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Aedes albopictus in urban and forested areas of Malaysia: A study of mitochondrial sequence variation using the *CO1* marker

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Abstract. This study explores the use of a long fragment of the mitochondrial *cytochrome oxidase subunit 1* (*CO1*) marker to elucidate the genetic diversity of *Aedes albopictus* sampled from urban and forested regions in Peninsular and East Malaysia. A total of 36 samples were collected from 5 localities from which its genetic variability was analysed. 33 distinct mtDNA haplotypes were identified following the amplification and sequencing of the concatenated *CO1* gene. The analysed region of the *CO1* gene identified substantial levels of genetic diversity among mosquitoes in urban populations and revealed unique genealogical relationships between local isolates as revealed in the haplotype network. This study highlights the reliability of the long *CO1* fragment to identify genetic divergence of *Aedes albopictus* which can be utilized in forthcoming studies.

INTRODUCTION

Aedes albopictus (Skuse) (Diptera: Culicidae), commonly referred to as the "Asian tiger mosquito" has spread from its native range in Southeast Asia to various regions of the world (Gratz, 2004; Delatte *et al.*, 2011; Vaux & Medlock, 2015) thus posing great risks to human health. Besides exhibiting strong ecological plasticity and adaptability in a new niche (Paupy *et al.*, 2009; Bonizzoni *et al.*, 2013), this day-time biting mosquito is a competent vector for dengue viruses (CDC, 2012) and is also responsible for the emergence of several documented arboviral outbreaks, namely chikungunya (Reiter *et al.*, 2006; Vazeilla *et al.*, 2007; Pages *et al.*, 2009; Burt *et al.*, 2012), eastern equine encephalitis (Saxton-Shaw *et al.*, 2015), West Nile (Sardelis *et al.*, 2002), Japanese encephalitis (CDC, 2012), and Zika viruses (Wong *et al.*, 2013; Zanluca *et al.*, 2015) and also filarial nematodes.

Ae. albopictus can thrive in a wide range of habitats especially in densely populated urban and suburban areas (Gratz, 2004; Paupy *et al.*, 2009; Kamgang *et al.*, 2011; Medlock *et al.*, 2012). The magnitude of vector-related disease outbreaks has increased dramatically in the past years (Medlock *et al.*, 2012; Medlock *et al.*, 2015) due to the expansion of breeding sites of *Aedes* mosquitoes as a consequence of globalization and unplanned urban growth (Raharimalala *et al.*, 2012; Bonizzoni *et al.*, 2013; Yugavathy *et al.*, 2016) The persistence and progressive spread of

insecticide resistance and the ineffective implementation of suitable control strategies against the vector complicates the matter (Tantely *et al.*, 2010; Kawada *et al.*, 2010). Insecticide resistance (Vontas *et al.*, 2012; Marcombe *et al.*, 2014), vector competency (Lourenco de Oliveira *et al.*, 2013) and feeding preferences (Delatte *et al.*, 2010) of Aedes mosquitoes are influenced by its geographical and evolutionary origins (Ismail *et al.*, 2015; Manni *et al.*, 2015).

An exploration towards the genetic diversity of *Ae. albopictus* (Ismail *et al.*, 2015; Adilah-Amrannudin *et al.*, 2016; Yugavathy *et al.*, 2016) is of paramount importance in unraveling its evolutionary origins (Hewitt, 1983), genealogical relationships (Birungi and Munstermann, 2002; Maia *et al.*, 2009; Zitko *et al.*, 2011; Navarro *et al.*, 2013, Zawani *et al.*, 2014; Ismail *et al.*, 2015; Adilah-Amrannudin *et al.*, 2016; Yugavathy *et al.*, 2016) and to aid novel strategies in controlling disease transmission (Ayres *et al.*, 2013; Raharimalala *et al.*, 2012). Genetic divergence in mosquitoes and its dispersal can be investigated by using the maternally inherited gene of the mitochondrial DNA (Khambhampati & Rai, 1991; Kambhampati, 1995; Tang *et al.*, 1996; Brelsfoard & Dobson, 2012). Specifically, the *cytochrome oxidase subunit 1 (CO1)* has been used in the past to study population genetics (Zawani *et al.*, 2014; Ismail *et al.*, 2015; Yugavathy *et al.*, 2016) due to its robustness and polymorphic features (Hebert *et al.*, 2003; Derycke *et al.*, 2010). Previous studies have used the short partial *CO1* gene to reveal phylogeography and evolutionary origins of *Ae. albopictus* (Mousson *et al.*, 2005; Kamgang *et al.*, 2011; Poretta *et al.*, 2012; Raharimalala *et al.*, 2013; Zhong *et al.*, 2013; Ismail *et al.*, 2015). Nevertheless, studies that utilize a longer fragment of the *CO1* gene that can reveal more meaningful information in terms of its genetic constituents and diversity (Goubert *et al.*, 2016) of *Ae. albopictus* isolated from the different environmental population is currently lacking and forms the basis of this study.

MATERIALS AND METHODS

Sample sites and collection method

Mosquito eggs were collected using ovitraps set at 5 localities within two regions in Peninsular Malaysia (Fig. 1) that represented forested and urban areas between October 2013 and December 2015. The sampling localities and geographical coordinates are described in Table 1. Collection of *Ae. albopictus* were primarily conducted in the urban state of Selangor in the western central region of peninsular Malaysia that represented dengue cluster areas based on its high duration, intensity, and frequency indices as described by Dom *et al.* (2013). For forested areas, specimens were collected from Perak, northern regions of Malay Peninsula and Sabah, Malaysian Borneo. In addition, the laboratory strain of *Ae. albopictus* (F135) from Vector Control Research Unit, Universiti Sains Malaysia (USM) was obtained and reared. The colonies of *Ae. albopictus* were derived from a <25-year-old laboratory colony established from wild pupae that originated from USM Penang field areas. Eggs were hatched into larvae and reared into adulthood under insectary conditions, with temperature and relative humidity maintained at 28±2°C and 70±10%, respectively. The photoperiod condition was maintained with 12h of light and 12h of darkness (12:12) as described by Gerberg (1970). Adult mosquitoes were morphologically identified as *Ae. albopictus* based on the Centre for Disease Control and Prevention pictorial keys (Stojanovich and Scott, 1965).

Genomic DNA extraction

Total genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Extracted DNA was stored at -20°C until required.

PCR amplification and sequencing

Partial sequences of the *CO1* mitochondrial gene was amplified to examine sequence

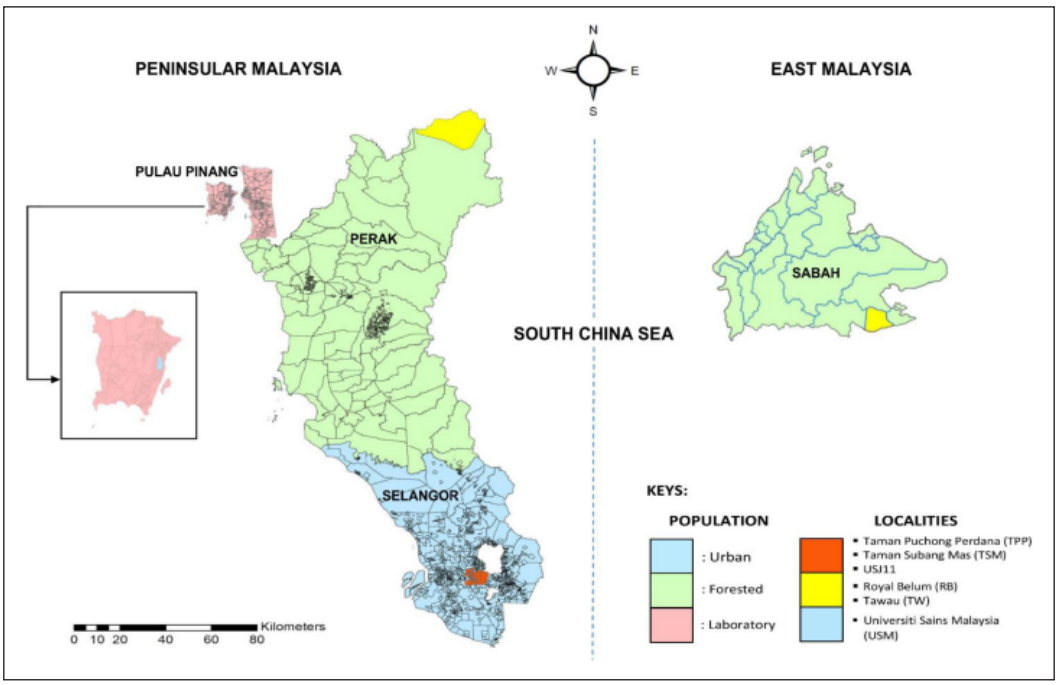

Figure 1. Mosquito collection sites in the 5 localities that consisted of different environmental areas in Peninsular and East Malaysia.

Table 1. Sample localities, regions and geographic coordinates for 5 sampled populations and labstrain of *Ae. albopictus*

Population	Locality	State	Region	Geographic coordinates
Urban	Taman Puchong Perdana (TPP)	Selangor	West Central (Peninsular Malaysia)	3°00'26.6"N 101°36'05.8"E
	USJ 11	Selangor	West Central (Peninsular Malaysia)	3°02'26.0"N 101°34'43.8"E
	Taman Subang Mas (TSM)	Selangor	West Central (Peninsular Malaysia)	3°03'14.4"N 101°33'26.8"E
Forested	Royal Belum (RB)	Perak	Northern (Peninsular Malaysia)	5°47'9.93"N 101°30'51.44"E
	Tawau (TW)	Sabah	Southern (East Malaysia)	4°14'40.74"N 117°53'28.27"E
Laboratory	Universiti Sains Malaysia (USM)	Pulau Pinang	Northern (Peninsular Malaysia)	5°22'8.82"N 100°18'34.92"E

polymorphism among mosquito samples using conventional polymerase chain reaction (PCR). Primers were adapted from Zhong *et al.* (2013) and Porretta *et al.* (2012) and synthesized commercially. A total of 25µl reaction mix containing 100ng DNA template, PCRBIO Taq DNA polymerase, 10µM primers, 6mM $MgCl_2$, and 2mM dNTPs was used in each PCR, and amplification was performed in a A6 Thermal Cycler (NYX Technik, Inc., USA). The PCR cycling conditions consisted of an initial denaturation step at 95°C for 1 min, followed by 40 cycles at 95°C for 15 sec, 55°C for 15 sec, 72°C for 1 min, with a final extension at 72°C for 1 min. The PCR products of the

mtDNA *CO1* were subjected to electrophoretic separation and visualized on 1.5% (w/v) agarose gels prestained with RedSafe™ Nucleic Acid Staining Solution (INTRON Biotechnology, Korea). The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced in an automated Abi 3730xl Big Dye Terminator version 3.1 cycler (Applied Biosystems, Foster City, CA).

_CO1_ sequence concatenation

All three sequence fragments of the *CO1* gene from each individual mosquito were aligned and manually concatenated using BioEdit v7.2.5 software to obtain the full coverage of a single sequence (Hall, 1999). The overlapped upstream and downstream sequences from each fragment, with corresponding characters were connected to construct the sequence for a longer fragment for each sample (Zhong *et al.*, 2013; Futami *et al.*, 2015) and compared with published sequences in the GenBank database using Basic Local Alignment Search Tool (BLAST).

Genetic diversity analysis

The concatenated sequences from TPP, USJ11, TSM, RB, TW and USM laboratory strains were aligned and edited using CHROMAS v.2.5.1 and CLUSTAL X v2.1 (Thompson *et al.*, 1997). Indices of sequence statistics including the number of haplotypes (h), polymorphic sites (#), haplotype diversity (Hd), nucleotide diversity (ð) and the average number of nucleotide differences (k) were computed with DnaSP v.5.10.1 software (Rozas, 2009). Neutrality test based on Tajima's D (D) and Fu's *Fs* (*Fs*) statistics were conducted with DnaSP (Rozas, 2009) to ascertain the neutrality in sample selection as well as in the detection of evolutionary forces (Fu, 1997).

Genetic distance

Average of pairwise genetic distance between populations of *Ae. albopictus* were estimated based on p-distance method by using Molecular Evolutionary Genetic Analysis v6.0 (MEGA6) software to assess the genetic relatedness among the sampled-populations.

Haplotype network

A haplotype network was constructed using Population Analysis with Reticulate Trees (PopART) (http://popart.otago.ac.nz/index.shtml) based on concatenated sequences of *CO1* genes to assess the genealogical relationship of *Ae. albopictus* mitochondrial haplotypes. Haplotypes were connected from the shortest to the longest distance until all the haplotypes were integrated completely. Population divergence for probability value was constructed using the TCS networks within the PopART software.

RESULTS

Concatenated CO1 sequence

The concatenation of three fragments of short *CO1* sequences of *Ae. albopictus* yielded 1516 nucleotides of a single long fragment that covered most of the coding DNA sequence (cds) of partial *CO1* sequence, 1537-base-pair (bp) in complete mitochondrion genome of *Ae. albopictus* as illustrated in Fig. 2.

Figure 2. Comparison of the different mtDNA *CO1* regions amplified for genetic diversity and phylogeographic studies of *Ae. albopictus*, which is indicated by different colours. The blue line represents the linear sequence of the *CO1* gene (numbers are the number of base pairs from the origin (NCBI sequence NC_006817.1)). The green and yellow lines show the partial *CO1* fragments used for gene concatenation. The red line is the long concatenated *CO1* sequence utilized in this study.

Genetic diversity

The alignment of the 1516 bp concatenated *CO1* gene sequences of 46 individual local *Ae. albopictus* revealed 306 polymorphic sites (Fig. 3). Analysis of the *CO1* sequences showed a nucleotide diversity, $\pi = 0.05030$ and haplotype diversity of Hd = 0.975. The average number of nucleotide differences among individual mosquito was 76.25797. In total, 33 haplotypes were identified and designated as H1-H33 (Table 2). The overall value for D and *Fs* was -0.31873 and 2.340, respectively. Interpopulation analysis of the urban, forested and laboratory sampled *Ae. albopictus* sequences were performed to compare the genetic diversity between different environments. The 1516 bp of 6 sequences that represent per population (randomly selected from 46 sequences) were aligned and revealed genetic diversity's statistic indices as summarized in Table 3. The urban-sampled population had the greatest genetic diversity compared to others.

Genetic distance

Average of pairwise genetic distance between sampled-populations of *Ae. albopictus* are documented in Table 4. Sampled-population with the value of genetic distance that is close to 0.00 indicates the low genetic distance or approach towards similar genetic constituents. The overall average of all sampled-populations is 0.05395.

Haplotype network analysis

The genealogical network of the 33 haplotypes is shown in Fig. 4. The haplotype network revealed several distinct groups, designated as Groups 1 and 2. These distinct groups of haplotypes are separated by several mutational events as well as extinct haplotypes, and are likely to

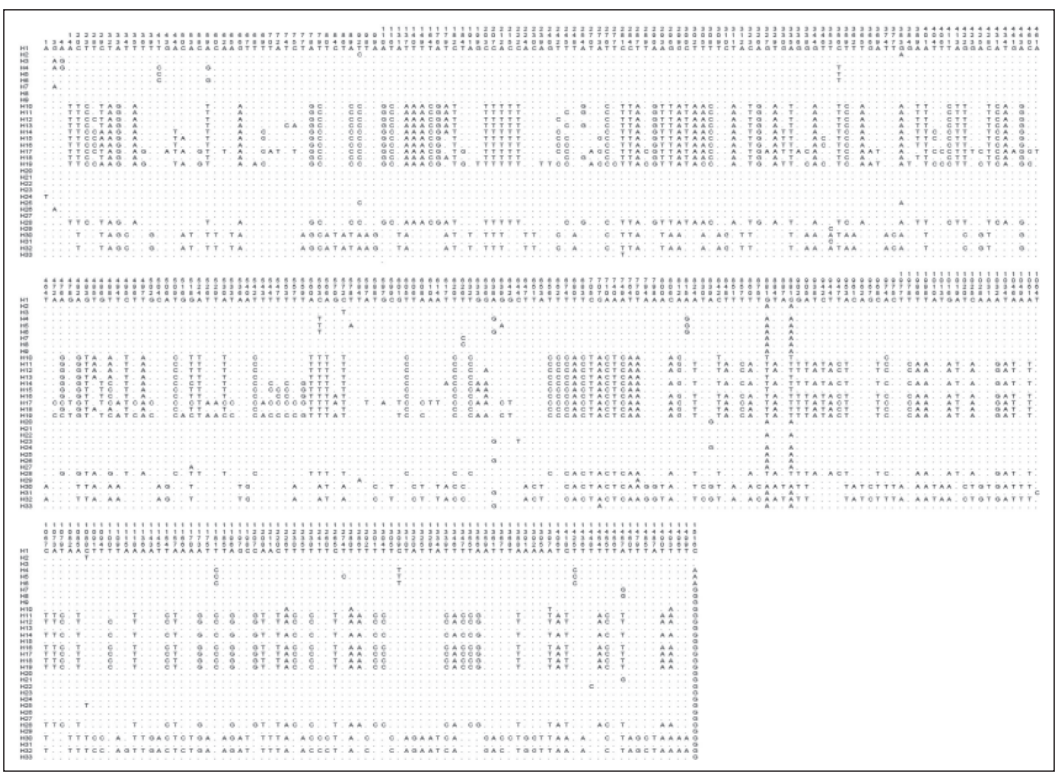

Figure 3. Polymorphism sites of *cytochrome oxidase subunit 1* gene alignments documented among haplotype (H1-H33) sequences from this study. Numbers at the top of the figure denote polymorphic sites found at nucleotide positions 1-1516. Dots represent nucleotides that were similar to the reference sequence.

Table 2. Haplotypes of local *Ae. albopictus* sequences based on the partial *cytochrome oxidase subunit 1 (CO1)* marker

Haplotype	No.[1]	Location	Specimens	Accession no.
H1	1	Royal Belum	RB_1	KY982334
H2	1	Royal Belum	RB_2	KY982335
H3	1	Royal Belum	RB_3	KY982336
H4	1	Tawau	TW_1	KY982337
H5	1	Tawau	TW_2	KY982338
H6	1	Tawau	TW_3	KY982339
H7	3	USM Labstrain	LS_1, LS_4, LS_5	KY982340
H8	5	USM Labstrain	LS_2, LS_7, LS_8,	KY982341
H9	4	USM Labstrain	LS_3, LS_6, USJ11_3, TSM_5	KY982342
H10	1	Taman Puchong Perdana	TPP_1	KY982343
H11	1	Taman Puchong Perdana	TPP_2	KY982344
H12	1	Taman Puchong Perdana	TPP_3	KY982345
H13	1	Taman Puchong Perdana	TPP_4	KY982346
H14	1	Taman Puchong Perdana	TPP_5	KY982347
H15	1	Taman Puchong Perdana	TPP_6	KY982348
H16	1	Taman Puchong Perdana	TPP_7	KY982349
H17	1	Taman Puchong Perdana	TPP_8	KY982350
H18	1	Taman Puchong Perdana	TPP_9	KY982351
H19	1	Taman Puchong Perdana	TPP_10	KY982352
H20	4	USJ11	USJ11_1, USJ11_7, TSM_2, TSM_10	KY982353
H21	1	USJ11	USJ11_2	KY982354
H22	1	USJ11	USJ11_4	KY982355
H23	1	USJ11	USJ11_5	KY982356
H24	1	USJ11	USJ11_6	KY982357
H25	1	USJ11	USJ11_8	KY982358
H26	1	USJ11	USJ11_9	KY982359
H27	1	USJ11	USJ11_10	KY982360
H28	1	Taman Subang Mas	TSM_1	KY982361
H29	1	Taman Subang Mas	TSM_3	KY982362
H30	1	Taman Subang Mas	TSM_4	KY982363
H31	2	Taman Subang Mas	TSM_6	KY982364
H32	1	Taman Subang Mas	TSM_8	KY982365
H33	1	Taman Subang Mas	TSM_9	KY982366

[1] Number of sequences per haplotype.

Table 3. Summary statistics of *CO1* gene diversity in *Ae. albopictus*

Population	Urban	Forested	Laboratory	*P*
Sample size, n	6	6	6	–
Nucleotide diversity, π	0.09332	0.00646	0.00110	–
Number of haplotypes, h	6	6	3	–
Haplotype diversity, Hd	1.000	1.000	0.733	–
Average number of nucleotide difference, k	141.46667	9.80000	1.66667	–
Tajima's D	1.07975	0.74242	1.38606	Not significant
Fu's *Fs*	2.204	-0.988	0.688	Not significant
Presence of mutation	Yes	No	No	–

P>0.10; Not significant.

Table 4. Average of pairwise genetic distance matrix between sampled-populations of *Ae. albopictus* based on *CO1* gene

Population	Forested	Laboratory	Urban
Forested (n=6)	0.00000		
Laboratory (n=6)	0.00579	0.00000	
Urban (n=6)	0.07611	0.07333	0.00000

n; sample size of each population. Bold; the average of genetic distance between same populations.

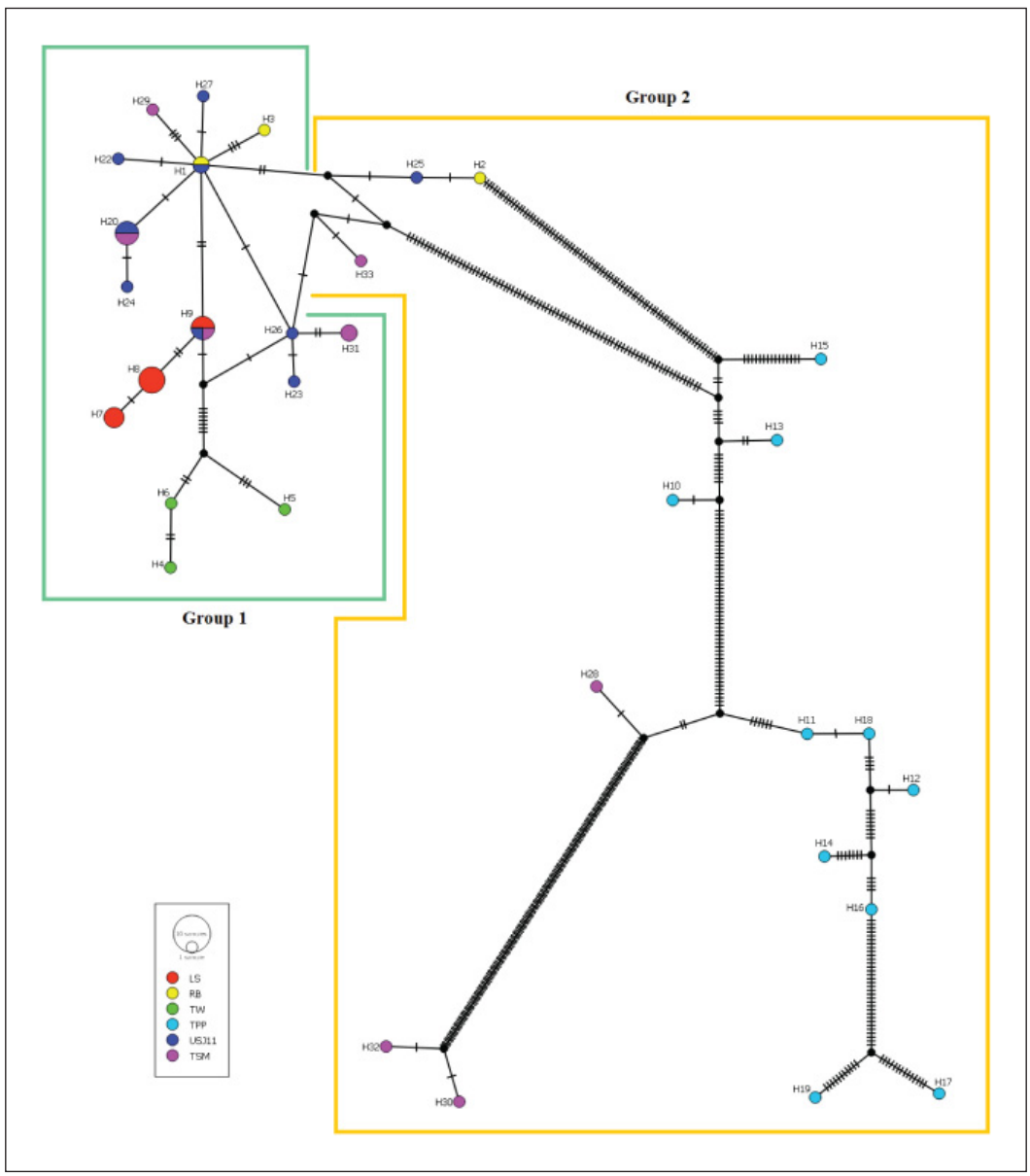

Figure 4. Genealogical network showing the relationship among 33 *CO1* haplotypes of *Ae. albopictus*. The diameter of the circle is proportional to the number of individual sequences in each connection. The perpendicular bars along the branches revealed the number of mutational events.

represent at least 2 separate lineages of *Ae. albopictus* in the sampled population. Group 1 is composed of the most common haplotype (H9). Notably, the level of genetic diversity within Group 1 is low as it is the only descendant from H1, H9 and H26, which are very closely related haplotypes and are only separated by a few mutational steps. On the other hand, Group 2 which consisted of haplotypes H10, H11, H12, H13, H14, H15, H16, H17, H18, H19, H25, H28, H30, H32 and H33 demonstrated a more complex evolutionary pattern, with numerous mutational steps. Interestingly, most of them are from the urban-sampled population and all samples from TPP are included in this group (H10-H19). Group 2 had a higher diversity.

DISCUSSION

Genetic heterogeneity and geographical distribution of mosquitoes can lead to genetic drift, concomitantly affecting their phenotypic variations (Delatte *et al.*, 2011; Tabachnick, 2013). Decrypting the genetic diversity among *Ae. albopictus* population in different environments, particularly in a tropical country such as Malaysia is pivotal in providing a better understanding of its ecology and intra- and inter-population relationships.

The present study utilized a long partial *CO1* fragment to characterize the genetic variation of *Ae. albopictus* in different environments, namely urban and forested regions in comparison to the laboratory strain. Our findings revealed high variable sites in the analysed *CO1* gene. Utilizing a longer partial *CO1* gene enabled a more holistic analysis of the genetic diversity and genealogical relationships of a species (Goubert *et al.*, 2016). The study conducted by Futami *et al.* (2015) supports the fact empirically by discovering five new haplotypes with high nucleotide diversity of *Ae. albopictus* from the Costa Rica population by utilizing a long *CO1* gene (1390 bp) as a marker. In addition, the *CO1* gene is a phylogenetic informative marker (Avise, 1994; Arif *et al.*, 2012) making it a reliable platform for exploring genetic variation in *Ae. albopictus*. This notion is also reflected by the high nucleotide diversity and the greater number of haplotypes obtained which can be attributed to the longer fragment of the *CO1* gene that was amplified and sequenced (>95% of the entire *CO1* gene in length, i.e., 1516 of 1537 bp). This outcome is also consistent with the findings of Zhong *et al.* (2013) and Futami *et al.* (2015), who revealed greater insights in terms of genetic polymorphism when using longer fragments of the *CO1* gene (1433 bp and 1390 bp, respectively) in *Ae. albopictus*. However, limitation of this gene has also documented in other species such as *Culex* spp. A separate study by Low *et al.* (2014) reported dissimilar findings in relation to low genetic variation in *Culex quinquefasciatus* from residential areas in Malaysia based on the *CO1* gene (624 bp). Such study could be furthered explored by using a longer gene of interest which will is more comprehensive in revealing additional variable sites and haplotypes.

Environmental factors such as types of breeding sites, habitat preferences, human settlements, and the use of insecticides may influence the distribution of *Ae. albopictus* (Kamgang *et al.*, 2013) giving rise to genetic variation as described by Paupy *et al.* (2004). In general, our findings agree with similar studies conducted in other countries (Poretta *et al.*, 2012; Zhong *et al.*, 2013; Ismail *et al.*, 2015; Yugavathy *et al.*, 2016). Our results indicated high levels of genetic diversity in *Ae. albopictus* from different environments especially in urban population. Our locality included dengue cluster areas (Dom *et al.*, 2013) with high human activities and movement that may have resulted in multiple introduction of Aedes mosquitoes from various sources (Zitko *et al.*, 2011) giving rise to genetically distinct population to maintain its existence in nature (Dlugosch & Parker, 2008). This finding could also be associated with phenotypic variations within a species that makes it susceptible to arbovirus infection as described in previous studies (Chepkorir *et al.*, 2014; Goncalves *et al.*, 2014). This nature vs nurture phenomenon

influencing mosquito competence for arbovirus infection is a subject of interest that requires further exploration.

In this study, contrasting patterns of genetic diversity were seen in *Ae. albopictus* from different environmental settings. *Ae. albopictus* is natively a sylvatic mosquito (Gratz, 2004), thus high genetic variation of this species in forested-population was anticipated (Frankham, 1995). Nevertheless, genetic variation could be impeded by several factors, such as deforestation, small population size, migration of native population and large clonal of the species population, resulting in genetic drift and bottleneck effect (Frankham, 1995; Gaublomme *et al.*, 2013; Razak *et al.*, 2016). The low genetic variation observed among *Ae. albopictus* in the forested areas shown in this study may display the evidence of the successful invasion and adaptation of a species in a new favourable niche (Prentis *et al.*, 2008). This is supported by studies conducted by Stout *et al.* (2014), which stated that bottleneck effects and genetic drifts in small colonizing areas may result in low genetic diversity incidences. In addition, low genetic diversity and heterogeneity were also observed among laboratory-adapted strains of *Ae. albopictus*, indicating that a certain amount of inbreeding or selection has occurred in the laboratory-colony population (Bush, 1975). Comparable findings were also documented by Poretta *et al.* (2012).

The genetic distance based on the *CO1* gene of *Ae. albopictus* from different types of environmental populations ranged from 0.00579 to 0.07611. A genetic distance of urban population that is comparable to forested population indicates the high genetic divergence which is associated with high geographic distance of sampling locations between these populations (Sousa *et al.*, 2017). However, the evidence of significant correlations could be further analysed with a greater sample size. Meanwhile, a relatively low genetic distance between *Ae. albopictus* colonized in the laboratory from the wild population is more likely to be attributable to the low genetic differentiation in laboratory environment regardless of the longer gap of sampling period of F0 generation of *Ae. albopictus* labstrain that was performed 20 years ago. Nevertheless, the genetic divergence of *Ae. albopictus* from disparate wild populations might have been explicably higher if sampling had been conducted over a longer period of time, and this speculation can only be verified by conducting additional sampling efforts with greater sample size over a longer duration in Malaysia.

Haplotype networks is a visualization tool that was employed in this study to envisage relationships between individual genotypes at the population level. Analysis of the network indicates the presence of two major lineages of *Ae. albopictus*. The haplotype distribution in Group 1 consisted of all the three-sampled population with a few mutational events which signifies low diversity. The presence of a single predominant haplotype (H9) with low diversity is possibly an indication of either bottleneck effect, founder effect or inbreeding among closely related species. Most of the forested and all laboratory sampled population were assembled in Group 1. Despite its low diversity, Tawau sampled population were descendants from two extinct haplotypes, in which DNA lineages went extinct by generation 10 (Templeton, 2005) due to several mutational events. Meanwhile, Group 2 haplotype distribution was separated from H1 and H26 in Group 1, and comprised of local haplotypes that diverged from the extinct haplotype and H2 in a common ancestor, making it unique compared to other groups. Interestingly, all sequences in TPP were clustered in this group. TPP is a highly populated urban area with active public and commercial transportation, which could indirectly affect mosquito ecology, distribution and genetic makeup as reported by Huber *et al.* (2004). Mutations at high frequency as displayed in the haplotype network suggests that the sampled population in this group had undergone evolution to challenge insecticide-based interventions and other types of human-induced stress (Ngoagouni *et al.*, 2015). Moreover, there is mounting evidence to suggest that the use of pesticides often leads to the adaption of organisms to evolutionary challenges giving rise to an adaptive genetic

variation. In these instances, adaptive alleles sweep through the population at the same time, either because the alleles were present as standing genetic variation or arose independently by *de novo* mutations (Messer & Petrov, 2013).

CONCLUSIONS

This preliminary study reports the genetic variability and genealogical relationship of *Ae. albopictus* collected from different environments based on the *CO1* genetic marker. It is hoped that the findings from this study would supplement the existing body of knowledge regarding the genetic diversity of *Ae. albopictus* in an effort to better understand the invasive potential of this vector, which could in turn aid the development of strategies targeted to impede its invasion and spread. To corroborate our findings, it is recommended that an ongoing assessment of the population genetic structure of field and colony populations of *Ae. albopictus* be done using a larger sample size.

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