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LOW GENETIC VARIABILITY IN THE RECOVERING URBAN BANDED LEAF MONKEY POPULATION OF SINGAPORE

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ABSTRACT. — The banded leaf monkey (*Presbytis femoralis femoralis*) is critically endangered in Singapore and affected by widespread deforestation in southern Peninsular Malaysia. The Singapore population has recovered from a low of 15–20 to more than 40 individuals, but prior to our study it was unclear how severely the past bottleneck had depleted the genetic diversity of the population. Here, we provide the first analysis of the genetic variability based on seven samples (ca. 20% of population) collected over two years of fieldwork. We find only two haplotypes that differ only in one variable site for the hypervariable region I (HV-I) of the mitochondrial d-*loop*. Compared to available population-level data for other colobines (proboscis monkey, Yunnan snub-nosed monkey, Sichuan snub-nosed monkey, Angolan black and white colobus), the banded leaf monkey population in Singapore has the lowest number and the most similar haplotypes. This low genetic variability is the next challenge for the conservation of the population. Protected habitats in prospering urban environment may become important sanctuaries for endangered species, but reintroductions may have to be considered in order to restore genetic variability that was lost during past bottlenecks.

KEY WORDS. — *Presbytis femoralis femoralis*, Asian colobine, mitochondrial HV-I, urban environment, reintroduction

INTRODUCTION

The banded leaf monkey (*Presbytis femoralis*) is found on the Malay Peninsula and Sumatra (Meijaard & Groves, 2004; Fig. 1). At least three subspecies are currently recognised, but the taxonomy is far from settled (see Md.-Zain et al., 2008; Vun et al., 2011). The type locality for *P. f. femoralis* is Singapore, but the same subspecies also occurs in Johor, a southern state of Malaysia. *Presbytis f. robinsoni* is known from the northwest Malay Peninsula extending north to Thailand and Myanmar. *Presbytis f. percura* is only found in eastern Sumatra. Although globally only listed as Vulnerable (IUCN Red List B1ab(ii,iii,iv,v); Nijman et al., 2008), *P. f. femoralis* is Critically Endangered (D) in its type locality

Singapore due to small population size (Lim et al., 2008). Widespread on the island in the last century (Chasen, 1924), they were still common in the 1920s (Chasen, 1940). Unfortunately, deforestation related to economic development destroyed much of their suitable habitat and eventually *P. f. femoralis* was confined to the Bukit Timah Nature Reserve (BTNR) and Central Catchment Nature Reserve (CCNR). In 1983, the construction of an expressway separated the two reserves, effectively stopping all gene flow, and the banded leaf monkeys disappeared from the BTNR (Yang & Lua, 1988).

A recent 2-year study provided evidence that the population is recovering and now consists of ca. 40 individuals in 5–6 groups inhabiting 455 ha of secondary and swamp forests in CCNR (Ang et al., 2010). At least six births were reported between 2008 and 2010 (Ang et al., 2010), but the population viability remains in doubt given the genetic constraints and the social organisation of leaf monkeys that affect fertility, reproduction, and group formation. Genetic diversity has long been recognised as an important component of fitness and population viability (e.g., Spielman et al., 2004; Charpentier et al., 2008) and most surviving individuals are suspected to be genetically closely related given that small populations rapidly lose genetic diversity through genetic drift and inbreeding (Lande & Barrowclough, 1987). We here assess the genetic variability of Singapore's banded leaf monkey population using the hypervariable region I (HV-I) of the displacement loop (d-*loop*) that is often considered to be the most rapidly evolving part of the mitochondrial genome (Lopez et al., 1997). HV-I is a widely used marker in population studies of vertebrates, but data for Asian colobines have only become available recently. We here examine the intra-population variability using HV-I for seven samples which are likely to represent ca. 20% of the population. Note that despite years of absence of hunting pressure, *P. f. femoralis* remains very shy in Singapore and hence it took two years of fieldwork to obtain these samples. Preliminary observations also indicate that the species is difficult to observe in Panti forest (Johor, Malaysia, AA, pers. obs.). We then compare our findings with what is known about the population-level variability of the same marker for four other colobines (Table 1).

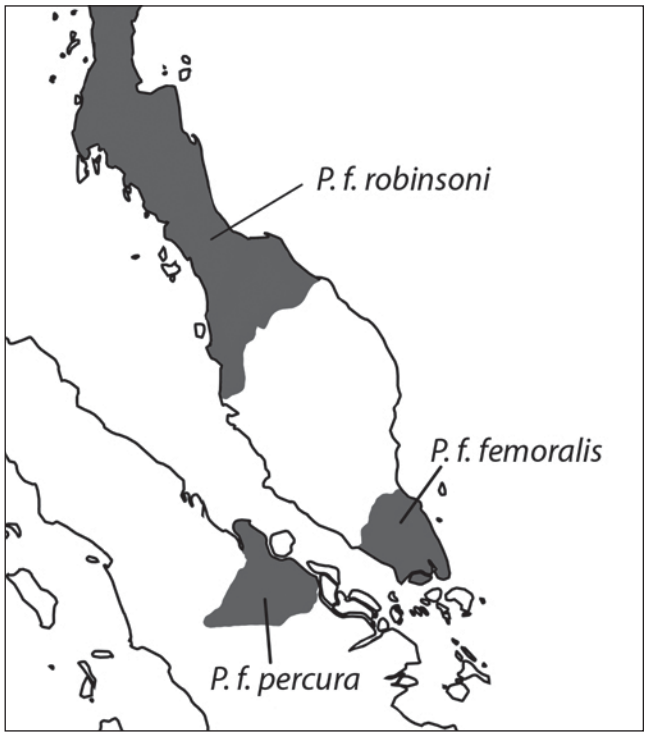

Fig. 1. Distribution of *Presbytis femoralis*.

MATERIAL AND METHODS

Faecal collections and DNA extraction. — Faecal samples were collected in the field during Aug.2009 – Mar.2011 (Table 2) and stored at –70°C. Genomic DNA was extracted from 50 mg of feces using QIAamp® DNA Stool Mini Kit (QIAGEN, Singapore). DNA was recovered in 40 µl of elution buffer (instead of 200 µl) in order to obtain a higher concentration of DNA, and was stored at –20°C. The recovery and sample purification of genomic DNA from a dry blood stain of a roadkill specimen (17 Jan.2011, Upper Thomson Road) was carried out using buccal brushes following the protocol of Puregene® Buccal Cell Core Kit (QIAGEN, Singapore; note that the carcass could not be recovered and is only known from a photograph). The DNA was dissolved in 20 µl of DNA hydration solution and also stored at –20°C.

DNA amplification and sequencing. — Specific HV-I primers for *Presbytis* were published by Meyer et al. (2011), but the forward primer failed and a new one had to be designed (new primer: hf_dloop_F 5'-GCCCTTATGTAATTCGTGCATTAC-3'; published primer: 6234 HV-I_r 5'-TGATAGACCCGTGATCCATC-3'). This primer was used in PCRs using the following recipe: 25 µl reaction (2.5 µL buffer, 2 µL dNTPs, 1.0 µL MgCl, 0.1 µL Takara Ex-Taq, 1.2 µl of each primer, 1.5–3 µl template DNA). Amplification conditions were as follows: denaturation at 95°C: 5 mins, 40 cycles of 1 min at 94°C, 1 min annealing at 58°C–60°C, 1 min at 72°C, final extension: 5 mins at 72°C). Amplification products were purified using Bioline SureClean (Randolph, MA) or gel extraction (QIAquick, QIAGEN, Singapore). Sequencing was carried out in both directions using BigDye ver. 3.1 (Applied Biosystems, Foster, CA) after purification with CleanSEQ® kit (Agencourt Bioscience Corporation, Beverly, MA) followed by direct sequencing in an ABI PRISM® 3100 Genetic Analyzer (Perkin Elmer Applied Biosystems, Norwalk, CT). The sequences were edited in Sequencher 4.6.

Comparison with other colobines. — In order to assess the genetic variability of *P. f. femoralis* population in Singapore, we would ideally compare it to genetic data for populations in Southern Malaysia. However, unfortunately these data are not available and we therefore have to compare the Singapore data to the variability observed in all published population-level studies on colobines using the same marker (Table 1). The sequences were downloaded from GenBank, aligned using MAFFT, and only the region overlapping with our sequences was retained. Based on the original publications (Liu et al., 2007; Pan et al., 2009; McDonald & Hamiliton, 2010; Munshi-South & Bernard, 2011), we identified which

Table 1. Population genetic studies of colobine species using HV-I.

Species	Populations	No. of sequences	No. of haplotypes	Expected no. of haplotypes* (significance)	Min. (%)	Max. (%)
Presbytis femoralis femoralis[1]	CCNR	7	2	2	0.19	0.19
Colobus angolensis palliatus[2]	Diani	22	4	3.44 ± 0.66 (**0.014**)	0.87	4.37
	Shimoni	18	4	3.06 ± 0.65 (0.051)	0.22	5.68
	Udzungwa	30	4	3.2 ± 0.78 (0.062)	0.22	5.68
	Southern Highlands	33	7	4.06 ± 0.92 (**0.013**)	0.22	1.31
Nasalis larvatus[3]	Garama	12	4	3.4 ± 0.59 (**0.008**)	0.67	3.12
	Weston	9	8	6.5 ± 0.53 (**<0.001**)	0.57	4.43
Rhinopithecus bieti[4]	North	40	12	5.34 ± 0.93 (**<0.001**)	0.30	12.17
	Central and Southwest	97	18	5.39 ± 0.97 (**<0.001**)	0.25	10.75
	Southeast	20	2	2 ± 0 (N/A)	1.48	1.48
Rhinopithecus roxellana[5]	Qinling	12	5	3.76 ± 0.75 (**0.01**)	0.19	0.95
	Minshan	15	4	3.46 ± 0.52 (**0.003**)	1.53	6.12

*Average of the expected number of haplotypes when only seven sequences were randomly selected 100 times from original populations (95% significance level based on Z-score). Min.: minimum divergence between haplotypes. Max.: maximum divergence between haplotypes. [1]Data from this study. [2]McDonald & Hamiliton, 2010. [3]Munshi-South & Bernard, 2011. [4]Liu et al., 2007. [5]Pan et al., 2009.

Table 2. Faecal and blood sample collections and length of sequences.

Date	Sample	Group (Group size)	Base pairs (bp)
5 Apr.2009	Faecal	A (8)	502
29 Aug.2009	Faecal	B (6)	422
27 Jan.2010	Faecal	C (5)	433
30 Mar.2010	Faecal	D (3)	522
2 Dec.2010	Faecal	E (2)	507
17 Jan.2011	Blood	D (3)	495
30 Mar.2011	Faecal	B (6)	506

sequences were from separate populations. The sample size was generally larger than ours. We therefore rarefied the samples 100 times for all populations other than the Weston population of *Nasalis larvatus* (Kalinowski, 2004) to match our sample size. For the latter, we rarefied the samples only 10 times given that there are only 36 possible ways of choosing subsets of 7 samples from 9 sequences available for the population (Table 1). We then determined the number of haplotypes for each rarefied sample and used the z-score to determine if the number of haplotypes in our study deviated significantly from the distribution. Note that by obtaining the distribution through rarefaction, some sequences are sampled multiple times; i.e., the data points for obtaining the distribution are not completely independent. We also calculated the intraspecific sequence divergences using TaxonDNA (v 1.6.2) (Meier et al., 2006).

RESULTS

All six faecal samples and the blood sample yielded usable DNA. The seven samples collected over two years of fieldwork represent ca. 20% (seven out of 40 individuals) of the Singapore population of banded leaf monkeys as long as no individual was sampled twice. This cannot be ruled out, but is unlikely given the low probability of duplicate sampling across five different localities. All samples were collected on different days (see Table 2), and the sample locations were separated by man-made barriers (e.g., military infrastructure) which helped us determined which groups were sampled. Hence we believe that our small samples should not influence the conclusions of the genetic results.

Seven mtDNA HV-I sequences ranging from 422–522 bp (Table 2) were obtained and varied only with regard to one site (position 190 in relation to full HV-I region of *P. melalophos* (DQ355299); Fig. 2). Four sequences showed evidence for an A/C polymorphism at this site (see discussion); two sequences had an adenine, and one sequence a cytosine. When compared to 11 populations of four other colobine species, all 11 populations have higher average genetic variability and all but one have a larger number of haplotypes (Table 1).

DISCUSSION

The banded leaf monkeys in Singapore are critically endangered with only 40 individuals left in the wild. Due to the small population size and elusive nature of the monkeys, ca. 20% of the population were sampled. The genetic variability of the banded leaf monkey population in Singapore is extraordinarily low. We find only two haplotypes differing by one nucleotide for the hypervariable HV-I control region. After adjusting for sample size (Kalinowski, 2004), this is the lowest average variability observed for all colobine species with population-level data (*N. larvatus*, *R. bieti*, *R. roxellana*, *C. a. palliatus*). With one exception, the number of haplotypes is also lower than for all other colobine species with data (Table 1).

Our study also reveals mtDNA heteroplasmy for four individuals within this population. Heteroplasmy refers to the presence of two or more mtDNA molecules in an individual. The two mtDNA molecules may be different in size or differ due to base substitutions (Lutz et al., 2000; Lo et al., 2005). Previous studies have reported mammalian mtDNA heteroplasmy which is particularly common near the hypervariable region (Lutz et al., 2000; Schwarz & Vissing, 2002; Bayona-Bafaluy et al., 2003; Lo et al., 2005). Here we present another case of point mutation heteroplasmy in primates. Since more than one individual has the polymorphic site, this suggests that the heteroplasmy may have been maintained for more than a generation (Hayasaka et al., 1991).

The low genetic variability is probably due to the population's recent history. Since becoming a British colony in 1819, >95% of the estimated 540 km^2 of original vegetation in Singapore was lost and at least a third of all plant and animal species became extinct (Brook et al., 2003). Once common throughout the island, banded leaf monkeys declined rapidly and after the extirpation of the BTNR population in 1987, it was estimated that only 10–15 individuals remained in the CCNR (Yang et al., 1990). This led to the assessment that the banded leaf monkey was a "living dead" (Brook et al., 2003) with a population too small to be viable (Pitra et al., 1995). Today, the population size has approximately doubled (>40), but the extremely low genetic variability of the HV-I region indicates a lack of genetic recovery. The after-effects of the bottleneck of the 1970s and 1980s are clearly recognisable which makes the population extremely vulnerable to environmental disturbances such as disease (Spielman et al., 2004; Charpentier et al., 2008).

In order to overcome this lack of genetic variability, translocation from genetically compatible populations in Malaysia may be considered given that such measures can restore the reproductive potential of endangered populations (e.g., Westemeier et al., 1998). Of the different types of translocation characterised by IUCN (1987), "augmentation" and "reintroduction" would be most appropriate. However, before translocation can be seriously considered, more research needs to be carried out. It should start with genetic comparison of the populations based on both mitochondrial and nuclear markers. In addition, fieldwork should compare the autecology of the populations. Lastly, the risks will have to be considered. For example, disease introduction through translocated animals can pose serious threats to the survival of populations (Viggers et al., 1993). Similarly, outbreeding depression (Weeks et al., 2011) through the loss and/or dilution of local adaptations can have a negative impact on population recovery although the importance of outbreeding depression is controversial (see Edmands, 2007). All this research will require close collaboration between the wildlife authorities of the two countries before reintroductions can be considered.

Urban reserves as sanctuaries of inbred rare species. — We suspect that the fate of the banded leaf monkey in Singapore is typical to what will be commonly observed in the urban environments in developing countries. Development usually starts in densely populated areas and after a period of rapid growth with little concern for natural resources, sustainable development and conservation of wildlife will become policy goals. The populations of those species that survives start to recover, but considerable genetic damage will have been done. In Singapore it was the BTNR and, in particular, the CCNR, with its 455 ha of secondary and primary forests that have become important wildlife refuges (Ng et al., 2011) during the rapid economic development of Singapore in the 20th century. With increased affluence, the negative impacts of human activities (e.g., deforestation, hunting) on the remaining forests disappeared and more resources were invested in maintaining the valuable habitats, creating green buffers, and promoting the conservation of biodiversity. This includes an ecological corridor ("Eco-Link") to be completed by the year 2013, which will reinstate the connectivity between BTNR and CCNR (Ng et al., 2011). This will also increase habitat availability and food resources for the banded leaf monkey. However, due to the localised extinction of banded leaf monkeys in BTNR, it will not help with the genetic recovery of the population.

Conclusions. — In order to ensure long-term persistence of banded leaf monkeys in Singapore, translocation of individuals from Malaysia may have to be considered in order to restore genetic variability and to increase the genetic adaptive potential. Natural forests within urban environment in Singapore can serve as a sanctuary for the recovery of the type population of *P. f. femoralis* population; i.e., even a small,

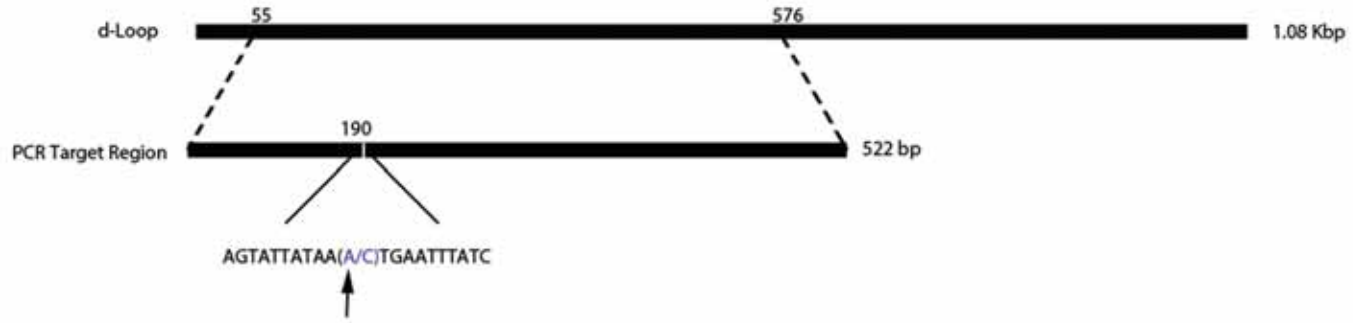

Fig. 2. Full length d-*loop* of *Presbytis melalophos* (1.08kbp) and target region of d-*loop* (variable site for *P. femoralis* is position 190). The complete mitochondrial genome of this specimen is published under Sterner et al. (2006), and the specimen is identified as *P. melalophos* following Groves (2001) and Brandon-Jones et al. (2004).

resource-poor, and highly urbanised country is not anathema for the conservation and recovery of a native species.

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