

Genetic Diversity and Distinctiveness of the Proboscis Monkeys (*Nasalis larvatus*) of the Klias Peninsula, Sabah, Malaysia

JASON MUNSHI-SOUTH AND HENRY BERNARD

From the Department of Natural Sciences, Baruch College, City University of New York (CUNY), New York, NY 10010 (Munshi-South); and the Unit for Primate Studies-Borneo, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia (Bernard).

Address correspondence to Jason Munshi-South at the address above, or e-mail: jason.munshi-south@baruch.cuny.edu.

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In this study, we sequenced a partial segment of the mitochondrial control region from 21 proboscis monkeys of the Klias peninsula, the last large population remaining on the west coast of Sabah, Malaysia. Our results showed that this population retains substantial genetic variation, and subpopulations from different river systems in the central and southern portions of the Klias share multiple haplotypes. We also compared our data with previously generated sequences from 2 eastern populations of proboscis monkeys in Sabah and found little evidence of regional genetic structure. Based on these results, we argue that conservation efforts should focus on restoring connectivity between central and southern Klias peninsula proboscis monkeys and discuss future analyses needed to better understand the mitochondrial structure of proboscis monkeys in Sabah.

Key words: control region, noninvasive genetics, peat swamp, phylogeography, population structure, primates

Introduction

The proboscis monkey (*Nasalis larvatus*) is endemic to the island of Borneo and primarily inhabits mangroves, peat swamps, and riverine forests. Agriculture, particularly oil palm plantations, logging, forest fires, and hunting have dramatically reduced proboscis monkey populations in recent decades (Bennett 1988; Meijaard and Nijman 2000), leading to the species' listing as Endangered on the IUCN Red List of Threatened Species (Meijaard and Supriatna 2010) and Appendix I of CITES (UNEP-WCMC 2003). Systematic surveys of *N. larvatus* populations throughout Borneo have not been attempted, but reviews of occurrence records indicate that the largest populations are in Kalimantan (Meijaard and Nijman 2000). Proboscis monkeys in Kalimantan may also be the most endangered and at least

one population was recently extirpated through habitat destruction (Meijaard and Nijman 2009).

Recent boat surveys in the Malaysian state of Sabah in NE Borneo recorded a nearly 2-fold greater number of proboscis monkeys (minimum $N = 5907$) than previous estimates (Sha et al. 2008). However, the distribution of *N. larvatus* in Sabah is highly fragmented, comprising 4 population clusters along eastern coastal waterways, a single west coast cluster in the Klias peninsula, and smaller isolated populations. The western Klias populations comprise the third largest, but most geographically isolated, cluster in Sabah. In Klias, the main concentrations of *N. larvatus* are located within the Padas Damit Forest Reserve and the Weston area, that is, the central and southern portions of the Klias peninsula, respectively. The Klias peninsula is separated from the western clusters by the Crocker Range highlands, although the presence of remnant isolated populations on the west coast indicate that proboscis monkeys may have once occurred along the entire Sabah coastline (Bernard and Hamzah 2006). In this study, we sequenced a segment of the mitochondrial DNA (mtDNA) control region from *N. larvatus* scat sampled from 2 Klias river systems to examine 1) genetic diversity within the Klias peninsula and 2) genetic differentiation of Klias populations from other Sabah populations in Labuk Bay and the Kinabatangan river.

Proboscis monkeys are typically organized into stable groups consisting of an adult male plus several females and their offspring (Yeager 1990; Murai 2004). Groups in Sabah exhibit home ranges as large as 220 ha (Boonratana 2000) and travel up to 1.7 km daily (Matsuda et al. 2009). Female transfer between groups is a frequent occurrence (Murai et al. 2007). However, it is unknown whether these behaviors have maintained genetic connectivity between river systems separated by agricultural lands in the Klias peninsula. The genetic structure of proboscis monkeys

throughout Sabah is also poorly understood. Orangutans (*Pongo pygmaeus*) exhibit considerable genetic differentiation between opposite sides of major rivers in Sabah (Jalil et al. 2008; Goossens et al. 2005), but proboscis monkeys can readily swim across waterways (Boonratana 2000). The dispersal capabilities of proboscis monkeys may be adequate to counteract genetic structuring of major populations in Sabah. Alternatively, historical isolation of the Klias peninsula may have resulted in considerable genetic drift and population-specific mtDNA haplogroups.

Materials and Methods

Fecal samples for mtDNA analysis were collected from proboscis monkey groups in the Klias peninsula using a boat survey method (Bernard and Hamzah 2006) because *N. larvatus* roost in trees by river banks in the early morning and late evening. As part of a monthly survey of Klias proboscis monkeys in August 2008, we cruised 18 km of the Garama and Klias rivers within the Padas Damit Forest reserve (lat 05°24.622' N, long 115°31.909' E) in central Klias, and 8 km of the Kukup and Nabahan rivers near Weston (lat 05°13.698' N, long 115°34.949' E) in southern Klias (Figure 1). When a group was encountered, we observed them from a distance until they moved away from the area. Then, we collected fresh fecal samples from underneath roosting trees and stored them in RNALater

(Ambion, Austin, TX). We encountered 4 groups in central Klias ($N = 11$ fecal samples collected) and 3 groups in southern Klias ($N = 14$ fecal samples). Additional fecal samples in ethanol were available from facultative collections in central Klias ($N = 23$ samples) and Labuk Bay ($N = 3$ samples; Figure 1) during surveys from January–March 2008.

Fecal DNA was extracted using the QIAamp DNA Stool Kit (Qiagen, Valencia, CA). We sequenced 264 bp of the mtDNA control region from 17 of 25 samples (68%) collected in August 2008 using the primers NL2F (5'-ACCCAACACCCAAAATTG-3') and NL2R (5'-TAA-GAACCAGATGTCCGT-3'). Four additional sequences from Klias (17.4% successfully sequenced) and one from Labuk Bay (33.3% success) were obtained from samples collected in January–March 2008. Polymerase chain reaction conditions consisted of denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 30 s, and primer extension at 72 °C for 1 min. Forward and reverse sequences were obtained using a Beckman-Coulter CEQ 8000 automated sequencer and then aligned and checked for accuracy using SEQUENCHER 4.8 (Gene Codes Corp., Ann Arbor, MI). Pairwise alignment of edited sequences from different individuals was performed using CLUSTALW implemented in BIOEDIT 7 (Hall 1999) and visually inspected for accuracy. Sequences from this data set were then aligned with 6 unique haplotypes previously obtained from the Lower Kinabatangan and one haplotype from Labuk Bay (Figure 1; Jalil MF, unpublished data) to

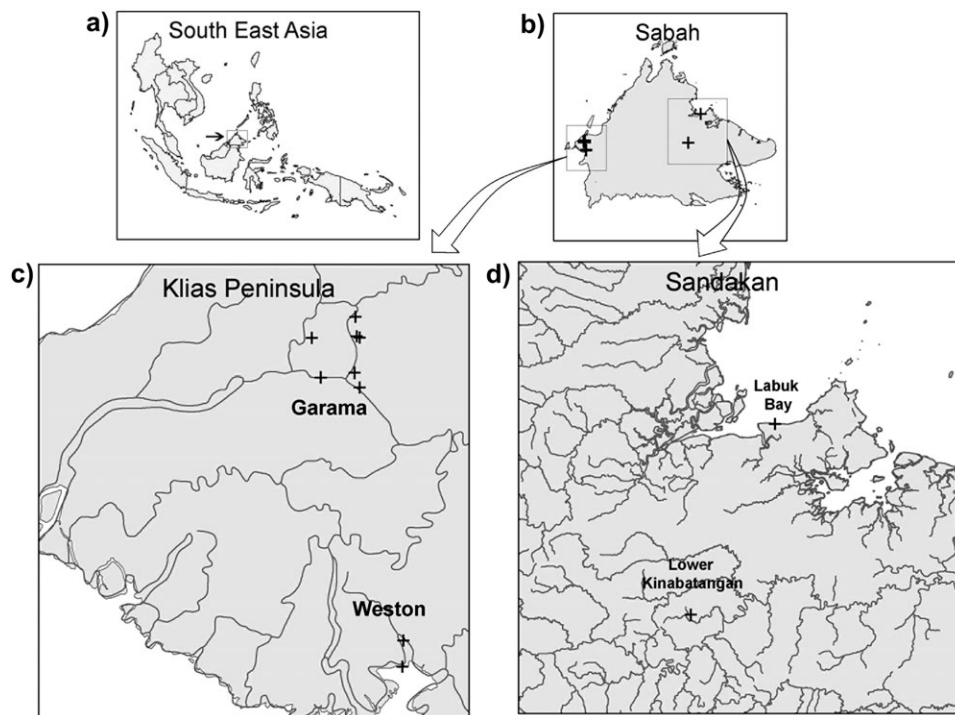


Figure 1. Sampling locations from this study: (a) Sabah, Malaysia; (b) Klias peninsula, Labuk Bay, and the Lower Kinabatangan in Sabah; (c) location of samples obtained from the central (Garama, $n = 12$) and southern Klias peninsula (Weston, $n = 9$); and (d) close-in view of Labuk Bay ($n = 2$ mtDNA sequences) and Lower Kinabatangan ($n = 2$).

identify unique haplotypes from the 3 populations. All unique mtDNA sequences have been deposited in GenBank (accession nos: HQ260729–HQ260744).

Diversity measures of mtDNA control region sequences from 2 Klias sampling areas, Klias overall, and Sabah overall were estimated using the number of haplotypes, haplotype diversity h , and nucleotide diversity π , in DNASP 5.1 (Rozas et al. 2003). To statistically test for nonneutral evolutionary dynamics of our haplotypes, we calculated Tajima's D and Fu's F_S using 10 000 coalescent simulations in DNASP to calculate significance. We calculated pairwise F_{ST} from haplotype frequencies (P values calculated from 1000 permutations) and an exact test of population differentiation in Arlequin 3.5 (Excoffier et al. 2005) to examine the degree of genetic difference between our 2 Klias sampling areas. Finally, we calculated a median-joining haplotype network based on weighted maximum likelihood distances in NETWORK 4.5 (Bandelt et al. 1999) to visually examine the evolutionary relationships between haplotypes from different populations.

Results

From 21 mtDNA control region sequences obtained from Klias proboscis monkeys, we identified 16 variable sites and 9 haplotypes. Eight haplotypes were detected from only 9 individuals sampled from southern Klias. The number of haplotypes, haplotype diversity, and nucleotide diversity were all higher for the southern Klias versus central Klias samples (Table 1). Tests of neutrality were not significant for any sample. Both an exact test of population differentiation ($P = 0.19$) and $F_{ST} = 0.04$ ($P = 0.18$) based on haplotype frequencies were not statistically significant for southern and central Klias samples.

When we combined the 21 Klias sequences with 2 haplotypes from Labuk Bay and 6 haplotypes from the Lower Kinabatangan, we identified a total of 16 unique haplotypes in Sabah (Table 1). These haplotypes and their population frequencies are available on Dryad (doi:10.5061/dryad.8564). A median-joining network identified 3 clusters of haplotypes, but sequences were only partially clustered by population (Figure 2). Three haplotypes were present in both the central and southern Klias peninsula (PM02, PM03, and PM05). Four of the 6 Kinabatangan haplotypes (PM08, PM13–15) were found in one cluster, and both

Table 1 Genetic diversity among the proboscis monkeys of the Klias peninsula based on 264 bp of the mtDNA control region.

Study area	N	V	H	h	π	Tajima's D	Fu's F_S
Garama (central Klias)	12	12	4	0.65	0.018	0.82	3.44
Weston (southern Klias)	9	15	8	0.97	0.023	0.42	-2.23
Total Klias sample	21	16	9	0.81	0.020	0.57	0.19
Total Sabah sample	29	21	16	0.90	0.022	0.20	-3.82

N , sample size; V , number of variable sites; H , number of haplotypes; h , haplotype diversity; π , nucleotide diversity.

Labuk Bay haplotypes (PM04 and PM11) were found in a different cluster. However, at least one haplotype from each of the 3 clusters was present in both the Klias peninsula and Kinabatangan. One haplotype was also shared between the Kinabatangan and southern Klias (PM08).

Discussion

The analysis of mtDNA control region sequences in this study indicates that proboscis monkeys have maintained considerable genetic variation at mtDNA loci in the Klias peninsula. Even with a moderate sample size of 21 sequences from 7 groups, we identified 9 haplotypes and 3 distinct haplogroups in Klias (Figure 2). The minimum population size of *N. larvatus* in Klias was recently estimated as 818 individuals, with 578 individuals (71%) in the central Garama region, 114 individuals (14%) in the southern Weston region, and small groups scattered elsewhere (Sha et al. 2008). Surprisingly, we found nearly twice as much genetic variation in the smaller Weston sample despite the considerably larger Garama subpopulation. Southern Weston may harbor significant numbers of undetected individuals or haplotypes or higher rates of female transfer between groups. Alternatively, this pattern may be an artifact of small sample size.

The high mtDNA haplotype diversity reported here is similar to estimates from other primate species with female transfer between groups, such as Tana River red colobus (*Procolobus rufomitratus*, Mborora and McPeck 2010) or Hamadryas baboons (*Papio hamadryas*, Hapke et al. 2001). In these cases, migration of females may maintain high mtDNA diversity across all subpopulations by slowing the extinction of mtDNA haplotypes due to genetic drift in small fragmented populations. Species exhibiting female philopatry are expected to exhibit lower variability within groups but greater genetic differentiation between (sub-)populations. The Tana mangabey (*Cercocebus galeritus*), for example, exhibits lower mtDNA diversity than the red colobus mentioned above, although both species exhibited considerable genetic structure between habitat patches (Mborora and McPeck 2010). Genetic structure can develop at nuclear loci in isolated primate populations after recent anthropogenic fragmentation (Liu et al. 2009), even when genetic diversity remains high (Quéméré et al. 2009). Proboscis monkeys may still exhibit genetic structure and lower genetic diversity at nuclear loci such as microsatellites if males are relatively philopatric and female transfer explains the lack of structure at mtDNA loci described here.

Proboscis monkeys exhibited little mitochondrial structure within the Klias peninsula, suggesting that female migration between the 2 large Klias subpopulations has occurred in recent times. Thus, conservation efforts in the Klias peninsula should focus on extending preexisting forest reserves (Bernard and Hamzah 2006) to maintain or restore connectivity between the central Garama and southern Weston areas. Tropical peat swamps are one of the most endangered habitats globally (Yule 2010), and the Klias peninsula is the last stronghold on the

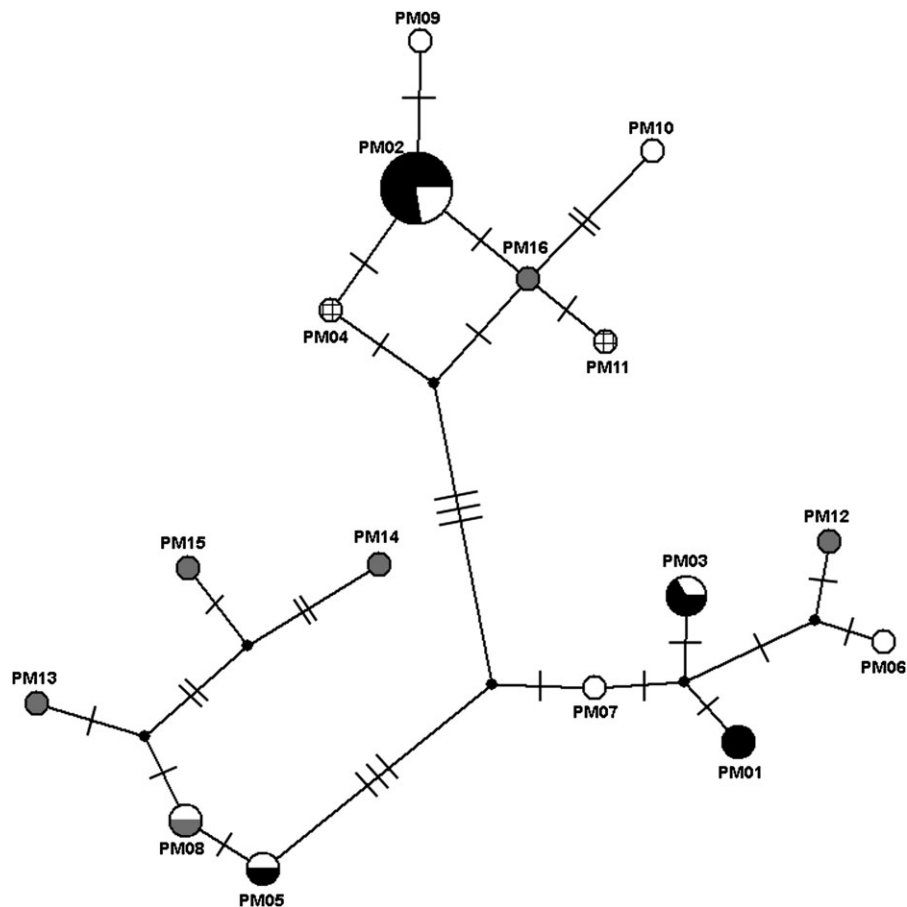


Figure 2. Median-joining network of 16 proboscis monkey mtDNA control region haplotypes from Sabah, Malaysia. Each circle represents a unique haplotype colored according to its presence in the central Klias peninsula (black), southern Klias (white), Lower Kinabatangan (gray), and Labuk Bay (cross-hatched). The diameter of the circles correspond to haplotype frequency, and the smallest circles represent singletons. Mutational steps between haplotypes are represented by black bars perpendicular to lines connecting haplotypes. Three clusters of haplotypes were identified (clockwise from top): Cluster 1 (PM02, PM04, PM09–11, PM16); Cluster 2 (PM01, PM03, PM06–07, PM12); and Cluster 3 (PM05, PM08, PM13–15). Haplotype sequences and frequencies are available on Dryad (doi:10.5061/dryad.8564).

west coast of Sabah. Though the 2 areas are connected by narrow strips of mangrove and riverine forest along the banks of the Klias river, it is currently unknown whether proboscis monkeys disperse between the central and southern Klias. However, groups have been recorded near human habitation outside of forest reserves (Ali et al. 2009). The recent development of microsatellite markers for *N. larvatus* (Salgado-Lynn et al. 2010) will facilitate noninvasive estimates of contemporary migration.

The Klias peninsula is isolated from eastern Sabah by the Crocker Range, but we did not find substantial genetic differentiation between the Klias and 2 eastern populations. The association with waterways and swimming ability of proboscis monkeys (Boonratana 2000) suggests that they could use intact riverine forests as regional dispersal corridors. However, no contemporary records exist of proboscis monkeys in the Crocker Range, and thus the Klias population has been isolated from the eastern Sabah populations for several decades or longer. Sharing of haplotypes between

eastern and western *N. larvatus* may represent relictual variation from recolonization out of Crocker range mountain refugia after the last glacial maximum (Tanaka et al. 2001). Alternatively, a continuous distribution along the west coast may have facilitated some gene flow and isolation by distance. Expanded sampling throughout Sabah could be used to test the hypothesis that the Klias peninsula contains greater genetic variation than eastern populations more distant from putative refugia (Jalil et al. 2008).

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