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Molecular Phylogenetics and Evolution 46 (2008) 683–694
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Molecular phylogeny and biogeography of langurs and leaf monkeys of South Asia (Primates: Colobinae)

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Received 1 June 2007; revised 25 October 2007; accepted 28 November 2007

Abstract

The two recently proposed taxonomies of the langurs and leaf monkeys (Subfamily Colobinae) provide different implications to our understanding of the evolution of Nilgiri and purple-faced langurs. Groves (2001) [Groves, C.P., 2001. Primate Taxonomy. Smithsonian Institute Press, Washington], placed Nilgiri and purple-faced langurs in the genus *Trachypithecus*, thereby suggesting disjunct distribution of the genus *Trachypithecus*. [Brandon-Jones, D., Eudey, A.A., Geissmann, T., Groves, C.P., Melnick, D.J., Morales, J.C., Shekelle, M., Stewart, C.-B., 2003. Asian primate classification. Int. J. Primatol. 25, 97–162] placed these langurs in the genus *Semnopithecus*, which suggests convergence of morphological characters in Nilgiri and purple-faced langurs with *Trachypithecus*. To test these scenarios, we sequenced and analyzed the mitochondrial cytochrome *b* gene and two nuclear DNA-encoded genes, lysozyme and protamine P1, from a variety of colobine species. All three markers support the clustering of Nilgiri and purple-faced langurs with Hanuman langur (*Semnopithecus*), while leaf monkeys of Southeast Asian (*Trachypithecus*) form a distinct clade. The phylogenetic position of capped and golden leaf monkeys is still unresolved. It is likely that this species group might have evolved due to past hybridization between *Semnopithecus* and *Trachypithecus* clades.

Keywords: Molecular phylogeny; *Semnopithecus*; Langurs; *Trachypithecus*; Cytochrome *b*; Protamine P1; Lysozyme; Biogeography; Disjunct distribution

1. Introduction

The Old World colobine monkeys (Subfamily Colobinae) are unique among the primates, in that they predominantly eat hard fruits and leaves and have evolved a complex foregut in which microbes ferment these otherwise indigestible plant materials (Chivers and Hladik, 1980). Colobine monkeys are currently found in forested areas of sub-Saharan Africa, throughout much of the South and Southeast Asia.

According to the species distribution map in Oates et al. (1994), there are at the least five species of colobines in South Asia. These include the langurs found in the Indian subcontinent—Hanuman langur (*Semnopithecus entellus*), Nilgiri langur (*Trachypithecus johnii*), and purple-faced langur (*T. vetulus*); the leaf monkeys found in the far north-eastern states of India, in Bhutan, and in Bangladesh (referred to here as the Northeast)—golden leaf monkey (*T. geei*) and capped leaf monkey (*T. pileatus*). Additionally, the genus *Trachypithecus* consists of at the least five more species that are found predominantly in Southeast (SE) Asia—Phayre's leaf monkey (*T. phayrei*), ebony leaf monkey (*T. auratus*), silvered leaf monkey (*T. cristatus*), Francois' leaf monkey (*T. francoisi*), and dusky leaf monkey (*T. obscurus*) (Fig. 1). The Northeast biota has greater

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doi:10.1016/j.ympev.2007.11.026

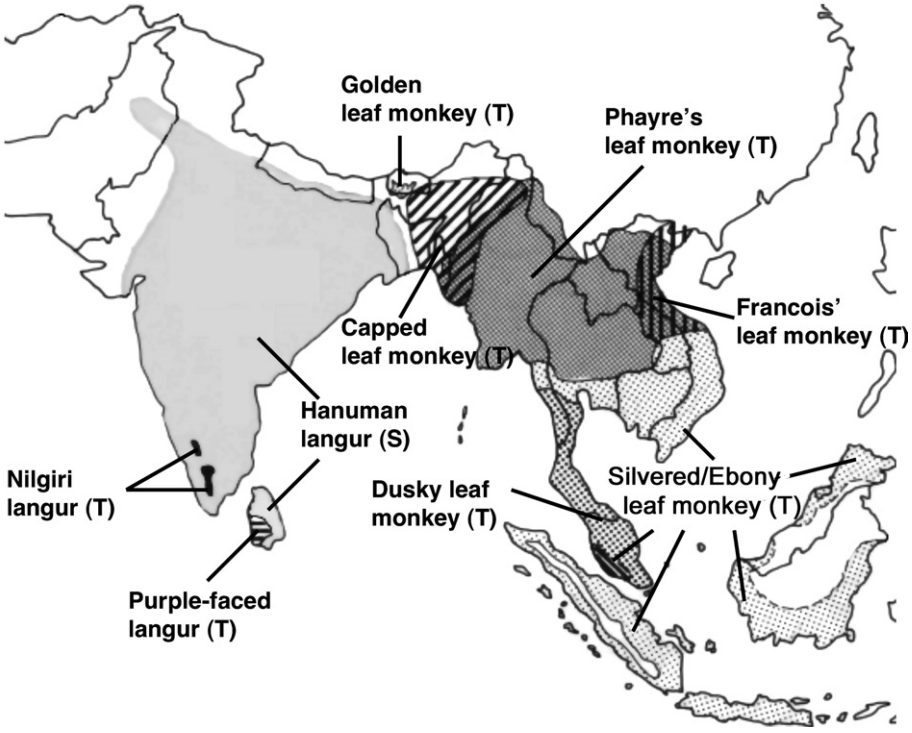

Fig. 1. Distribution of the langurs and leaf monkeys. Groves (2001), classification scheme suggests disjunct distribution of the genus *Trachypithecus* (T). Species classified in this genus are found in southwestern India and Sri Lanka, but are not found in the rest of the Indian subcontinent. They are also found in Northeast India and throughout SE Asia. Thus, there is a gap in the distribution of the species currently classified in the genus *Trachypithecus*. Whereas Hanuman langur, genus *Semnopithecus* (S), is distributed predominantly in South Asia (including Sri Lanka). Figure modified from Oates et al. (1994).

affinity with SE Asian biota than with the rest of the Indian subcontinent. Therefore, species distributed in the Northeast (such as capped and golden leaf monkeys) have not been included in the Indian subcontinent.

The generic level classification of these langurs and leaf monkeys have been in a flux for a long time (Table 1). Most authors place the Hanuman langurs in the genus *Semnopithecus* distinct from other langurs and leaf monkeys (Hill, 1939; Pocock, 1939; Groves, 1993; Oates et al., 1994; Groves, 2001) or in a separate species group [*entellus* group (Napier and Napier, 1985)]. Hanuman langurs are unique among Asian colobines in that they are widely distributed and are found in a range of habitats predominantly in the dryer parts of South Asia (Roonwal and Mohnot, 1977). Whereas most other Asian colobines tend to be confined to moist and wet forests (Oates et al., 1994). The two wet evergreen forest species of the Indian subcontinent, the Nilgiri and purple-faced langurs, have been placed either in the genus *Semnopithecus* (Brandon-Jones et al., 2003) or in the genus *Trachypithecus* (Oates et al., 1994; Brandon-Jones, 1996; Groves, 2001). Additionally, some authors considered Nilgiri and purple-faced langurs as sister taxa, and classified them in a separate genus *Kasi* (Hill, 1939; Pocock, 1939) or species group [*vetulus* group (Napier and Napier, 1985; Groves, 2001)]. The rest of the leaf monkeys have been placed in the genus *Trachypithecus* (Hill, 1939; Pocock, 1939; Oates et al., 1994; Brandon-Jones, 1996; Groves, 2001) or separate species group [*cristatus* group (Napier and Napier, 1985)] (Table 1). Thus one of the major disagreements between these classification schemes is with respect to the taxonomic position of Nilgiri and purple-faced langurs. Are Nilgiri and purple-faced langurs sister taxa? Additionally, are these two species more closely related to genus *Semnopithecus* or are they related to genus *Trachypithecus*?

These taxonomic scenarios have interesting biogeographic implications. For example, placing Nilgiri and purple-faced langurs in the genus *Trachypithecus* suggests there is a disjunct distribution of the genus *Trachypithecus* (Fig. 1). This implies that species classified as *Trachypithecus* are found in the wet evergreen forests of southwest India (Nilgiri langur, *T. johnii*) and Sri Lanka (purple-faced langur, *T. vetulus*) but not in the rest of the Indian subcontinent; they are again found in the wet evergreen forests of Northeast and SE Asia.

There are several known cases of disjunct distributions of seemingly closely related taxa in the wet zone (areas of high rainfall) of the Indian subcontinent, including certain macaques (Morales and Melnick, 1998), other mammals (Kurup, 1974), birds (Ali, 1969), fresh-water fishes and amphibians (Jayaram, 1974), and reptiles (Das, 1996). Mani (1974), points out that the discontinuity in the distribution of these taxa can be explained by two quite different models. In the first, for some species the current discontinuity represents a relict of a former continuous distribution; in that the prior extensive and continuous range of these

Table 1
Generic level classification of langurs and leaf monkeys put forward by various authors

	Brandon-Jones et al. (2003)	Groves (2001)	Brandon-Jones (1996)	Oates et al. (1994)	Napier and Napier (1985)	Pocock (1939)	Hill (1939)
Hanuman langur	S	S	S	S	P1	S	S
Purple-faced langur	S	T+	S(T)	T	P2	K	K
Nilgiri langur	S	T+	S(T)	T	P2	K	K
Capped leaf monkey	T	T*	S(T)	T	P3	T	T
Golden leaf monkey	T	T*	S(T)	T	P3	—	—
Phayre's leaf monkey	T	T	S(T)	T	P3	T	T
Silvered leaf monkey	T	T	S(T)	T	P3	—	T
Dusky leaf monkey	T	T	S(T)	T	P3	T	T
Francois' leaf monkey	T	T	S(T)	T	P3	—	T
Ebony leaf monkey#	T	T	S(T)	T	—	—	—

S = *Semnopithecus*; T = *Trachypithecus*; K = *Kasi*; P = *Presbytis*; P1 = *Presbytis entellus* group; P2 = *Presbytis vetulus* group; P3 = *Presbytis cristatus* group; (T) = subgenus *Trachypithecus*; * = *pileatus* group; + = *vetulus* group. # species not included in the current work.

wet forest forms recently has been broken into isolated refugial patches, mainly due to climatic changes (Mani, 1974). Ripley et al. (1986) invoked this model (Refugial model) to explain the disjunct distributions of certain wet zone species of birds found on the Indian subcontinent. This is a likely scenario for many species, given that much of South and SE Asia was once covered with a continuous belt of wet forest (Meher-Homji, 1983). Eudey (1980) points out that as the wet forests retreated during glacial events in the Pleistocene the range of Nilgiri langurs may have been reduced to relict populations making the rapid spread of Hanuman langurs possible throughout the Indian subcontinent. In support of the refugial model, most of the SE Asian leaf monkeys are wet forest species like the Nilgiri and purple-faced langurs. In contrast, Hanuman langurs are adapted to drier habitats, predominantly deciduous woodlands (Roonwal and Mohnot, 1977) suggesting the Hanuman langurs could have dispersed into the Indian subcontinent independently of the other langur lineage. This scheme would require two independent migrations of colobines into the Indian subcontinent, one of the humid forest species (Nilgiri and purple-faced langurs) and the other of the dry zone species (Hanuman langurs).

Based on limited molecular data, Brandon-Jones et al. (2003) placed Nilgiri and purple-faced langurs in the genus *Semnopithecus* along with Hanuman langur. This classification scheme suggests that the langurs of Indian subcontinent are closely related to each other to the exclusion of SE Asian leaf monkeys (Table 1). As a result of this rearrangement, the genus *Trachypithecus* no longer exhibits disjunction. Thus, morphological characters, such as cranial morphology, neonate pelage color, and sexually dichromatic pubic integument, that unite Nilgiri and purple-faced langurs with *Trachypithecus* (Brandon-Jones et al., 2003), might reflect independent evolution of these characters in these two species. Mani (1974) noted that for a number of other species, their discontinuous distribution might be due to distantly-related forms showing convergent or parallel evolution in widely separated areas (here referred to as convergence model). Thus, it is essential to resolve the phylogenetic relationships of the langurs of the Indian subcontinent to determine if Nilgiri and purple-faced langurs are affiliated to *Trachypithecus*, there by supporting disjunct distribution of the genus *Trachypithecus*. In contrast, if they are related to *Semnopithecus* this would suggest convergence of morphological characters in Nilgiri and purple-faced langurs with *Trachypithecus*.

Most previous taxonomic studies on colobines have used morphological and behavioral characters and have not been able to fully resolve their evolutionary relationships or devise a stable phylogeny-based taxonomy (reviewed in Disotell, 2000). Messier and Stewart (1997) found two major clades among langurs and leaf monkeys based on their work on the nulearDNA (nDNA)-encoded lysozyme gene. The first clade consisted of Hanuman langur and purple-faced langur (both of which are found in the Indian subcontinent), and the second clade consisted of two SE Asian species, dusky and Francois' leaf monkeys. Similar results were obtained from the analysis of 424 bp mitochondrial $tRNA^{Thr}$ gene and cytochrome *b* gene fragment by Zhang and Ryder (1998). These authors found support for the monophyly of Hanuman langur, Nilgiri langur, and purple-faced langur, to the exclusion of the clade consisting of Francois' and Phayre's leaf monkeys reaffirming the classification scheme by Brandon-Jones et al. (2003). However, none of these previous molecular phylogenetic studies have used nDNA markers in conjunction with mitochondrial DNA (mtDNA) on all the species of langurs of South Asia. These studies also lacked extensive sampling of the widely distributed and highly variable Hanuman langur. In this paper, we attempt to resolve the evolutionary relationships between the various species of langurs and leaf monkeys, particularly those from South Asia, through phylogenetic analysis. We used two unlinked nDNA markers (lysozyme and protamine P1) and one mtDNA (cytochrome *b*) marker to better understand disjunct distribution among the langurs and to determine if the classification of Nilgiri and purple-faced langurs by Brandon-Jones et al. (2003) is consistent with the molecular phylogeny.

2. Materials and methods

2.1. Sample collection

Three kinds of samples—namely hair, blood, and muscle tissue—were collected from wild and captive animals. Blood samples were stored in digestion buffer (0.01 M Tris–HCl, pH 8; 0.01 M EDTA; 0.1 M NaCl; 10 μl 1 M DTT) in a 1:1 ratio. Tissue samples were stored in 95% ethanol.

Hair samples were collected from captive individuals by rubbing a bamboo pole with sticky tape at the end on the animal's back. The hairs that stuck to the tape were removed and transferred to a clean paper envelope, which was labeled with collection site and species. In the case of hair samples from wild animals, all the collections were performed on Hanuman langurs. These monkeys are fairly well-habituated to humans, and they are periodically fed by local people throughout most of their range. We usually employed a local person to pluck some hair from such habituated monkeys while they were being fed. As we plucked only a few hairs per individual, this appeared to be a relatively painless procedure. In the laboratory, the hair samples were stored at −20 °C until use. Samples collected for this study and their sources are listed in Table 2.

2.2. DNA extraction

The protocol used here was modified from Refs. Vigilant et al. (1989), Meyer et al. (1995), and Li (1997). Using a sterile razor blade, one to three plucked hairs were cut 1 cm from the root (follicle) and the shafts were discarded. The resulting hairs, including follicles, were washed with distilled water, followed by a wash in 95% ethanol. The hair sample was then digested in 250 μl of digestion buffer and 2–5 μl of 20 μg/ml Proteinase-K (Fisher Biotech, Cat. No. 972159) for 12 h in a shaking incubator at 37 °C (digestion step). After the hair was completely digested, 250 μl of 5 M lithium chloride and

Table 2
Sequences used for the phylogenetic analysis, their accession numbers, and sources of the samples

	Code	Sample type	Source	GenBank accession number		
				Cyt-*b*	prml	Lzm
Hanuman langur	S2	Hair	HZ	AF293952	AF294852	—
	S5	Hair	CZ	AF293953	AF294852	AF294862
	N16	Blood	UCB	AF012470	AF294851	(1)
	N17			AF295576	—	—
Nilgiri langur	1	Hair	W	AF294619	AF294853	AF294863
	2	Hair	HZ	AF294620	AF294853	—
	3[a]	DNA	EZ	—	AF294854	AF294863
Golden leaf monkey	1	Hair	BZ	—	AF294857	AF294865
	2	Hair	HZ	AF294618	AF294857	AF294865
Phayre's leaf monkey	I1	Tissue	W	AF294621	AF294858	AF294866
	I2	Hair	SZ	—	AF294859	—
	V1[b]	DNA	EPRC	AF294622	AF294860	AF294867
	V2[b]	DNA	EPRC	—	AF294860	—
Capped leaf monkey	1	hair	HZ	—	—	AF294864
	2	Hair	SZ	AF294626	AF294856	AF294864
Purple-faced langur	1	Hair	CZ	—	AF294855	—
	2	See Ref. (Blacket et al., 1999)		AF295577	AF119236	(1)
Francois' leaf monkey	1	See Ref. (Blacket et al., 1999)		AF295578	AF119234	AF294869
Dusky leaf monkey		See Ref. (Blacket et al., 1999)		AF295579	AF119238	(1)
Silvered leaf monkey		Blood	NYZS	AF295580	AF294861	AF294868
Guereza colobus		See Ref. (Bossuyt and Milinkovitch, 2000)		U38264	AF119233	—
Red colobus		Tissue	LSU	AF294625	AF29485O	—
Proboscis monkey		See Ref. (Blacket et al., 1999)		U62663	AF119237	(1)
Patas monkey				(5)	(6)	(1)
Rhesus monkey		See Ref. (Blacket et al., 1999)		U38272	AF119240	(1)
Baboon		See Ref. (Blacket et al., 1999)		Y16590	AF119239	(1)

Note. Hanuman langur samples N16 and N17 are from North India, S2 and S5 are from South India and Sri Lanka, respectively. Phayre's langurs samples, I1 and I2 are from India and V1 and V2 from Vietnam. HZ = Hyderabad Zoo, South India; CZ = Colombo Zoo, Sri Lanka; UCB = University of California, Berkeley, USA (P. Dolhinow); HZ = Hyderabad Zoo, South India; EPRC = Endangered Primate Rescue Center, Vietnam (C. Roos); SZ = Shipahijala Zoo, Northeast India; BZ = Bannerghatta Zoo, South India; EZ = Erfurt Zoo, Germany; W(Nilgiri langur) = Wild from Anamalai Hills, South India (M. Singh); LSU = Louisiana State University, USA (P.A. Marx and P. Telfer); NYZS = New York Zoological Society, USA (D. Wharton); W (Phayre's langur) = Wild from Shipahijala national park, Northeast India. 1, Messier and Stewart (1997); 2, Zhang and Ryder (1998); 3, Messier (1998); 4, Collura and Stewart (1995); 5, sequence provided by Todd Disotell (NYU); 6, Queralt and Oliva (1991); samples collected in India and Sri Lanka were sequenced in Dr. Lalji Singh's laboratory.

The CDC import permit number for all the samples 98-0398.

[a] CITES export permit A—0011/98.

[b] CITES import permit US838407, CITES export permit W—0414/98.

500 μl chloroform–isoamylalcohol (24:1) were added to the sample and mixed thoroughly. The sample was centrifuged for 15 min at 10,000*g*. The supernatant was transferred into a new tube. DNA in the supernatant was precipitated in 900 μl of 95% ethanol and 10 μl of 2 M sodium acetate at −20 °C for 1 h. After DNA precipitation, the tube was centrifuged at 11,000*g* for 15 min. The resulting pellet was washed with 500 μl of 70% ethanol and centrifuged again at greater than 11,000*g* for 15 min. The ethanol was discarded and the pellet was dried and then re-dissolved in 50 μl TE (10 mM Tris–Cl, pH 8, 1 mM EDTA) at 65 °C for 1 h. The protocol described above was also used for DNA extraction from 0.5 g of tissue and 250 μl of blood (stored in digestion buffer) samples.

2.3. PCR amplification and sequencing of various markers

Previous work on the mitochondrial cytochrome *b* (*Cyt-b*) gene showed that it accurately resolves the known mtDNA phylogeny of the hominoids (Collura and Stewart, 1995), which radiated around the same time as the langurs. Thus, the *Cyt-b* gene was used to infer the mtDNA phylogeny of the langurs. For high quality DNA samples (samples from blood and tissue), the full-length (1140 bp) *Cyt-b* gene was PCR-amplified using primer pair L15915 & H14724 (Table 3). The cycling parameters used were as follows: initial denaturation for 5 min at 94 °C; 35 cycles of 94 °C for 40 s, 54 °C for 30 s, and 72 °C for 90 s; and a final extension at 72 °C for 10 min. A semi-nested PCR approach was followed for those species for which high quality DNA was not available (particularly in the case of DNA from hair samples). This procedure involved running a regular PCR as mentioned above (first PCR), followed by two separate semi-nested PCRs using primers L15915 & H15575 and L15369 & H14724. One microliter of the PCR product from the first PCR was used as template for the second semi-nested PCR. The cycling parameters used for the semi-nested PCRs were initial denaturation for 5 min at 94 °C; 35 cycles of 94 °C for 40 s, 55 °C for 30 s, and 72 °C for 90 s; and a final extension at 72 °C for 20 min. Various internal primers (Table 3) were used to sequence directly from PCR products (both full length and semi-nested PCRs).

The presence of nuclear copies of mitochondrial genes is a major problem in phylogenetic studies employing mitochondrial genes (reviewed in Zhang and Hewitt, 1996; Bensasson et al., 2001). These mtDNA-derived nuclear pseudogenes, also called as "numts", are known to occur in primates (Collura and Stewart, 1995; van der Kuyl et al., 1995; Collura et al., 1996; Mundy et al., 2000; Olson and Yoder, 2002). The likelihood of sequencing numts can be higher when mtDNA-encoded genes are being amplified from DNA extracted from hair follicles (Greenwood and Pääbo, 1999). For most species we did not have access to high enough quality samples to prepare RNA for RT-PCR (Collura et al., 1996) or for the extraction and purification of mtDNA.

Table 3
Primers used in this study for amplification and sequencing of various markers

Primer	Sequence (5′ → 3′)	Reference
Cytochrome b (Cyt-b)		
L14724	CGAGATCTGAAAAACCATCGTTG	1
H15915	AACTGCAGTCATCTCCGGTTTACAAGA	1
L15369[a]	TTCCTACACGAAACAGGATCAAAYAAYCC	2
H15347[a]	GGGGTTGTTTGATCCTGTYTCGTG	2
H15575[a]	ATTAGGGACGGATCGTAAGATYGCGTABGC	2
L15081[a]	TCATTYCTCCTCMTTGAAAC	2
H15251[a]	GGTAGCTGTTAGGTTGGGA	2
Protamine P1 (Prm1)		
Quer1	ACCTGCTCACAGGTTGGCTG	3
ProtL	TTACAGGTTGGCTGGCTC	4
ProtR	TTGACAGGTCGGCATTGTTC	4
Lysozyme c (Lzm)		
WM8	GAATCAGTAGATCAATACACAGT	5
WM9	CCTAGCACTAGAACAGTACA	5

1, Collura et al. (1996); 2, this study; 3, Queralt and Oliva (1991); 4, Retief et al. (1993); 5, Messier (1998).
[a] Indicates 'internal' primers.

When *Cyt-b* PCR products were directly sequenced, multiple sequences were detected for some samples suggesting the presence of numts. For these samples the PCR products were cloned using a TA Cloning kit (Invitrogen) and between 4 and 10 clones per sample were sequenced using the *Cyt-b* primers listed in Table 3. We used a combination of approaches (see Collura et al., 1996; Zhang and Hewitt, 1996) to identify the numts and the presumed mtDNA-encoded *Cyt-b* gene among these sequences. The putative mtDNA-encoded genes were used in subsequent phylogenetic and other analyses.

The protamine P1 (*Prm1*) gene has been used to help resolve the phylogeny of primate (Retief and Dixon, 1993) and other mammalian groups (Retief et al., 1995; Krajewski et al., 1997; Blacket et al., 1999). Therefore, we have used *Prm1* gene as a marker to infer the nDNA phylogeny of the langurs. Full-length *Prm1* sequences (380 bp) were obtained by a semi-nested PCR approach. The primer pair Quer1 & ProtR (Table 3) was used for the first round of PCR. One microliter of PCR product from the reaction was then used as template for the second round PCR, using primer pair ProtL & ProtR. Both the first and second PCRs were carried out using the following parameters: initial denaturation for 5 min at 94 °C; 30 cycles of 94 °C for 40 s, 56 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. This PCR product was directly sequenced using primers ProtL & ProtR. For samples that were heterozygous at this locus, the PCR products were cloned using an Invitrogen TA Cloning kit to determine the sequences of the alleles. Minimums of four positive clones were sequenced.

A 387 bp length fragment composed of exon 1 and part of intron 1 of the lysozyme (*Lzm*) gene was amplified using primers WM8 and WM9 (Table 3). This fragment includes a phylogenetically informative segment of the *Lzm* protein

that has two unique amino acid replacements within the langurs (Messier, 1998). The cycling parameters used were as follows: initial denaturation for 2 min at 95 °C; 38 cycles of 94 °C for 50 s, 52 °C for 50 s, and 72 °C for 1 min; and a final extension at 72 °C for 2 min. This PCR product was directly sequenced using the PCR primers.

All sequencing was performed using the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied BioSystems), following the manufacturer's instructions. Reactions were analyzed using either an ABI Model 373A or an ABI Model 377 Automated DNA Sequencer. Sequences were edited using the program SeqEd (Applied BioSystems), and aligned manually in *PAUP** (Swofford, 2001).

2.4. Phylogenetic analysis of the Cyt-b and Prm1 gene sequences

Three different tree building methods—maximum likelihood (ML), minimum evolution (ME), and maximum parsimony (MP)—were used in *PAUP** (Swofford, 2001) to determine the mitochondrial and nuclear phylogenies of the langurs. Sequences used in these analyses are listed in Table 2. The program MODELTEST (Posada and Crandall, 1998) was used to choose substitution models that best fits these two datasets (*Cyt-b* and *Prm1*), to estimate the transition–transversion ratios, gamma shape parameters, and base frequencies through likelihood ratio tests (Swofford et al., 1996). In the case of *Cyt-b* dataset the selected model along with the estimated parameters were used to derive likelihood trees through two different heuristic searches, one where a molecular clock was enforced and the other where a molecular clock was not enforced. For the *Prm1* dataset, the selected model along with the estimated parameters was used to derive likelihood trees through a heuristic search where molecular clock assumption was not imposed. This is because primate *Prm1* genes are known to evolve rapidly (Retief et al., 1993) and are also under selection particularly among the hominoids (Rooney and Zhang, 1999). Gaps in the *Prm1* dataset were treated as "missing data" for the distance and likelihood analyses because these algorithms in *PAUP** do not use a model that incorporates such phylogenetic information. The selected models were also used to derive ME trees through multiple heuristic searches. Support for various nodes were determined by executing 10,000 bootstrap replications with full heuristic search in *PAUP**. For the MP analysis, heuristic searches were performed with 100 replicates of random addition option. Support for various nodes were determined through 1000 bootstrap replications where each bootstrap replication did 10 additional replications with different input order of the taxa. Gaps in the *Prm1* dataset were treated as "new states" for parsimony analysis, gaps were weighted 3:1. Unique amino acid deletions or changes in the *Prm1* coding sequence in various species were also considered as important phylogenetically informative characters. The cercopithecine sequences (Rhesus monkey, baboon, and Patas monkey) were used as a outgroup to root these trees.

Shimodaira–Hasegawa tests (Shimodaira and Hasegawa, 1999) were performed for multiple comparisons of likelihood scores of various trees (derived through different tree building methods) in *PAUP**. The program *MacClade* (Maddison and Maddison, 1992) was used to infer the amino acid sequences and to build hypothetical phylogenetic trees based on current classifications of these monkeys (Table 1). These hypothetical trees were used as constraint trees in *PAUP** to derive alternative parsimony trees. The likelihood scores of these alternative parsimony trees were compared with each other by implementing the Shimodaira–Hasegawa test in *PAUP**.

2.5. Analysis of lysozyme amino acid sequence

The *Lzm* exon 1 amino acid sequence was compared with published sequence to determine the evolutionary affinities of various langurs at this locus. A part of intron 1 (174 bp) of the *Lzm* gene was also sequenced from all the species of langurs to determine if it contains phylogenetically informative characters. The program *MacClade* (Maddison and Maddison, 1992) was used to infer the amino acid sequences for the coding regions of the *Lzm* gene. Sequences used in this analysis are listed in Table 2.

3. Results

3.1. Cyt-b dataset

In this dataset, transitions were empirically estimated to be around 11 times more frequent than transversions. To account for this difference in base substitution, various weighting schemes (7:1, 11:1, and 22:1) were used in three different heuristic searches under the parsimony option. Since all weighting schemes gave identical trees, a weighting scheme of 11:1 was used for all subsequent parsimony analysis. The overall tree topologies for different tree building methods were the same. The topology of the most-parsimonious tree ($L = 3063$, Fig. 2) was identical to one of the three equally likely likelihood trees (HKY85+G+I substitution model), when the molecular clock assumption was not enforced ($-\ln L = 6696.4978$). In the other two ML trees capped and golden leaf monkeys were not monophyletic but were basal to the langurs of the Indian subcontinent (trees not shown). The ML tree (when molecular clock assumption was enforced with HKY85+G+I substitution model) and the ME tree (with HKY85+G+I distance option) were slightly different from the parsimony tree. The parsimony (Fig. 2) tree has both the silvered leaf monkey and capped-golden leaf monkey clades basal to the SE Asian and Indian clades, respectively. However, in the ML (when molecular clock assumption was enforced) and the ME trees, they fall within the two species groups (not shown). From these analyses, as well as from the low bootstrap for these clades it is clear that the exact phy-

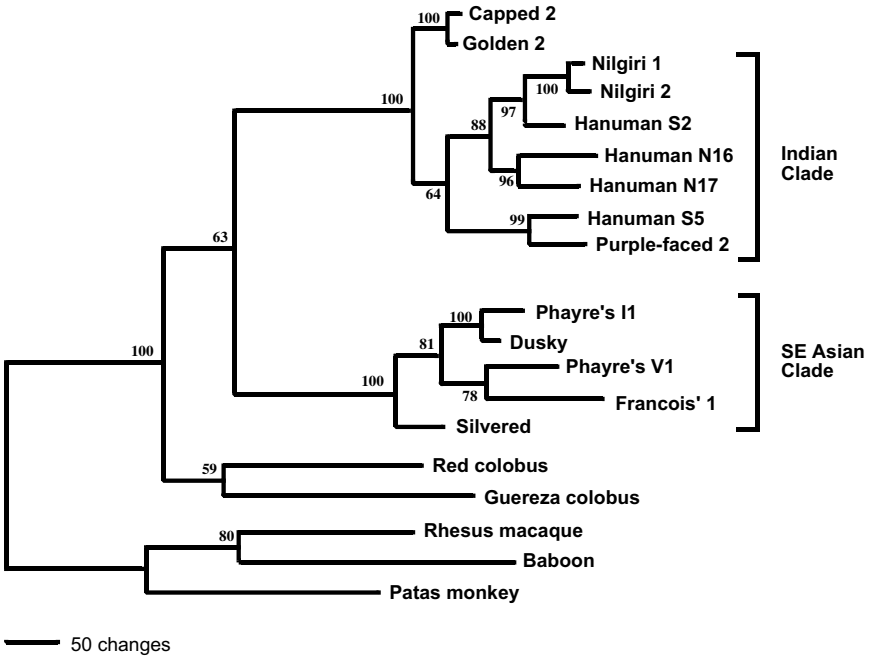

Fig. 2. The *Cyt-b* MP tree (length = 3063). Numbers above the branches indicate bootstrap support from 1000 replications with 10 random stepwise additions. The same overall tree topology (see text for details) was obtained by ML option (HKY85 G+I, −clock, $-\ln L = 6696.4978$). In the case of minimum evolution tree (HKY85 G+I) there were minor rearrangements of taxa within the Indian clade and the SE Asian clade (see text). The bootstrap support for various nodes in the minimum evolution tree (not shown) was similar to the parsimony bootstrap values.

logenetic positions of these taxa (capped-golden leaf monkey clade and silvered leaf monkey) are not resolvable with this dataset. Both the MP and ML methods support the monophyly of the African colobines, although with low boostrap support. In the ME tree the red colobus was basal to the Asian colobines and the guereza colobus was basal to all the colobines. By the likelihood criteria, the parsimony tree (Fig. 2) has a slightly lower likelihood score than the ML and ME trees but the difference in likelihood scores is not significant ($p > 0.05$, Shimodaira–Hasegawa one tail test).

Except for these minor rearrangements mostly with respect to capped, golden, and silvered leaf monkeys the overall *Cyt-b* tree topology is the same for various tree building methods. As seen in previous studies (Messier and Stewart, 1997; Zhang and Ryder, 1998), the *Cyt-b* gene tree splits the langurs into two major clades (Fig. 2). The first clade contains the langurs of the Indian subcontinent—Hanuman langur, Nilgiri langur, and purple-faced langur (called here the Indian clade). The golden and capped leaf monkeys also group with the Indian clade, with high bootstrap support. The second clade consists of leaf monkeys from SE Asia, and is thus called the SE Asian clade; these include Phayre's, silvered, Francois', and dusky leaf monkeys (Fig. 2). Within the Indian clade, there are two interesting results. Firstly, golden and capped leaf monkeys cluster together with a very high bootstrap value. Indeed, there is only 0.5% nucleotide sequence difference between the *Cyt-b* sequences from these two supposed species, in stark contrast to up to 10% sequence difference between some Hanuman langur *Cyt-b* sequences. Secondly, the mtDNA genomes from the so-called Hanuman langurs are polyphyletic with respect to those from the purple-faced and Nilgiri langurs in the Indian clade. Within the SE Asian clade, the mtDNA from the so-called Phayre's leaf monkeys from India is more closely related to that of the dusky leaf monkey than to that of the Phayre's leaf monkeys from Vietnam (Fig. 2). The likelihood score of the parsimony tree where Nilgiri and purple-faced langurs were constrained to be in *Trachypithecus* was significantly lower than the tree where these species were placed in *Semnopithecus* ($P < 0.05$, Shimodaira–Hasegawa one tail test).

3.2. The Prm1 gene

In this dataset, transitions were empirically found to be around two times more frequent than transversions. To account for this difference in base substitution, various weighting schemes (1:1, 2:1, and 4:1) were used in three different heuristic searches under the parsimony option. All weighting schemes gave identical trees, therefore an weighting scheme of 2:1 was used for all subsequent parsimony analysis. The parsimony search resulted in three equally parsimonious trees. A strict consensus of these three trees is shown in Fig. 3 ($L = 90$). The same overall tree topology was obtained under ML option (with HKY85 substitution model and molecular clock assumption not enforced, $-\ln L = 838.5277$). These trees split the colobines into two clades, the African colobines (represented by red colobus and guereza colobus) and the Asian colobines (represented by the langurs, leaf monkeys and the proboscis monkey). Among the langurs and leaf monkeys, the

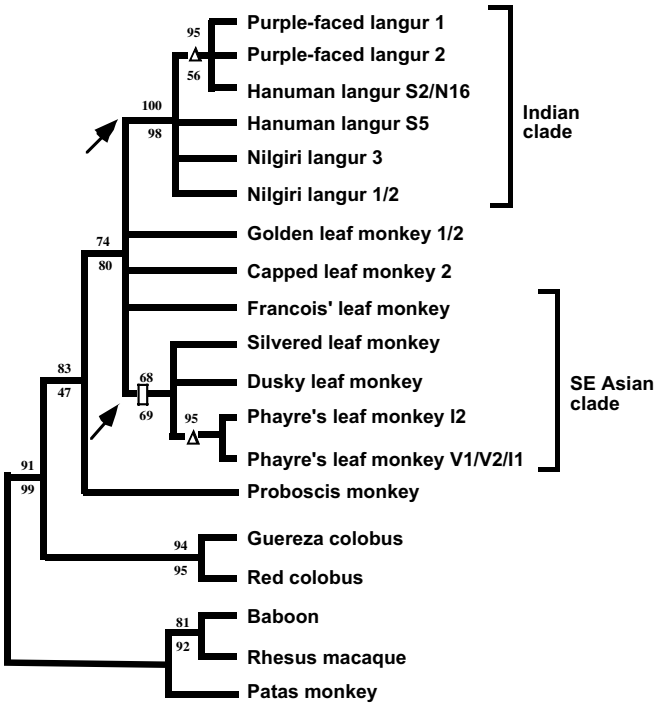

Fig. 3. *Prm1* phylogeny of the langurs and leaf monkeys. This is a strict consensus of three equally parsimonious trees (length = 90). The numbers above and below the branches indicate bootstrap support for the MP and ME trees, respectively. Arrows indicate the species groups that were always monophyletic irrespective of the method used for tree building. The symbols "□" and "△" indicate shared unique amino acid replacement and shared unique amino acid deletion, respectively. Identical sequences are indicated by "/". Refer to Table 2 for sample information and abbreviations.

Prm1 gene tree supports the clustering of Nilgiri and purple-faced langurs with Hanuman langur with high bootstrap support (Fig. 3). Dusky, silvered, and Phayre's leaf monkeys also form a clade (but with low bootstrap support). However, it is interesting to note that they share one unique amino acid replacement in the protamine P1 protein sequences (Fig. 4). The topology of the ME tree was similar to that in Fig. 3 with the exception of the position of proboscis monkey which in the ME tree clusters with the African colobines albeit with very low bootstrap support. The *Prm1* gene sequences of Phayre's leaf monkeys from Vietnam (V1 and V2) and from India (I1) are identical, additionally *Prm1* protein of Phayre's leaf monkeys from India and Vietnam share a unique amino acid deletion (Fig. 4). The parsimony tree also supports the monophyly of Phayre's leaf monkeys from Vietnam (V1/V2/I1) and India (I2) (Fig. 3). This is in contrast to the mtDNA tree wherein Phayre's leaf monkey from India and Dusky leaf monkey cluster together. The *Prm1* gene does not resolve the phylogenetic positions of Francois', golden, and capped leaf monkeys (Fig. 3). The likelihood score of the parsimony tree where Nilgiri and purple-faced langurs were constrained to be in *Trachypithecus* was significantly lower than the tree where these species were placed in *Semnopithecus* ($P < 0.05$, Shimodaira–Hasegawa one tail test).

3.3. The Lzm gene

The langurs of the Indian subcontinent (Hanuman, Nilgiri, and purple-faced langurs) share a unique amino acid replacement (Thr_5) in the leader sequence of the Lysozyme protein (Fig. 5). This result reinforces the *Cyt-b* and *Prm1* results in that these three species are closely related. However, golden and capped leaf monkeys share a unique amino acid replacement (Arg_2) with the SE Asian leaf monkeys, and do not have the Thr_5 replacement shared (Fig. 5). The *Lzm* gene shows very little variation within the Asian colobines, either in the intron or the exon regions of the gene (Messier, 1998). Therefore, the two unique amino acid changes we observe in the leader sequence are strong phylogenetic signals that can be used to classify the langurs and leaf monkeys into these two groups: in addition, these changes are not observed in any other primate including the hominoids (Messier, 1998) and the cercopithecines

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INDIAN CLADE
                          #                        #
Hanuman S2          ARYRRCRSQS RSRCCRPRRR CRRRRRRSCR ARRRATRCCR RRYRLRCRRY
Hanuman N16,S5      ARYRRCRSQS RSRCCRPRRR CRRR-RRSCR ARRRATRCCR RRYRLRCRRY
Purple-faced 1,2    ARYRRCRSQS RSRCCRPRRR CRRR-RRSCR ARRRATRCCR RRYRLRCRRY
Nilgir 1,2          ARYRRCRSQS RSRCCRPRRR CRRRRRRSCR ARRRATRCCR RRYRLRCRRY
Nilgir 3            ARYRRCRSQS RSRCCRPRRR CRRRQRRSCR ARRRATRCCR RRYRLRCRRY

SE ASIAN CLADE
                                                                    ##
Silvered            ARYRCCRSQS RSRCCRPRRR CRRRRRRSCR ARRRATRCCR RRYRLRSRRY
Dusky               ARYRCCRSQS RSRCCRPRRR CRRRRRRSCR ARRRATRCCR RRYRLRSRRY
Phayre's I2         ARYRCCRSQS RSRCCRPRRR CRRRRRRSCR ARRRATRCCR RRYRL-SRRY
Phayre's V1-2,I1    ARYRCCRSQS RSRCCRPRRR CRRRRRRSCR ARRRATRCCR RRYRL-SRRY

UNRESOLVED SPECIES

Francois'           ARYRCCRSQS RSRCCRPRRR CRRRRRRSCR ARRRATRCCR RRYRLRCRRY
Capped              ARYRCCRSQS RSRCCRPRRR CRRRRRRSCR ARRRATRCCR RRYRLRCRRY
Golden 1,2          ARYRCCRSQS RSRCCRPRRR CRRRRRRSCR ARRRATRCCR RRYRLRCRRY
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Fig. 4. Protamine P1 amino acid sequences from the langurs and leaf monkeys. Amino acids in bold are uniquely shared; (—) indicates amino acid deletion; (#) indicates phylogenetically informative sites.

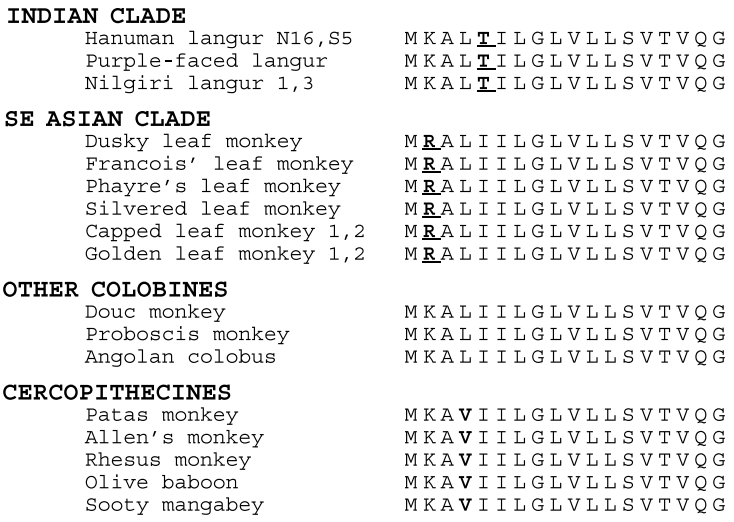

Fig. 5. Leader sequence of the lysozyme protein from various primates. Amino acids in bold are uniquely shared. Underlined amino acids are uniquely shared among the langurs and leaf monkeys. The leader sequences of additional colobine and cercopithecine lysozymes were obtained from Messier (1998).

(Fig. 5). Golden and capped leaf monkeys also share a three base insertion (TTT) in the first intron of this gene, that is not seen in any other species sequenced (data not shown). This reinforces the *Cyt-b* result that golden leaf monkey and capped leaf monkey are very closely related.

We also undertook a combined analysis of the individual datasets by concatenating the three markers into one large dataset. The tree topology derived from the combined analysis was very similar to the mtDNA tree (tree not shown). This finding is not surprising give that disproportionately large number of informative characters in the combined dataset are from cytochrome *b* (379) followed by Protamine P1 (24) and Lysozyme (2). Additionally, the mtDNA and nuclear datasets were highly incongruent with each other ($p = 0.001$, partition homogeneity test). Thus combining the individual datasets did not give us any new insights or generate a better resolved tree, on the contrary information regarding unique evolutionary history of certain species was lost (see discussion). For these reasons the individual datasets were treated separately.

4. Discussion

Results from this and other studies (Messier and Stewart, 1997; Zhang and Ryder, 1998) indicate that the langurs of the Indian subcontinent are closely related and therefore, must be classified in the same genus separate from the leaf monkeys of SE Asia. To this end, the Nilgiri and purple-faced langurs should be classified in the genus *Semnopithecus* with the Hanuman langurs as suggested by Brandon-Jones et al. (2003). This classification scheme predicts only one dispersal of colobines into the Indian subcontinent as opposed to two independent dispersal events (Eudey, 1980). These results also suggest that the apparent disjunct distribution of the species in the genus *Trachypithecus* supported by Groves (2001) classification scheme is due to the incorrect placement of Nilgiri and purple-faced langurs in with the SE Asian leaf monkeys.

As mentioned earlier, many of the wet zone faunal species of the Indian subcontinent show disjunct distributions. It is quite likely that for many of these species groups, the overall morphological similarity that one sees between species from the wet zones of southwest India and Sri Lanka on one side, and the Northeast and SE Asia on the other, might be due to convergence (as they occupy similar ecological zones) rather than recent shared ancestry. Thus, the closest relatives of these wet zone species might be their immediate neighbors from the dry zones of the subcontinent and not the species found thousands of kilometers away in the Northeast and in SE Asia (as appears to be the case with the langurs of the Indian subcontinent). For example, Bossuyt and Milinkovitch (2000) published a phylogenetic analysis of ranid frogs from Madagascar and Asia using both nuclear and mitochondrial markers. Among the ranid frogs, the genus *Limnonectes* (fanged frogs) showed a discontinuous distribution, in that these species are found from Northeast India to New Guinea, with a single species known from Sri Lanka. However, fanged frogs are absent from the rest of the Indian subcontinent and surprisingly the molecular phylogeny of the fanged frogs does not support the monophyly of this group. In fact, the species of fanged frog from Sri Lanka is more closely related to some of the non-fanged ranid species endemic to South India than they are to the fanged frogs from SE Asia (Bossuyt and Milinkovitch, 2000). Clearly, in the case of the ranid frogs of Sri Lanka, the convergence model explained the so-called "discontinuity" in their distribution. This also appears to be the case for Nilgiri and purple-faced langurs. The two models described earlier have very different phylogenetic predictions and can be applied to test the refugial verses convergence scenarios in a variety of species exhibiting disjunct distribution (Karanth, 2003).

The molecular phylogeny of the langurs and leaf monkey presented here is also consistent with their geographic distributions, in that langurs of the Indian subcontinent (genus *Semnopithecus*) form a clade (Indian clade) and the leaf monkeys of SE Asia (genus *Trachypithecus*) are also monophyletic (SE Asian clade). The phylogenetic position of the capped and golden leaf monkeys remains unresolved. It is clear from both nDNA and mtDNA data that these two species are closely related. However, the mtDNA (Fig. 2) tree strongly suggests that they belong to the India clade (*Semnopithecus*), whereas the nuclear-encoded lysozyme gene suggests that they may belong to the SE Asian clade (*Trachypithecus*). Interestingly, these two species are distributed in an area that is sandwiched between the distributions of *Semnopithecus* and *Trachypithecus*. One explanation that can reconcile these two opposing results is that the capped-golden leaf monkeys group evolved from past hybridization between the Indian and SE Asian clades (Karanth, 2000). However, more molecular genetic work is needed to test this hypothesis and to resolve the phylogenetic position of the capped and golden leaf monkeys.

4.1. Lack of monophyly of mtDNA haplotyes among langurs and leaf monkeys

With respect to the relationships between various langurs and leaf monkeys within the Indian and SE Asian clades, the *Cyt-b* tree does not support the monophyly of Phayre's leaf monkey in the SE Asian clade or of Hanuman langur in the Indian clade. In the case of the Phayre's leaf monkey, the *Prm1* results indicated that Phayre's leaf monkey from India and those from Vietnam are closely related (Figs. 3 and 4). In contrast, the mtDNA results suggested that Phayre's leaf monkey from India is more closely related to the dusky leaf monkey but Phayre's leaf monkey from Vietnam is more closely related to Francois' leaf monkey (Fig. 2). This discordance between the *Prm1* result and the *Cyt-b* result might have a reasonable explanation. Like most Old World monkeys, the majority of colobines species live in matrilineal or female-bonded societies (Newton and Dunbar, 1994). In matrilineal societies, males leave their natal troops at puberty, but females stay in the natal troop. Most of the gene flow between populations is of nuclear genes due to male movement, whereas mtDNA tends to be more highly substructured between populations, presumably due to female natal philopatry. Evidence of biogeographical events and instances of intraspecific paraphyly can be preserved in the mitochondrial genome (Melnick and Hoelzer, 1992) but might have been obliterated in the nuclear genome by subsequent gene flow through male migration and recombination.

Phayre's leaf monkey from India and Vietnam have the same *Prm1* allele (based on protein sequence, Fig. 4) and cluster together in the *Prm1* parsimony tree (Fig. 3). In comparison, Phayre's leaf monkey has at least two highly divergent mtDNA haplotypes, each of which branch with the dusky and Francois' leaf monkeys (Fig. 2). Male mediated gene flow might have homogenized the nuclear genome and female natal philopatry might have resulted in highly substructured mtDNA. Such discordance in topology between trees derived from nuclear and mitochondrial markers have also been reported among macaques (Tosi et al., 2000). In the case of macaques, the authors contend that the preservation of "ancestral" mtDNA accurately depicts the origin of these species, whereas trees based on nuclear markers are more representative of their current biological relationships. Similarly, in the case of the leaf monkeys, it is possible that the dusky and Francois' leaf monkeys evolved from a Phayre's leaf monkey-like ancestral species that contained deep mtDNA polymorphism. This ancestral mtDNA polymorphism has been retained in the current populations resulting in poly/paraphyly. The results from the *Prm1* locus suggest the current relationship between Phayre's leaf monkeys from India and Vietnam. Another possible reason for Phayre's leaf monkeys from India and Vietnam to have the same *Prm1* allele might be the fixation of this allele due to a selective sweep. It is difficult to distinguish between these two scenarios (male mediated gene flow and selective sweep), moreover, male mediated gene flow might have facilitated the selective sweep of *Prm1* allele in Phayre's leaf monkey. A similar result is obtained in the case of mtDNA from the so-called Hanuman langurs, which is polyphyletic with respect to that from Nilgiri langur and purple-faced langur (Fig. 2). Here again, Hanuman langur-like ancestral species might have diverged into Nilgiri and purple-faced langurs in the wet zones of South India and Sri Lanka. These two species are not sister taxa in the mtDNA tree, thus the mitochondrial data does not support placing them in the genus *Kasi*.

5. Conclusions

Based on the results reported in this paper, we suggest that the observed "disjunct distribution" of the genus *Trachypithecus* is an artifact of incorrect classification. Our molecular data support the classification of the langurs of the Indian subcontinent (Hanuman, Nilgiri, and purple-faced langurs) in the genus *Semnopithecus* and the leaf monkeys from SE Asia in the genus *Trachypithecus*. The phylogenetic position of the capped and golden leaf monkeys is still unresolved and it is likely that this species group might have evolved due to past hybridization between *Semnopithecus* and *Trachypithecus* clades. Additionally, for widely distributed species such as the Hanuman langur and Phayre's leaf monkey, it is important to collect samples from a wide range to capture the whole spectrum of mitochondrial diversity within a species.

Acknowledgments

The authors thank Dr. R. Pethiagoda, Dr. M. Singh, Dr. S. Siva, Dr. S.N. Nigam, C. Roos, Dr. K. Launhardt, Dr. P. Dolhinow, R.S. Harave, A. Chaudary, Dr. S.M.

Mohnot, and Dr. N.K. Lohiya for helping us with sample collection. Fieldwork for this study was supported through SUNY benevolent, SUNY GSO, and Primate Conservation Inc. grants to K.P.K.

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