Molecular phylogeny and divergence time of *Trachypithecus*: with implications for the taxonomy of *T. phayrei*

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Abstract: The genus *Trachypithecus* is the most diverse langur taxon, distributed in southwestern China, south and southeastern Asia. In this study, we include 16 recognized *Trachypithecus* species to reconstruct the phylogeny with particular concern to the taxonomy of the three subspecies of *T. phayrei* using multiple genes. Our results support a sister-relationship between *T. p. phayrei* and *T. p. shanicus*. However, the mitochondrial *CYT B* gene supported *T. p. crepuscula* as a distinct species, but the nuclear *PRM1* gene suggested a closer relationship between *T. p. crepuscula* and *T. p. phayrei*. The incongruence between nuclear and mitochondrial genes suggests that hybridization may have occurred, a fact that would benefit from re-examination using multiple unlinked nuclear genes.

Keywords: Non-invasive sampling; Partitioned Bayesian phylogenetic analyses; Relaxed molecular clock; *Trachypithecus phayrei*

The genus *Trachypithecus* is the most diverse langur taxon, having a broad distribution including India, Sri Lanka, Bangladesh, Southwestern China, and Southeast Asia (Groves, 2005; Wang et al, 1999). It is phylogenetically embedded within the Family Cercopithecidae and closely related to *Semnopithecus* (Perelman et al, 2011; Wang et al, 2012). Groves (2005) assigned full species status to 17 taxa, which he clustered into 5 species groups. While 16 of these species have been assessed in other phylogenetic contexts (Bleisch et al, 2008; Geissmann et al, 2004; Karanth et al, 2008; Liedigk et al, 2009; Nadler et al, 2003; Wang et al, 2012; Zhang & Shi, 1993), which has greatly improved our understanding of *Trachypithecus* evolution, there has been no dedicated molecular analysis of the complex species structure of *Trachypithecus*. Of particular concern is the disputed taxonomy of the endangered Phayre's leaf monkey (*T. phayrei*). Three putative subspecies inhabit Bangladesh, northeastern India, Myanmar, Southwestern China, Thailand, Laos, and northern Vietnam (Bleisch et al, 2008). Recent genetic analyses have demonstrated that *T. p. crepuscula* and *T. p. phayrei* do not form a monophyletic clade. *T. p. phayrei* from India is the sister taxon of *T. barbei* and *T. obscures,* but *T. p. crepuscula* from Vietnam represents a distinct lineage, being a close relative of the *T. francoisi* species group (Karanth et al, 2008; Nadler et al, 2003). Accordingly, Nadler et al (2003) and Liedigk et al (2009) suggested that *T. p. crepuscula* from Vietnam should be given full species status. However, a lack of genetic information from *T. p. crepuscula* and *T. p. shanicus* (southwestern China) has prevented agreement on the purported taxonomy (Bleisch et al, 2008).

In this study, we sampled *T. p. crepuscula* and *T. p. shanicus* from southwestern China (Yunnan) and northern Myanmar and sequenced both mitochondrial

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and nuclear genes to resolve the phylogenetic relationships of *T. phayrei*. The sequences of *T. p. shanicus* are represented for the first time. By including langur sequences from GenBank into our analysis, we test the validity of the species groups proposed by Groves (2005). Furthermore, we estimated divergence times simultaneously with phylogenetic reconstruction to test the diversification pattern of *Trachypithecus* through time (Drummond et al, 2012). This effort will provide further evidence to clarify the nebulous classification of *T. phayrei* and the putative *Trachypithecus* species-group divisions.

MATERIALS AND METHODS

Sampling and lab work

Four samples of *T. p. crepuscula* and five of *T. p. shanicus* were collected from northern Myanmar, Yunnan, and Guangxi, China. One sample of *T. germaini* was obtained from the Kunming Cell Bank of the Chinese Academy of Sciences (Table 1). These samples are tissues from deceased individuals, museum skins, or noninvasive feces collections. All animal samples were obtained following the regulations of China for the implementation of the protection of terrestrial wild animals (State Council Decree [1992] No. 13) and approved by the Ethics Committee of Kunming Institute of Zoology, Chinese Academy of Sciences, China.

Total DNA was extracted from tissues and museum skins using the phenol/proteinase K/sodium dodecyl sulphate method (Sambrook & Russell, 2001). Four fecal samples were collected from the Gaoligong Mountain National Nature Reserve in 2008. Samples were stored using the "two-step" storage procedure (Nsubuga et al, 2004). We extracted total DNA from fecal samples using the 2CTAB/PCI method (Vallet et al, 2008).

Table 1 Sample and sequences used in this study

Species	Collection code	Code	Sample Locality	*CYT B*	*PRM1*	*LZM*
Trachypithecus phayrei crepuscula	Myanmar01	NM	Northern Myanmar	KC285863	KC285882	KC285875
	YN_WLS0301001	WL	Mt.Wuliang,Yunnan,China	KC285866	KC285883	KC285876
	YN_XSBN1	BN1	Xishuangbanna,Yunnan,China	KC285864	KC285884	KC285873
	GX_KCB89	BN2	Xishuangbanna, Yunnan,China	KC285865	KC285885	KC285874
T. p. shanicus	YN_GLG0912002	G1	Mt.Gaoligong,Yunnan,China	KC285867	KC285886	KC285877
	YN_GLG08002	G2	Mt.Gaoligong,Yunnan,China	KC285868	KC285887	-
	YN_GLG08003	G3	Mt.Gaoligong,Yunnan,China	KC285869	−	KC285878
	YN_GLG08004	G4	Mt.Gaoligong,Yunnan,China	KC285870	−	KC285879
	YN_GLG08005	G5	Mt.Gaoligong,Yunnan,China	KC285871	−	KC285880
T. germaini	KCB92007	V2	Northern Vietnam	KC285872	KC285888	KC285881

We sequenced the mitochondrial *CYT B* and nuclear protamine P1 (*PRM1*) and lysozyme (*LZM*) genes in this study. *CYT B* has been widely used in mammalian phylogenetic and phylogeographic studies (Bradley & Baker, 2001). This gene was amplified using L14724_hk6 (CCGTGATATGAAAAACCATCGTTG) and H15915 (Irwin et al, 1991). L14724_hk6 was modified from the universal primer L14724 (Irwin et al, 1991) to avoid amplification of nuclear pseudogenes of *CYT B* (Karanth, 2008). *PRM1* and *LZM* have been used previously in the phylogenetic study of other primates, including langurs (Karanth et al, 2008). These two genes were amplified following (Karanth et al, 2008). All PCR products were purified using the UNIQ-10 spin column DNA gel extraction kit (Sangon, Shanghai, China). Purified products were directly sequenced with PCR primers using the BigDye Terminator Cycle kit v3.1 on an ABI 3730xl sequencer by Tiangen Biotech Co, LTD. (Beijing, China).

Phylogenetic and molecular dating analyses

Nucleotide sequences were edited using SeqMan and EditSeq in the DNASTAR package v7.1 (DNASTAR, Inc., USA) and aligned with MUSCLE (Edgar, 2004). *CYT B* and coding regions of the two nuclear genes were translated into amino acids following the identification of any premature stop codon. All these sequences were submitted to GenBank (Accession numbers: KC285863 - KCKC285888). 1141 bp of *CYT B*, 387 bp of *PRM1*, and 135 bp of *LZM* were used in further analyses. Additional sequences of Catarrhini were downloaded from GenBank and added to the alignments (Supplementary Table 1, available online). In total, 70 *CYT B*, 35 *PRM1* and 32 *LZM* sequences representing 36 species including all three subspecies of *Trachypithecus phayrei* were used in this study.

We performed partitioned Bayesian analyses (Brandley et al, 2005) to reconstruct the phylogenetic relationships using BEAST v1.7.2 (Drummond et al, 2012). The analyses were performed on both the *CYT B* data set as well as the three-gene combined data set. Missing data were coded as "?". The model of DNA evolution was determined by a Bayesian Information Criterion (BIC) (Schwarz, 1978) in jModelTest v0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). Because

there are missing data in the *CYT B* alignment, this gene was divided into two partitions (partition 1: 1−423 bp, 826−1 141 bp; partition 2: 424−825 bp). Because partition 2 contains no missing data, it was used for the estimation of the time of molecular divergence (see below). The evolutionary model for each codon position of partition 1 and partition 2 of *CYT B*, *LZM*, and the coding and non-coding regions of *PRM1* were determined separately. We did not calculate the evolutionary model for each codon position of *LZM* or the coding regions of *PRM1* to avoid error caused by overpartitioning (Brown & Lemmon, 2007). In jModelTest, three substitution schemes were selected and a proportion of invariant sites were not included in the model selection, following He et al (2012). The partition information and models selected are shown in Figure 1.

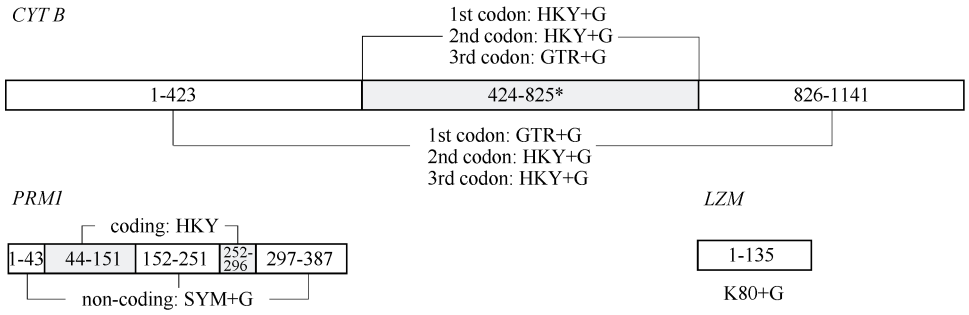

Figure 1 Models of molecular evolution used for each gene/partition for phylogenetic reconstruction

*: partition was used for divergence time estimation.

We performed a MCMC search of twenty million generations, sampling every 2 000 generations. We constrained the monophyly of *Semnopithecus* + *Trachypithecus* according to previous analyses (Osterholz et al, 2008; Perelman et al, 2011; Wang et al, 2012). All analyses were repeated twice. Tracer v1.5 was used to make sure all analyses reached the same posterior distributions and estimated the convergences by calculating effective sample sizes (ESSs) (Rambaut & Drummond, 2009). Posterior probabilities (PP) ≥ 0.95 were considered as strongly supported (Huelsenbeck & Rannala, 2004).

Molecular divergence times were estimated simultaneously with the multi-gene phylogenetic reconstruction. Because missing data will mislead estimates of branch lengths and thus affect the result of divergence time estimation (Lemmon et al, 2009), our molecular dating analyses were limited to partition 2 of the *CYT B* alignment (402 bp, Figure 1). We used a "relaxed" molecular approach (Drummond et al, 2006) and used fossil records and secondary calibration for dating. Six calibration points were applied as lognormal or exponential distributions (Ho, 2007). *Saadanius hijazensis* dated to 29−28 Ma for the hominoid-cercopithecoid divergence between 29−28 and 24 Ma (Zalmout et al, 2010), so that we set the prior as lognormal and offset=24, *SD*=0.98. The oldest known hominoid (*Morotopithecus bishopi*) is dated to 20.6 Ma (Gebo et al, 1997). We set the prior as lognormal. The earliest sample age was set to 20.6 Ma (offset=20.6) and the older 95% CI to the beginning of the Miocene (24.1 Ma, *SD*=0.76). The Homo-Pan divergence occurred between 7.2−5.6 Ma (Aiello & Collard, 2001; Senut et al, 2001), so we set the prior as lognormal and offset=5.6, *SD* =0.29. The oldest Colobinae was dated to 9.8 Ma (Benefit & Pickford, 1986; Nakatsukasa et al, 2010), but the molecular divergence estimation indicated a much more ancient divergence (Chatterjee et al, 2009), so we set the prior as exponential. The earliest possible age was set to 9.8 Ma, and the older 95% CI to 20 Ma (mean=3.4). The oldest African Colobinae was dated to 6.1 Ma (Gilbert et al, 2010). We set the prior as lognormal, the earliest possible age to 6.1 Ma, and the 95% CI to the beginning of the Late Miocene (11.6 Ma, *SD*=1.04). The most recent common ancestor (MRCA) of *Semnopithecus* and *Trachypithecus* was estimated to have existed at about 4.05 Ma (95% CI=2.93-5.36) (Perelman et al, 2011), so we set the prior as lognormal (mean=4.03, *SD*=0.18). Fossils of *Trachypithecus* have been found in Southeast Asia and southern China, most of which date to the Pleistocene (Jablonski, 2002). The oldest known fossil is *T. auratus sangiranensis* with an age of 1.9±0.5 Ma (Jablonski & Tyler, 1999); however, the site stratigraphy remains in doubt (Larick et al, 2000). Other fossils are not suitable for calibration, because of taxonomic uncertainty.

The minimum-spanning tree of *PRM1* were derived from Network v4.5 using the median-joining approach (Bandelt et al, 1999).

RESULTS

Phylogenetic relationships

The combined phylogenetic tree is shown in Figure 2, and the mitochondrial phylogenetic topology is identical to that based on multiple genes. *Trachypithecus*

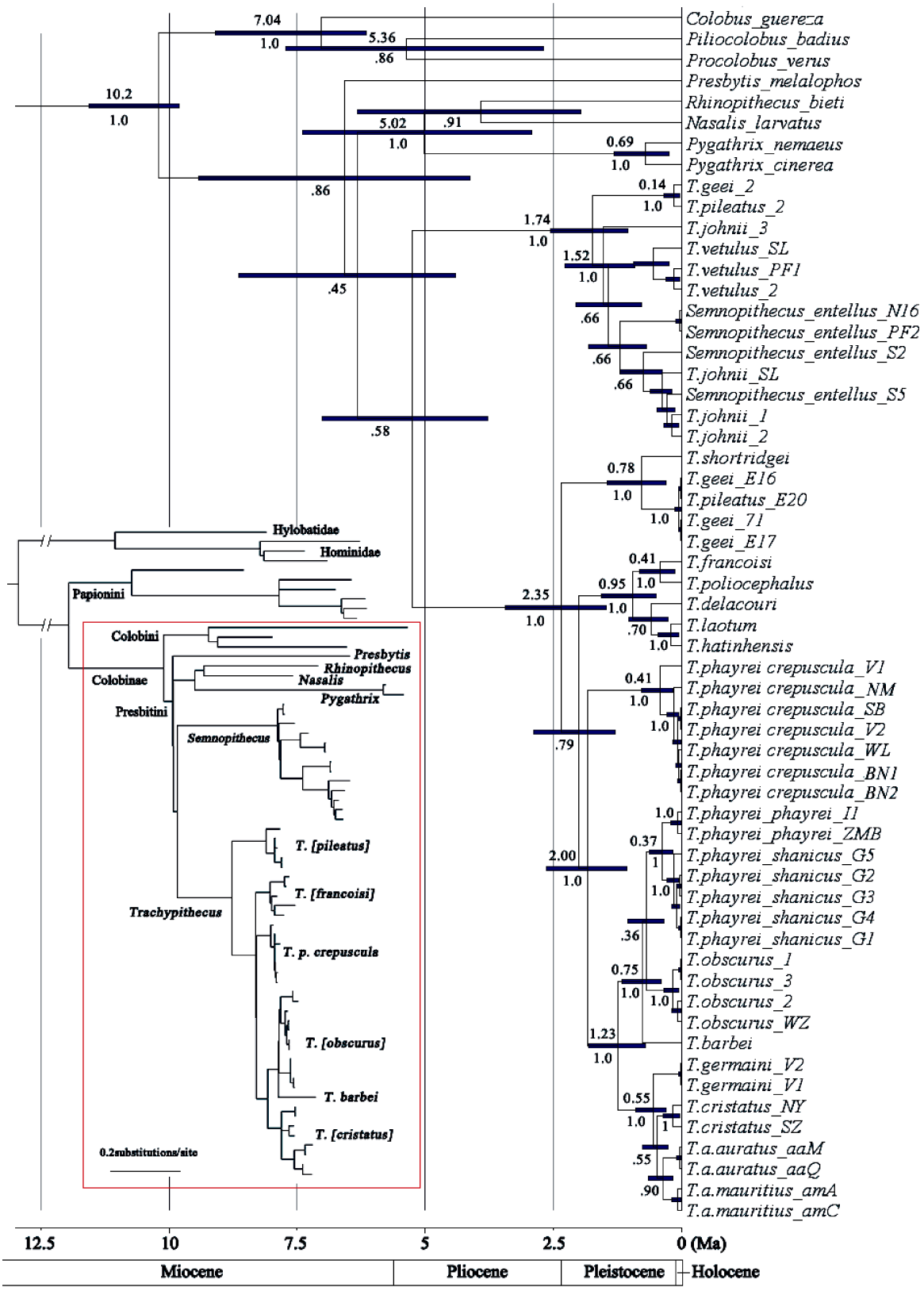

Figure 2 Phylogenetic relationships of Cartarrhini based on combined mitochondrial and nuclear genes and chronogram of Cercopithecidae based on partial *CYT B* gene

Node numbers above branches indicate mean divergence time; numbers below branches indicate Bayesian posterior probabilities; node bars indicate the 95% CI for the clade age.

taxa fell into two clades. *T. pileatus, T. geei* from India, *T. vetulus,* and *T. johnii* cluster with *Semnopithecus* (PP=1.0), which is consistent with previous studies (Chatterjee et al, 2009; Karanth et al, 2008; Osterholz et al, 2008; Wang et al, 2012), suggesting that *T. vetulus* and *T. johnii* should be merged into the genus *Semnopithecus*. The rest of the *Trachypithecus* taxa form a monophyletic clade (PP=1.0) with five strongly supported lineages recognized (PP=1.0). Our results strongly support that *T. p. phayrei* and *T. p. shanicus* are sister-taxa (PP=1.0) and are close relatives to *T. barbei* and *T. obscures* (PP=1.0). *T. p. crepuscula* from southwestern China and Vietnam form a distinct lineage (PP=1.0) as the sister taxon to the species group *T. Obscurus* +*T. cristatus* (PP=1.0). *T. geei* and *T. pileatus* from Bhutan are strongly supported as the sister taxa of *T. shortridgei* (PP=1.0).

LZM shows low variation in *Trachypithecus* and *Semnopithecus*. Only two mutations that lead to amino acid substitutions were observed (Table 2). As shown previously (Karanth et al, 2008), our results indicate that the *T. pileatus* and *T. geei* from India, which are members of the genus *Semnopithecus* in the phylogenetic tree (Figure 2), have the same *LZM* amino acid sequences as the species in the *Trachypithecus* clade, indicating possible hybridization.

Nucleotide substitutions and insertions/deletions (indels) were found in the PRM1 alignment in both coding and noncoding regions. In the Network tree, the *T. geei* from India and *T. francoisi* share the central haplotype, which differs from other haplotypes by two to nine mutations (Figure 3). These mutations also lead to amino acid substitutions/deletions. *T. p. shanicus* shares the same haplotype with *T. germaini* and *T. obscurus* and differs from the haplotype shared by *T. p. phayrei* and *T. p. crepuscula* by one amino acid deletion.

Table 2 The first ten amino acid sequences of the lysozyme protein of presbytini species

Nasalis larvatus	M	K	A	L	I	I	L	G	L	V
Pygathrix nemaeus	M	K	A	L	I	I	L	G	L	V
Semnopithecus entellus	M	K	A	L	**T**	I	L	G	L	V
Trachypithecus johnii	M	K	A	L	**T**	I	L	G	L	V
T. vetulus	M	K	A	L	**T**	I	L	G	L	V
T. pileatus (India)	M	**R**	A	L	I	I	L	G	L	V
T. geei (India)	M	**R**	A	L	I	I	L	G	L	V
T. francoisi	M	**R**	A	L	I	I	L	G	L	V
T. phayrei phayrei	M	**R**	A	L	I	I	L	G	L	V
T. phayrei shanicus	M	**R**	A	L	I	I	L	G	L	V
T. phayrei crepuscula	M	**R**	A	L	I	I	L	G	L	V
T. obscurus	M	**R**	A	L	I	I	L	G	L	V
T. germaini	M	**R**	A	L	I	I	L	G	L	V
T. cristatus	M	**R**	A	L	I	I	L	G	L	V

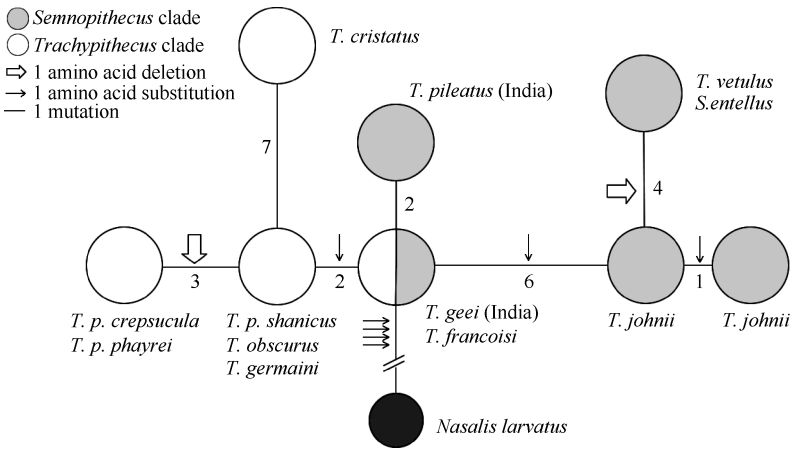

Figure 3 The median-joining network of the genus *Trachypithecus* based on partial *PRM1*

Numbers by the branches indicate the number of mutations.

Divergence time estimation

The estimates of divergence time including 95% credible intervals are shown in Figure 2. The *Trachypithecus* clade diverged from outgroups at about 5.25 Ma (7.01−3.77 Ma). Diversifications within this clade started at about 2.35 Ma (3.43−1.46 Ma) and occurred throughout the Pleistocene. *T. p. phayrei* and *T. p. shanicus* diverged at about 0.37 Ma (0.17−0.63 Ma). The MRCA of *T. p. crepuscula* occurred at about 0.41 Ma (0.16−0.77 Ma).

DISCUSSION

As has been discussed previously, the conflicting phylogenetic information observed in *Trachypithecus* is likely related to hybridization, incomplete lineage sorting, and ancestral polymorphism (Karanth et al, 2008; Liedigk et al, 2009; Wang et al, 2012). Pleistocene glaciations may have caused large scale deforestation

throughout southeast Asia, isolating formerly widespread species in refugia, and the subsequent recolonization of these territories during interglacial periods may have allowed for the hybridization of these isolates (Brandon-Jones, 1996; Meijaard & Groves, 2006). Consistent with the karyotype stability within the subfamily Colobinae (O'Brien et al, 2006), hybridization between different species of *Trachypithecus* and between *Trachypithecus* and closely related genera has been observed in captivity and could explain the paraphyly of *T. pileatus* and *T. geei* (Que et al, 2007; Schempp et al, 2008; Wangchuk, 2005; Wangchuk et al, 2008). Coupled with hybridization, the observed discrepancies between autosomal and mitochondrial genes might also have resulted from maternal philopatry and male dispersal from natal groups, common in living *Trachypithecus* and possibly the ancestral state of the genus, which could have produced these different patterns of genetic inheritance (Karanth et al, 2008).

Previous study including *T. p. crepuscula* from Vietnam and *T. p. phayrei* from India have found *Trachypithecus phayrei* to be paraphyletic (Karanth et al, 2008). As a result, it remained unclear whether the subspecies *T. p. crepuscula* is itself monophyletic and whether *T. p. shanicus* is close a relative to either of the other two subspecies. For the first time, we found *T. p. crepuscula* from Vietnam, southwestern China, and Myanmar are monophyletic and share a young MCRA at about 0.41 Ma and *T. p. shanicus* diverged from *T. p. phayrei* at about 0.37 Ma. However, *T. p. shanicus* is represented by a different and probably more ancient *PRM1* haplotype than the other two subspecies. This might be the result of nuclear introgression from *T. p. shanicus* to the ancestor of *T. p. crepuscula* due to male dispersal and female philopatry. Additional taxon sampling of other species and multiple unlinked genes are needed for reconstructing these robust evolutionary histories.

Although the incongruences between nuclear and mitochondrial genes undoubtedly increase the difficulty for estimating the "real" species tree and taxonomy, our combined phylogenetic tree (most likely reflecting the mitochondrial relationships) supports the species-group division proposed by Groves (2005) except for *T. barbei* and *T. p. crepuscula*. *T. barbei*, which was included in the *T. cristatus* group, is strongly supported to be a member of the *T. obscurus* group, being congruent with previous study and supported by morphological characters as well (Geissmann et al, 2004). Our results are consistent with this subspecies representing a distinct maternal genealogy with nuclear introgression from *T. p. shanicus,* suggesting a possible species status, namely *T. crepusculus*, which could also be a new species group (Liedigk et al, 2009).

Our study largely supports the proposed species-group division of Groves (2005) except for two taxa (*T. barbei* and *T. p. crepuscula*), both of which have been discussed in previous studies. We support the sister-relationship of *T. p. shanicus* and *T. p. phayrei*, whereas *T. p. crepuscula* may represent a distinct species throughout its distribution range, although hybridization may have occurred between it and *T. p. phayrei*. The incongruences between nuclear and mitochondrial genes imply complicated evolutionary histories of the three subspecies and the classification would benefit from re-examination using multiple nuclear genes.

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