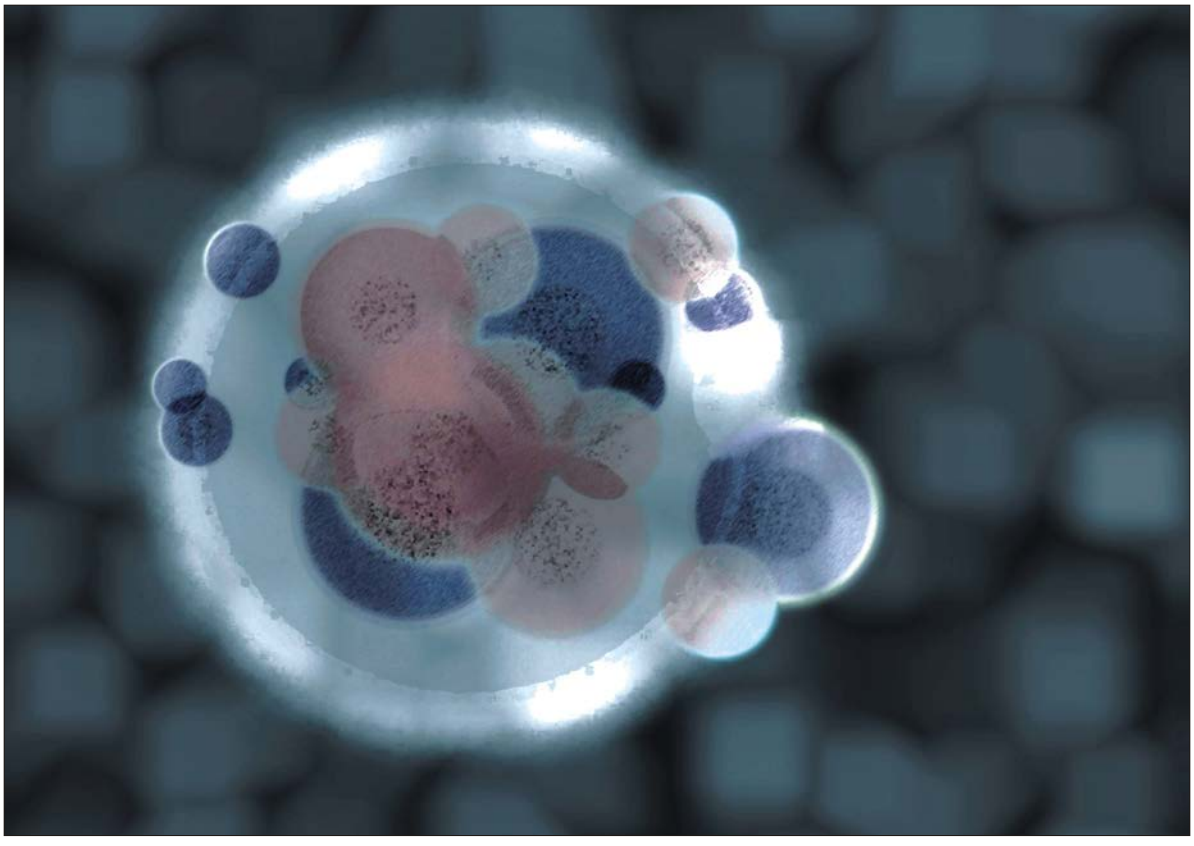

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Phylogenetic Position of *Tarsius bancanus* Based on Partial Cytochrome *b* DNA Sequences

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Abstract: This study was carried out to ascertain the molecular phylogenetic position of *Tarsius bancanus* among Malaysian primates based on the partial of Cytochrome *b* (cyt *b*) gene sequences. A total of five samples of *Tarsius bancanus* from Sabah, Malaysia, were used in this study. Several other Malaysian primates were also included in the analysis (Leaf monkeys (*Presbytis* and *Trachypithecus*), Macaques (*Macaca*), Siamang (*Symphalangus*) and Slow loris (*Nycticebus*). We also included DNA sequences of several prosimians (*Galago*, *Cheirogaleus*, *Daubentonia*, *Indri*, *Avahi*, *Lemur* and *Lepilemur*) from GenBank. In addition, one individual of orang utan (*Pongo pygmaeus*) and human (*Homo sapiens*) were used as outgroups to root the tree. All taxa were analysed using character method (Maximum Parsimony, MP) and distance method (Neighbor-Joining, NJ). From the 375 examined characters, 43.2% were constant characters while 4.8% characters were parsimony uninformative whereas 52.0% characters were parsimony informative. Tree topologies discriminated three major clades in which primitive primates, Old World Monkeys and Anthropoids belongs to their own monophyletic clades. Both MP and NJ trees showed that *T. bancanus* was placed in primitive primates group.

Key words: *Tarsius bancanus*, molecular systematics, Malaysian primates, cytochrome *b*, Tarsiers

INTRODUCTION

The Bornean tarsier, *Tarsius bancanus* is a nocturnal primate (Napier, 1970), which is classified under the family Tarsiidae (Brandon-Jones *et al*., 2004; Szalay and Delson, 1979). They can be found on the island of Borneo (Groves, 2001). Tarsiers have sometimes been called 'living fossils'. The proportions of the limbs, which indicate their tree-hopping gait, are very similar to those of early primates of the Eocene period (Napier, 1970). Tarsier possesses problem in the primate systematics. Its evolutionary position is hard to be clarified. Mittermaier *et al.* (1999) stated that there are evidence suggesting tarsiers to be most closely related to the monkeys and apes than prosimians. For example, like anthropoid, tarsiers have a short muzzle rather than typical prosimians that externally have relatively long muzzles terminating in a naked, moist snout (Mafham and Mafham, 1992).

Recent developments in molecular techniques allow evolutionary biologists with an additional tool for making phylogenetics inferences, by comparing DNA sequences between homologous DNA segments using mitochondrial genes (Shahrom *et al*., 2005; Lim *et al*., 2010). These mitochondrial DNA (mtDNA) genes are widely used to infer phylogenetic relationships on primates (Masters *et al*., 2007; Roos *et al*., 2008). Cyt *b* gene of the mitochondrial is well-known as rapidly evolve gene and can shows variations within and between species and has been used in phylogeny and biogeography studies (Caine´ *et al*., 2006; Karanth *et al*., 2008).

Many molecular systematic researches have studied on primates focusing on Great Apes (Steiper, 2006; Gonder *et al*., 2006) and Old World Monkeys (Karanth *et al*., 2008; Osterholz *et al*., 2008). In addition, there are also many molecular studies on primitive primates particularly tarsiers (Raina *et al*., 2005; Uddin *et al*., 2008). Andrew *et al.* (1998) have looked on cyt *b* gene of *T. bancanus* and Murayama *et al.* (1998) worked on D4 dopamine receptor (D4DR) genes of prosimians (including tarsier) and tree shrew. In addition, Schmitz *et al.* (2002) had sequenced the entire mitochondrial genome of *T. bancanus*. Most of the molecular systematic studies on Malaysian primates focused only at the genus level, for instance the genus

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Presbytis, *Trachypithecus* and *Nasalis* (Ernie *et al*., 2005; Md-Zain *et al*., 2005, 2008). Other molecular studies from neighbouring country (Thailand) also incorporated many samples of cercopithecidae (Chaveerach *et al*., 2007; Tanee *et al*., 2009). Therefore, very limited inferences can be made on the molecular systematics of Malaysian primitive primates. Data obtained from this research is paramount important as to resolve difficulties in prosimian primate classifications. This study was carried out to determine phylogenetic position of *T. bancanus* from Sabah, Malaysia, compared to the other primates particularly the Malaysian primates using partial DNA sequences of cyt *b* gene.

MATERIALS AND METHODS

Taxa sampled: There were six *T. bancanus* tissue samples used in this study (Table 1). All the tarsier samples were obtained from Maklarin Lakim of Sabah Parks. Several institutions (Sabah Parks, Department of Wildlife and National Parks and Sarawak Forestry Department) provided the necessary facilities and assistance for tissue sampling collection of the other Malaysian primates since, 1998.

DNA extraction, amplification and sequencing: The cyt *b* gene was chosen to reflect sequence variation in mitochondrial DNA (mtDNA). Total DNA was extracted from tissue and skin using Gene All Tissue SV (Plus!) Mini Extraction Kit (Gene All). L14724 and H15149 (Table 2) were used as primers to amplify the cyt *b* gene (Irwin *et al*., 1991; Kocher *et al*., 1989). We managed to get the optimum condition of PCR components ($MgCl_2$, *Taq* DNA polymerase, dNTP mix, PCR buffer, DNA template) in DNA amplification of cyt *b* gene (Table 3). Estimated fragment size of the cyt *b* gene for all amplified samples, observed after electrophoresis on 1.5% agarose gel is approximately 500 bp (Fig. 1). The PCR products were purified using QIAquick gel purification kit protocol (QIAGEN) and sent to company (1st Base, Kuala Lumpur) for sequencing purposes.

Sequence alignment and data analysis: Sequences were aligned using the ClustalW program, with minor adjustments made manually. We added several other Malaysian primate DNA sequences that are available in our laboratory database and other primate sequences from GenBank (Table 4). *Pongo pygmaeus* and *Homo sapiens* were used as outgroup to properly rooting the generated tree. Data matrix was analyzed by excluding 15 characters

Table 1: Tissue samples of *Tarsius bancanus* used in this study

Accession No.	Locality	Date	Specimenstype
SP52	Tawau Hill Park, Sabah, HQ	15/11/1998	Skin (Dry)
SP18337	Poring, Kinabalu Park, Sabah	18/07/1996	Wet (Formalin)
SP18431	Tawau Hill Park, Sabah, HQ	07/04/1997	Wet (Formalin)
SP18508	Meroyai, Tawau Hill Park, Sabah	15/08/1997	Wet (Formalin)
SP19190	Poring, Kinabalu Park, Sabah	23/09/1999	Skin (Dry)
SP19480	Tawau Hill Park, Sabah	23/06/2002	Wet (Formalin)

Table 2: Oligonucleotide primer pair and PCR conditions

No. of cycles	Duration (min)	Temperature (°C)	Phase
35	3	94	Pre-denaturation
	1	94	Denaturation
	1	55	Annealing
	1	72	Elongation
	10	72	Post-elongation

Forward/Reverse Primer Sequences.
L14724: 5'-CGAAGCTTGATATGAAAAACCATCGTTG-3'
H15149:5'-AAACTGCAGCCCCTCCGAATGATATTTGTCCTCA-3'

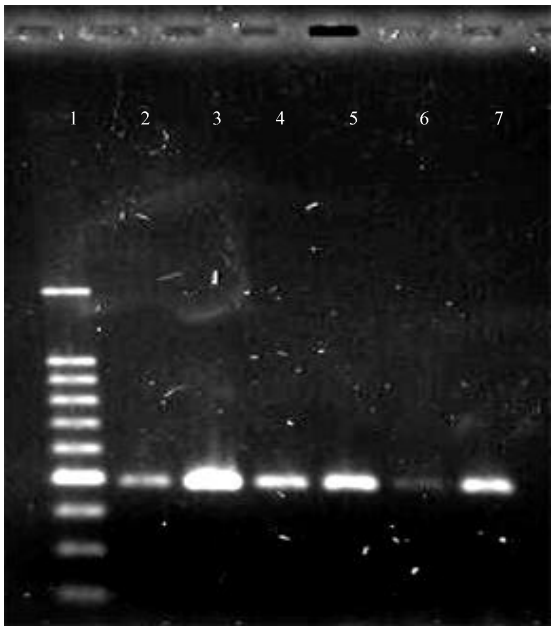

Fig. 1: Amplified products of *T. bancanus* of mtDNA cyt *b* gene. Lanes: 1, 100 bp ladder; 2, *T. bancanus* (SP52); 3 *T. bancanus* (SP18337); 4, *T. bancanus* (SP18431); 5, *T. bancanus* (SP18508); 6, *T. bancanus* (SP19190); 7, *T. bancanus* (SP19480)

Table 3: PCR components in DNA amplification of cyt *b* gene for tissue samples

PCR components	Concentration	Volume (μL)
DNA template		4.0-5.0*
ddH_2O	-	11.7-13.7
PCR buffer	5 X	2.5
$MgCl_2$	25 mM	3.0-4.0
dNTP Mix	1 mM	0.5
CytB (L14724)	20 pmol μL^{-1}	0.5
CytB (H15149)	20 pmol μL^{-1}	0.5
Taq DNA polymerase	5 U μL^{-1}	0.3
Total		25.0

*The quality depends on DNA concentration from DNA extraction

Table 4: Details of taxa used in the phylogenetic analysis

Code	Species	Locality
PmsBM23	*Presbytis melalophos siameusis*	Ulu Besut, Terengganu
PmsBM24	*P. m. siameusis*	Ulu Besut, Terengganu
PmrBM27	*P. m. robinsoni*	Ulu Kenas, Perak
PmrBM31	*P. m. robinsoni*	Ulu Kenas, Perak
PhBM67	*P. hosei*	Tawau Hill Park, Sabah
PhBM70	*P. hosei*	Lembah Danum, Sabah
PrBM102	*P. rubicunda*	Unknown
PrMK01	*P. rubicunda*	Tawau Hill Park, Sabah
TcBM01	*Trachypithecus cristatus*	Kota Kuala Muda, Kedah
TcBM03	*T. cristatus*	Kota Kuala Muda, Kedah
TcAA01	*T. cristatus*	Sarawak
ToBM09	*T. obscurus*	Changloon, Kedah
ToBM10	*T. obscurus*	Zoo Taiping, Perak
ToL01	*T. obscurus*	Langkawi
MnBM102	*Macaca nemestrina*	Zoo Melaka
MnBM103	*M. nemestrina*	Zoo Melaka
MaBM104	*M. arctoides*	Zoo Melaka
MaZZ01	*M. arctoides*	Unknown
MfMF02	*M. fascicularis*	Pulau Sapi, Sabah
MfSP50L	*M. fascicularis*	Tawau Hill Park, Sabah
HyZZ090	*Symphalangus syndactylus*	Zoo Melaka
HyZZ096	*S. syndactylus*	Pahang
HyZZ095	*S. syndactylus*	Ipoh, Perak
NcZZ098	*Nycticebus coucang coucang*	Selangor
NcZZ099	*N. c. coucang*	Selangor
NcSP32	*N. coucang*	Ranau, Sabah
NcAY687889	*N. coucang*	GenBank
NcmAY878361	*N. c. menageusis*	GenBank
NcjAY878365	*N. c. javanicus*	GenBank
NbAY441477	*N. bengaleusis*	GenBank
NpAY6878892	*N. pygmaeus*	GenBank
TbSP18508	*Tarsius bancanus*	Tawau Hill Park, Sabah
TbSP18337	*T. bancanus*	Poring, Sabah
TbAB011077	*T. bancanus*	GenBank
TbAF348159	*T. bancanus*	GenBank
TbNC002815	*T. bancanus*	GenBank
GsAY441471	*Galago senegaleusis*	GenBank
CmAY441457	*Cheirogaleus major*	GenBank
MgDQ979930	*Microcebus griseorufus*	GenBank
DmAY441444	*Daubentonia madagascarieusis*	GenBank
IiAY441455	*Indri indri*	GenBank
AlEF103327	*Avahi laniger*	GenBank
LcAY441445	*Lemur catta*	GenBank
LmDQ444303	*Lepilemur mittermeieri*	GenBank
PpU38274	*Pongo pygmaeus*	GenBank
HsEF495222	*Homo sapieus*	GenBank

with the remaining 375 characters. Two primary methods were used to discern phylogenetic relationships, Maximum Parsimony (MP) and Neighbor Joining (NJ). The analysis were conducted using PAUP version 4.0. For unweighted MP, trees were obtained by heuristic searches treating all nucleotide substitutions as unordered. Data were also subjected to bootstrap analysis with 1000 replications. Tree topology constructed using the NJ method employing the Kimura 2 parameters distance option of PAUP. Homoplasy was quantified using the consistency index (CI) and the homoplasy index (HI).

RESULTS

All DNA from the six samples of *T. bancanus* were successfully amplified (Fig. 1). However, only two of them

Table 5: Summary of variations along the sequences across taxa

Analysis	No. of character	Percentage
Total characters examined	375	
Constant Characters	162	43.2
Parsimony-uninformative characters	18	4.8
Parsimony-informative characters	195	52.0
Tree length = 968		

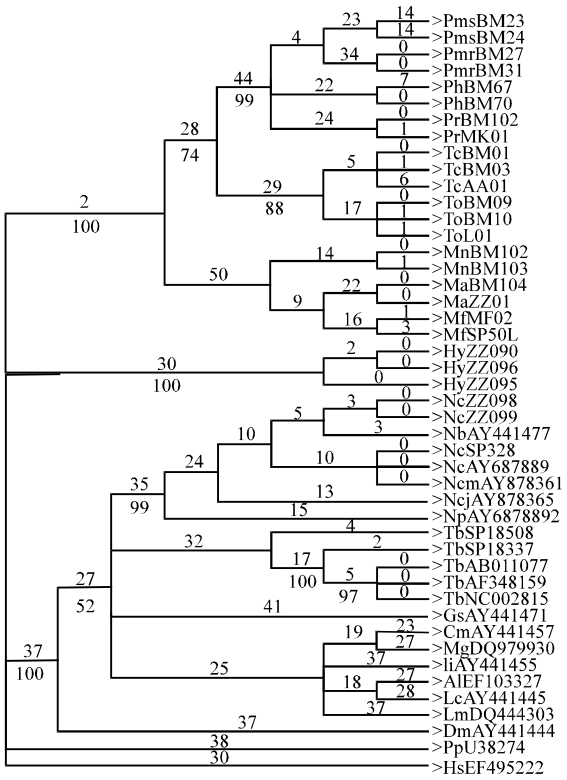

Fig. 2: Maximum Parsimony tree of partial cyt *b* gene sequences obtained from heuristic searches. The bootstrap values are shown below the branches of the parsimony tree

(SP18337 and SP18508) were successfully sequenced with the length of more than 400 bp. The other samples failed to produce any sequence that can be analysed. This problem was due to the degradation of samples where most of the samples have been stored for a long period and those samples are the formaldehyde-fixed tissue.

In phylogenetic tree obtained from the analysis of partial cyt *b* gene sequences, 15 characters were excluded because of missing data during data generated and the sequences do not represent variation. Thus, 375 remaining characters were examined with 43.2% constant characters. 4.8% characters are parsimony uninformative and 52.0% characters are parsimony informative (Table 5).

Figure 2 and 3 are useful in indicating the tree topologies obtained from Unweighted Maximum

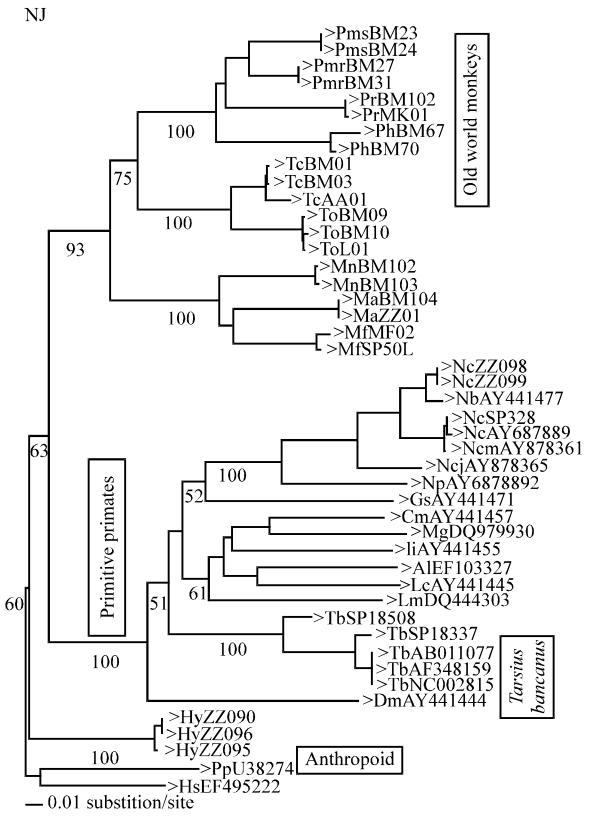

Fig. 3: Neighbor-Joining phylogram with bootstrap values

Table 6: Average percentages of genetic distance among anthropoids, old world monkeys and primitive primates using kimura 2 parameters

Groups	Anthropoids	Old world monkeys	Primitive primates
Anthropoids	12.282		
Old World Monkeys	26.023	19.130	
Primitive primates	30.776	37.199	22.676

Parsimony and Neighbor-Joining analyses of the partial cyt *b* gene sequences. Unweighted MP analysis produced a bootstrap tree (length = 968, CI = 0.3428, HI = 0.6572) with *T. bancanus* to form its own monophyletic clade with supported 100% of bootstrap value. NJ tree performed clearer topology, which also distinguish *Tarsius* in its own monophyletic clade supported by 100% bootstrap value. Both MP and NJ placed *Tarsius* within primitive primate clade and discriminated them from Old World Monkeys and Hylobatidae. Besides looking at tree topologies and bootstrap value, support for distinct monophyletic relationship among these three major clades can also be derived from Kimura 2 Parameters distance matrix. Table 6 summarized the average percentage of genetic distance among three major groups: anthropoids, Old World Monkeys and primitive primates (including *Tarsius*). Among these three groups, genetic distance between Old World Monkeys and primitive primates is the greatest, which is 37.199%, followed by anthropoids-primitive primates, 30.776%. The Old World Monkeys and anthropoids are the closest; their average percentage of genetic distance is 26.023%.

DISCUSSION

Tarsiers placed at a special position among the primates, in that they are generally intermediate between lemur and loris groups and also between monkeys and apes (Martin, 1990). In this study, both MP and NJ analysis (Fig. 2 and 3) indicated that *T. bancanus* is more closely related and group together with primitive primates (Strepsirrhini) rather than Old World Monkeys and anthropoid (*Symphalangus*). Results in this study support findings of many previous studies (Eizirik *et al*., 2001; Murphy *et al*., 2001; Jow *et al*., 2002; Schmitz *et al*., 2002; Hudelot *et al*., 2003) in which tarsiers have a close relationship with strepsirrhini. The results support Murphy *et al*. (2001), which grouped tarsiers with lemurs and separated them from New World Monkeys and great apes. While comparison of DNA sequences of the α A-crystalline gene (Jaworski, 1995) and γ-globin genes (Hayasaka *et al*., 2003) also indicated a difference between tarsiers and simians. In Schmitz *et al*. (2002) study, phylogenetic trees reconstruction based on amino acid and DNA sequences support a cluster of *Tarsius* and slow loris.

The position of tarsier depends on the molecular and morphological data sets (Matsui *et al*., 2009). Some previous data have been contradicted with our findings and even suggested tarsiers to be a close to anthropoidea (Schmitz *et al*., 2001; Poux and Douzery, 2004; Gibson *et al*., 2005) or as a basal group of primates (Arnason *et al*., 2002). At morphological level, Shoshani *et al*. (1996) stated that *Tarsius* is grouped with anthropoidea based on of 264 morphological characters on 18 primate taxa and four outgroups. Ross *et al*. (1998) also concluded that cranial evidence supports the tarsier-anthropoid hypothesis. Phylogenetic trees resulted from molecular analysis of the gene encoding Interphotoreceptor Retinoid Binding Protein (IRBP) and ε-globin sequences indicated that tarsier is grouped with Haplorhini (Stanhope *et al*., 1993). Moreover, a study of the existence pattern from short interspersed elements (SINEs), successfully showed a sister group relationship of *T. bancanus* with anthropoids to the exclusion of the Strepsirhini representatives (Schmitz *et al*., 2001). Mitochondrial cytochrome *c* oxidase subunit II reveals only a weak affinity of tarsiers to anthropoids (49.6% of bootstrap value) (Adkins and Honeycutt, 1994). In

the study by Madsen *et al.* (2003), which utilised a new phylogenetic marker, apolipoprotein B (APOB) had showed a strong support (84-98% bootstrap value) in maximum likelihood for an association of tarsier and anthropoidea within primates.

Schmitz *et al.* (2002) stated that there is a general conflict exists at the molecular level between nuclear and mitochondrial DNA data. Phylogenetic analysis based on nuclear DNA sequences traditionally represent *Tarsius* as a sister group to anthropoids. In contrast, mitochondrial DNA data only marginally support this affiliation or even exclude *Tarsius* from primates. They suggested two possible scenarios causing this to be in conflict: a period of adaptive molecular evolution or a shift in the nucleotide composition of higher primate mitochondrial DNA through directional mutation pressure. Andrew *et al.* (1998) suggested that an episode of adaptive evolution might have taken place on the lineage leading to higher primates after *Tarsius* diverged. This is based on a higher relative rate of non-synonymous to synonymous substitutions. These evolutionary forces caused tarsiers to shift from their historical place in the mitochondrial DNA tree.

Results of this study indicate the phylogenetic problems concerning tarsiers. However, this study has increased our understanding of phylogenetic relationships among Malaysian primates particularly *T. bancanus*. Results showed that *T. bancanus* form its own monophyletic clades and clustered together with other primitive primates (strepsirrhini) towards Old World Monkeys and ape. Thus, *T. bancanus* belongs to primitive primates as supported by tree topologies, bootstrap values and genetic distance of cyt *b* DNA sequences. What now needs to be done is further work to define the molecular phylogenetic relationships of *T. bancanus* among other members of tarsiers as have been previously well described using morphological data (Groves, 1998; Merker and Groves, 2006). More population genetic studies should also incorporated other tarsier species in the Southeast Asian region as previously conducted by Merker *et al.* (2007, 2008) using microsatellite markers.

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