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Phylogenetic relationships of leaf monkeys (*Presbytis*; Colobinae) based on cytochrome *b* and 12S rRNA genes

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ABSTRACT. Little is known about the classification and phylogenetic relationships of the leaf monkeys (*Presbytis*). We analyzed mitochondrial DNA sequences of cytochrome *b* (Cyt *b*) and 12S rRNA to determine the phylogenetic relationships of the genus *Presbytis*. Gene fragments of 388 and 371 bp of Cyt *b* and 12S rRNA, respectively, were sequenced from samples of *Presbytis melalophos* (subspecies *femoralis*, *siamensis*, *robinsoni*, and *chrysomelas*), *P. rubicunda* and *P. hosei*. The genus *Trachypithecus* (Cercopithecidae) was used as an outgroup. The Cyt *b* NJ and MP phylogeny trees showed *P. m. chrysomelas* to be the most primitive, followed by *P. hosei*, whereas 12S rRNA tree topology only indicated that these two species have close relationships with the other members of the genus. In our analysis, *chrysomelas*, previously classified as a subspecies of *P. melalophos*, was not included in either the *P. m. femoralis* clade or the *P. m. siamensis* clade. Whether or not there should be a separation at the species level remains to be clarified. The tree topologies also showed that *P. m. siamensis* is paraphyletic with *P. m. robinsoni*, and *P. m. femoralis* with *P. rubicunda*, in two different

clades. Cyt *b* and 12S rRNA are good gene candidates for the study of phylogenetic relationships at the species level. However, the systematic relationships of some subspecies in this genus remain unclear.

Key words: *Presbytis*; Cytochrome *b* gene; 12S rRNA gene; Colobinae; Leaf monkeys

INTRODUCTION

Presbytis, the genus of the leaf monkey, subfamily Colobinae, superfamily Cercopithecidae, is the Asian langur confined to the Sundaland (Figure 1) such as the Malay Peninsula, Thailand, Indonesia (Sumatra, Java, Natuna Island, Riau Island, and Mentawai Island) and Borneo (Oates et al., 1994; Meijaard and Groves, 2004; Ampeng and Md-Zain, 2007). Generally, the number of species in *Presbytis* range from four to eleven, depending on the researcher (Md-Zain, 2001). Our research, however, closely follows the classification of Oates et al. (1994) and Md-Zain et al. (2008), who classified *Presbytis* into seven species, namely *P. melalophos*, *P. rubicunda*, *P. frontata*, *P. potenziani*, *P. thomasi*, *P. hosei*, and *P. comata*. To date, most of the phylogenetic relationships of the proposed *Presbytis* species were based on morphological traits such as coat coloration, head pattern, vocals of the adult male, behavioral studies such as social structure of the group, and ecological studies (Napier, 1985; Peng et al., 1993; Oates et al., 1994). Brandon-Jones (1996a), using coat coloration, suggested that the most primitive form of *Presbytis* is *P. potenziani* found on Mentawai Island, Indonesia, followed by the intermediate forms (*P. comata*, *P. thomasi* and *P. hosei*) and the derived forms (*P. melalophos*, *P. femoralis*, *P. frontata*, and *P. rubicunda*). According to him, the most primitive form has a dark coloration, intermediate with grey coloration, and the derived forms have the greatest color variation, ranging from red to brown. For the derived forms, a special affinity between the *P. melalophos* and *P. rubicunda* was proposed by Brandon-Jones (1996b) in view of their behavioral similarity. The evolutionary relationships of the derived forms also showed that *P. rubicunda* was a derivation of *P. siamensis* (based on head pattern). Based on the shared external cephalic structure of *P. frontata* and *P. comata sabana*, these two species were also thought to have a close relationship (Brandon-Jones, 1996b).

Medway (1970), on the other hand, taking into consideration the entry point of *Presbytis*, which is from mainland Asia, discovered that the Mentawai Island fauna had similar characteristics as those of Sumatra from which it was derived. Thus, it was proposed that the *P. potenziani* was the descendent of the first primate that inhabited Sumatra. The Sumatran primate later separated and underwent speciation due to geographical movement. Md-Zain (2001) and Md-Zain et al. (2008), adopting various molecular techniques, including maternal (mitochondrial ND3, ND4L, ND4 and tRNAs), paternal (TSPY and SRY) and biparental autosomal (IRBP intron 3) markers in their study on several *Presbytis* species found that the *P. comata*, *P. thomasi* and *P. hosei* are respectively independent, and that *P. rubicunda* and *P. melalophos* are not closely related, with the *P. rubicunda* grouped into a clade containing *P. comata* and *P. thomasi*. Therefore, it was concluded that *P. rubicunda* has a close relationship with *P. comata* and *P. thomasi*. Contrary to the theoretical ancestry put forward by Brandon-Jones, the Borneo *Presbytis* also underwent cladogenic splits that separated the *P. hosei* from all other *Presbytis*, and the latter underwent a further division into the *melalophos* group and *comata-thomasi-rubicunda* group (Md-Zain, 2001; Meijaard and Groves, 2004).

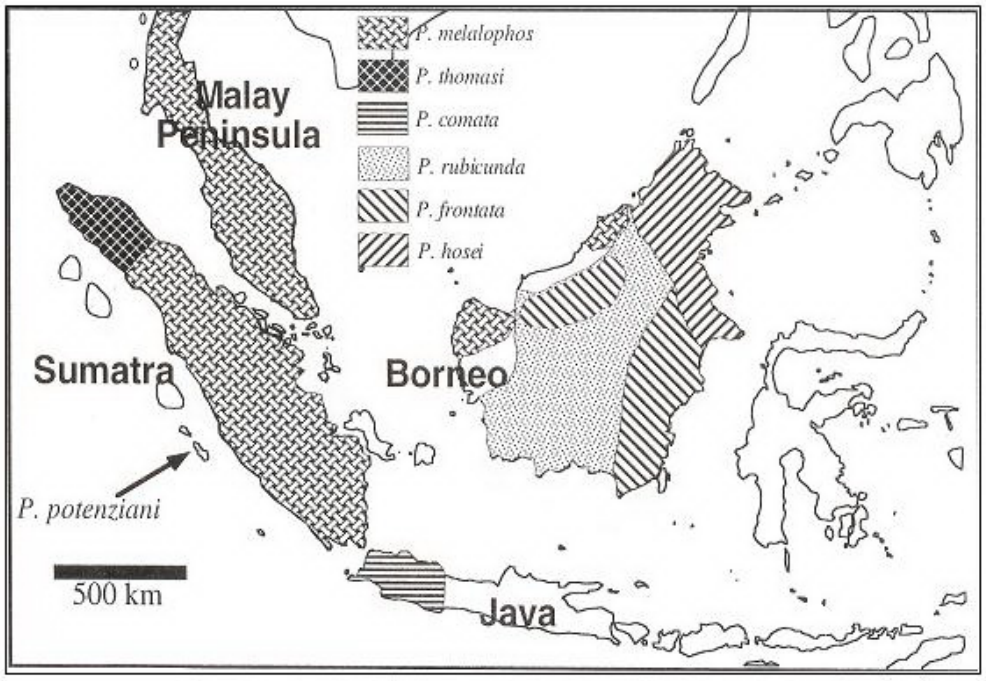

Figure 1. The geographical distribution of the seven *Presbytis* species according to Oates et al. (1994) and adapted from Md-Zain et al. (2008).

Mitochondrial DNA (mtDNA) has been widely used to infer phylogenetic relationships of closely related species of both vertebrates and invertebrates due to its unique characteristics. It has a high evolutionary rate as compared to the nuclear genome, and is maternally inherited, resulting in the lack or absence of DNA recombination, coupled with its relatively small size that give it an advantage for manipulation in the laboratory (Melnick and Hoelzer, 1993). Consequently, a large comparative database of mitochondrial sequences is easily available and accessible nowadays (Shahrom et al., 2005; Sterner et al., 2006; Karanth, 2008; Khan et al., 2008; Lim et al., 2010; Md-Zain et al., 2010a,b). In the present study, mtDNA cytochrome *b* (Cyt *b*) and 12S rRNA were sequenced and analyzed to obtain a full understanding as well as to clarify the controversies associated with *Presbytis* classification and phylogeny.

MATERIAL AND METHODS

Samples

Thirty-seven genetic samples of *P. melalophos* (subspecies *femoralis*, *siamensis*, *robinsoni*, and *chrysomelas*), *P. rubicunda*, *P. hosei*, and the outgroup (*Trachypithecus obscurus*, *T. cristatus*, and *T. auratus*) were used (Table 1).

Table 1. Samples of species used in this study.

Sample code	Species	Location
PmsBM 21	*P. m. siamensis*	Ulu Besut, Terengganu, Malaysia
PmsBM 22	*P. m. siamensis*	Ulu Besut, Terengganu, Malaysia
PmsBM 23	*P. m. siamensis*	Ulu Besut, Terengganu, Malaysia
PmsBM 24	*P. m. siamensis*	Ulu Besut, Terengganu, Malaysia
PmsBM 25	*P. m. siamensis*	Kuala Krai, Kelantan, Malaysia
PmsBM 26	*P. m. siamensis*	Kuala Krai, Kelantan, Malaysia
PmrBM 27	*P. m. robinsoni*	Kuala Kangsar, Perak, Malaysia
PmrBM 28	*P. m. robinsoni*	Kuala Kangsar, Perak, Malaysia
PmrBM 31	*P. m. robinsoni*	Kuala Kangsar, Perak, Malaysia
PmrBM 30	*P. m. robinsoni*	Selama, Perak, Malaysia
PmrBM 33	*P. m. robinsoni*	Selama, Perak, Malaysia
PmfBM 35	*P. m. femoralis*	Kluang, Johor, Malaysia
PmfBM 36	*P. m. femoralis*	Kluang, Johor, Malaysia
PmfBM 37	*P. m. femoralis*	Kluang, Johor, Malaysia
PmfBM 38	*P. m. femoralis*	Kluang, Johor, Malaysia
PmfBM 39	*P. m. femoralis*	Mersing, Johor, Malaysia
PmfBM 40	*P. m. femoralis*	Mersing, Johor, Malaysia
PmfBM 41	*P. m. femoralis*	Mersing, Johor, Malaysia
PmfBM 42	*P. m. femoralis*	Mersing, Johor, Malaysia
PmfBM 43	*P. m. femoralis*	Kota Tinggi, Johor, Malaysia
PmfBM 45	*P. m. femoralis*	Kota Tinggi, Johor, Malaysia
PmfBM 46	*P. m. femoralis*	Kota Tinggi, Johor, Malaysia
PmfBM 47	*P. m. femoralis*	Pontian, Johor, Malaysia
PmcrAA 04	*P. m. chrysomelas*	Tanjong Datu, Sarawak, Malaysia
PmcrAA 05	*P. m. chrysomelas*	Tanjong Datu, Sarawak, Malaysia
PrBM 101	*P. rubicunda*	Tawau, Sabah, Malaysia
PrMK 01	*P. rubicunda*	Tawau, Sabah, Malaysia
PhBM 67	*P. hosei*	Tawau, Sabah, Malaysia
PhBM 70	*P. hosei*	Danum Valley, Sabah, Malaysia
TcAA 01	*T. cristatus*	Tanjong Datu, Sarawak, Malaysia
TcBM 01	*T. cristatus*	Kota Kuala Muda, Kedah, Malaysia
TcBM 02	*T. cristatus*	Kota Kuala Muda, Kedah, Malaysia
TaBM 99	*T. auratus*	East Java
ToBM 07	*T. obscurus*	Sik, Kedah, Malaysia
ToBM 09	*T. obscurus*	Changloon, Kedah, Malaysia
ToBM 15	*T. obscurus*	Suluk Paya Dalam, Melaka, Malaysia
ToBM 17	*T. obscurus*	Suluk Paya Dalam, Melaka, Malaysia

DNA extraction and PCR amplification

Genomic DNA from the muscles was extracted using the GeneAll™ kit (General Biosystem (GBS), Inc., Korea). Primer pairs of L14724 and H15149 (Pääbo and Wilson, 1988; Kocher et al., 1989) and L1091 and H1478 (Kocher et al., 1989) were used to amplify the Cyt *b* and 12S rRNA partial gene sequences, respectively. Polymerase chain reaction (PCR) amplification was performed in the GeneAmp PCR system 2400 (Perkin Elmer) and PTC-100™ Programmable Thermal Controller (MJ Research Inc., USA) in a 50-µL reaction volume containing 10X buffer, 1 µL 10 mM dNTPs, 1 µL (10 pmol) of each primer, 2.5 U/µL *Taq* polymerase (Invitrogen) and ddH_2O. Double-stranded PCR amplifications were performed using the following conditions: 30-35 cycles of denaturation at 94°C, annealing at 55°C for Cyt *b* and 53.6°C for 12S rRNA, and extension at 72°C, with final extension at 72°C for 10 min. Cycles of denaturation, annealing and extension were performed for 1 min, respectively. The PCR product was purified using QIAquick PCR Purification and Gel Purification kit (QIAGEN Inc., USA) and sequenced for both strands by First Base Laboratories, Sdn Bhd, Malaysia.

Alignments and phylogenetic analyses

The alignment of the sequences were performed via ClustalX (Thompson et al., 1997) and the resulting alignment was adjusted visually. Prior to the multiple alignment, the sequences were manually edited by visual analysis and then using computer software, Chromas (Technelysium Pty., Ltd.) and/or BioEdit version 7.0.2. The genetic distance was tabulated using the Kimura-2-parameter method (Kimura, 1980) in PAUP 4.0b10 (Swofford, 2002). Phylogenetic reconstructions were obtained via the neighbor joining (NJ) and maximum parsimony (MP) methods in PAUP 4.0b10 packages (Swofford, 2002). Heuristic search with the stepwise addition method was performed for 1000 replicates in MP analysis for Cyt *b* and 12S rRNA data for all the samples. The confidence of the NJ and MP tree was assessed via bootstrap resampling (based on 1000 resampling events) to estimate the stability of the tree topologies. A strict consensus tree was constructed for all arrangements. The transition and transversion ratio and the position of these substitutions were analyzed by MEGA version 3.1 (Kumar et al., 2004). An outgroup of *Trachypithecus* species was used to root the phylogenetic tree in this study.

RESULTS

Amplification products of 388 and 371 bp were obtained for Cyt *b* and 12S rRNA gene fragments, respectively. Sequence analysis indicated that of the 156 variable sites within the Cyt *b* gene, 124 were parsimony informative. Meanwhile, among 54 variable sites within 12S rRNA, 49 were found to be parsimony informative. The nucleotide substitutions for the Cyt *b* and 12S rRNA revealed that both genes were rich in transition, with 38 transitions among 44 substitutions for Cyt *b* and 14 transitions among a total of 22 substitutions in 12S rRNA sequences. The Ti/Tv ratio for Cyt *b* was 9.3 after excluding the outgroups. The Ti/Tv ratio of 12S rRNA, however, was significantly lower than for Cyt *b*, which was 1.6 after the exclusion of the outgroups. An MP tree with tree length of 236 (CI = 0.6398, HI = 0.3602, RI = 0.9101) was chosen as the best tree for Cyt *b*, whereas for 12S rRNA, the best MP tree length was 79 (CI = 0.7848, HI = 0.2152, RI = 0.9433).

Analysis of the pairwise sequences using the Kimura-2-parameter of Cyt *b* for each individual in *Presbytis* showed a low distance value (0.159), indicating that these individuals were closely related to each other. The average sequence divergence within all *P. m. femoralis* was 0.00590, within *P. m. siamensis* 0.02776, within *P. m. robinsoni* 0.04139, within *P. m. chrysomelas* 0.00258, within *P. hosei* 0.01826, and within *P. rubicunda* 0.00258 (Table 2). The average genetic distance of individuals within a species was relatively very low, that is, <4.1%. At the interspecies level, the average pairwise genetic distances revealed relatively distant relationships among the species except for *P. m. siamensis* and *P. m. robinsoni*. Analysis of the distance matrices generated by 12S rRNA also showed an average distance divergence at the intraspecies level that was relatively lower, which is less than 1.6%. 12S rRNA also gave relatively low average sequence divergence as compared to the average sequence divergence of Cyt *b* at the interspecies level (Table 3). Therefore, the results indicated that all the individuals in this genus have very close relationships.

Table 2. Average pairwise genetic distances for Cyt *b* gene between species and subspecies of *Presbytis*.

	femoralis	*siamensis*	*robinsoni*	*chrysomelas*	*hosei*	*rubicunda*
femoralis	0.006					
siamensis	0.111	0.028				
robinsoni	0.100	0.058	0.041			
chrysomelas	0.124	0.133	0.122	0.003		
hosei	0.126	0.147	0.135	0.131	0.018	
rubicunda	0.095	0.134	0.128	0.114	0.146	0.003

Table 3. Average pairwise genetic distances for 12S rRNA gene between species and subspecies of *Presbytis*.

	femoralis	*siamensis*	*robinsoni*	*chrysomelas*	*hosei*	*rubicunda*
femoralis	0.002					
siamensis	0.050	0.011				
robinsoni	0.049	0.025	0.016			
chrysomelas	0.042	0.052	0.053	0.003		
hosei	0.037	0.051	0.045	0.034	0.003	
rubicunda	0.030	0.056	0.054	0.051	0.036	0.000

The phylogenetic relationships among the species and subspecies of *Presbytis* are shown in the NJ trees for Cyt *b* (Figure 2) and 12S rRNA (Figure 3). The MP trees obtained for both Cyt *b* and 12S rRNA (Figures 4 and 5) were very similar to the NJ trees obtained. The Cyt *b* phylogram (Figures 2 and 4) showed that the three species endemic to Peninsular Malaysia (*P. m. femoralis*, *P. m. siamensis* and *P. m. robinsoni*) diverged from the Borneo *P. hosei*. The peninsular species were further separated into two clades, with one clade including the individuals of *P. m. robinsoni* and *P. m. siamensis* (clade I) and the second consisting of *P. m. femoralis*. Our analyses showed that *P. m. robinsoni* diverged earlier than *P. m. siamensis* and that there is an ongoing evolutionary process currently between these two subspecies with the presence of individuals with intermediate characters (PmrBM33, PmrBM30, PmsBM26, and PmsBM25), which showed very high NJ and MP bootstrap values of 98 and 94, respectively. These individuals were found to share 10 intermediate nucleotide characters between the species with four unique nucleotide characters, which were only found in PmrBM33, PmrBM30, PmsBM26, and PmsBM25 individuals. In addition to the 10 intermediate and four unique characters, the two subspecies generally clustered together based on 13 other characters, which were only found within these two subspecies. Similary, the 12s RNA NJ and MP trees also showed a grouping of *robinsoni* and *siamensis* in a single clade (Figures 3 and 5, Clade I). Our analysis of the 12S rRNA nucleotide sequences of *robinsoni-siamensis* revealed that these two species were clustered together based on five unique nucleotides only present in these species and a shared intermediate character. In alignment with the Cyt *b* tree topologies, the 12S rRNA tree topologies also showed an ongoing evolutionary process between the *robinsoni* and *siamensis* with the presence of the same four individuals (PmrBM33, PmrBM30, PmsBM26, and PmsBM25) sharing three unique nucleotide characters just amongst them. The bootstrap values obtained for the intermediate individuals were 85 for NJ and 82 for MP. High NJ and MP bootstrap values obtained for both Cyt *b* and 12S rRNA showed that the ongoing evolution of *robinsoni-siamensis* was quite consistent.

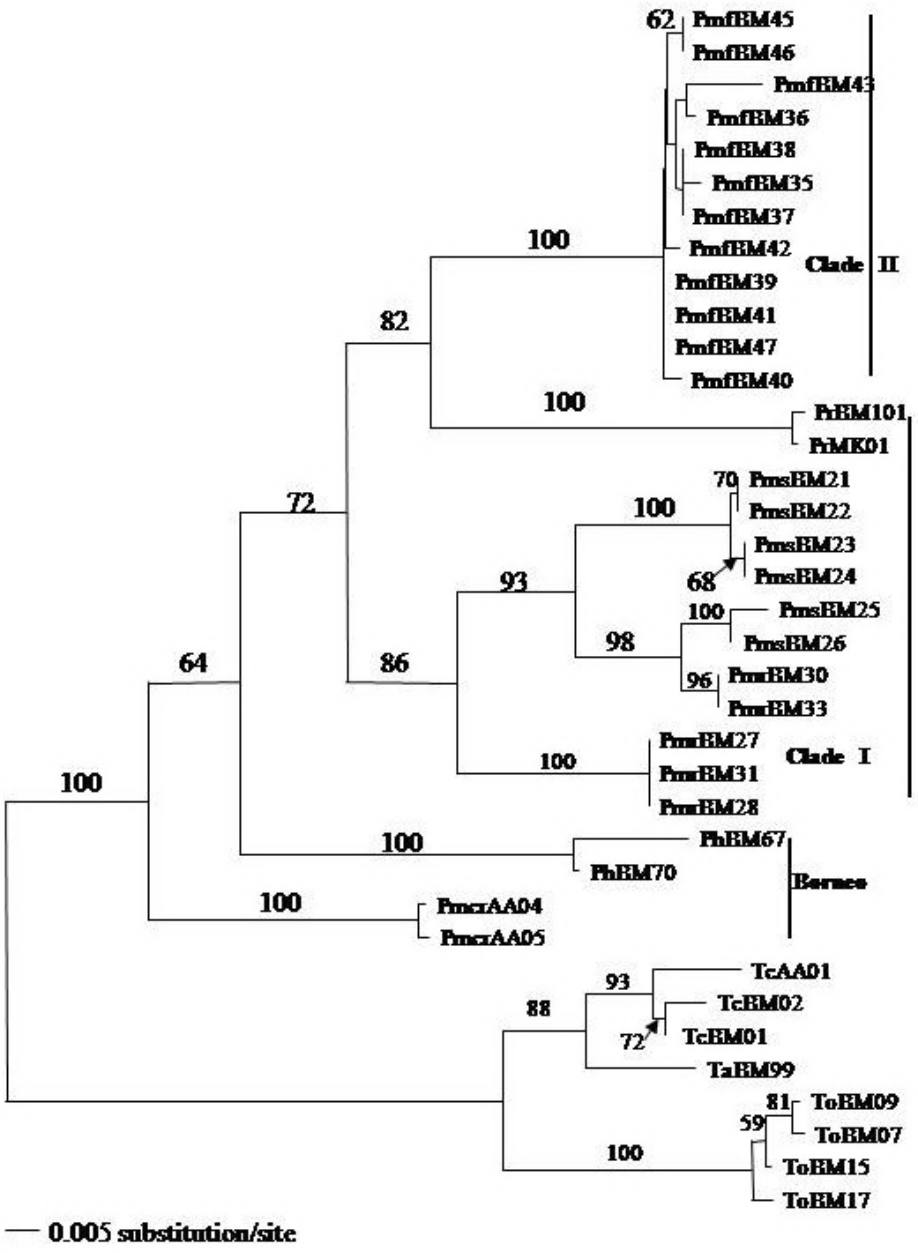

Figure 2. Neighbor joining phylogram of Cyt *b* based on the Kimura-2-parameter. Number above branches is the bootstrap value (1000 replications).

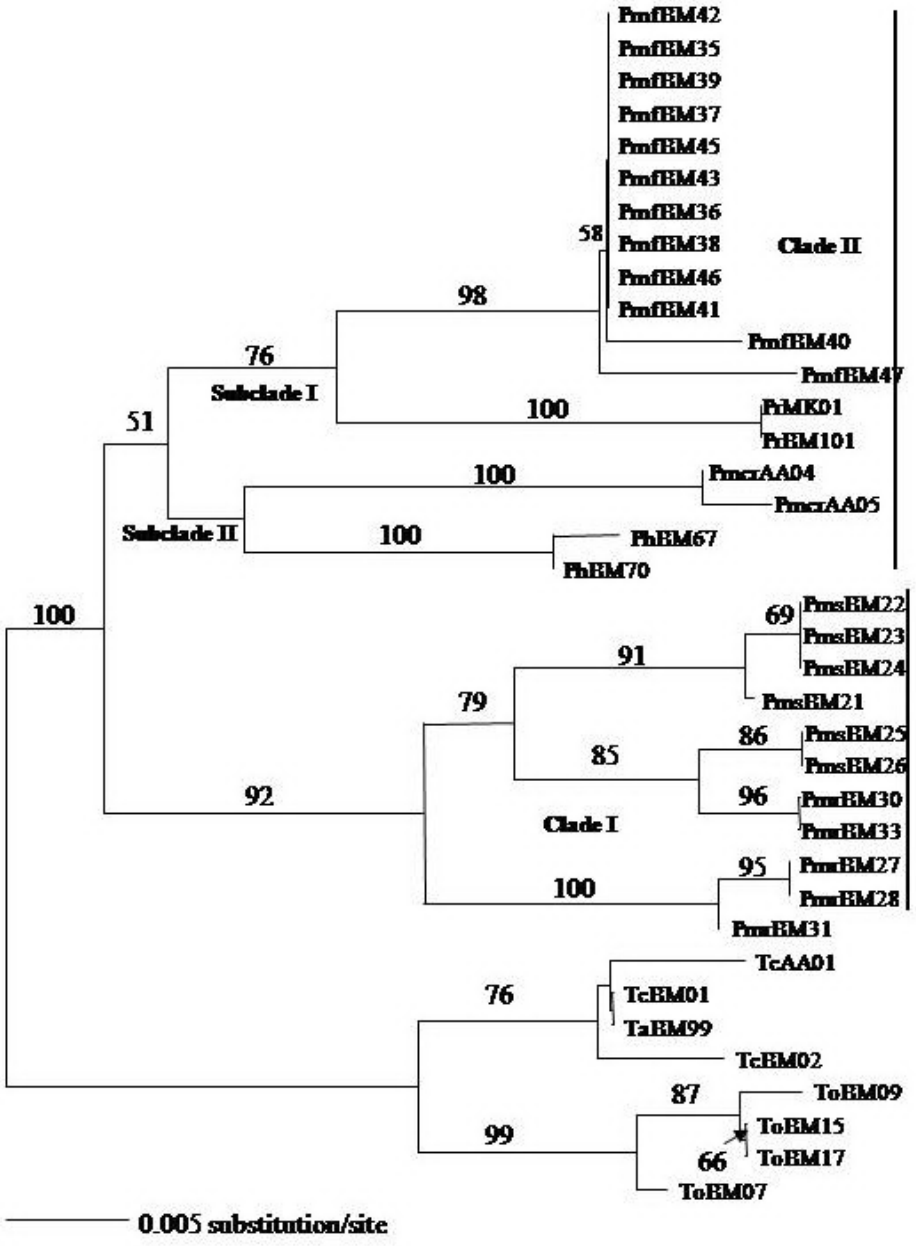

Figure 3. Neighbor joining phylogram of 12S rRNA based on the Kimura-2-parameter. Number above branches is the bootstrap value (1000 replications).

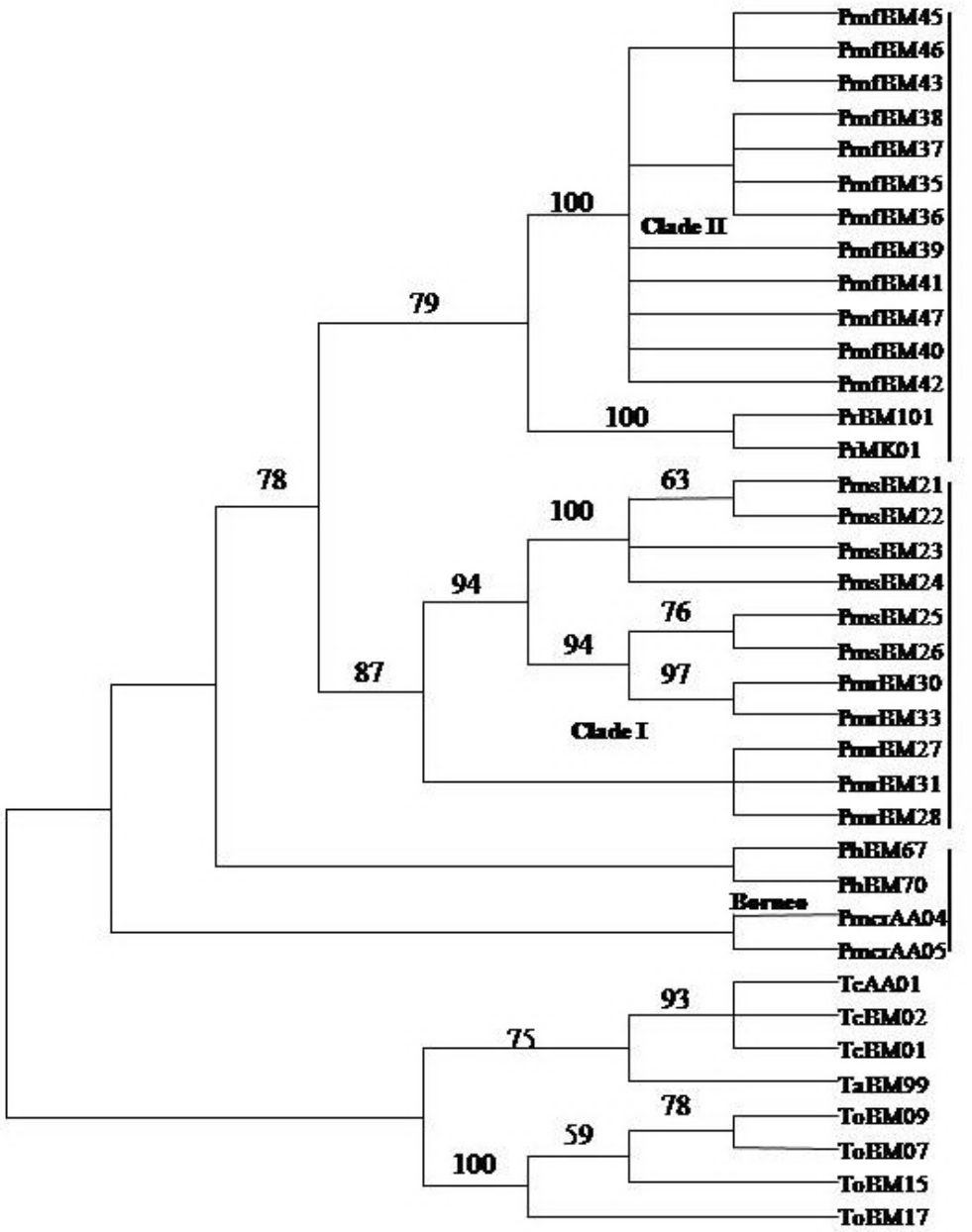

Figure 4. The maximum parsimony heuristic bootstrap tree of Cyt *b*. The bootstrap support values are shown above the branches of the parsimony tree.

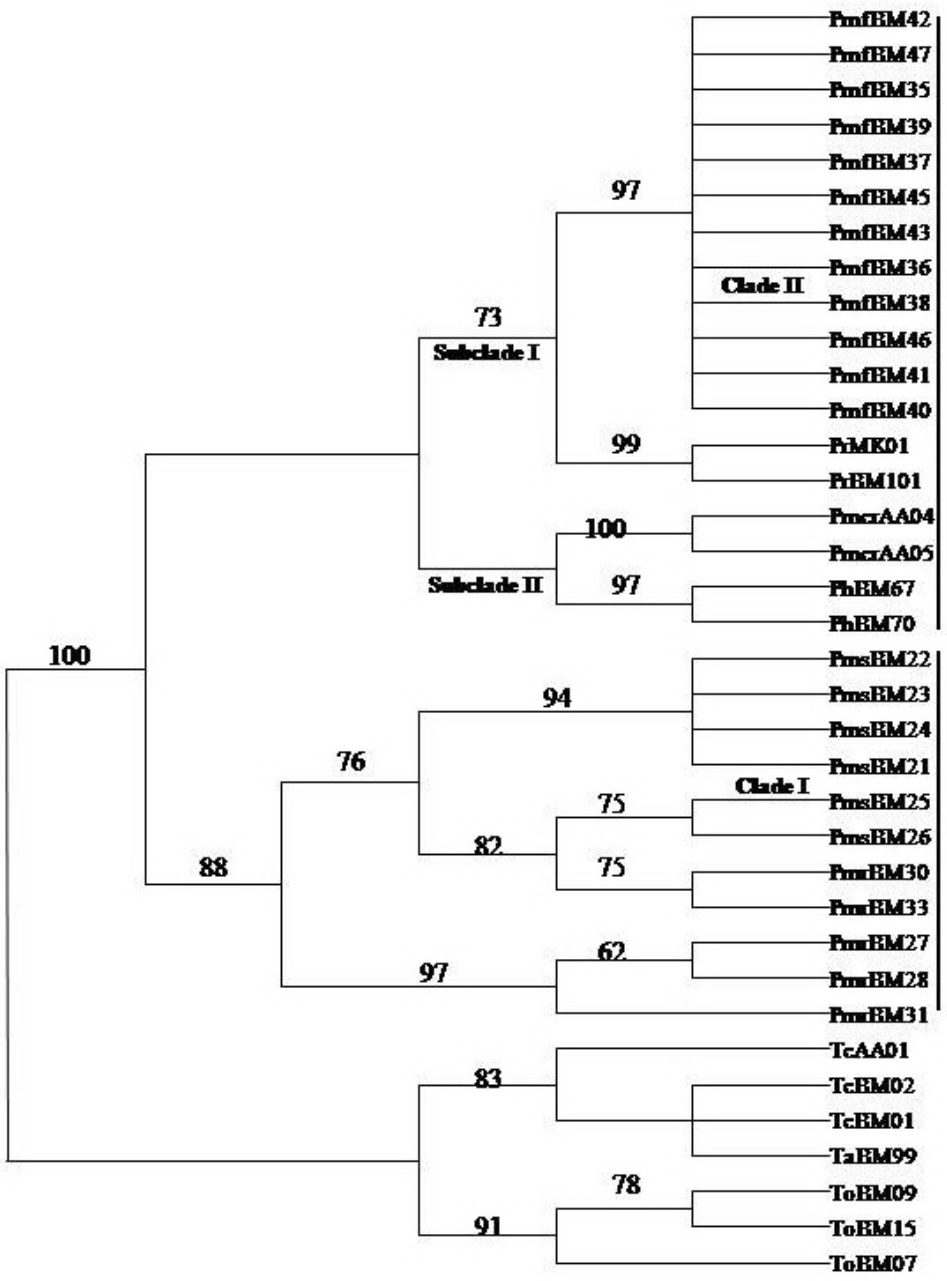

Figure 5. The maximum parsimony heuristic bootstrap tree of 12S rRNA. The bootstrap support values are shown above the branches of the parsimony tree.

Interestingly, both the NJ and MP tree topologies for both the Cyt *b* and 12S rRNA genes also revealed the clustering of Borneo *P. rubicunda* with *P. m. femoralis* of Peninsular Malaysia (Clade II), with high bootstrap values (Cyt *b*: NJ = 82 and MP = 79, 12S rRNA: NJ = 76 and MP = 73). Based on the Cyt *b* NJ and MP trees, the Borneo endemic *P. hosei* and *P. m. chrysomelas* were found to be more primitive than those of Peninsular Malaysia, with *P. m. chrysomelas* as the most basal to the other *Presbytis*. Our 12S rRNA NJ and MP trees, however, did not give similar results as for Cyt *b* due to poor bootstrap value. The 12S rRNA tree topologies only showed a close relationship of the *chrysomelas-hosei* (subclade II) with *rubicunda-melalophos* (subclade II).

In order to obtain more DNA information about the *chrysomelas* individuals, that had previously not been included in any molecular study (Md-Zain et al., 2008) and thought to be a subspecies of *melalophos*, a close examination of the nucleotide sequences of the *chrysomelas* was conducted and surprisingly, Cyt *b* and 12S RNAs both showed a high number of mutations in these individuals. As many as 19 point mutations were found in the Cyt *b* nucleotide sequence of the two *chrysomelas*. These point mutations were not found in any of the *melalophos*. Meanwhile, comparison of *chrysomelas* 12S rRNA nucleotide sequences with all the *melalophos* showed that one of the *chrysomelas* (AA05) had a very large number of point mutations (39.4%). However, the causes of the high mutation rate in *chrysomelas* individuals could not be ascertained.

DISCUSSION

The results of the present study of partial Cyt *b* and 12S rRNA genes provide evidence to clarify the classification of *Presbytis* and the relationships among taxa in *Presbytis*. Based on the morphological data, it was found that *Presbytis* generally had the most variable fur coloration across the taxa, thus complicating the recognition task. Classification based solely on morphological traits could not give conclusive results, as the morphological traits were derived or influenced by the environment surrounding the particular animal.

Traditionally, a subspecies is said to be of the same species when they share the same geographical location or habitat, same morphological and behavioral charateristics, and evolutionary history, especially if capable of interbreeding within the same taxa (O'Brian and Mayr, 1991). Based on the morphological, ecological and behavioral data, Oates et al. (1994) suggested that *P. chrysomelas* be classified as a subspecies of the Borneo *P. melalophos*. However, Brandon-Jones et al. (2004) and Groves (2001, 2005) disagreed with this suggestion. Instead of clustering the *chrysomelas* into any existing taxa, Groves (2001, 2005) awarded this primate species status, including under it another subspecies, namely *P. c. cruciger*. Thus, the *chrysomelas* became a two subspecies taxon. Meanwhile, Brandon-Jones et al. (2004) classified the *chrysomelas* as a subspecies of *femoralis*. Our analysis of the partial Cyt *b* and 12S rRNA gene sequences revealed extra-specific point mutations occurring within the nucleotide sequences of *chrysomelas*, which were not found in any of the *melalophos*. Therefore, our findings on the classification closely resemble those of Groves (2001, 2005). However, it is still too early to conclude that the *chrysomelas* is a distinct species due to unavoidable factors, such as only two representative samples of *P. m. chrysomelas* and the lack of *cruciger* in this study.

Our data, however, did not concur with *Presbytis* divergence as proposed by Brandon-Jones (1996a). He hypothesized that the divergence of *Presbytis* originated from Mentawai Island of Sumatra, Indonesia, which was solely based on the coat coloration of the animal.

Based on the molecular evolution of the Cyt *b* gene, *chrysomelas* was found to have the earliest divergence, which is in accordance with the findings of Peng et al. (1993), indicating that two major evolution events may have happened in the history of Colobinae. The first was centered around the geographical area of Heng Duan, China, whereas the second was centered in the Sundaland including Borneo, Peninsular Malaysia, Sumatra and Java, Indonesia. The latter was supported by molecular data (Md-Zain, 2001). Based on molecular data, Md-Zain (2001) hypothesized that the divergence originated in Borneo, and later moved towards Peninsular Malaysia and Sumatra. The divergence was further complicated by the fact that Borneo remained as part of Asian mainland (linked to the Asian mainland through Peninsular Malaysia and Sumatra) before the Miocene or Pliocene (Wilson and Moss, 1999; Hall, 2001). The paleographical data obtained also showed that the migration of life forms on the Asian mainland and Sundaland was enabled through land bridges during the Pleistocene period (Sartono, 1973; Heaney, 1986; Tjia, 1996). Therefore, the cladogenic activity in Borneo made it possible for the primates to migrate to Sumatra and Java, Indonesia and Peninsular Malaysia through the land bridges connecting them.

The *P. melalophos* taxa (*femoralis*, *siamensis* and *robinsoni*) were clustered in two different clades, with one consisting of *P. m. siamensis* and *P. m. robinsoni*, while *P. m. femoralis* clustered together with *P. rubicunda*, which is in contrast with the findings of Md-Zain (2001). However, the observation by Brandon-Jones (1977) based on coat coloration provides useful information to explain the current findings. The most primitive color, as suggested by Brandon-Jones (1977), would be the dark color, followed by the intermediate color and lastly the derived color, which has the most variable color, ranging from brown to maroon. According to him, both *melalophos* and *rubicunda* are of the same category of coat color, that is the derived color. Brandon-Jones (1996b) further suggested a special affinity of *P. melalophos* with *P. rubicunda* based on the behavioral similarity between the two species. Thus, our findings on the clustering of *P. melalophos* and *P. rubicunda* were well supported by both morphological and behavioral data.

P. m. siamensis and *P. m. robinsoni*, as shown in the NJ and MP tree topology, were not clustered together with *P. m. femoralis*. According to Aimi and Bakar (1992), *P. melalophos* is a sister species to *P. femoralis* (*P. m. femoralis* in the present study) and *P. thomasi* (not included in the present study). The field observation carried out by the authors in Sumatra showed significant differences with regard to the morphological aspect as well as geographical area. Based on these observations, both *femoralis* and *melalophos* were recognized as distinct species. However, their study did not include any primate individual available in Malaysia. Bennett and Bennett (1988), who worked on the Malaysian primates, found that the vocalization of *P. femoralis* and *P. melalophos* were not as consistent as observed by Aimi and Bakar (1992), and proposed that both the species are actually conspecific, which means they are of the same species but highly variable. The separation of *femoralis* from *melalophos* was also supported by Wilson and Wilson (1977) based on both the coat color and vocalization of adult individuals.

Brandon-Jones et al. (2004) classified *P. femoralis* and *P. siamensis* as distinctive species that consist of six subspecies and five subspecies, respectively. According to authors, *robinsoni*, *chrysomelas* and *femoralis* are subspecies of *femoralis*. Our constructed Cyt *b* tree topology, however, did not meet such classification. Instead, our analysis showed a classification closer to that of Oates et al. (1994) and Md-Zain (2001). Meanwhile, *chrysomelas* showed a specific affinity that follow Groves (2001, 2005) but not Brandon-Jones et al. (2004). Thus, its status remains unclear. The efforts to clarify the current status of *Presbytis* were further

complicated by the difficulties to obtain specimens for the complete species list of the genus. Even though there is still some doubt, the present study has generally provided us with a clearer picture of *Prebytis* classification. Further studies should incorporate more species samples and more loci and relationships with other Asian leaf monkeys as previously described in *Trachypithecus* and *Semnopihecus* and odd noses (Karanth et al., 2008; Osterholz et al., 2008; Roos et al., 2008; Ting et al., 2008; Md-Zain et al., 2008, 2010b).

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