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Molecular Phylogenetic Analysis of Non-Invasive Samples for the Endangered Phayre's Leaf Monkey (*Trachypithecus phayrei*) in Popa Mountain Park, Central Myanmar

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Abstract: Phayre's leaf monkey (*Trachypithecus phayrei*) is an endangered endemic species distributed predominantly in Southeast Asia. Although, three subspecies (*T. p. phayrei*, *T. p. crepusculus* and *T. p. shanicus*) are known, molecular phylogenetic studies on this monkey are still limited. In Myanmar, there was a controversy for the species identity of Phayre's leaf monkey inhabiting the Popa Mountain Park (PMP). Here, 32 non-invasive fecal samples and one non-destructive bone sample were used to infer the phylogenetic status of *T. phayrei* from PMP. Two DNA markers, nuclear Protamine P1 (Prm1) and mitochondrial Cytochrome b (Cyt-b) were selected for PCR and sequencing. Three haplotypes for *Cyt-b* gene and two unique sequences for *Prm1* gene were detected from 33 samples. The *Cyt-b* phylogenetic trees showed that the population of Phayre's leaf monkey in PMP is more closely related to the subspecies *T. p. shanicus*. However, the Prm1 phylogenetic trees could not resolve the phylogenetic position of *T. phayrei* subspecies. The results suggest the population from PMP as the subspecies *T. p. shanicus* but further taxonomic studies for all populations of this threatened monkey in Myanmar should be recommended for the species' conservation and management.

Key words: Cytochrome b, non-invasive sample, phylogeny, protamine P1, *Trachypithecus phayrei*, Myanmar

INTRODUCTION

Phayre's leaf monkey (*Trachypithecus phayrei*) is a threatened endemic species distributed predominantly in Southeast Asia. This species is categorized as Endangered by IUCN (Bleisch *et al*., 2008) and listed in CITES appendix II (CITES, 2011). The global population is declining due to a combination of habitat degradation and anthropogenic activities (Molur *et al*., 2003). Bangladesh and India each host about 1,000 individuals (Gittins and Akonda, 1982; Gupta, 2001) but the status is less known for Myanmar, China, Vietnam, Thailand and Laos (Bleisch *et al*., 2008). In Myanmar this species is protected under the national wildlife law (MoF, 1994).

Phayre's leaf monkey is a medium-sized primate (6-7 kg for adults) and the striking feature of this monkey is a pale patch surrounding the mouth and eyes (Bhattacharya and Chakraborty, 1990). Three subspecies (*T. p. phayrei*, *T. p. crepusculus* and *T. p. shanicus*) have been identified (Pocock, 1939; Bleisch *et al*., 2008). *T. p. phayrei* distributes in Bangladesh, Northeastern India and Western Myanmar (Bleisch *et al*., 2008) and its distribution was also recorded at Popa Mountain Park (PMP) in central Myanmar (Pocock, 1939). *T. p. crepusculus* can be found in Southwestern China, Laos, Myanmar (South of the range of *T. p. phayrei*), Thailand and Northern Vietnam (Pocock, 1939; Geissmann *et al*., 2004; Bleisch *et al*., 2008; He *et al*., 2012). *T. p. shanicus* distributes in Southwestern China, Northern and Eastern Myanmar (Bleisch *et al*., 2008). The geographical distribution of *T. phayrei* group in Myanmar is visualized in Fig. 1 according to Pocock (1939) but the current distribution in Myanmar is still unclear at subspecies level.

Due to the lack of detailed studies at molecular level, the identification of *T. phayrei*, particularly in Myanmar, continues to be based on morphological features and geographical distribution (Pocock, 1939). Moreover, after an intensive field survey in PMP in 1995 there was an erroneous identification for Phayre's leaf monkey as Dusky leaf monkey (*T. obscurus*) which distributes in Southern part of Myanmar (Pocock, 1939;

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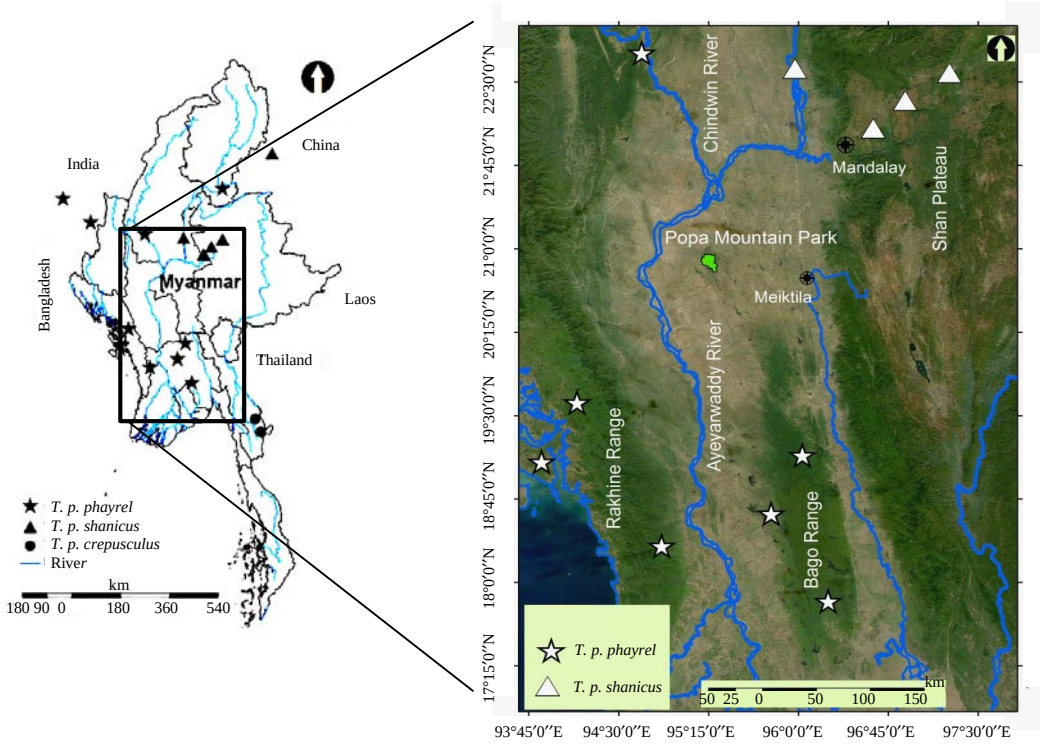

Fig. 1: Maps showing the subspecies distribution of Phayre's leaf monkey (★: *T. p. phayrei*; ▲: *T. p. shanicus* and ●: *T. p. crepusculus*) in Myanmar and its neighboring countries, India and China (left). The study site, Popa Mountain Park (right) showing the distribution of *T. p. phayrei* and *T. p. shanicus* along with the rivers (Ayeyarwaddy, Chindwin) and mountain ranges (Rakhine, Bago, Shan Plateau). Maps were created according to existing information for localities of Phayre's leaf monkey (Pocock, 1939; Gupta, 2001; He *et al*., 2012)

Geissmann *et al*., 2004). Both species have similar morphological characteristics, especially pale eye-patches (Pocock, 1939; Geissmann *et al*., 2004). Although, the population of Phayre's leaf monkey in PMP was previously recorded as subspecies *T. p. phayrei*, no comprehensive molecular studies have been yet conducted to confirm the identity of this population, at both species and subspecies level.

This study is the first molecular work using non-invasive (feces) and non-destructive (bone) samples to assess the phylogenetic status of threatened Phayre's leaf monkey inhabiting PMP located in central Myanmar. Although, DNA extracted from non-invasive samples tend to be of low quantity and quality it has been used to produce reliable results such as high rate of amplification success for sex identification of apes using faeces (Bradley *et al*., 2001) and clarification the historical distribution of wolves using 16th century skull fragments (Rutledge *et al*., 2010). Molecular analysis using the non-invasive samples could especially be preferred for wild primates which are endangered, elusive or both (Vigilant and Groeneveld, 2012). Wildlife management aimed at species conservation generally needs the adequate information of species identities and their phylogenetic positions among species, subspecies as well as closely related taxa (Isaac *et al*., 2007; Kuntner *et al*., 2010; Medina-Romero *et al*., 2012). This study aimed to clarify the molecular status of Phayre's leaf monkey in PMP both at species and subspecies levels using nuclear Protamine P1 (Prm1) and mitochondrial Cytochrome b (Cyt-b) in order to provide better species conservation plan.

MATERIALS AND METHODS

Study site: Popa Mountain Park (PMP) is located in central Myanmar lying between 20°47'-20°56'N and 95°11'-95°19'E (Fig. 1). The government of Myanmar declared the park as a protected area (about 150 km^2) in 1989. Its elevation ranges from about 300-1,500 m above sea level and vegetation types vary including dry mixed deciduous forest, dry hill forest or semi-evergreen forest, Burma Than-dahat forest and dry dipterocarp forests (Htun *et al*., 2011). Although, PMP is an isolated extinct

volcanic mountain area rising abruptly out of the plains in the central, there might have been some past vegetative connectivity with Bago Range to the South and Shan Plateau to the East (Fig. 1) where the historical distributions of Phayre's leaf monkey was recorded (Pocock, 1939). There is no precise evidence as to the age of Mt. Popa but it was estimated at approximately 2-3 million years ago and the existence of Phayre's leaf monkey in PMP was first recorded in 1913 (FD, 1981).

Sample collection: Researchers conducted field surveys of the Phayre's leaf monkey in the study site from June 2010 to July 2012. The number of the monkey in the study site was estimated about 100 individuals consisting of three groups (mean = 41±0.67, 41±0.58 and 21±0.67 individuals for group 1, 2 and 3, respectively). Thirty two non-invasive fecal samples and one non-destructive bone sample were collected in July 2012 (Appendix 1). The bones (head, femur and tibia) were obtained from local people who had stored them from a monkey killed by a landslide in 2006 and were kept in a clean plastic bag in ambient temperature. Although, an extentive fecal sampling was supposed to conduct for all groups it was difficult in practical due to the steep terrains where the monkeys mostly stay, especially group 2 and 3. Researcher collected fecal samples from one group (group 1) within 1 h post-defecation and then stored in 2 mL tubes containing absolute ethanol. All samples were stored at -20°C until DNA extraction.

DNA extraction, PCR and sequencing: Genomic DNA was extracted using the LaboPass™ Tissue Mini kit (COSMO GENETECH Corp., Korea) from bone sample and the QIAamp DNA Stool Mini kit (Qiagen Corp., Germany) from fecal samples.

The *Prm1* gene was used to infer the nuclear DNA (nDNA) phylogeny of Phayre's leaf monkeys (Karanth *et al*., 2008; He *et al*., 2012). A nested PCR approach was used because high DNA quality was not available particularly for the the fecal samples. Through two sequential PCRs, full-length Prm1 sequences (343 bp) were obtained by PCR as follows: a total 25 μL reaction volume containning 1 μM of each primers, 1X PCR buffer (iNtRON Inc., Korea), 2.5 μg of BSA (Promega Inc., USA), 1.5 mM of Mg^{2+} (iNtRON Inc.), 0.2 mM of each dNTP (iNtRON Inc.), 1 μL of DNA templates and 2 U of i-Star Taq polymerase (iNtRON Inc.). A primer pair, Quer1 and ProtR was used for the first PCR (Table 1). The first PCR was carried out using the following protocol: initial denaturation for 5 min at 94°C, followed by 30 cycles (94°C for 40 sec, 56°C for 30 sec and 72°C for 1 min) and a final extension at 72°C for 5 min. The second nested PCR was conducted using a newly designed primer set, ProtL1 and ProtR1 with 1 μL of the first PCR template (Table 1). The second PCR was carried out using the same protocol for the first PCR except for running 50 cycles.

The *Cyt-b* gene was used to infer the mitochondrial DNA (mtDNA) phyolgeny (Geissmann *et al*., 2004; Karanth *et al*., 2008; He *et al*., 2012). Two sequential PCRs were carried out by a nested PCR approach. Including primer regions, 514 bp of *Cyt-b* gene was obtained by PCR as follows: a total 25 μL reaction volume containing 1 μM of each of primers, 1X PCR buffer, 2.5 mM of Mg^{2+}, 0.2 mM of each dNTP, 1 μL of DNA templates and 2 U of i-Star Taq polymerase. The first PCR was conducted using a set of primers, Cytb-F and Cytb-R. A set of newly designed species-specific primers, CytbTP-F and CytbTP-R (Table 1) was used for second PCR with 1 μL of the first PCR template (Table 1). Both PCRs were carried out using the following protocol: initial denaturation for 3 min at 94°C, followed by 50 cycles (94°C for 1 min; 50°C for 45 sec and 72°C for 1 min) and a final extension at 72°C for 5 min.

The PCR products were purified using the Zymoclean™ Gel DNA Recovery kit (Zymo Research Corp., Korea). The purified Prm1 PCR products were bi-directionally sequenced using the forward ProtL1 and reverse ProtR1 primers. The Cyt-b PCR products were bi-directly sequenced using the primer pair (CytbTP-F

Table 1: Primers used for amplification and sequencing of nuclear protamine P1(*Prm 1*) and mitochondrial cytochrome b (*Cyt-b*) genes of Phayre's leaf monkey

Primers	Direction	Sequence (5'-3')	Annealing (°C)	Reference
Protamine P1 (Prm1)				
Quer1	Forward	ACC TGC TCA CAG GTT GGC	62	1
ProtR	Reverse	TTG ACA GGT CGG CAT TGT TC	60	1
ProtL1-F	Forward	TGG TGC CCT GCT CTG AGC	60	3
ProtR1-R	Reverse	GGA TGG TGG CAT TTT CAA GA	56	3
Mitochondrail Cytochrome b (Cyt-b)				
Cytb-F	Forward	CTC CTC ATT GAA ACA TGA AAT AT	55	2
Cytb-R	Reverse	CTT TGT TGT TTG GAT TTG TG	56	2
CytbTP-F	Forward	TTA CTC ATA ACC ATA GCA ACA G	56	3
CytbTP-IF	Inner	TTC ATT ATT GCA ACC CTA ACA	50	3
CytbTP-R	Reverse	GCT AAG ATA AGA ATG GAT AGA	57	3

1: Karanth *et al*. (2008); 2: Geissmann *et al*. (2004); 3: This study

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

Common name	Scientific name	Code	Sample type/Reference	Source	Accession No.	
					Cyt-b	Prm1
Hanuman langur	*S. entellus*	-	Hair	HZ	AF293952	AF294852
Nilgiri langur	*S. johnii*	-	Hair	W	AF294619	AF294853
Purple-faced langur	*S. vetulus*	-	Karanth *et al.* (2008)	-	AF295577	AF119236
Golden leaf monkey	*T. geei*	-	Hair	HZ	AF294618	AF294857
Phayre's leaf monkey	*T. phayrei*	I	Tissue	W	AF294621	AF294858
	T. p. phayrei	V	DNA	EPRC	AF294622	AF294860
	T. p. phayrei	M	Museum skin	ZMB	AY51946	-
	T. p. crepusculus	V	Hair	EPRC	AY519461	-
	T. p. crepusculus	M	He *et al.* (2012)	-	KC285863	KC285882
	T. p. crepusculus	C	He *et al.* (2012)	-	KC285864	KC285884
	T. p. shanicus	C	He *et al.* (2012)	-	KC285868	KC285886
	T. p. shanicus (this study)	M	Bone	Popa	KC751745	KC833502
	T. p. shanicus (this study)	M	Feces	Popa	KC833499	KC833501
	T. p. shanicus (this study)	M	Feces	Popa	KC833500	-
Tenasserim lutung	*T. barbei*	-	Hair	BZ	AY519462	-
Capped leaf monkey	*T. pileatus*	-	Hair	SZ	AF294626	AF294856
Francois' leaf monkey	*T. francois*	-	Karanth *et al.* (2008)	-	AF295578	AF119234
Dusky leaf monkey	*T. obscurus*	-	Karanth *et al.* (2008)	-	AF295579	AF119238
Silvered leaf monkey	*T. cristatus*	-	Blood	NYZS	AF295580	AF294861
Guereza colobus	*C. guereza*	-	Karanth *et al.* (2008)	-	U38264	AF119233
Red colobus	*P. badius*[a]	-	Tissue	LSU	AF294625	AF29485O
Rhesus monkey	*M. mulatta*	-	Karanth *et al.* (2008)	-	U38272	AF119240
Baboon	*P. cynocephalus*[b]	-	Karanth *et al.* (2008)	-	Y16590	AF119239

C = Colobus; M = Macaca; [a] P = Piliocolobus; [b] P = Papio; S = Seminopithecus; T = Trachypithecus; Phayre's langurs samples; C from China (K. He); I from India (K.P. Karanth); V from Vietnam (K.P. Karanth) and M from Myanmar (T. Geissmann). HZ = Hyderabad Zoo; South India (K.P. Karanth); EPRC = Endangered Primate Rescue Center; Vietnam (C. Roos); SZ = Shipahijala Zoo; Northeast India (K.P. Karanth); BZ = Bangkok Zoo; Thailand (T. Geissmann); W (Nilgiri langur) = Wild from Anamalai Hills; South India (M. Singh); W (T. phayrei) = Wild from Shipahijala National Park; Northeast India (K.P. Karanth); LSU = Louisiana State University; USA (P.A. Marx and P. Telfer); NYZS = New York Zoological Society; USA (D. Wharton); ZMB: Zoologisches Museum der Humboldt Universitat Berlin (T. Geissmann); Popa = Popa Mountain Park, Myanmar

and CytbTP-R) but showed no success for all the reverse sequences suggesting probably the presence of numts. Instead of the CytbTP-R, a newly designed species-specific inner primer (CytbTP-IF) was used for sequencing. All sequences were deposited in GenBank (accession Nos. KC751545 and KC833499-833502). PCR and sequencing successes were shown in Appendix 1.

Phylogenetic analysis: A total of eight sequences were analyzed in this study. All sequences of Prm1 and Cyt-b were aligned by AlignIR program Version 2.1 (LI-COR Inc., USA) and manually examined at least three times. Since, the nuclear *Prm1* gene is inherited from both sexes (Melnick and Hoelzer, 1993), the appearances of heterozygote were carefully check by eyes for both the forward and reverse sequences. After aligning Prm1 sequences from each sample, researchers assigned all genotypes to homozygotes.

In total, 20 Cyt-b and 17 Prm1 reference sequences of closely related taxa including all three subspecies of *T. phayrei* were used in this analysis (Table 2) and other reference sequences of Baboon (*Papio cynocephalus*), Guereza colobus (*Colobus guereza*), Red colobus (*Piliocolobus badius*) and Rhesus monkey (*Macaca mulatta*) were also used. Genetic distances (p_s) between nucleotide sequences were analyzed using MEGA Version 5.1 (Tamura *et al.*, 2011) under the Kimura-2-Parameter (K-2-P) Model (Kimura, 1980). The phylogenetic tree reconstructions between haplotypes of all monkey species were carried out using the Neighbor-Joining (NJ) (Saitou and Nei, 1987) Method under the K-2-P Model. The Maximum Likelihood (ML) phylogenetic trees were also reconstructed (Kimura, 1980). To select the best-fit model, maximum likelihood analysis was subjected to the Akaike Information Criterion (AIC; Akaike, 1974) using j Model test Version 0.1 (Posada and Crandall, 1998). The HKY+G and TIM3+I+G Model were determined to be most appropriate for Prm1 and *Cyt-b* gene, respectively. ML heuristic searches were conducted using procedure with a Tree-Bisection-Reconnection (TBR) branch swapping algorithm with 100 random addition sequence replications. As outgroup a corresponding sequence of the Patas monkey (*Saguinus imperator*) from GenBank (accession No. X61678 and HM368019 for Prm1 and Cyt-b, respectively) was chosen to root the phylogenetic trees. Confidence in estimated relationship was determined using the bootstrap approach obtained through 1,000 (for NJ) or 100 (for ML) replications incorporating the

same model as above (Felsenstein, 1985). Both bootstrap analysis and phylogeny reconstruction were conducted using PAUP 4.0b10 (Swofford, 2001).

RESULTS AND DISCUSSION

Nuclear protamine P1 (*Prm1*) gene: Out of 33 collected samples, 39% (13/33) for PCR products and 24% (8/33) for sequencing were successful. The sequences of Prm1 were highly similar to those of closely related colobines deposited in GenBank. Two haplotypes (Popa 1 and 2) of *Prm1* gene were detected from a total of eight sequences analyzed. Both of haplotype diversity (h) and nucleotide diversity (π) between two haplotypes were low (h = 0.429 and π = 0.25%, respectively). Nucleotide sequence distance between Popa 1 and 2 was also relatively low (p_s = 0.6%). The NJ and ML trees showed a similar topology for *Prm1* gene (Fig. 2a). The *Prm1* gene trees supported monophyly of Phayre's leaf monkey from PMP (Popa 1 and 2) and all other *T. phayrei* groups from India, Vietnam, Myanmar and China with low genetic distances (p_s = 0-0.6%, Fig. 2a). However, Dusky leaf monkey (*T. obscurus*) also clustered together with *T. phayrei* groups (p_s = 0-0.3%, Fig. 2a). Thus, researcher concluded that the *Prm1* gene could not resolve the phylogenetic position of *T. phayrei*. It is possibly due to the slow fixation time for *Prm1* gene evolution (Wilson *et al*., 1995; Ting *et al*., 2008) and therefore *Prm1* gene shows little or no difference among closely related taxa (Ruvolo, 1997).

Mitochondrial cytochrome b (*Cyt-b*) gene: Collected samples and success percentages for PCR and sequencing were same as *Prm1* gene. Out of eight sequences, three haplotypes (Popa1, 2 and 3) of *Cyt-b* gene were assessed. Haplotype diversity was relatively high with overall h = 0.61±0.16 among three haplotypes but nucleotide diversity was low (π = 0.13±0.04%). Pairwise genetic distances among three haplotypes were also low (p_s = 0.2% for between Popa 1 and 2; p_s = 0.2% for between Popa 1 and 3; p_s = 0.4% for between Popa 2 and 3). The NJ and ML trees show a similar topology with minor differences (Fig. 2b). Phayre's leaf monkey from PMP is more closely related to the subspecies *T. p. shanicus* from Southwest China (p_s = 1.8-2.0%) rather than the subspecies *T. p. phayrei* from Southwest Rakhine Range in Myanmar (p_s = 2.0-2.2%). The *Cyt-b* gene trees also supported the monophyletic group of *T. p. phayrei* from India and *T. p. phayrei* from southwest Myanmar (p_s = 0.4%, Fig. 2b). These are possibly the result of contiguous geographical range between the two countries because populations along the mountain range showed little genetic differences and shared common alleles for mitochondrial gene (Tennessen and Zamudio, 2008). In Myanmar, *T. p. phayrei* distributes along the Western Rakhine mountain range (Fig. 1) extending to Northeast India where distribution of *T. p. phayrei* have been previously reported (Bleisch *et al*., 2008; Karanth *et al*., 2008). Similarly, *Cyt-b* gene supported monophyletic group of subspecies *T. p. crepusculus* from Myanmar, Vietnam and China with high bootstrap values (Fig. 2b) but low genetic distance (p_s = 0.2-1.0%). This result is consistent with the previous study reporting the monophyletic group of *T. p. crepusculus* for these three countries with low genetic distances (He *et al*., 2012). The Cyt-b trees revealed that Dusky leaf monkey (*T. obscurus*)

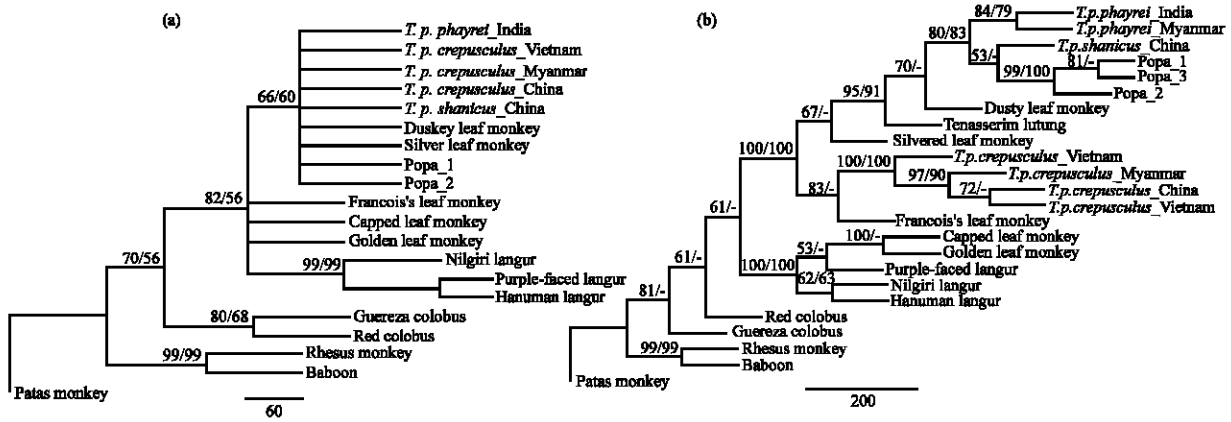

Fig. 2: a) Prm1 and b) Cyt-b phylogeny trees. Numbers on the branches indicate bootstrap values obtained from the neighbor-joining (NJ, 1,000 replications) and Maximum-Likelihood (ML, 100 replications) analyses. Both phylogenetic trees are rooted with Patas monkey (Saguinus imperator, GenBank accession No. X61678 and HM368019 for Prm1 and Cyt-b, respectively)

forms a clade with the subspecies *T. p. phayrei*, *T. p. shanicus* and the study populations from PMP but the genetic distance was high with overall p_s = 3.6-4.5% across the taxa. Moreover, the genetic distance between *T. obscurus* and the subspecies *T. p. crepusculus* was relatively high (p_s = 10.1%).

Comparison of the results from Prm1 and Cyt-b analyses: The Prm1 trees revealed that the study population of Phyare's leaf monkey from PMP clustered together with all subspecies of *T. phayrei* groups as well as *T. obscurus*. In contrast, the Cyt-b trees showed that the study population is more closely related to the subspecies *T. p. shanicus* but showed no clustering of *T. phayrei* groups and *T. obscurus*. This incongruence between nuclear and mitochondrial phylogenetic trees might reflect the result of different gene flows of male and females of the species. Maternally inherited *mtDNA* genes tend to be highly substructured between populations due to female natal philopatry in mammals (Melnick and Hoelzer, 1993; Karanth *et al*., 2008) and also *mtDNA* genes evolve rapidly (Ruvolo, 1997). However, the evolution of nuclear genes is very slow (Ting *et al*., 2008) and consequently little or no variation of *Prm1* gene has been assessed among closely related taxa (Ruvolo, 1997). The results of phylogenetic incongruence between two DNA markers in this study supported that the previous phylogenetic works using the nuclear and mitochondrial markers for langurs and leaf monkeys (Ting *et al*., 2008), common vampire bat (Martins *et al*., 2009) and macaques (Tosi *et al*., 2000).

CONCLUSION

This study is the first molecular phylogenetic analysis on Phayre's leaf monkey using non-invasive and non-destructive samples for the remnant population of Popa Mountain Park in the Central Myanmar. Based on the results reported in this study, researchers suggest that the population from the study area could be identified as the *T. phayrei* and it is more closely related to the subspecies *T. p. shanicus* which distributes at the eastern Shan Plateu (Fig. 1). It is likely that there may have past migration between the study area and the eastern ranges. Since the study area hosts the only remnant population of Phayre's leaf monkey in Myanmar it is urgently needed to give high priority for species conservation together with habitat protection. However, further studies on the current distribution and molecular phylogeny for all populations of *T. phayrei* within Myanmar would be recommended to resolve the whole spectrum of phylogenetic position as well as migration among all subspecies.

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APPENDIX 1

The sample number, sample ID, sample type, PCR and sequencing success, haplotype for Protamine P1 (*Prm1*) and Cytochrome b (*Cyt-b*) genes use in this study. O: Success; X: Fail; N/A: No Account; H: Haplotype

Sample No.	Sample ID	Sample type	PCR		Sequencing		Haplotype	
			Prm1	Cyt-b	Prm1	Cyt-b	Prml	Cyt-b
1	Popa-B	Bone	O	O	O	O	H1	H1
2	Popa-01	Feces	O	O	O	O	H1	H1
3	Popa-02	Feces	O	O	O	O	H2	H1
4	Popa-03	Feces	X	X	N/A	N/A	N/A	N/A
5	Popa-04	Feces	X	X	N/A	N/A	N/A	N/A
6	Popa-05	Feces	X	X	N/A	N/A	N/A	N/A
7	Popa-06	Feces	X	X	N/A	N/A	N/A	N/A
8	Popa-07	Feces	O	O	O	O	H1	H2
9	Popa-08	Feces	X	X	N/A	N/A	N/A	N/A
10	Popa-09	Feces	O	O	X	X	N/A	N/A
11	Popa-10	Feces	X	X	N/A	N/A	N/A	N/A
12	Popa-11	Feces	X	X	N/A	N/A	N/A	N/A
13	Popa-12	Feces	X	X	X	X	H1	H1
14	Popa-13	Feces	O	O	O	O	N/A	N/A
15	Popa-14	Feces	O	O	O	O	H1	H2
16	Popa-15	Feces	X	X	N/A	N/A	N/A	N/A
17	Popa-16	Feces	O	O	O	O	H1	H2
18	Popa-17	Feces	X	X	N/A	N/A	N/A	N/A
19	Popa-18	Feces	O	O	O	O	H2	H3
20	Popa-19	Feces	X	X	N/A	N/A	N/A	N/A
21	Popa-20	Feces	X	X	N/A	N/A	N/A	N/A
22	Popa-21	Feces	X	X	N/A	N/A	N/A	N/A
23	Popa-22	Feces	X	X	N/A	N/A	N/A	N/A
24	Popa-23	Feces	X	X	N/A	N/A	N/A	N/A
25	Popa-24	Feces	X	X	N/A	N/A	N/A	N/A
26	Popa-25	Feces	O	O	N/A	N/A	N/A	N/A
27	Popa-26	Feces	O	O	N/A	N/A	N/A	N/A
28	Popa-27	Feces	X	X	N/A	N/A	N/A	N/A
29	Popa-28	Feces	O	O	N/A	N/A	N/A	N/A
30	Popa-29	Feces	X	X	N/A	N/A	N/A	N/A
31	Popa-30	Feces	X	X	N/A	N/A	N/A	N/A
32	Popa-31	Feces	X	X	N/A	N/A	N/A	N/A
33	Popa-32	Feces	O	O	N/A	N/A	N/A	N/A
-	-	-	39%	39%	24%	24%	-	-
-	-	-	(13/33)	(13/33)	(8/33)	(8/33)	2	3

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