PHYLOGENETIC RELATIONSHIPS OF *Heterotrigona itama* IN MALAYSIA BASED ON *COI* DNA SEQUENCES

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ABSTRACT

In Malaysia, stingless bee *Heterotrigona itama* is one of the bee species that is actively reared and mostly utilized in meliponiculture practice. In this preliminary study, we described the phylogenetic relationship scenario among selected localities of *H. itama* in Peninsular Malaysia and Borneo. Genomic DNA from 16 samples of five localities in Peninsular Malaysia (Kedah, Kelantan, Melaka and Johor) and Borneo (Sarawak) was successfully extracted and sequenced for phylogenetic analysis. The 489 bp mitochondrial DNA of *COI* sequences was generated for phylogenetic tree reconstruction and population genetic analysis. Tree topology indicated two monophyletic clades which belong to Peninsular Malaysia and Sarawak specimens with high bootstrap support. Five population genetic parameters were studied including nucleotide diversity (π), net nucleotide divergence (Da), nucleotide subdivision (Nst), population subdivision (F_{ST}) and gene flow (N_m). The Sarawak samples had the highest scores for all five parameters indicating its genetic affinity to the population in Peninsular Malaysia. Haplotype analysis showed that Kedah-Kelantan samples shared the same haplotype while Sarawak samples had its own unique haplotype with no sharing of haplotypes between other localities. Minimum-Spanning Network generated a visual representation of the relationship reflect a clear separation between Sarawak and Peninsular Malaysia stingless bees samples. This preliminary study shows the importance of understanding the systematic data towards conservation efforts of Malaysian stingless bee's diversity.

Keywords: Stingless bee, *Heterotrigona itama, COI,* DNA barcode

ABSTRAK

Di Malaysia, kelulut *Heterotrigona itama* merupakan salah satu dari lebah yang diternak dan digunakan dalam penghasilan madu. Dalam kajian awal ini, kami mentafsirkan senario pertalian filogenetik beberapa lokasi terpilih *H. itama* di Semenanjung Malaysia dan Borneo. DNA genomik dari 16 sampel melibatkan lima lokasi Semenanjung Malaysia (Kedah, Kelantan, Melaka dan Johor) dan Borneo (Sarawak) telah berjaya diesktrak dan dijujuk untuk analisis filogenetik. Sebanyak 489 pb jujukan DNA mitokondria *COI* telah dihasilkan

untuk analisis pembinaan pohon filogeni dan genetik populasi. Topologi pohon menunjukkan dua klad monofiletik dimiliki spesimen dari Semenanjung Malaysia dan Sarawak telah disokong oleh nilai butstrap yang tinggi. Lima parameter genetik populasi telah dikaji termasuk kepelbagaian nukleotid (π), pencapahan nukleotid akhir (Da), pemecahan nukleotid (Nst), pemecahan populasi (F_{ST}) dan aliran gen (N_m). Sampel Sarawak mempunyai nilai paling tinggi dalam kesemua parameter menunjukkan pengukuhan genetik berbanding populasi Semenanjung Malaysia. Analisis haplotip menunjukkan sampel Kedah-Kelantan berkongsi haplotip yang sama manakala sampel Sarawak mempunyai haplotip unik tersendiri tidak berkongsi dengan lokasi lain. Minimum-Spanning Network telah menunjukkan pertalian jelas yang memisahkan sampel lebah kelulut Sarawak dari Semenanjung Malaysia. Hasil akhir ini menunjukkan kepentingan untuk memahami data sistematik kearah usaha pemuliharaan kepelbagaian lebah kelulut di Malaysia.

Kata kunci: Kelulut, *Heterotrigona itama*, *COI*, barkod DNA

INTRODUCTION

Stingless bees are eusocial insects that are widely distributed throughout all tropical and subtropical regions except some oceanic islands and also act as important pollinators in tropical dipterocarp forests (Momose et al. 1998; Nagamitsu et al. 1999). Stingless bees belong to family Apidae and are closely related to honey bees, carpenter bees, orchid bees, and bumblebees (Roubik 1989). Malaysia is a tropical country with high species diversity of stingless bee. However, the diversity of stingless bees throughout Peninsular Malaysia is poorly documented (Salim et al. 2012). Currently, the number of species of stingless bees has increased up to 45 species (Rasmussen 2008). Mohd Fahimee et al. (2016) reported a total of 29 species in Peninsular Malaysia and 19 were set up in the primary forest. Thus, biodiversity of stingless bee in Malaysia is something unique to explore and learn.

In this study, *Heterotrigona itama* was chosen as the main subject. *Heterotrigona* is common in Malaysia as they are easily to find in many places. According to Kelly et al. (2014), out of 17 to 32 species that have been identified, only *H. itama* and *Geniotrigona thoracica* were cultivated in the meliponiculture. These two species also produce propolis containing high anti-bacterial properties (Ibrahim et al. 2016). *H. itama* are distributed along the Sundaland, ranging from northern Thailand to the South and East Java, Borneo (Rasmussen 2008). Previous stingless bee studies in Malaysia have been carried out in Sabah, Negeri Sembilan, Selangor, Perak and Terengganu (Eltz et al. 2003; Salim et al. 2012). However, their molecular systematic and population genetics have yet to be discovered comprehensively for Malaysian population.

Many molecular systematic studies have employed mitochondrial markers for species identification and relationships (Abdul-Latiff et al. 2014a, b; Ang et al. 2011; Rosli et al. 2014). The region with the high potential for species identification at the species level is the cytochrome oxidase subunit I (*COI*) gene (Kandil et al. 2010: Md-Zain et al. 2018). *COI* as a conserved gene with lower mutation rate as compared to other mitochondrial genes could also be employed in molecular systematics and population genetic studies (Patwardhan et al. 2014; Rosli et al. 2011a). This study explains the phylogenetic relationships among the *H. itama* populations in different localities representing north, east, west and south part of Peninsular Malaysia and Borneo Sarawak.

MATERIALS AND METHODS

Stingless Bee Samples

The study of phylogenetic relationships of stingless bee, *H. itama* consist of 16 individuals from five sampling sites in Peninsular Malaysia (Kedah, Kelantan, Melaka and Johor) and Sarawak (Table 1). The samples used as a genetic source are fresh insect samples. Samples were not damaged, and remain available for morphological re-examination or re-used for taxonomic work. DNA was extracted from the whole body using the QIAGEN DNeasy® Blood & Tissue Kit (Aifat et al. 2016).

Table 1 List of samples and localities.

Sample Code	Sample code correspond on phylogeny tree	Location
BMHIK01	K01	Kg. Padang, Baling, Kedah
BMHIK03	K03	Kg. Padang, Baling, Kedah
BMHIK04	K04	Kg. Padang, Baling, Kedah
NRHIM02	M02	Kg. Sg. Buloh, Alor Gajah, Melaka
NRHIM03	M03	Kg. Sg. Buloh, Alor Gajah, Melaka
NRHIM04	M04	Kg. Sg. Buloh, Alor Gajah, Melaka
NRHIM05	M05	Kg. Sg. Buloh, Alor Gajah, Melaka
NRHIJ01	J01	Kg. Mawai, Kota Tinggi, Johor
NRHIJ02	J02	Kg. Mawai, Kota Tinggi, Johor
NRHIJ04	J04	Kg. Mawai, Kota Tinggi, Johor
NRHIJ05	J05	Kg. Mawai, Kota Tinggi, Johor
NRHIB01	B01	Bachok, Kelantan
NRHIB02	B02	Bachok, Kelantan
NRHIB03	B03	Bachok, Kelantan
IRHIS03	S03	Kg. Bungin, Betong, Sarawak
IRHIS04	S04	Kg. Bungin, Betong, Sarawak

DNA Amplification

The PCR amplification of COI gene was carried out using one set of primers, forward C1-J-1718F GGGGGGTTTGGAAATTGATTAGTGCC and reverse, C1-N-2191R CCCGGT AAAATTAAAATATAAACTTC (Simon et al. 1994). PCR condition was carried out with an annealing temperature of 42°C and a further elongation of 10 minutes (Table 2) with total volume of 25 µl (Table 3) according to Nik-Rashidi (2017). The PCR products were examined through electrophoresis process using 1.5% agarose gel in 1X TAE buffer. PCR products were sent to First Base Sdn. Bhd. (Shah Alam, Malaysia) for DNA sequencing.

Table 2 Details on PCR profiles.

Parameter	**Temp(°C)**	**Duration (second)**	**cycle**
Initial denaturation	95	180	-
Denaturation	95	60	30
Annealing	42	60	30
Extension	72	30	30
Post-extension	70	600	-

Table 3 PCR component concentration and volume.

PCR cocktail	**Final concentration**	**Volume (µL)**
Distilled water (ddH2O)		8.5
GoTaq Green Master Mix		12.5
Forward primer	1.0 µl (20.0 uM)	1.0
Reverse primer	1.0 µl (20.0 uM)	1.0
DNA template		2.0
Total	**-**	**25.0**

Data Analysis

DNA sequence analysis involved editing and aligning using BioEdit Sequence Alignment Editor 7.2.5, MEGA7 and BLAST (Basic Local Alignment Search Tool) online software (Abdul-Latiff et al. 2017; Kumar et al. 2016; Rosli et al. 2011b). The phylogenetic tree was reconstructed using Neighbor Joining (NJ) with the Kimura-2 parameter (K2P) model and *Apis melifera* (MF543437) as the outgroup. Character analyses were performed using Maximum parsimony. Haplotype data was generated using DnaSP v6 (Rozas et al. 2017) and MEGA7. Meanwhile, Network 4.6.1.2 was used to generate a minimum-spanning network (MSN) to illustrate the relationships among populations (Bandelt et al. 1999). Five parameters were studied in the analysis of population genetics among populations of *H. itama* which are nucleotide diversity (π), net nucleotide divergence (Da), nucleotide subdivision (Nst), estimate of population subdivision (F_{ST}) and gene flow (N_m).

RESULT

Sequence Analysis

DNA of 16 samples *H. itama* which consists of 14 individuals from Peninsular Malaysia (Kedah, Kelantan, Johor and Melaka) and 2 individuals from Sarawak have been amplified with the size of 489 bp. Characters of COI sequence analysis were summarized in the Table 4. It was found that 184 (37.6%), out of 489 characters in the sequences were constant, leaving 305 (62.3%) variable characters. In addition, out of 305 variable characters, 97 sites were parsimony informative characters (19.8%), while the 42.5% remaining characters were parsimony uninformative. However, after the outgroup is removed from the analysis, it was found that 345 (70.5%) out 695 characters were constant, meanwhile 144 (29.4%) were variable characters. Parsimony informative characters recorded were 90 (18.4%). In addition, nucleotide A has the highest frequency of 45.6% while nucleotide G has the lowest frequency of 8.3%.

Genetic distance based on kimura-2 parameter (K2P) algorithm was also calculated using MEGA7.0. Table 5 showed the highest genetic distance is between samples from Sarawak and Peninsular Malaysia. Meanwhile, genetic distance between the Kelantan and Kedah samples of Peninsular Malaysia showed the lowest value of 0.000.

Table 4 Character of COI sequence analysis.

Type of character	**Total/Value**	
	With outgroup	**Without outgroup**
Total characters	489	489
Constant character	184	345
Variable character	305	144
Parsimony informative character	97	90
Parsimony uninformative character	208	54
Percentage of informative character	19.8%	18.4%
Bias ti/tv (R)	0.4	0.3
Nucleotide Frequency A	33.1%	33.0%
Nucleotide Frequency T	45.4%	45.6%
Nucleotide Frequency C	13.2%	13.1%
Nucleotide Frequency G	8.3%	8.3%

Table 5 Genetic distance for the *H. itama* samples based on *COI* gene.

Locality	Kelantan	Kedah	Johor	Melaka	Sarawak
Kelantan					
Kedah	0.000				
Johor	0.008	0.008			
Melaka	0.002	0.002	0.006		
Sarawak	0.279	0.279	0.285	0.280	

Phylogenetic Analysis

Neighbor-Joining tree topology was generated using the distance method through MEGA7. Phylogenetic analysis produced a NJ tree topology (Figure 1) that diverged into two main clades, clade A and clade B which are Peninsular Malaysia and Sarawak respectively. The formation of clades between taxa in Peninsular Malaysia and Sarawak was supported with high bootstrap value of 100% and 94%. Meanwhile, clade A shows a clear separation between north and south part in Peninsular Malaysia with bootstrap value of 64% and 84% respectively.

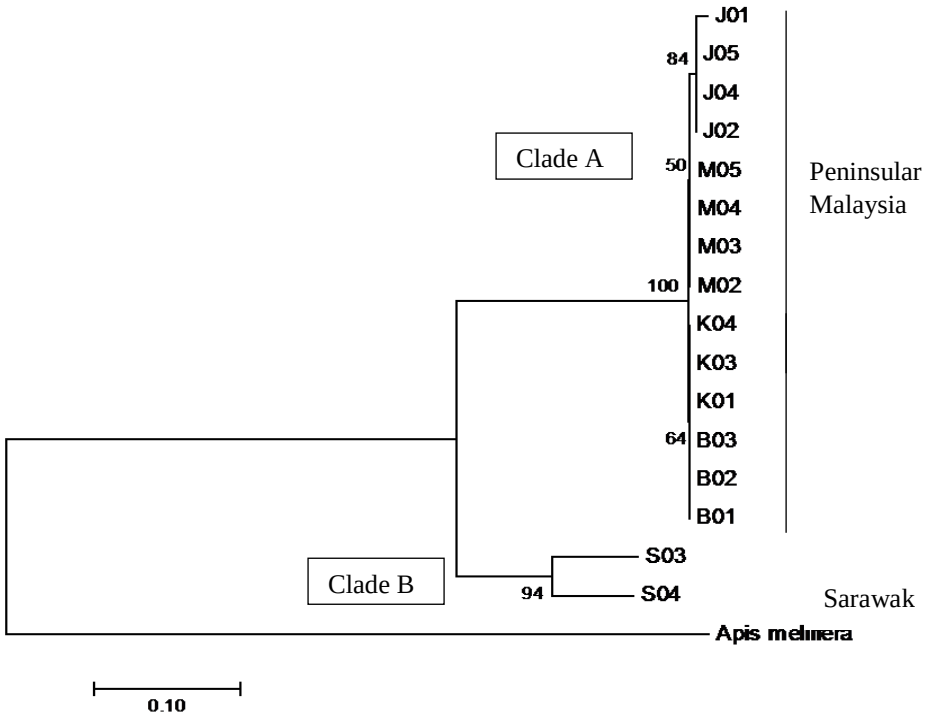

Figure 1 Neighbor-Joining tree of *COI* sequences. Numbers above and below the branches are bootstrap values (in percentage at 1000 replicates).

Population Genetic Analysis

Based on the measures (Table 6), populations in Sarawak showed the highest nucleotide diversity (π) with samples in Kedah and Kelantan, at 0.1500, followed by Sarawak-Johor and Sarawak-Melaka with the value of 0.1347 and 0.1310 respectively. Within samples in Peninsular Malaysia, Kedah and Johor exhibited high nucleotide diversity at 0.0070. However, samples from Kedah and Kelantan showed 0.0000 π value. Net nucleotide divergence (Da) among populations of *H. itama* is consistent with the π value obtained. Samples in Sarawak-Johor exhibited high value of Da, at 0.1821 followed by Sarawak with Kedah, Kelantan and Melaka of 0.1803. In the meantime, Kedah and Johor also showed high Da value, at 0.0082.

Genetic differentiation (F_{ST}, Nst, N_m) values were calculated to further elucidate the relationships among *H. itama* samples in Peninsular Malaysia and Sarawak. *Heterotrigona itama* from Sarawak showed high division with samples in Peninsular Malaysia (Sarawak-Melaka), (Sarawak-Kedah), (Sarawak-Kelantan) at 0.77533. Meanwhile, the samples within Peninsular Malaysia portray the highest F_{ST} between Kedah and Johor with value of 0.8000. However, samples from Kedah-Kelantan shows no value of F_{ST} as there is no genetic difference and was considered to have the same genetic composition.

Nst value can be used to estimate a population's subdivision at the nucleotide level and completely parallel with $F_{ST.}$ Two populations within Peninsular Malaysia from Melaka-Kedah and Melaka-Kelantan exhibited the highest Nst value with 1.00000. Samples between Sarawak and Peninsular Malaysia (Melaka, Kedah and Kelantan) still showed high Nst value,

at 0.79788 followed by Sarawak-Johor, with 0.79462. In addition, for gene flow measure (N_m), samples between Sarawak-Kedah and Sarawak-Kelantan showed the same value at 0.82 and followed by Sarawak-Melaka which exhibit N_m value, at 0.73.

Table 6 Measures of nucleotide diversity (π), net nucleotide divergence (Da), nucleotide subdivision (Nst), estimate of population subdivision (F_{ST}), and gene flow (number of migrants, N_m) among samples of *H. itama*.

Locality	π	Da	F_{ST}	Nst	N_m
Melaka – Johor	0.00439	0.00410	0.66667	0.66667	0.33
Melaka –Kedah	0.00117	0.00205	1.00000	1.00000	0.00
Melaka – Kelantan	0.00117	0.00205	1.00000	1.00000	0.00
Johor – Kedah	0.00701	0.00818	0.80000	0.80044	0.39
Johor – Kelantan	0.00701	0.00818	0.80000	0.80044	0.39
Kedah – Kelantan	0.00000	0.00000	-	-	-
Sarawak – Melaka	0.13101	0.18033	0.77533	0.79788	0.73
Sarawak – Johor	0.13470	0.18212	0.77031	0.79462	2.67
Sarawak – Kedah	0.15000	0.18033	0.77533	0.79788	0.82
Sarawak - Kelantan	0.15000	0.18033	0.77533	0.79788	0.82

Haplotype Analysis and Minimum Spanning Network

Haplotype analysis was performed by using DNAsp5. Six haplotypes with a size of 143 bp were defined from the five localities of *H. itama* (Table 7) and obtained from 16 analyzed sequences (excluding outgroup). Kedah and Kelantan are the only localities that share the same haplotypes (Hap_1). In contrast, Johor, Melaka, and Sarawak showed there is no sharing of haplotypes with other localities. Melaka samples only contain one unique haplotype, (Hap_4); Johor and Sarawak on the other hand exhibit two haplotypes, (Hap_2, Hap_3) and (Hap_5, Hap_6) respectively. Samples originating from five localities exhibited high haplotype diversity (Hd) with 0.8000.

Table 7 Segregating sites (143 bp) in 489-bp segment of *COI* gene. Peninsular Malaysia (haplotype 1 – 4) and Sarawak (haplotype 5 – 6).

Haplotype	Nucleotide Positions (Sequence 5' – 3')																																
											111	111	111	111	111	111	111	111	111	111	111	111	112	222	222	222	222	222	222	222	222	222	222
		1	112	223	344	556	677	778	889	999	000	011	222	223	333	444	455	556	667	777	788	999	990	000	000	111	122	233	344	445	566	777	777
	124	573	450	367	956	892	634	680	563	459	137	905	134	581	234	012	501	291	582	348	929	013	491	345	789	012	312	718	907	890	138	056	789
Hap_1	AGA	TAT	ATT	CCT	TTT	CTT	ACC	ATC	ACA	ACA	ACT	TTT	TAT	AAC	TTC	CTG	GTA	CTC	TTA	CTA	TCT	TTG	GTA	ATT	TAT	TGT	ATG	TAC	TCA	CTT	CTA	GAT	CAC
Hap_2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	TGG	.G.	.A.	G.T	...	...	...	...	...	...
Hap_3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.	G.T	...	...	...	...	...	...
Hap_4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	...	...	...	...	...
Hap_5	TCC	CGG	CAC	GTA	GGA	TAA	TAT	CG.	GA.	.A.	T.A	.AA	A.A	.TT	CCG	AG.	AA.	T.T	.AG	AAC	AAG	.AC	AA.	...	..A	ACA	..A	ATA	ATC	AAA	.CT	A.A	ATA
Hap_6	TCC	.GG	CAC	GTA	GGA	TAA	TAT	.GA	GAG	T.C	TT.	C..	.CA	TTT	...	AGA	AAT	TAT	AAG	AA.	AAG	GAC	.AT	.A.	G.A	ACA	.AA	A.A	ATC	.AA	TCT	ACA	AT.

Haplotype															
	222	223	333	333	333	333	333	333	333	334	444	444	444	444	44
	889	990	001	111	223	444	455	677	788	894	555	566	777	788	88
	364	573	450	128	892	034	767	335	836	907	127	857	035	613	89
Hap_1	TTT	ATT	ATA	CTT	TGC	TTA	TTA	TTA	TTT	ATA	TTT	TAC	GGT	TTT	TT
Hap_2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..
Hap_3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..
Hap_4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..
Hap_5	AGA	TAA	.CC	T.A	GAG	AAC	C.C	.AC	CAA	TCC	AAG	AGA	TAA	.AA	A.
Hap_6	AGA	TAA	TC.	.A.	.AG	AAC	CAC	AAC	C.A	...	..G	AGA	T.A	AAA	AA

Minimum-Spanning Network was generated to portray a visual representation of the relationship between locality haplotypes of *H. itama*. Figure 2 shows that there are haplotypes sharing by samples from Kedah and Kelantan, while samples from Melaka, Johor and Sarawak have their own unique haplotype. However, Kelantan and Kedah samples represent a close relationship with each other compared to the samples from the southern part, Johor which is completely separated. In addition, samples from Sarawak were separated from Peninsular Malaysia's samples significantly.

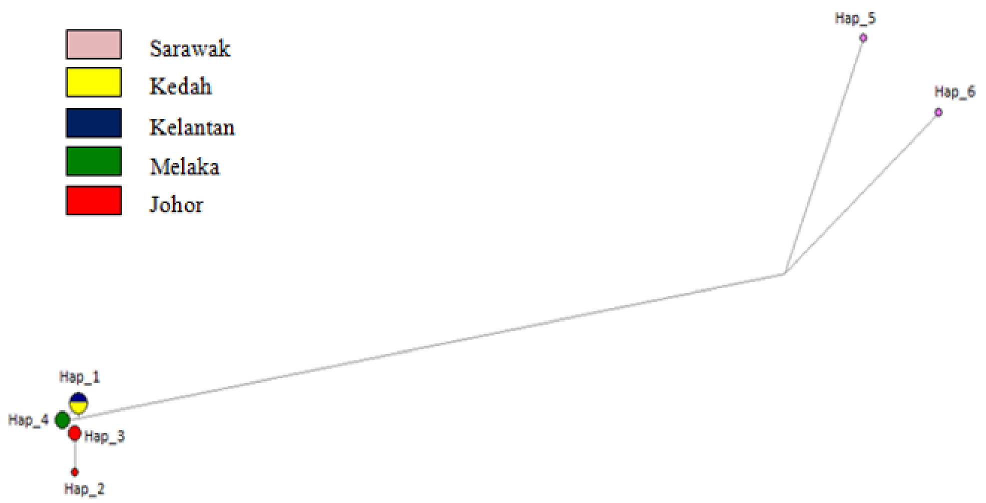

Figure 2 The minimum-spanning network (MSN) illustrating the relationships of *H. itama* in five localities. Each circle represents a haplotype, and the diameter is scaled to the haplotype frequency.

DISCUSSION

The presence of variable character in an organism is due to the mutation rate that occurs in the nucleotide sequence. The higher the variable character present in the DNA sequence, the higher the genetic distance between the samples. High genetic distances will result in more significant tree topology. The genetic distance analysis of this study has shown that samples in Sarawak with the samples in Peninsular Malaysia had illustrates a distant relationship between one another. Meanwhile, the genetic distance obtained between samples of Kedah and Kelantan shows that there is a similarity in terms of genetic composition and is said to be inherited from the same ancestor. Phylogenetic analysis produced a tree topology that diverged into two main clades, clade A and clade B which are Peninsular Malaysia and Sarawak that formed a monophyletic clade. The grouping of the taxa *H. itama* in this study was supported by high bootstrap confidence value indicating a significant separation between the samples in Peninsular Malaysia and Sarawak.

Based on the finding of population genetic parameters, the samples in Sarawak displayed high scores for all five parameters and indicates that much of genetic affinity to the samples in Peninsular Malaysia. However, samples of Kedah-Kelantan had value of 0.0000 for π and Da because there is no nucleotide diversity as both localities are inherited from the same ancestor. Moreover, these two localities also have failed to show their F_{ST} value because there are no genetic differences and are considered to have the same genetic composition. In contrast, F_{ST} value for other localities showed a value greater than 0.25 which are statistically significant. Only F_{ST} values >0.25 strongly indicate a genetic differentiation of populations (Lowe et al. 2004). High estimate of population distribution is related to the living character of *H. itama.* Stingless bee could not be scattered at long distances or large water strains due to inability of the stingless bees to fly at long distance, as shown by the study of the correlation between body size and flight distance in stingless bee (Araújo et al. 2004).

On the other hand, N_m describes the average number of individuals per generation migrating between localities. Based on Table 4, N_m value between samples in Sarawak and Peninsular Malaysia were not consistent and samples of Melaka-Kedah and Melaka-Kelantan were recorded N_m at 0.00, indicating that gene flow between these two localities has cut off over time. Theoretically, the value of N_m is inversely proportional to the value of F_{ST} and N_{ST}. Waples (1987) mentioned that probably the number of population samples should be five or more individuals to obtain a more reliable and relevant estimation value. One of the potential sources of bias in estimating gene flow is the sampling scheme. Because of the estimation value of N_m is taken as average, there is a value differences. Therefore, low N_m value may be caused by one or several separate populations (Pashley et al. 1985).

It was found that only Kedah-Kelantan samples shared the same haplotypes. Haplotype sharing between Kedah-Kelantan samples can be attributed to the value of nucleotide diversity (π) and the final value of net nucleotide divergence (Da), both of which exhibited 0.000. Kedah-Kelantan did not show any pattern of genetic relationships due to maternal inheritance. Some individuals in the Johor and Sarawak show a unique haplotype formation, but still do not share haplotypes with other localities. The unique haplotype formation is due to the differences of mutation site in the sequences obtained. Overall, segregation site (143 bp) of haplotype in COI sequences over 489 bp indicates the generation of DNA barcoding in *H. itama* samples in Peninsular Malaysia and Sarawak.

Minimum-spanning network analysis (Figure 2), showed there is no haplotype sharing between samples in Sarawak, Johor and Melaka. Only samples in Kelantan and Kedah shared the same haplotype and probably they were inherited from the same ancestor or the colony accidentally separated through land transportation. The geographical distance that separates Kedah and Kelantan samples also has the potential to be among the main reasons to this pattern of relationships. Samples in Sarawak shows a difference of 489 mutation sites to the haplotype in Peninsular Malaysia. In addition, genetic distances and population genetic analysis also discovered that populations from Sarawak reflect a clear separation and relationships that much of the samples in Peninsular Malaysia.

This study portrays the effectiveness of *COI* gene in clarifying the genetic relationships of *H. itama* of selected localities in Malaysia. Even though with limited sample numbers, our phylogenetic and population analysis could still be able to indicate a clear separation between *H. itama* samples of Malay Peninsular and Borneo. This preliminary study suggests future studies to include more individuals with more localities, alternative loci and additional phylogeography analyses to detect and provide valid explanation on the *H. itama* systematics in Malaysia.

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REFERENCES

Abdul-Latiff, M.A.B., Ruslin, F., Faiq, H., Hairul, M.H., Rovie-Ryan, J.J., Abdul-Patah, P., Yaakop, S. & Md-Zain, B.M. 2014a. Continental monophyly and molecular divergence of Peninsular Malaysia's *Macaca fascicularis fascicularis. BioMed Research International* 2014: 897682.

Abdul-Latiff, M.A.B., Ruslin, F., Vun, V.F., Mohd-Hashim, A., Rovie-Ryan, J.J., Abdul-Patah, P., Lakim, M., Roos, C., Yaakop, S. & Md-Zain, B. M. 2014b. Phylogenetic relationships of Malaysia's long-tailed macaques, *Macaca fascicularis*, based on cytochrome *b* sequences. *ZooKeys* 407: 121-139.

Abdul-Latiff, M.A.B., Aifat, N.R., Yaakop, S. & Md-Zain, B.M. 2017. A noninvasive molecular approach: exploiting species-locus-specific PCR primers in defeating numts and DNA cross-contamination of cercopithecidae. *The Journal of Animal & Plant Sciences* 27: 1015-1023.

Aifat, N. R., Yaakop, S. & Md-Zain, B. M. 2016. Optimization of partial Cyt *b* gene sequence from selected ancient *Presbytis* museum skin specimens. *Malaysian Applied Biology* 45: 93-96.

Ang, K.C., Leow, J.W.H., Yeap, W.K., Hood, S., Mahani, M.C. & Md-Zain, B.M. 2011. Phylogenetic relationships of the Orang Asli and Iban of Malaysia based on maternal markers. *Genetics and Molecular Research* 10: 640-649.

Araújo, E.D., Costa, M., Chaud-Netto, J. & Fowler, H.G. 2004. Body size and flight distance in stingless bees (Hymenoptera: Meliponini): Inference of flight range and possible ecological implications. *Brazilian Journal of Biology* 64: 563-568.

Bandelt, H.J., Forster, P. & Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37-48.

Eltz, T., Bruhl, C.A., Imiyabir, Z. & Linsenmair, K.E. 2003. Nesting and nest trees of stingless bees (Apidae: Meliponini) in lowland dipterocarp forests in Sabah, Malaysia, with implications for forest management. *Forest Ecology and Management* 172: 301-313.

Ibrahim, N., Zakaria, A.J., Ismail, Z. & Mohd, K.S. 2016. Antibacterial and Phenolic Content of Propolis Produced by Two Malaysian Stingless Bees, *Heterotrigona itama* and *Geniotrigona thoracica*. *International Journal of Pharmacognosy and Phytochemical Research* 8: 156-161.

Kandil, O.M., Mahmoud, M.S., Allam, N.A. & Namaky, A.H. 2010. Mitochondrial Cytochrome C Oxidase Subunit 1 (*cox 1*) Gene Sequence of the *Hymenolepis* Species. *J. American Science* 6: 640-647.

Kelly, N., Farisya, M.S.N., Kumara, T.K. & Marcela, P. 2014. Species Diversity and External Nest Characteristics of Stingless Bees in Meliponiculture. *Tropical Agricultural Science* 37: 293-298.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33:1870-1874.

Lowe, A., Harris, S. & Ashton, P. 2004. Ecological genetics: design, analysis, and application. New Jersey: John Wiley & Sons.

Md-Zain, B.M., Abid-Kamal, S.N.A., Aifat, N.R., Abdul-Latiff, M.A.B., Mohd-Hashim, A., Ampeng, A., Yaakop, S. & Samat, A. 2018. Molecular identification of shark fins in Malaysian Borneo's local markets. *Biodiversitas* 19: 1035-1043.

Mohd Fahimee, J., Rosliza, J., Muhamad Razali, M. 2016. *Lebah Kelulut Malaysia – Biologi dan Penternakan*. Serdang: Penerbit Institut Penyelidikan dan Kemajuan Pertanian Malaysia (MARDI).

Momose, K., Yumoto, T., Natamitsu, T., Kato, M., Nagamasu, H., Sakai, S., Harrison, R.D., Itioka, T., Hamid, A.A. & Inoue, T. 1998. Pollination biology in a lowland dipterocarp forest in Sarawak, Malaysia. Characteristics of the plant-pollinator community in a lowland dipterocarp forest. *American Journal of Botany* 85: 1477-1501.

Nagamitsu, T., Momose, K., Inoue, T. & Roubik, D.W. 1999. Preference in flower visits and partitioning in pollen diets of stingless bees in an Asian tropical rain forest. *Researches on Population Ecology* 41: 195-202.

Nik-Rashidi, N.A.R. 2017. DNA barkod dan genetik populasi lebah kelulut (*Heterotrigona itama)* di Malaysia menggunakan jujukan mtDNA COI. Tesis SarjanaMuda. Universiti Kebangsaan Malaysia.

Pashley, D.P., Johnson, S.J. & Sparks, A.N. 1985. Genetic Population Structure of Migratory Moths: The Fall Armyworm (Lepidoptera: Noctuidae). *Annals of the Entomological Society of America* 78: 756-762.

Patwardhan, A., Ray, S. & Roy, A. 2014. Molecular markers in phylogenetic studies – a review. *Journal Phylogenetics and Evolutionary Biology* 2: 1-9.

Rasmussen, C. 2008. Catalog of the Indo-Malayan/Australasian stingless bees (Hymenoptera: Apidae: Meliponini). *Zootaxa* 1935:1-8.

Rosli, M.K.A., Zamzuriada, A.S., Syed-Shabthar, S.M.F., Mahani, M.C., Abas-Mazni, O. & Md-Zain, B.M. 2011a. Optimizing PCR Conditions to Amplify Cyt *b*, COI and 12S rRNA Gene Fragments of Malayan Gaur (*Bos gaurus hubbacki*) mtDNA. *Genetics and Molecular Research* 10: 2554-2568.

Rosli, M.K.A., Zakaria, S.S., Syed-Shabthar, S.M.F., Zainal, Z.Z., Shukor, M.N., Mahani, M. C., Abas-Mazni, O. & Md-Zain, B.M. 2011b. Phylogenetic relationships of Malayan gaur with other species of the genus *Bos* based on cytochrome *b* gene DNA sequences. *Genetics and Molecular Research* 10: 482-493.

Rosli, M.K.A., Syed-Shabthar, S.MF., Abdul-Patah, P., Abdul-Samad, Z., Abdul, S.N., Burhanuddin, M. N., Zulkifli, N. A., Shukor, M.N., Budsabong, K., Changtragoon, S., Sekiguchi, T., Sasaki, H. & Md-Zain, B. M. 2014. A New Subspecies Identification and Population Study of the Asian Small-Clawed Otter (*Aonyx cinereus*) in Malay Peninsula and Southern Thailand Based on Fecal DNA Method. *The ScientificWorld Journal* 2014: 457350.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E. & Sánchez-Gracia, A. 2017. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Molecular Biology and Evolution* 34: 3299-3302.

Roubik, D.W. 1989. *Ecology and Natural History of Tropical Bees*. Cambridge: Cambridge University Press.

Salim, H.M., Dzulkiply, A.D., Harrison, R.D., Fletcher, C., Kassim, A.R. & Potts, M.D. 2012. Stingless bee (Hymenoptera: Apidae: Meliponini) diversity in dipterocarp forest reserves in Peninsular Malaysia. *Raffles Bulletin of Zoology* 60: 213-219.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* 87: 651-701.

Wapples, R. 1987. A Multispecies Approach to the Analysis of Gene Flow in Marine Shore Fishes. *Evolution* 41: 385-400.