Pertanika J. Trop. Agric. Sci. 35 (3): 485 - 510 (2012)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Phylogeny and Phylogeography of *Aethalops* from Sundaland using Mitochondrial 12S rRNA Gene

Tingga, R. C. T.[1, 2]* and Abdullah, M. T.[1]

[1] *Molecular Ecology Laboratory, Department of Zoology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*
[2] *Centre for Pre-University Studies, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*

ABSTRACT

One of the smallest fruit bats in Pteropodidae is *Aethalops*. This genus is known to be confined in montane forest, which is generally above 1000 meters above sea level (m.a.s.l.). Bornean *Aethalops* is generally known as *Aethalops alecto* in most previous literature. This study aimed at constructing the phylogenetic relationship of *A. alecto* and *A. aequalis* in Sundaland and determining gene flow within Bornean *A. aequalis* using partial mitochondrial 12S rRNA gene. Seven populations of *A. aequalis,* representing Sabah and Sarawak and a single population from Kalimantan were observed, whereas *A. alecto* were represented by four populations from Indonesian islands. From the phylogenetic analyses and minimum spanning network, there were two major clusters within the genus, with *Aethalops*. *A. aequalis* in Borneo were clearly distinguished from *A. alecto* from the islands of Indonesia. However, phylogenetic analyses within *A. aequalis* were unresolved at the population levels in Sabah and Sarawak. Therefore, it can be concluded that *A. aequalis* is the species found only in Borneo. High genetic similarities were detected among the populations of *A. aequalis* in Sabah and Sarawak. Hypothetically, the Kalimantan harbors ancestral populations of *A. aequalis* in Borneo, with high genetic divergence from Sabah and Sarawak populations.

Keywords: Aethalops, populations, phylogeny, phylogeography, Sundaland, 12S rRNA

ARTICLE INFO

Article history:
Received: 20 May 2010
Accepted: 14 September 2011

E-mail addresses:
rchaya84@gmail.com (Tingga, R. C. T.), tabbulla@frst.unimas.my (Abdullah, M. T.)
* Corresponding author

INTRODUCTION

The montane bat *Aethalops* is among of the smallest Old World fruit bat (Pteropodidae), which is also known as Pigmy Fruit Bat or tailless fruit bat. *Aethalops* is confined in the montane forest above 1000 m (Payne *et*

ISSN: 1511-3701

al., 1985; Mickleburgh *et al*., 1992; Francis, 2005) and has a low widespread area (Kitchener *et al*., 1993). The fur is grey-brown to reddish brown, and thick and long on the dorsal surface. The muzzle is narrow and pointed and forearm length is between 42 – 46 mm (Payne *et al*., 1985). The distinctive characteristics that differentiate this genus from its sister genus, *Balionycteris* (Ryan *et al*., 2008) is that the *Aethalops* are tailless, spotless on the wings and have a pair of lower incisors (Payne *et al*., 1985). *Aethalops* are found throughout Peninsular Malaysia, Sundaland, and other islands in Indonesia. Sundaland refers to Sumatra, Java, Lombok, Borneo and Peninsular Malaysia. There are two species within the genus, namely *A. alecto* and *A. aequalis*, and both are endemic to the mountainous areas. Previous studies have indicated that there is a distribution boundary between the two species (Kitchener *et al*., 1993; Maharadatunkamsi *et al*., 2006). However, some authors still consider *Aethalops* in Borneo as *A. alecto* rather than *A. aequalis* (Payne *et al*., 1985; Francis, 2005). Bornean *Aethalops* or Bornean Pigmy Fruit Bats (*A. a. aequalis*) are considered as a sub-species

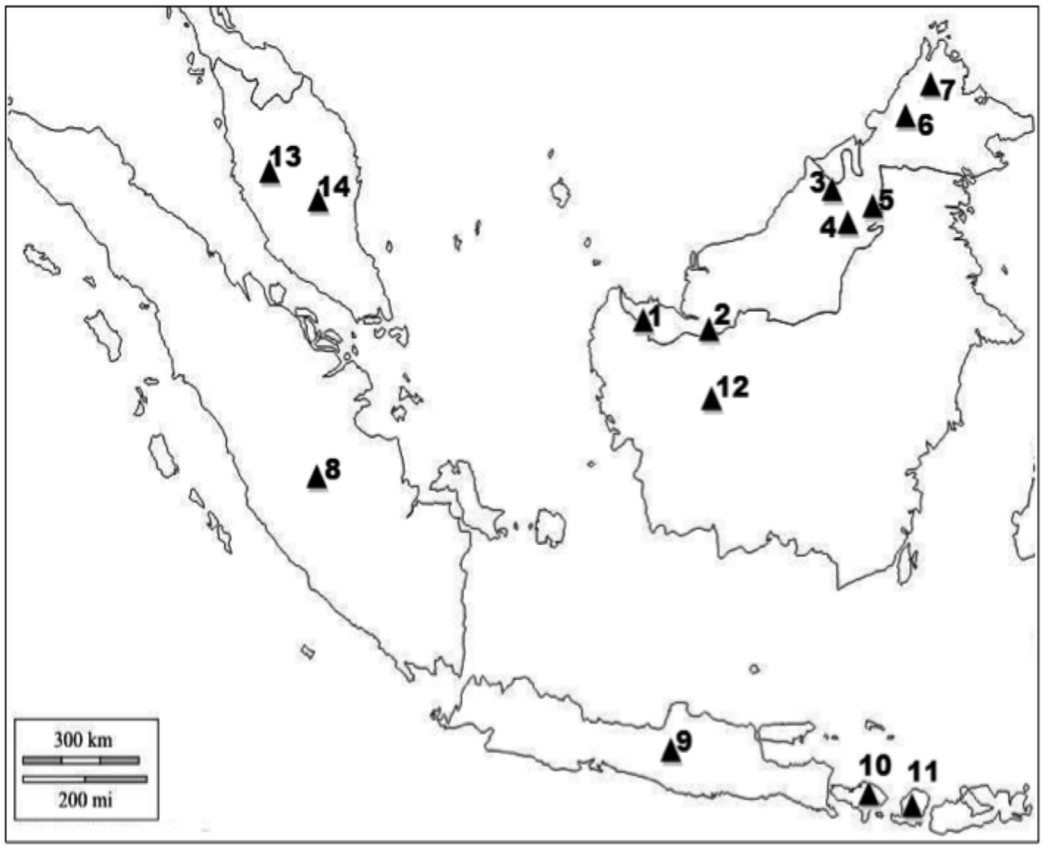

Fig. 1: Distributions of *A. aequalis* based from the specimens used in this study (1-12) and the sampling sites for *A. alecto* (13-14). 1-Mt Pueh; 2-Mt Penrissen; 3-Mt Mulu; 4-Bario; 5-Mt Murud; 6-Mt Trus Madi; 7- Mt Kinabalu; 8-Sumatra; 9- Java; 10-Bali; 11-Lombok; 12- Kalimantan; 13-Fraser's Hill; 14-Mt Benom

of *A. alecto* (Hill, 1961, 1966; Hill, 1983; Boeadi & Hill, 1986; van Strien, 1986; Koopman, 1989).

In this paper, the phylogenetic relationships of Sundaland *Aethalops* were assessed using 12S rRNA on the *Aethalops* of Sundaland. The aims of this study were to construct the phylogenetic relationship of *A. alecto* and *A. aequalis* and determine the patterns of gene flow of *A. aequalis* within Borneo, as evident in the partial mitochondrial 12S rRNA gene.

TABLE 1
List of the specimens, museum reference, location, abbreviation (Abbr.) and GenBank accesion numbers

Locality	Elevation	FA	Sex	Abbr.	Field No.	UNIMAS Voucher No.	Accession No.
Mt Kinabalu	1527 m a.s.l	44.50	M	TK4	TK004	UNIMAS 00371	HM067793
		42.18	F	TK20	TK152920	-	HM067816
		44.31	F	TK21	TK152921	-	HM067774
		42.94	M	TK22	TK152922	-	HM067775
		44.25	F	TK23	TK152923	-	HM067814
		43.92	M	TK24	TK152924	-	HM067776
		42.54	F	TK25	TK152925	-	HM067777
		46.98	F	TK26	TK152926	-	HM067778
		45.53	M	TK27	TK152927	-	HM067779
		43.82	F	TK28	TK152928	-	HM067780
		44.29	F	TK30	TK152930	-	HM067815
Mt Trus Madi	1446 m a.s.l	43.16	F	TM1	TM001	-	HM067781
		44.17	F	TM2	TM011	-	HM067785
		44.59	F	TM3	TM012	-	HM067782
		43.59	F	TM4	TM013	-	HM067786
		41.53	F	TM5	TM014	-	HM067801
		43.77	F	TM6	TM015	-	HM067783
Mt Murud	1335-2113 m a.s.l	44.35	M	MRD1	RV018	UNIMAS 01015	HM067760
		32.84	F	MRD2	RV042	UNIMAS 01016	HM067804
		43.73	M	MRD3	MRT004	UNIMAS 01127	HM067787
		45.1	M	MRD5	MRT010	UNIMAS 01129	HM067788
		44.22	F	MRD7	RV 019	UNIMAS 01361	HM067802
		40.25	F	MRD9	RV 032	UNIMAS 01363	HM067803
		45.46	F	MRD10	RV 041	UNIMAS 01364	HM067798
		43.68	F	MRD11	RV 027	UNIMAS 01365	HM067762
		41.86	F	MRD12	RV 013	UNIMAS 01366	HM067807

Table 1 (*continued*)

Locality	Elevation	FA (mm)	Sex	Abbr.	Field No.	UNIMAS Voucher No.	Accession No
Mt Murud	1335-2113 m a.s.l	45.82	F	MRD13	RV 008	UNIMAS 01367	HM067805
		41.48	F	MRD14	RV 012	UNIMAS 01368	HM067789
		42.16	F	MRD15	RV 011	UNIMAS 01369	HM067811
		46.18	F	MRD16	RV 027	UNIMAS 01370	HM067817
		41.48	F	MRD17	RV 029	UNIMAS 01371	HM067790
		43.27	F	MRD18	RV 005	UNIMAS 01372	HM067791
		43.27	F	MRD19	RV 010	UNIMAS 01373	HM067758
		44.39	F	MRD20	RV 006	UNIMAS 01374	HM067812
		45.34	M	MRD21	RV 007	UNIMAS 01375	HM067808
		45.12	F	MRD22	RV 009	UNIMAS 01376	HM067809
		44.9	M	MRD23	Mrd004	-	HM067763
		41.96	F	MRD24	Mrd007	-	HM067764
		43.64	F	MRD25	Mrd008	-	HM067765
		42.38	F	MRD26	Mrd009	-	HM067766
		42.77	M	MRD27	Mrd015	-	HM067767
Mt Mulu	1764 m a.s.l	45.40	F	MU1	Berta1	-	HM067768
		42.23	F	MU2	Berta2	-	HM067769
		42.55	F	MU3	Berta3	-	HM067800
		42.26	F	MU4	Berta4	-	HM067770
		41.63	F	MU5	Berta5	-	HM067771
		44.22	M	MU6	Berta6	-	HM067772
		42.67	M	MU7	MMB3	-	HM067795
		43.69	M	MU8	MMB4	-	HM067796
		43.45	F	MU9	MMB5	-	HM067797
Bario	1100-1250 m a.s.l	44	M	Bar3	BD016	UNIMAS 00053	HM067810
Mt Penrissen	746-1000 m a.s.l	45.7	M	MP2	MP03	UNIMAS 00590	HM067794
		43.09	M	MP3	MP06	UNIMAS 00591	HM067761

Table 1 (*continued*)

Locality	Elevation	FA	Sex	Abbr.	Field No.	UNIMAS Voucher No.	Accession No
Mt Penrissen	746-1000 m a.s.l	43.75	F	MP4	MP001	-	HM067759
		-	F	MP5	MP016	-	HM067813
		44.94	F	MP6	MP020	-	HM067784
		42.03	M	BOH1	PB 035	UNIMAS 00678	HM067806
				BOH2		UNIMAS 00679	HM067792
		44.62	M	BOH3	BH 76	UNIMAS 01525	HM067799
Mt Pueh	845m a.s.l	-	F	PUEH1	1046	UNIMAS 01632	HM067773

MATERIALS AND METHODS

Samples were collected from nine sites, namely, Southwest Sarawak group [Mount (Mt) Penrissen and Mt Pueh], Northeast Sarawak group (Mt Murud, Mt Mulu and Bario) and Sabah group (Mt Kinabalu and Mt Trus Madi) and Peninsular Malaysia (Fraser's Hill and Mount Benom) (Table 1 and Fig.1). Mist nets were set along the forest trail, near streams and on the forest edge. Captured bats were identified and measured following Payne *et al*. (1985) and deposited in the Zoological Museum of Universiti Malaysia Sarawak (Abdullah *et al*., 2010). Selected bats were preserved either as wet or dry specimens, and the others were released with marked bands. Tissue samples were taken from the pectoral part of the body and preserved either in lysis buffer or ethanol.

Total genomic DNA of *A. aequalis* was then extracted using the modified 2X cetil – trimethylammonium bromide (CTAB) method, following Grewe *et al*. (1993). Partial mitochondrial 12S rRNA gene was amplified with primer 12SA-L 5' – aaa ctg gga tta gat acc cca – 3' and and 12SA-H 5' – atg ttt ttg ata aac agg – 3' (Palumbi *et al*., 1991). The template DNA was amplified in 25 µl of the reaction mixture containing 5 µl of 5x buffer (Promega), 1.5 µl of 25 mM $MgCl_2$ (Promega), 0.2 µl of dNTP (10 mM) (Promega), 0.1 µl of each primer (10 mM) and 0.2 units of *Taq* polymerase (Promega). The cycle parameters consisted of 30 cycles of denaturation (at 94°C for 1 minute), annealing (55 – 58°C for 1 minute) and extension (at 72°C for 2 minutes). The amplified products were visualised on 2% agarose gels containing ethidium bromide, run on gel electrophoresis for 30 minutes at 90V, and photographed under the ultraviolet light. GeneRuler™ 100 bp DNA ladder was used as a standard size marker (Promega). Purified products were sent to private laboratories for sequencing using ABI prism ™ Big dye ™ terminator cycle sequencing Ready kit version 3.1, or using the ABI PRISM® 377 DNA Sequencer with the BigDye® Terminator v3.0 Cycle Sequencing Kit and the sequencing product was run using ABI 3730 XL capillary DNA sequencer (50 cm capillary).

TABLE 2
List of the 12S rRNA sequences of *A. aequalis* and *A. alecto* from Indonesia (obtained from the GenBank, as well as the longitude and latitude estimated from Google Map)

Species	Locality	Accession number (Field no.)	Longitude/ Latitude	Elevation (m) a.s.l
A. alecto	Gunung Rinjani, Lombok	DQ845089 (GR1)	116° 28'00"E 08° 25'00"S	3726
	Gunung Rinjani, Lombok	DQ845088 (GR2)	116° 28'00"E 08° 25'00"S	3726
	Batang Toru, Sumatra	DQ845091 (BT1)	98° 53' – 99° 26'E 02° 03' – 01° 27'N	400 - 1803
	Kebun Raya Eka Karya, Bali	DQ845086 (KREK1)	115° 22' 30"E 8° 14' 30"S	1400
	Taman Nasional Gunung Halimunan, Java	DQ845081 (TNGH1)	106° 21' – 106° 31'E 06° 37' – 06° 51'S	500 - 1929
	Taman Nasional Gunung Halimunan, Java	DQ845080 (TNGH2)	106° 21' – 106° 31'E 06° 37' – 06° 51'S	500 - 1929
	Kebun Raya Cibodas, Java	DQ845082 (KRC5)	107° 37' 53.4"E 06° 51' 53.2794"S	1250
	Kebun Raya Cibodas, Java	DQ845083 (KRC6)	107° 37' 53.4"E 06° 51' 53.2794"S	1250
	Kebun Raya Cibodas, Java	DQ845084 (KRC2)	107° 37' 53.4"E 06° 51' 53.2794"S	1250
	Tahura Raden Soeryo, Java	DQ845085 (TRS3)	31°'44.69"E 7° 44'12.58"S 112	2227
	Tahura Raden Soeryo, Java	DQ845087 (TRS4)	31°'44.69"E 7° 44'12.58"S 112	2227
	Tahura Raden Soeryo, Java	DQ845090 (TRS2)	31°'44.69"E 7° 44'12.58"S 112	2227
A.aequalis	Taman Nasional Bukit Baka, Kalimantan	DQ845096 (BBBR1)	112° 50'E 0° 47'S	150 - 2278
	Taman Nasional Bukit Baka, Kalimantan	DQ845092 (BBBR2)	112° 50'E 0° 47'S	150 - 2278
	Taman Nasional Bukit Baka, Kalimantan	DQ845093 (BBBR3)	112° 50'E 0° 47'S	150 - 2278
	Taman Nasional Bukit Baka, Kalimantan	DQ845094 (BBBR4)	112° 50'E 0° 47'S	150 - 2278
	Taman Nasional Bukit Baka, Kalimantan	DQ845095 (BBBR5)	112° 50'E 0° 47'S	150 - 2278

The fluorescence-based DNA sequences were displayed using Chromas version 1.45 (McCarthy, 1996). CLUSTAL X version 1.8 (Thompson *et al.,* 1997) was used to align the DNA sequences. After the alignment, the DNA sequences were blasted in NCBI Blast for species confirmation. Additional sequences of 12S rRNA were obtained from GenBank (Table 2). Pair-wise distance between the populations was performed in Molecular Evolutionary Genetic Analysis (MEGA) 4.0 using Kimura-2-parameter (K2P) model (Kimura, 1980). Evolutionary model for 12S rRNA gene was conducted from Modeltest 3.7, and the best model was selected by Akaike Information Criterion (AIC) (Pasoda & Crandall, 1998). Phylogenetic trees was constructed using Neighbour Joining (NJ), while Maximum Parsimony (MP) and Maximum Likelihood (ML) were implemented in Phylogenetic Analysis Using Parsimony (PAUP version 4.0 beta; Swofford, 1998), and the Bayesian tree was constructed in MrBayes (Huelsenbeck & Rosquist, 2001). The Bootstrap method with NJ search (Saitou & Nei, 1987) was conducted using PAUP version 4.0 beta with 1000 replicates. For character-based method, the MP and ML methods were applied to estimate the phylogenetic relationship study for discrete data. Meanwhile, the Heuristic searches for MP analysis were performed with 10 random additions of taxa. The reliability of the nodes defined by the phylogenetic trees was assessed using 1000 bootstrap iterations in the fast heuristics modes.

The ML analysis was performed based from the best fit evolutionary model selected by AIC. The Heuristic search option was used in PAUP* with tree-bisection-reconnection (TBR) branch swapping and 10 random addition sequence replicates. Tree-bisection-reconnection (TBR) was used as the branch-swapping algorithm. The consensus tree from a parsimony heuristic search was used to evaluate the ML tree.

The Bayesian analysis (MrBayes 3.1.2, Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) was performed with 100^{th} generations implementing Metropolis-coupled Markov chain Monte Carlo (MCMC) under best selected model by AIC, each with four independent incrementally heated Markov chains, sampling every 100^{th} generation and burn-in of 1000 for summary parameter values and trees. The convergence of the two runs was assumed when the average standard deviation of the split frequencies has reached less than 0.1 and the potential scale reduction factor approached 1.00.

Haplotype and nucleotide diversity, Pi (p) (Nei, 1987) were calculated in DnaSP (Rozas *et al*., 2003) by using Nei's (1987) indices. The nucleotides divergence among the populations was estimated in DnaSP (Rozas *et al*., 2003). Meanwhile, the number of haplotype, segregating sites and total number of mutations were estimated using DnaSP (Rozas *et al*., 2003).

Genetic differentiation (F_{st}, N_{st} and N*m* values) was implemented in DnaSP (Rozas *et al*., 2003), whereas a hierarchical

analysis (Analysis of Molecular Variance or AMOVA), and Mantel test were estimated using Arlequin software (Excoffier, 2005).

The significance level of the F_{st} values was determined by a permutating test between the localities ($p < 0.05$). F_{st}, which is the population subdivision index, was calculated to describe the reduction in heterozigosity relative to the total population which are due to selection or drift. In fact, F_{st} is the most common measurement used to describe the genetic differentiation of the populations and was developed by Wright (1951). F_{st} is the value of probability of two random gametes which were drawn from two populations that are identical by descent, and relative to gametes taken from the entire populations. The F_{st} values ranging from of 0.00 – 0.05 are commonly considered as having little genetic differentiation, whereas 0.05 – 0.25 commonly indicates moderate genetic differentiation, and the values > 0.25 signify a pronounced level of genetic differentiation (Lowe *et al*., 2004).

N_{st} is used to estimate the degree of populations' subdivision at the nucleotide level, with the values ranging from 0 (no population subdivision) to 1 (complete population subdivision) (Bouga *et al*., 2005), of which, it describes the genetic differentiation within the species (Riginos *et al*., 2010).

The Mantel test was conducted in Arlequin (Excoffier, 2005) to estimate isolation by distance. A statistical method that uses permutations to test the null hypothesis, i.e. two variables were independent of each other and a statistical approach, was used to compare the geographical distance and genetic differentiation among the populations; in other words, to test for the isolation by distance. The significance level was tested using 1000 permutations.

Gene flow, N*m* i.e. the number of migrants per generation, was also implemented in DnaSP (Rozas *et al*., 2003). When the value of Nm is less than 1 ($F_{st} = 0.2$), the population is expected to genetically diverge over time. However, if N*m* is more than 1, the populations are expected to retain genetic connectivity.

The 12S rRNA gene constant transversion rate for mammals (bats) was estimated following Mindell *et al*. (1991), which is 0.27%. Formula divergence time (Rustchmann, 2006) is T = % net mean PD / 2r (T = time of divergence; PD = pair-wise distance; r = constant transversion rate).

RESULTS

Fig.1 shows the sampling sites of *A. aequalis* and *A. alecto* used in this study. However, *A. alecto* was unsuccessfully to be captured at Fraser's Hill and Mt Benom. From 72 individuals, including two outroups, 69 were successfully sequenced and aligned for a total of 290 bp of 12S rRNA gene. The highest nucleotide frequencies in 12S rRNA of genus *Aethalops* were adenine (A), with the average value of 38.0%, followed by thymine (T) with 21.6%, cytosine (C) with 20.6% and guanine (G) with 19.8%. The nucleotide composition showed an anti-G bias with the least frequencies of C and G (40.5%), as compared to A and T (59.5%), a

TABLE 3
Above diagonal is the mean pairwise distance within (bold) and between the populations of *A. aequalis*. The maximum and minimum ranges of the pair-wise distance are shown in parenthesis. Below diagonal is the geographical distance between localities

	Kinabalu	Trus Madi	Murud	Mulu	Bario	Penrissen	Pueh	Kalimantan
Kinabalu	**0.68 (0.0-3.6)**	0.35 (0.0-2.1)	0.49 (0.0-2.8)	0.52 (0.0-2.8)	3.9 (3.5-5.8)	0.79 (0.0-2.1)	0.35 (0.0-2.1)	4.79 (3.5-6.1)
Trus Madi	58.3	**0 (0.0)**	0.12 (0.0-0.7)	0.23 (0.0-0.7)	3.5 (3.5)	0.43 (0.0-0.7)	0.0 (0.0)	4.49 (3.5-5.0)
Murud	268	224	**0.32 (0.0-1.4)**	0.34 (0.0-1.4)	3.72 (3.5-4.3)	0.59 (0.0-1.4)	0.17 (0.0-0.7)	4.61 (3.5-5.4)
Mulu	279	242	46.8	**0.38 (0.0-0.7)**	3.5-4.3	0.67 (0.0-1.4)	0.23 (0.0-0.7)	4.65 (3.5-5.4)
Bario	284	238	30.5	79.1	**0.0**	3.99 (3.5-4.3)	3.5 (3.5)	8.25 (7.3-8.8)
Penrissen	868	830	626	591	629	**0.35 (0.0-0.7)**	0.43 (0.0-0.7)	4.95 (3.5-5.7)
Pueh	901	865	670	636	676	85.9	**0.0 (0.0)**	4.49 (3.5-4.6)
Kalimantan	845.4	791.4	576.6	560.4	541.3	344.7	414.2	**1.61 (0.0-2.1)**

TABLE 4
List of the haplotypes found in each population in Sundaland

	Kinabalu (12)	Trus Madi(6)	Murud (24)	Mulu (9)	Bario (1)	Penrissen (8)	Pueh (1)	Kalimantan (5)	Lombok (2)	Bali (1)	Sumatra (1)	Java (8)
Aa1									1			
Aa2									1			
Aa3										1		2
Aa4												1
Aa5												1
Aa6												3
Aa7												1
Aa8											1	
Aae1								1				
Aae2								1				
Aae3								1				
Aae4								2				
Aae5						4						
Aae6			1			2						
Aae7				1								
Aae8	9	6	17	5		2	1					
Aae9			1									
Aae10			1									
Aae11					1							
Aae12	1											
Aae13	1		4	2								
Aae14	1											
Aae15				1								

*Figures in parenthesis indicate the total number of individuals from each population.
Shaded represents the haplotype for *A. alecto*.

characteristic indicating mitochondrial gene. From 290 bp, 219 (84.5%) were conserved sites and 71 (14.5%) were variable sites, with 33 parsimoniously informative sites.

The genetic distance of *A. aequalis* populations within Malaysian Borneo ranged from 0.0 – 5.8%, and this was 0.0 – 2.1% (mean divergence = 1.6%) within the populations in Kalimantan. Within the populations in Sabah, it encounters 0.0 – 3.6% (mean divergence = 0.5%), and this ranged from 0.0 – 4.3% (mean divergence = 0.6%) of genetic divergence for the populations in Sarawak. Overall, the genetic distance among the species *A. aequalis* ranging from 0.0 to 8.8% (Table 3). For *A. alecto*, the divergence values for this species ranged from 0.0 – 3.9% distance. The genetic distance between the two species was between 5.4 – 12.1%. Overall, the mean divergence for the whole populations in Sabah and Sarawak was small, i.e. 0.3 – 0.7% as compared to 1.6% for the population in Kalimantan.

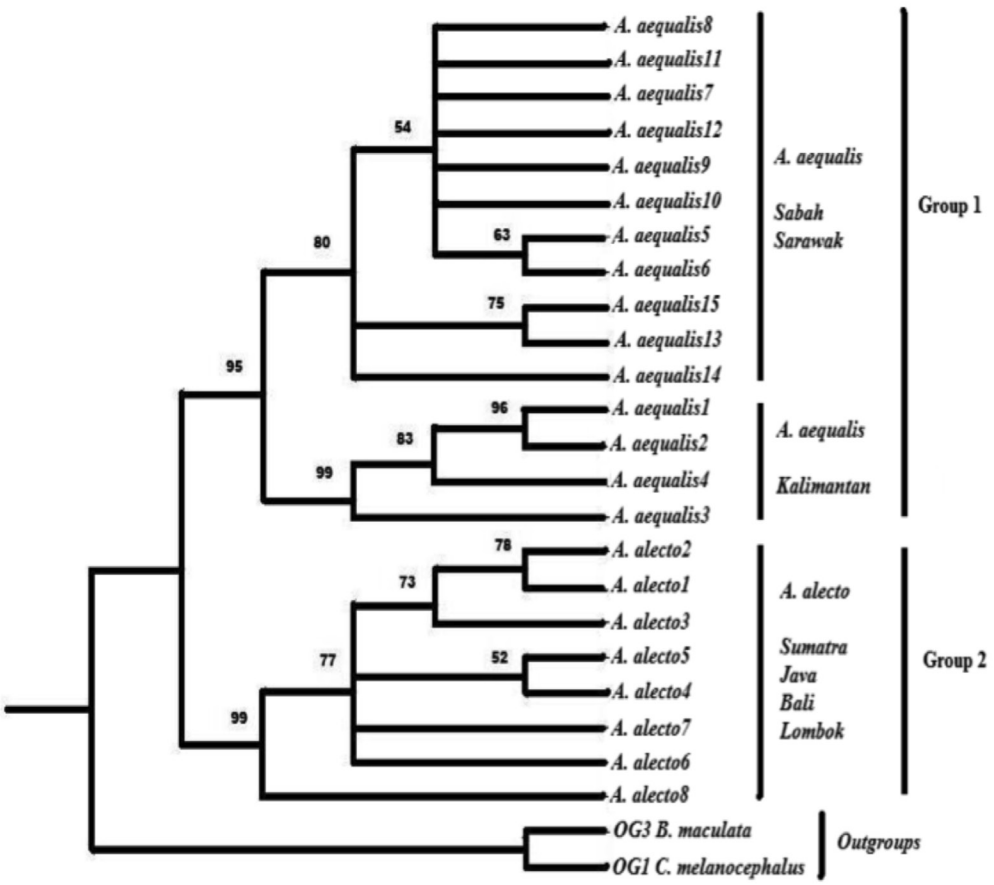

Fig. 2: Neighbour joining (NJ) the tree of genus *Aethalops* based on 290 bp 12S rRNA gene. Values on the branches were the NJ bootstrap estimates based on 1000 replicates and only >50% of the values are shown

TABLE 5
A summary of the variable sites, haplotype diversity, nucleotide diversity and the distribution of 15 haplotypes of 12SrRNA among the Bornean *A. aequalis* populations

Hap	Variable site	Populations				Whole populations
		Sabah	NSwak	SSwak	Kalimantan	
	1 1111111112 2222222223 3333333334 44] 1234567890 1234567890 1234567890 1234567890 12]					
Aae13	CCCTTGGGGC CCTCAAGTAC TAACTCTCGA GAGCGAACAC GC	(1)0.0556	(6)0.176			
Aae14	.... .T........ ....C. ..	(1)0.0556				
Aae5	....G....T C.G.......			0.444		
Aae6	....G..... C.G.......		(1)0.0294	(2)0.222		
Aae7	....G..... ..G.C.....		(1)0.0294			
Aae8	....G..... ..G.......	(17)0.833	(17)0.647	(3)0.333		
Aae12	.... T.C.GGAC.. ..G....... ....T.. ..	(1)0.0556				
Aae9	....G..... ..G...G...		(1)0.0294			
Aae11	TTTAAAACTA ....G..... ..G.......		(1)0.0294			
Aae10	....TG...G. ..G.......		(1)0.0294			
Aae15	....G.......		(1)0.0294			
Aae1	....G..... .CCT.T.GAG AG.AC.G..G ..				(1)0.2	
Aae2	....G..... .CCT.T.GAG AG.AC....G AG				(1)0.2	
Aae3	....G..... .CCT.T.GAG A..GA..... ..				(1)0.2	
Aae4	....G..... .CCT.T.GAG AGC.AG...G ..				(1)0.2	
K		1.307	1.519	1.056	4.600	3.225
Hd		0.31373±0.138	0.561±0.092	0.722±0.097	0.90000±0.161	0.615±0.067
Pi†		0.00451±0.00232	0.00524±0.00193	0.00364±0.00054	0.01586±0.00289	0.01096±0.00262

Note:
Dots indicate similar with Aea13 haplotype sequence
Numbers in the parenthesis indicate the number of individuals possessing that haplotype
NSwak – Northeast Sarawak; SSwak – Southwest Sarawak
N – number of sequence analysed; H – number of haplotypes; S – segregating sites; Sdiv – pairwise distance (estimated using Kimura-2-parameter) (Kimura, 1980); Hd – haplotype diversity; Pi – nucleotide diversity; K – average number of nucleotide differences.
† - sites with gaps were excluded

The alignment of the partial 12S rRNA gene successfully extracted eight haplotypes of *A. alecto* (Aa) and 15 haplotypes were *A. aequalis* (Aae) (Table 4). *A. alecto*1 to *A. alecto*8 were haplotypes of *A. alecto* from the populations in Lombok, Bali, Sumatra and Java. *A. aequalis*1 to *A. aequalis*4 were unique haplotypes of *A. aequalis* from the populations in Kalimantan. The remaining haplotypes of *A. aequalis* (*A. aequalis*5 – *A. aequalis*15) were the haplotypes from the mixed populations from Sabah and Sarawak. In particular, *A. aequalis* had three shared haplotypes and the most common haplotype was *A. aequalis*8 which was shared by the populations inhabiting Mt Kinabalu, Mt Trus Madi, Mt Murud, Mt Mulu, Mt Penrissen and Mt Pueh.

All the phylogenetic trees produced similar results by grouping the *Aethalops* into two different major clades (NJ, 83% MP, 79% ML, 89% BPP). Group 1 consists of *A. alecto* from Lombok, Java, Bali and Sumatra, while Group 2 consists of individuals from Borneo. Both NJ and MP separated Sabah and Sarawak into different groups from the population in Kalimantan, as supported by 95% and 92% of bootstrap value respectively in Group 2 (see Fig.2 and Fig.3).

Both ML and Bayesian trees (see Fig.4 and Fig.5) were constructed based on the

TABLE 6
The analysis of molecular variance (AMOVA) on the geographical population differentiation in Bornean *A. aequalis* using 12S rRNA gene

	Variance component	Variation (%)	Fixation Index, Φ	p[a]
Among groups	1.24494	53.36	Φ_{ct} = 0.53357	0.32454
Among populations within groups	0.28843	12.36	Φ_{sc} = 0.26503	0.00098*
Within populations	0.79987	34.28	Φ_{st} = 0.65718	0.00000*

* significant ($p < 0.05$)
[a] Probability of finding a more extreme variance component
Φ index than the observed by chance alone after 1000 permutations

TABLE 7
The Genetic differentiation matrix of the populations as measured by Φ_{st} and p-value (parenthesis) among the populations of *A. aequalis*

	Sabah	Northeast Sarawak	Southwest Sarawak	Kalimantan
Sabah	-			
Northeast Sarawak	-0.00734 (0.52252)	-		
Southwest Sarawak	0.32445 (0.00000)*	0.29194 (0.00000)*	-	
Kalimantan	0.84382 (0.00000)*	0.84956 (0.00000)*	0.83169 (0.00000)*	-

*significant ($p < 0.05$) with 1000 permutations

TABLE 8
Above diagonal are the measures of population subdivision (F_{st})* and gene flow (number of migrant, N*m*)* in parenthesis. Below diagonal are the measures of the nucleotide subdivision (N_{st})** among the populations of *A. aequalis*

	Sarawak		Sabah	Kalimantan
	Southwest Swak	Northeast Swak		
Southwest Swak		0.32092** (0.53)	0.33548 **(0.50)	0.79376* (0.06)
Northeast Swak	0.32101		-0.00550ns(-45.73)	0.76905 ***(0.08)
Sabah	0.34578	-0.00528		0.77756 **(0.07)
Kalimantan	0.79829	0.77357	0.77987	

Probability test (Chi-squared): *p < 0.05, **p < 0.01, ***p < 0.001, ns – not significant based on 1000 permutations of the sequence datasets.
*F_{st} and N*m* following Lynch and Crease (1990).
**N_{st} following Hudson *et al*. (1992).
Swak – Sarawak.

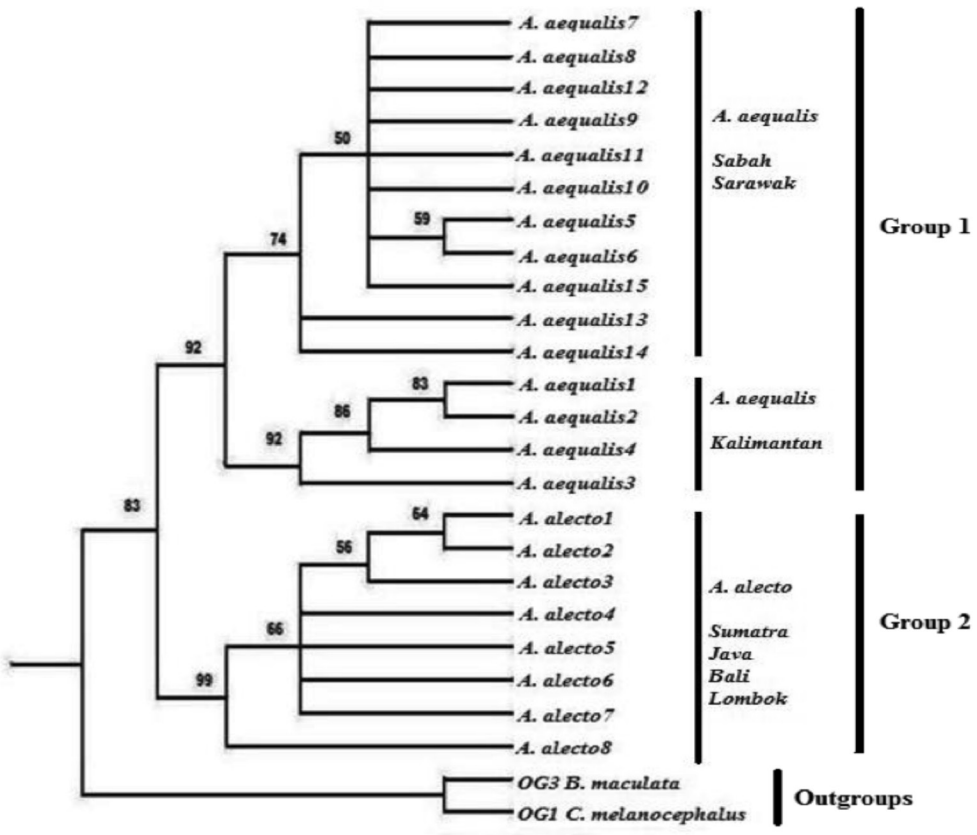

Fig.3: An equally weighted and rooted maximum parsimony (MP) tree of the genus *Aethalops,* based on 290 bp of 12S rRNA gene (tree length = 103; Consistency index, CI = 0.8447; retention index, RI = 0.9121). Values shown on the branches were the MP bootstrap estimates, based on 1000 replicates (only >50% of the values are shown)

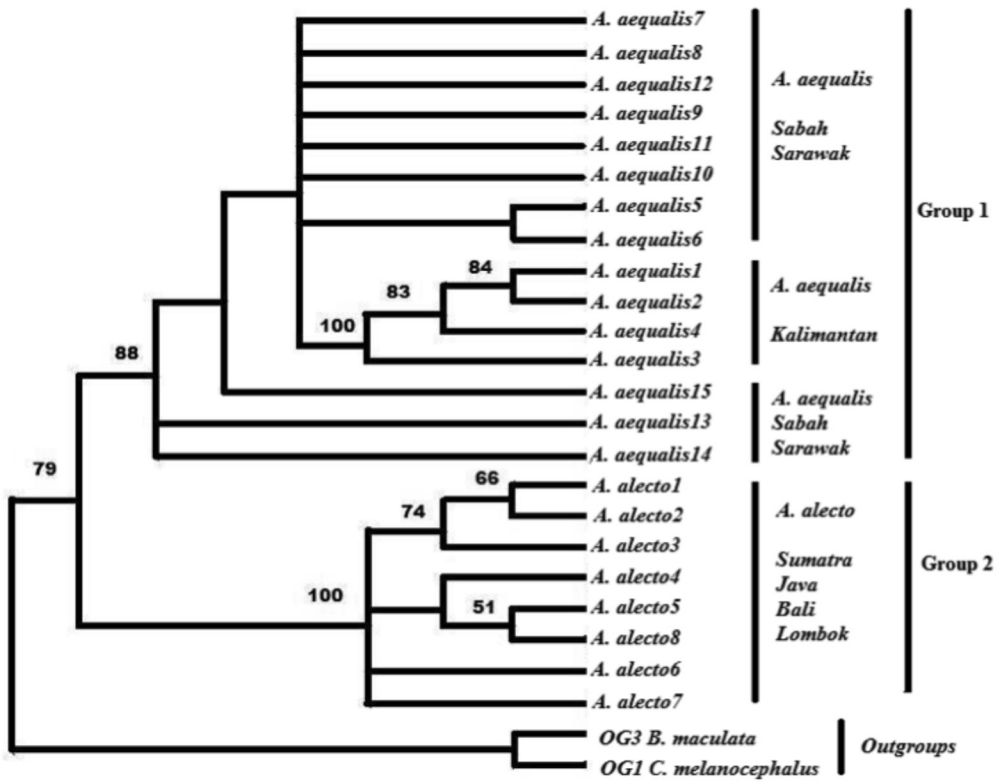

Fig.4: The maximum likelihood (ML) tree of 290bp 12S rRNA of genus *Aethalops* in Borneo with -Ln likelihood = 982.9361. Values shown on the branches represent the ML bootstrap value estimates, with 100 replicates (only >50% values are shown)

HKY+G substitution model, i.e. the best fit evolutionary model given by AIC in Modeltest 3.07 (Pasoda, 2005) with (-1nL = 982.9361; Ti/tr ratio = 2.0538; invariable sites = 0; among-site rate heterogeneity = 0.3642). Both ML and the Bayesian trees produced slightly different topologies from the NJ and MP trees. The Kalimantan group was clustered in between the Sabah and Sarawak clades. However, the grouping of Kalimantan in a group was strongly supported by high bootstrap value in all the phylogenetic trees (99% NJ, 92% MP, 100% ML, 1.00 BPP). As a conclusion, individuals that were obtained from Sabah and Sarawak were found to clade together with *A. aequalis* of Kalimantan. Therefore, it can be concluded to confirm that *Aethalops* from Sabah and Sarawak are *A. aequalis*.

The phylogenetic structure among the *Aethalops* was revealed by clustering in a minimum spanning network (MSN) (Fig.6). Based on this unrooted network of 12S rRNA gene, the *A. aequalis* from Borneo were successfully separated into two groups. Most of the haplotypes were

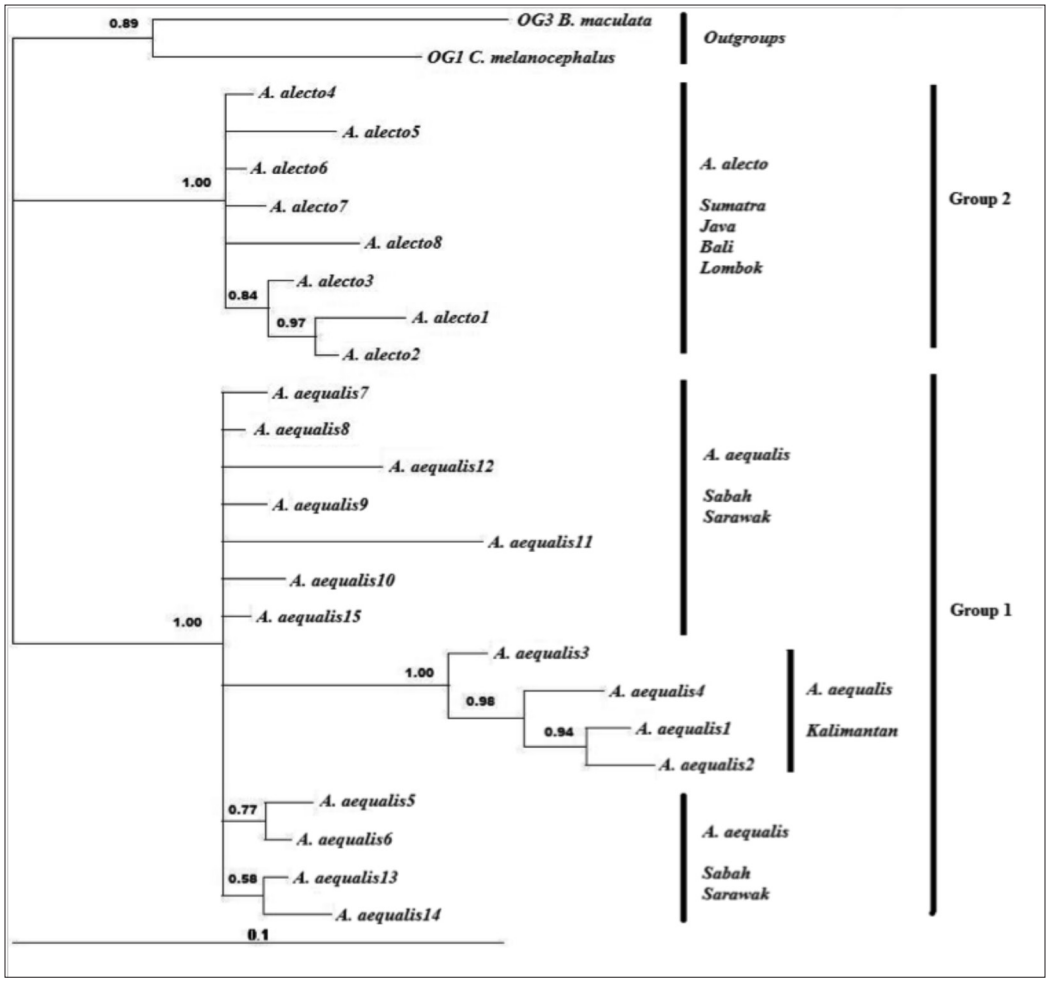

Fig.5: The Bayesian inference with 50% majority rule consensus tree of 12S rRNA gene of genus *Aethalops* in Borneo. Values of the Bayesian posterior probabilities (BPP) are shown on the branch nodes

unique haplotype for single population. *A. aequalis*8, *A. aequalis*6 and *A. aequalis*13 were shared by a few individuals from different populations. The frequencies of haplotype for each species were denoted by the proportional size of their haplo-nodes. In particular, *A. aequalis*11 from Bario was deviated from *A. aequalis*8 by 14 mutational steps. Nonetheless, the population in Kalimantan does not have sharing haplotypes with other populations of *A. aequalis,* either from Sabah or Sarawak. The genetic linkage between the two groups was deviated from *A. aequalis*3 of the Kalimantan population by 10 mutational steps before reaching the *A. aequalis* populations of Sabah and Sarawak.

The Genetic Analysis within the Population of the Bornean Pigmy Fruit Bats

Nucleotide divergence from 12S rRNA gene intrapopulation was also low

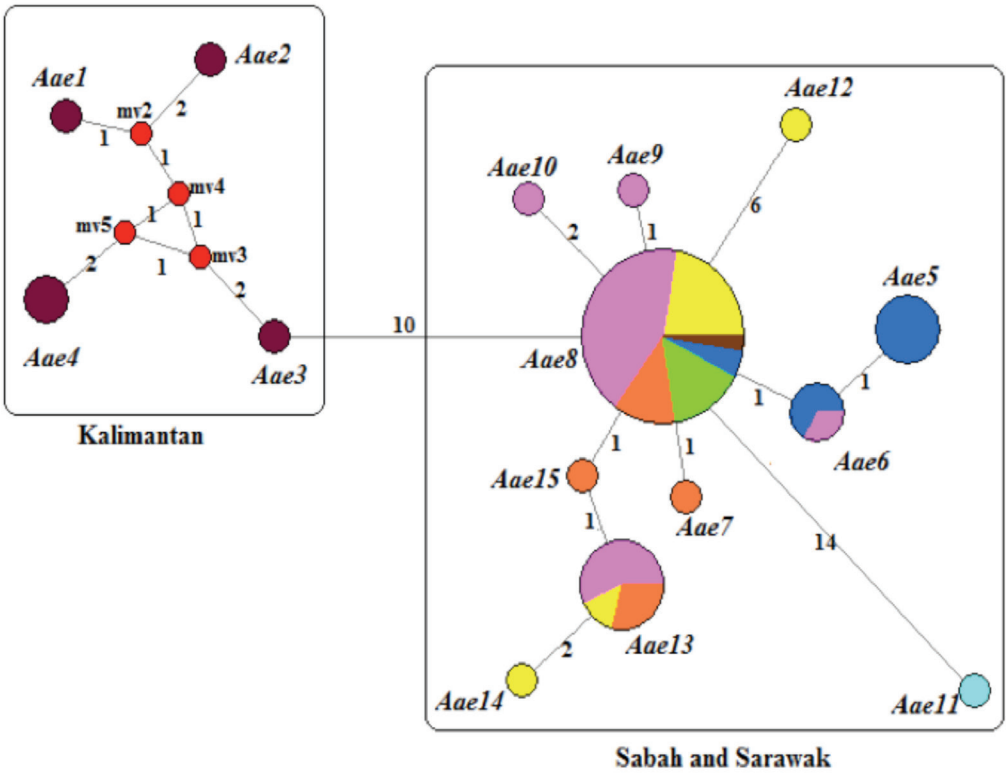

Fig.6: The haplotype mapping of 15 assigned haplo-nodes within eight populations of *A. aequalis* from Borneo. Each coloured nodes represents each population. Yellow node-Mt Kinabalu, green-Mt Trus Madi, pink-Mt Murud, orange-Mt Mulu, turquoise-Bario, blue-Mt Penrissen, brown-Mt Pueh, and purple-Kalimantan. The red nodes represent missing or unsampled haplotypes in this analysis. Note that each node represents a unique haplotype and the 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. The minimum-spanning network (MSN) was generated by Network 4.5.1.6 programme (Fluxus Tech., 2004-2009)

as it ranged from 0.4 – 0.5% and net nucleotide divergence (0.003 – 0.2%) from the populations of Sabah and Sarawak, suggesting that these two populations had very high genetic similarities. The haplotype diversity of 12S rRNA was high, i.e. varying from 31.3% (Sabah) to 90.0% (Kalimantan). Nucleotide diversity of 12S rRNA gene interpopulation was low, ranging from 0.3 – 1.5%. Overall, 12S rRNA gene had a low level of genetic differences which may due to high frequency of haplotype *A. aequalis8* in the populations of both Sabah and Sarawak (33.3 – 83.3%) but not the populations of Kalimantan (Table 5). Another possible reason was that the samples used in this present analysis were small in number. According to Esa *et al*. (2008), the small sample size may underestimate the actual haplotype distribution among the study species.

A lack of significant relationship was observed between the geographical distance and net percent nucleotide divergence, Da (r = 0.023237; p = 0.622), among the populations of *A. aequalis* in Borneo (Fig.7). Hence, it indicated that the distance between the populations was not a factor that contributed to the divergence of the sequences in *A. aequalis*.

Population Subdivision

In the AMOVA analysis, the populations were grouped into three which consisted of Sabah population (Group 1), Northeast and Southwest Sarawak (Group 2) and Kalimantan (Group 3). The results showed that the among group has the highest variation with 53.36% and was not siginificantly differentiated (p = 0.32454), followed by within the populations with 34.28%, which were to be highly significant (p = 0.0000), and lastly among the populations with 12.36% (p = 0.00098) of the variation differentiated the individuals (Table 6). The estimated Φ_{st} values among the grouped populations were significant for the genetic differentiation matrix of the populations (Table 7).

The levels of nucleotide (N_{st}), population subdivision (F_{st}) and migrants per generation (Nm) are presented in Table 8. The results show that the Bornean populations of *A. aequalis,* except for those in Kalimantan, have low levels of N_{st} and F_{st}, with high

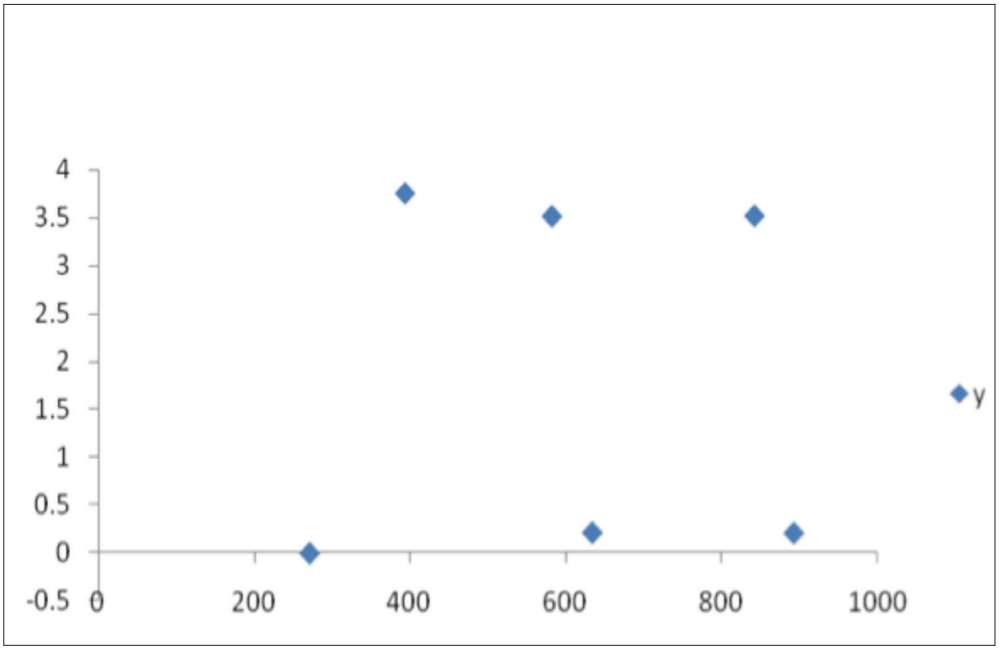

Fig.7: Scatter plots showing the relationships of the geographical distance and net nucleotide divergence, and Da (%) between the populations of *A. aequalis* in Borneo. Regression statistic: y = 0.00054; correlation coefficient, r = 0.023237)

levels of N*m*. Meanwhile, the lowest level of N_{st} and F_{st}, and the highest level of N*m* are shown by the Sabah-Northwest Sarawak population, indicationg that the gene flow between these populations is the highest. The Kalimantan population appeared to have the lowest gene flow, suggesting that this population was isolated from Sabah and Sarawak, despite being on the same island (N*m* = 0.06 – 0.08). The comparisons of genetic differentiation among the populations of Bornean *A. aequalis* were significant ($p < 0.01$ and and $p < 0.001$), except for the genetic differentiation between Northeast Sarawak and Sabah. A highly significant genetic differentiation ($p < 0.001$) was also observed between Kalimantan – Northeast Sarawak.

DISCUSSION

Overall, the phylogenetic trees NJ, MP, ML and Bayesian successfully resolved genus *Aethalops* into two major monophyletic groups corresponding to *A. alecto* and *A. aequalis,* with high bootstrap values (69% NJ, 83% MP, 79% ML, 1.00 Bayesian). The interpopulation relationships of *A. aequalis* from Sabah and Sarawak were mixed up; however, the separation between Malaysian Borneo (Sabah and Sarawak) from those in Kalimantan was clearly distinct, except for ML and Bayesian in which Kalimantan was clustered in between two clades consisting of Sabah and Sarawak. Thus, it is conclusive that the species in Sabah and Sarawak were confirmed to be *A. aequalis*, and should no longer be referred to as *A. alecto*, and *A. aequalis* is classified as a distinct species from *A. alecto*. The findings of the present study support those of Kitchener *et al*. (1993) and Maharadatunkamsi *et al*. (2006). The genetic distance within *A. aequalis* among the Malaysia Borneo is small, with a mean divergence (0.3 – 0.7%) that is almost consistent with that of Faisal (2008) with 0.2% using Cyt. *b*.

The interpopulations mixing of Sabah and Sarawak indicated that the populations of *A. aequalis* have had high genetic similarities. Among the *A. aequalis* haplotypes from Sabah and Sarawak, *A. aequalis8* or Aae8 is the most common shared haplotypes among the individuals, ranging from southwest Sarawak to northeast Sarawak and Sabah. Apart from being genetically similar, they are considered as a single morphotype based on the same samples taken from the populations in Sabah and Sarawak, as indicated by another study using skull morphometric analysis. The slight morphological difference between southwest Sarawak and northern Borneo populations of these bats is possibly due to the adaptation to food resources to survive in the species competition. In more specific, the skull of the bats may have evolved to adapt into optimised form to meet the demand of holding and masticating of different food sources, depending on what habitat provides (Tingga, 2010).

Based on coalescent theory, the most common haplotype may be the oldest, with the expectation that the haplotype should be geographically widespread. Therefore, *A. aequalis8* is predicted as the ancestral haplotype. However, since *A. aequalis8*

is not identified as basal by the outgroup rooting, it is suggested that this may not be the absolute oldest haplotype, but it can relatively be considered as one of the ancestral haplotype as compared to other observed haplotypes of *A. aequalis*. A relatively similar case was also observed within the song sparrow haplotypes (Fry & Zink, 1998), where two common haplotypes were observed to be widely distributed but not placed at the basal clade to be considered as the oldest haplotype. The present study also showed that the common haplotypes were not rooted at the basal; however, *A. aequalis13* and *A. aequalis14* were rooted at the basal of the monophyletic group of *A. aequalis* from Sabah and Sarawak.

*A. aequalis*11 (Bar3) was genetically distance from other haplotypes (genetic distance 3.9 – 5.8%). Bario (Kelabit Highland) is situated at the geographical boundary between Kalimantan and Sarawak. This individual is genetically unique and has high genetic distance ranging from 1.1 – 4.6%). Moreover, this individual may still retain its ancestral haplotype which diverged million of years ago (mya). Such a divergent individual from this species is regarded to be associated with distinct geographic ranges which reflect a long-term zoogeographic barrier to gene flow that is largely independent of glaciations events (reviewed by Avise *et al*., 1987, as cited in Dobson *et al*., 1995).

Using the constant transversion rate of 0.27% per million years (Mindell *et al.,* 1991), the separation time between *A. aequalis* and *A. alecto* was estimated approximately 12 mya, which fell during the mid Miocene period. The data obtained for the speciation of *A. aequalis* were not consistent with the Pleistocene speciation hypothesis. Therefore, distributional of this species was apparently due to dispersal rather than vicariance, changes of sea level or vegetational change. According to Haq *et al*. (1993), however, there was a relatively low sea level even before the last 2 mya.

Thus, the question now is that which of the observed islands in Indonesia was the earliest population of *A. alecto* after its separation from *A. aequalis*? As discussed earlier, the divergence time between *A. alecto* and *A. aequalis* was ~12 mya, which predated the Pleistocene period in Lombok (the earliest ~13.2 mya), followed by Java and Bali (~12.53 mya) and Sumatra (~10.96 mya). Hence, Lombok was predicted to be the ancestor population among all the *A. alecto* populations that had been observed. Hypothetically, Lombok was the first colonised island of *A. alecto* after this particular species had diverged from *A. aequalis*. Nonetheless, it is still undetermined whether the two forms of *Aethalops* arose from Borneo or Lombok before it diverged.

It is possible that *A. alecto* from Sumatra, Java, Bali and Lombok, following 12S rRNA constant transversion rate (Mindell *et al*., 1991) started to disperse from Lombok approximately ~1.9 mya, based on the 12S rRNA gene mammals divergence time by Mindell *et al*. (1991) to Sumatra, and it then spread to Java, Bali and Lombok (Lombok-Java = ~0.89 mya, Lombok-Bali =

~0.89 mya, Lombok-Sumatra = ~1.98 mya, Sumatra-Java = ~0.74 mya). The prediction time for *A. aequalis* in Java is supported by the findings of van der Bergh *et al*. (2001), who found no evidence indicating mammals present on Java prior to 2.4 mya. After that time, intermittent land bridges allowed colonisation to occur (van der Bergh *et al*., 2001). It was during the early Pleistocene that the presence of the fauna characteristic of open woodlands found in the vertebrate fossil record of Java (van der Bergh *et al*., 2001). At this stage, there was a connecting tract of open vegetation from the Asian mainland to Java. According to Bird *et al*. (2005), the earlier separation would have likely caused the island to retain a group representative from the populations frequenting the area during the glacial times.

In the analysis of the current data, all the phylogenetic trees showed a close genetic relationship between the populations in Lombok and Bali. A drop of sea level in the Strait of Lombok would have likely facilitated the dispersal of *A. alecto* to Bali across the Lombok Strait. Similarly, this condition has also been observed in other species of bats, such as *Myotis muricola* and *Cynopterus brachyotis*. A recent study by Wiantoro (2010) indicated that in Bali and Krakatua, *M. muricola* was not clustered accordingly to the population groups based on Wallace's Line. Similarly, Wiantoro (2010) also stated that the low sea level in the Strait of Lombok had provided the possibility of gene flow within *M. muricola* Eastern and other populations on Krakatau and Bali islands to the populations on other Lesser Sunda islands.

Historical Population of the Bornean Pigmy Fruit Bats

Using the constant transversion rate of 0.27% per million years (Mindell *et al.,* 1991), *A. aequalis* was found to have diverged from its sister species *A. alecto* approximately 12 mya during the mid-Miocene period. Therefore, it was hypothesised that the widespread distribution of *A. aequalis* in Borneo was most probably due to the colonisation event that occurred before the Pleistocene and was not caused by the changes in the sea level (Dobson *et al*., 1995). It was also suggested that the species is endemic to the island and this originated > 2 mya. However, the species has not undergone repeated extinction and recolonisation, and it is more likely to have persevered at a particular island since its origin (Steppan *et al*., 2003). Thus, Kalimantan (West Central Kalimantan at Taman Nasional Bukit Baka) has been predicted to be the location of the original population of *A. aequalis*. Furthermore, among the populations of *A. aequalis* in Borneo, the population in Kalimantan was found to be the ancestor towards the other populations observed in Borneo.

There was a very high genetic difference between the populations in Sabah and Sarawak compared to the one in Kalimantan, with a high genetic distance of 3.5 – 8.8% and very low level of gene flow. The Tamo Abo Range is the boundary that separates Sarawak and Sabah from Kalimantan and thus limits the gene flow between the population of Malaysian Borneo and Kalimantan. Hypothetically, the specimens from Sabah and Sarawak could also be a

sub-species of the population in Kalimantan. Nevertheless, this has yet to be investigated as there were no secondary data available in the present study to support this hypothesis. According to Fry and Zink (1998), DNA polymorphism has been used as an inference on the historical patterns of population expansion.

Based on the flow of the divergence time from 12S rRNA gene, it was hypothesised that the pattern of movements of this particular species went from Kalimantan to southwest Sarawak to the northeast of Sarawak and Sabah. Since the divergence from *A. alecto* (12 mya), the population of *A. aequalis* from Kalimantan dispersed to southeast Sarawak after approximately 5 million years (7.2 mya) and later from southwest to northeast of Sarawak (370 ka) and Sabah (370 ka). Both the populations of the northeast Sarawak and Sabah were recently diverged from the one in southeast Sarawak (see Fig.8). Meanwhile, age estimation between northeast Sarawak and Sabah groups suggests that the northern populations of Borneo are sister populations that are supported by very close genetic relationship and high gene flow.

The divergence time between southwest Sarawak and northeast Sarawak –Sabah groups occurred during the Pleistocene. This suggests that the haplotypes from Mt Mulu, Mt Murud, Mt Kinabalu and Mt Trus Madi have recently diverged from one another. In fact, this could be the reasons why these populations are highly genetically similar. The population in southwest Sarawak was predicted to be the ancestral group because during the Pleistocene period, The Northern Borneo was suggested to act as a refugium for the lowland rainforest species during the late Pleistocene (Brandon-Jones, 1998; Garthorne-Hardy *et al*., 2002). The Pleistocene facilitated the dispersal and genetic exchange of *A. aequalis* populations that are now confined to mountaintops.

In general, the mountains in Sabah and Sarawak form the backbone of a highland ranging from Mount Kinabalu (Crocker range) through Kelabit Highland (Bario) to Madi Plateau and the Schwaner Range of Kalimantan. It is assumed that the dispersal of a montane bat is similar to a montane bird, where dispersal to another mountain or range occurs along a spinal chain that connects the mountains. Most mountains in Sabah and Sarawak are connected along a chain, with an elevation of more than 1500 m a.s.l. Along this chain, the ridge breaks away to form a long stretch of lowland (Lubok Antu) between south western Sarawak and Mount Lawit. The separation of this ridge could be one of the reasons that had led to genetic divergence between the population at Mt Penrissen and those in the northern part of Sarawak and Sabah.

CONCLUSIONS

In conclusion, 12S rRNA was found to be able to resolve the interspecific relationships of *A. aequalis* and *A. alecto*. The current findings conclude that *A. aequalis* is a single unit panmictic population in Borneo and thus support the previous studies that *A. aequalis* is no longer known as a subspecies of *A. alecto*. Moreover, the

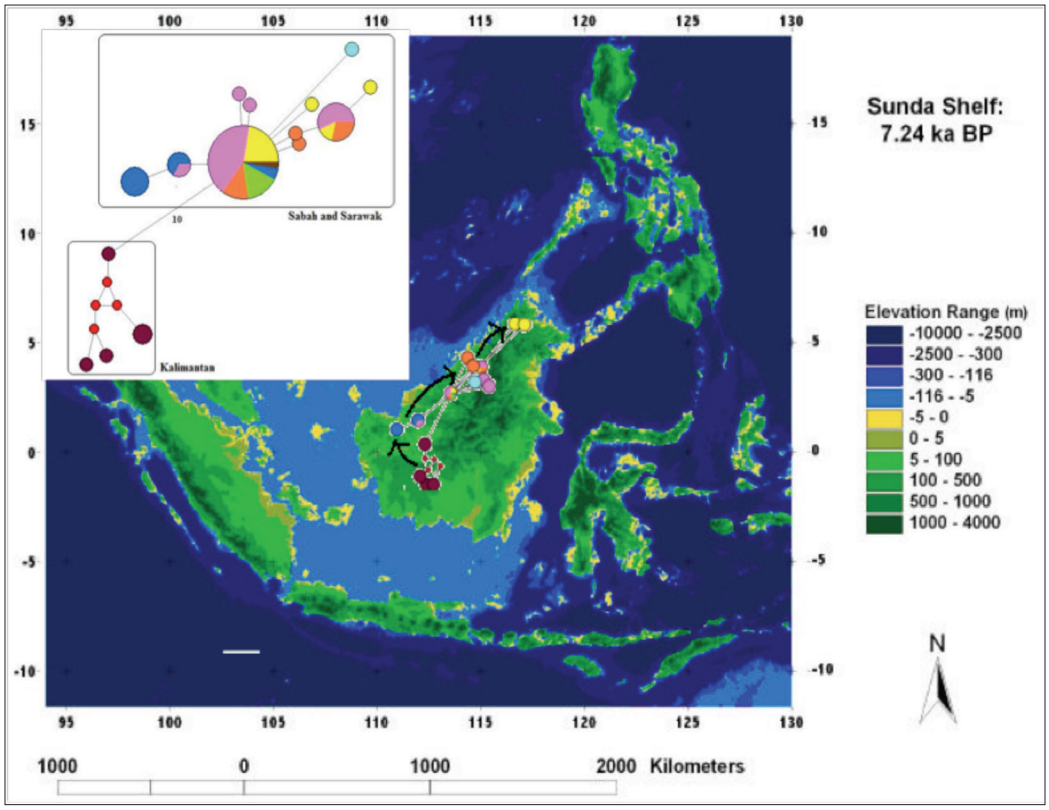

Fig.8: The Minimum Spanning tree of 12S rRNA haplotypes (left) and the hypothetical origin population of *A. aequalis* and its route dispersal, as indicated by the arrows (right). The map was adapted from Sathiamurthy and Voris (2006)

genetic distance between *A. aequalis* from Malaysian Borneo and Kalimantan is rather high and this is supported by the high value of population and nucleotide subdivision, which produced a new hypothesis on this particular species, with the possibility of two subspecies within Borneo. However, this gene is unable to resolve the intraspecific relationships of *A. aequalis* in Sabah and Sarawak. The intermixing population among the populations of *A. aequalis* in Sabah and Sarawak indicates high genetic similarities, whereby the dispersal was hypothesised from southern Sarawak to the northern Borneo, and that this dispersal was facilitated by Pleistocene climatic fluxes. The population in Kalimantan was also postulated as the possible ancestral for *A. aequalis* of Borneo.

ACKNOWLEDGEMENTS

The authors would like to extend their greatest gratitude to Zoological Museum of UNIMAS (MZU) for allowing them to examine the specimens and tissue samples used in this study. Sincerely appreciations also go to the Faculty Resource Science and Technology for various administrative

and logistic supports throughout this study. This study was partially funded by MOHE FRGS 06(08)6602007 led by MTA. The authors would also like to thank Sarawak Forestry Corporation and Sarawak Forestry Department for permitting the license number 07412 under the State Wild Life Protection Rules 1998 for the researchers to work at the protected areas, using the research permit number NPW.907.4.2(III)-3 and also the permit to enter park number 3/2008. Our thanks also go to Sabah Park for permitting us to collect the samples at Mount Trus Madi and Kinabalu Park. The team would also acknowledge Dr. Ramlah Zainuddin for her comments and suggestions on the Minimum Spanning Network and AMOVA analyses, as well as members of the Department of Zoology in assisting sample collections during the field and laboratory works.

REFERENCES

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

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A., & Saunders, N. C. (1987). Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. *Annual Reviews Ecology and Systematics*, *18*, 489-522.

Bird, M. I., Taylor, D., & Hunt, C. (2005). Palaeoenvironments of insular Southeast Asia during the last Glacial Period: A savannah corridor in Sundaland? *Quarternary Science Reviews*, *24*, 2228-2242.

Brandon-Jones, D. (1996). The Asian Colobinae as indicators of Quaternary climatic change. *Biological Journal of the Linnean Society*, *59*, 327-350.

Boeadi, & Hill, J. E. (1986). A new subspecies of *Aethalops alecto* (Chiroptera: Pteropodidae) from Java. *Mammalia*, *50*, 263-266.

Bouga, M., Harizanis, P. C., Kilias, G., & Aldiotis, S. (2005). Genetic divergence and phylogenetic relationships of honey bee Apis mellifora (Hymenoptera: Apidae) populations from Greece and Cypnes using PCR-RFLP analysis of mtDNA segments. *Biochemical Genetics*, *36*, 335-344.

Dobson, J. J., Colombani, F., & Ng, P. K. L. (1995). Hyogeographic structure in mitochondrial DNA of a South – east Asian freshwater fish, *Hemigbagrus nemurus* (Siluroidei:Bagridae) and Pleistocene sea level changes on the Sunda Shelf. *Molecular Ecology*, *4*, 331-346.

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

Excoffier, L. (2005). Editorial. *Human Genomics*, *2*(3), 155-7.

Faisal, A. K. (2008). *Diversification of Old World Bats in Malaysia: An evolutionary and phylogeography, hypothesis tested through Genetic species concept (Msc. thesis dissertation)*. Texas Tech University, Lubbock.

Francis, C. M. (2005). *Guide of Mammals of South east Asia*. London: New Holland Publishers (UK) Ltd.

Fry, A. J., & Zink, R. M. (1998). Geographic analysis of nucleotide diversity and song sparrow (Aves: Emberizidae) population history. *Molecular ecology*, *7*, 1303-1313.

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 the Linnean Society, 75, 4*53-466.

Grewe, P. M., Krueger, C. C., & Aquadro C. F. (1993). Mitochondrial variation among lake trout (*Salvelinus namaycush*) strains stocked into Lake Ontario. *Canadian Journal of Fisheries and Aquatic Sciences*, *50*, 2397-2403.

Haq, B. U., Hardenbol, J., & Vail, P. R. (1993). Chronology of fluctuating sea levels since the Triassic. *Science*, *235*, 1156-1167.

Hill, J. E. (1961). Fruit bats from Federation of Malaya. *Proceedings of the Zoological Society of London*, *136*, 629-642.

Hill, J. E. (1966). A collection of Bats from Sarawak. *Sarawak Museum Journal*, *14*, 237-246.

Hill, J. E. (1983). Bats (Mammalia: Chiroptera) from Indo – Australia. *Bulletin of the British Museum Natural History (Zoology), 45*, 103-208.

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

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

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.

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

Koopman, K. F. (1989). Distributional patterns of Indo–Malayan Bats (Mammalia: Chiroptera). *American Museum Novitates*, *2942*, 1-19.

Lowe, A., Harris, S., & Ashton, P. (2004). *Ecological Genetics. Design, analysis and application*. Oxford: Blackwell Publishing.

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

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

Mickleburgh, S. P., Hutson, A. M., & Racey, P. A. (1992). *Old World Fruit Bats, An Action Plan for their Conservation.* IUCN/ SSC Chiroptera Specialist Group, International Union for Conservation of Nature, Gland, Switzerland.

Maharadatunkamsi, & Syamsul Arifin Zein, M. (2006). Genetic Variation of Bat in the Genus *Aethalops* (Chiroptera: Pteropodidae) from Indonesia: Analysis of 12S rRNA Gene of Mitochondrial DNA. *Journal Biologi Indonesia*, *5*(2), 75-86.

Mindell, D. P., Dick, C. W., & Baker, R. J. (1991). Phylogenetic relationships among megabats, microbats and and primates. *Proceedings of the National Academy of Sciences*, *88*, 10322-10326. USA.

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

Palumbi, S., Martin, A., Romano, S., McMillan, W. O., Stice, L., & Grobowski, G. (1991). *The simple tool's guide to PCR*. Department of Zoology and Kewalo Marine Laboratory. Honolulu: University of Hawaii.

Pasoda, D., & Crandall, K. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics*, *14*, 817-818.

Payne, J., Francis, C. M., & Philipps, K. (1985). *A Field Guide to the Mammals of Borneo.* The Sabah Society, Kota Kinabalu.

Riginos, C., & Victor, B. C. (2010). Larval spatial distribution and other early life-history

characteristic predict genetic differentiation in eastern Pacific blennoid fishes. *Proceeding of Royal Society London B*, *268*: 1931-1936.

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.

Ronquist, F., & Huelsenbeck, J. P. (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, *19*, 1572-1574.

Rustchmann, F. (2006). Molecular dating of phylogenetic trees: A brief reviewing current methods that estimate divergence time. *Diversity and Distribution*, *12*, 35-48.

Ryan, J. R, Guan, A. K. H., Kumaran J. V., Esa Y., Sallehin A. A., & Abdullah, M. T. (2008). Malaysian Fruit Bats Phylogeny Inferred Using Ribosomal RNA. *Pertanika Journal of Tropical Agricultural Science*, *31*(1), 67-77.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*, 406-425.

Steppan, S. J., Zawadzki, C., & Heaney, L. R. (2003). Molecular phylogeny of the endemic Philippine rodent Apomys (Muridae) and the dynamics of diversification in an oceanic archipelago. *Biological Journal of the Linnean Society*, *80*, 699-715.

Swofford, D. L. (1998). *PAUP, Phylogenetic Analysis Using Parsimony (and Other Methods) Version 4*. Massachusetts: Sinauer Associates.

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.

Tingga, R. C. T. (2010). *Morphology and Genetic Variation of Aethalops (Chiroptera: Pteropodidae) using Mitochondrial and Nuclear Genes*. (MSc thesis dissertaion). Universiti Malaysia Sarawak, Kota Samarahan.

van den Bergh, G. D., de Vos, J., & Sondaar P. Y. (2001). The Late Quaternary palaeogeography of mammal evolution in the Indonesian Archipelago. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *171*, 385-408.

Van strien, N. J. (1986). *Abbreviated checklist of the Mammals of the Australian Archipelago*. School of Environmental Conservation Management, Bogor.

Wiantoro, S. (2010). *Biogeography and variation of* Myotis muricola *(Gray, 1846) (Chiroptera: Vespertilionidae) from the west and east of Wallace's line*. (MSc thesis dissertaion). Universiti Malaysia Sarawak, Kota Samarahan.

Wright, S. (1951). The genetical structure of populations. *Annuals of Eugenics*, *15*, 323-354.