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Genetic Relationships of Cercopithecidae in Thailand as Inferred from rDNA ITS Regions

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Abstract: Genetic relationships of cercopithecoid species in Thailand are determined based on sequence analysis of the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). Aligned sequences of the complete ITS regions obtained from the six taxa and an outgroup, *Pan paniscus* resulted in 634 characters. Within the Cercopithecidae, ITS sequences are highly conserved with 97.48% homology. The resulting comparisons of ITS sequences were used for analysis of genetic distance and a dendrogram was constructed based on the Neighbor Joining Method. From the dendrogram, *Pan paniscus* is solely separated. The Colobinae species, *Trachypithecus cristatus*, *T. phayrei*, *T. obscurus*, were placed inside the Cercopithecinae species, *Macaca arctoides*, *M. mulatta*, *M. nemestrina*. The dendrogram of these species does not completely match the former classification based on morphological characters. Genetic distances are very low (0.002 to 0.013) among Thai cercopithecoid species. The results suggest that the ITS regions of rDNA is highly conserved in Thai cercopithecoids.

Key words: Cercopithecids, dendrogram, genetic distance, ITS sequences, ribosomal DNA

INTRODUCTION

The old world monkeys are a group of primates in the family Cercopithecidae. The family contains two extant subfamilies, Cercopithecinae (cercopithecines; the cheek-pouch monkeys) and Colobinae (colobines; the leaf monkeys). The cercopithecines are omnivorous, having cheek pouches and simple stomachs while the colobines are folivorous, lacking cheek pouches but having complex stomachs (Swindler, 1998).

The Colobinae comprises seven genera. Of these, five have a solely Asian distribution and two have a solely African distribution (Page *et al.*, 1999). The phylogenetic separation of the African colobines has been clearly studied, whereas, there is a lack of information about the Asian group. In Thailand, two genera and four species are recognized, *Presbytis femoralis*, *Trachypithecus cristatus*, *T. phayrei* and *T. obscurus* (Lekagul and McNeely, 1988; Francis, 2001) and the diploid chromosome number is 44 in the extant genera (Chaveerach *et al.*, 2007).

The Cercopithecinae comprise 11 extant genera. Genus *Macaca* (macaques) is the most widely

distributed. Fifteen species have been found in Asia and one more species is found in Africa. The other ten genera are solely African (Page *et al.*, 1999). In Thailand, there are five species, *Macaca arctoides*, *M. assamensis*, *M. fascicularis*, *M. mulatta* and *M. nemestrina* (Lekagul and McNeely, 1988; Francis, 2001). They have diploid chromosome number of 42 (Tanee *et al.*, 2006).

However, in recent years, the genetic relationships among different cercopithecoid species are still controversially discussed because of the limited material. The currently accepted classification of cercopithecoids is mainly based on morphological studies, while their cytogenetics and molecular genetics have become important tools (Page *et al.*, 1999; Tanee *et al.*, 2006, 2007; Chaveerach *et al.*, 2007). However, few analysis have included information from internal transcribed spacer (ITS) sequence data. As part of a study on the complete genetic relationships of Cercopithecidae, the old world monkeys in Thailand play a key role, because of the high number of endemic species and the low amount of currently available data. Moreover, the genetic relationships within wildlife cercopithecoids in Thailand have never been addressed using the sequences of ITS regions.

The internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) is one of the most extensively sequenced molecular markers (Alvarez and Wendel, 2003). The two internal transcribed spacers, ITS1 and ITS2, of rDNA gene normally exhibit high rates of variability and have been widely used for phylogenetic studies of both closely and distantly related species (Insua *et al.*, 2003; Sumida *et al.*, 2004; Chaveerach *et al.*, 2008).

From the advantage of ITS analysis above, the genetic relationships of six old world monkeys in Thailand, *Macaca arctoides*, *M. mulatta*, *M. nemestrina*, *Trachypithecus cristatus*, *T. obscurus*, *T. phayrei*, were investigated using the sequences of ITS regions. The results will be added into scientific data and will be useful for long-term management of these primates.

MATERIALS AND METHODS

Sample collection: Since December 2006, blood samples from three macaque species (*Macaca arctoides*, *M. mulatta*, *M. nemestrina* from Northeastern Thailand) and three leaf monkey species (*Trachypithecus cristatus* and *T. phayrei* from Northeastern Thailand, *T. obscurus* from Southern Thailand) were collected. The collected locations are shown on a map of Thailand (Fig. 1).

DNA isolation: The genomic DNA was extracted from blood samples using the Genomic DNA extraction kit (RBC Bioscience, USA). The quality and quantity of extracted DNA was assessed by 0.8% agarose gel electrophoresis.



Fig. 1: Map of Thailand indicating sample collection of cercopithecids (•). N: Northern, NE: Northeastern, C: Central, W: Western, E: Eastern, S: Southern

Amplification of ITS regions: Internal transcribed spacers (ITS1, ITS2) and 5.8s rDNA regions were amplified using primers IT1 (5'-TCGTAACAAGGTTTCCGTAGGT-3') and IT2 (5'-GTAAGTTTCTTCTCCTCCGCT-3') described by Tsai *et al.* (2004). PCR amplifications were performed in a total volume of 25 µL, containing PCR master mix (Promega), 0.5 µM each primer and 10 ng genomic DNA. Amplification reactions were performed in a thermal cycler (Gene Amp PCR System 9700) as follows: (1) 5 min initial denaturation step at 94°C; (2) 40 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, extension at 72°C for 1 min; (3) a final extension for 10 min at 72°C. The size of PCR products was determined by agarose gel electrophoresis (1.0% w/v in TAE) with ethidium bromide staining.

DNA sequencing: The PCR products with expected size were examined for their nucleotide sequences with either IT1 or IT2 primer. The sequencing was done by using BigDye Version 3.1 (Applied Biosystems, USA) with MyGenie96ThermalBlock (Bioneer, Korea) follow by 3100 Genetic Analyzer with BigDye terminator v.3.1 cycle sequencing kit (Applied Biosystems).

Data analysis: Genetic relationships of the cercopithecoid samples based on ITS sequences were analyzed. ITS sequence of pygmy chimpanzee (*Pan paniscus*), used as outgroup was taken from GenBank (accession number EF647787). The genetic distance matrix was calculated by the two-parameter method of Kimura and then was used to construct a phylogenetic tree using the Neighbor-Joining Method (NJ) with interior branch tests of 1000 replicates. All these analysis were done using MEGA software version 4.0 (Tamura *et al.*, 2007).

RESULTS

Genomic DNAs of six cercopithecoid species, namely *Macaca arctoides*, *M. mulatta*, *M. nemestrina*, *Trachypithecus cristatus*, *T. obscurus* and *T. phayrei*, were amplified for their ITS regions with IT1-IT2 primers. The amplification yielded single fragments of approximately 750 bp on the gel for all samples. The fragments were analyzed for their sequences. From the sequencing results, the length of the ITS regions of six cercopithecoids varied from 587 to 591 bp. All of these sequences were submitted to GenBank. The accession numbers were shown in Table 1. All of these sequences were aligned and resulted in 634 characters (Fig. 2). Among six of the Thai cercopithecoid species, the number of different sites in the ITS regions is 16 (2.52%) sites and number of common sites is 618 (97.48%) sites.

A dendrogram obtained from comparisons of ITS sequences using Neighbor-Joining showed that the six cercopithecoid species are clustered together, whereas *Pan paniscus* is separated as an outgroup. Within the cercopithecoid cluster, *T. obscurus* is more closely related to *T. phayrei* than other cercopithecoids (Fig. 3). The genetic distances computed from sequence alignment among all taxa are shown in Table 2. The genetic distance values vary from 0.002 (between *M. nemestrina* and *T. cristatus*) to 0.013 (between *M. arctoides* and *T. phayrei*) among cercopithecoid species.

DISCUSSION

ITS sequence comparisons are becoming an increasingly popular tool for genetic relationship studies (Schlötterer *et al.*, 1994; Insua *et al.*, 2003; Sumida *et al.*, 2004; Tsai *et al.*, 2004; Chaveerach *et al.*, 2008). In this study, the genetic relationships among cercopithecoid species in Thailand have been determined by using the ITS regions including ITS1, 5.8s ribosomal DNA and the ITS2. From the alignment of ITS sequences (Fig. 2), the ITS regions are highly conserved with 97.48% homology. The results suggest that the ITS regions in family Cercopithecidae are very highly conserved sequences reflecting the close genetic relationships among cercopithecoid species, whereas this region in other animals shows great variability such as in mollusks (Insua *et al.*, 2003), amphibians (Sumida *et al.*, 2004) and carnivores (Chaveerach *et al.*, 2008). Moreover, the genetic distances (Table 2) and dendrogram (Fig. 3) show that the genetic relationships within cercopithecoids are closely related. The results are supported by other works using morphology (Lekagul and McNeely, 1988; Swindler, 1998; Falk, 2000; Francis, 2001) and g-Globin DNA sequences (Page *et al.*, 1999).

The results from ITS alignment (Fig. 2) and dendrogram (Fig. 3) show that, within langur species, *Trachypithecus phayrei* is more closely related to *T. obscurus* than *T. cristatus*. This agrees with the morphological study (Brandon-Jones, 2006) and DNA fingerprinting study (Chaveerach *et al.*, 2007) viewpoint, i.e., the *T. phayrei* is not a valid separate species. It is better to regard *T. phayrei* as a subspecies of *T. obscurus*.

Table 1: The GenBank accession number and ITS length of six cercopithecoid species and *Pan paniscus*

Species	Accession number	Length (bp)
<i>Macaca arctoides</i>	EU938063	587
<i>M. mulatta</i>	EU938064	588
<i>M. nemestrina</i>	EU938065	591
<i>Trachypithecus cristatus</i>	EU938066	587
<i>T. obscurus</i>	EU938067	588
<i>T. phayrei</i>	EU938068	589
<i>Pan paniscus</i>	EF647787	599

	ITS1	69
Macaca arctoides	--- --- --- --- --- --- --- TCA TTA CCG AGT TTA GAA CTC OCA AAC OCA TG- TGA ACA TAC CTT T-C	
M. mulatta	--- --- --- --- --- --- --- *** **	
M. nemestrina	--- --- --- --- --- --- --- *** **	
Trachypithecus cristatus	--- --- --- --- --- --- --- *** **	
F. obscurus	--- --- --- --- --- --- --- *** **	
F. phayrei	--- --- --- --- --- --- --- *** **	
Pan paniscus	TGG AAT CCT GCG ATA GCA AAA *G* CCC G*T *A* G*G T** ACA *AT TGG G** A*C *TC GGG GGG *** *TG	
		138
Macaca arctoides	CTT TCC TTC GGC GGA G-C GGC CCC GGC GGC C-- --G GAA -CC TCT TCG GTT TCG CGG CCC GG- AAC CAG	
M. mulatta	*** **	
M. nemestrina	*G* **	
Trachypithecus cristatus	*G* **	
F. obscurus	*C* **	
F. phayrei	*G* **	
Pan paniscus	*C* CTT G** T*T *A* CC* *AG G*A **T *G* *CT TC* *GG TG* C*A C** *C* *AC GAA AT* *** -G* GC*	
		207
Macaca arctoides	GCG CCC GCC GGA GGC CAC AAA CTC TTT TGT TTT TAC AGT TTC TTC TGA GTG TGC CGC AAG G-- CAA AAT	
M. mulatta	*** **	
M. nemestrina	*** **	
Trachypithecus cristatus	*** **	
F. obscurus	*** **	
F. phayrei	*** **	
Pan paniscus	*AA AG* *** AAC *AA T*T C** A-- --** GAA *** CG* CCG *G* *** *T- **T C** T*C *** CGT *GT C**	
	5.8s rDNA	276
Macaca arctoides	ACA AAT GAA TCA A-A ACT TTC AAC AAC GGA TCT CTT GGT TCT GGC ATC GAT GAA GAA GGC AGC GAA ATG	
M. mulatta	*** **	
M. nemestrina	*** **	
Trachypithecus cristatus	*** **	
F. obscurus	*** **	
F. phayrei	*** **	
Pan paniscus	T*T G*A AC* CA* *C* *** C** GG* *** **A* *CC **C *** C** *** **T *** **	
		345
Macaca arctoides	CGA TAA GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC ATT GCG CCC GCC AGA	
M. mulatta	*** **	
M. nemestrina	*** **	
Trachypithecus cristatus	*** **	
F. obscurus	*** **	
F. phayrei	*** **	
Pan paniscus	*** *C T*G G** *** ** *C* C** *** C** *** *G* *** ** *A* G** *** ** -- *G*	
	ITS2	414
Macaca arctoides	ATT CTG GC- --G GGC ATG CCT GTT CGA GCG TGA --- --- TTT GAA CCC TCG AGC TCC OCT CTT TTT GGG	
M. mulatta	*** **	
M. nemestrina	*** **	
Trachypithecus cristatus	*** **	
F. obscurus	*** **	
F. phayrei	*** **	
Pan paniscus	CC* TA* **T AA* **T *C* T** *CC T*G *T* *** CCG ATC *G* TCG *** A*T GA* CA* TAA **C CC* *A*	
		483
Macaca arctoides	AGA GCC CCG CGT TGG GGA CCC GGC GCT AAC ACC GGC GGC CCC GAA ATG GAG TGG CCG CCC GTC CCG GG-	
M. mulatta	*** **	
M. nemestrina	*** **	
Trachypithecus cristatus	*** **	
F. obscurus	*** **	
F. phayrei	*** **	
Pan paniscus	G*G AA* ATA G*C *** T-- TT* **G **G G*T *AT *G* CT* *TC TCC C*A *C* GT* *** TTG **G *AA AAA	
		552
Macaca arctoides	CGA CCT CTG CGT AGT AAT ATC CAC TCG CAC CCG GAC CCG -GG GGC GGC CAC GCC G-T TAA ACA CCC CAC	
M. mulatta	*** **	
M. nemestrina	*** **	
Trachypithecus cristatus	*** **	
F. obscurus	*** **	
F. phayrei	*** **	
Pan paniscus	T** GA* T*T G*C G*C GGA CGT *G* G-A **T *** TGG TT* TAA AAA *A* *CT CTT *TC *TG T*G TG* **G	
		621
Macaca arctoides	CTT CCG AAT GTT GAC CTC GAA TCA GGT AGG -AA TAC CCG CTG AAC TTA AGC ATA TCA ATA AGC GGA GGA	
M. mulatta	*** **	
M. nemestrina	*** **	
Trachypithecus cristatus	*** **	
F. obscurus	*** **	
F. phayrei	*** **	
Pan paniscus	TGC *** TCA CC* A*G *GA *CT CA* **A CCC A*G GTG **A *GT *TT *G T** GCT *G G-- --- --- ---	
	634	
Macaca arctoides	GAA -GA AAC TTA C	
M. mulatta	*** **	
M. nemestrina	*** A** **	
Trachypithecus cristatus	*** A** **	
F. obscurus	*** **	
F. phayrei	*** **	
Pan paniscus	--- --- --- ---	

Fig. 2: ITS sequence alignments from six cercopithecoid species and *Pan paniscus* as an outgroup. Dots (•) indicate identical nucleotides, gaps (-) mean deletions of the bases

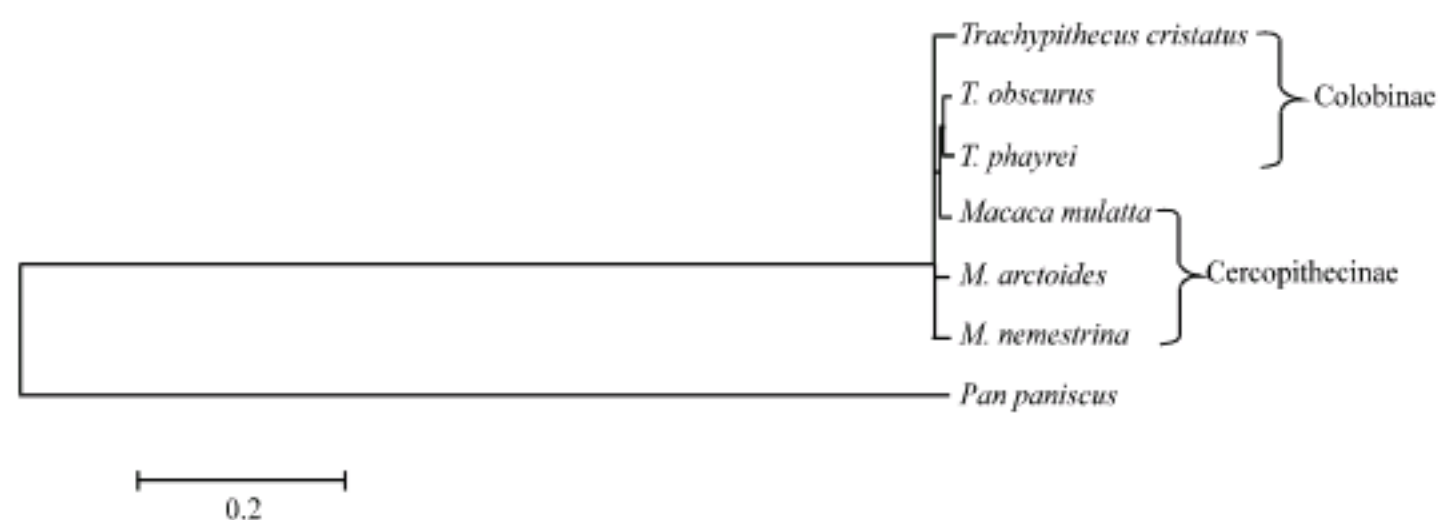


Fig. 3: A dendrogram of six cercopithecoid species and *Pan paniscus* as an outgroup constructed from sequence comparisons of the ITS regions using the Neighbor Joining Method (NJ)

Table 2: Genetic distance values based on ITS sequences of six cercopithecoid species and *Pan paniscus*

Sample study species	<i>M. arctoides</i>	<i>M. mulatta</i>	<i>M. nemestrina</i>	<i>T. cristatus</i>	<i>T. obscurus</i>	<i>T. phayrei</i>	<i>Pan paniscus</i>
<i>Macaca arctoides</i>	0.000						
<i>M. mulatta</i>	0.005	0.000					
<i>M. nemestrina</i>	0.004	0.007	0.000				
<i>Trachypithecus cristatus</i>	0.004	0.005	0.002	0.000			
<i>T. obscurus</i>	0.004	0.005	0.005	0.005	0.000		
<i>T. phayrei</i>	0.013	0.011	0.011	0.009	0.011	0.000	
<i>Pan paniscus</i>	0.804	0.806	0.803	0.804	0.798	0.807	0.000

Based on the morphology, the Cercopithecidae are divided into two extant subfamilies, Colobinae (the leaf monkeys) and Cercopithecinae (the cheek-pouch monkeys). They differ in morphological characters as described below. The colobine monkeys are distinguished by their specialized stomachs, which have been modified for a highly folivorous, or leaf-eating, diet. Along with this specialized diet, these leaf-eating monkeys have ruminant stomachs and a dental morphology suited for shearing leaves. Colobines are almost exclusively arboreal and possess a reduced or absent pollux and long, nonprehensile tails (Fleagle, 1999). In contrast, cercopithecines have well-developed thumbs and tails of varying lengths. These cheek-pouched monkeys are omnivorous, concentrating on fruit, but are also known to hunt on occasion (Swindler, 1998). They possess sacs in the buccal region of the oral cavity which are used to store food. Moreover, from the cytogenetic data, the diploid chromosome number of colobine species is 44 (Chaveerach *et al.*, 2007) whereas the macaques have 42 as the diploid chromosome number (Tanee *et al.*, 2006). Therefore, although the morphology and cytogenetics show high variability, the ITS region is highly conserved in these species. However, from the comparison between cercopithecids and the *Pan paniscus* (Hominoidea), the ITS1 and ITS2 regions show highly variable sequences.

From all of these results, the cercopithecoid species have lower evolution than other species. This suggests that the rate of evolution may be different in different species groups. In addition, the low ITS variation suggested that the cercopithecoid species may be at risk of extinction. Currently, the loss of specific, genetic and

ecological diversities caused by human disturbance is a serious problem in Thailand. Forest destruction negatively affects wild animals such as cercopithecids. It reduces habitats for wild animals and causes population fragmentation. These isolated populations rapidly lose genetic heterogeneity and become vulnerable to environmental change and risk extinction. One more factor leading to genetic loss is the ability of interspecific intercross caused by close evolutionary relationship (Brown *et al.*, 1986). The genetics of a species might be lost. In order to resolve the genetic relationships among cercopithecoid species properly, it is necessary to study a larger sample, as well as to consider comparative behavioral studies.

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