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Phylogenetic incongruence between nuclear and mitochondrial markers in the Asian colobines and the evolution of the langurs and leaf monkeys

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Abstract

Evidence of incongruence between mitochondrial and nuclear gene trees is now becoming documented with increasing frequency. Among the Old World monkeys, this discordance has been well demonstrated in the Cercopithecinae, but has not yet been investigated in the Colobinae. The mitochondrial relationships between the colobine genera have recently been clarified and cluster *Presbytis* and *Trachypithecus* as sister taxa to the exclusion of *Semnopithecus*. This is incongruent with previous morphological hypotheses that suggest the latter two are sister taxa, and perhaps even congeneric. In addition to analyzing a previously published 10,896 bp mitochondrial dataset, we sequenced and analyzed a 4297 bp fragment of the X-chromosome in order to test the competing mitochondrial and morphological phylogenetic hypotheses. The results from the mitochondrial dataset again support a *Presbytis* + *Trachypithecus* group while the X-chromosomal dataset supported a *Semnopithecus* + *Trachypithecus* group. A Shimodaira–Hasegawa test performed on both datasets indicates that the mitochondrial and X-chromosomal trees are significantly better at explaining their respective datasets than alternative topologies ($p < 0.05$). We suggest that differential lineage sorting or ancient hybridization may be the cause of this strong discordance between the mitochondrial and X-chromosomal markers in these taxa.

Keywords: Colobine; Colobine systematics; Colobine phylogeny; Leaf monkey; Langur; Phylogeny; Systematics; Incongruence; Discordance; Hybridization; Lineage sorting

1. Introduction and background

The extant Old World monkeys have been divided into two major groups—the cercopithecines and the colobines—which diverged from one another in the mid-Miocene (Delson, 1994; Sterner et al., 2006). The colobines are primarily distinguished from their cercopithecine relatives by several derived morphological traits, including a multi-chambered ruminant-like stomach, that are adaptations to a more folivorous diet (Strasser and Delson, 1987). While the cercopithecines (macaques, guenons, and baboons) have been well characterized in many aspects of their natural history, the colobines are not as well known. This is especially true concerning their molecular relationships. While nuclear and mitochondrial gene tree incongruence has been well documented in the macaques and guenons (e.g., Evans et al., 1999; Tosi et al., 2002, 2003, 2004), the mitochondrial relationships of the colobines have only recently been clarified (Sterner et al., 2006; Whittaker et al., 2006; Ting, in press), and a comprehensive colobine phylogenetic study based on a nuclear marker has yet to be produced.

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The colobines have traditionally been split into an African clade and an Asian clade based on both morphology and geographical distribution (Delson, 1975), and among the Asian colobines there exists the "odd-nosed" group and the "langur and leaf monkey" group. The odd-nosed monkeys consist of the genera *Nasalis*, *Simias*, *Pygathrix*, and *Rhinopithecus*. Meanwhile, the genera *Presbytis*, *Semnopithecus*, and *Trachypithecus* comprise the langurs and leaf monkeys, which is a diverse group with an unstable alpha taxonomy. In the most recent overview (Brandon-Jones et al., 2004), 10 species are recognized within *Presbytis*, another 10 within *Trachypithecus*, and 3 within *Semnopithecus*, although the genus level membership of two of these *Semnopithecus* taxa are in question (see below Section 5). These animals are found throughout Southeast Asia, including Southern China and the Indian subcontinent (Fig. 1), occupying a variety of ecological niches ranging from arboreal habitats in tropical rainforest to the harsh semi-terrestrial habitats in the Himalayan foothills (Bennet and Davies, 1994).

The langurs and leaf monkeys have traditionally been considered to form a monophyletic group. With the exception of Pocock (1928, 1939), who separated the three genera from one another based on neonatal coloration, pelage patterns, and cranial morphology, most 20th century classifications retained all three groups within the genus *Presbytis* (e.g., Groves, 1970; Delson, 1975). Brandon-Jones (1984) separated *Semnopithecus* from *Presbytis* and recognized *Trachypithecus* as a morphological subgenus of the former based on overall appearance. Classifications now follow either Pocock in separating all three at genus rank or use Brandon-Jones's two-genus arrangement, and phylogenetically link *Semnopithecus* with *Trachypithecus* to the exclusion of *Presbytis*. However, recent molecular evidence from mitochondrial data strongly suggest that *Presbytis* and *Trachypithecus* are sister taxa to the exclusion of *Semnopithecus* (Sterner et al., 2006). We sequenced and analyzed a nuclear marker in addition to reanalyzing a published mitochondrial dataset in order to test these competing hypotheses and further elucidate the evolutionary history of these animals.

2. Materials and methods

2.1. Samples

This study includes a representative from all but one of the commonly recognized colobine genera (sensu Brandon-Jones et al., 2004; Grubb et al., 2003) (Table 1). Only *Simias*, which is expected to cluster as the sister taxon of *Nasalis* based on morphological and mitochondrial data (Groves, 1970; Delson, 1975; Whittaker et al., 2006), is

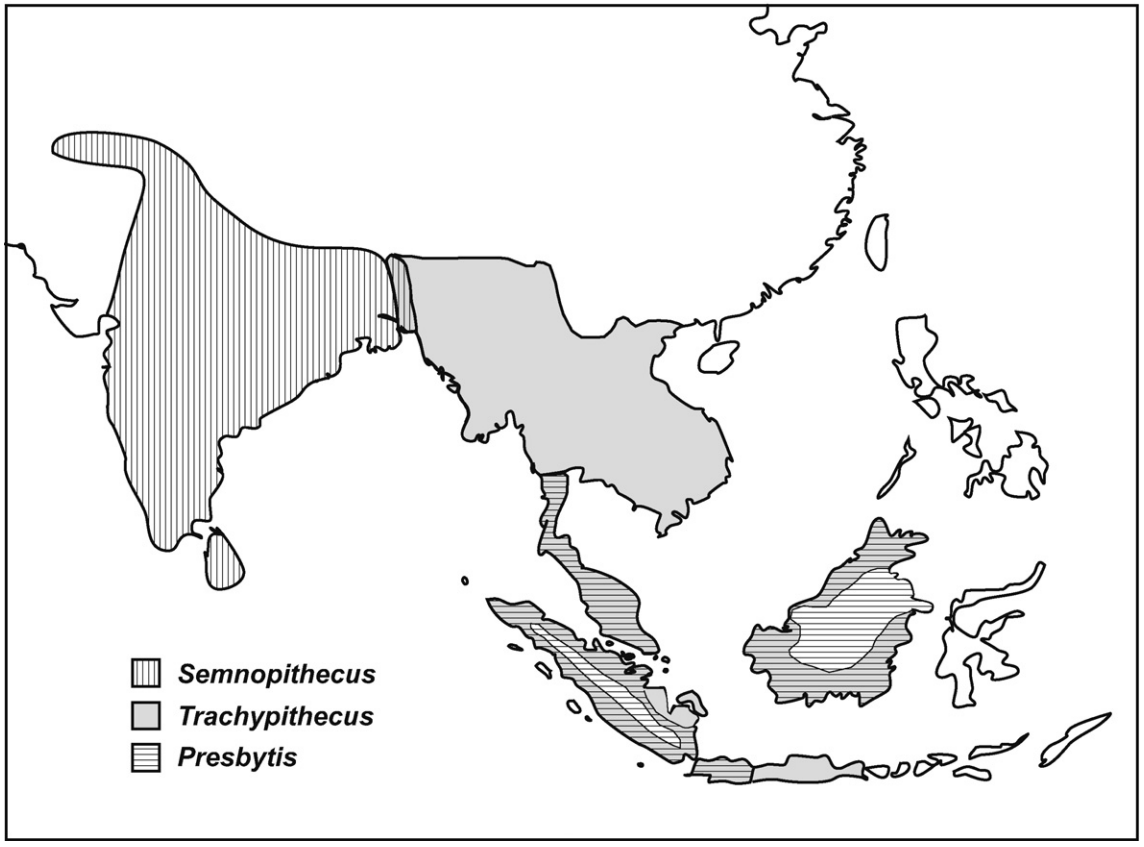

Fig. 1. Distribution of the langurs and leaf monkeys (genera *Presbytis*, *Semnopithecus*, and *Trachypithecus*) in Southeast Asia. Adapted from Oates et al. (1994). Classification follows Brandon-Jones et al. (2004).

Table 1
Species sampled in the X-chromosomal and mitochondrial datasets

	Common name	GenBank Accession No.	
		X-data	Mt-data
Taxon (colobine)			
*Colobus guereza**	Eastern black and white colobus	AY899240	AY8633427
Procolobus badius	Western red colobus	EU342361	DQ355301
*Nasalis larvatus**	Proboscis monkey	EU342359	DQ355298
Rhinopithecus avunculus	Tonkin snub-nosed monkey	EU342363	—
Rhinopithecus roxellana	Sichuan golden monkey	—	DQ355300
*Pygathrix nemaeus**	Red-shanked douc	EU342362	DQ355302
*Presbytis melalophos**	Mitered leaf monkey	EU342360	DQ355299
*Semnopithecus entellus**	Hanuman langur	EU342364	DQ355297
*Trachypithecus obscurus**	Dusky or spectacled leaf monkey	EU342365	AY8633425
Taxon (outgroup)			
Papio hamadryas	Hamadryas baboon	AY899234	NC_001992
Theropithecus gelada	Gelada baboon	AY899236	—
Macaca sylvanus	Barbary macaque	—	NC_002764
Macaca mulatta	Rhesus macque	AY899239	—
Pan troglodytes	Common chimpanzee	—	NC_001643
Homo sapiens	Human	AJ241091.1	NC_001807
Cebus albifrons	White-fronted capuchin	—	NC_002763

Colobine species between the two were constant except for representatives of *Rhinopithecus*. Taxa with asterisks indicate data from the two markers are from the same individual.

not included. All but two of the colobine individuals used (*Procolobus*, *Rhinopithecus*) were held constant in the X-chromosomal and mitochondrial datasets. Therefore, differences in the resulting topologies are directly comparable and likely due to differences in the evolutionary history of the loci rather than the individual animals. Effort was made to keep the outgroup taxa between the two datasets consistent as well, although they did differ slightly due to availability of biomaterials. The X-chromosomal outgroup taxa consisted of *Papio hamadryas*, *Theropithecus gelada*, *Macaca mulatta*, and *Homo sapiens*. The mitochondrial outgroup taxa were *Papio hamadryas*, *Macaca sylvanus*, *Pan troglodytes*, *Homo sapiens*, and *Cebus albifrons*. All colobine X-chromosomal data except those for *Colobus guereza* are presented here for the first time. Males (XY), who carry only one copy of the X-chromosome as opposed to females (XX) who carry two, were used whenever possible to avoid polymorphic sites that might dampen phylogenetic signal. The only female samples used were *Trachypithecus obscurus* and *Semnopithecus entellus*. All other data were obtained from GenBank.

2.2. Molecular markers

The X-chromosomal marker surveyed here is a 4297 bp fragment homologous to a portion of human Xq13.3 (Kaessmann et al., 1999). This is an intergenic region and therefore unlikely to be the direct target of any selective forces. The mitochondrial marker analyzed is a 10,896 bp concatenation consisting of the protein-coding regions on the heavy strand of the mitochondrial genome.

2.3. Extraction, amplification, and sequencing

Total genomic DNA was extracted with the QIAamp DNA Blood Mini kit (Qiagen, cat. No. 51104) or the DNeasy Tissue kit (Qiagen, cat. No. 69504). Amplification was performed using standard *Taq* polymerase (Promega, Madison, WI) and PCR primers for four overlapping X-chromosomal amplicons (numbers 3–6 of Tosi et al., 2005). Amplified products were cleaned using exonuclease I and shrimp alkaline phosphatase (Hanke and Wink, 1994). Cycle sequencing was performed with primers based upon human and Old World monkey sequences (available upon request) and the Big Dye kit (Big Dye v3.1, ABI, cat. No. 4337456) following the manufacturer's protocol for diluted reactions. Products were run on an ABI PRISM 3730 DNA Sequencer. Complementary strands were sequenced from multiple PCR products to ensure the fidelity of the data, and the sequences were edited and assembled using Sequencher v4.5 (Gene Codes Corp.). Polymorphic sites were given the appropriate IUPAC ambiguous code.

2.4. Phylogenetic analysis

X-chromosomal sequences were aligned using the program ClustalW (Chenna et al., 2003) and then adjusted by eye to correct for spurious insertions/deletions. Large insertions/deletions, including a 1.73 kb insert composed of a LINE1 element in *Procolobus badius* and a 326 bp Alu in *Nasalis larvatus*, were removed from the analysis. Two single-nucleotide repeat (poly-A) regions totaling 34 bp were also removed. The total length of the fragment analyzed was 4297 bp. Recombination was not detected in

this dataset when subjected to the Maximum χ^2-squared test (Maynard Smith, 1992). The mitochondrial alignment analyzed was downloaded from the supplementary material of Sterner et al. (2006) (Fig. S2, heavy proteins).

Maximum likelihood (PAUP 4.0b10, Swofford, 2001) and Bayesian methods (Mr. Bayes 3.1, Ronquist and Huelsenbeck, 2003) were used to infer mitochondrial and X-chromosomal gene trees. The evolutionary model that best fit the data was determined using Modeltest 3.6 (Posada and Crandall, 1998). When analyzed under the Akaike Information Criterion (AIC), the X-chromosomal data were best fit by the transversional model (TVM) with a gamma distribution (G) of site-specific rates, and the mitochondrial data were best fit by the Hasegawa, Kishino, and Yano model (HKY) with invariant sites (I) and a gamma distribution (G) of site-specific rates. These models were employed in the maximum likelihood analysis. The general time reversible (GTR) model with a gamma distribution (G) of site-specific rates, which is the model most similar to the TVM + G and HKY + I + G models available in Mr. Bayes 3.1, was used in the Bayesian analysis.

For maximum likelihood, 1000 bootstrap replicates were performed under a heuristic search with the taxon addition set to random and all other parameters left as default values. For the Bayesian analysis, the Markov Chain Monte Carlo (MCMC) chain was run for 1,000,000 generations and sampled every 100 generations. Two burn-in periods were calculated to ensure an appropriate number of samples were discarded. The first was determined by dividing the number of generations it took for the log likelihood of the cold chain to stabilize by the sampling frequency. In both dataset analyses, the log likelihood of the cold chain had stabilized by 30,000 generations, and dividing this by the sample frequency (100) gave burn-in values of 300. The second burn-in corresponded to 25% of the sample, which was calculated to be 2500 (250,000 generations divided by a sampling frequency of 100). All nodes that were supported by bootstrap values of less than 85 and posterior probabilities lower than 0.95 were collapsed.

2.5. Tree comparison tests

The mitochondrial and X-chromosomal datasets were not combined because they represent two different genetic systems that possess different evolutionary histories. The mitochondrial genome is maternally inherited, evolves at a much faster rate, and has a smaller effective population size compared to the X-chromosome, and thus has a shorter time to lineage fixation. It is therefore possible for these markers to follow different evolutionary trajectories (Moore, 1995; Avise, 2000). The Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) was implemented in PAUP (4.0b10) on both mitochondrial and X-chromosomal datasets to determine whether alternative trees can explain the data as well as the ones inferred from the initial phylogenetic analyses. Specifically, we were interested to see if a tree with the *Semnopithecus* + *Trachypithecus* (X-chromosomal) group could explain the mitochondrial dataset, and if a tree with the *Presbytis* + *Trachypithecus* (mitochondrial) group could explain the X-chromosomal dataset. All possible Asian colobine topologies involving an odd-nosed group, *Presbytis*, *Semnopithecus*, and *Trachypithecus* were tested against both datasets. These trees were built by hand in MacClade 4.08 (Maddison and Maddison, 2005) and then imported into PAUP (4.0b10) for the SH test.

3. Results

3.1. Phylogenetic analysis and tree comparison tests

3.1.1. Mitochondrial data

Maximum likelihood and Bayesian analyses produced the same mitochondrial topology and both Bayesian runs showed identical results (Fig. 2A). These are congruent with those from Sterner et al. (2006) and show reciprocally monophyletic Asian and African colobine clades, a monophyletic odd-nosed clade, and a sister–taxon relationship between *Presbytis* and *Trachypithecus*. The SH test (Fig. 3) shows that trees possessing a *Presbytis* + *Trachypithecus* pair are significantly better at explaining the data than other trees ($p \leqslant 0.05$), though the placement of *Semnopithecus* cannot be determined.

3.1.2. X-chromosomal data

Maximum likelihood and Bayesian analyses produced the same X-chromosomal topology (Fig. 2B). Both burn-in values showed identical results in the Bayesian analysis. Support was found for reciprocally monophyletic Asian and African colobine clades. Within the Asian clade, *Semnopithecus* and *Trachypithecus* are sister taxa and are united by a relatively long branch length. There is also a monophyletic odd-nosed group, and *Presbytis* is sister to all other Asian colobines. The SH test (Fig. 3) shows that trees possessing a *Semnopithecus* + *Trachypithecus* group are significantly better at explaining the data than other trees ($p \leqslant 0.05$), though the placement of *Presbytis* cannot be determined.

4. Discussion

The inferred X-chromosomal and mitochondrial trees differ mainly in the relationships between the langurs and leaf monkeys. The mitochondrial tree finds *Presbytis* and *Trachypithecus* as sister taxa, while the X-chromosomal tree groups *Semnopithecus* and *Trachypithecus* together. The relatively long X-chromosomal branch supporting this latter relationship suggests that the two taxa shared a common ancestry for a long period of time. The X-chromosomal tree also places *Presbytis* as sister to all other Asian colobine genera; however, the SH test reveals that placement of *Presbytis* in multiple positions within the Asian colobine clade yields topologies that are not signifi-

cantly different from the initial (shortest) X-chromosomal tree. Therefore, based on the present X-chromosomal dataset, relationships among the Asian colobines are best considered an unresolved trichotomy between *Presbytis*, an odd-nosed group, and the *Semnopithecus*/*Trachypithecus* pair. The SH test also revealed that the initial X-chromosomal tree is significantly better at explaining the X-chromosomal dataset than a tree clustering *Presbytis* and *Trachypithecus* together (i.e., the topology of the mitochondrial tree) ($p = 0.00$). Conversely, the tree inferred from mitochondrial data is significantly better at explaining the mitochondrial dataset than any topology containing the *Semnopithecus* + *Trachypithecus* X-chromosomal group ($p < .018$). In other words, trees inferred from one dataset are not statistically equivalent explanations of the other dataset.

The X-chromosomal *Semnopithecus* + *Trachypithecus* relationship is congruent with morphological hypotheses concerning the phylogeny of these two taxa. One may reason that such convergence among multiple datasets indicates that a true sister-relationship has been uncovered. However, the morphology supporting a *Semnopithecus* and *Trachypithecus* relationship is based on overall similarity of appearance. To be clear, there are no known synapomorphic morphological traits or fossil specimens that unite the two to the exclusion of *Presbytis*; rather, some researchers have suggested the traits that unite the two are symplesiomorphic (Strasser and Delson, 1987; Groves, 2001). Therefore, until more extensive morphological work is performed, or nuclear loci are surveyed, it is unclear whether the X-chromosomal pattern (*Semnopithecus* + *Trachypithecus*) or the mitochondrial pattern (*Presbytis* + *Trachypithecus*) is correctly tracing the organismal phylogeny.

Several hypotheses have been proposed to explain gene tree discordance between mitochondrial and nuclear markers (for reviews, see Funk and Omland, 2003; Ballard and Whitlock 2004); however, we consider some to be more likely than others in the present case. For example, we believe neither nuclear pseudogenes nor rate saturation to be affecting the mitochondrial tree of Sterner et al. (2006). Sterner and colleagues amplified mitochondrial genomes in two large fragments that overlapped with one another on their ends. The homologous regions were identical between the two amplicons, thus increasing the likelihood of obtaining a target template that is circular and of true mitochondrial origin rather than a combination of nuclear pseudogenes (Thalmann et al., 2004; Raaum et al., 2005). Sterner and colleagues also performed a comparative phylogenetic analysis including

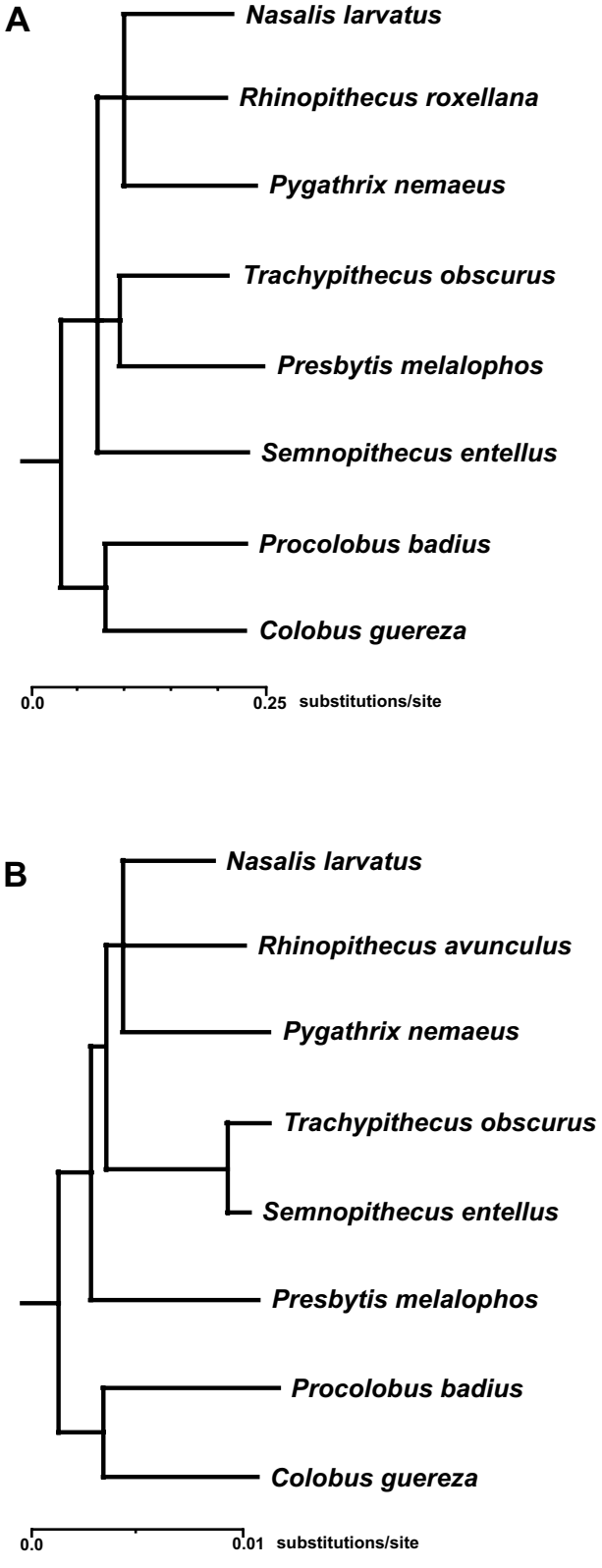

Fig. 2. (A) Mitochondrial tree of colobine relationships as inferred by maximum likelihood and Bayesian analyses. Non-colobine taxa not shown. All clades supported by bootstrap values above 85 (1000 replicates) and posterior probabilities of 1.0, with the *Presbytis* + *Trachypithecus* clade supported by a bootstrap value of 99. (B) Xq13.3 tree of colobine relationships as inferred by maximum likelihood and Bayesian analyses. Non-colobine taxa not shown. All clades are supported by bootstrap values above 85 (1000 replicates) and posterior probabilities of 1.0, with the *Semnopithecus* + *Trachypithecus* clade supported by a bootstrap value of 100. Note the incongruence concerning the relationships of *Presbytis melalophos*, *Trachypithecus obscurus*, and *Semnopithecus entellus*.

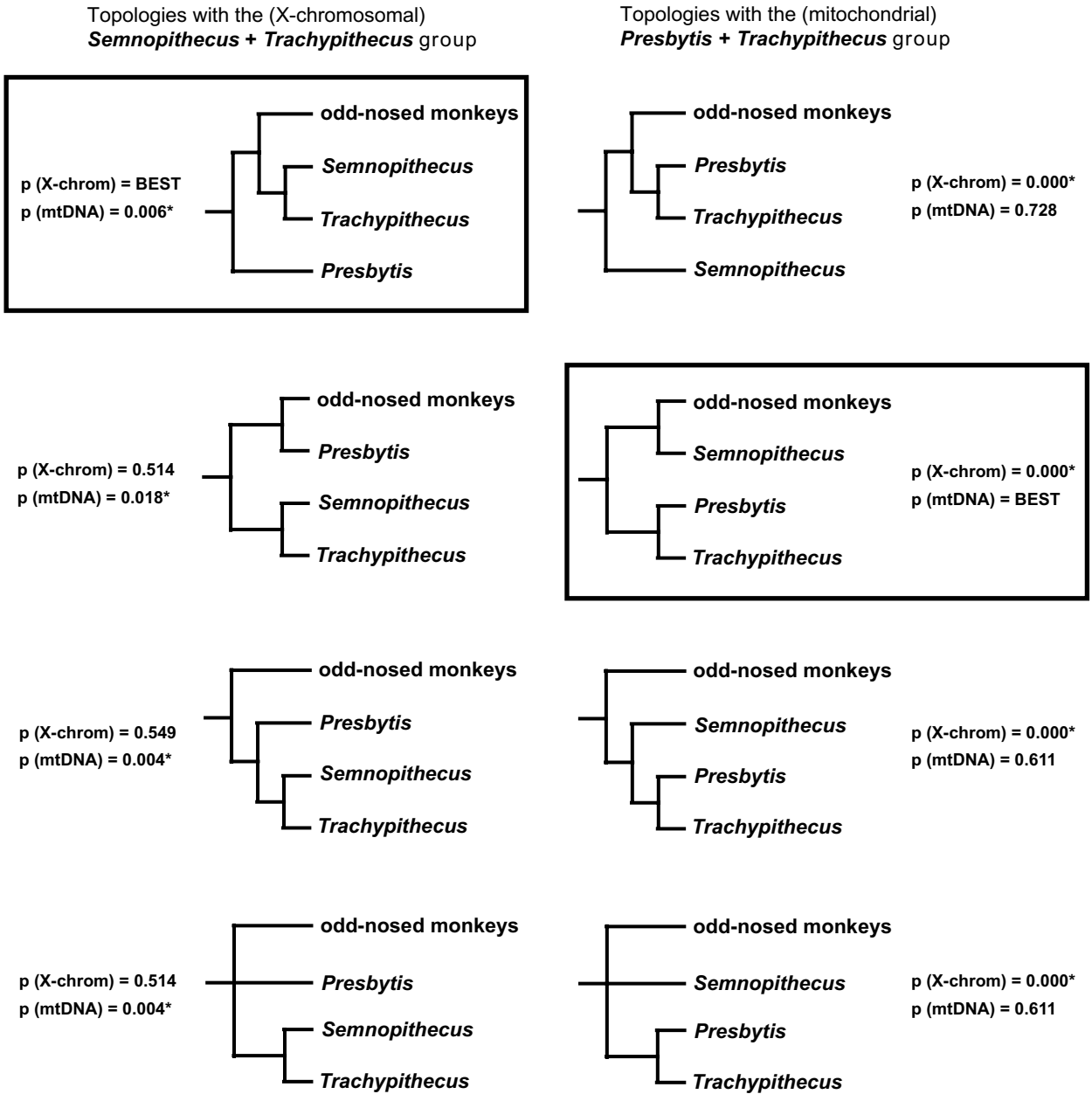

Fig. 3. Shimodaira-Hasegawa (SH) test results. All possible Asian colobine tree topologies involving an odd-nosed group, *Presbytis*, *Semnopithecus*, and *Trachypithecus* were tested to see how well they explained the two datasets. p(X-chromosomal) = p value for the X-chromosomal data. p(mtDNA) = p value for the mitochondrial data. $^{*}p < 0.05$. Trees inferred from the X-chromosomal data (left side, those with *Semnopithecus* + *Trachypithecus*) are poor explanations of the mitochondrial data, while trees inferred from the mitochondrial data (right side, those with *Presbytis* + *Trachypithecus*) are poor explanations of the X-chromosomal data. Boxed tree on the left represents the topology that best explains the X-chromosomal data, while boxed tree on the right represents the topology that best explains the mitochondrial data. Trees with *Presbytis* + *Semnopithecus* as sister taxa (not shown) are poor explanations of both datasets ($p < 0.05$).

and excluding third base positions of codons, which may become rapidly saturated due to a higher incidence of synonymous mutations (Li, 1997). If their dataset were affected by rate saturation, the exclusion of third base positions would provide a more accurate gene tree, possibly with a different topology. However, support for the *Presbytis* + *Trachypithecus* grouping was not affected by such exclusion.

The gene tree incongruence revealed here (Fig. 2A vs. B) is more likely explained by either differential lineage sorting or ancient hybridization. Differential lineage sorting may have occurred if mitochondrial or X-chromosomal polymorphisms were retained through colobine speciation events and randomly sorted into patterns of allelic relationships that do not match the organismal phylogeny (Avise, 2000). Alternatively, ancient hybrid-

ization, which has possibly shaped the evolutionary history of numerous primate taxa (see review by Arnold and Meyer, 2006), may have allowed for the exchange of mitochondrial or X-chromosomal alleles between langur and leaf monkey taxa, thus resulting in gene tree incongruence. Further research employing a synthesis of genetics, morphology, and ethology will help to distinguish between patterns of differential lineage sorting and hybridization that may have occurred. Especially useful would be divergence dates estimated from nuclear DNA loci, but these are most reliably inferred from long stretches of DNA (e.g., tens of thousands of base pairs). Until these additional studies are performed, there remain four viable hypotheses to explain the X-chromosomal and mitochondrial discordance witnessed here.

4.1. Differential sorting among mitochondrial lineages

This could have occurred if the langur and leaf monkey common ancestor carried multiple divergent mitochondrial lineages, and the same mtDNA lineage fixed—at random—in both *Presbytis* and *Trachypithecus* stocks. Consequently, mitochondrial lineages of *Trachypithecus* would today cluster most closely with those of *Presbytis*, although the majority of its genome (reflected in nuclear DNA and morphology) may affiliate most closely with that of *Semnopithecus*. This scenario would gain indirect support if *shared*, *derived* morphological traits were found between *Semnopithecus* and *Trachypithecus*. Additional nuclear loci that cluster these two together would also support this hypothesis. However, if their time of divergence estimated from these data postdates the mitochondrial divergence between *Presbytis* and *Trachypithecus* (7.2 ± 0.71 Ma, Sterner et al., 2006) by a considerable amount, this hypothesis would seem implausible. It is unlikely that nuclear alleles would sort before mitochondrial ones due to their larger effective population sizes and slower fixation times.

4.2. Differential sorting among X-chromosomal lineages

Due to differences in effective population size and time to fixation, differential lineage sorting is more likely to occur among X-chromosomal lineages than those of mitochondria. This may lead to the assumption that the mitochondrial tree is more likely to reflect the true organismal phylogeny, thus linking *Presbytis* and *Trachypithecus* as true sister taxa. In this scenario, multiple divergent X-chromosomal lineages would have existed in the common ancestor of the langurs and leaf monkeys, and the same lineage would have randomly fixed in both *Semnopithecus* and *Trachypithecus* stocks. However, if true, this hypothesis would require the morphological similarities that link *Semnopithecus* and *Trachypithecus* to be sympleisiomorphic.

4.3. Introgressive hybridization between Semnopithecus and Trachypithecus

Ancient hybridization between these two genera may have led to exchange of X-chromosomal alleles and a resultant X-chromosomal tree (Fig. 2B) that is incongruent with ancestral relationships depicted in the mitochondrial tree (Fig. 2A). If this were the case, some nuclear loci would show the mitochondrial topology, and the morphology shared between *Semnopithecus* and *Trachypithecus* is sympleisiomorphic. Alternatively, hybridization could have been uni-directional with backcrossing over a long period of time so that components of the nuclear genome of one lineage have displaced the other, and thus they are now phenotypically alike. This could occur if males of one taxon were strongly selected for over males of the other so that they monopolized all mating opportunities, and hybrid males saw reduced fitness. If this happened, further molecular surveys would show nearly all nuclear loci carrying the X-chromosomal topology. Evidence of this type of hybridization has been documented in African elephants (Roca et al., 2005) and warblers (Rohwer et al., 2001; Bensch et al., 2006), demonstrating that phenomena such as "nuclear swamping" do indeed occur. Ancient hybridization between *Semnopithecus* and *Trachypithecus* would gain indirect support if nuclear markers linking these two taxa show divergence dates that are much more recent than the mitochondrial dates linking *Presbytis* and *Trachypithecus*.

4.4. Introgressive hybridization between Presbytis and Trachypithecus

It is also possible that hybridization between ancestral stocks of *Presbytis* and *Trachypithecus* led to exchange of mitochondrial lineages, thus resulting in a mitochondrial topology (Fig. 2A) that is incongruent with the ancestral relationships depicted in the X-chromosomal tree (Fig. 2B). This could occur if a limited number of females from one taxon transferred into a group of the other and subsequently produced female offspring who held a strong selective advantage over the resident females. Over time, the invasive mitochondrial lineages would become fixed while the contributions of the associated nuclear lineages would decrease every generation. Loss of resident mitochondrial lineages through drift could also play a factor. This process is different than the one described above because it can only occur with the movement of a limited number of female individuals, although it produces the same resultant pattern (movement of numerous females over many generations would result in replacement of both mitochondrial and nuclear lineages). Nuclear divergence dates between *Semnopithecus* and *Trachypithecus* that considerably predate the mitochondrial divergence of *Presbytis* and *Trachypithecus* would be consistent with a hypothesis of ancient hybridization between the latter pair.

5. Future research in langur and leaf monkey molecular systematics

Semnopithecus sensu stricto (i.e., excluding *Trachypithecus*) has traditionally been comprised of one species, the Hanuman langur (*S. entellus*). Brandon-Jones et al. (2004) reclassified two other langur species (Nilgiri black langur [*T. johnii*], purple-faced langur [*T. vetulus*]) into *Semnopithecus* based primarily on mitochondrial relationships (Zhang and Ryder, 1998) despite their morphological similarity to other *Trachypithecus* species. If nuclear genes do not follow this pattern, then this move by Brandon-Jones et al. (2004) is invalid. However, if they do follow this pattern, then *Trachypithecus* sensu lato is paraphyletic and the morphological traits that unite this genus are sympleisiomorphic. Adding those two species to the present X-chromosomal dataset, or *Semnopithecus* to the Xing et al. (2005) dataset, would aid in clarifying this issue.

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