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Phylogenetic Analysis of the Malaysian *Rhinolopus* and *Hipposideros* Inferred from Partial Mitochondrial DNA Cytochrome *b* Gene Sequences

Sazali, S.N.*, Besar, K. and Abdullah, M.T.
Department of Zoology, Faculty of Resource Science and Technology,
Universiti Malaysia Sarawak,
94300 Kota Samarahan, Sarawak, Malaysia
**E-mail: ssnurlydia@frst.unimas.my*

ABSTRACT

The phylogenetic relationships among 10 species of *Rhinolophus* and 10 species of *Hipposideros* from Borneo and Peninsular Malaysia were successfully inferred from the partial mitochondrial DNA (mtDNA) cytochrome (cyt) *b* sequences. Of the 413 nucleotide positions examined, there were 171 positions (41.4%), of which 164 positions (95.9%) were parsimoniously informative. The phylogenetic trees reconstruction using neighbour-joining (NJ), unweighted maximum parsimony (MP) and maximum likelihood (ML) methods suggest the monophyletic clustering of these families. The interspecific relationships within Rhinolophidae were completely resolved, while those within Hipposideridae were not fully resolved, as supported by the low bootstrap values. Overall, the phylogenetic analysis using partial mtDNA cyt *b* gene was useful to discriminate these complicated taxa and successfully revealed the misidentification of several specimens before due to their similar morphologies.

Keywords: Cytochrome *b*, *Hipposideros*, mitochondrial DNA, phylogenetics, *Rhinolophus*

INTRODUCTION

Rhinolophus (Horseshoe bats) and *Hipposideros* (Roundleaf bats) are widely distributed throughout the tropic, sub-tropic and temperate zones of the Old World region (Corbet & Hill, 1992; Feldhamer *et al*., 1999; Hutson *et al*., 2001). In Malaysia, there are currently 22 *Rhinolophus* species recorded with 18 species found in Peninsular Malaysia and 11 species in Borneo. Generally, the rhinolophids are small to medium in size, having an elaborate complex noseleaf and a raised portion called sella that is very useful for identification among the species of this genus (Payne *et al*., 1985; Corbet & Hill, 1992). The ears are sorted from moderate to large sized with a moderate long tail that is completely enclosed within their interfemoral membrane (Payne *et al*., 1985; Vaughan, 1986; Corbet & Hill, 1992).

On the other hand, 17 *Hipposideros* species are currently recorded, in which 16 species are distributed in Peninsular Malaysia and 10 species are found in Borneo (Payne *et al*., 1985; Corbet & Hill, 1992; Khan, 1992; Koopman, 1994). Varying from small to moderate large in size with no sella, the hipposiderids have an elaborate noseleaf with a horse-shoe shaped anterior leaf while the posterior leaf is low and rounded that is divided into several pockets by vertical septa (Payne *et al*., 1985; Corbet & Hill, 1992; Francis, 2001). Their ears range from moderate small to large size with a low antitragus, having very

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*Corresponding Author

small eyes and the tail is short to moderate long, which is completely enclosed in the interfemoral membrane (Payne *et al*., 1985; Corbet & Hill, 1992).

The rhinolopids and the hipposiderids are generally found roosting in caves, tunnels, buildings, hollow trees and foliage including rock crevices recorded mostly from the tall forest understorey (Payne *et al*., 1985; Corbet & Hill, 1992). The closed association between the two families has led to many arguments in their classification and grouping. Some authors, including Vaughan (1986), Findley (1993), Wilson & Reeder (1993) and Koopman (1994), had classified both *Rhinolophus* and *Hipposideros* into a single family of Rhinolophidae alone, while Corbet & Hill (1992), Hutson *et al*. (2001) and Simmons (2005) had grouped these genera separately into each distinct family of Rhinolophidae and Hipposideridae.

On top of that, there is a lack of current information on the taxonomic and phylogenetic relationships of Rhinolophidae and Hipposideridae, particularly in Malaysia. A similar study was done by Wang *et al*. (2003), but using only the specimens from China with mostly different target species. A preliminary study by Besar *et al*. (2005) successfully revealed the phylogenetic relationships of only five Bornean *Rhinolophus* species. Paul (2007), however, was unable to resolve the interspecific relationships of *Hipposideros* in Borneo, using the combined 12S and 16S mtDNA sequences, due to unstable phylogenies supported by low bootstraps values.

Therefore, there is a need to evaluate the interspecific and intraspecific relationships of Rhinolophidae and Hipposideridae in Malaysia, as well as to examine the monophyletic status of these two families. *Nycteris tragata* (family: Nycteridae) and *Megaderma spasma* (family: Megadermatidae) were used as the outgroups as they are classified together with these two families in the superfamily of Rhinolophoidea. This is to reveal the relationship at the family level for both Rhinolophidae and Hipposideridae.

MATERIALS AND METHOD

Tissue samples were collected from 10 species of *Rhinolophus* and 10 species of *Hipposideros*, including other representatives from *Nycteris tragata* and *Megaderma spasma* as the outgroups. The bats were collected using standard mist-nets and four-bank harp traps (Mohd-Azlan *et al*., 2004). The selected bats were euthanised using chloroform and tissue samples were preserved in 95% ethanol. Some additional samples were taken from alcohol preserved specimens from Universiti Malaysia Sarawak (UNIMAS) Zoological Museum and the Department of Wildlife and National Park (DWNP) Museum. The DNA extractions of the tissues samples were made following Grewe *et al*. (1993) and the amplification was done in polymerase chain reaction (PCR) *et al*using a pair of cyt *b* primer; GludG-L: 5'-TGACTTGAARAACCAYCGTTG-3' and CB2-H: 5'- CCTCAGAATGATATTTGTCC TCA-3' (Palumbi *et al*., 1991).

A total reaction volume of 25µl comprising of 2.5 µl 10× PCR buffer was used, 1.5 µl magnesium chloride (25mM), 0.5 µl dNTP (10mM), 1.25 µl of each forward and reverse primers (10µM), 15.8 µl deionised water, 2.0 µl DNA template and 0.2 µl *Taq* DNA polymerase. The amplification process included initial denaturation at 93°C for 2 minutes, denaturation for 30 cycles at 93°C for 1 minute, primer annealing at 56°C for 1 minute, polymerase extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes. The PCR products were loaded into 1% agarose gel containing ethidium bromide and run for about 45 minutes at 90V. Fragment sizes of the amplified products were estimated to be between 400 bp to 500 bp length using a low range DNA ladder (100 bp). The PCR products were then purified using a purification Kit following the protocol provided by the manufacturer (Fermentas), purposely to remove any trace of contamination that might be present in the PCR products including salt, PCR reagents and primer-dimer before being sent to a private laboratory (First Base Sdn. Bhd.) for DNA sequencing. Only

the forward strands (GludG-L) were sequenced using the ABI ® 377 DNA automated sequencer with the ABI PRISM BigDye® Terminator version 3.0 Cycle Sequencing Kit.

All the sequences were uploaded into GenBank and each specimen has been provided with accession number for future revisions (EF095237 and EF108140 to EF108177)and were aligned using the Clustal X 1.81 program (Thompson *et al*., 1997) and saved in clustal and nexus formats. The nucleotide compositions and genetic pairwise distances among the examined species were calculated using the Kimura 2-parameter model (Kimura, 1980), whereas the phylogenetic relationships of the species were analysed using the Phylogenetic Analysis Using Parsimony (PAUP*) program version 4.0 beta (Swofford, 1998) and constructed using the neighbour-joining (NJ), unweighted maximum parsimony (MP) and maximum likelihood (ML) methods.

The NJ clustering was performed using the Kimura 2-parameter evolution model (Kimura, 1980) and the MP method was conducted using full heuristic search while ML analysis corresponded to the Hasegawa, Kishiro and Yano of HKY85 evolutionary model (Hasegawa *et al*., 1985). All trees were rooted with *N. tragata* and *M. spasma* as the outgroups. Phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replications of data sets for both NJ and MP, whereas for ML, 100 replications (Hedges, 1992) of data sets were applied.

According to Miyamoto & Boyle (1989) and Irwin *et al*. (1991), the transversion substitutions in mammals showed a linear relationship with time. The estimation of divergence (Saitou & Nei, 1987) between the ingroups (Rhinolophidae and Hipposideridae) and the outgroups (Nycteridae and Megadermatidae) of the mtDNA cyt *b* gene was calculated using a constant transversion rate of 0.2% per million years ago (mya) (Bastian *et al*., 2001), where each species was grouped into their respective families and the rate calculated were multiplied by 0.2%.

RESULTS

Eighty five specimens from 10 *Rhinolophus* species, 10 *Hipposideros* species, *N. tragata* and *M. spasma* (Table 1) were successfully sequenced and analysed, together with a sequence of *H. armiger* (AF451332), where the sequences began at 7 bp until 419 bp of the 1140 bp complete mtDNA cyt *b* sequence (*H. armiger*; DQ297585) representing 36.2% of the total cyt *b* gene sequence.

Of the total 413 bp length nucleotide sequences analysed, 171 positions (41.4%) were variable, in which 164 positions (95.9%) of the variable sites were parsimoniously informative. The nucleotide translation into a total of 137 amino acid sequences produced 25 variable positions (18.25%), in which 24 positions (96.00%) of the variable sites were parsimoniously informative. The empirical base compositions of the mtDNA cyt *b* among the examined species were T (26.8%), C (31.1%), A (27.6%) and G (14.5%). The frequencies of T and A (54.4%) were slightly higher than those of C and G (45.6%), resulting in anti-G bias sequenced, which is a characteristic for the mitochondrial gene (Cantatore *et al*., 1994; Briolay *et al*., 1998).

Meanwhile, the genetic pairwise distances calculated using the model of Kimura 2-parameter among the species of *Rhinolophus*, *Hipposideros*, *Nycteris*, and *Megaderma* are shown in Table 2. Generally, the percentage of genetic distance within the genus *Rhinolophus* ranged from 3.7% (between *R. luctus* and *R. trifoliatus*) to 14.2% (between *R. sedulus* and *R. acuminatus*), with a mean of 9.5% genetic distance. In *Hipposideros*, the percentage of genetic distance ranged from 7.0% (between *H. armiger* and *H. larvatus*) to 16.5% (between *H. bicolor* and *H. cervinus; H. bicolor* and *H. coxi*), with a mean of 12.0% genetic distance.

Overall, the average genetic distance between *Rhinolophus* and *Hipposideros* was 17.0%. The average genetic distance between *Rhinolophus* and *Nycteris* was 22.1% and that between *Hipposideros* and *Nycteris* was 20.8% respectively, whereas the average genetic

TABLE 1
Scientific and local name of rhinolophid and hipposiderid species, sample collection, sample size and GenBank accession numbers used in the study

		Species	Common name	Sample collection			n	Genbank accession no.
				Swk	Sbh	PM		
Rhinolophidae	***Rhinolophus***	*R. acuminatus*	Acuminate horseshoe bat		√		2	EF108154, EF108155
		R. affinis	Intermediate horseshoe bat	√		√	9	EF108156 to EF108160
		R. borneensis	Bornean horseshoe bat	√			3	EF108161, EF108162
		R. creaghi	Creagh's horseshoe bat		√		2	EF108163, EF108164
		R. luctus	Great woolly horseshoe bat	√		√	5	EF108165, EF108166
		R. philippinensis	Philippine horseshoe bat	√	√		5	EF108167 to EF108169
		R. pusillus	Least horseshoe bat	√			2	EF108170, EF108171
		R. sedulus	Lesser woolly horseshoe bat	√		√	5	EF108172 to EF108174
		R. stheno	Lesser brown horseshoe bat			√	1	EF108175
		R. trifoliatus	Trefoil horseshoe bat	√		√	4	EF108176, EF108177
Hipposideridae	***Hipposideros***	*H. armiger*	Great roundleaf bat				1	AF451332
		H. ater	Dusky roundleaf bat	√			3	EF108139, EF108140
		H. bicolor	Bicolored roundleaf bat	√		√	6	EF108142, EF108143
		H. cervinus	Fawn roundleaf bat	√			4	EF108141, EF108144, EF108146
		H. cineraceus	Ashy roundleaf bat	√		√	5	In progress.
		H. coxi	Cox's roundleaf bat	√			5	EF108145, EF108147, EF108148
		H. diadema	Diadem roundleaf bat	√			4	EF108149
		H. dyacorum	Dayak roundleaf bat	√			4	EF108150, EF108151
		H. galeritus	Cantor's roundleaf bat	√			5	In progress.
		H. larvatus	Intermediate roundleaf bat	√			3	EF108152, EF108153
		H. ridleyi	Ridley's roundleaf bat	√			4	EF095237
Outgroups								
		Nycteris tragata	Hollow-faced bat	√		√	2	In progress.
		Megaderma spasma	Lesser false vampire	√			2	In progress.
						Total	86	

Swk = Sarawak, Sbh = Sabah, PM = Peninsular Malaysia, n = number of samples

distance between *Rhinolophus* and *Megaderma* was 15.1% and that between *Hipposideros* and *Megaderma* was 16.2%, respectively. Additionally, the estimation on the times of divergence is shown in the NJ topology (*Fig. 1*). Meanwhile, the divergence times between Rhinolophidae and Hipposideridae was predicted at around 31.5 mya ± 4.5 mya.

Phylogenetic tree constructions using the NJ (*Fig. 1*), MP (*Fig. 2*), and ML (*Fig. 3*) methods suggested that Rhinolophidae and Hipposideridae formed their own monophyletic group, with relatively low to moderate bootstrap support (68% in NJ; 73% in MP; 80% in ML), although the groupings of the examined species in both the families were slightly different where the arrangements were similar, as inferred by the different methods.

In NJ (*Fig. 1*), Rhinolophidae was divided into four sub-groups; where Group 1 consisted of

TABLE 2
Genetic pairwise distance (%) among 10 *Rhinolophus* species, 10 *Hipposideros* species, *N. tragata* and *M. spasma* analysed in this study based on mtDNA cyt b gene sequences. Distances were calculated using the Kimura 2-parameter model (Kimura, 1980)

Species	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]
[1] *R. acuminatus*																							
[2] *R. affinis*	10.5																						
[3] *R. borneensis*	12.2	11																					
[4] *R. creaghi*	8.6	7.7	11.8																				
[5] *R. luctus*	12	11.7	12.1	10.6																			
[6] *R. philippinensis*	10.6	10.5	6.8	10.5	12.2																		
[7] *R. pusillus*	10.8	11.2	5.6	9.4	11.5	3.9																	
[8] *R. sedulus*	14.2	12.4	11.8	12.5	7.1	12.1	11.7																
[9] *R. stheno*	8.3	7.1	11.5	4.6	11.8	11.1	10.3	12.6															
[10] *R. trifoliatus*	12.1	10.8	11.9	11.1	3.7	12.4	11.3	7.7	12.3														
[11] *H. armiger*	14.9	14.8	19.6	13.7	16.1	16.4	15.7	16.6	13.6	17.2													
[12] *H. ater*	17.2	19	21.8	18.4	17.9	18.9	18.9	20.4	16.9	18.7	13.6												
[13] *H. bicolor*	16.8	17.9	18.8	18	19.8	18.3	17.3	20.4	16.5	19.2	14.5	10.7											
[14] *H. cervinus*	16.3	16.2	18	14.5	17.1	17.1	16.2	17.9	13.9	17	14.4	16.8	16.5										
[15] *H. cineraceus*	16.6	17.5	17.8	15.6	16.3	15.7	15.1	17.1	14	17.3	12.7	10.8	11	14.4									
[16] *H. coxi*	17.4	18.8	19.5	16.2	15.5	18	17.6	18.2	16.3	15.7	14.8	14.4	16.5	13.8	16.2								
[17] *H. diadema*	17.5	17.7	17.7	16.2	16.6	16.4	15.1	17.4	16.2	18.1	10.5	12.8	15.1	13.6	11.5	15.9							
[18] *H. dyacorum*	14.8	18.1	19.3	15.7	17.4	17.3	16.1	19.2	15.3	17.9	11.9	9.6	10.3	12.1	8.8	14.4	9.8						
[19] *H. galeritus*	14	14.9	16.9	15.4	17.3	14.8	13.8	15.7	13.5	16.6	13	13.9	14.3	11	12.2	14.2	12.4	11.3					
[20] *H. larvatus*	13.1	13.1	16	14.4	15	14.2	14.2	15.9	13	15.4	7	13.5	13.8	14.3	13.5	16.1	10.9	12.2	12.7				
[21] *H. ridleyi*	14.7	16.7	18.5	16.6	16.3	16.4	15.8	17.7	13.7	17.2	14.1	8.8	10.5	13.9	9.4	15.4	11.2	8.5	12.6	13.5			
[22] *N. tragata*	21.2	22.4	23.8	20.3	21.1	22.7	22	22.3	19.5	22.3	22.3	21.1	21.2	19.8	21.9	21.4	21.2	18.9	18.9	21.3	21.9		
[23] *M. spasma*	16.1	14.1	16.3	13.6	15.3	15.2	13.4	15.7	13.2	16.3	14.9	17.2	16.9	16.3	15	17.6	16.4	15.4	15.3	13.6	17.7	17.4	

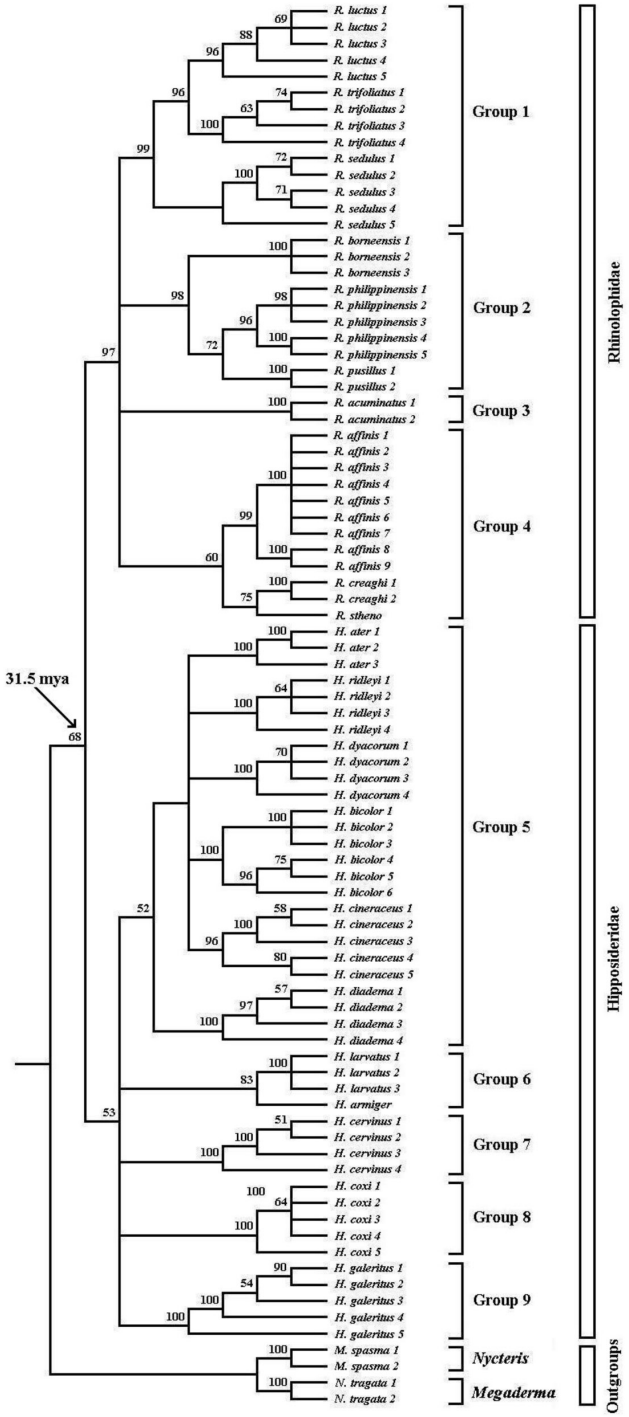

Fig. 1: Phylogentic relationships of rhinolophids *and* hipposiderids *under study based on 413 mtDNA cyt* b *gene sequences. The phylogeny is a single tree recovered using NJ analysis. Values on the branches represent NJ bootstrap estimates, based on 1000 replicates. Only bootstrap values >50% are shown*

R. luctus, R. trifoliatus and *R. sedulus*, Group 2 consisted of *R. borneensis, R. philippinensis* and *R. pusillus*, Group 3 consisted of *R. acuminatus* alone and Group 4 consisted of *R. affinis, R. creaghi*, and *R. stheno*. The clustering result within this family was supported by 97% of the bootstrap values. In Hipposideridae, five sub-groups were identified, in which, *H. ater, H. ridleyi, H. dyacorum, H. bicolor, H. cineraceus*, and *H. diadema* were clustered in Group 5, whereas *H. larvatus* and *H. armiger* were categorized in Group 6, *H. cervinus* in Group 7, *H. coxi* in Group 8, and *H. galeritus* in Group 9. However, the grouping within Hipposideridae was only supported by 53% of the bootstrap values.

Using the MP analysis with the unweighted characters, the tree was 673 bp with a consistency index (CI) of 0.3507 and a retention index (RI) of 0.8544 (*Fig. 2*). The phylogeny and branching within Rhinolophidae was similar to the NJ clustering, supported by 84% of the bootstrap values. Within Hipposideridae, four sub-groups were obtained. The fifth group was formed by *H. ater, H. ridleyi, H. dyacorum, H. bicolor* and *H. cineraceus*, while the sixth group was formed by *H. diadema, H. larvatus* and *H. armiger*, the seventh group comprised of *H. cervinus* and *H. coxi* and the eighth group consisted of *H. galeritus* alone. Similarly, the clustering within this family was supported by only 55% of the bootstrap values.

Using the ML procedure (-Ln likelihood = 1461.22137) (*Fig. 3*), the groupings within Rhinolophidae (83% of bootstrap value) were similar to those obtained using the NJ and MP. The ML analysis, however, produced different groupings within Hipposideridae (75% of the bootstrap value), in which three sub-groups were formed. Group 5 consisted of *H. ater, H. ridleyi, H. dyacorum, H. bicolor* and *H. cineraceus*, whereas Group 6 was represented by *H. diadema, H. larvatus* and *H. armiger*, and the remaining species of *H. cervinus, H. coxi* and *H. galeritus* were clustered together in Group 7.

DISCUSSION

All the phylogenies of NJ, MP and ML methods, inferred from 413 bp of the mtDNA cyt *b* gene, resulted in the monophyletic clustering of *Rhinolophus* and *Hipposideros*. The genetic pairwise distances obtained from the present study were also comparable to those of Wang *et al*. (2003) in classifying the two groups into different families. The separation of Rhinolophus and Hipposideros was further supported by the allozyme variability (Maree & Grant, 1997).

Other molecular results revealed that the Rhinolophidae and Hipposideridae are sister taxa, as reported by Maree & Grant (1997), Jones *et al*. (2002), Teeling *et al*. (2002; 2005), Guillén *et al*. (2003) and Gunnell & Simmons (2005). Similar results were also obtained through karyotypical and morphological analyses presented by Bogdanowicz & Owen (1992; 1998), as well as Hand & Kirsch (1998). However, the present study was unable to reveal the ancestral lineage of the family, as they were supported by only moderate bootstrap values which might be due to the short sequence length analysed, small sample sizes, as well as incomplete representatives of the whole Malaysian rhinolophids and hipposiderids, respectively.

According to Hand *et al*. (1994), the oldest bat fossils of Rhinolophidae were recorded from the late Oligocene-early Miocene in Lake Eyre Basin, Australia. Guillén *et al*. (2003) reported that the bat fossils of Hipposideridae had been recorded from the Oligocene of Africa and the Miocene of Africa, Australia and South East Asia. In the present study, Rhinolophidae and Hipposideridae were predicted to have diverged from each other around 31.5 mya ± 4.5 mya, whereas Guillén *et al*. (2003) estimated the event happened approximately 35 mya. Guillén *et al*. (2003) also suggested that the origin of the *Rhinolophus* species was from Europe, which contradicted with the findings of Bogdanowicz (1992), and Bogdanowicz & Owen (1992), who suggested that the origin of Rhinolophidae was from Asia.

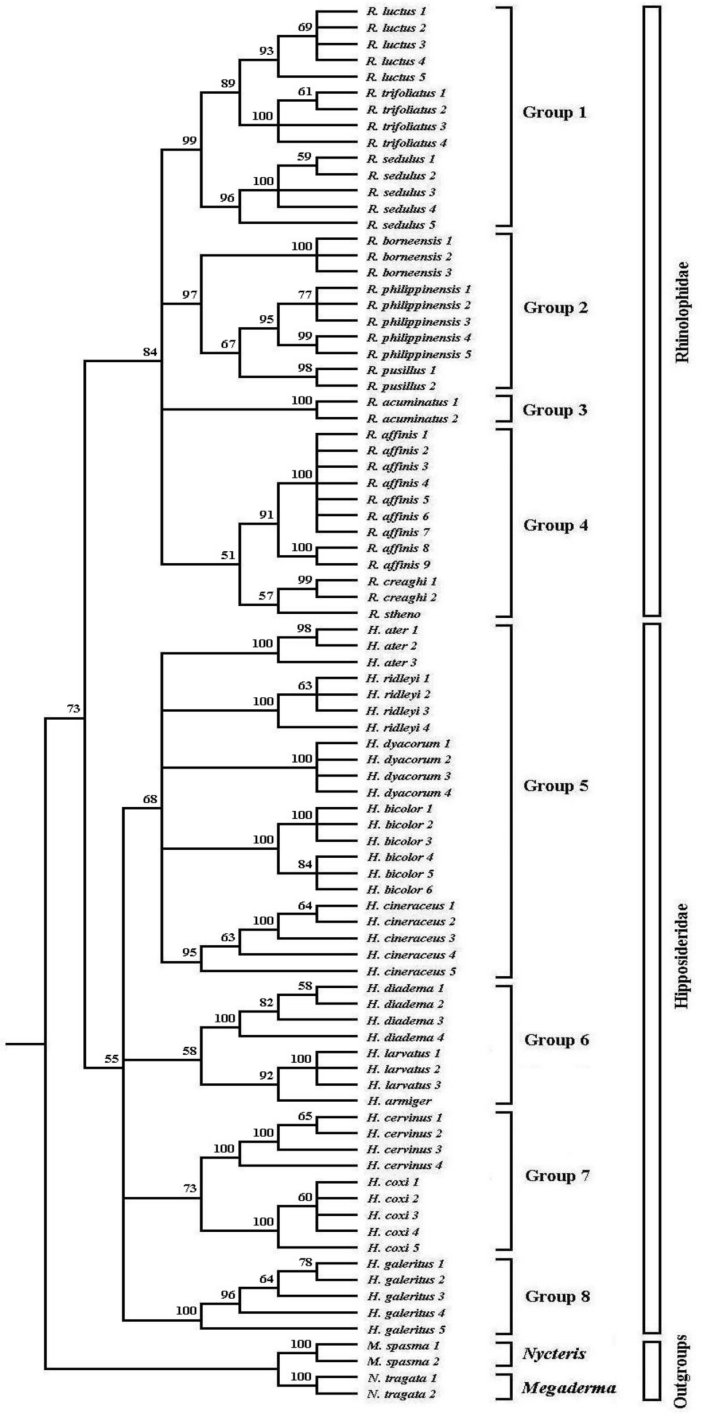

Fig. 2: The unweighted and rooted MP tree based on nucleotide data set of partial mtDNA cyt b *gene (tree length=673; CI=0.3507; RI=0.8544). The values on the branches represented the MP bootstrap estimates, based on 1000 replicates. Only bootstrap values >50% are shown*

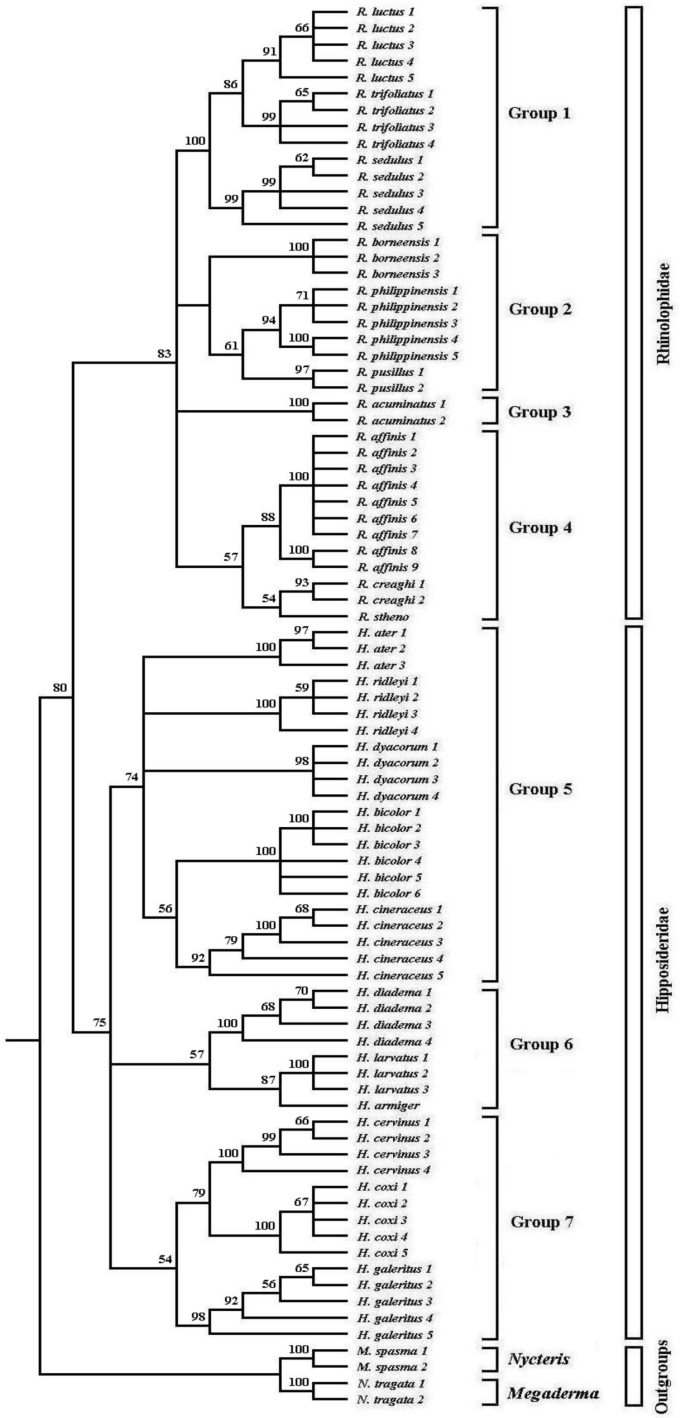

Fig. 3: Rooted ML tree (-Ln likelihood=1461.22137) generated based on the nucleotide data set of partial mtDNA cyt b *gene. Values on the branches represented the ML bootstrap estimates, based on 100 replicates. Only bootstrap values >50% are shown*

In Group 1, the grouping of *R. luctus, R. trifoliatus* and *R. sedulus* showed similar arrangement, as reported in phenetic clustering by Bogdanowicz (1992) and the phylogenetic studies by Bogdanowicz & Owen (1992) and Guillén *et al*. (2003). The arrangement of *R. luctus* and *R. trifoliatus* in a sub-group, which was derived from *R. sedulus*, was fully supported by high bootstrap value. Bogdanowicz & Owen (1992), through their ordination method, had classified these species into the *trifoliatus* group that further assigned it as the sub-genus, *Aquias* by Guillén *et al*. (2003). Meanwhile, Payne *et al*. (1985) stated that this group was morphologically similar with long body fur and the presence of lateral lappets, with *R. luctus* being the easiest species to identify, as they are the largest rhinolophid.

The clustering of *R. borneensis, R. philippinensis*, and *R. pusillus* in Group 2 is congruence to the species grouping proposed as the sub-genus *Rhinophyllotis* by Guillén *et al*. (2003). However, Bogdanowicz (1992) and Bogdanowicz & Owen (1992) placed *R. philippinensis* into its own group, together with *R. marshalli* and *R. macrotis* that were not examined in the present study. According to Payne *et al*. (1985), *R. borneensis* shares similarities of their external morphological characters with *R. pusillus*, but the latter possesses shorter forearm length and has a very small noseleaf. Beside that, *R. philippinensis* is easily distinguished from the other two species by its larger body size.

In addition, Guillén *et al*. (2003) also included *R. acuminatus* as the basal species within this sub-genus. However, the *R. acuminatus* analysed in this study was independently clustered in Group 3, although it was found to be morphologically similar to the other rhinolophid species in Group 4 (Payne *et al*., 1985; Corbet & Hill, 1992). Bogdanowicz (1992) and Bogdanowicz & Owen (1992) also placed this species out of the group consisting of *R. affinis, R. creaghi* and *R. stheno*.

The association between *R. affinis, R. creaghi* and *R. stheno* in Group 4 is similar to Bogdanowicz's (1992) phenogram, Bogdanowicz & Owen (1992) and Guillén *et al*. (2003), who categorised these species into *Coelophyllus* sub-genus, together with the presence of *R. arcuatus*. However, using the robust analysis from the ordination technique, Bogdanowicz (1992) classified *R. affinis* and *R. acuminatus* into the *rouxi* group, where the researcher separated *R. arcuatus, R. creaghi* and *R. stheno* into the *euryotis* group.

From this study, some specimens that were primarily assigned as *R. arcuatus (R. affinis 1, R. affinis 2, R. affinis 3* and *R. affinis* 5) and *R. acuminatus (R. affinis* 6) in the field were misidentified (*Figs. 1*, *2* and *3*) and clustered together into the *R. affinis clade*. They were only differed by a value of less than 2% divergence that further confirmed the recognition of these specimens as the *R. affinis*. Bradley & Baker (2001) noted that a genetic distance of less than 2% in cyt *b* sequences of mammals was typical of population and intraspecific variation.

For the Hipposideridae in the present study, the species grouping proposed by Hill (1963), Corbet & Hill (1992) and Simmons (2005) was not supported, as the members of the *bicolor* group seemed to be paraphyletic, where the species of *H. cervinus, H. coxi*, and *H. galeritus* were clustered in Group 7. This finding was also at variance with the phylogenetic studies of Bogdanowicz & Owen (1998) and Paul (2007), who also used similar species. These discrepancies could have occurred due to the different data implemented by each author. Only the ML analysis was further discussed due to its moderate bootstrap support of 75% that was regarded as sufficiently resolved topology (Huelsenbeck & Hillis, 1993).

The clustering among *H. ater, H. ridleyi, H. dyacorum, H. bicolor*, and *H. cineraceus* in Group 5 were generally supported by Payne *et al*. (1985), Corbet & Hill (1992) and Bogdanowicz & Owen (1998). Generally, these species share similar characters of not possessing lateral leaflets, and having similar facial ornamentation with simple noseleaves (except for *H. ridleyi* that possesses large noseleaf) (Payne *et al*., 1985; Khan, 1992). Moreover, the darker brown noseleaf colour and the pointed ear tips of

H. dyacorum are useful for discriminating this particular species from the others.

In Group 6, the close relationship between *H. larvatus* and *H. armiger* as sister clades was supported, and the phylogeny showed a similar arrangement as that described by previous authors, including Allen (1938) and Wang *et al*. (2003), together with the presence of *H. diadema* in the group (Bogdanowicz & Owen, 1998). In addition, this arrangement showed a similar result to Paul (2007), who used a combination of the 12S and 16S mtDNA sequence.

The grouping of *H. diadema, H. larvatus* and *H. armiger* was also supported by Payne *et al*. (1985), Khan (1992), Koopman (1994) and Kingston *et al*. (2006), as these species have three or more lateral leaflets. However, *H. larvatus* can be easily recognised by the length of the forearm, which ranges from 52 to 65 mm, whereas *H. armiger* (FA: 85-97 mm) and *H. diadema* (FA: 76-87 mm) can be identified using their body coloration (Payne *et al*., 1985; Khan, 1992; Kingston *et al*., 2006).

The remaining species of the bicolor group, including *H. cervinus, H. coxi* and *H. galeritus* were independently clustered in Group 7, although the arrangement was poorly supported with 54% of the bootstrap value. Previously, Hill (1963) had missed several diagnostic characters that were obviously useful in differentiating *H. cervinus* and *H. galeritus*. Later, Jenkins & Hill (1981) revealed that both *H. cervinus* and *H. galeritus* were wrongly classified as the same species due to the absence of several possible characters which might be useful for discriminating these species, as applied by Kitchener *et al*. (1993a,b). This included the details of the nose leaf structure and the measurements of the second phalanx on the third digit.

Both Payne *et al*. (1985) and Corbet & Hill (1992) supported the correlation among *H. cervinus, H. coxi* and *H. galeritus* as these species have two lateral leaflets and a similar facial structure. In addition, *H. cervinus* and *H. galeritus* can be differentiated from each other through the noseleaves structure and tail length, while *H. coxi* possesses darker body coloration and larger, more complex noseleaf than the other two species.

Overall, the findings of this study have shown that the partial mtDNA cyt *b* gene is useful to resolve the interspecific relationships within selected species of Rhinolophidae, but was unsuccessful in completely reviewing the phylogenetic relationships among the selected Malaysian *Hipposideros*.

CONCLUSION AND RECOMMENDATIONS

In conclusion, the phylogenetic relationships inferred from the partial mtDNA cyt *b* gene supported the monophyletic grouping of Rhinolophidae and Hipposideridae, as two different families have provided new information on the limited knowledge regarding the microchiropterans in Malaysia. The taxonomy and systematic of *Rhinolophus* are similar to the metric phenetic clustering shown by Bogdanowicz (1992) and the phylogenetic studies by Bogdanowicz & Owen (1992) and Guillén *et al*. (2003). However, the phylogenetic relationships within *Hipposideros* were incompletely resolved.

It also revealed that the misidentification of specimens in the field was common among closely related species, as the morphological characteristics of some species are similar and overlapping. Thus, correct field identification of species is important in order to infer an accurate biological diversity of the fauna and to avoid incorrect conclusions (Sazali *et al*., 2008). Although the monophyletic status of these families is currently reviewed, further molecular studies should be conducted using larger sample sizes, the complete mtDNA cyt *b* gene (approximately 1140 bp length) or other coding regions (e.g. COI, ND2), including other species, to fully assess the phylogenetic relationships of the horseshoe bats and roundleaf bats. Hopefully, the findings of this research can be applied for effective future management and conservation of these insectivorous bats, particularly in relation to the Malaysian specimens.

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