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Genetic variations and population structure of the genus *Cynopterus* in Malaysia

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ABSTRACT

Nuclear microsatellite analysis of *Cynopterus* was aimed at characterising the microsatellite genotypes and the population structure of this genus especially in the large form of *C. brachyotis* and the small form of *C. brachyotis*. Nine pairs of existing microsatellite primers isolated from Indian *C. sphinx* were used. A total of 51 alleles and 97 genotypes were documented from four forms of *Cynopterus*. Genetic variations revealed from AMOVA analysis showed that there was low genetic variation among the four forms. The interspecies Global AMOVA comparison analysis showed that the genetic variation between the large and small forms of *C. brachyotis* was the lowest among interspecies comparisons. This resulted in low genetic structure in the UPGMA tree, and species boundary of each form was not clearly defined. This might due to the microsatellite primers that were isolated from Indian *C. sphinx* being low in sensitivity to detect variations in Malaysian cynopterans.

Keywords: Microsatellite analysis, *Cynopterus*

INTRODUCTION

Microsatellites or Simple Sequence Repeats (SSRs) are nuclear markers, as well as co-dominant Mendelian markers (DeWoody and Avise, 2000; Srikwan *et al.*, 2002; Scandura, 2004). In microsatellites, sequences are composed of repeated units of sequences; these repeats are generally two to five base pairs in length and are called di-, tri-, tetra- or pentanucleotides (Srikwan *et al.,* 2002). The dinucleotide CA repeats are most commonly found in many eukaryotes (Page & Holmes, 1998; Scandura, 2004).

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The mutation rate of microsatellites was estimated between 10^{-2} and 10^{-5} per gamete, per generation (Page and Holmes, 1998; Wright *et al.,* 2005), and these mutations directly influence the changes of repeat units (Degnan *et al.,* 1999). They vary greatly in copy number between individuals (Page and Holmes, 1998). This high level of diversity, together with characteristics such as neutral evolution, codominance and simple Mendelian inheritance allows microsatellites to be used to identify closely related individuals, recent migration events and sex dispersal patterns (Hedrick, 1999; Flagstad *et al.,* 2003; Hansson *et al.,* 2003).

The difference in the repeat units of nucleotides carries the information that was passed through from generations to generations from their common ancestral alleles. Recent common ancestry is shown by the similarity of the repeat number, thus the microsatellite approach can be used to examine some phylogenetic applications in mtDNA (Degnan *et al.,* 1999). The highly polymorphic nuclear loci can also be used to identify population specific polymorphisms (Bruford & Wayne, 1993).

Analysis of microsatellites can provide both paternal and maternal information (Burg *et al.,* 1999). These are important in providing more reliable genetic information such as evolutionary lineages between closely related species which cannot be answered or revealed using mtDNA based genetic markers. In one instance, Campbell *et al.* (2004) stated that without comparison of nuclear markers, they cannot rule out the possibility that *C. brachyotis* lineage may have a significantly longer and more complex evolutionary history than what was revealed from mitochondrial haplotype data.

In Malaysia, research focused on the species complex of *C. brachyotis* has been ongoing for many years. The first indication on the existence of two forms of *C. brachyotis* dates back to 1985 where Payne *et al.* (1985) noticed that there were two forms of *C. brachyotis*, namely, large-sized *C. brachyotis*, which can be found in forest edges and gardens, and small-sized *C. brachyotis*, which can be found in tall forests. Later, Francis (1990) mentioned the size difference of *C. brachyotis* captured in primary versus secondary forests. They stated that there was some morphological overlap and that the taxonomic situation of *C. brachyotis* was unresolved (Payne *et al.*, 1985; Francis, 1990).

Abdullah *et al*. (2000) and Abdullah (2003) were the first to present significant genetic data on the existence of two forms of *C. brachyotis*, with clear indication that these two forms were found in distinct habitats – *C. brachyotis* I (large-sized) in open habitat and *C. brachyotis* II (small-sized) in closed habitat – but also exist sympatrically in ecotones between forests (closed habitat) and open areas (open habitat). Abdullah (2003) also speculated the existence of hybrids in the ecotonal areas based on clustered individuals that were found in his phylogenetic analysis based on 635bp partial cytchrome *b* gene in the mtDNA. His conclusion on the existence of the two forms of *C. brachyotis* was later confirmed in similar studies by Campbell *et al.* (2004,

2006). Campbell *et al.* (2004) used 690bp partial mitochondrial cytchrome *b* gene and 576bp of partial mitochondrial control region to infer phylogenetic relationships of the cynopterans in Malaysia. Subsequently, Campbell *et al*. (2006) used 567bp of partial mitochondrial control region as mtDNA molecular marker to examine evolutionary relationships of these species. Later, these two forms of *C. brachyotis* were referred as *C.* cf. *brachyotis* "Forest" for the small size and *C.* cf. *brachyotis* "Sunda" for the large size (Campbell *et al*., 2004; 2006; Francis, 2008).

Morphological evidence was also presented by Abdullah (2003) using five external morphological characters. Furthermore, Jayaraj *et al*. (2004) collected data on 28 morphological characters and used multivariate analyses to assess the morphometrics of the *Cynopterus* complex based on Abdullah's (2003) results. Jayaraj *et al*. (2004) observed congruent results with Abdullah (2003); both studies were in-agreement with the existence of two forms of *C. brachyotis*. Jayaraj *et al.* (2004) found that the large-sized *C. brachyotis* and the small-sized *C. brachyotis* can be differentiated using forearm length as stated by Abdullah (2003), and measurements of the palatal length (Jayaraj, 2009). Later, Jayaraj *et al*. (2012) developed two predictive models with eight measurements from the skull, dental and external characters to discriminate these two forms of *C. brachyotis*. Furthermore, Abdullah and Jayaraj (2006) deduced that the type specimen was a large form of *C. brachyotis* based on limited data from the original descriptions and morphological measurements by Müller (1938).

According to Francis (2008), the small-sized *C. brachyotis* consumes mostly fruits especially figs, while the large-sized *C. brachyotis* consumes a wider range of food as compared to the small-sized *C. brachyotis*. The large-sized *C. brachyotis* consumes small fruits, bananas, nectar, pollens, and soft pulp, whereby it sucks out the juice from the pulp. In terms of habitat type, the large-sized *C. brachyotis* and small-sized *C. brachyotis* occupy different habitat types. The large-sized *C. brachyotis* is found in open habitats, such as secondary forests, agricultural lands, forest fringes and swamps, while the small-sized *C. brachyotis* is found in closed habitats, such as primary forests, old regenerated forests, and forest fringes near primary forests. However, as indicated by Abdullah (2003), these two forms of *C. brachyotis* are sympatric at forest edges where hybridisation could occur. Unfortunately, this cannot be investigated using mtDNA molecular markers as mtDNA only reflects the gene flow and dispersal pattern of the female founders in the population (García-Moreno *et al.,* 1996).

To study the species boundary and genetic variations between *C. sphinx* and *C. brachyotis* in Peninsular India, Storz (2000) developed nine pairs of microsatellite primers from *C. sphinx* (n = 413 individuals). In the preliminary study of taxonomic relationship between *C. sphinx* and *C. brachyotis*, a total of 300 cynopterans (additional 189 *C. sphinx* and 111 *C.*

brachyotis) were screened using five pairs of the microsatellite primers (CSP1, CSP2, CSP5, CSP7 and CSP9). As a result, CSP2 was found monomorphic in 20 individuals of *C. brachyotis* and the allele segregations were relatively shorter in *C. brachyotis* compared to *C. sphinx* (Storz, 2000). Besides, he also used these microsatellite primers to investigate the polygyny and social structure of *C. sphinx* using the population genetic structure (Storz, 2001; Storz *et al*., 2001a, 2001b).

This study aimed to investigate the utility and reproducibility of the existing microsatellite markers designed by Storz (2000) for population studies of *Cynopterus* in Malaysia; and subsequently to include more samples of this taxon from Malaysia especially from Sabah and Sarawak. Second, this study aimed to elucidate the possibility of hybridisation of the two forms of *C. brachyotis* occurring in the ecotone as speculated by Abdullah (2003) by using microsatellite analysis.

MATERIALS AND METHOD

Sample Identification and Collection

The samples for this study were from two sources, namely, the sample collection from the field and museum deposits in Malaysia. The distinction of the large form and small form was based on the *C. brachyotis* forearm measurements (> 60 mm = large form, < 60 mm = small form) as described in previous studies (see Abdullah, 2003; Campbell *et al.,* 2004, 2006, 2007; Jayaraj *et al.,* 2004, 2005; Francis, 2008; Jayaraj, 2009). *C. sphinx* and *C. horsfieldii* were identified based on forearm measurements and dentition as described in Payne *et al.* (1985), Lekagul and McNeely (1988), as well as Corbet and Hill (1992). Sampling locations were selected from open habitats (i.e. agricultural areas, secondary forests and swamp areas), closed habitats (i.e. primary forests and old regenerated forests) and forest edges in Peninsular Malaysia and Borneo. Museum samples were collected from the Sarawak Museum, Kinabalu Park Museum, the zoological museum of the Faculty of Resource Science and Technology (FRST) in Universiti Malaysia Sarawak (UNIMAS) (Abdullah *et al*., 2010) and the zoological museum of the Department of Wildlife and National Parks (DWNP) at Bukit Rengit, Pahang.

DNA Extraction and Microsatellite Genotyping

All instruments were sterilised by autoclave to avoid cross-contamination of DNA. DNA extractions were done using the standard cetyl trimethyl ammonium bromide (C-TAB) protocol following Grewe *et al.* (1993). Nine pairs of microsatellite primers designed by Storz (2000) were used to screen cynopterans in this study. The PCR amplifications were performed following the standard protocol as described by Sambrook *et al.* (1989). The amplifications were performed in a 25 µl of PCR mixture containing 3.5 µl of 10× PCR buffer, 1.5 µl of magnesium chloride ($MgCl_2$) (25 mM), 0.5 µl of dNTP mixture (10 mM), 1.25 µl of each primer (10 µM), 0.2 µl of

Taq polymerase and 1.0 to 2.0 µl of DNA template. Negative control (a reaction without a DNA template) was included in each PCR amplification to ensure that no DNA cross-contamination occurred during the preparation of PCR reagents.

PCR amplifications for all microsatellite primers used were done using a programmable thermal cycler (MyCycler™ by Bio-Rad) with standard-3 PCR protocol (Fong, 2011). The PCR programme was set as follows: a preliminary denaturation at 94°C for 2 minutes and 30 seconds, followed by denaturation of double stranded DNA at 94°C (30 seconds), primer annealing at optimised temperature (45 seconds), and primer extension at 72°C (50 seconds) for one complete cycle. Each cycle was repeated for 30 times and subsequently a final extension at 72°C for 2 minutes and 30 seconds was included to avoid generation of incomplete double stranded DNA.

Three percent of TBE-agarose gel was used to screen the DNA products in microsatellite analysis. All the results of the gel runs were photographed under UV illumination.

Microsatellite Data Analysis

The numbers of alleles per locus and between species were directly calculated. Overall allele frequencies for each species across loci were generated using POPGENE (version 1.31; Yeh *et al*., 1999). Probability tests for Hardy-Weinberg equilibrium (p-value < 0.05) were carried out for each locus for each species by using the algorithm of Levene (1949) in POPGENE (version 1.31; Yeh *et al*., 1999). GENEPOP (version 3.2; Raymond and Rousset, 1995) online analysis was used to test for linkage equilibrium across loci for each species.

The numbers of genotypes for each population, interspecies as well as intraspecies genotype sharing and gene diversity were generated using Arlequin (version 3.1; Excoffier *et al*., 2005). The Ewens-Watter neutrality test was performed to test the selection pressure for each locus using POPGENE (version 1.31; Yeh *et al*., 1999). The statistics in the neutrality test were calculated using 1000 simulated samples.

Global analysis of molecular variance (AMOVA) was used to perform the hierarchical analysis for overall species and inter-species level (Excoffier *et al*., 2005). Four covariance components from the total variance of the hierarchical analysis were used to compute four fixation indices generated using Arlequin (version 3.1; Excoffier *et al*., 2005). A total of 1000 permutations were used to carry out the significance tests between the covariance components and the correlated fixation indices with p-value less than 0.05.

A dendogram based on the unweighted pair group method with arithmetic mean (UPGMA) was constructed using the F_{ST} estimates between populations across the four species implemented in Molecular Evolutionary Genetics Analysis (MEGA) (version 4.0; Tamura *et al.*, 2007). The number of migrants per generation, N_m for

interspecies as well as between populations was calculated using the F_{ST} estimates as follows:

$$N_m = \frac{1 - F_{ST}}{4F_{ST}}$$

where, F_{ST} is the fixation index, and N_m is the number of migrants per generation.

RESULTS AND DISCUSSION

Allelic Diversity in Cynopterus

Overall, 173 individuals of cynopterans comprising 64 large form of *C. brachyotis*, 41 small form of *C. brachyotis*, 38 *C. horsfieldii*, and 30 *C. sphinx* were tested using nine pairs of microsatellite primers (Fig.1). Seven out of the nine microsatellite primer pairs produced distinct and reproducible genotypic patterns and showed polymorphism in all the four forms of *Cynnopterus* examined. The two remaining primer sets (CSP2 and CSP8) were excluded from the analysis as these primers did not generate any band. Similarly, the failure of both primer sets to generate bands was also observed in a similar study carried out in Peninsular Malaysia (Campbell *et al*., 2006). All nine pairs of primers utilised in the study were originally isolated from *C. sphinx* caught in India (Storz, 2000). Thus, it is safe to presume that the two primer sets failed to amplify the targeted region in the cynopterans due to inter and intraspecies variations that occurred between both sources of samples (India and Malaysia).

In reference to locus CSP4, the populations of *C. horsfieldii* and small form of *C. brachyotis* were found to be monomorphic with only two alleles produced (Table 1). In contrast, Campbell *et al.* (2006) found that CSP4 showed monomorphism in *C. brachyotis* Sunda only (referred here as large form of *C. brachyotis*) in contrast with this study where there were three alleles produced in the large form of *C. brachyotis* from Peninsular Malaysia. The allele was contributed by a sample captured from Gua Batu Puteh, Pulau Langkawi, previously not sampled by Campbell *et al.* (2006). Thus, inclusion of samples from other areas in the population as well as phylogeography study would show not only the distribution of the genotypes and gene flow of the studied species but also reveal more alleles that may not be found by restricted sampling efforts especially in bats which are highly mobile.

The allele frequencies for each locus in each species are presented in Table 1. Several private alleles were found in loci CSP3, CPS4, CSP6, CSP7 and CSP9 in each species, except the large-seized *C. brachyotis* (Table 1). This indicates that the remaining cynopterans in this study diverged from the large form of *C. brachyotis*. The founder population would have the greatest number of alleles, haplotypes, as well as genetic diversity, and shares its haplotypes with its descent groups. The descent group might hold their own unique haplotypes or private alleles as well as loss of certain haplotypes inherited from the founders due to genetic drift and other evolutionary processes during speciation (Kaestle & Smith, 2001; Merriwether *et al.*, 1996).

TABLE 1
Allele frequencies, observed heterozygosities (Obs Het), and expected heterozygosities (Exp Het) at each locus in representatives of *Cynopterus*. Bold values indicate private alleles in each species.

CSP 1

Species\Allele	200	210	220	230	240	250	Obs Het	Exp Het
Large *C. brachyotis*	0.0690	0.0345	0.5172	0.2155	0.1034	0.0603	0.3966	0.6715
Small *C. brachyotis*	0.1316	0.0789	0.2632	0.3684	0.0658	0.0921	0.7895	0.7688
C. sphinx	0.1000	0.0000	0.2333	0.2833	0.1000	0.2833	0.3000	0.7780
C. horsfieldii	0.1184	0.0000	0.2500	0.4079	0.1447	0.0789	0.3947	0.7396

CSP 3

Species\Allele	100	110	120	130	140	150	Obs Het	Exp Het
Large *C. brachyotis*	0.5678	0.0763	0.2797	0.0339	0.0424	0.0000	0.2881	0.5957
Small *C. brachyotis*	0.6148	0.1447	0.1053	0.0789	0.0526	0.0000	0.4734	0.5842
C. sphinx	0.3500	0.2333	0.3000	0.1167	0.0000	0.0000	0.2000	0.7316
C. horsfieldii	0.7895	0.0000	0.0000	0.1053	0.0000	**0.1053**	0.3158	0.3593

CSP4

Species\Allele	100	120	130	140	150	Obs Het	Exp Het
Large *C. brachyotis*	0.0169	0.0000	0.9068	0.0085	0.0678	0.1017	0.1743
Small *C. brachyotis*	0.0000	0.0000	0.9474	0.0000	0.0526	0.1053	0.1011
C. sphinx	0.0172	**0.0172**	0.2586	0.0690	0.6379	0.4138	0.5299
C. horsfieldii	0.0000	0.0000	0.9342	0.0000	0.0658	0.1316	0.1229

CSP 5

Species\Allele	130	140	150	160	170	180	190	200	Obs Het	Exp Het
Large *C. brachyotis*	0.1525	0.0085	0.4576	0.0932	0.2203	0.0169	0.0508	0.0000	0.6610	0.7132
Small *C. brachyotis*	0.1974	0.0132	0.2632	0.0395	0.3026	0.0526	0.1316	0.0000	0.7368	0.7888
C. sphinx	0.0000	0.1379	0.4483	0.1724	0.1034	0.0517	0.0345	0.0517	0.2759	0.7459
C. horsfieldii	0.0000	0.0526	0.1447	0.1974	0.2368	0.1711	0.0000	0.1974	0.7105	0.8239

CSP 6

Species\Allele	130	140	150	160	170	180	190	200	220	Obs Het	Exp Het
Large *C. brachyotis*	0.0085	0.1690	0.4237	0.0170	0.2458	0.0000	0.0508	0.1525	0.0000	0.5593	0.7297
Small *C. brachyotis*	0.0921	0.0526	0.3421	0.0921	0.1053	0.0000	0.2368	0.0789	0.0000	0.5263	0.8004
C. sphinx	0.0769	0.0000	0.6346	0.0000	0.0769	0.0577	0.0000	0.0769	**0.0769**	0.3077	0.5814
C. horsfieldii	0.0588	0.1029	0.2647	0.1029	0.3235	0.0735	0.0735	0.0000	0.0000	0.2647	0.8016

CSP 7

Species\Allele	220	230	240	250	260	270	280	290	300	310	Obs Het	Exp Het
Large *C. brachyotis*	0.0000	0.0000	0.0169	0.3220	0.1780	0.0763	0.2203	0.0424	0.1441	0.0000	0.7627	0.7641
Small *C. brachyotis*	0.0000	0.0000	0.0000	0.1447	0.2237	0.1316	0.3026	0.0132	0.1711	**0.1320**	0.7895	0.8011
C. sphinx	**0.0167**	0.0000	0.0167	0.3833	0.1667	0.0833	0.1667	0.1000	0.0667	0.0000	0.8333	0.7887
C. horsfieldii	0.0000	**0.1184**	0.0263	0.1711	0.0395	0.4079	0.2105	0.0000	0.0263	0.0000	0.4474	0.753

CSP 9

Species\Allele	280	290	300	310	320	330	350	Obs Het	Exp Het
Large *C. brachyotis*	0.2797	0.1864	0.4407	0.0254	0.0339	0.0339	0.0000	0.2203	0.6958
Small *C. brachyotis*	0.4242	0.1667	0.3182	0.0455	0.0000	0.0303	**0.0152**	0.1515	0.6984
C. sphinx	0.4821	0.0357	0.4107	0.0000	0.0714	0.0000	0.0000	0.5000	0.6032
C. horsfieldii	0.2763	0.1842	0.4211	0.0526	0.0263	0.0395	0.0000	0.2368	0.7168

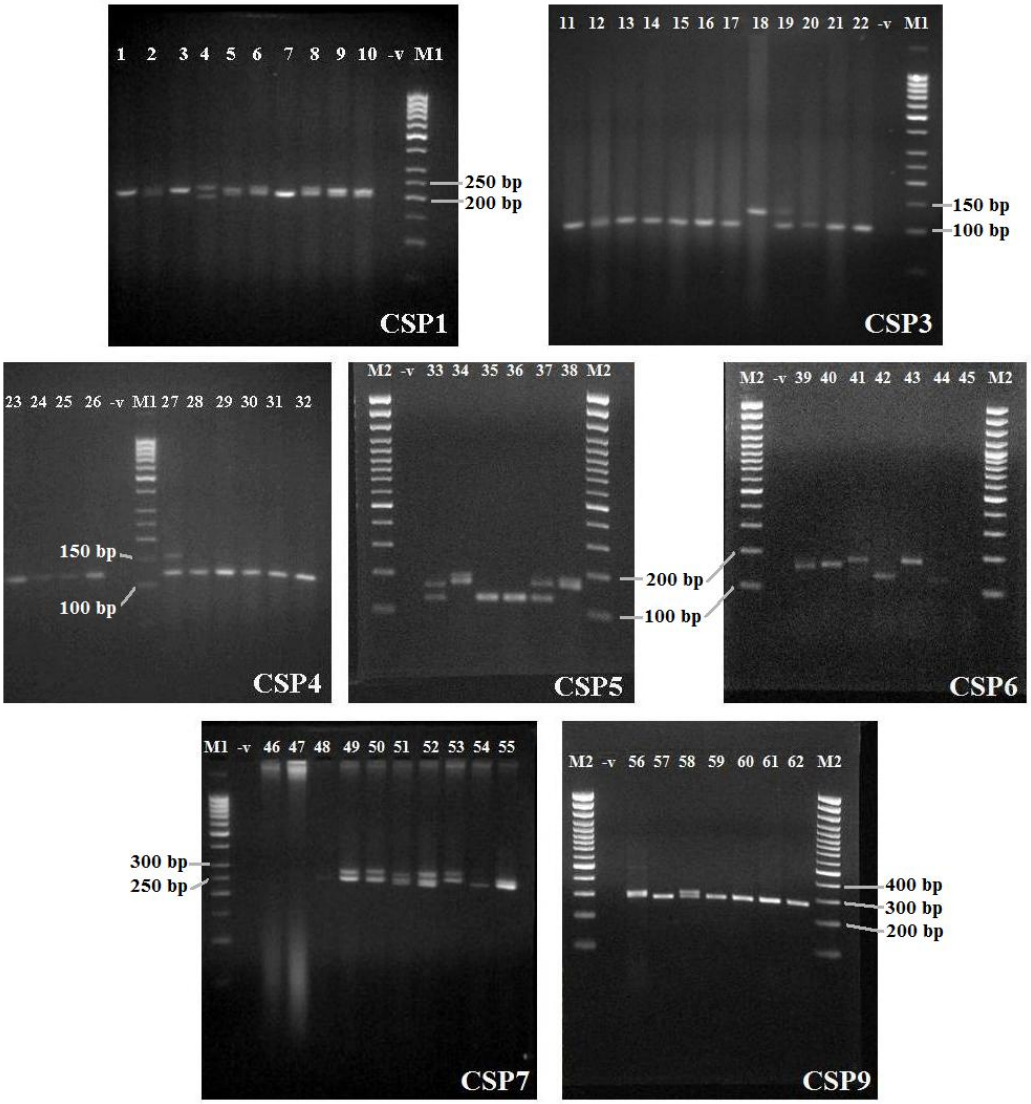

Fig.1: Representative gel pictures of each microsatellite marker used in this study. M1 indicates the GeneRuler™ 50 bp DNA Ladder, M2 is the GeneRuler™ 100 bp DNA Ladder Plus and –v is negative control. Each lane in the gels is labelled by number following the sample ID: 1 = I19; 2 = I20, 3 = I21; 4 = I22; 5 = I23; 6 = I24; 7 = I26; 8 = I29; 9 = I30; 10 = I31; 11 = H3; 12 = H4; 13 = H5; 14 = H6; 15 = H7; 16 = H8; 17 = H9; 18 = H10; 19 = H11; 20 = H12; 21 = H13; 22 = H14; 23 = K31; 24 = K32; 25 = K40; 26 = K92; 27 = I19; 28 = I20; 29 = I21; 30 = I22; 31 = I23; 32 = I24; 33 = PB026; 34 = PB012; 35 = B1; 36 = B2; 37 = B12; 38 = 01274; 39 = I20; 40 = I21; 41 = I23; 42 = I26; 43 = I30; 44 = H2; 45 = H7; 46 = FK1; 47 = FK2; 48 = FK3; 49 = FK4; 50 = FK5; 51 = FK6; 52 = FK7; 53 = FK8; 54 = FK9; 55 = FK10; 56 = S131; 57 = S184; 58 = S192; 59 = S185; 60 = S220; 61 = S221; 62 = S222.

There were 51 alleles documented in this study, where the highest number of alleles was found in the large form of *C. brachyotis* (42 alleles), while the lowest number of alleles was found in *C. horsfieldii* (36 alleles). The average observed heterozygosity ranged from 0.3574 in *C. horsfieldii* up to 0.5103 in the small form of *C. brachyotis* populations (Table 2). All loci in this study deviated from Hardy-Weinberg equilibrium across the four *Cynopterus* species (data not shown). No linkage disequilibrium was detected in all loci across all four cynopterans.

Deviation from Hardy-Weinberg expectations was detected across species over seven screened loci. However, not all genotype frequencies over seven loci deviated from Hardy-Weinberg expectation when observed within the populations of all species in this study. In this study, the populations with small sample size (less than or equal to seven individuals) were prone to follow Hardy-Weinberg equilibrium. Nevertheless, larger sample size for each region is still required to show the actual situation in the test of Hardy-Weinberg equilibrium. Non-deviation from Hardy-Weinberg equilibrium does not mean there were no evolutionary forces acting on these populations (Halliburton, 2004). Hardy-Weinberg principle is a null hypothesis to describe an ideal population; however, in reality, there are many evolutionary processes or factors that may influence genotype frequencies and cause deficiency and excess of heterozygosity in a population of a particular species (Snustad & Simmons, 2010; Halliburton, 2004).

A total of 97 unique genotypes were found across the four forms of *Cynopterus* in 15 regions (28 sites) of this study (Table 3). There were three common genotypes across the four forms. Among the four forms of cynopterans, the large and small forms of *C. brachyotis* shared the most genotypes compared to others. Besides, null alleles were found in all cynopteran bats (Table 3).

Population Structure

High genotype numbers were detected in the populations of large and small forms of *C. brachyotis* from southern Sarawak. *C. sphinx* from Perak had higher number of genotypes (44 genotypes) compared to the population from Perlis (38 genotypes), whereas in *C. horsfieldii*, the population from southern Peninsular Malaysia had

TABLE 2
Genetic variability of cynopterans across seven microsatellite loci and average observed (Obs) and expected (Exp) Hardy-Weinberg heterozygosities (Het)

Species	Total visible alleles	Average allelic genotypes	Average Obs Het	Average Exp Het
Large *C. brachyotis*	42	118	0.4271	0.6206
Small *C. brachyotis*	40	75	0.5103	0.6490
C. sphinx	39	58	0.4044	0.6798
C. horsfieldii	36	75	0.3574	0.6167

TABLE 3
Genotype distribution of nuclear microsatellite in 15 populations of four forms of *Cynopterus*

Genotype	KLCb (6)	NSLCb (20)	SSLCb (21)	NPMLCb (10)	SPMLCb (7)	NPMSCb (5)	SSSCb (23)	NSSCb (10)	SPMSCb (3)	NSCh (2)	SSCh (13)	NPMCh (7)	SPMCh (16)	PLCs (16)	PKCs (14)
100/100	6	8	8	5	3	2	8	4	3	2	5	7	10	4	5
110/100			4	1			3	2							
110/110		2			2	1	2	1						2	4
120/100			3	1	1		2	3							
110/120														1	
120/120		6	6		1		1							6	2
120/150														1	
130/100							4				4			2	2
130/110															1
130/120		1		1										1	
130/130	7	18	21		4	7	24	10	3	2	15	6	14	1	5
130/160			1	10			1								
130/170							1								
140/100		2		2		1	2								
140/120		1					1								
140/130								1							
140/140		1	1				1				1	1	2	1	3
150/100											2		6		
150/130	4	3	3	1	3	2	5	3			1	1	3	3	5
150/140			1	1		1								1	1
150/150	1	11	7	4	4	1	5	4	4	2	4	1	4	24	13
160/140							1							1	
160/150		2	6				2	1							1
160/160	1	2		2			1				1	1	4	1	2
170/130	1						1								
170/140										1			2		1
170/150	1	9	9	6	5		6	2			1	2			2
170/160						1	1						4		
160/160		1	5	1	2		2				3	1	4	1	2
180/160		3						2			3		1	3	1
180/160				1								2	1		2
180/170				1			3						3		
180/180								1				2	1		
190/130		1													
190/150			1			2	1	1							
190/160			1	1			1								
190/170			6		2	2	5	3	2	1	2		2		

TABLE 3
Genotype distribution of nuclear microsatellite in 15 populations of four forms of *Cynopterus* (cont.)

190/180							1									
190/190							5							1		
200/150	2	4	1	2	1		2							1	1	
200/160		1									3					
200/170		1									1	3	2			
200/180		1									1		1			
200/200	1	7			1		1	4			6			2	1	
210/210		1	1						1							
220/150															1	
220/170															1	
220/200			2	1			3	1							1	
220/220	6	5	11				3				1		4	2	4	
230/200						1	1	1					1			
230/210						2			2							
230/220		2	1		1		4	4			1		6		1	
230/230		6	2	1		1	2			1	7		1	5	2	
250/230							1									
260/230													1			
270/230													1			
240/200		1			1		1							2		
240/220				4	3		1					2				
240/230							2	1		1		1	3			
240/240					1							1	1		2	
250/200							1							1		
250/220			2	1			1	1						1	1	
250/230			2	1		1	2								2	
250/240				1												
250/250	2	2	1	2	1		3		1		2	3	3	6	4	
260/220														1		
260/250				1	1	1										
260/260		2	1	1	1				2					1		
270/230											1		2			
270/240											1		1		1	
270/250		1						2					2	2	1	
270/260							1									
270/270				1				2			5	4	2			
280/230											3		1			
280/240			2													
280/260	2	7	6		2		1	1		1				8	1	
280/260		4	3			2	4	3		1		1			1	
280/270							2						1			
280/280	6		3	6			9	6			2	3	9	6	2	

TABLE 3
Genotype distribution of nuclear microsatellite in 15 populations of four forms of *Cynopterus* (cont.)

290/250														1	2
290/260		2	2											1	2
290/270			1				1								
290/290		6	8	1			3	3		2	2	1	1		
300/250				3											
300/260	1		1	1			1							1	2
300/270	1	2	1	1	1	1	1	2							1
300/280			4	2			8				1			4	4
300/290														1	1
300/300		12	4		6	4	6	1			4	5	5	3	3
310/300											1				
310/260						1	3								
310/290			2								2				
320/300		1		1	2								2	1	2
330/300		1	3				1				1		1		1
330/310											1				
350/330							1								
Null alelle				1		1	1		3		3	1		7	1

the highest number of genotypes among all the other populations of *C. horsfiledii* in this study (Table 4). Average gene diversity over the loci of the large form of *C. brachyotis* showed that the population in southern Peninsular Malaysia had the highest diversity (0.6421), while the lowest diversity was recorded in the population from Kalimantan. The highest average gene diversity among seven loci was found in southern Sarawak populations of the small form of *C. brachyotis*. The average gene diversity over the loci in the population from Perak was higher than the population from Perlis in *C. sphinx*. The populations of *C. horsfieldii* from southern Sarawak had the highest average gene diversity in the overall loci among the remaining three populations.

The Ewens-Watterson neutrality test indicated that loci CSP1 and CSP5 were under selection pressure as the observed F-values were lower than 95% (L95) confidence limits of expected F-values in all seven microsatellite loci of cynopterans in this study. However, these two loci were included in further analysis as the observed F-values were within the interval of standard errors (Table 5).

The interspecies Global AMOVA comparison analysis showed that there was low genetic variation (0.53%) in the populations between the large and small forms of *C. brachyotis*. This was followed by genetic variation for the populations between the small form of *C. brachyotis* versus *C. horsfieldii* (3.12%) and the populations between the large form of *C. brachyotis* versus *C. horsfieldii* (3.59%). Although there were significant differences between the small form of *C. brachyotis*

TABLE 4
Number of sample size, genotypes, gene diversity, average gene diversity over loci (with standard deviation in brackets) for each region across four species

Species	Region	Sample size	Number of genotype	Gene diversity	Average gene diversity over loci
Large form *C. brachyotis*	northern Sarawak	20	37	0.9977 (0.0094)	0.5307 (0.3068)
	southern Sarawak	21	39	0.9872 (0.0114)	0.5817 (0.3360)
	northern Peninsular Malaysia	10	34	0.9895 (0.0193)	0.5667 (0.3380)
	southern Peninsular Malaysia	7	22	1.0000 (0.0270)	0.6421 (0.3775)
	Kalimantan	6	15	0.9848 (0.0403)	0.2966 (0.2006)
Small form *C. brachyotis*	northern Sarawak	10	28	1.0000 (0.0270)	0.5981 (0.3548)
	southern Sarawak	23	58	0.9990 (0.0048)	0.6707 (0.3713)
	northern Peninsular Malaysia	5	20	1.0000 (0.0625)	0.5306 (0.3410)
	southern Peninsular Malaysia	3	9	1.0000 (0.0625)	0.4167 (0.2854)
C. sphinx	Perlis	16	38	0.9960 (0.0090)	0.6820 (0.4133)
	Perak	14	44	1.0000 (0.0095)	0.7297 (0.4137)
C. horsfieldii	northern Sarawak	2	10	1.0000 (0.1768)	0.3095 (0.2557)
	southern Sarawak	13	33	0.9938 (0.0126)	0.5882 (0.3445)
	northern Peninsular Malaysia	7	21	1.0000 (0.0270)	0.4524 (0.2867)
	southern Peninsular Malaysia	16	37	1.0000 (0.0078)	0.6005 (0.3406)

TABLE 5
The overall Ewens-Watterson test for neutrality. The k-values were the number of visible alleles in each locus.

Locus	k	Min F	Max F	Mean*	L95*	U95*	Obs. F	SE*
CSP1	6	0.1667	0.9700	0.4920	0.2519	0.8885	0.2480**	0.0284
CSP3	6	0.1667	0.9702	0.4964	0.2519	0.8723	0.3988	0.0298
CSP4	5	0.2000	0.9759	0.5517	0.2844	0.9402	0.6802	0.0327
CSP5	8	0.1250	0.9582	0.4063	0.2029	0.7510	0.2007**	0.0213
CSP6	9	0.1111	0.9503	0.3683	0.1841	0.7068	0.2323	0.0185
CSP7	10	0.1000	0.9469	0.3392	0.1743	0.6702	0.1839	0.0169
CSP9	7	0.1429	0.9627	0.4498	0.2330	0.8558	0.3099	0.0279

* These statistics were calculated using 1000 simulated samples;
**Deviation from neutrality.

versus *C. horsfieldii* and the large form of *C. brachyotis* versus *C. horsfieldii,* variations between the populations were less than 5%. This may indicate that the populations' split was significant but the variations were not meaningful. This might be due to the small sample size, resulting in insufficient alleles and genotypes generated to show meaningful variations between them.

Both forms of *C. brachyotis* showed that they were more distantly related to *C. sphinx* as compared to *C. horsfieldii*. The percentage of variation showed that the populations of *C. sphinx* and *C. horsfieldii* had the highest genetic variation among the four species (Table 6).

A UPGMA tree was constructed based on the F_{ST} estimates values between 15 populations across four forms of cynopterans as listed in Table 7 (Fig.2). Among the 15 populations, *C. brachyotis* from Kalimantan was distantly related to other populations. Clustering of populations of the large and small forms of *C. brachyotis* did not conform to expected regions and forms (large versus small form), instead they were clustered into two clades (Clade A and Clade C). Precisely, Clade A consisted of the large form of *C. brachyotis* from northern Peninsular Malaysia, the small form of *C. brachyotis* from northern Sarawak, the small form of *C. brachyotis* from southern

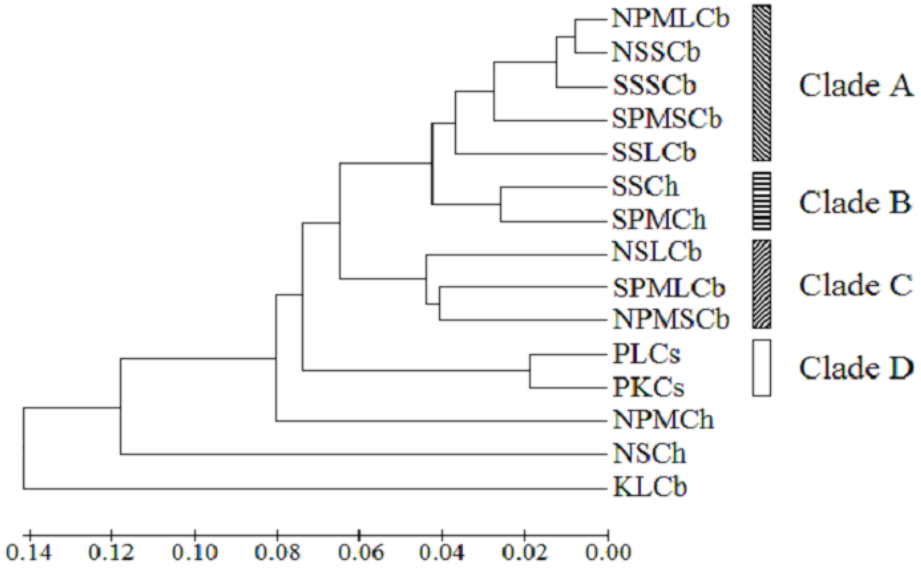

Fig.2: Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree based on interbreed genetic distance, F_{st} estimates. Branches labelled by region of each species and populations; NPMLCb = northern Peninsular Malaysia large form *C. brachyotis*, NSSCb = northern Sarawak small form *C. brachyotis*, SSSCb = southern Sarawak small form *C. brachyotis*, SPMSCb = southern Peninsular Malaysia small form *C. brachyotis*, SSLCb = southern Sarawak large form *C. brachyotis*, SSCh = southern Sarawak *C. horsfieldii*, SPMCh = southern Peninsular Malaysia *C. horsfieldii*, NSLCb = northern Sarawak large form *C. brachyotis*, SPMLCb = southern Peninsular Malaysia large form *C. brachyotis*, NPMSCb = north Peninsular Malaysia small form *C. brachyotis*, PLCs = Perlis *C. sphinx*, PKCs = Perak *C. sphinx*, NPMCh = north Peninsular Malaysia *C. horsfieldii*, NSCh = northern Sarawak *C. horsfieldii*, KLCb = Kalimantan *C. brachyotis*.

TABLE 6
Interspecies Global AMOVA based on seven loci

Source of variation	Sum of squares	Variance components	Variation (%)	Fixation Indices	p-value
Large form *C. brachyotis* vs Small form *C. brachyotis*					
Among species	6.470	0.01019	0.52967	F_{CT}: 0.00530	0.13978
Within species	29.418	0.11185	5.81455	F_{SC}: 0.05846	0.00000*
Within populations	183.572	0.28847	14.996	F_{IS}: 0.16012	0.00000*
Within individuals	146.50	1.51316	78.659	F_{IT}: 0.21340	0.00000*
Total	365.960	1.92367			
Large form *C. brachyotis* vs *C. sphinx*					
Among species	24.307	0.19059	7.54064	F_{CT}: 0.07541	0.01271*
Within species	41.252	0.23548	9.31699	F_{SC}: 0.10077	0.00000*
Within populations	221.492	0.64708	25.60197	F_{IS}: 0.30793	0.00000*
Within individuals	127.500	1.45432	57.54040	F_{IT}: 0.42460	0.00000*
Total	414.487	2.52748			
Large form *C. brachyotis* vs *C. horsfieldii*					
Among species	16.321	0.08369	3.59461	F_{CT}: 0.03595	0.01173*
Within species	51.839	0.23742	10.19767	F_{SC}: 0.10578	0.00000*
Within populations	228.011	0.61860	26.56971	F_{IS}: 0.30821	0.00000*
Within individuals	133.500	1.38849	59.63801	F_{IT}: 0.40362	0.00000*
Total	429.670	2.32820			
Small form *C. brachyotis* vs *C. sphinx*					
Among species	22.042	0.27156	11.79640	F_{CT}: 0.11796	0.00196*
Within species	14.026	0.05786	2.51354	F_{SC}: 0.02850	0.00098*
Within populations	149.713	0.48287	20.97591	F_{IS}: 0.24479	0.00000*
Within individuals	100.000	1.48973	64.71415	F_{IT}: 0.35286	0.00000*
Total	285.781	2.30202			
Small form *C. brachyotis* vs *C. horsfieldii*					
Among species	9.180	0.06119	3.11801	F_{CT}: 0.03118	0.00587*
Within species	21.492	0.08641	4.40305	F_{SC}: 0.04545	0.00000*
Within populations	146.624	0.37623	19.17190	F_{IS}: 0.20731	0.00000*
Within individuals	108.500	1.43860	73.30704	F_{IT}: 0.26693	0.00000*
Total	285.796	1.96243			
C. sphinx vs *C. horsfieldii*					
Among species	30.262	0.37074	13.81301	F_{CT}: 0.13813	0.00098*
Within species	21.582	0.11204	4.17452	F_{SC}: 0.04844	0.00000*
Within populations	184.744	0.87952	32.77125	F_{IS}: 0.39959	0.00000*
Within individuals	88.000	1.32162	49.24122	F_{IT}: 0.50759	0.00000*
Total	324.588	2.68396			

*Significantly different at 95% confident interval.

TABLE 7
F_{ST} estimates (below diagonal) as a measure of genetic distance between four species of cynopterans and number of migrants per generation, N_m (above diagonal and bolded)

Pop	NSLCb	SSLCb	NPMLCb	SPMLCb	KLCb	NSSCb	SSSCb	NPMSCb	SPMSCb	PLCs	PKCs	NSCh	SSCh	NPMCh	SPMCh
NSLCb		**2.3447**	**1.3519**	**2.5899**	**0.5558**	**1.2048**	**2.5625**	**2.6382**	**1.6374**	**1.0504**	**1.5866**	**0.8031**	**1.4674**	**1.1879**	**1.3110**
SSLCb	0.0964		**2.6853**	**1.6086**	**1.1984**	**3.0543**	**8.6027**	**1.4156**	**2.1319**	**0.9549**	**1.7772**	**1.7629**	**1.8419**	**1.8737**	**2.0211**
NPMLCb	0.1561	0.0852		**1.7073**	**2.1962**	**15.6029**	**7.9360**	**1.2709**	**6.5881**	**1.2714**	**2.9692**	**0.8913**	**2.0131**	**2.4167**	**3.8618**
SPMLCb	0.0880	0.1345	0.1277		**0.4624**	**1.0784**	**2.5919**	**2.8243**	**2.0834**	**1.6422**	**5.0321**	**0.6447**	**1.3105**	**1.4209**	**1.4526**
KLCb	0.3103	0.1726	0.1022	0.3510		**1.9386**	**1.2819**	**0.3347**	**0.4472**	**0.4881**	**0.8228**	**0.2295**	**0.5793**	**0.6154**	**0.9874**
NSSCb	0.1719	0.0757	0.0158	0.1882	0.1142		**12.5247**	**1.2504**	**2.2267**	**1.1847**	**1.9286**	**0.9989**	**2.1114**	**1.7837**	**2.7570**
SSSCb	0.0889	0.0282	0.0305	0.0880	0.1632	0.0196		**3.5645**	**8.8608**	**1.4183**	**2.5315**	**1.6754**	**3.9503**	**4.1283**	**3.9467**
NPMSCb	0.0866	0.1501	0.1644	0.0813	0.4276	0.1666	0.0655		**3.5151**	**0.8052**	**1.8537**	**0.5977**	**2.0736**	**2.0344**	**1.6790**
SPMSCb	0.1325	0.1050	0.0366	0.1071	0.3586	0.1009	0.0274	0.0664		**0.9383**	**2.2368**	**0.5549**	**2.3089**	**1.6664**	**1.7666**
PLCs	0.1923	0.2075	0.1643	0.1321	0.3387	0.1743	0.1499	0.2369	0.2104		**6.3831**	**0.5319**	**0.8179**	**0.7962**	**0.8105**
PKCs	0.1361	0.1233	0.0777	0.0473	0.2330	0.1148	0.0899	0.1188	0.1005	0.0377		**0.7418**	**1.6146**	**1.6594**	**1.9708**
NSCh	0.2374	0.1242	0.2191	0.2794	0.5214	0.2002	0.1298	0.2949	0.3106	0.3197	0.2521		**1.2726**	**0.8837**	**0.7253**
SSCh	0.1456	0.1195	0.1105	0.1602	0.3015	0.1059	0.0595	0.1076	0.0977	0.2341	0.1341	0.1642		**3.4055**	**3.2445**
NPMCh	0.2107	0.1684	0.1431	0.1659	0.3754	0.1809	0.0935	0.1349	0.2069	0.2814	0.1645	0.3240	0.1228		**5.9643**
SPMCh	0.1497	0.0922	0.0496	0.1444	0.1839	0.0690	0.0470	0.1206	0.1003	0.2197	0.1044	0.2149	0.0517	0.0626	

NSLCb = northern Sarawak large form *C. brachyotis*, SSLCb = southern Sarawak large form *C. brachyotis*, NPMLCb = northern Peninsular Malaysia large form *C. brachyotis*, SPMLCb = southern Peninsular Malaysia large form *C. brachyotis*,KLCb = Kalimantan *C. brachyotis*, NSSCb = northern Sarawak small form *C. brachyotis*, SSSCb = southern Sarawak small form *C. brachyotis*, NPMSCb = north Peninsular Malaysia small form *C. brachyotis*, SPMSCb = southern Peninsular Malaysia small form *C. brachyotis*, PLCs = Perlis *C. sphinx*, PKCs = Perak *C. sphinx*, NSCh = northern Sarawak *C. horsfieldii*, SSCh = southern Sarawak *C. horsfieldii*, NPMCh = north Peninsular Malaysia *C. horsfieldii*, SPMCh = southern Peninsular Malaysia *C. horsfieldii*.

Sarawak, the small form of *C. brachyotis* from southern Peninsular Malaysia, and the large form of *C. brachyotis* from southern Sarawak. Meanwhile, Clade C comprised the large form of *C. brachyotis* populations from northern Sarawak, the large form of *C. brachyotis* from southern Peninsular Malaysia, and the small form of *C. brachyotis* from northern Peninsular Malaysia. *C. horsfieldii* from southern Peninsular Malaysia and *C. horsfieldii* from southern Sarawak were clustered in Clade B, whereas both populations of *C. sphinx* were clustered together in Clade D.

Based on the UPGMA tree constructed using the seven pairs of microsatellite primers, all cynopterans within this genus were very closely related to each other. There was a lack of distinct clusters for each form, dissimilar with what was revealed in mtDNA data. Besides, greater number of migrants per generation (N_m) between the large form and small form of *C. brachyotis* was observed compared with other forms of cynopterans (Table 7). Thus, the populations of both forms of *C. brachyotis* were mixed up, while *C. sphinx* and *C. horsfieldii* were grouped within their own phylogroups in the tree. This basically shed some light on the paternal inheritance in the genus of *Cynopterus*.

The low genetic structure in the bi-parental phylogenetic tree in the genus *Cynopterus* might be caused by low genetic diversity and limited Y chromosome inheritance in the male germ line. Several studies have shown that there is low genetic diversity of Y chromosome in most mammalians including humans (Hellborg & Ellegren, 2004; Handley *et al*., 2006; Goetting-Minesky & Makova, 2006). The low genetic variations in the Y chromosome are caused by the non-recombinant nature in the Y chromosome, where only the common genealogy is shared in the male germ line (Charlesworth & Charlesworth, 2000; Hellborg & Ellegren, 2004; Handley *et al*., 2006). In addition, selections and mutations, especially favourable mutations, may contribute to the reduction of the diversity of Y chromosome where these mechanisms could reduce the effective population size of the Y chromosome in a random mating population (Boissinot & Boursot, 1997; Charlesworth & Charlesworth, 2000; Hellborg & Ellegren, 2004).

Besides, low structuring in the microsatellite data might be caused by low sensitivity of microsatellite primers used in this study to examine Malaysian cynopterans. The existing primers used in this study were designed by Storz (2000) using *C. sphinx* from India, and currently, there are no existing microsatellite primers for Malaysian cynopterans. Different evolutionary forces may have been acting on Indian *C. sphinx* as compared to Malaysian *C. sphinx*. The study done by Campbell *et al*. (2006) on Malaysia *Cynopterus* spp. showed a similar result, where no distinctive genetic structure was generated using the same microsatellite loci used in this study. Several factors such as geographical barriers, the climate and forest ecology between India and Malaysia might be the possible explanation to the different genetic

profiles in the cynopterans. In Chen *et al*. (2010), little genetic variation of *C. sphinx* was found using eight out nice microsatellite loci by Storz (2000) although there was greater genetic mtDNA differentiation between the *C. sphinx* samples from India and those from China and Vietnam. In addition, the sample size used in this study for each form of cynopterans was unequal and small, thus the generated allelic profiles might result in the loss of other alleles as well as private alleles. Large and equal population sizes have great influence in determining the number of private alleles and distinct alleles in a population, which in turn can reveal genetic diversity and relationships (Szpiech *et al*., 2008).

CONCLUSION

The objective of this study was to investigate the utility of the existing microsatellite markers designed by Storz (2000) for the population study of *Cynopterus* in Malaysia. We found that seven out of the nine existing microsatellite markers isolated from *C. sphinx* can be utilised on cynopterans in Malaysian and Kalimantan samples. These seven primer pairs produced mixed results of distinctiveness, polymorphism and monomorphism as opposed to the two remaining primers that did not produce any band.

Subsequently, the utility of these microsatellite markers on *Cynopterus* was tested using the samples from Malaysia, especially from Sabah and Sarawak. The results showed that there were a total of 51 alleles and 97 genotypes generated from four forms, large and small forms of *C. brachyotis*, *C. sphinx* and *C. horsfieldii*. Although these primers were able to produce microsatellite bands, the data generated were not enough to clearly differentiate both forms of *C. brachyotis*, and thus, these primers cannot be used to clearly define the species boundary of these two forms of *C. brachyotis* as suggested by previous authors.

Second, on elucidating the possibility of hybridisation of both forms of *C. brachyotis* occurring in the ecotone as the contact zone that was speculated by Abdullah (2003), intermixing between the two forms of *C. brachyotis* could not be observed as most of the microsatellite alleles were shared amongst the species. Besides, genetic variations between the species were also low without any significant genetic difference. Thus, no distinctive genetic structure was exhibited between the four species using microsatellite analysis and without clear indication of hybridisation happening between the small and large forms of *C. brachyotis*.

Based on the results of previous and the current studies, the existing microsatellite primers isolated from *C. sphinx* by Storz (2000) could not clearly define the species boundary of both forms of *C. brachyotis*. Most of the alleles generated in this study were common among all cynopterans leading to no genetic structuring using the bi-parental genetic data. Thus, it is recommended that new microsatellite primers should be designed using local cynopterans especially the large form of *C. brachyotis*, which is the founder and

its cryptic species in this region. More polymorphisms between the two forms of *C. brachyotis* as well as *C. sphinx* and *C. horsfieldii* may be detected and can be used to further elucidate the genotype and genetic structure from bi-parental inheritance.

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