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Taxonomic Revision and Evolutionary Phylogeography of Dusky Langur (*Trachypithecus obscurus*) in Peninsular Malaysia

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Dusky langur, *Trachypithecus obscurus*, inhabits tropical rainforests in Peninsular Malaysia, Thailand, and Myanmar. Morphologically, five subspecies are distributed in Peninsular Malaysia, but few studies have used genetic data to verify the classification. It is difficult to differentiate subspecies based on morphological characteristics, so this study used molecular data to differentiate subspecies of *T. obscurus*. The issue was addressed by analyzing 723 and 649 base pairs of the mitochondrial D-loop region and *COI*, respectively. DNA amplifications were performed using species-specific primer toward 35 individuals representing different populations. Phylogenetic analyses showed that two main clades representing populations in southern and northern Peninsular Malaysia. The results demonstrate that subspecies of *T. obscurus* in Peninsular Malaysia does not support classification based on the morphology that recognizes five subspecies. Previous study based on morphology that classified the subspecies on Perhentian Island, Terengganu, as *T. obscurus styx* is not recognized in this study. This subspecies happened to merge with the population in northern Peninsular Malaysia. *Trachypithecus o. styx* probably inhabited the southern peninsula and, due to the terminal Pleistocene sea level rise, spread to the east coast but could not spread farther because the subspecies was situated on offshore islands during the period. This assumption was supported by the molecular clock, which showed that subspecies on Perhentian Island spread after the Perlis population (*T. obscurus flavicauda*).

Key words: *Trachypithecus obscurus*, Dusky leaf monkey, Dusky langur, Colobinae, Primate phylogeography.

BACKGROUND

Trachypithecus obscurus, also known as dusky leaf monkey or spectacled langur (Fig. 1), is a langur that is distributed in Myanmar, Thailand, and Peninsular Malaysia (Fig. 2), including surrounding islands (Groves 2001; Md-Zain and Ch`ng 2011). Primatologists do not agree on the total number of subspecies in this langur species. Groves (2001) suggests that there are seven subspecies of *T. obscurus*, which are *T. obscurus obscurus* (Peninsular Malaysia to northern Perlis), *T. obscurus halonifer* (restricted to Penang Island), *T.*

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obscurus carbo (Langkawi Island), *T. obscurus styx* (restricted to Perhentian Island), *T. obscurus flavicauda* (northern Perlis), *T. obscurus sanctorum* (Myanmar), and *T. obscurus seimundi* (Phangan Island, Thailand). Meanwhile, Brandon-Jones et al. (2004) suggested 11 subspecies, seven of which are similar to those of Groves (2001) with four additional subspecies: *T. obscurus corax*, *T. obscurus phayrei*, *T. obscurus shanicus*, and *T. obscurus smithi*. Roos et al. (2014), the latest study to classify *T. obscurus* subspecies, identified seven subspecies based on Groves (2001). *Trachypithecus o. phayrei*, suggested by Brandon-Jones et al. (2004), has been upgraded to species level as *T. phayrei*. This subspecies was upgraded to the species level based on the fur coloration of the tail, leg, and pelage being lighter than that of the *obscurus* group (Blyth 1847; Groves 2001; Roos et al. 2014).

The biogeography of *T. obscurus* is poorly defined because the geography and environment of its habitats change regularly. During the Pliocene and Pleistocene, drastic climate change had influenced the species distribution in certain tropical forest habitats (Bird et al. 2005; Meijaard 2003; Morley 2000). The Asian colobine's ancestor most likely invaded Southeast Asia via an emerging land bridge connecting Africa and the Arabian Peninsula during the late Miocene (Stewart and Disotell 1998; Whybrow 1992). Habitat isolation that happened around 3.17 ± 0.31 million years ago (MYA) caused other species and subspecies of *Trachypithecus* to emerge (Karanth et al. 2008). Subspecies in Thailand are classified as *T. o. seimundi*, and *T. o. sanctorum* was classified as a subspecies in Myanmar. *Trachypithecus obscurus* in Peninsular Malaysia was classified into five subspecies: *T. o. obscurus*, *T. o. halonifer*, *T. o. carbo*, *T. o. styx*, and *T. o. flavicauda* (Brandon-Jones et al. 2004; Groves 2001; Roos et al. 2014). However, there are discrepancies around the distributions of these subspecies. Groves (2001) proposed that *T. o. halonifer* is endemic to Penang Island, but Brandon-Jones et al. (2004) expanded the distribution of the subspecies to Langkawi Island because they considered the subspecies' pelage colour to be nearly the same as that of the population in Langkawi. Meanwhile, Groves (2001) argued that the population in Langkawi has darker hair dorsal to that of *T. o. halonifer* in Penang. Therefore, he proposed a subspecies in Langkawi: *T. o. carbo*. These subspecies are said to have been distributed at Terutau, Langkawi Island, and Dayang Bunting Island as well as the west coast of Peninsular Malaysia in both the Thai-Malaysia boundary areas (Groves 2001).

Morphological characters in taxonomic studies were used to hypothesize *T. obscurus* subspecies classification, but no previous study has used genetics to classify subspecies. Page et al. (1999) studied the phylogenetic relationships of 16 species in family Cercopithecidae using genetic samples of *T. obscurus* and other Old World monkeys. In order to prove that *Presbytis* and *Trachypithecus* are indeed two separate genera, Md-Zain et al. (2005 2008) used *T. obscurus* as a representative of *Trachypithecus.* Md-Zain et al. (2008) sequenced two loci in the Y chromosome—testis-specific protein and sex-determining region—to explain the separation. They precisely separated

Fig. 1. Spectacled langur owing to its interesting looks. Photographed by Ridwan Rahman.

Trachypithecus and *Presbytis* supported by genetic data, behavior, morphology, and ecology (Abdul-Latiff et al. 2019; Md-Zain et al. 2010; Ruslin et al. 2019; Vun et al. 2011). Raaum et al. (2005) were the first to use complete mtDNA sequences from four main tribes in Cercopithecoid, and *T. obscurus* was used to represent Asian Colobinae. Osterholz et al. (2008) used *T. obscurus* to represent *Trachypithecus* to study the phylogenetic position of the langur from *Semnopithecus* and *Trachypithecus* among Asian colobines. Genetic relationships of langur species of *Trachypithecus* and *Presbytis* were determined by Chaveerach et al. (2007) using amplified fragment length polymorphism markers. This study used blood samples from five species of *T. obscurus* from Southern Thailand. Perelman et al. (2011) presented a molecular phylogeny of living primates comprising approximately 90% of all living genera, including *Trachypithecus*, but *T. obscurus* was highlighted to represent *Trachypithecus* in this study.

Groves (2001) and Brandon-Jones et al. (2004) classified *T. obscurus* in Malaysia. However, from their classification, there is a conflict for certain subspecies. Based on the findings from previous research, using *T. obscurus* as the sole answer to the taxonomic position of *T. obscurus* in Peninsular Malaysia does not accurately estimate its position. Thus, we aimed to determine the molecular systematics and biogeographical history of *T. obscurus* in Peninsular Malaysia using D-loop and *COI* genes of mtDNA sequences. Based on the analysis of the samples derived predominantly from wild living animals of known location, our results enabled us to a) provide the phylogeny for *T. obscurus*, b) estimate divergence times between lineages, and c) provide a reliable taxonomic classification for *T. obscurus*. This is the first study to test classifications of subspecies *T. obscurus* using genetic tools.

MATERIALS AND METHODS

DNA Extraction, Polymerase Chain Reaction (PCR), and Sequencing

A total of 35 genetic samples of *T. obscurus* were used in this study (Table 1). All 35 samples were collected across mainland Peninsular Malaysia including surrounding islands such as Perhentian Island, Langkawi Island, and Penang Island (Fig. 2). Fecal samples were collected fresh in the field and were kept in alcohol 97% to preserve the DNA, whereas tissue samples were collected from roadkill accidents. DNA was extracted from 0.5–1.0 g of fecal samples using the innuPREP Stool DNA Kit (Analytik Jena, Germany) and 0.04 g of tissue samples using the innuPREP DNA Mini Kit (Analytik Jena, Germany) following the manufacturer's protocol (Aifat et al. 2016a).

About 800 bp of mtDNA D-loop region and *COI* were amplified through a polymerase chain reaction (PCR) using a Mastercycler Nexus (Eppendorf North America, Inc.). PCR reactions were generated using MyTaq Red Mix (Bioline). The primers used in this study were designed to be species specific to maximize the extent of both region sequences and to avoid amplifying Numts. The primers for the D-loop region were designed by Abdul-Latiff et al. (2017), and those for *COI* by the authors in this study (Table 2). The parameters for the PCR reaction were as follows: initial denaturation for 3 min at 95°C; followed by 30 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 52°C (D-loop) and 49°C (*COI*), extension for 10 s at 72°C; and a final extension stage for 10 min at 72°C (Aifat et al. 2016b). A double PURE kit was used to purify the PCR products, and the samples were sent to Apical Scientific Sdn. Bhd. (Malaysia) for sequencing.

Sequence and Phylogenetic Analyses

The sequences from both regions were obtained and edited using Bioedit Sequence Alignment Editor. All *T. obscurus* DNA sequences were deposited into GenBank under accession numbers MT193169–MT193202. The edited sequences were validated using sequence similarity searches (GenBank BLASTn) to ensure that the targeted species and loci were obtained. The sequences were treated separately to detect any incongruence and each individual sequence was evaluated. The datasets were then combined, and phylogenetic analysis was constructed. All 35 sequences of *T. obscurus* and one sequence representing *Trachypithecus cristatus* (NC023971: Jiang and Huang 2016), one sequence of *T. auratus* (KY117596: Mohd-Salleh et al. 2017), three sequences of *T. obscurus* (AY863425, NC006900: Raaum et al. 2005; MF872234: Roos et al. 2019) from GenBank were aligned using the MEGA 7 ClustalW multiple alignment algorithm (Kumar et al. 2016).

The sequence information from GenBank was unknown as samples were obtained from captivity from Wuppertal Zoo, Germany and Houston Zoological Gardens, USA. The aligned sequences were analyzed at three levels: sequence analysis, phylogenetic analysis, and haplotype analysis. The analyses were performed using PAUP 4.0b10 (Swofford 2002) and DnaSP 4.0 (Rozas et al. 2003). Sequence analysis was done to reveal genetic distances and single-nucleotide polymorphisms (Md-Zain et al. 2018).

Phylogenetic analysis was used to construct three types of phylogenetic trees using three different

softwares: distance based (Neighbor-Joining, NJ) using MEGA 7 (Kumar et al. 2016), character based (Maximum Parsimony, MP) using PAUP 4.0b10 (Swofford 2002), and Bayesian inference (BI) using MrBayes 3.1 (Huelsenbeck and Ronquist 2001). NJ tree reconstruction was tested with bootstrap values of 1,000 using the Kimura two-parameter model. For the MP trees, tree bisection reconnection algorithms were used. The divergence times of *T. obscurus* were estimated using BEAST v 1.10.4 and BEAUti v 1.10.4 (Drummond et al. 2012). As calibration point, *T. cristatus* was used as a second calibration point according to Perelman et al. (2011), whereas *T. auratus* was used as a fossil record around 1.9 ± 0.05 MYA (Jablonski and Tyler 1999).

RESULTS

Amplification products 723- and 649-bp long were obtained for D-loop and *COI* gene fragments, respectively. The combined analysis, about 1372 bp, was used for phylogenetic and haplotype analyses. Of about 49 variable sites from sequence analyses, 35 were parsimony informative. Analysis of the pairwise sequences using the Kimura two-parameter for the northern population of *T. obscurus* in Langkawi Island-Kedah, Perlis, and Penang Island was 0.011 to 0.015. The Perhentian Island-Terengganu population showed a low genetic distance value for northern population (0.011–0.013) (Table 3). The eastern population of *T. obscurus* in Selangor, Johor, and Melaka showed genetic distances of 0.007–0.016.

The NJ, MP, BI, and molecular clock phylogenetic

Table 1. Details on the samples used in this study

No.	Sample Code	Locality	Type of sample
1	ALTOK408	Langkawi Island	Fecal
2	ALTOK409	Langkawi Island	Fecal
3	ALTOK410	Langkawi Island	Fecal
4	ALTOK411	Langkawi Island	Fecal
5	ALTOK412	Langkawi Island	Fecal
6	ALTOK416	Langkawi Island	Fecal
7	ALTOK417	Langkawi Island	Tissue
8	BM19	Kedah	Tissue
9	MMTOK595	Kedah	Fecal
10	ALTOP512	Penang Island	Fecal
11	ALTOP514	Penang Island	Fecal
12	ALTOP522	Penang Island	Fecal
13	ALTOP509	Penang Island	Fecal
14	ALTOP508	Penang Island	Fecal
15	ARTOT525	Perhentian Island	Fecal
16	ARTOT527	Perhentian Island	Fecal
17	ARTOT607	Terengganu	Fecal
18	ALTOR497	Perlis	Fecal
19	ALTOR498	Perlis	Fecal
20	ALTOR499	Perlis	Fecal
21	FRTOB09	Selangor	Tissue
22	FRTOB442	Selangor	Fecal
23	FRTOB443	Selangor	Fecal
24	FRTOB444	Selangor	Fecal
25	FRTOB446	Selangor	Fecal
26	FRTOB447	Selangor	Fecal
27	FRTOB448	Selangor	Fecal
28	ALTOJ544	Johor	Fecal
29	ALTOJ545	Johor	Fecal
30	ALTOJ546	Johor	Fecal
31	ALTOJ547	Johor	Fecal
32	ARTOM611	Melaka	Fecal
33	ARTOM612	Melaka	Fecal
34	ARTOM615	Melaka	Fecal
35	BM17	Melaka	Tissue

trees exhibited identical topological formations and were summarized in a single phylogenetic tree (Fig. 3). All the phylogenetic trees showed absolute division between *T. obscurus* from GenBank and other *obscurus* individuals supported with 0.99 (BI), 99% (NJ) and 100% (MP) bootstrap values. Excluding *obscurus* samples from GenBank, *T. obscurus* diverged to form two separate major clades supported by 0.8 (BI), 88% (NJ) and 90% (MP) bootstrap values. The first major clade comprised all samples from southern populations of *T. obscurus*—Selangor, Johor, and Malacca. The second major clade comprised samples from the north-east population. Sampel ARTOT607 from Terengganu was the earliest to diverge from the clade, a split that was supported by 0.6 (BI), 70% (NJ) and 77% (MP) bootstrap values. The molecular divergence phylogenetic tree was constructed using the uncorrelated lognormal relaxed-clock model. This was done to estimate the

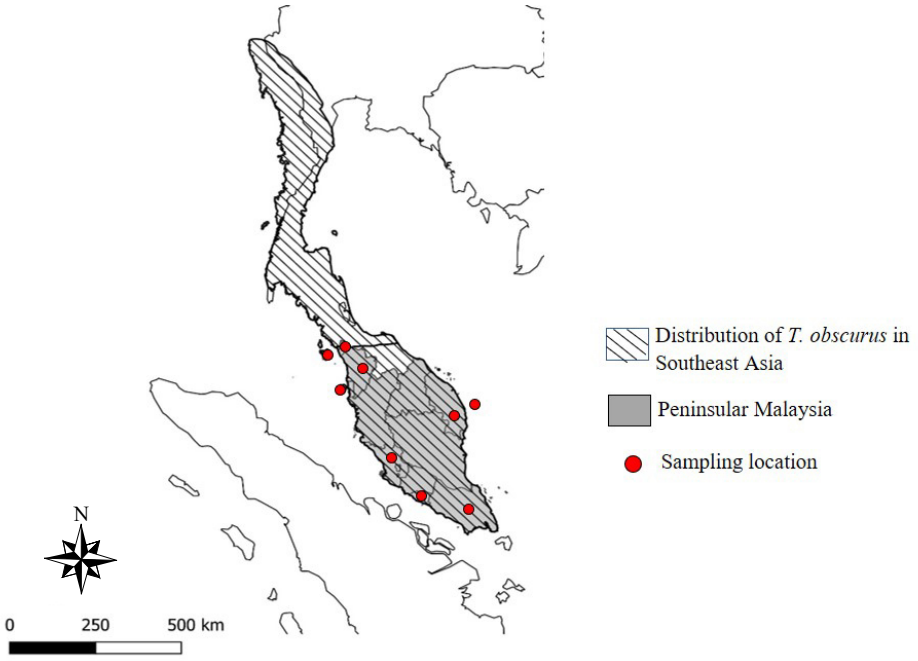

Fig. 2. Map of *T. obscurus* distribution in Southeast Asia and the sampling locations in Peninsular Malaysia.

Table 2. Sequence primers that were designed to be species specific to the target locus

Primer name	Primer sequence (5'-3')	Region	Annealing temperature (°C)
LATIFF1978_F	AAGTGCTTAACCGTCCATAG	D-loop	52.0
LATIFF1909_R	AAGGCAAATTATGTGGGAGT		
AIFAT0326_F	AACAGATCGTAATCTCAACA	COI	49.0
AIFAT0109_R	GTTCATCAAATGTGTGGTAG		

Table 3. Average pairwise genetic distances among populations of *T. osbcurus*

	[1]	[2]	[3]	[4]	[5]	[6]	[7]
[1] Langkawi Island-Kedah	-						
[2] Perlis	0.014	-					
[3] Penang Island	0.011	0.015	-				
[4] Perhentian Island-Terengganu	0.012	0.013	0.011	-			
[5] Selangor	0.019	0.025	0.022	0.020	-		
[6] Johor	0.018	0.025	0.021	0.019	0.007	-	
[7] Melaka	0.022	0.026	0.024	0.025	0.016	0.013	-

substitution rate for all nodes in the tree and establish the divergence dates of the *T. obscurus* population in Peninsular Malaysia. The analysis indicated that the *T. obscurus* group diverged from *T. cristatus* around 2.12 MYA. Molecular clock separation between the southern and north-east populations of *T. obscurus* occurred around 0.15 MYA.

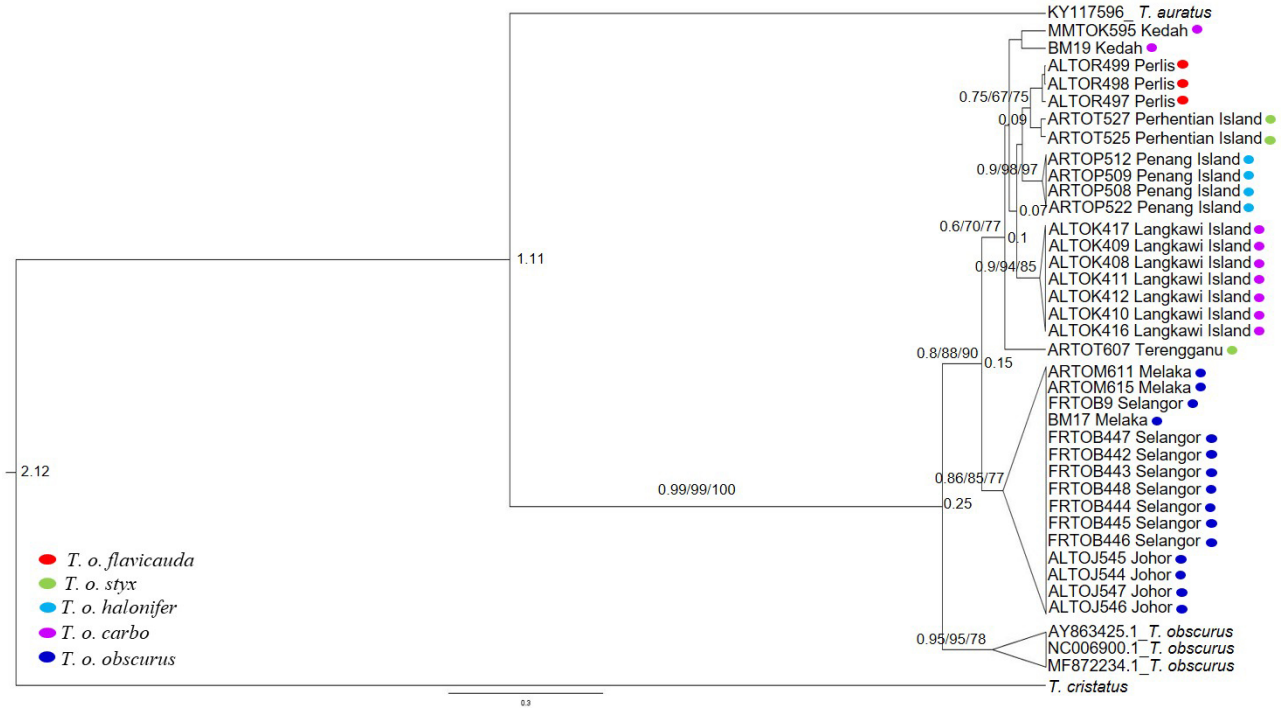

Fig. 3. Summary of Neighbor-Joining, Maximum Parsimony, Bayesian Inference, and Molecular clock phylogenetic tree of *T. obscurus*. Values above the branch represent posterior probability for BI/bootstrap values for NJ/bootstrap values for MP. Values on nodes represent divergence times of the clade.

Table 4. Haplotype analysis with segregation sites for *T. obscurus*

Hap	Segregation sites				
[		1111	1111111111	1122223333	555555667]
[	1222333355	6666880000	1113445788	8901284555	134478552]
[	0236036956	0129041789	1563123801	7051329267	996834132]
#Hap_1	TTTCGGTATC	ACTGGATGTT	CTACGATTAT	TATGACCTAA	GCATTATCG
#Hap_2	....A.....	.T.....AC.	..G.....G.	..C...T.G.	....
#Hap_3	....A.....	....A..AC.	.....G....	CG....T...	.......T.
#Hap_4	....A.....	.T..A.CA..	...T......	........G.	.T.C.....
#Hap_5	....A.....	.T..A.CA..	...T......	........G.	.TGC.....
#Hap_6	....A....T	....AG.A..	....	..C....C..	....
#Hap_7	.C..A.....	....A.CA..	....	..C..A....	.T...G...
#Hap_8	....A.....	....A.CA..	....	..C.......	.T...G...
#Hap_9	..CTA..G..	..C....A..	....	......T...	....
#Hap_10	....A.....	..C....A.C	T...A.CC..	....G.T..G	......C..
#Hap_11	..C.A..G..	G.CA...A.C	......CC..	...A..T...	....
#Hap_12	....AAC...	G.CA..CA..	....A.CC.C	...A..T..G	.T..C....
#Hap_13	..C.A.....	G.CA...A.C	....A.CC..	...A..T...	....
#Hap_14	....AAC.CT	GTCA...A.C	.C..A.C...	......T.G.	A........
#Hap_15	....AAC.CT	GTCA...A.C	.C..A.C...	......T.G.	........A
#Hap_16	C...A.....	G.CA...A.C	....A.CC..	.........G	....

Haplotype analysis and Minimum Spinning Network (MSN)

Data were collected for a total of 16 *T. obscurus* haplotypes (Table 4), with a haplotype diversity of 0.9234. Based on the analysis, the populations in Langkawi-Kedah, Perhentian-Terengganu, Selangor, and Melaka were recorded to have four haplotypes: Hap 1–3, Hap 7–9, Hap 10–12, and Hap 14–16. The population in Perlis was recorded to have two haplotypes: Hap 4 and Hap 5. Meanwhile, both of populations in Penang Island and Johor were recorded to have one haplotype each: Hap 6 and Hap 13, respectively.

Based on the haplotype analysis, a minimum spanning network (MSN) was used to picture the relationships among haplotypes (Fig. 4). The populations on Perhentian Island-Terengganu were found to differ at 14 mutational sites, even though both populations were in the same state. The population on Terengganu (Hap 9) was closer to the population in Langkawi Island, with seven mutational sites. Meanwhile, the population on Perhentian Island (Hap 7 and Hap 8) was closest to the population in Perlis, with six mutational sites in the same sequence.

DISCUSSION

Taxonomy status of *Trachypithecus obscurus* in Peninsular Malaysia

The results from this study support the classification in Chasen (1940), which found that *T. o. obscurus* is distributed in southern Peninsular Malaysia. According to Brandon-Jones et al. (2004), Groves (2001), and Roos et al. (2014), *T. o. obscurus* is found all over Peninsular Malaysia, except along the north coast or south of Perlis. The genetic distance analysis supported this by its results that the southern and north-eastern populations had higher genetic distances. The tree topologies indicated a separation between the south and north populations, and *T. o. obscurus* is found in the southern Peninsular Malaysia.

The classification distributions of *T. o. carbo* by Groves (2001) and Roos et al. (2014) are different from that of Brandon-Jones et al. (2004), which can be seen in this study. Pocock (1935) was the first to recognize *T. o. carbo* on Langkawi Island based on morphology—fur color; this subspecies has a darker pelage than *T. o. halonifer* on Penang Island (Fig. 5). Meanwhile, Brandon-Jones et al. (2004) argued that a darker pelage

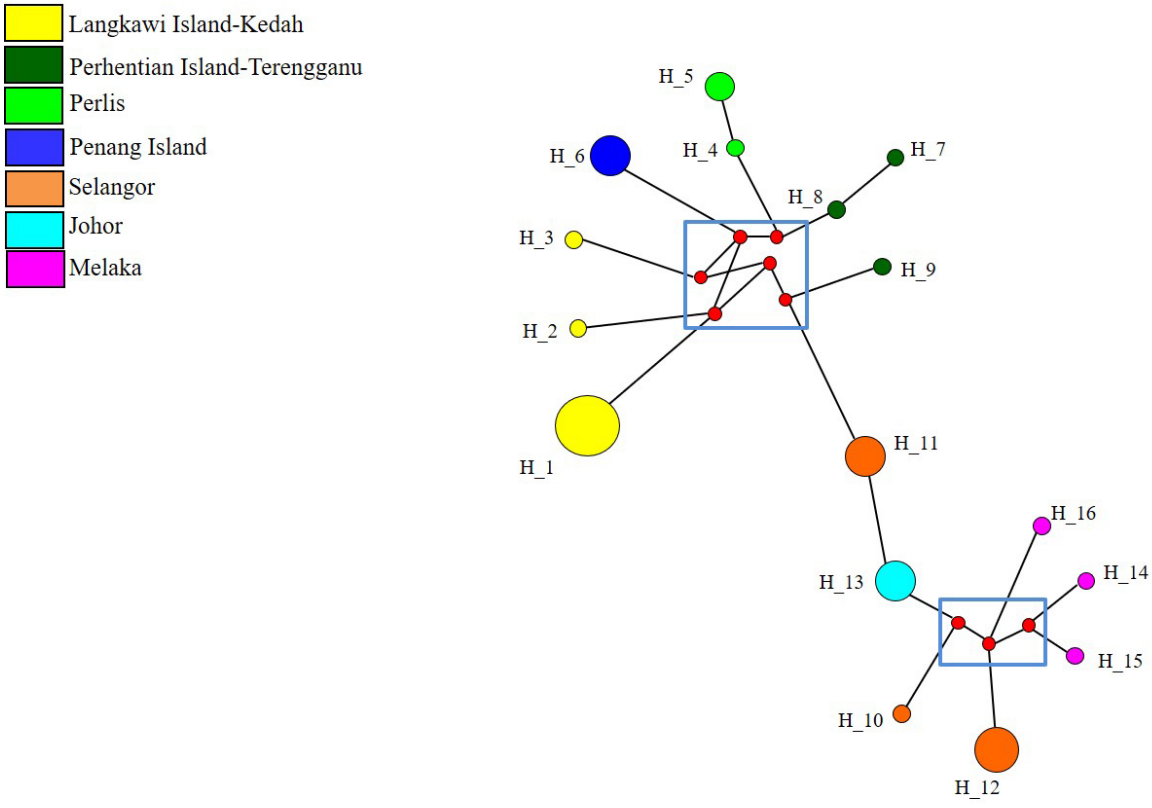

Fig. 4. MSN network for both regions for populations of *T. obscurus*. The size of the circle is directly proportional to haplotype frequency. Red nodes represent the median vector, which is often hypothesized to be the ancestral sequence.

than *T. o. halonifer* keeps *T. o. carbo* from being discriminated, but this subspecies is distributed on Terutau Island in Thailand and not on Langkawi Island. Groves (2001) stated that subspecies of *T. obscurus* in Terutau Island, Thailand, has a darker coloration than the subspecies on Langkawi Island and Dayang Bunting Lake as it is likely the result of the fur coloration becoming darker along a gradient from south to north. This study provides the classification based on genetic data, and the results are found to agree with those of

Fig. 5. Morphology of *T. obscurus* in Peninsular Malaysia. A, Langkawi Island (*T. o. carbo*), B, Penang Island (*T. o. halonifer*), C, Perlis (*T. o. flavicauda*), D, Selangor (*T. o. obscurus*), E, Perhentian Island (*T. o. styx*). Photographed by Md-Zain, Aifat Rahman and Abdul-Latiff.

Groves (2001) and Roos et al. (2014).

In this study, the population of *T. obscurus* from Penang Island formed its own clade and did not shared the same haplotype with the population on Langkawi Island. Thus, this study rejected the classification of subspecies by Brandon-Jones et al. (2004), who stated that subspecies in Penang and Langkawi Island are *T. o. halonifer*. The results from this study supports the morphological classifications by Groves (2001) and Roos et al. (2014). Chasen (1935 1940) was the earliest to identify subspecies *T. obscurus* on Langkawi Island as *T. o. carbo* and stated that *T. o. halonifer* is endemic to Penang Island. This statement is supported by Groves (2001) and Roos et al. (2014).

The population of *T. obscurus* on Perhentian Island was represented on an unstable clade in the phylogeny. In the NJ phylogenetic trees for both regions, this population formed a paraphyletic clade with the population in Perlis and Penang Island (63% bootstrap value). Meanwhile, in the MP and BI phylogenetic trees of both regions, the population on Perhentian Island formed monophyletic and polyphyletic clades with the populations in Perlis. Subspecies of *T. obscurus* on Perhentian Island has been classified as *T. o. styx* (Kloss 1911). Pocock (1935) did not differentiate *T. o. styx* and *T. o. carbo* (Langkawi Island population) based on fur coloration. However, both of these subspecies have opposite geographical areas and are separated by *T. o. obscurus* and *T. o. halonifer*. Groves (2001) states that *T. o. styx* may have had a darker pelage and inhabited the northern Peninsular Malaysia (*T. o. flavicauda*) (Fig. 5) until the late Pleistocene. During this time, sea level rose to the north of Peninsular Malaysia and caused the species to migrate to the south of Peninsular Malaysia. At that time, most of Peninsular Malaysia was underwater, and the species could not migrate or inhabit the area because it was an isolated island (Groves 2001).

Biogeography of *T. obscurus* in Peninsular Malaysia

Roos et al. (2011) suggested that the primitive ancestor of the Asian Colobinae migrated to Asia via a land route connecting Africa and the Arabian Peninsula during the late Miocene (Stewart and Disotell 1998; Whybrow 1992). This is supported by the discovery of Colobinae primate fossils dated to the last days of the Miocene, which were found in the Tibetan plateau via the northern road of the Himalayas (Davies and Oates 1994). The Hengduan Mountain, which is now a border area between Myanmar and China, is said to be the hotspot of this genetic diversity (Peng et al. 1993; Thinh et al. 2010). The rise in wide river waters in Southeast Asia such as the Mekong River, Salween River, and Yangtze River caused *Trachypithecus* to migrate south of the Asia mainland, which includes Thailand and Peninsular Malaysia (Hallet and Mollnar 2001).

According to Abdul-Latiff (2015), species diversification from this genus formed four groups of species: those in South Asia, center of Southeast Asia, southern Southeast Asia, and northern Southeast Asia. This situation illustrates that the ancestors of extant species are likely to survive in these four separate groups. The cool climate during the Pliocene led to declines in the area of evergreen forest (Batchelor 1979; Morley 2000) and caused these four groups to separate (Wang 1994). In this study, we proposed that *T. obscurus* invaded Peninsular Malaysia around 0.25 MYA. This process caused the species to separate into north-east and south populations. Separation of *T. obscurus* occurred in the mainland before the species were distributed in the archipelago area around Peninsular Malaysia. *Trachypithecus o. styx* diverged after separation from the Perlis population *T. o. flavicauda*. The molecular clock results showed that the hypothesis of Groves (2001) is acceptable.

CONCLUSIONS

Classification of the *Trachypithecus obscurus* populations in Peninsular Malaysia showed that the north-eastern and southern populations are distinct. Based on the studies conducted, genetic data show that the subspecies classification in Peninsular Malaysia is not fully resolved for the north-eastern populations, whereas for southern population, the classification of *T. o. obscurus* is acceptable. Classification of subspecies *T. o. styx* in Perhentian Island that is based on morphology is not relevant as this subspecies is nested with populations in the north, such as Perlis and Langkawi Island. The population on Perhentian Island was a continuation of the northern population before separation occurred because of geographical factors. The molecular clock phylogenetic tree for both sequences was effective in classifying subspecies and evolutionary histories. The results of this study should be considered as preliminary study for the taxonomic revision of subspecies *T. obscurus* using a molecular approach. In the future, additional samples of *T. obscurus* from other states—especially Terengganu, Pahang and Kelantan—should be used to confirm the subspecies for *T. obscurus* populations in the east. From the conservation standpoint, genetic information helps the Department of Wildlife and National Parks (DWNP) manage dusky leaf monkey populations via translocation. This leaf monkey is believed to have a

conflict with humans by disturbing their crops. The information from this study is expected to prevent gene mixing in the original habitat of the dusky leaf monkey. Further taxonomic and phylogenetic work is needed to fully resolve the classification of *T. obscurus* in Peninsular Malaysia.

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