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Malaysian Fruit Bats Phylogeny Inferred Using Ribosomal RNA

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ABSTRACT

Fourteen species of the Malaysian fruit bats (Pteropodidae) were used in this DNA taxonomy using 1,334bp of the 12S ribosomal RNA (rRNA), transfer RNA (tRNA) valine and 16S rRNA gene segments. Previous studies using DNA found contradictions between morphology and molecular data in inferring the phylogeny of the fruit bats proposed by Andersen (1912). Our phylogenetic analysis using the neighbor-joining and the maximum parsimony methods did not support the monophyly of the subfamily Macroglossinae and the cynopterine group of the subfamily Pteropodinae as proposed by classical taxonomists. This is congruent with previous molecular studies. Here, we provide the first registered 12S rRNA, tRNA valine and 16S rRNA sequence records for Dyacopterus spadiceus in the GenBank database. This study represents the first attempt to infer the phylogenetic relationship of fruit bats from Malaysia using molecular methods.

Keywords: DNA phylogenetic relationship, pteropodids, ribosomal and transfer RNAs

INTRODUCTION

The suborder of Megachiroptera consists of only one family, the Pteropodidae, containing 42 genera and 166 species recorded worldwide (Corbet and Hill, 1992; Nowak 1994; Wilson and Reeder, 2005). They live in subtropical and tropical areas of Africa, through southern Asia to Australia and on the islands in the Indian and western Pacific Oceans (Mickleburgh *et al.*, 1992). Juste *et al.* (1999) recognised the Malaysian-Indonesian rainforest along with the African rainforest belt across the Congo basin as the two areas with the highest diversity of fruit bats. In Borneo, there are 11 genera of pteropodids (Payne *et al.*, 1985) while Peninsular Malaysia has 17 species (Kingston *et al.*, 2006), making it the fourth highest country in terms of their worldwide diversity.

Pteropodids are relatively small to very large bats with the forearm length ranging from 4 cm to 22 cm. They feed on plant products such as fruits, flowers, nectar and pollens. They become active in the late evening and at night when they may fly long distances in search of food. Pteropodids consist of all flying foxes and Old World fruit bats which are further divided into four subfamilies, namely, (i) the diverse subfamily Pteropodinae, (ii) subfamily Macroglossinae which consists of six genera of blossom bats, dawn bats, long-tongued fruit bats, and relatives, (iii) the aberrant subfamily Harpyionycterinae and (iv) the subfamily Nyctimeninae (Corbet and Hill, 1992). The Malaysian pteropodids consist of 18 species from two subfamilies, the Pteropodinae, which are specialised fruit and flower eating bats and the Macroglossinae, which

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contains the genera that are principally adapted to feed on nectar and pollen (Corbet and Hill, 1992).

There remains considerable uncertainty in both the subordinal and the superfamilial classification within bats (Teeling *et al.*, 2005) and many traditionally recognised groups are not monophyletic (Simmons, 2005), particularly in the pteropodids. Recent molecular data have indicated the requirement for substantial revisions of the phylogeny of pteropodids based on the morphological characters (Kirsch *et al.*, 1995; Juste *et al.*, 1999; Romangnoli and Springer, 2000; Colgan and da Costa, 2002). However, no complete classification of bat families based on molecular data yet exists and the present classifications are based on morphology that is not at all congruent with the new data (Simmons, 2005).

The classical taxonomy by Andersen (1912) categorised the subfamily Pteropodinae into three sections (or tribes) of rousettine (consisting the genera *Rousettus, Pteropus* and *Dobsonia*), epomophorine (African fruit bats) and cynopterine (genera *Myonycteris, Balionycteris, Nyctimene, Sphaerias* and *Cynopterus*); and in the subfamily Macroglossinae are the eonycterine and notopterine bats. Cladistic re-analysis of Andersen's (1912) characters supported a Macroglossinae monophyly, but monophyly of rousettine, cynopterine and epomorphorine was less clear (Springer *et al.*, 1995).

Nevertheless, the classifications of pteropodids by Andersen (1912) remain the most comprehensive of the evolutionary framework reference for the relationships among approximately 200 species of pteropodids described (Koopman, 1994; Hollar and Springer, 1997). However, relationships among pteropodid genera are not yet fully resolved and the positioning of several Southeast Asian endemic genera is still problematic (Simmons, 2005). Due to much contradiction between the morphological and genetic data, the current taxonomic status and phylogenetic relationship of the Malaysian pteropodids remains unclear. In this study, we attempt to infer the phylogenetic relationship and to reconstruct the taxonomic relationships among the pteropodids of Malaysia using the 12S ribosomal RNA (rRNA), transfer RNA (tRNA) valine and 16S rRNA gene segments.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

A list of the species of fruit bats, their voucher numbers and collection localities are shown in Table 1. A total of 14 pteropodids (out of 18 species existing in Malaysia) samples were used in this study which were representatives of the different regions in Peninsular Malaysia, Sabah and Sarawak. The samples were preserved in either vials containing 95% ethanol or stored at -20°C for fresh samples prior to analysis. Bats were captured using standard mist nets set at under storey level, and across forest trails and over water bodies (Hall *et al.*, 2004). Some samples were acquired from the museums at the Department of Wildlife and National Parks of Peninsular Malaysia (Kuala Lumpur) and Sabah Parks and were re-identified following Payne *et al.* (1985). Four species were not included in this study due to their scarcity: *Megaerops wetmorei*, *Rousettus spinalatus*, *Eonycteris major*, and *Pteropus hypomelanus*. *Pteropus dasymallus*, *Rhinolophus pumilus* and *Mystacina tuberculata* (AB042770, NC005434 and AY960981, respectively) were used in this study as a comparison to determine the position of the mitochondrial DNA (mtDNA) sequences of the fruit bats studied.

Total genomic DNA was extracted from muscle tissues from both fresh and ethanol samples following a modified cetyltrimethylammonium bromide (CTAB) procedure as described by Grewe *et al.* (1993) with the presence of Proteinase K. The quality and approximate yield was determined by electrophoresis of 2 μL of genomic DNA on a 1% agarose gel in 50 ml of 1X TAE buffer containing ethidium bromide at 90V for 30 min. Isolated genomic DNA was used for further mtDNA analysis.

Polymerase Chain Reaction and DNA Sequencing

Approximately 1,400-base pairs (bp) of the 12S rRNA, tRNA valine and 16S rRNA regions were amplified using the standard polymerase chain reaction (PCR) procedures. A set of 12S and 16S rRNA primers were used: 5'-ATG TTT TTG ATA AAC AGG CG-3' known as 16SA-H (Palumbi *et al.,* 1991) and 5'- AAA CTG GGA TTA GAT ACC CCA CTA T-3' known as 12SA-L (Palumbi *et al.*, 1991). Thermal cycle amplification was performed in a 25 μL reaction volume containing

TABLE 1
Species names, specimen voucher and localities of the pteropodids used in this study

	SubFamily	Species	Specimen Voucher	Park/Museum Collection	Localities/Sources	GenBank Accession No.
INGROUP	Pterpodinae	*Aethalops alecto*	TK004	SP	Kinabalu Park, Sabah	DQ002939
		Balionycteris maculata	NNP135	UNIMAS ZM	Niah National Park, Sarawak	DQ002940
		Chironax melanocephalus	MTA96041	UNIMAS ZM	Rayu River, Kubah, Sarawak	DQ002941
		Cynopterus brachyotis	WK1	DWNP ZM	Wang Kelian, Perlis	DQ002942
		C. horsfieldi	MTA96318	UNIMAS ZM	Rayu River, Kubah, Sarawak	DQ002943
		C. sphinx	CS29	DWNP ZM	Taiping, Perak	DQ002944
		Dyacopterus spadiceus	DS10	UNIMAS ZM	Balambangan Island, Sabah	DQ002945
		Megaerops ecaudatus	BD013	UNIMAS ZM	Lelang Dam, Bario, Sarawak	DQ002949
		Penthetor lucasi	NNP084	UNIMAS ZM	Niah National Park, Sarawak	DQ002950
		Pteropus dasymallus	-	-	GenBank (Nikaido *et al.*, 2000)	AB042770
		P. vampyrus	P. V. 1	UNIMAS ZM	Serian, Sarawak	DQ002952
		Rousettus amplexicaudatus	1017	UNIMAS ZM	Balambangan Island, Sabah	DQ002951
	Macroglossinae	*Eonycteris spelaea*	P015	UNIMAS ZM	Pueh Mountain, Sematan, Sarawak	SDQ002946
		Macroglossus minimus	UMS/Bf/00104	UNIMAS ZM	Niah, Miri, Sarawak	DQ002947
		M. sobrinus	MTA96376	UNIMAS ZM	Kuala Gandan, Pahang	DQ002948
	OUTGROUP	*Rhinolophus pumilus*	-	-	GenBank (Nikaido *et al.*, 2001)	NC_005434
		Mystacina tuberculata	-	-	GenBank (Sandbrook *et al.*, unpublished)	AY960981

SP: Sabah Park; UNIMAS ZM: Universiti Malaysia Sarawak Zoological Museum; DWNP ZM: Department of Wildlife and National Parks Zoological Museum

15.5 µL of sterilised distilled water, 0.5 µL of *Taq* DNA polymerase (Promega), 2.5 µL of 10X reaction buffer (Promega), 0.5 µL of dNTP (10mM), 1.5 µL of magnesium chloride (25 mM), 1.25 µL of each primer (10 µM) and 2.0 µL of the genomic DNA. The cycle profile was 5 min at 96°C for initial denaturation, followed by 35 cycles of 45 sec at 95°C for denaturation, 1 min 30 sec at 56°C for annealing and 1 min 30 sec at 72°C for elongation, and finally 7 min at 72°C for final elongation. The amplified products were later visualised on 1% agarose gel containing ethidium bromide, ran for approximately 30 min at 90 V and photographed under UV trans-illuminator (Bio-Rad). 1 kb DNA ladder (Promega) was used as a standard size marker to quantify the size of the PCR products. The amplified DNA products were purified using a commercial kit (Fermentas) and subsequently sent for sequencing. Sequencing of each sample was carried out on both the forward and reverse strands by using the same primers as for the PCR amplification on the ABI PRISM® 377 DNA Sequencer in a private laboratory (First BASE Laboratories Sdn. Bhd.).

Sequence Alignment and Phylogenetic Analyses

Multiple alignments of the nucleotide sequences were done using the program CLUSTAL X 1.81 (Thompson *et al.*, 1997) and subsequently aligned by eye. Pairwise distance calculations were conducted using the two-parameter model of Kimura (1980) to estimate genetic distances among the species of pteropodids under study using *MEGA* (version 2.1, Kumar *et al.*, 2001). Nucleotide compositions (% of A, C, T and G bases) were also estimated for each species using *MEGA*. Phylogenetic trees were reconstructed using the neighbour joining (NJ) and unweighted maximum parsimony (MP) as well as the maximum likelihood (ML) methods implemented in PAUP (version 4.0b 10; Swofford, 1998). The NJ clustering was performed using the two-parameter model of Kimura (1980) while the ML analysis corresponded to the HKY85 evolutionary model (Hasegawa *et al.*, 1985). All trees were rooted with two Microchiroptera sequences from GenBank: *R. pumilus* (NC_005434) and *M. tuberculata* (AY960981) as outgroups. Phylogenetic confidence was estimated by bootstrapping (Felsenstien, 1985) with 1000 replicate data sets for the NJ and MP methods while for the ML method there were 100 replicate data sets. All the sequences were submitted to GenBank with Accession Numbers: DQ002939-DQ002952.

According to Miyamoto and Boyle (1989) and Irwin *et al.* (1991), transversion substitutions in mammals showed a linear relationship with time. Similar to the calculations done by Bastian *et al.* (2001), we calculated the estimate of divergence between the pteropodids and the outgroup species using a constant transversion rate of 0.2% per mya (million years ago) (Miyamoto and Boyle, 1989; Irwin *et al.*, 1991).

RESULTS AND DISCUSSION

Sequence Analysis and Estimate of Divergence

Partial sequences with the length of 1334-bp comprising the 12S rRNA, tRNA valine and 16S rRNA gene segments from 14 species of Malaysian pteropodids were successfully sequenced and aligned (including gaps). By comparing with the complete mitochondrion genome (using *R. pumilus*, *M. tuberculata* and *P. dasymallus* taken from GenBank with accession number, NC_005434, AY960981 and AB042770, respectively), our sequences begin at the 547-bp until the 1880-bp of the complete mitochondrial sequence. The base composition showed an anti-G bias (data not shown), which is characteristic for the mitochondrial gene (Cantatore *et al.*, 1994; Briolay *et al.*, 1998; Ryan and Esa, 2006). From the 1334-bp sequence, 519 (38.9%) variable or polymorphic (segregating) sites were observed. In addition, among the 519 variable sites, 373 (71.9%) were parsimoniously informative sites.

The pairwise genetic distances (number of nucleotide substitutions per site) calculated by using the Kimura two-parameter model (Kimura 1980) are shown in Table 2. Pairwise comparisons among all the sequences range from 0.2% to 24.5% of differences. Within the subfamily Pteropodinae, the distances range from 0.2% to 18.1% of differences, with the least differences observed between *Cynopterus brachyotis* and *C. sphinx* within the genus *Cynopterus*. The differences between the subfamilies ranged from 11.8% to 17.9%. Within the subfamily Macroglossinae the difference between *Macroglossus minimus* and *M. sobrinus* was 1.6% while *Eonycteris spelaea* was 15.7% and 15.0% different from *M. minimus* and *M. sobrinus*, respectively. All the pteropodid sequences were

TABLE 2
Pairwise distances with all sites considered (below the diagonal; including gaps) and transversion pairwise distances (above the diagonal) in percentage using the two-parameter model of Kimura (1980) among the pteropodids species used in this study

No.	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1.	*Aethalops alecto*		*4.0*	*3.4*	*4.6*	*4.6*	*4.6*	*5.0*	*5.6*	*2.5*	*5.0*	*4.9*	*5.1*	*4.5*	*6.9*	*6.7*	*8.4*	*9.4*
2.	*Balionycteris maculata*	10.8		*5.1*	*6.0*	*6.0*	*6.0*	*6.6*	*7.6*	*4.5*	*7.1*	*7.0*	*6.8*	*6.4*	*7.8*	*7.6*	*8.9*	*11.2*
3.	*Chironax melanocephalus*	9.9	14.4		*5.4*	*5.5*	*5.4*	*5.7*	*6.6*	*2.7*	*5.4*	*5.3*	*5.8*	*5.2*	*7.1*	*6.7*	*9.1*	*10.0*
4.	*Cynopterus brachyotis*	12.3	16.1	14.4		*0.2*	*0*	*5.4*	*2.4*	*4.3*	*3.9*	*4.0*	*4.4*	*3.6*	*5.5*	*5.3*	*7.6*	*8.9*
5.	*Cynopterus horsfieldi*	11.9	15.8	14.1	3.3		*0.2*	*5.4*	*2.4*	*4.3*	*4.1*	*4.2*	*4.4*	*3.6*	*5.3*	*5.1*	*7.6*	*8.9*
6.	*Cynopterus sphinx*	12.2	15.9	14.4	0.2	3.3		*5.4*	*2.4*	*4.3*	*3.9*	*4.0*	*4.4*	*3.6*	*5.5*	*5.3*	*7.6*	*8.9*
7.	*Dyacopterus spadiceus*	11.5	15.5	13.0	12.9	12.9	12.7		*6.2*	*4.3*	*5.7*	*5.6*	*5.8*	*4.8*	*6.4*	*6.4*	*8.6*	*10.4*
8.	*Megaerops ecaudatus*	12.2	15.4	14.5	7.5	7.3	7.4	13.2		*5.5*	*4.9*	*5.0*	*5.2*	*4.2*	*6.5*	*6.5*	*7.7*	*10.1*
9.	*Penthetor lucasi*	8.1	12.6	9.4	11.7	11.7	11.6	10.5	11.6		*4.6*	*4.5*	*4.4*	*4.3*	*5.8*	*5.5*	*8.0*	*9.2*
10.	*Pteropus dasymallus*	12.7	17.6	14.6	13.2	12.9	13.3	12.9	13.5	12.7		*0.1*	*4.2*	*3.2*	*5.5*	*5.3*	*7.0*	*10.6*
11.	*Pteropus vampyrus*	12.0	17.4	14.9	12.6	12.7	12.7	12.4	12.7	12.0	2.3		*4.3*	*3.3*	*5.5*	*5.4*	*7.1*	*10.5*
12.	*Rousettus amplexicaudatus*	12.7	16.7	14.0	12.3	12.3	12.1	13.2	12.0	11.4	13.0	12.7		*2.8*	*6.1*	*5.7*	*8.1*	*10.5*
13.	*Eonycteris spelaea*	12.8	18.1	14.5	12.7	12.5	12.5	13.1	13.0	13.8	12.0	12.3	11.8		*5.5*	*5.3*	*7.2*	*10.2*
14.	*Macroglossus minimus*	14.8	17.9	15.8	15.6	14.6	15.6	14.0	16.0	15.5	13.0	12.4	15.2	15.7		*0.3*	*8.5*	*11.2*
15.	*Macroglossus sobrinus*	14.8	17.8	15.6	15.9	14.5	15.7	13.9	16.1	15.2	12.7	12.1	15.3	15.0	1.6		*8.1*	*11.2*
16.	*Rhinolophus pumilus*	19.6	22.6	21.8	22.0	21.2	22.0	21.3	20.9	20.4	19.6	19.3	20.9	21.0	22.1	22.0		*11.3*
17.	*Mystacina tuberculata*	20.3	24.5	21.3	21.4	20.6	21.3	22.4	20.8	21.7	23.7	23.2	22.1	23.1	21.9	22.3	21.8	

distantly related to both the outgroup sequences with an average distance value of 21.6% (data not shown).

Our estimation on the divergence time between the outgroups (Microchiroptera) and the pteropodids dates back to around 45-mya ± 4.75-mya. This estimation is very close to the earliest fossil record of bats back in the Early Eocene period (about 50 to 55-mya) and is also comparable to the estimation by Bastian *et al.* (2001), which suggests that the megachiropterans diverge from the microchiropterans at 50.2-mya. We estimated that the speciation of *Cynopterus* occurred 12-mya ± 2.5-mya, which is comparable with the divergence time of *Cynopterus* from *Ptenochirus* as calculated by Bastian *et al.* (2001) (divergence time of 12.3-mya). *Dyacopterus spadiceus* which is an endemic species of Borneo, diverged out from the other cynopterine group (*Aethalops*, *Balionycteris*, *Chironax* and *Penthetor*) around 27-mya ± 3.5-mya. The divergence time for the Malaysian fruit bats predates the Pleistocene epoch glaciations during the Quaternary Period (about 2 million to 10,000 years ago) in the Sunda Shelf. The divergence time between the subfamily Pteropodinae and Macroglossinae was however unclear.

Phylogenetic Tree Analyses

The topologies of the tree reconstructions are similar among all the three methods with high confidence levels (based on 1000 bootstrap replicates for NJ and MP and 100 replicates for ML). The NJ tree is presented in *Fig. 1* while MP and ML produced matching topologies which are combined and presented in *Fig. 2*. The relationship of the pteropodids as proposed by Andersen (1912) was also shown in both figures. Megachiroptera is monophyletic with bootstrap values between 93 to 100% for the NJ, MP and ML methods.

The NJ method formed three major groups (*Fig. 1*). The first group was formed by the genera *Macroglossus* and *Pteropus*, the second group consisted of the genera *Aethalops*, *Balionycteris*, *Chironax*, *Penthetor* and *Dyacopterus* while the genera *Cynopterus*, *Megaerops*, *Eonycteris* and *Rousettus* formed the third group.

Using the MP method, where all the characters were weighted equally, the tree length was 1465 with consistency index (CI) of 0.5468 and retention index (RI) of 0.4943. The ML tree (-ln likelihood = 8595.50874) produced a similar topology with the MP tree with only minor differences. The tree topology separated the pteropodids into five clades with a 100% bootstrap value (*Fig. 2*). Members of the family Pteropodinae formed two major groups. The first group consisted of the *Cynopterus* genera (*C. brachyotis*, *C. horsfieldi* and *C. sphinx*) and the genus *Megaerops* and the second group consisted of the genera *Aethalops*, *Balionycteris*, *Chironax*, *Penthetor* and *Dyacopterus*. Interestingly, the members of the family Macroglossinae (*Eonycteris* and *Macroglossus*) were not grouped together and did not form a monophyletic clade as proposed earlier by morphological studies. The genus *Pteropus* was grouped with the species *M.*

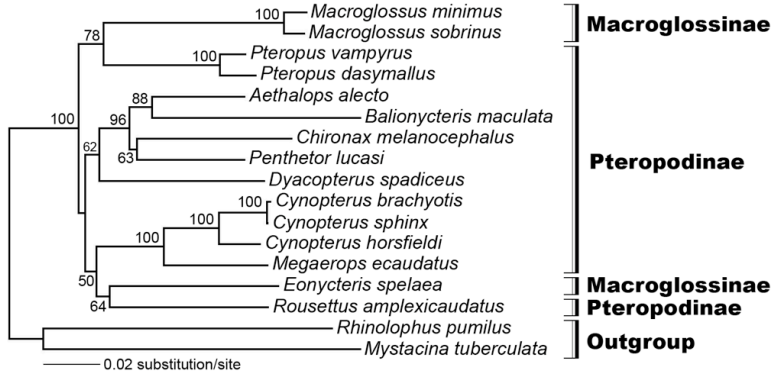

Fig. 1: NJ tree generated using the 12S rRNA, tRNA valine, and 16S rRNA gene segments of the pteropodid species used in this study (only bootstrap values >50% are shown). Values on the branches represent NJ bootstrap estimates, based on 1000 replicates.

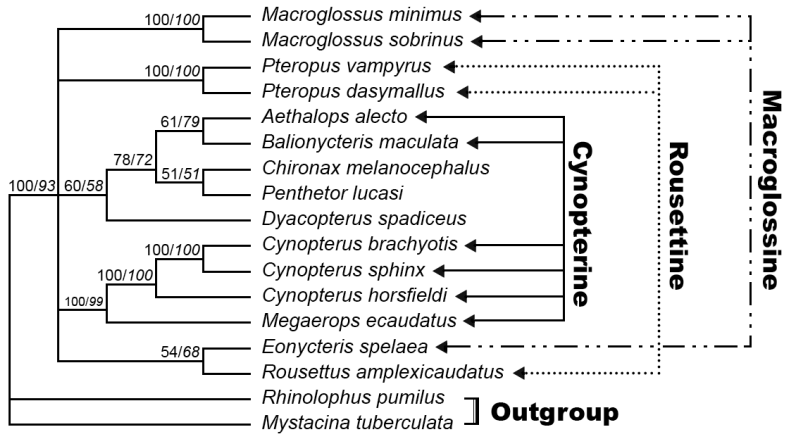

Fig. 2: Combined phylogenetic tree of unweighted MP (tree length=1465; CI=0.5468; RI=0.4943) and ML (-ln likelihood = 8595.50874) tree generated using the 12S rRNA, tRNA valine, and 16S rRNA of the megachiropteran species used in this study (only bootstrap values >50% are shown). Regular font values on the branches represent MP and italic font values represent ML bootstrap estimates, based on 1000 replicate for MP and 100 replicate for ML.

minimus and *Rousettus amplexicaudatus* was seen grouped with the species *E. spelaea*.

Considering all three phylogenetic trees, the clade consisting of the genera *Aethalops*, *Balionycteris*, *Chironax*, *Penthetor* and *Dyacopterus*, and *Cynopterus* group (all three species of *Cynopterus*) and the single species of *Megaerops ecaudatus*, was the most consistent. The NJ tree grouped together the genera *Macroglossus* and *Pteropus* into a clade with moderate support (78% bootstrap value) while MP and ML did not support the groupings. Additionally, even though *E. spelaea* and *R. amplexicaudatus* was placed together in one clade for all the three methods used, their relationship was poorly supported with low bootstrap values (64%, 54% and 68% for NJ, MP and ML, respectively).

Andersen's (1912) monograph remains the most comprehensive treatment of the pteropodids. However, recent studies using molecular approaches challenged his morphological classification and proposed a re-organisation on their taxonomic status. The contradictions between classical and molecular data on the phylogenetic relationships of the pteropodids are well documented. A study of 19 genera based on single-copy (sc) DNA hybridisation contradicted the monophyly of the cynopterine section (Kirsch *et al.*, 1995). The authors also suggested that the rousettine section and the subfamily Macroglossinae respectively are not monophyletic groupings. Colgan and Flannery (1995) used 23 informative Restriction Fragment Length Polymorphism (RFLP) markers for their analysis and included *Eonycteris* within a paraphyletic cynopterine section, thus challenging both cynopterine and macroglossine monophyly. Hollar and Springer (1997) used 12S rRNA and tRNA valine gene sequences and their results agree with the scDNA hybridisation work by Kirsch *et al.* (1995) in contradicting both rousettine and macroglossine monophyly. Romangnoli and Springer (2000) later used additional 16S rRNA gene segment apart from the one used by Hollar and Springer (1997) and further confirmed the non-monophyletic state of the macroglossine section. Next, Colgan and da Costa (2002) studied the evolution of the African pteropodid clade using 12S rDNA and *c-mos* DNA sequences. Their results confirmed the non-monophyletic state of Macroglossinae and weakly supported the cynopterine section as a monophyletic group. Similarly, Juste *et al.* (1999) conducted a study on the phylogenetic relationships among the African pteropodids using a combined 16S rRNA and cytochrome *b* (cyt *b*) gene region. Their results contradicted the classical morphology-based subdivisions of the pteropodids. Furthermore, they discovered that the Asian cynopterine group (*Cynopterus*, *Megaerops*, *Aethalops*, *Balionycteris*) did not form a monophyletic group, which also contradicted with the traditional classification (Andersen, 1912; Mickleburgh *et al.*, 1992). In the Southeast

Asia region, a study by Bastian *et al.* (2001) analysed five species of pteropodids in the Philippines using the complete sequence of the cyt *b* gene and found that the genetic divergence between *R. amplexicaudatus*, *E. spelaea*, and *C. brachyotis* was small. Recent studies by Abdullah *et al.* (2000), Abdullah (2003) and Campbell *et al.* (2004) revealed at least two cryptic species within the *C. brachyotis* complex. In this study, some aspects of the positioning of the pteropodids at the suprageneric level was clarified (e.g. grouping of the cynopterine group) but failed to elucidate at the subfamilial categories (i.e. relationships between Pteropodinae and Macroglossinae).

The Malaysian pteropodids consist of two subfamilies of the Pteropodinae and Macroglossinae with 18 species in 11 genera which are widespread in both Borneo and Peninsular Malaysia on the Asian mainland. Only one species, *E. major* is distributed in Borneo (Payne *et al.,* 1985). According to Andersen (1912), the genus *Rousettus* and *Pteropus* are assembled within the group rousettines. Rousettines has a mixed diet that includes soft fruits and / or fruit juices as well as nectar (Nowak, 1994). However, from our phylogenetic trees, both genera did not from a cluster together, which is in concordance with Ahmad (2005), Bastian *et al.* (2001), Juste *et al.* (1999), and Hollar and Springer (1997). Instead, *Rousettus* clustered with *Eonycteris* while *Pteropus* clustered with *Macroglossus*, where both *Eonycteris* and *Macroglossus* are from the subfamily Macroglossinae. The clustering of *Rousettus* and *Eonycteris* were in agreement with the findings by Rickart *et al.* (1989) who observed a close similarity of the morphological features and identical chromosome number, $2n = 36$ between *Rousettus* and *Eonycteris*. In conclusion, our phylogenetic analyses reject the sister-group relationship of *Rousettus* and *Pteropus* within the rousettine section.

Within the subfamily Pteropodinae, the clustering of the three species of *Cynopterus* with *Megaerops* and between *Aethalops* and *Balionycteris* in our study is similar with the results of the analysis done by Juste *et al.* (1999). According to traditional classification (Rickart *et al.*, 1989; Mickleburgh *et al.*, 1992), the cynopterine group (consisting of the genera *Aethalops*, *Balionycteris*, *Cynopterus* and *Megaerops*) are grouped in a monophyletic clade. However, our phylogenetic trees divided these genera into two well-supported clades (*Figs. 1* and *2*) which appeared to be non-monophyletic.

CONCLUSIONS

Overall, the phylogenetic analysis in this study was able to clarify some confusion on the relationships among the pteropodids. Our results reconfirmed some of the findings by several authors (using molecular approaches) particularly about the obscure monophyletic status of *Pteropus* and *Rousettus*, and also the monophyletic status of the cynopterine group. Here, we provide the first registered 12S rRNA, tRNA valine and 16S rRNA sequence records for *D. spadiceus* in the GenBank database. Finally, the 12S rRNA, tRNA valine and 16S rRNA genes are highly conserved and unable to discriminate the evolutionary relationships among the Malaysian pteropodids at the species level. Further studies should explore using fast evolving genes (e.g. control region and cytochrome *b*) and to include all of the pterodid species found in Malaysia and the rest of the Sunda region. Also, each species should be represented by more than one sample which is the major weakness of this study.

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