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Population Genetics of the Cave-dwelling Dusky Fruit Bat, *Penthetor lucasi,* Based on Four Populations in Malaysia

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ABSTRACT

The population genetics of *P. lucasi* was inferred using 1,061 base pairs (bp) of the Cytochrome *b* mitochondrial gene. A total of 77 individuals were classified *a priori* according to their localities, namely, Miri, Kuching, Sri Aman and Kelantan. Results showed that the populations of *P. lucasi* were separated into two haplogroups, namely, Haplogroup 1 (found in Miri and Kuching populations) and Haplogroup 2 (Miri, Kuching, Sri Aman and Kelantan populations). This separation was supported by bootstrap values in the phylogenetics analyses (94.9% in the maximum likelihood and 100% in Bayesian). A high level of genetic divergence was detected between two haplogroups (3.88%) and this separation could be related to historical events which include multiple colonisation and Pleistocene refugia during the Last Glacial Maximum ice age period. High genetic divergence within Miri (4.93%) and Kuching (4.72%) populations could be due to the presence of a species complex within the *P. lucasi* populations. The presence of haplotypes from both the populations in Haplogroup 1 and Haplogroup 2 might be due to the ability of this particular species of bats to perform long-distance flight for foraging. A high gene flow between these populations suggests a widespread female gene flow of *P. lucasi,* judging from the distance of both localities. Meanwhile, the absence of a deep structure from the haplotype trees further proves that *P. lucasi* may have had a wide dispersal ability since the Pleistocene has allowed for genetic exchange to occur between the regions in Malaysia.

Keywords: Penthetor lucasi, population study, genetic diversity, mitochondrial DNA

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INTRODUCTION

An understanding of a species population structure typically provides significant

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information to address questions relating to both past and present evolutionary and behavioural processes of organism. Thus, the introduction of molecular techniques is a great breakthrough in the pursuit of such understandings. This is especially true for studies in which traditional methods, such as the direct observation of individuals or populations, are greatly restricted (Burland & Worthington-Wilmer, 2001). Numerous studies on intraspecific phylogenetics and phylogeography of organisms have also positively impacted the current level of knowledge of species evolution and speciation.

The use of genetic markers has led to the description and a better understanding on social life (Bryja *et al.,* 2009). Today, studies on population genetics in bats have further revealed that phylogeographic variations are affected by various factors, such as seasonal migrations, geographical barriers, and past processes (Burland & Worthington-Wilmer, 2001; Bryja *et al.,* 2009). In the Indo-Malayan region, such studies have been conducted by various authors (e.g. Kitchener *et al.*, 1993a, 1993b; Schmitt *et al.*, 1995; Hisheh *et al*., 1998; Abdullah, 2003; Mahadatunkamsi *et al*., 2003; Imelda, 2007; Tingga, 2010). Other than bats, population genetics studies on other taxa in this region have also been documented, including on birds (Rahman, 2000), fish (Esa *et al.,* 2008) and frogs (Ramlah, 2009). These studies have utilised various genetic markers, such as allozymes, RNA, mtDNA and nuclear DNA.

Isolation is one of the major factors facilitating evolutionary changes. A cave is a good example of habitat isolation, which is surrounded by mosaic habitat types. However, the presence of gene flow between populations over long distances will decrease differentiation, and it is assumed that genetic structuring is weak across the macrogeographical range in migratory bats (McCracken *et al.,* 1994; Webb & Tidemann, 1996; Hisheh *et al.,* 1998; Russell *et al.,* 2005). In contrast, the non-migratory ghost bat (*Macroderma gigas*) shows a clear genetic structuring among the populations in Australia (Worthington-Wilmer *et al.,* 1994).

The dusky fruit bat or *Penthetor lucasi* was selected for this study as it is known to live specifically near total darkness in isolated caves. This particular species has gone through several taxonomic reviews from *Cynopterus* (*Ptenochirus*) *lucasi* Trouessart (1897) to *Ptenochirus lucasi* Trouessart (1904), and is presently placed in the genus *Penthetor* (Andersen, 1912; Maryanto, 2004). This bat is medium in size, with dark grey brown upperpart and pale buffy underpart. Sometimes, the specimens are observed to have a distinct dark shade at the centre of the head and paler near the eyes. It is widely distributed throughout the southern part of Thailand, Peninsular Malaysia, the Riau Archipelago, Borneo (Payne *et al.,* 1985; Corbet & Hill, 1992; Abdullah *et al.,* 2007; Francis, 2008; Abdullah *et al*., 2010) and Sumatra (Maryanto, 2004). A morphological

study on the species in Sarawak showed differences in the body and skull sizes (Sri Aman, Kuching and Miri populations). It was suggested that different ecological factors, such as breeding, crowding effect, foraging behaviour, resource availability and selective pressure, are the possible causes of the morphological variation among *P. lucasi* populations (Abd Rahman & Abdullah, 2010).

This study aimed to examine the phylogenetic relationships, diversification and genetic variation within the *P. lucasi* populations in Malaysia, inferring from the mtDNA Cytochrome *b* (Cyt *b*) gene. It was hypothesised that *P. lucasi* had high site fidelity for roosting. Thus, there would be low gene flow and high genetic divergence among the isolated roosts in Malaysia.

MATERIALS AND METHODS

Samples Collection and DNA Extraction

A total of 77 individuals of *P. lucasi* from four populations, namely Miri (33 individuals), Kuching (33 individuals), Sri Aman (six individuals) and Kelantan (five individuals), were used in this study (see Figure 1). The specimens were collected using mist nets and then euthanized using chloroform, and preserved in 95% ethanol prior to genetic analysis. Museum samples from the zoological collections at Universiti Malaysia Sarawak (Abdullah *et al.,* 2010) and the Department of Wildlife and National Park or DWNP (Pahang) were also included in this study. All the specimens used are listed in **Appendix 1.** DNA extraction was done using the cetyltrimethylammonium bromide (CTAB) method (Grewe *et al.,* 1993), with the presence of proteinase K. Extracted DNA was visualized on 1% agarose gels containing ethidium bromide, run for approximately 30 minutes at 90 V, and then photographed under ultraviolet (UV) illumination. The isolated DNA was used for further mtDNA analyses.

Polymerase Chain Reaction (PCR) and DNA Sequencing

Approximately 1061 base pairs (bp) of Cyt *b* were amplified following the standard protocol as described by Sambrook *et al*. (1989). A pair of Cyt *b* primers were used, 5'-CGAAGTTGATATGAA AAACCATCGTTG-3', and known as L14724 (forward) (Irwin *et al.,* 1991) and 5'-AACTGCAGTCATCTCCGGT TTACAAGAC-3' known as H15915 (reverse) (Irwin *et al.,* 1991). A total volume of 25 µl master mix was made comprising of 5.0 µl 5X colourless GoTaq® Flexi buffer, 1.5 µl of $MgCl_2$ solution (25 mM), 0.5 µl of dNTP mix (10 mM), 1.0 µl of each forward and reverse primers (10 mM) 15.5 µl of deionised distilled water, 1.0 µl of DNA template and 0.5 µl GoTaq® DNA polymerase (5u/µl). PCR was carried out using a thermocycler with 30 cycles inclusive of one initial denaturation at 94ºC and final extension at 72ºC for three and five minutes, respectively. The other 29 cycles consisted of denaturation at 94ºC for one minute, annealing at 40ºC for one minute and an extension at 72ºC for two minutes. Amplification products were then visualised using the agarose gel electrophoresis

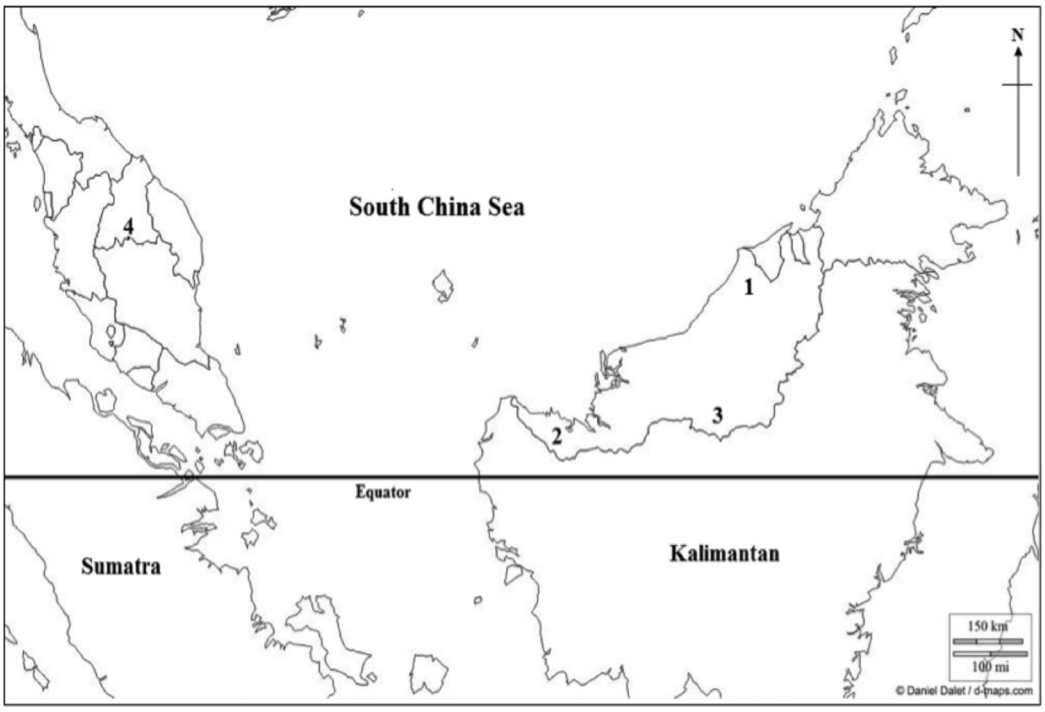

Fig. 1: Maps showing the type locality of *P. lucasi* specimens used in the molecular analyses; 1- Miri; 2 - Kuching; 3 - Sri Aman; 4 - Kelantan. Map was modified from Dalet (2010).

method. DNA Purification was done using the Promega Wizard SV Gel and PCR Clean Up System (Promega Co.). The purified samples were then sent for sequencing at a private laboratory using ABI prism ™ Big dye ™ terminator cycle sequencing Ready Reaction Kit version 3.1 or using the ABI PRISM ® 377 DNA Sequencer with the BigDye®Terminator v3.0 Cycle Sequencing Kit. The sequencing product was run using ABI 3730 XL capillary DNA sequencer (50 cm capillary).

Sequence Alignment and Phylogenetic Analyses

The DNA sequence results were displayed using the CHROMAS version 1.45 software (McCarthy, 1996). The multiple alignments of DNA sequences were done using CLUSTAL X (Thompson *et al.*, 1997) software. The pair-wise distance between the populations were computed using the Molecular Evolutionary Genetic Analysis (MEGA) software version 3.0 (Kumar *et al.*, 2004), with correction using a Kimura 2-parameter (K2P) model (Kimura, 1980). The time of divergence of bats was estimated following Brown *et al.* (1982), which was based on an evolutionary rate of Cyt *b* gene at 2% substitution rate per million years and calculated using Kimura-2 parameter distance matrix implemented in MEGA version 3.0 (Kumar *et al*., 2004).

A maximum likelihood (ML) tree was constructed by using phylogenetics analysis using Parsimony (PAUP) version 4.0beta

(Swofford, 1998), whereas a Bayesian tree was constructed using MrBayes version 3.0 (Huelsenbeck & Ronquist, 2001). The Akaike Information Criterion (AIC) was used to determine the best-fit-model of sequence evolution in the species by using Modeltest 3.7 (Pasoda & Crandall, 1998). The Maximum Likelihood (ML) and Bayesian trees were constructed based on the General Time reversible (GTR) model (Tavare, 1986), as determined by AIC. For ML, the heuristic search option was used in PAUP* with Tree-bisection-reconnection (TBR) branch swapping and 10 random additional sequence replicates. The Bayesian analysis was performed with 2 745 000 generations implementing Metropolis-coupled Markov chain Monte Carlo (MCMC) with 100 generation and burn in=1000 for summary parameter values and trees. The trees were rooted with two outgroups, namely, *Cynopterus brachyotis* (TK152458; Abd Rahman, 2010) and *Rhinolophus philippinensis* (TK152938; Abd Rahman, 2010). To obtain a graphical representation of the Cyt *b* gene variation, minimum spanning networks (MSN) of haplotypes were constructed by allowing all the required mutational steps that would eventually link the different sub-networks. These haplotype networks were generated using the programme, Network 4.5.0.2 (Fluxus Technology 2004-2008).

Population Genetic Analyses

Haplotype (*h*) and nucleotide (π) diversities (Nei & Tajima, 1981; Nei, 1987), nucleotide divergence (Da), the number of polymorphic sites (S) and the mean number of nucleotide differences (K) were calculated using the DnaSP version 4.5 (Rozas *et al.*, 2003). The Mantel test was conducted in Arlequin Version 3.0 (Exoffier *et al.,* 2005). Permutations of size 1000 were used to examine the effect of isolation-by-distance (IBD) by testing the correlation between geographical distance and genetic differentiation among the populations. The neutrality tests of Tajima's, *D* (Tajima, 1989), Fu and Li's *D** and *F** (Fu & Li, 1993) and Fu's F_s (Fu, 1997) were used to test the hypothesis that all mutations are selectively neutral (Kimura, 1983). Tajima *D* is based on the differences between the number of segregating sites and the average number of nucleotide differences (Tajima, 1989). Fu and Li's *D** and *F** tests are based on molecular polymorphism data (Fu & Li, 1993). Fu's F_s (Fu, 1997) assessment of the haplotype structure on the haplotype frequency distribution was used as an additional neutrality test. The level of population subdivision (F_{st}) (Hudson *et al.,* 1992), nucleotide subdivision (N_{st}) (Lynch & Crease, 1990), and the number of female migrant (N*m*) (Hudson *et al.,* 1992) for determining the gene flow were calculated using DnaSP version 4.5 (Rozas *et al.*, 2003). The analysis of Molecular Variance (AMOVA) was used to estimate F-statistic (Φ_{st}) (Weir & Cockerham, 1984) values in order to assess further differentiation among the populations. The significance was tested using 10 000 permutations, as performed using the Arlequin Version 3.0 software (Excoffier *et al.,* 2005).

RESULTS

Analysis of Sequence

A total of 1,061 bp of cyt *b* of 77 *P. lucasi* individuals were successfully sequenced. Out of the total, 95 were variable sites (8.95%) comprising 28 singleton sites (29.47%) and 67 parsimony informative sites (70.53%). On the average, the nucleotide composition consisted of adenosine (A) = 29.6%, thymine (T) = 24.3%, cytosine (C) = 32% and guanine (G) = 14.1%. The overall frequency distributions of nucleotides at the first, second and third codon positions [values in percentages (%); A = 26.1, 20.1, 42.6, T = 23.0, 41.2, 8.7, C = 27.0, 24.6, 44.3 and G = 23.9, 14.1, 4.3]. All the sequences were submitted to the GenBank with the accession numbers GU724879-GU724957.

Haplotypes Distribution of P. lucasi

Haplotype trees of *P. lucasi* were constructed using the maximum likelihood (ML) and the Bayesian methods (see Fig.2 and Fig.3). Generally, both trees showed the same grouping of *P. lucasi*, with only slight differences in their topology. These trees revealed the monophyly of *P. lucasi* (94.9% ML of bootstraps support; and 100% in BPP) with respect to the out-groups, *C. brachyotis* and *R. philippinensis*. Two clades were constructed from the phylogenetics trees, namely, Haplogroup 1 and Haplogroup 2. Haplogroup 1 comprised 31 haplotypes of *P. lucasi* from Miri and Kuching, while Haplogroup 2 consisted of 14 haplotypes of *P. lucasi* from Miri, Kuching, Sri Aman and Kelantan.

Haplotype Network

The phylogenetic structure among the samples from the four populations of *P. lucasi* was revealed by haplotype clustering on a minimum-spanning network (MSN) (Fig.4). Based on the unrooted network of mtDNA cyt *b*, the MSN showed a 'star-like' phylogeny in the *P. lucasi* populations in Malaysia. Furthermore, the MSN topology pattern is similar to other haplotype trees (ML and Bayesian), which include two groups of sequences from the populations of Miri-Kuching (Haplogroup 1) and Kuching-Miri-Sri-Aman-Kelantan (Haplogroup 2), respectively. Within both sub-networks, most of the haplotypes were

TABLE 1
Number of haplotypes and nucleotide diversity within each population of *P. lucasi*.

Localities	N	No. of haplotypes	Haplotype diversity (*h*)†	Nucleotide diversity (π)*†	% Pairwise divergence*†
Miri	33	26	0.985 ± 0.011	0.01584 ± 0.00321	0.00 - 4.72
Kuching	33	17	0.938 ± 0.023	0.01316 ± 0.00343	0.00 - 4.93
Sri Aman	6	3	0.733 ± 0.155	0.00082 ± 0.00023	0.00 - 0.19
Kelantan	5	4	0.900 ± 0.161	0.00528 ± 0.00105	0.00 - 0.76

N=Number of individuals
*Estimated using Kimura two-parameter distance (Kimura, 1980)
†Sites with gaps were completely excluded.

unique to individuals (30/45), while 15 haplotypes were associated with more than one individual. Haplotype frequencies were denoted by the proportional size of haplonodes. Thirty-seven mutational steps link the two haplogroups.

Both the haplogroup sub-networks were rather complex with divergent branches marked with grey nodes, indicating hypothetical haplotypes (missing haplotypes). Within haplogroup 1, five haplotypes (namely, haplotypes 1, 10, 12, 13 and 25) were shared between Miri and Kuching populations, with a high frequency suggesting the female gene flow. All the haplotypes from Miri and Kuching populations were divergent with the mutational step ranging from one to four. Within haplogroup 2, the Miri population diverged by one to five mutational steps. The Kuching population was divergent with mutational steps ranging from one to three, while the Kelantan population diverged by one to four mutational steps. All Sri Aman haplotypes were divergent with a single mutational step.

Nucleotide Divergence within and among the Populations

A total of 95 segregating sites were detected from 45 haplotypes that were distributed within and among the four populations of *P. lucasi*. From the total of 77 individuals, six haplotypes were shared between the populations, namely; H1, H10, H12, H13 and H25 and all were shared between Miri and Kuching. The population from Miri showed the highest frequency of unique haplotypes, with 26 haplotypes from a total of 33 individuals sampled (Table 1).

The genetic divergence between the haplogroups is 3.88%. The genetic divergence within the population of *P. lucasi* ranged from 0.0% to 4.9% (Table 1), whereas the divergence among population ranged from 0.003% to 0.14% (Table 2). The haplotype diversity (*h*) within the population ranged from 0.73 to 0.99

TABLE 2
Analysis of nucleotide diversity (π), net nucleotide divergence and divergence time estimates (age) among the four populations of *P. lucasi*.

Localities	Distance (KM)	% Pair-wise divergence*†	Nucleotide diversity (π)*†	Net Nucleotide divergence (D_a)	Age of divergence (Kya)#
Miri-Kuching	516.5	0.003	0.01439	-0.00220	7.5
Miri–Sri Aman	420.8	0.13	0.02073	0.02696	325
Miri-Kelantan	1324.4	0.14	0.02061	0.02626	350
Kuching-Sri Aman	210.6	0.14	0.01895	0.02878	350
Kuching-Kelantan	996.9	0.14	0.01877	0.02832	350
Sri Aman-Kelantan	1178.2	0.01	0.00463	0.00327	25

* Estimated using Kimura two-parameter distance (Kimura, 1980).
†Sites with gaps were completely excluded.

TABLE 3
Summary analysis of mtDNA cyt *b* sequences variation among the four populations of *P. lucasi* in Malaysia.

Population	N	H	S	% sdiv	*h*†	π†	K	*D*	F_s	*D**	*F**	r
Miri	33	26	73	0.00 -0.04729	0.985 ± 0.011	0.01584 ± 0.00321	16.80114	-0.29301	**-20.5431***	-0.12175	-0.21304	0.0115
Kuching	33	17	63	0.00 -0.04931	0.938 ± 0.023	0.01316 ± 0.00343	13.96212	-0.37283	**-23.0524***	0.59156	0.31485	0.0220
Sri Aman	6	3	2	0.00 -0.00189	0.733 ± 0.155	0.00082 ± 0.00023	0.86667	-0.05002	**-7.09607***	0.06221	0.03984	0.3467
Kelantan	5	4	12	0.00 -0.00759	0.900 ± 0.161	0.00528 ± 0.00105	5.60000	-0.20090	-1.16655	-0.20090	-0.21293	0.2300
Whole population	77	45	95	0.00 -0.4931	0.978 ± 0.006	0.01964 ± 0.00177	20.83288	0.22450	-6.467	-1.06638	-0.65307	0.0081

N = number of sequence; H = number of haplotypes, S = number of segregating sites, % sdiv = percentage of pair-wise sequence divergence (estimated by K2P distance (Kimura, 1980)); *h* = haplotype diversity; π = nucleotide diversity, K = average number of nucleotide differences; *D* = Tajima's statistics (Tajima 1989), F_s = Fu's statistics (Fu 1997), *D** and *F** = Fu and Li's statistics (Fu & Li, 1993); r = raggedness statistics.
**P* < 0.05, Significance was calculated using coalescent simulation in DnaSP version 4.0 (Rozas *et al.*, 2003).
† Sites with gap were completely excluded.

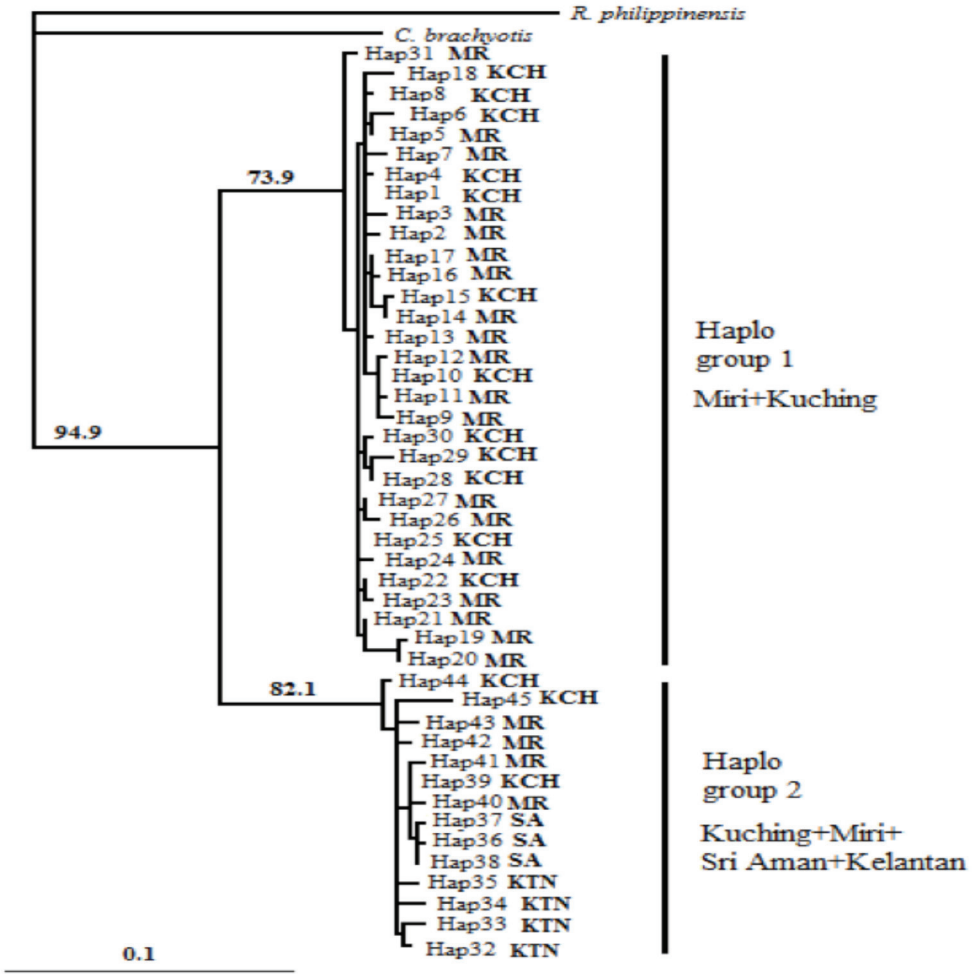

Fig. 2: A maximum likelihood 50% majority rule consensus tree of mtDNA cyt *b* of *P. lucasi*. Bootstrap values above 50 % are indicated below branch. KCH - Kuching; KTN - Kelantan; MR - Miri; SA - Sri Aman.

TABLE 4
Measures of geographical population differentiation in *P. lucasi* based on the analysis of molecular variance (AMOVA)

	Variance component	Percentage % of variation	F-statistic (Φ)	Significant(*P*)
Among groups	9.23414	46.42	Φ_{ct} = 0.46417	0.49970
Among population within groups	3.73415	18.77	Φ_{sc} = 0.35030	0.00000*
Within population	6.92574	34.81	Φ_{st} = 0.65187	0.00000*

*Significant P <0.05

TABLE 5
Genetic differentiation matrix of the populations calculated by Φ_{st} and *P* values is shown in parenthesis.

	Miri	Kuching	Sri Aman	Kelantan
Miri	-			
Kuching	- 0.01525 (0.55856)	-		
Sri Aman	0.65238 (0.0000)*	0.70842 (0.0000)*	-	
Kelantan	0.6335 (0.0000)*	0.69223 (0.0000)*	0.54475 (0.00293)*	-

*Significant $P < 0.05$ with 1000 permutation.

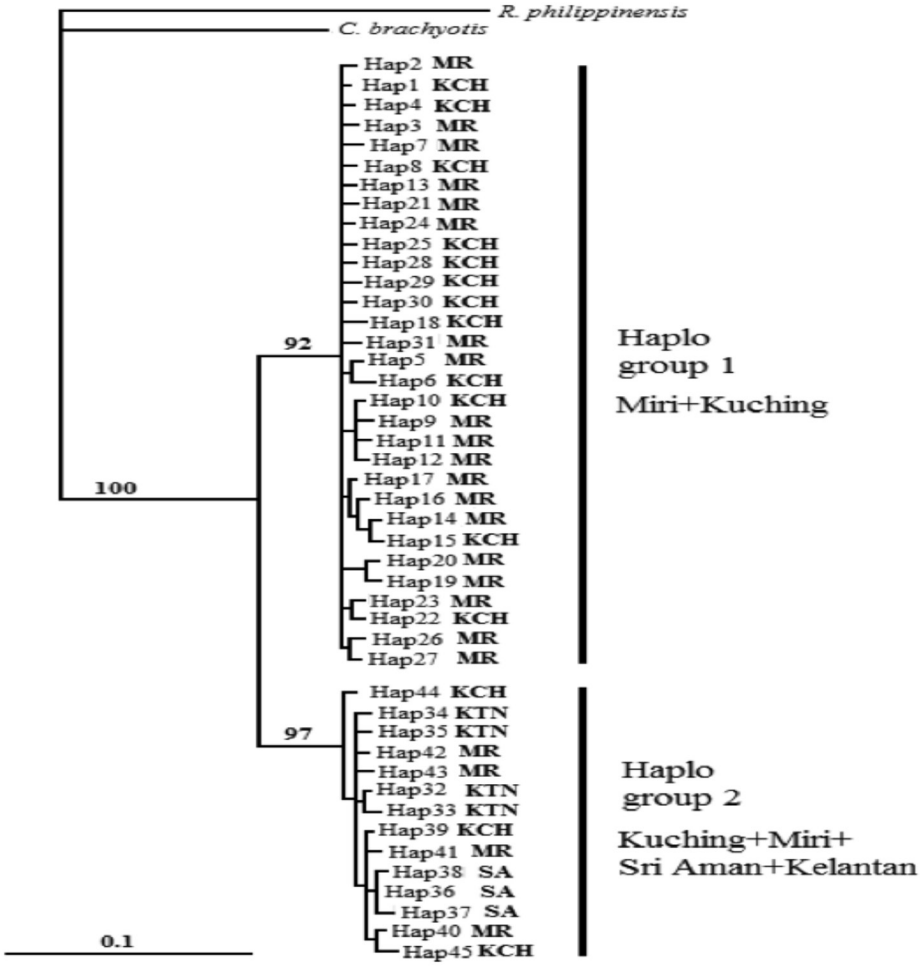

Fig. 3: A Bayesian 50% majority rule consensus tree of mtDNA cyt b of *P. lucasi*. The Bayesian posterior probabilities (BPP) are indicated beside the tree branch nodes: KCH - Kuching; KTN - Kelantan; MR - Miri; SA - Sri Aman.

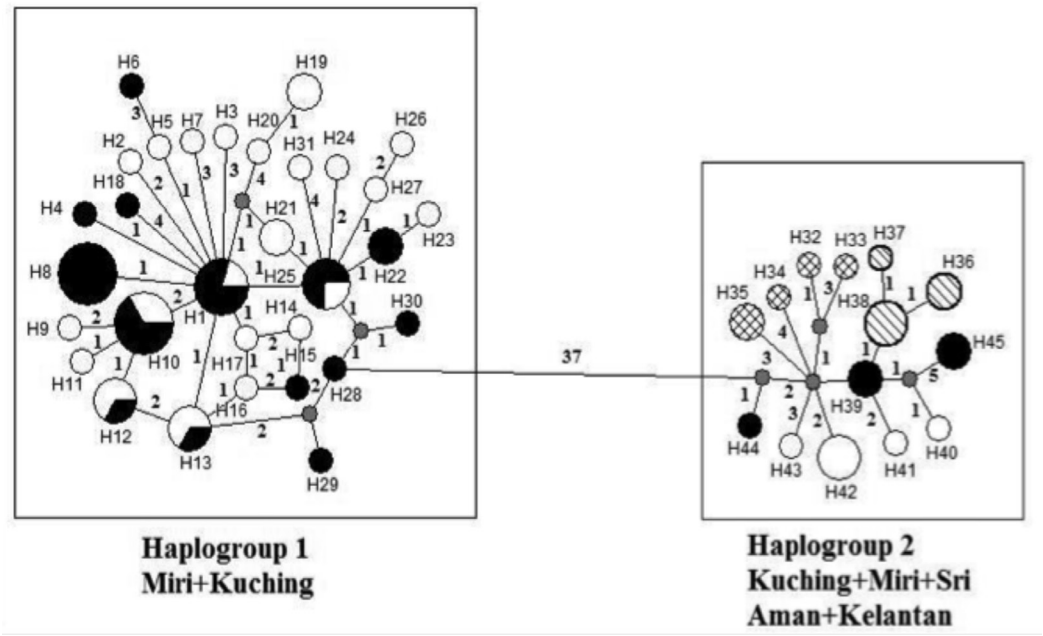

Fig. 4: Haplotype mapping of 45 assigned haplo-nodes within the four populations of *P. lucasi* in Malaysia. All the nodes for the populations of Miri, Kuching, Sri Aman and Kelantan are represented by white, black, forward diagonal and diagonal cross, respectively. The grey nodes represent missing or unsampled haplotypes in this analysis. Note that each node represents unique haplotype and node sizes are proportional to the haplotype frequencies of the given population. Bold numbers indicated at the node branches are the number of mutational steps to connect the nodes. Minimum-spanning network (MSN) was generated by Network 4.5.1.6 program (Fluxus Tech., 2004-2009).

(Table 1). The intra-population nucleotide diversity (π) was high in the Miri population with 0.016.

Among the populations, the nucleotide diversity (π) ranged from 0.004 to 0.02, with an average nucleotide substitutions per site between populations (nucleotide divergence, D_a) ranging from 0.002 to 0.029. A comparison between Miri and Sri Aman showed the highest nucleotide diversity with 0.021 and a divergence (D_a) of 0.027, while the lowest nucleotide diversity of 0.004 was observed between Sri Aman and Kelantan, along with a divergence (D_a) value of 0.003 (Table 2).

The Mantel analysis revealed a lack of significant relationship between nucleotide divergence and geographic distance (correlation coefficient, r = 0.0189, significant P = 0.928) among the four populations of *P. lucasi*. This indicated that the geographical distance was not a contributing factor in the nucleotide divergence within *P. lucasi*.

Neutrality Test and Population Expansion

The neutrality tests of Tajima's D, Fu and Li's, D^* and F^* and Fu's F_s, suggested that there were expansion events within all the *P. lucasi* populations. This was

also supported by a 'star-like' shape of the network of *P. lucasi*. This 'star-like' pattern can be attributed to an expanding population (Slatkin & Hudson, 1991; Rahman, 2000). Tajima's *D* was positive for the total overall population, indicating a lack of recently derived haplotype (Table 3) (Fu & Li, 1993). The negative values of Fu and Li's *D**(-1.06638), Fu and Li's *F**(-0.65307) and Fu's F_s (-6.467) were observed for the total overall population, suggesting the presence of rare haplotypes or polymorphism in the population (Akey *et al.*, 2004; Ramlah, 2009). The analysis for each population also showed a highly significant value of Fu's F_s for the Miri, Kuching and Sri Aman populations (F_s = -20.0525, P = 0.000; F_s = -23.5413, P = 0.000; F_s = -7.0960, P = 0.000, respectively), indicating excess of the recent mutations, while the non-significant value of Fu and Li's *D** and *F** (*D** = -0.1218, P = 0.404; *F** = -0.2130, P = 0.423; *D** = 0.5916, P = 0.7460; *F** = 0.3149, P = 0.678; *D** = 0.0622, P = 0.640; *F** = 0.0398, P = 0.58, respectively) indicated a demographic expansion for each of the populations. However, this was not observed for the Kelantan population.

Population Subdivision

AMOVA was used to determine the extent of population differentiation in *P. lucasi* (Table 4). Population structuring was investigated by grouping the four populations into two broad geographical groups (namely, East and West Malaysia). The grouping was made based on the geographical distance between these two regions within Malaysia which are separated by the South China Sea. A high variation was observed among the groups (46.42%), but was not significantly supported (P = 0.49970). Both the variation among the population within the groups (18.77%) and the variation within (34.81%) the populations were highly significant (P = 0.000). On other hand, the estimated Φ_{st} values among the grouped populations showed a high significance in the pair-wise differentiation (Table 5).

The analysis between the populations revealed high levels of nucleotide (N_{st}) and population subdivision (F_{st}), with low level of migrant per generation (N*m*) between the populations, and the exception between the Miri and Kuching populations. In particular, the *P. lucasi* of both the populations showed a high gene flow (N*m* = 31.72). Despite the closer distance, both the populations in Kuching and Sri Aman showed low levels of migrant per generation (N*m* = 0.30), indicating low female gene flow. Overall, the analyses from the gene flow estimator gave a low level of female migrant per generation of *P. lucasi* in all the populations, except for the population from Miri.

DISCUSSION

Genetic and Population History

Overall, the analysis of 1,061 bp sequences of *P. lucasi* revealed low levels of nucleotide and haplotypes variation. The populations with low level of genetic diversity might have experienced a prolonged or severe demographic bottleneck in the recent times (Avise, 2000). A potential cause for such a bottleneck effect could be due to the

multiple glaciations during Pleistocene epoch (Roques & Negro, 2005; Piaggio *et al.,* 2009). The low levels of genetic variation within *P. lucasi* populations also suggest that they might be recovering from catastrophic or stochastic events during their recent history (Ojeda, 2010). Meanwhile, climatic change and habitat loss may also contribute to reductions in genetic variability of the populations (Hadly *et al.,* 2004; Chan *et al.,* 2005). A study by Chan *et al.* (2005) found that rodent species lost genetic variability as a response to major climatic changes and habitat changes during the Holocene. These conditions may also decrease the population size and range the species (Chan *et al.,* 2005; Roques & Negro, 2005; Piaggio *et al.,* 2009).

Two haplogroups were observed for the *P. lucasi* populations, based on all the haplotype trees and network analyses with a high statistical support, suggesting that the isolation of the haplogroups was not a recent event (Piaggio *et al.,* 2009). A high genetic divergence was found between the two haplogroups (3.88%) in this study. The separation of the haplogroups might be explained in relation to the historical events (Ross *et al.,* 1997; Ramlah, 2009). High mutational steps (37 times) in MSN also suggest that the separation is an ancient event (William *et al.,* 2005). A similar pattern of separation was also found in other taxa, including anurans (Ramlah, 2009) and birds (Ramji, 2010).

Although the historical glacial events appeared to have influenced the genetic structure of the *P. lucasi*, different patterns of colonisation events and refugia could exist between the haplogroups (William *et al.,* 2005; Robert, 2006). The divergence between the haplogroups has a possibility of dating back to 1.95 Mya, which was within the Pleistocene epoch. The mammalian history was typically associated with the Pleistocene event, as it has been known as an important determinant for historical migration. Theoretically, the Sunda Shelf islands, namely, Borneo, Sumatra and Java, had repeatedly merged with Peninsular Malaysia to form a large landmass a number of times (Ruedi & Fumagalli, 1996; Bird *et al.,* 2005). The changing of the sea levels and the fluctuating temperature of the Malay Archipelago during Pleistocene had led to the repeated tropical rain forest isolation and fragmentation, which consequently affected the forest-associated taxa (Ruedi & Fumagalli, 1996; Anthony *et al.,* 2007).

It was hypothesised that some individuals of *P. lucasi* had migrated from their maternal roosts to establish new colonies. These colonies were expected to be surrounded by adequate food resources and secure places for shelter and breeding. As the colonies reached their carrying capacity, the initiator bats were forced to find more fragmented habitats to form new colonies. This stepping stone migration was repeated several times during the Pleistocene climate change period. Eventually, colonies with a common ancestor were assumed to be genetically mixed at intermediate refugia near the water bodies. The northern parts of Borneo (Miri and Sabah) were suggested as the main Quaternary rain forest refugia

in Borneo, as described by many authors (e.g., Ashton, 1972; Brandon-Jones, 1998; Cranbrook, 2000; Morley, 2000; Hunt *et al.,* 2007). The discovery of pollens from Kalimantan also provided the evidence for the existence of the tropical rain forests during LGM (Anshari *et al.,* 2004).

Furthermore, the reduction of moist rainforest, which was concentrated near water bodies, provided refugia for the animals (MacKinnon *et al*., 1996; Morley, 2000). The populations of *P. lucasi* were assumed to be isolated into these refugia over a long period of time. It was further speculated that *P. lucasi* colonised into the tropical rainforest during the interglacial dry period of Pleistocene maximum and dispersed during the cool wet period of Pleistocene minima (Gathorne-Hardy *et al*., 2002), with the spread of the tropical rainforest. Therefore, repeated contraction and expansion of the rainforest during Quaternary would have resulted in two broad haplogroups in the northern and south-western Borneo. It could be hypothesised that such occurrences might have affected the bats in terms of their movement and dispersal abilities. Based on the data obtained in the current study, it could be postulated that the age of divergence for all the populations of *P. lucasi* occurred between 7.5 - 350 kya. The late Pleistocene era dated back to 128 to 11 kya, while the Holocene era began 11 kya and has continued to the present (Cranbrook, 2000). Therefore, part of the divergence events of *P. lucasi* would have occurred from the Holocene to the Late Glacial Maximum (LGM) of Pleistocene epoch.

The placement of haplotypes from Miri and Kuching in both Haplogroup 1 and Haplogroup 2 had led to the occurrence of a species complex which might be present within these populations. A high level of genetic divergence was detected between the haplotypes from all the *P. lucasi* populations (4.9%). Faisal (2008) also found a high divergence of 5% within the populations of *P. lucasi* from Borneo. The author has further suggested that a comprehensive genetic study is needed to verify the divergence. Meanwhile, recent reviews have also suggested that a criterion of 5% sequence divergence in the Cyt *b* gene is considered as an existence of the subspecies, whereas the values exceeding 10% are considered in bats as indicatives of species-level divergence (Bradley & Baker, 2001; Baker & Bradley, 2006). However, the levels of genetic divergence at mtDNA markers alone are not necessarily sufficient to identify the possible cryptic species (Ruedi & McCracken, 2009). Meanwhile, Ibanez *et al.* (2006) proposed species level recognition only to those mtDNA lineages of highly differentiated species (>10%), which also showed morphological differentiation and or ecological isolation. Nonetheless, the assumptions that are solely based on mtDNA markers have been criticised because they reflect only an incomplete part of the natural history of the organisms (Ballard & Whitlock, 2003), or may be misled by the presence of pseudogenes

(Bensasson *et al*., 2001), and/or are affected by the natural limitations of mtDNA markers (Hudson & Turelli, 2003). Due to these possible disadvantages, a cross-validation with independent nuclear markers is highly recommended (Zhang & Hewitt, 2003).

According to Jayaraj (2008), the misclassification of nectarivorous bats into different geographical clades in Malaysia might be due to their ability to perform long-distance flight for foraging. Therefore, this kind of behaviour might explain the misclassification of *P. lucasi* haplotypes from Miri and Kuching present in both haplogroups. The Old World fruit bats can travel up to hundreds of kilometres, both within the mainland and across the ocean barriers (Shilton *et al.,* 1999). Some good examples of the local species are *Eonycteris spelaea* and *C. brachyotis*, which can travel up to 50 km for foraging in a single night (Fukuda *et al.,* 2009). The high mobility of these species has made them very successful in terms of distribution; they can be found to inhabit various types of vegetations, from the lowland dipterocarp forest, peat swamp forest, kerangas, and up to montane forest (Payne *et al.,* 1985; Francis, 2008). As a megabat, *P. lucasi* is capable of travelling long distances and foraging in more places. This enables individuals to migrate from the north to the south of Sarawak, or *vice versa*. This is further demonstrated by the colonisation of bats in Krakatau Island, which proves that the bodies of water or oceans are not an effective barrier to impede the dispersion of the species of fruit bats (Whittaker & Jones, 1994; Thornton *et al.,* 1996).

Population Partitioning and Gene Flow

Gene flow

The level of gene flow is expected to decrease with the increase of distance between two or more populations (Karuppudurai *et al*., 2007). Consequently, the nearest population is more similar at the neutral loci (Storz, 2002). This relationship refers to the isolation by distance, and assumes a stepping stone model of gene flow, which will provide a sufficient time for the population to reach a condition of equilibrium (Kimura & Weiss, 1964). However, the levels of gene flow are not only dependent on the distance between the populations, but also on the environment of the surrounding landscape between the populations (Storz, 2002). Thus, a high level of genetic variation within a population could result in a high level of gene flow, specifically for the populations in Miri and Kuching (Karuppudurai *et al*., 2007). This can be assumed since the sharing of haplotypes has been observed only (between) in the populations in Miri and Kuching, despite their notable distance from each other. This could have resulted from the continuous distribution of the *P. lucasi* population.

In sedentary species, extrinsic barriers to gene flow and historical events may determine the extent of genetic partitioning among the populations (Karuppudurai *et al.,* 2007). A barrier such as a developed area separating these localities has been suggested as a factor contributing to the failure of this particular species to be connected with each other and hence, impedes any gene flow between the populations (Storz, 2002).

Fluctuations in the world's temperature and a series of lowering and rising of sea levels during the late Pleistocene might have somehow affected this particular species since it depended on the forest for food. These phenomena have also allowed for the formation of different types of forest (Campbell *et al.,* 2006). According to Hudson *et al*. (1992), a significant differentiation between the populations would be expected only if the N*m* value was < 1.0. Similar results have been reported in *P. poliochepalus* and *P. alecto* (Webb & Tideman, 1996); *Plecotus auritus* (Burland *et al.,* 1999); *M. lyra* (Rajan & Marimuthu, 2006) and *C. sphinx* (Karuppudurai, 2007). As for the populations of *P. lucasi*, only one population interaction showed a deviated value with its N*m*>1, i.e. the Miri-Kuching populations. The non-significant correlation between the geographical distance and the genetic diversity among the populations of *P. lucasi* in Malaysia has led to the rejection of genetic isolation by geographic distance. Therefore, factors other than the distance between the populations are responsible for the differentiation observed in the populations of *P. lucasi*.

CONCLUSION

The findings of the current study indicated that the age of divergence for all the populations of *P. lucasi* occurred between 350 – 7.5 kya. The divergence within the populations in Miri (4.9%) and Kuching (4.7%) could have led to the occurrence of a species complex within *P. lucasi*. The presence of the haplotypes from both the populations in Haplogroup 1 and Haplogroup 2 is due to the ability of the dusky fruit bats to perform long-distance flights for foraging. A high gene flow was detected between these populations, suggesting continuous "stepping-stone" distributions of *P. lucasi,* despite the existing considerable distance between both localities. Meanwhile, the absence of a deep structure from the haplotype trees suggested that *P. lucasi* has a wide dispersal ability. The populations of *P. lucasi* were also expected to experience interpopulation genetic divergence, which could be classified into different evolutionary significant units (ESU) for management purposes. This study provided some useful insights into the phylogeoraphic relationships, genetic uniqueness, and population structure of *P. lucasi* in Malaysia. However, further studies should be carried out using larger sample sizes per population and samples from other cave areas (e.g. Mulu in Sarawak, Gomantong and Madai in Sabah) within their geographical distribution for conservation management strategies of the populations of *P. lucasi,* which are highly dependent on the cave system for breeding and shelter, and the surrounding forested areas for food resources. Additionally, information based on the nuclear DNA markers and fast evolving mtDNA genes (microsatellites) is necessary to elucidate the complex status of *P. lucasi*.

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REFERENCES

Abdullah, M. T. (2003). *Biogeography and variation of Cynopterus brachyotis in Southeast Asia*. (Doctoral Thesis dissertation). The University of Queensland, St Lucia, Australia.

Abdullah, M. T., Wong, S. F., & Besar, K. (2010). *Catalogue of mammals of UNIMAS Zoological Museum*. Kota Samarahan: Penerbitan Universiti Malaysia Sarawak.

Abdullah, M. T., Jusanit, P., Di, P. W. H., Zabani Ariffin, M., & Hall, L. S. (2007). Observations on bats in three national parks in Thailand. *Tigerpaper*, *34*(4), 5-10.

Abd Rahman, M. R. (2010). *Biogeographical status of dusky fruit bat, Penthetor lucasi in Malaysia inferred by morphological and genetics analyses*. MSc Thesis. Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan.

Abd Rahman, M. R. & Abdullah, M. T. (2010). Morphological variation of dusky fruit bat, *Penthetor lucasi* in Sarawak, Malaysia. *Tropical Natural History*, *10*(2), 141-158.

Andersen, K. (1912). *Catalogue of the Chiroptera in the collections of the British Museum*. Megachiroptera. British Museum of Natural History. London.

Anshari, G., Kershaw, A. P., van der Kasrs, S., & Jacobsen, G. (2004). Environmental change and peatland forest dynamics in the Lake Sentarum area, West Kalimantan. *Indonesia Journal of Quarternary Science*, *19*(7), 637-655.

Anthony, N. M., Johnson-Bawe, M., Jeffery, K., Clifford, S. L., Abernethy, K. A., Tutin, C. E., Lahm, S. A., White, L. J. T., Utley, J. F., Wickings, E. J., & Bruford, M.W. (2007). The role of Pleistocene refugia and rivers in shaping gorilla genetic diversity in central Africa. *Proceedings of the National Academy of Sciences, 104*(51), 20432-20436.

Ashton, P. S. (1972). The quaternary geomorphological history of Western Malesia and lowland forest phylogeography. In P. Ashton, & M. Ashton (Eds.). *Transactions of the Second Aberdeen-Hull Symposium and Malesian Ecology*. Hull.

Avise, J. C. (2000). *Phylogeography: The History and Formation of Species*. Cambridge: Harvard University Press.

Baker, R. J., & Bradley, R. D. (2006). Speciation in mammals and the Genetic Species Concept. *Journal of Mammalogy*, *87*(4), 643-662.

Ballard, J. W., & Whitlock, M. C. (2003). The incomplete natural history of mitochondria. *Molecular Ecology*, *13*, 729-744.

Bensasson, D., Zhang, D. X., Hartl, D. L., & Hewitt, G. M. (2001). Mitochondrial pseudogenes: evolution's misplaced witness. *Trends in Ecology and Evolution*, *16*, 314-321.

Bird, M. I., Taylor, D., & Hunt, C. (2005). Palaeoenvironments of insular Southeast Asia during the Last Glacial Period: a savanna corridor in Sundaland? *Quartenary Science Review*, *24*, 2228-2242.

Bradley, R. D., & Baker, R. J. (2001). A test of the genetic species concept: Cytochrome *b* sequences and mammals. *Journal of Mammalogy*, *82,* 960–973.

Brandon-Jones, D. (1998). Pre-glacial Bornean primate impoverishment and Wallace's Line. In J. D. Holloway, & R. Hall (Eds.). *Biogeography and geographical evolution of Southeast Asia*. Backhuy, Leiden.

Brown, W. M., Prager, E. M., Wang, A., & Wilson, A. C. (1982). Mitochondrial DNA sequence of primates: tempo and mode of evolution. *Journal of Molecular Evolution*, *18*, 225-239.

Bryja, J., Kanuch, P., Fornuskova, A., Bartonicka, T., & Rehak, Z. (2009). Low population genetic structuring of two cryptic bat species suggests their migratory behaviour in continental Europe. *Biological Journal of the Linnean Society, 96*, 103-114.

Burland, T. M., & Worthington-Wilmer, J. (2001). Seeing in the dark: molecular approaches to the study of bat populations. *Biological Reviews, 76*, 389-409.

Campbell, P., Schneider, C. J., Adnan A. M., Zubaid, A., & Kunz, T. H. (2006). Comparative population structure of *Cynopterus* fruit bats in Peninsular Malaysia and southern Thailand. *Molecular Ecology, 15*, 29-47.

Chan, Y. L., Lacey, E. A., Pearson, O. P., & Hadly, E. A. (2005). Ancient DNA reveals Holocene loss of genetic diversity in a South American rodent. *Biology Letters*, *1*, 423-426.

Corbet, G. B., & Hill, J. E. (1992). *The mammals of the Indomalayan region: a systematic review*. New York: Oxford University Press.

Cranbrook, E. (2000). Northern Borneo environments of the past 40,000 years. *Sarawak Museum Journal, 76,* 61-109.

Dalet, D. (2010). *Map of Malaysia*. Retrieved 18 January 2010 from http://d-maps.com/m/malaisie/malaisie08.gif.

Esa, Y. B., Siraj, S. S. Daud, S. K., Rahim K. K. A., Japning J. R. R., & Tan, S. G. (2008). Mitochondrial DNA diversity of *Tor tambroides* Valenciennes (Cyprinid) from five natural populations in Malaysia. *Zoological Studies*, *47*(3), 360-367.

Excoffier, L., Laval, G., & Schneider, S. (2005). Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, *1*, 47-55.

Faisal, A. K. (2008). *Diversification of Old World Bats in Malaysia: An Evolutionary and Phylogeography. Hypothesis tested through Genetic Species Concept*. MSc Thesis. Texas Tech University, Lubbock.

Francis, C. M. (2008). *A field guide to the mammals of Southeast Asia: Thailand, Peninsular Malaysia, Singapore, Myanmar, Laos, Vietnam and Cambodia*. London: New Holland Publishers.

Fu, Y. X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, *147*, 915-925.

Fu, Y. X., & Li, W. H. (1993). Statistical tests of neutrality of mutations. *Genetics*, *133*, 693-709.

Fukuda, D., Tisen, O. B., Momose, K., & Sakai, S. (2009). Bat diversity in the vegetation mosaic around a lowland dipterocarp forest of Borneo. *The Raffles Bulletin of Zoology*, *57*(1), 213-221.

Gathorne-Hardy, F. J., Syaukani, Davies, R. G., Eggleton, P., & Jones, D. T. (2002). Quaternary rainforest refugia in Southeast Asia: Using termites (Isoptera) as indicators. *Biological Journal of Linnean Society*, *75*, 453-466.

Grewe, P. M., Krueger, C. C., Aquadro, C. F., Bermingham, E., Kincaid, H. L., & May, B. (1993). Mitochondrial variation among lake trout (*Salvenilus namaycush)* strains stocked into Lake Ontario. *Canadian Journal of Fish and Aquatic Science, 50,* 2397-2403.

Hadly, E. A., Ramakrishnan, U., Chan, Y. L., Van Tuinen, M., O'Keefe, K., Spaeth, P. A., & Conroy, C. J. (2004). Genetic response to climatic change: insights from ancient DNA and phylochronology. *Public Library of Science Biology*, *2*, 1600-1609.

Hisheh, S., Westerman, M., & Schmitt, L. H. (1998). Biogeography of the Indonesian archipelago: mitochondrial DNA variation in the fruit bats, *Eonycteris spelaea*. *Biological Journal of the Linnean Society*, *65*, 329-345.

Hudson, R. R., Slatkin, M., & Maddison, W. P. (1992). Estimation of levels of gene flow from DNA sequence data. *Genetics*, *132*(2), 583-589.

Hudson, R. R., & Turelli, M. (2003). Stochasticity overrules the three times rule: genetic drift, genetic draft and coalescence times for nuclear versus mitochondrial DNA. *Evolution*, *57*, 182-190.

Huelsenbeck, J. P., & Ronquist, F. (2001). MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics*, *17*, 754-755.

Hunt, C. O., Gilbertson, D. D., & Rushworth, G. (2007). Modern human in Sarawak, Malaysia Borneo, during Oxygen Isotope Stage 3: palaeenvironmental evidence from the Great cave of Niah. *Journal of Archaeological Science*, *34*(11), 1953-1969.

Ibanez, C., Garcia-Mudarra, J. L., Ruedi, M., Stadelmann, B., & Juste, J. (2006). The Iberian contribution to cryptic diversity in European bats. *Acta Chiropterologica*, *8*, 277-297.

Imelda, V. P. (2007*). Molecular phylogenetic and phylogeography studies of microchiroptera in Malaysia Borneo*. MSc Thesis. Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan.

Irwin, D. M., Kocher, T. D., & Wilson, A. C. (1991). Evolution of the cytochrome *b* gene of mammals. *Journal of Molecular Evolution*, *32*, 128-144.

Jayaraj, V. K. (2008). *The phylogenetic relationship of megachiroptera in Malaysia inferred from morphological and DNA analyses*. MSc Thesis. Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan.

Karuppudurai, T., Sripathi, K., Gopukumar, N., Elangovan, V., & Marimuthu, G. (2007). Genetic diversity within and among populations of the Indian short-nosed fruit bat, *Cynopterus sphinx* assessed through RAPD analysis. *Current Science*, *93*(7), 942-950.

Kimura, M., & Weiss, G. H. (1964). The stepping-stone model of population structure and the decrease of genetic correlation with distance. *Genetics*, *49*, 561-576.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotides sequence. *Journal of Molecular Evolution*, *16*, 111-120.

Kimura, M. (1983). *The neutral theory of molecular evolution*. Cambridge: Cambridge University Press.

Kitchener, D. J., Hisheh, S., Schmitt, L. H., & Maryanto, I. (1993a). Morphological and genetic variation in *Aethalops alecto* (Chiroptera, Pteropodidae) from Java, Bali and Lombok Is, Indonesia. *Mammalia,* 57, 255-272.

Kitchener, D. J., Schmitt, L. H., Hisheh, S., How, R. A., Cooper, N. K., & Maharadatunkamsi. (1993b). Morphological and genetic variation in the bearded tomb bats (*Taphozous*: Emballonuridae) of Nusa Tenggara, Indonesia. *Mammalia, 57*(1), 63-83.

Kumar, S., Tamura, K., & Nei, M. (2004). MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, *5*, 150-163.

Lynch, J. M., & Crease, T. J. (1990). The analysis of population survey data on DNA sequence variation. *Molecular Biology and Evolution*, *7,* 377-394.

Maharadatunkamsi, Hisheh, S., Kitchener, D. J., & Schmitt, L. H. (2003). Relationships between morphology, genetics and geography in the cave fruit bat *Eonycteris spelaea* (Dobson, 1871) from Indonesia. *Biological Journal of the Linnean Society*, *79*, 511-522.

Maryanto, I. (2004). Taxonomic status of dusky short nosed fruit bat *Penthetor lucasi* (Dobson, 1880) from Sumatra, Indonesia. *Tropical Biodiversity*, *8*(1), 51-62.

MacCarthy, C. (1996). *CHROMAS 1.45 program*. Queensland, Australia.

MacKinnon, K., Hatta, G., Halim, H., & Mangalik, A. (1996). *The ecology of Kalimantan*. London: Oxford University Press.

McCracken, G. F., McCracken, M. K., & Vawter, A. T. (1994). Genetic structure in migratory populations of the bat *Tadarida brasiliensis mexicana*. *Journal of Mammalogy, 75*, 500-514.

Morley, R. J. (2000). *Origin and evolution of tropical rainforests*. Leicester: John Wiley and Sons Ltd.

Nei, M., & Tajima, F. (1981). DNA polymorphism detectable by restriction endonucleases. *Genetics*, *97*, 145-163.

Nei, M. (1987). *Molecular evolutionary genetics*. New York: Columbia University Press.

Payne, J., Francis, C. M., & Phillips, K. (1985). *A field guide to the mammals of Borneo*. The Sabah Society and WWF Malaysia, Kota Kinabalu.

Piaggio, A. J., Navo, K. W., & Stihler, C. W. (2009). Intraspecific comparison of population structure, geneticdiversity, and dispersal among three subspecies of Townsend's big-eared bats, *Corynorhinus townsendii townsendii*, *C. t. pallescens*, and the endangered *C. t. virginianus*. *Conservation Genetic*, *10*, 143-159.

Posada, D., & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics, 14*(9), 817-818.

Rahman M. A. (2000). *Biogeography of avifauna and patterns of variation in the little spiderhunter (Arachnothera longirostra) in Southeast Asia* (Doctoral Thesis dissertation). University of Queensland, St Lucia, Australia.

Rajan, K. E., & Marimuthu, G. (2006). A Preliminary examination of genetic diversity in the Indian false vampire bat *Megaderma lyra*. *Animal Biodiversity and Conservation, 29*(2), 109–115.

Ramji M. F. S. (2010). *Patterns of plumage colouration, genetic and morphological variation in mountain blackeye(Cholorocharis emiliae) from Malaysian Borneo*. MSc Thesis. Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan.

Ramlah Z. (2009). *Ecology and molecular phylogenetics of frogs from the genus Rana*

Linnaeus 1758 in Sarawak. PhD Thesis. Universiti Kebangsaan Malaysia, Bangi.

Roberts, T. E. (2006). History, ocean channels, and distance determine phylogeographic patterns in three widespread Philippines fruit bats (Pteropodidae). *Molecular Ecology*, *15*, 2183-2199.

Roques, S., & Negro, J. J. (2005). MtDNA genetic diversity and population history of dwindling raptorial bird, the red kite (*Milvus milvus*). *Biological Conservation, 126*, 41-50.

Ross, K. G., Krieger, M. J. B., Shoemaker, D. D., Vargo, E. L., & Keller, L. (1997). Hierarchical analysis of genetic structure in native fire ant populations: results from three classes of molecular markers. *Genetics*, *147*, 643-655.

Rozas, J., Sanchez-Delbarrio, J. C., Messeguer, X., & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, *19*, 2496-2497.

Ruedi, M., & Fumagalli, L. (1996). Genetic structure of Gymnures (genus *Hylomys*; Erinaceidae) on continental islands of Southeast Asia: historical effects of fragmentation. *Journal of Zoological Systematic and Evolutionary Research, 34,* 153-162.

Ruedi, M., & McCracken, G. F. (2009). Genetics and evolution: phylogeographic analysis of bats. In T. H. Kunz, & S. Parsons (Eds). *Ecological and behavioral methods for the study of bats* (2nd Edition). Boston: Johns Hopkins University Press.

Russell, A. L., Medellin, R. A., & McCracken, G. F. (2005). Genetic variation and migration in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*). *Molecular Ecology*, *14*, 2207-2222.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (2nd Edition). New York: Cold Spring Harbor Laboratory Press.

Schmitt, L. H., Kitchener, D. J., & How, R. A. (1995). A genetic perspective of mammalian variation and evolution in the Indonesian archipelago: biogeographic correlates in the fruit bat Genus *Cynopterus*. *Evolution, 49*(3), 399 - 414.

Shilton, L. A., Altringham, J. D., Compton, S. G., & Whittaker, R. J. (1999). Old world fruit bat can be long distance seed dispersers through extended retention of viable seeds in the gut. *Proceeding of the Royal Society of London B*, *266*, 219-223.

Slatkin, M., & Hudson, R. R. (1991). Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics*, *129*, 555-562.

Storz, J. F. (2002). Constrasting pattern of divergene in quatitative traits and neutral DNA markers: Analysis of clinal variation. *Molecular Ecology*, *11*, 2537-2551.

Swofford, D. L. (1998). PAUP*. *phylogenetic analysis using parsimony (*and other methods) Version 4.* Sinauer Associates, Massachusetts.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics, 123*, 585–595.

Tavare, S. (1986). Some probabilistic and statistical problems in the analysis of DNA sequences. In R. M. Miura (Ed.). *Some mathematical questions in biology - DNA sequence analysis*. Providence, Rhode Island: American Mathematical Society.

Thompson, J. D., Gibson, T. J., & Plewniak, F. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by the quality analysis tools. *Nucleic Acids Research, 24*, 4876-4882.

Thornton, I. W. B., Compton, S. G., & Wilson, C. N. (1996). The role of animals in the colonisation of the Krakatau Islands by fig trees (*Ficus* species). *Journal of Biogeography*, *23*, 577-592.

Tingga, T. R. C. (2010). *Morphological and genetic variation of Aethalops aequalis using*

mitochondrial and nuclear gene in Malaysian Borneo. (MSc Thesis dissertation). Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan.

Trouessart, E. L. (1897). *Catalogue mammalium tam viventium quam fossilium*. Nova editie (Prima completa) Vol 1. Friedlander, Berlin.

Trouessart, E. L. (1904). *Catalogue mammalium tam viventium quam fossilium*. Quinquennale supp. Pt 1. Friedlander, Berlin.

Webb, N. J. & Tidemann, C. R. (1996). Mobility of Australian flying–foxes, *Pteropus* spp. (Megachiroptera): evidence from genetic variation. *Proceedings of Royal Society of London (B), 263*, 497-502.

Weir, B. S., & Cockerham, C.C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution*, *38*, 1358-1370.

Whittaker, R. J., & Jones, S. H. (1994). The role of frugivorous bats and birds in there building of a tropical forest ecosystem, Krakatau, Indonesia. *Journal of Biogeography*, *21*, 245-258.

Williams, H. C., Ormerod, S. J., & Bruford, M. W. (2006). Molecular systematics and phylogeography of the cryptic species complex *Baetis rhodani* (Ephemeroptera, Baetidae). *Molecular Phylogenetics and Evolution*, *40,* 370-382.

Worthington-Wilmer, J., Moritz, C., Hall, L., & Toop, J. (1994). Extreme population structuring in the threatened ghost bat, *Macroderma gigas*; evidence from mitochondrial DNA. *Proceedings of Royal Society of London (B),* 257: 193-198.

Zhang, D. X., & Hewitt, G. (2003). Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology*, *12*, 563–584.

APPENDIX 1

List of samples of *P. lucasi* used in the genetic analyses.

No	Species	Voucher/ Museum. No	Locality	Habitat	GenBank Acc. No.
1	*P. lucasi*	MZU/M/02120	Niah NP, Miri, Sarawak	Limestone forest	GU724886
2	*P. lucasi*	MZU/M/02122	Niah NP, Miri, Sarawak	Limestone forest	GU724906
3	*P. lucasi*	MZU/M/02123	Niah NP, Miri, Sarawak	Limestone forest	GU724887
4	*P. lucasi*	MZU/M/02124	Niah NP, Miri, Sarawak	Limestone forest	GU724932
5	*P. lucasi*	MZU/M/02125	Niah NP, Miri, Sarawak	Limestone forest	GU724933
6	*P. lucasi*	MZU/M/02127	Niah NP, Miri, Sarawak	Limestone forest	GU724888
7	*P. lucasi*	MZU/M/02128	Niah NP, Miri, Sarawak	Limestone forest	GU724889
8	*P. lucasi*	MZU/M/02130	Niah NP, Miri, Sarawak	Limestone forest	GU724890
9	*P. lucasi*	MZU/M/02131	Niah NP, Miri, Sarawak	Limestone forest	GU724891
10	*P. lucasi*	MZU/M/02133	Niah NP, Miri, Sarawak	Limestone forest	GU724934
11	*P. lucasi*	MZU/M/02134	Niah NP, Miri, Sarawak	Limestone forest	GU724892
12	*P. lucasi*	MZU/M/02135	Niah NP, Miri, Sarawak	Limestone forest	GU724907
13	*P. lucasi*	MZU/M/02153	Niah NP, Miri, Sarawak	Limestone forest	GU724935
14	*P. lucasi*	MZU/M/02154	Niah NP, Miri, Sarawak	Limestone forest	GU724908
15	*P. lucasi*	MZU/M/02155	Niah NP, Miri, Sarawak	Limestone forest	GU724936
16	*P. lucasi*	MZU/M/02156	Niah NP, Miri, Sarawak	Limestone forest	GU724937
17	*P. lucasi*	MZU/M/02157	Niah NP, Miri, Sarawak	Limestone forest	GU724893
18	*P. lucasi*	MZU/M/02163	Niah NP, Miri, Sarawak	Limestone forest	GU724909
19	*P. lucasi*	MZU/M/02169	Niah NP, Miri, Sarawak	Limestone forest	GU724894
20	*P. lucasi*	TK152463	Niah NP, Miri, Sarawak	Limestone forest	GU724895
21	*P. lucasi*	TK152468	Niah NP, Miri, Sarawak	Limestone forest	GU724896
22	*P. lucasi*	TK152470	Niah NP, Miri, Sarawak	Limestone forest	GU724897
23	*P. lucasi*	TK152481	Niah NP, Miri, Sarawak	Limestone forest	GU724910
24	*P. lucasi*	TK152482	Niah NP, Miri, Sarawak	Limestone forest	GU724929
25	*P. lucasi*	TK152483	Niah NP, Miri, Sarawak	Limestone forest	GU724911
26	*P. lucasi*	TK152933	Niah NP, Miri, Sarawak	Limestone forest	GU724898
27	*P. lucasi*	TK152953	Niah NP, Miri, Sarawak	Limestone forest	GU724899
28	*P. lucasi*	TK152954	Niah NP, Miri, Sarawak	Limestone forest	GU724900
29	*P. lucasi*	TK152964	Niah NP, Miri, Sarawak	Limestone forest	GU724912
30	*P. lucasi*	TK152965	Niah NP, Miri, Sarawak	Limestone forest	GU724901
31	*P. lucasi*	TK152966	Niah NP, Miri, Sarawak	Limestone forest	GU724902
32	*P. lucasi*	TK152971	Niah NP, Miri, Sarawak	Limestone forest	GU724930

33	*P. lucasi*	MZU/M/01685	Lambir NP, Miri, Sarawak	Lowland Dipterocarp Forest	GU724954
34	*P. lucasi*	TK152883	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724938
35	*P. lucasi*	TK152884	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724939
36	*P. lucasi*	TK152885	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724940
37	*P. lucasi*	TK152887	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724941
38	*P. lucasi*	MZU/M/02173	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724942
39	*P. lucasi*	MZU/M/02180	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724943
40	*P. lucasi*	MZU/M/02207	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724904
41	*P. lucasi*	MZU/M/02209	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724914
42	*P. lucasi*	MZU/M/02210	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724905
43	*P. lucasi*	MZU/M/02211	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724915
44	*P. lucasi*	MZU/M/02212	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724916
45	*P. lucasi*	MZU/M/02214	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724917
46	*P. lucasi*	MZU/M/02216	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724918
47	*P. lucasi*	MZU/M/02217	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724919
48	*P. lucasi*	MZU/M/02226	Wind Cave NR, Kuching, Sarawak	Secondary forest	GU724920
49	*P. lucasi*	MZU/M/02227	Wind Cave NR, Kuching, Sarawak	Secondary forest	GU724921
50	*P. lucasi*	MZU/M/02232	Wind Cave NR, Kuching, Sarawak	Secondary forest	GU724922
51	*P. lucasi*	MZU/M/02229	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724923
52	*P. lucasi*	MZU/M/02233	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724927

53	*P. lucasi*	MZU/M/02235	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724925
54	*P. lucasi*	MZU/M/02236	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724926
55	*P. lucasi*	MZU/M/02234	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724924
56	*P. lucasi*	MZU/M/02238	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724928
57	*P. lucasi*	MZU/M/01716	Kubah NP, Kuching, Sarawak	Mixed Dipterocarp Forest	GU724903
58	*P. lucasi*	MZU/M/02239	Padawan, Kuching, Sarawak	Limestone forest	GU724953
59	*P. lucasi*	MZU/M/02240	Padawan, Kuching, Sarawak	Limestone forest	GU724885
60	*P. lucasi*	MZU/M/02241	Padawan, Kuching, Sarawak	Limestone forest	GU724931
61	*P. lucasi*	MZU/M/00568	Mount Penrissen, Kuching, Sarawak	Montane forest	GU724952
62	*P. lucasi*	MZU/M/00569	Mount Penrissen, Kuching, Sarawak	Montane forest	GU724913
63	*P. lucasi*	MZU/M/00570	Mount Penrissen, Kuching, Sarawak	Montane forest	GU724950
64	*P. lucasi*	MZU/M/02242	Bako NP, Kuching, Sarawak	Mixed Dipterocarp Forest	GU724948
65	*P. lucasi*	MZU/M/02243	Bako NP, Kuching, Sarawak	Mixed Dipterocarp Forest	GU724955
66	*P. lucasi*	MZU/M/02244	Bako NP, Kuching, Sarawak	Mixed Dipterocarp Forest	GU724949
67	*P. lucasi*	MZU/M/01192	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724881
68	*P. lucasi*	MZU/M/01193	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724882
69	*P. lucasi*	MZU/M/01190	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724951
70	*P. lucasi*	MZU/M/01194	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724883

71	*P. lucasi*	MZU/M/01191	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724947
72	*P. lucasi*	MZU/M/01195	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724884
73	*P. lucasi*	DWNP 02142	Gua Musang, Kelantan	NA	GU724879
74	*P. lucasi*	DWNP 02143	Gua Musang, Kelantan	NA	GU724945
75	*P. lucasi*	DWNP 02144	Gua Musang, Kelantan	NA	GU724880
76	*P. lucasi*	DWNP 02145	Gua Musang, Kelantan	NA	GU724946
77	*P. lucasi*	DWNP 02375	Gua Musang, Kelantan	NA	GU724944
78	*C.brachyotis*	TK152458	Mount Murud, Miri, Sarawak	Montane forest	GU724956
79	*R. philippinensis*	TK152938	Niah NP, Miri, Sarawak	Limestone forest	GU724957

NA= Not available; NP= National Park; NR= Nature Reserve.