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Phylogenetic relationships of Malaysian monkeys, Cercopithecidae, based on mitochondrial cytochrome c sequences

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ABSTRACT. Mitochondrial DNA cytochrome c oxidase II (COII) gene sequences of Malaysian Cercopithecidae were examined to ascertain their phylogenetic relationships. Colobinae were represented by the genera *Presbytis*, *Trachypithecus* and *Nasalis*, while the genus *Macaca* represented Cercopithecinae. DNA amplification and sequencing of the COII gene was performed on 16 samples. *Symphalangus syndactylus* (Hylobatidae) was used as the outgroup. Data were analyzed using both character (maximum parsimony) and distance (neighbor-joining) methods. Tree topologies indicated that Colobinae and Cercopithecinae have their own distinct monophyletic clade. This result was well supported by bootstrap values and genetic distances derived from the Kimura-2-parameter algorithm. Separation of *Macaca nemestrina* from *M. fascicularis* was also well supported by bootstrap values. In addition,

tree topologies indicate a good resolution of the Colobinae phylogenetic relationships at the intergeneric level, but with low bootstrap support. The position of *Nasalis* remained problematic in both trees. Overall, COII is a good gene candidate for portraying the phylogenetic relationships of Malaysian primates at the inter- and intra-subfamily levels.

Key words: Malaysian primates; COII gene; Molecular phylogeny; Cercopithecinae; Colobinae

INTRODUCTION

Malaysian cercopithecids consist of two groups, the cercopithecine (subfamily Cercopithecinae) and colobine (subfamily Colobinae) (Md-Zain, 2001). Members of these subfamilies live sympatrically but are separated by their life styles, either arboreal or terrestrial, and their diets (Marsh and Wilson, 1981). *Macaca*, the only genus in cercopithecine representing the omnivorous group, includes three species: *Macaca fascicularis*, *M. nemestrina* and *M. arctoides*, whereas the wholly Malaysian vegetarian colobines, known as langurs or leaf monkeys, are represented by three genera: *Presbytis*, *Trachypithecus* and *Nasalis* (Oates et al., 1994). *Trachypithecus* is represented by two species (*T. obscurus* and *T. cristatus*) while *Nasalis* by a single species, *Nasalis larvatus* (Md-Zain et al., 2008). The number of species in the genus *Presbytis* varies according to primatologist lists (Groves, 2001; Brandon-Jones et al., 2004; Md-Zain et al., 2008). *P. hosei*, *P. rubicunda*, and *P. frontata* remain as the valid species. However, various classifications have been made on the *P. melalophos* groups involving *femoralis siamensis*, *robinsoni*, *chrysomelas*, and *cruciger*. Many primatologists still favor the retention of *P. melalophos* as a single polytypic species with numerous subspecies (Oates et al., 1994; Md-Zain, 2001). However, some subspecies have been assigned at the species level (Groves, 2001; Brandon-Jones et al., 2004). The differences in the classification of these species may cause difficulty in conservation efforts.

Cytochrome c oxidase subunit II (COII) is the third largest component of the cytochrome c oxidase complex. The evolution rates of the COII gene in both nucleotide and amino acid sequences are believed to be significantly higher than for COI and COIII (Ramharack and Deeley, 1987). Analysis of sequence data based on the COII gene has been successfully conducted with mammals (Adkins et al., 1996; Shevchuk and Allard, 2001). However, in many phylogenetic relationship studies, other genes of mitochondrial DNA are still favored as gene candidates (Shahrom et al., 2005; Khan et al., 2008; Lim et al., 2010; Md-Zain et al., 2010). In primate evolutionary studies, several COII gene data analyses have focused on hominoids and the New World Monkeys (Ruvolo et al., 1991, 1993). For New World Monkeys, the sequence of the COII gene has been studied on Platyrrhini including both Atelidae and Cebidae families (Ascunce et al., 2002): owl monkeys, genus *Aotus* (Ashley and Vaughn, 1995); spider monkeys, genus *Ateles* (Collins and Dubach, 2000) and marmoset species group (Sena et al., 2002).

For Old World Monkeys, molecular phylogeny studies have been focusing mainly on Africa. For example, the phylogenetic studies of the tribe Papionini, composed of baboons, mandrills, drills, and mangabeys were conducted on the African continent (Ruvolo et al., 1991; Disotell et al., 1992; Harris, 2000). Phylogenetic inference using COII gene in the Asian region remains less pronounced than in the African region. These studies include the macaques

(Ramharack and Deeley, 1987) and leaf monkeys (Ernie-Muneerah et al., 2005). These various molecular studies indicate the potential of the COII gene as a gene candidate in studying molecular evolution in primates.

Conservation efforts are compulsory, but conservation management plans are difficult to set up if the systematic relationships among species remain unclear. Previously, variable primate classifications have been proposed especially for *Presbytis* and *Trachypithecus* (Oates et al., 1994; Brandon-Jones et al., 2004; Md-Zain et al., 2008). In addition, both colobines and cercopithecines have been distinguished by certain features (Oates et al., 1994; Groves, 2001). Unfortunately, these classifications are based mostly on morphological, behavioral, ecological, and biogeographical information, and not genetic data (Brandon-Jones et al., 2004). The application of phylogenetics is important to improve the systematic classification of Malaysian primates. Constructing the phylogenetic tree that shows the relationship between Malaysian primate species, particularly Cercopithecinae and Colobinae subfamilies, is thus vital.

MATERIAL AND METHODS

Sample and genomic DNA extraction

Tissue samples of different Malaysian cercopithecoids used in this study are listed in Table 1. Genetic samples were obtained from several institutions such as the Department of Wildlife and National Parks, Sabah Parks, and the Sarawak Forestry Department. DNA was extracted from the tissue samples using the GENE ALL Tissue and Tissue (Plus!) SV mini-extraction kit.

Table 1. Samples of Malaysian primates used in this study.

No.	Code	Species	Locality
1	BM29	*P. m. robinsoni*	Selama, Perak
2	BM40	*P. m. femoralis*	Mersing, Johor
3	BM67	*P. hosei*	Tawau, Sabah
4	BM70	*P. hosei*	Danum Valley, Sabah
5	BM08	*T. obscurus*	Sik, Kedah
6	BM09	*T. obscurus*	Changloon, Kedah
8	BM19	*T. obscurus*	Tupah, Kedah
9	AA102	*N. larvatus*	Sarawak
10	SP156	*M. nemestrina*	Kinabalu Park, Sabah
11	SP206	*M. nemestrina*	Kinabalu Park, Sabah
12	MF01	*M. fascicularis*	Pulau Sapi, Sabah
13	MF03	*M. fascicularis*	Pulau Sapi, Sabah
14	MF05	*M. fascicularis*	Pulau Sapi, Sabah
15	MF06	*M. fascicularis*	Pulau Sapi, Sabah
16	MF07	*M. fascicularis*	Pulau Sapi, Sabah

Polymerase chain reaction

COII of the mtDNA gene was amplified by polymerase chain reaction (PCR) using the Perkin Elmer GeneAmp® PCR System 2400. Amplification was performed using 50 µL total volume per reaction with 50 ng (5 µL) DNA template, 0.2 pmol (0.5 µL) primer, 3.0 mM (6.0 µL) $MgCl_2$, 0.2 mM (1.0 µL) dNTP mix, 1X (6.0 µL) PCR buffer, and 2.5 U (0.5 µL) *Taq*

Polymerase. Two oligonucleotide primers L7553 (5'-AACCATTTCATAACTTTGTCAA-3') and H8320 (5'-CTCTTAATCTTTAACTTAAAAG-3') based on Adkins and Honeycutt (1994) were used. The temperature profile for 35 amplification cycles was pre-denaturation (94.0°C for 3 min), denaturation (94°C for 60 s), annealing (51-55°C for 60 s), extension (72.0°C for 70 s), and post-extension (72.0°C for 9 min). Amplified products were purified using the QIAquick gel purification kit protocol (QIAGEN) and visualized using 1.5% agarose gel electrophoresis. The purified DNA was then stored at -20°C and sent for DNA sequencing at First Base (First BASE Laboratories Sdn Bhd, Malaysia).

Data analysis

DNA sequences were aligned using CLUSTALW. Sequence data were analyzed using both character and distance methods in PAUP version 4.0 (Swofford, 2002). *Symphalangus syndactylus* (M580071) from Genbank was employed as the outgroup. Maximum parsimony (MP) and neighbor joining (NJ) analysis were conducted. Trees were obtained by heuristic or branch and bound searches. The data were also subjected to bootstrap analysis with 2000 replications in order to estimate the strength of support for each clade. Neighbor-joining analysis based on the distance measure of Kimura-2-parameter was obtained, as were branch lengths for the most parsimonious MP tree using the "describe tree" option of PAUP. Homoplasy was quantified using the consistency index (CI) and the homoplasy index (HI).

RESULTS

PCR products

For macaque and leaf monkey samples, the estimated DNA fragment size of the amplified COII gene was around 850 bp. Sample BM29 had the brightest DNA band indicating the highest concentration of PCR product. Other samples produced relatively faint DNA bands or lower concentrations of PCR products (Figure 1). Leaf monkey samples were succesfully amplified at 55°C while macaques needed annealing temperatures ranging from 51° to 55°C.

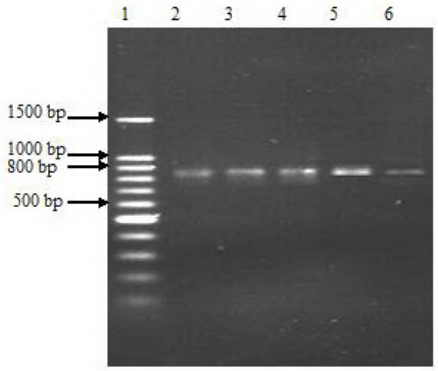

Figure 1. Amplification products of the COII gene for leaf monkeys. *Lane 1* = 100-bp ladder; *lane 2* = BM25; *lane 3* = BM30; *lane 4* = BM31; *lane 5* = BM29; *lane 6* = BM1.

Phylogenetic inference

MP analysis was conducted using a heuristic search in PAUP version 4.0 (Swofford, 2002). Table 2 shows variations along the sequences across taxa. Findings showed that 61.3% of the total characters examined were constant characters, 9.57% characters were parsimony uninformative and 29.13% characters were parsimony informative. Our MP analysis yielded a single bootstrap tree (length = 123, CI = 0.8049, HI = 0.1095) (Figure 2). Two distinct monophyletic clades are clearly shown on the phylogeny tree of Malaysian Cercopithecidae. These Cercopithecinae and Colobinae clades were fully supported by high bootstrap values, 90 and 100%, respectively. Within Cercopithecinae, *Macaca nemestrina* (SP156 and SP206) formed a clade with 100% bootstrap support. *M. fascicularis*, consisting of Borneo samples (MF01, MF05, MF06, MF07, and SP03), also formed a clade, with 99% bootstrap value support. In Colobinae, the node was resolved where leaf monkey samples clustered according to genus. *P. m. robinsoni* (BM29), *P. m. femoralis* (BM40) and *P. hosei* (BM67 and BM70) formed the *Presbytis* clade while all *T. obscurus* samples (BM08, BM09, and BM19) clustered in the *Trachypithecus* clade. *Nasalis*, which was only represented by a single sample (AA02), joined together in the Colobinae clade with its phylogenetic position remaining unclear. Although the genus node was resolved in the Colobinae clade, the supports on each clade were not high, 57 and 58% bootstrap values in *Presbytis* and *Trachypithecus*, respectively.

Table 2. Summary of variations along the sequences across taxa.

Locus	Nuclear gene		Mitochondrial gene			
	SRY[a]	IRBP[a]	ND3-ND4[a]	Cyt *b*[b]	12S rRNA[b]	COII[c]
Total characters examined	3060	1613	2080	388	371	230
Constant characters	2767	1518	1276	225	283	141
Parsimony-uninformative characters	111	33	154	6	7	22
Parsimony-informative characters	180	62	650	157	81	67
% Parsimony-informative characters	6.0	3.8	31.5	40.5	21.8	29.1
Tree length	334	109	1773	79	133	123

[a]Md-Zain, 2001; Md-Zain et al., 2008. [b]Vun, 2007. [c]This study.

The NJ tree is concordant with the MP tree as it shares a relatively similar tree topology of phylogenetic relationships (Figure 3). Cercopithecinae and Colobinae subfamilies formed their distinct monophyletic clades at 87 and 100% of bootstrap support, respectively. Both *M. nemestrina* and *M. fascicularis* were highly defined as distinct species with a 100% bootstrap value. Although the position of *Nasalis* in the NJ tree is problematic, the leaf monkey clade was almost resolved by NJ analyses by medium bootstrap value support.

An average genetic distance was generated using the Kimura-2-parameter algorithm. The genetic distance between and among genera is summarized in Table 3. The intra-generic averages of genetic distance for *Presbytis*, *Trachypithecus* and *Macaca* are 1.40, 1.33, and 8.76%, respectively. In the leaf monkeys, the average genetic distances between *Presbytis* and *Trachypithecus*; *Presbytis* and *Nasalis*, and *Trachypithecus* and *Nasalis* are 3.42, 5.36, and 5.47%, respectively. This result indicates that the intra-generic genetic distance of each genus is less than that in the inter-generic level. The genetic distances between Colobinae and Cercopithecinae are quite high: *Presbytis* (27.79%), *Trachypithecus* (27.70%) and *Nasalis* (31.87%). In macaques, the average intra-species genetic distances in *M. fascicularis* and *M. nemestrina* are 1.45 and 0.44%, respectively; while the average genetic distance between *M.*

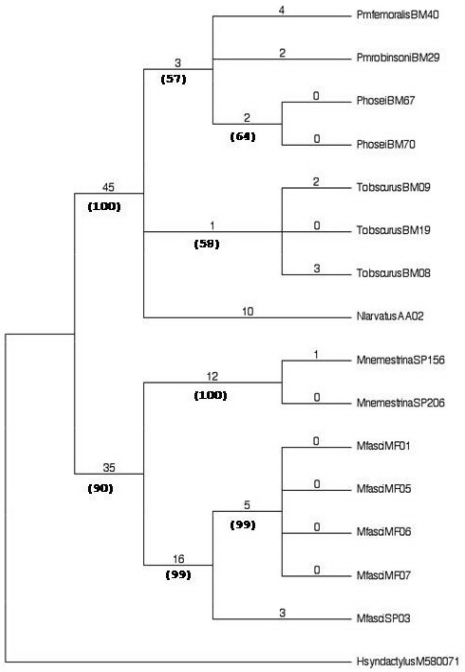

Figure 2. Maximum parsimony tree of partial COII mtDNA gene sequences. Numbers under branches (in parentheses) are the percentage of bootstrap values at 2000 replicates while numbers above branches are the branch lengths.

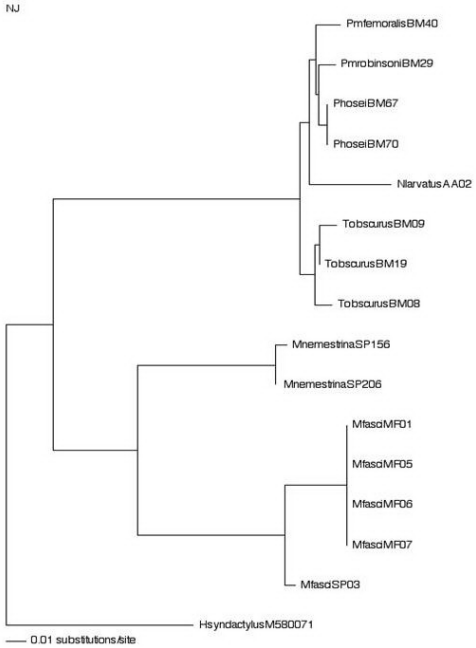

Figure 3. Neighbor-joining phylogram of partial COII mtDNA sequences.

Table 3. Average genetic distance between genera.

	Presbytis	*Trachypithecus*	*Nasalis*	*Macaca*
Presbytis	1.404	-		
Trachypithecus	3.421	1.326	-	
Nasalis	5.357	5.468	-	-
Macaca	27.785	27.704	31.869	8.755

Table 4. Average genetic distance among and between Malaysian macaque species using the Kimura-2-parameter.

	Macaca fascicularis	*Macaca nemestrina*
Macaca fascicularis	1.448	-
Macaca nemestrina	16.893	0.44

fascicularis and *M. nemestrina* is 16.90% (Table 4).

DISCUSSION

In order to obtain the best PCR conditions, optimization of annealing temperatures was carried out in the range of 51-55°C for macaque samples. All the annealing temperature trials produced the same results; the best based on band brightness was recorded at 51°C. This finding supports a previous study (Table 5) on the same gene and same subfamily Cercopithecinae in the tribe Papionini (Disotell et al., 1992), whereas for the PCR conditions of gorilla, siamang and macaques, as described by Ruvolo et al. (1991), the annealing process required 50-57°C for 1 min. A study by Ernie-Muneerah et al. (2005) on leaf monkeys required an annealing temperature of 54-55°C (1 min). This is in line with the range of annealing temperatures used for the leaf monkeys in this study. Md-Zain et al. (2004) stated that the right mixture of PCR components is important to obtain positive results with good and clear bands. The right volume of DNA template used, annealing temperature, extension time, and cycle numbers are all equally important.

Table 5. Comparison of annealing temperatures for the COII gene within the order Primates.

Primates	Annealing temperature	References
Old World Monkey Tribe Papionini	51°-55°C 1 min	Disotell et al., 1992
Gorilla, Siamang and Macaques	50°-57°C 1 min	Ruvolo et al., 1991
Apes	50°-57°C 1 min	Ruvolo et al., 1993
Malaysian leaf monkeys	54°-55°C 1 min	Ernie et al., 2005
Malaysian Cercopithecidae	51°-55°C 1 min	This study

The percentage of informative characters in the COII gene is compared with several loci of mtDNA and nuclear genes from previous studies (Table 2). The differences in percentage between mtDNA and the nuclear genes signify that the mtDNA region has evolved more rapidly compared to the nuclear regions of cercopithecids. The slower evolution of the nuclear regions is evident in the lower percentage of informative characters they possess. The ability of mtDNA to resolve phylogenetic relationships among the cercopithecids (Karanth et al., 2008; Osterholz et al., 2008; Roos et al., 2008) and other primates has been discussed by several researchers (Shahrom et al., 2005; Lim et al., 2010; Md-Zain et al., 2010). Thus, based on the reasonable percentage of informative characters, COII can also be regarded as a good locus candidate to be used in portraying the phylogenetic relationships

of Malaysian Cercopithecidae.

Partial COII mtDNA gene sequences used in this study seem to be effective in determining the phylogenetic relationships between the two subfamilies. This is because both MP and NJ tree topology showed very clear distinction between Cercopithecinae and Colobinae as they formed their own highly supported monophyletic clade. Furthermore, the average genetic distance is proof of the distinction of these subfamilies as their values fall into the range of 27 to 32%. This result supports previous studies on mitochondrial and nuclear genes (Page et al., 1999; Chatterjee et al., 2009). In macaques, the node is solved in MP and NJ tree as two highly supported monophyletic clades are formed, representing the species *M. fascicularis* and *M. nemestrina*. High genetic distance also supports their distinction by which both species come from different macaque groups, specifically *M. fascicularis* from the *fascicularis* group and *M. nemestrina* from the *silenus-sylvanus* group (Groves, 2001). Our results are congruent with other previous results based on DNA data (Chatterjee et al., 2009; Li et al., 2009). *Macaca*, the single genus of Malaysian Cercopithecinae, is clearly divided into two distinct groups representing the macaque species, *M. nemestrina* and *M. fascicularis*. This result is supported by high bootstrap values in both MP and NJ trees and by the average genetic distance.

The distinction of three genera of leaf monkeys (Colobinae), *Presbytis*, *Trachypithecus*, and *Nasalis*, is not as clear as the clade resolved by partial COII mtDNA gene sequences and is not highly supported by bootstrap values in MP and NJ tree. At the inter-generic level of leaf monkeys (Colobinae), the partial COII gene nucleotide sequences used resolved the node joining *Presbytis*, *Trachypithecus* and *Nasalis* but has low bootstrap support. In this case, *Presbytis* and *Trachypithecus* formed their own monophyletic clade distinct from each other but with low bootstrap values. Many morphologists and ecologists do not agree on a common delimitation of species within the *Presbytis* group (Groves, 1989; Brandon-Jones, 1995). Formerly, *Trachypithecus* was grouped into *Presbytis* (Peng et al., 1988; Li, 1993). However, the separation of *Trachypithecus* from *Presbytis* has been adopted by several other researchers (Oates et al., 1994; Brandon-Jones et al., 2004) based on ecological and morphological data. These findings are further validated by Md-Zain et al. (2008) and data based on mitochondrial DNA sequences in this study. Meanwhile the position of *Nasalis* is problematic for both MP and NJ trees. This problem is due to the limited number of *Nasalis* samples obtained.

The separation of the two subfamilies of Malaysian Cercopithecidae (Cercopithecinae and Colobinae) is supported by MP and NJ analysis as well as with the high average percentage of genetic distance among them based on the Kimura-2-parameter distance matrix method. Overall, partial COII gene nucleotide sequences utilized in this study are effective in clarifying the phylogenetic relationships between and within the Cercopithecinae and Colobinae subfamilies. For further studies, more samples should be used especially for the leaf monkeys. This is important to generate more reliable results on the molecular phylogeny of Malaysian Cercopithecidae.

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