for sample collection in Madagascar (specifically F. Gordon, L.G. Razanajatovo, C. Razafindravelo, E.E. Rakotoson, and J.P. Marolahy for fecal sample collection), the staff at the Grewcock Center for Conservation and Research at Omaha's Henry Doorly Zoo and Aquarium for help with genotyping, T. Wyman for help with maps, and M. Aylward for input regarding statistical analysis. Funding was provided by the Natural Sciences and Engineering Research Council of Canada (SMH), the government of Alberta (SMH), the University of Calgary (SMH, SEJ), the International Primatological Society (SMH), the Calgary Zoological Society (SMH), the National Science Foundation (ALB, DDIG BSC-0725975), J. William Fulbright Foundation (ALB), the L.S.B. Leakey Foundation (ALB), Stony Brook University (ALB), Conservation International and Margot Marsh Biodiversity Foundation's Primate Action Fund (SMH, ALB, SEJ), and Primate Conservation, Inc. (SMH, ALB, SEJ).

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