



Original article

Population structure and genetic diversity of *Aedes aegypti* and *Aedes albopictus* in Penang as revealed by mitochondrial DNA cytochrome oxidase I

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ABSTRACT

The population genetics study is crucial as it helps in understanding the epidemiological aspects of dengue and help improving a vector control measures. This research aims to investigate the population genetics structure of two common species of *Aedes* mosquitoes in Penang; *Aedes aegypti* and *Aedes albopictus* using Cytochrome Oxidase I (COI) mitochondrial DNA (mtDNA) marker. Molecular investigations were derived from 440 bp and 418 bp mtDNA COI on 125 and 334 larvae of *Aedes aegypti* and *Aedes albopictus* respectively, from 32 locations in Penang. All samples were employed in the BLASTn for species identification. The haplotype diversity, nucleotide diversity, neutrality test and mismatch distribution analysis were conducted in DnaSP version 5.10.1. AMOVA analysis was conducted in ARLEQUIN version 3.5 and the phylogenetic reconstructions based on maximum likelihood (ML) and neighbor-joining (NJ) methods were implemented in MEGA X. The relationships among haplotypes were further tested by creating a minimum spanning tree using Network version 4.6.1. All samples were genetically identified and clustered into six distinct species. Among the species, *Ae. albopictus* was the most abundant (67.2%), followed by *Ae. aegypti* (25.2%) and the rest were counted for *Culex* sp. and *Toxorhynchites* sp. Both *Ae. aegypti* and *Ae. albopictus* show low nucleotide diversity (π) and high haplotype diversity (h), while the neutrality test shows a negative value in most of the population for both species. There are a total of 39 and 64 haplotypes recorded for *Ae. aegypti* and *Ae. albopictus* respectively. AMOVA analysis revealed that most of the variation occurred within population for both species. Mismatch distribution analysis showed bimodal characteristic of population differentiation for *Ae. aegypti* but *Ae. albopictus* showed unimodal characteristics of population differentiation. Genetic distance based on Tamura-Nei parameter showed low genetic divergent within population and high genetic divergent among population for both species. The maximum likelihood tree showed no obvious pattern of population genetic structure for both *Ae. aegypti* and *Ae. albopictus* from Penang and a moderate to high bootstrap values has supported this conclusion. The minimum spanning network for *Ae. aegypti* and *Ae. albopictus* showed five and three dominant haplotypes respectively, which indicates a mixture of haplotypes from the regions analysed. This study revealed that there is no population genetic structure exhibited by both *Ae. aegypti* and *Ae. albopictus* in Penang. Mutation has occurred rapidly in both species and this will be challenging in controlling the populations. However, further analysis needed to confirm this statement. © 2020 The Authors. Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Molecular phylogeny and population genetics study can divulge evidence of past biogeographic events and suggest life history traits that contribute to shape the distribution of genetic variation among populations (Avisé, 2000). Such studies give information on genetic variation and by applying genetic model, one can make inferences about the biology of organisms (Sunnucks, 2000). Population genetics is a study of evolution and it uses a well-developed

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and ever-growing body of theoretical knowledge that allows quantitative predictions (Cavalli-Sforza, 1998). The field of population genetics is generating a great progress in recent years. However, relatively only few studies have focused on understanding the patterns of population genetics structure of *Aedes* species (Gupta and Preet, 2014) especially in Malaysia.

Mosquito within the genus *Aedes* belongs to Family Culicidae from Order Diptera. There are approximately 3500 species of mosquitoes occupy almost every continent in the world (Alshehri, 2013). Some species of mosquitoes are very dangerous and caused mosquito-borne diseases worldwide. They affect both children and adolescents, hence increase mortality rate worldwide. For example, malaria kills more than one million children every year, mostly in sub-Saharan Africa while Japanese encephalitis has expanded its widespread in the Indian subcontinent and Australasia, thus has raised a serious concern (Tolle, 2009). Another alarming mosquito-borne disease nowadays, especially in Southeast Asia is dengue fever, which has expanded its range over the past several decades (Tolle, 2009). It has become one of the most significant mosquito-borne viral diseases found in humans and is a leading cause of childhood mortality in many countries in the world (Alshehri, 2013).

Dengue fever caused the highest mortality threat due to viral infection in more than half of the world's population (Goswami et al., 2012). The World Health Organization (WHO) estimates that more than 2.5 billion people are at risk of dengue infection with 50 to 100 million dengue infections worldwide every year (WHO, 2014). Dengue fever and dengue hemorrhagic fever are caused by the four viral serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) (Goswami et al., 2012). Dengue is transmitted from viraemic to susceptible humans mainly by the bites of *Aedes aegypti* and *Aedes albopictus* (Guha-Sapir and Schimmer, 2005). *Aedes albopictus* is generally believed to be a less efficient vector of arboviruses than *Ae. aegypti*, the most important vector of dengue because it is not well adapted to urban domestic environments and is less anthropophilic than *Ae. aegypti*. However, a rapid change in its overall distribution made the species becoming more important vector in dengue outbreaks (Giovanni, 2012). In the Central America, *Ae. albopictus* is now replaced *Ae. aegypti* as the dominant species at the periphery of urban centers (Kamgang et al., 2010). In Hawaii, this species is now described as ubiquitous and has been the major vector for several dengue fever outbreaks (Effler et al., 2005).

The study of population genetics could provide significant information on the dispersal and population dynamics of a species (Gupta and Preet, 2014). In this context, genetic polymorphisms transmitted in strict mendelian fashion give useful information where the use of available markers is the key to the analysis (Cavalli-Sforza, 1998). Both *Ae. aegypti* and *Ae. albopictus* have brought considerable interest in multiple researches such as vector competence, insecticide resistance, ecological and evolutionary studies, spatial, temporal and geographical analyses and population genetics study (Kaplan et al., 2010). To date, research in Malaysia has focused on the distribution and abundance (Wan-Norafikah et al., 2012; Rozilawati et al., 2007), ecology and biology of *Aedes* mosquitoes (Sivanathan, 2006; Nur Aida et al., 2011), mixed breeding (Chen et al., 2006) and genetic engineering (Lacroix et al., 2012). The population genetics study is important as it helps in understanding the epidemiological aspects of dengue and help improving the vector control measures, primarily the genetic control, to prevent or reduce the epidemic impacts in Malaysia. This research aims to investigate the population genetics structure of two common species of *Aedes* mosquitoes in Penang namely *Aedes aegypti* and *Aedes albopictus* using Cytochrome Oxidase I mitochondrial DNA (mtDNA) marker.

2. Materials and methods

2.1. Sample collections

All mosquito samples in a form of instar (4th instar) were obtained from 31 locations that represents four zones in Penang namely North Seberang Perai (NSP), Central Seberang Perai (CSP), South West (SW) and North East (NE) (Fig. 1 and Supplementary Material 1). Sampling localities are shown in Table 1. The samples were gathered applying ovitrap and empty containers and/or cans during the year 2012–2014. There are 20 ovitraps were placed in each location for consecutively five days every month before collection and transported to the laboratory for further analysis. The ovitraps were left at bushy and housing area. In the laboratory, all specimens were stored in a sterile microcentrifuge tube contains 75% alcohol prior DNA extraction. The larvae of both *Ae. aegypti* and *Ae. albopictus* could be distinguished using morphological characteristics by looking at the abdomen and head (comb scale, setae and siphon) as described by Chung et al. (1997). Nevertheless, due to the size of larvae that is very tiny and some larvae have been broken during preservation in an alcohol, identification of mosquitoes were conducted based on genetic characteristics.

2.2. DNA isolation

The method of salt extraction (Aljanabi and Martinez, 1997) was used in DNA extraction for all samples. In summary, each sample was homogenated and placed into a sterile microcentrifuge tube (2 ml) contains approximately 400 μ l of TNES Urea and 10 μ l of proteinase-K. Then, the mixture was left for 18 h (overnight) inside an incubator that set to 60 °C. Approximately 100 μ l of 5 M NaCl was then added into the mixture prior to centrifuge for 6 min at 13000 rpm. Then, approximately 200 μ l of the mixture was removed and added into another 2 ml centrifuge tube. Then, 350 μ l of cold EtOH (ethanol) was poured in all microcentrifuge tube prior to flip over several times to well homogenate and mix the solution. All samples were recentrifuged at 13000 rpm for 30 min after being incubated for an hour at –16 °C. The specimens were then processed until the moisture less/dried DNA pellet was observed prior to mix in 150 μ l of TE (Tris-EDTA) and preserved at –20 °C.

2.3. PCR-amplified samples and DNA sequencing

The target position of mtDNA COI was amplified using Polymerase Chain Reaction (PCR) and the reaction mixtures contained 1.2 dNTP mix (1.6 mM/ μ l), 5.0 μ l of 10x buffer, 0.3 μ l of 2U DNA polymerase i-Taq+ (Intron, Korea), 5.0 μ l Magnesium Chloride (20 mM/ μ l), 1.5 μ l of each reverse and forward primers, 2.5 μ l of genomic DNA templates and distilled ddH₂O in 50 μ l of final volume. Probability of contamination was detected using negative control in all samples. The PCR condition comprises of 3 min initial denaturation at 96 °C followed by 35X (95 °C for 46 s, 45.5 °C for 50 s, 70 °C for a min with a final elongation at 70 °C for 10 min), employed in the BIORAD (USA) thermal cycler. All specimens were subject for electrophoresis in 1.8% agarose gel contained of EtBr (ethidium bromide). The pair of primers developed by Bonacum et al. (2001) was used to amplify the COI gene; C1-J-1718-5'-GGAG GATTGGAAATTGATTAGTTC-3' and C1-N-2191-5' CCCGGTAAAT-TAAAATATAAACTTC-3'. All samples were purified following protocol developed by the iNtRON Biotechnology (Korea). Approximately 30 μ l of the cleaned PCR products were selected and sent out to NHK Bioscience (Korea) for sequencing process.

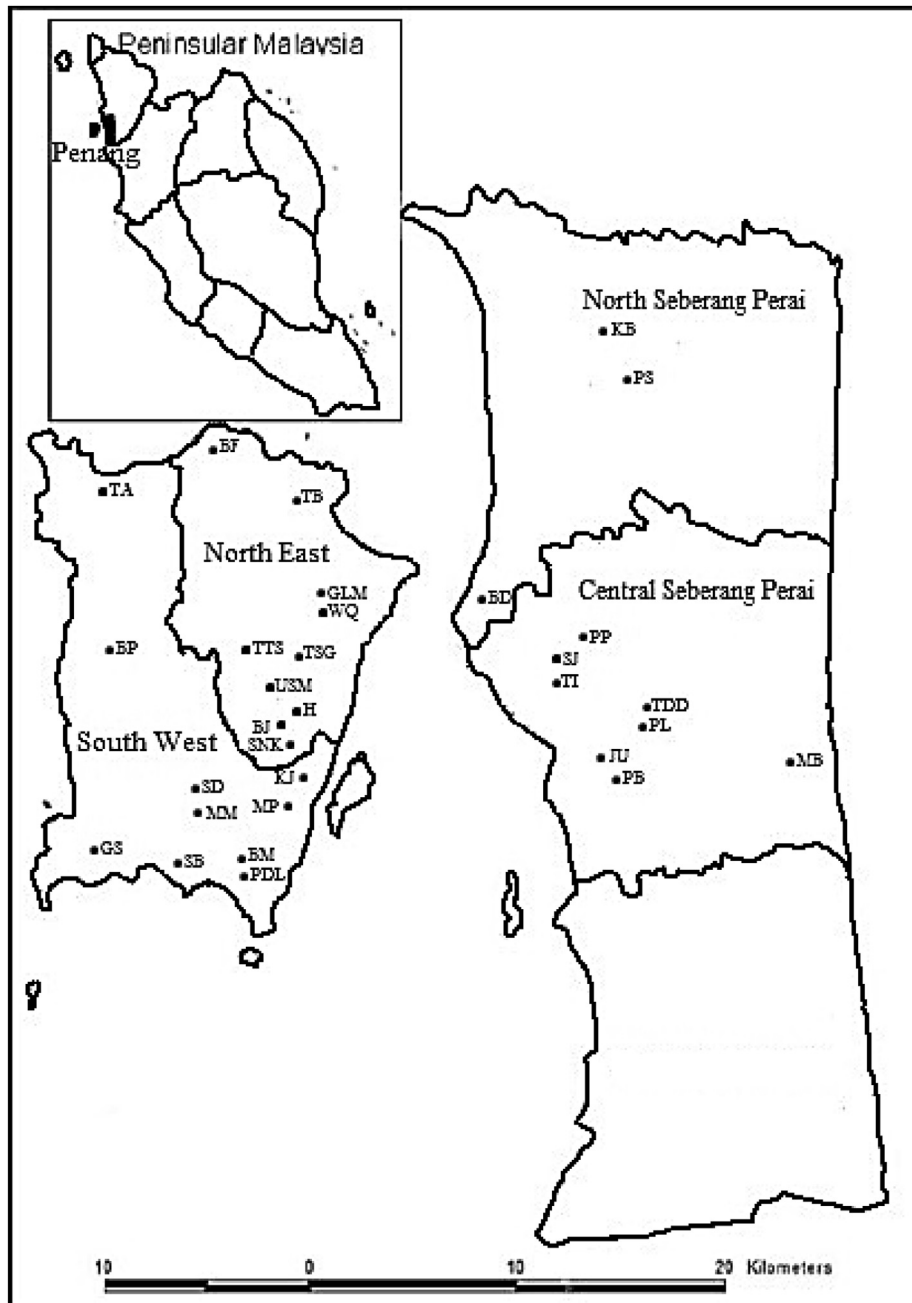


Fig. 1. Sampling localities of mosquitoes' populations analyzed in the present study. See Table 1 for sampling site abbreviation.

2.4. Species identification

The amplified samples were employed in the GenBank database based on the BLAST algorithm with all available respective genes of the similar species and employed in BLASTn (<http://blast.ncbi.nlm.nih.gov>). The previously deposited sequence of genus *Aedes* in GenBank was also used as a reference in this study. All sequences were aligned automatically and implemented in the Collapse version 1.2 (Provan et al., 2005). All sequences were then rearranged using MUSCLE (Edgar, 2004) with default parameters and all aligned haplotypes were then synchronized in MEGA X (Kumar et al., 2018). The sequence arrangement was then manually reassessed in an attempt to minimize the positional dissimilarity. All missing data and gaps within the sequences were removed.

2.5. Population genetics and differentiation

The aligned sequences were exported to DnaSP version 5.10.1 (Librado & Rozas, 2009) program to compute the nucleotide variable sites, conserve sites and parsimony informative sites. Haplotype diversity (h), nucleotide diversity (π) and neutrality test (Tajima's D and Fu's F_s) were computed using DnaSP version 5.10.1 program (Librado and Rozas, 2009). Tajima's D uses the information on mutation frequency (segregating sites) to detect deviation from neutrality due to population bottleneck or expansion, directional selection or introgression. A positive value of Tajima's D suggests balancing selection or population sub-structuring or recent population bottleneck whereas negative values suggest a recent directional selection (selection sweep) or recent population

Table 1
Sampling locations, number of *Aedes* mosquitoes individual (N) and the maximum identification (Max. Ident.) of each species in reference to GenBank.

Localities	<i>Ae. albopictus</i> (N)	Max. Ident.	<i>Ae. Aegypti</i> (N)	Max. Ident.	Others	Max. Ident.	Total no. of individuals
North East							
Pengkalan Quay (WQ)	8	99%	6	99%	0	–	14
Gat Lebu Macallum (GLM)	14	99%	0	–	0	–	14
Flat Hamna (H)	2	99%	13	99%	0	–	15
Sungai Nibong Kecil (SNK)	13	99%	1	99%	0	–	14
Bukit Jambul (BJ)	3	99%	15	99%	0	–	18
Taman Tun Sardon (TTS)	17	99%	1	99%	0	–	18
Universiti Sains Malaysia (USM)	16	99%	2	99%	0	–	18
Tingkat Sungai Gelugor (TSG)	17	99%	1	99%	0	–	18
Tanjung Bungah (TB)	11	99%	0	–	1 (<i>Culex minutissimus</i>)	88%	12
Batu Feringghi (BF)	12	99%	8	99%	0	–	20
South West							
Flat Seri Delima (SD)	0	–	13	99%	0	–	13
Medan Mahsuri (MM)	15	99%	0	–	0	–	15
Taman Sri Gertak Sanggul (GS)	7	99%	7	99%	0	–	14
Kampung Jawa (KJ)	12	99%	12	99%	0	–	24
Permatang Damar Laut (PDL)	18	99%	0	–	0	–	18
Balik Pulau (BP)	16	99%	0	–	0	–	16
Mayang Pasir (MP)	1	99%	12	99%	0	–	13
Sungai Batu (SB)	0	–	20	99%	0	–	20
Teluk Awak (TA)	14	99%	0	–	0	–	14
Batu Maung (BM)	18	99%	0	–	0	–	18
Central Seberang Perai							
Taman Desa Damai (TDD)	9	99%	1	99%	0	–	10
Perkampungan Berapit (PB)	6	99%	0	–	16 (<i>Culex gelidus</i>)	99%	22
Flat Teluk Indah (TI)	8	99%	6	99%	0	–	14
Padang Lalang (PL)	16	99%	0	–	0	–	16
Permatang Pauh (PP)	8	99%	0	–	0	–	8
Seberang Jaya (SJ)	0	–	2	99%	18 (<i>Culex pipiens</i>)	99%	20
Juru (JU)	9	99%	0	–	0	–	9
Macang Bubuk (MB)	12	99%	0	–	3 (<i>Toxorhynchites sp.</i>)	99%	15
North Seberang Perai							
Bagan Dalam (BD)	22	99%	2	99%	0	–	24
Pokok Sena (PS)	16	99%	3	99%	0	–	19
Kepala Batas (KB)	14	99%	0	–	0	–	14
Total	334		125		38		497

growth in excess of rare alleles (Tajima, 1989). Fu's F_s on the other hand used information on haplotype distribution to detect past population size fluctuation (Ramos-Onsins and Rozas, 2002).

To measure an extent of genetic structuring among samples, analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed using 1000 permutations implemented in the software ARLEQUIN version 3.5 (Excoffier and Lischer, 2010). Analysis was performed for both species, within and among populations for each region. Pairwise F_{st} values were computed by permutation tests from 1000 random permutations of haplotypes between populations based on the haplotype frequencies using the same program. Significant levels of pairwise F_{st} were obtained under the null hypothesis of no differentiation between populations. Mismatch distribution analysis was performed using DnaSP version 5.10.1 (Librado and Rozas, 2009) to identify patterns in nucleotide site differences between haplotype pairs.

2.6. Phylogenetic analysis and minimum spanning network

The phylogenetic tree reconstructions based on ML (maximum likelihood) and NJ (neighbor-joining) method were utilized to examine the evolutionary relationships and divergence among haplotypes and conducted in MEGA X (Kumar et al., 2018). Only samples that have a unique haplotype will be included in the analysis and represent the sampling locations. The Akaike's Information Criterion (AIC) that correspond with the Bayesian's Information Criterion (BIC) was used employed in MEGA X to examine the best fit model before phylogenetic tree reconstruction. In this current study the best model was Kimura 2-

parameter (Kimura, 1980) for both *Ae. albopictus* and *Ae. aegypti*. The significance of all phylogenetic nodes was evaluated with 1000 replicates and was rooted with *Culiseta bergrothi* (GenBank accession no: LC176745.1) as an outgroup. Genetic deviations within and among populations was calculated following Tamura-Nei (Tamura and Nei, 1993) distance and employed in MEGA X (Kumar et al., 2018).

The relationships among haplotypes were further tested by creating a minimum spanning tree using Network version 4.6.1.1 (available at <http://fluxus-engineering.com/sharenet.htm>), connecting all haplotype as nodes in a network connected by the least number of substitutions. Data for the minimum spanning tree was computed in the ARLEQUIN version 3.5 (Excoffier and Lischer, 2010) as a matrix of pairwise substitutions between all putative haplotypes. In addition, to provide a schematic representation of haplotype relationships, the network allowed inferences of the coalescent history among haplotypes relative to their common ancestry.

3. Results

3.1. Sampling data and species identification

Approximately 497 mosquito larvae were obtained from 31 locations that represents four districts/zones in Penang (Fig. 1) which consists of $n = 161$ (North East), $n = 165$ (South West), $n = 114$ (Central Seberang Perai) and $n = 57$ (North Seberang Perai) (Table 1). The sampling locations, species identification and maximum identification in reference to GenBank was presented in

Table 1. BLASTn analysis for all sequences showed that all samples have been correctly identified up to a species level, demonstrating that all samples preliminary identified based on the larvae morphological characteristic matched with the scientific names retrieved from the conspecific sequences deposited in GenBank (Table 1). Most of the mosquitoes found in Penang are from genus *Aedes* (92.4%) where *Ae. albopictus* was the most abundant (67.2%) and another 25.2% account for *Ae. aegypti*. Another genus found in Penang were *Culex* (7.04%) and *Toxorhynchites* (0.60%). Specifically, a total of 125 *Ae. aegypti* and 334 of *Ae. albopictus* larvae were collected and genotyped from 18 and 28 locations of Penang respectively (Table 1). However, for population genetics study, only 106 (from 9 locations) and 328 samples (from 25 locations) of *Ae. aegypti* and *Ae. albopictus* were included in the population genetic analysis as the rest of the locations only have one or two samples of larvae.

3.2. Genetic diversity and haplotype distribution

The COI gene amplified 440 bp sequence with 312 (73.1%) variable sites and 38 haplotypes for *Ae. aegypti* while for *Ae. albopictus*, there are 61 haplotypes and 48 variable sites (26.9%) as revealed by the 418 bp sequence of COI. All unique sequences have successfully deposited in GenBank (Acc. No: KPP122807 - KPP122845 for *Ae. albopictus* and KP122846 - KP122909 for *Ae. aegypti*). The nucleotide composition for *Ae. aegypti* was A + T rich; A = 40.3%, T = 27.5%,

G = 14.4% and C = 17.8%. Likewise, the nucleotide composition for *Ae. albopictus* was also A + T rich, in which A = 40.1%, T = 28.0%, G = 15.7% and C = 16.2%.

Table 2 showed the summary of the number of haplotypes, nucleotide diversity (π), haplotype diversity (h), Fu's F_S and Tajima's D statistics for *Ae. aegypti* and *Ae. albopictus* respectively. Low nucleotide diversity was recorded within a population (*Ae. aegypti*, $\pi = 0.002$ – 0.030 ; *Ae. albopictus*, $\pi = 0.002$ – 0.013) while haplotype diversity (h), showed the high estimation ($h = 0.667$ – 0.933 for *Ae. aegypti*, $h = 0.476$ – 0.929 for *Ae. albopictus*). Neutrality test, Fu's F_S for *Ae. aegypti* revealed negative values in most of the populations (e.g. Kg. Jawa, Bukit Jambul, Flat Hamna, Gertak Sanggul and Sungai Dua). The same pattern was also found for *Ae. albopictus* which shows negative values in all populations except the population from Tingkat Sungai Gelugor and Perkampungan Berapit (Table 2). Negative values of Tajima's D were also observed in some populations from both regions in *Ae. aegypti* (Bukit Jambul, Batu Feringhi, Gertak Sanggul and Kampung Jawa) while all populations showed negative values for *Ae. albopictus* (Table 2).

Table 3 shows a total of 39 haplotypes recorded from the 106 individuals of *Ae. aegypti*. The highest total number of haplotypes for *Ae. aegypti* was recorded in Bukit Jambul with nine haplotypes ($n = 15$), followed by Flat Hamna ($n = 13$) and Kampung Jawa ($n = 12$) with eight haplotypes respectively. Some haplotypes were shared among districts. For instance, six haplotypes were shared

Table 2
Number of haplotypes, nucleotide diversity (π) and haplotype diversity (h), Fu's F_S and Tajima's D statistics for *Aedes albopictus*. Bold indicate data for *Ae. aegypti*.

Locations	N	No. of haplotypes	$\pi \pm SD$	$h \pm SD$	F_S	D
<i>North East</i>						
WQ	8	6	0.009 ± 0.002	0.929 ± 0.007	-1.039	0.001
	6	5	0.030 ± 0.005	0.933 ± 0.122	1.287	0.770
GLM	14	9	0.013 ± 0.003	0.901 ± 0.062	-1.167	-0.579
SNK	13	5	0.003 ± 0.000	0.782 ± 0.079	-1.511	0.444
TTS	17	4	0.002 ± 0.000	0.640 ± 0.073	-0.223	-0.512
USM	16	7	0.003 ± 0.000	0.750 ± 0.107	-3.252	-1.422
TSG	17	4	0.004 ± 0.000	0.728 ± 0.060	0.998	0.367
TB	11	5	0.004 ± 0.001	0.764 ± 0.107	-0.665	-1.438
BF	12	7	0.004 ± 0.000	0.894 ± 0.063	-3.483	0.506
	8	4	0.004 ± 0.002	0.750 ± 0.139	0.119	-0.705
BJ	15	9	0.011 ± 0.002	0.886 ± 0.062	-1.237	-1.062
H	13	8	0.009 ± 0.002	0.910 ± 0.056	-1.362	0.113
<i>South West</i>						
MM	15	5	0.003 ± 0.001	0.476 ± 0.155	-0.841	-1.969
GS	7	5	0.006 ± 0.001	0.857 ± 0.137	-0.943	-0.963
	7	3	0.002 ± 0.000	0.667 ± 0.160	-0.438	-0.275
KJ	12	5	0.003 ± 0.000	0.758 ± 0.093	-1.105	-1.167
	12	8	0.013 ± 0.004	0.924 ± 0.057	-0.732	-1.152
PDL	18	7	0.003 ± 0.000	0.771 ± 0.083	-2.994	0.254
BP	16	9	0.005 ± 0.000	0.883 ± 0.061	-3.771	-1.007
TA	14	7	0.005 ± 0.001	0.846 ± 0.074	-2.074	-1.126
BM	18	7	0.003 ± 0.000	0.810 ± 0.070	-2.491	0.709
MP	12	7	0.013 ± 0.002	0.894 ± 0.063	0.233	0.177
SB	20	6	0.008 ± 0.001	0.716 ± 0.087	1.363	0.806
SD	13	8	0.005 ± 0.000	0.910 ± 0.056	-3.322	0.470
CSP						
TDD	9	6	0.011 ± 0.003	0.889 ± 0.091	-0.182	-0.503
PB	6	3	0.006 ± 0.001	0.733 ± 0.155	1.312	1.799
TI	8	5	0.003 ± 0.000	0.857 ± 0.108	-2.238	0.331
PL	16	9	0.004 ± 0.000	0.817 ± 0.095	-5.149	-1.175
PP	8	4	0.003 ± 0.000	0.821 ± 0.101	-0.785	0.331
JU	9	5	0.004 ± 0.000	0.861 ± 0.087	-1.338	0.385
MB	12	5	0.003 ± 0.000	0.788 ± 0.090	-1.449	0.672
<i>continue</i>						
Locations	N	No. of haplotypes	$\pi \pm SD$	$h \pm SD$	F_S	D
<i>NSP</i>						
BD	22	6	0.003 ± 0.000	0.788 ± 0.054	-1.081	0.095
PS	16	5	0.003 ± 0.000	0.800 ± 0.057	-0.899	0.072
KB	14	6	0.004 ± 0.000	0.769 ± 0.089	-1.828	-0.244

N = sample size.

Table 3
Haplotype distribution among nine populations of *Aedes aegypti* from two regions in Penang Island.

Locations	Haplotype																																																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	Total											
North east																																																			
BJ					1	4	4	1	1	1	1	1	1																																						
H	1	3	3											1		1	1	1	2																																
WQ																																																			
BF	1	4	1	2																																															
South west																																																			
MP	1	3			2															1	3																														
SB														3		10																																			
GS													4		2	1																																			
SD	2	3	1																																																
KJ	1	2	1																																																
Total	1	9	9	10	1	6	4	1	1	1	1	1	1	1	8	2	12	1	1	2	2	2	4	6	1	1	1	1	1	1	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	106		

among the two regions (North East and South West) of Penang Island (Hap 2, Hap 3, Hap 4, Hap 6, Hap 14, Hap 16) (Table 3). Three common haplotypes (Hap 2, Hap 3, Hap 4) were observed to occur in four or more populations; Hap 2 and 4 occurred in five populations (Hamna, Batu Feringhi, Mayang Pasir, Seri Delima, Kampung Jawa), while Hap 3 occurred in four populations (Flat Hamna, Batu Feringhi, Seri Delima, Kampung Jawa). There were 30 population-specific haplotypes observed with one to four haplotypes per population (Table 3).

Table 4 presents a total of 64 haplotypes from 328 individuals of *Ae. albopictus*. The highest total number of haplotypes was nine haplotypes recorded in Gat Lebu Macallum (n = 14), Balik Pulau (n = 16) and Padang Lalang (n = 16). Thirteen haplotypes were shared by two or more regions (Hap 1–6, Hap 8–9, Hap 11, Hap 14, Hap 27, Hap 36, Hap 40). Four common haplotypes (Hap 1, Hap 2, Hap 4, Hap 8) were observed to occur in 11 or more populations. Hap 1 and 4 occurred in 19 populations, Hap 2 occurred in 16 populations and Hap 8 was found in 11 populations. There were 48 population-specific haplotypes observed with one to seven haplotypes per population (Table 4).

3.3. Population structure

Analysis of molecular variance (AMOVA) (Table 5) for *Ae. aegypti* revealed that most of the variation in North East and South West areas occurred within the population with the percentage of variation were 61.48% and 59.75%, respectively. The same pattern was also shown by *Ae. albopictus* in which the within population percentage for North East, South West, Central Seberang Perai and North Seberang Perai were 74.05%, 78.07%, 86.44% and 95.53%, respectively (Table 5). The mismatch distribution analysis (Fig. 2) was carried out to further estimates the population size changes. The graph showed bimodal characteristics of population differentiation for *Ae. aegypti* (Fig. 2A) but *Ae. albopictus* showed unimodal characteristics of population differentiation (Fig. 2B).

Pairwise F_{st} values (Table 6) for *Ae. aegypti* showed no significant difference ($p > 0.05$) between all regions included in the analysis except for KJ and BF, SD and BF, SD and KJ, KJ and H. Meanwhile, the F_{st} values for *Ae. albopictus* (Table 7) showed a significant difference ($p < 0.05$) between 25 analysed regions in Penang. Genetic distance based on Tamura-Nei parameter showed low genetic divergent within a population (0.000–0.031) and high genetic divergent among population (0.019–0.835) for *Ae. aegypti* (Table 6). The same genetic divergence pattern was also observed for *Ae. albopictus* in which the low intrapopulation divergent ranging from 0.000 to 0.013. The interpopulation genetic divergent for *Ae. albopictus* was 0.009–0.514 (Table 7).

3.4. Phylogenetic analysis and minimum spanning network

Maximum likelihood (ML) and neighbor joining (NJ) tree showed similar topology, thus, only the ML tree will be presented and discussed as it is known to be the best method in the study of evolution (Edwards, 1995). The maximum likelihood tree showed no obvious pattern of population genetic structure for both *Ae. aegypti* (Fig. 3) and *Ae. albopictus* (Fig. 4) from Penang and a moderate to high bootstrap values has supported this conclusion. The result was further strengthened by the minimum spanning network analysis. The minimum spanning network for *Ae. aegypti* (Fig. 5) and *Ae. albopictus* (Fig. 6) showed five (Hap 2, Hap 3, Hap 4, Hap 14, Hap 16) and three (Hap 1, Hap 2, Hap 4) dominant haplotypes respectively which indicates a mixture of haplotypes from the regions analysed. Each of the related sequences was separated by a minimum of one mutational step.

Table 4
Haplotype distribution among 25 populations of *Aedes albopictus* in Penang.

Locations	Haplotype																																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36			
North east																																							
WQ		2		1																																			
GLM	3			4																																	1		
SNK		2	1				4							5																							1		
TTS		8	1		1		7																																
USM	8	2	1		1		2		1		1																												
TSG				5																																			
TB	5	3	1																																				
BF	3	3	1			2	1	1	1																														
South west																																							
MM		11						1																															
GS	1						3																																
KJ	5			4		1				1											1																		
PDL	2	8	1				4				1												1					1	1	1									
BP	5	5	3	1	1		1			1				2	1	1	1																						
TA	3	5		1																																			
BM	1			4			1	2			2	7	1																										
CSP																																							
TDD	2			3			1																																
PB	2			1																																			
TI	3	1		2			1																																1
PL	7			1	1																																		
PP	3	1																							2	2													
JU	2	1		1															2	3																			
MB	5	3					1																	2	1														
NSP																																							
BD	8	3	3	6	1	1																																	
PS	5	5		2																																		1	
KB	6			4	1														1	1	1																		
Total	74	63	4	46	5	4	4	25	4	1	3	2	7	8	1	1	1	2	3	1	1	1	1	4	3	1	4	1	2	1	1	1	1	1	1	1	3		
Locations	Haplotype																																						
	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	Total										
North east																																							
WQ									2	1	1	1																										8	
GLM													1	1	1	1	1	1																				14	
SNK																																						13	
TTS																																						17	
USM																																						16	
TSG				4	7	1																																17	
TB							1	1																														11	
BF																																						12	
South west																																							
MM																						1	1	1													15		
GS																							1	1	1												7		
KJ																																						12	
PDL																																						18	
BP																																						16	
TA	1	1	1	2																																		14	
BM																																						18	
CSP																																							
TDD																																						9	
PB																																						6	
TI																																						8	
PL																																						16	
PP																																						8	
JU																																						9	
MB																																						12	
NSP																																							
BD																																						22	
PS																																						16	
KB																																						14	
Total	1	1	1	6	7	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	328		

4. Discussion

4.1. Sampling data and species identification

All of the species collected has been successfully identified and data shows that the samples matched (99%) with conspecific

sequence from GenBank (Table 1). This inferred that mosquito larvae can be distinguished based on COI gene marker aside from traditional methods that relied on the morphological attributes. There is no doubt that the COI has been the chosen DNA barcode for species identification in many animals, including mosquitoes and this can be proved by an increasing number of researches conducted

Table 5
Analysis of molecular variance (AMOVA) among *Ae. aegypti* and *Ae. albopictus* collected from Penang.

Species	Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation index
<i>Ae. aegypti</i>	North East					
	Among population	3	56.269	1.607	38.52	$F_{ST} = 0.385$
	Within populations ns	38	97.421	2.564	61.48	
	South West					
	Among populations	4	69.838	1.252	40.25	$F_{ST} = 0.403$
	Within populations	59	109.646	1.858	59.75	
	NSP					
	Among populations	2	2.463	0.032	4.47	$F_{ST} = 0.045$
	Within populations	49	33.614	0.686	95.53	
CSP						
<i>Ae. albopictus</i>	Among populations	6	14.316	0.150	13.56	$F_{ST} = 0.136$
	Within populations	61	58.243	0.955	86.44	
	North east					
	Among populations	7	41.117	0.361	25.95	$F_{ST} = 0.260$
	Within populations	100	103.068	1.031	74.05	
	South west					
	Among populations	6	24.762	0.233	21.93	$F_{ST} = 0.219$
	Within populations	93	77.228	0.830	78.07	

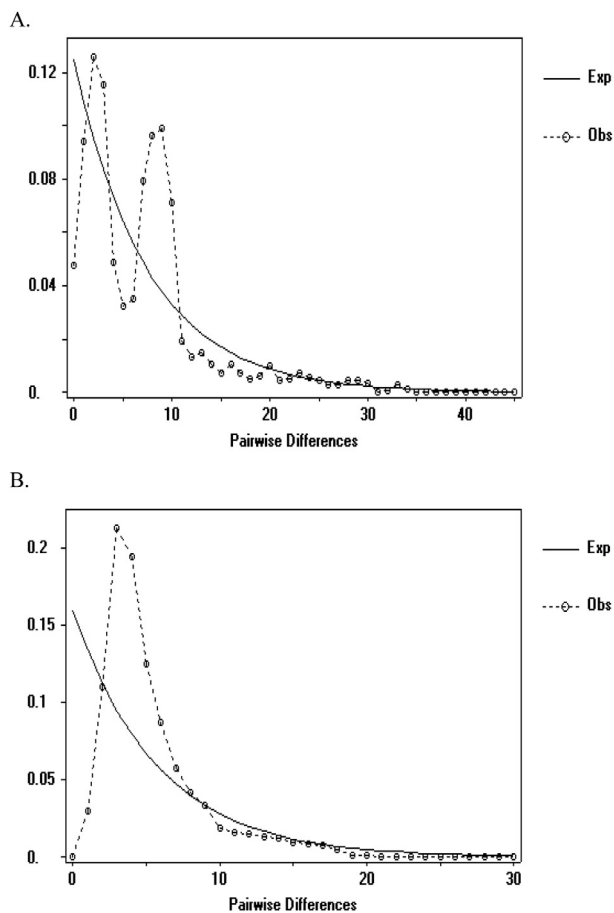


Fig. 2. Bimodal (A) and unimodal (B) pattern of mismatch distributions analysis for COI gene of *Ae. aegypti* (A) and *Ae. albopictus* (B) showing the expected and observed pairwise differences between the sequences with respective frequencies. The dash line shows the empirical pairwise-difference distribution whereas the solid line is an equilibrium distribution with the same mean.

worldwide (see e.g. Engdahl et al., 2014; Talbalaghi and Shaikovich, 2011).

This study demonstrated that the total number of *Ae. albopictus* larvae collected were more than the total number of *Ae. aegypti* in most of the places (Table 1). In some places (i.e., Flat Hamna, Bukit Jambul, Mayang Pasir), *Ae. aegypti* was found to be more abundant

while in other places (i.e., Sungai Nibong Kecil, Taman Tun Sardon, Universiti Sains Malaysia, Tingkat Sungai Gelugor, Bagan Dalam, Pokok Sena), *Ae. albopictus* conquered the area in which only one to three individuals of *Ae. aegypti* can be found. This may be due to interspecific larval competition, which has important effects on the growth, survivorship and reproductive success of the species (Juliano and Lounibos, 2005). A study conducted by Juliano (1998) has reported that interspecific resource competition is the most obvious explanation of the observed decline of *Ae. aegypti* in the United States after invasion of *Ae. albopictus*. Bagny-Beilhe et al. (2012) also suggested that interspecific larval competition resulted in the declination of *Ae. aegypti* towards *Ae. albopictus* invasion. These strengthen the fact that interspecific larval competition can also occur in *Aedes* species in Penang.

4.2. Genetic diversity and haplotype distribution

The analysis of nucleotide in *Ae. aegypti* and *Ae. albopictus* revealed that both species possessed 67.8% and 68.1% A + T rich composition respectively, even though the composition was moderately different between the species. Feng et al. (2003) has demonstrated that the A + T rich composition in a species will give rise to the diminution of synonymous position and will devastate the amino acid content, hence effects the substitution percentage of amino acid (Jukes and Bushan, 1986). This current study exhibited the minimum number of synonymous positions in *Ae. albopictus* (18) and *Ae. aegypti* (12) but the A + T composition range is comparable to the published study on mosquitoes based on COI DNA marker (e.g., Barbosa et al. 2014; Gutierrez et al. 2014; Pavana and Sebastian, 2012).

The haplotype data (Tables 3 and 4) showed the varied number of haplotypes among populations for both species of *Aedes*. Data for *Ae. aegypti* showed that the greatest number of individuals came from the Sungai Batu with 20 individuals collected. However, number of haplotypes recorded was among the lowest with only six haplotypes. Data for *Ae. albopictus* showed the same results where the highest number of individuals per site was recorded in Bagan Dalam ($n = 22$) but the haplotype number was only six. Thus, the haplotype number was not correlated to the number of individuals in a population.

This research revealed low levels of genetic variation within both *Ae. aegypti* and *Ae. albopictus* population with the range of nucleotide diversity, π is 0.002–0.030 and 0.002–0.013 respectively (Table 2). The observed pattern may be attributed to severe, repeated, or long periods of population bottleneck which is the

Table 6

Below diagonal: population divergence between samples (F_{ST}) based on 1000 permutations of the sequence dataset implemented in ARLEQUIN version 3.5. Above diagonal: pairwise Tamura-Nei genetic distances (D) among and within nine populations of *Ae. aegypti* implemented in MEGA X (Kumar et al., 2018).

	BF	BJ	GS	H	KJ	MP	SB	SD	WQ
BF	0.000	0.002	0.017	0.001	0.005	0.003	0.011	0.001	0.017
BJ	0.170	0.011	0.020	0.002	0.002	0.004	0.012	0.002	0.019
GS	0.835	0.694	0.002	0.012	0.013	0.006	0.002	0.017	0.021
H	0.053	0.177	0.630	0.009	0.001	0.001	0.006	0.001	0.019
KJ	0.029	0.136	0.582	0.018	0.013	0.001	0.007	0.001	0.016
MP	0.200	0.250	0.403	0.088	0.072	0.013	0.003	0.003	0.020
SB	0.594	0.560	0.200	0.416	0.426	0.256	0.008	0.011	0.028
SD	0.032	0.217	0.812	0.083	0.019	0.266	0.602	0.005	0.018
WQ	0.524	0.539	0.697	0.553	0.462	0.527	0.692	0.592	0.031

*Bold indicates significant at $p < 0.05$.

reduction of effective population size as a result of natural environmental events or induce by humans (Wright, 1938). Population bottleneck caused losses in genetic variation due to random genetic drift (Nei et al., 1975; Birungi and Munstermann, 2002) and may reduce genetic variability and correlative effects on fitness (Weber et al., 2004). As a vector, *Aedes* mosquitoes may experience periodic bottlenecks in population size due to changes in host abundance (human) and breeding sites. Apart from that, Birungi and Munstermann (2002) deduced that repeated control programs have caused the reduction of *Ae. albopictus* population in United States. In most areas in Penang, extensive and repeated insect control activities involving source reduction and insecticide application may lead to the reduction or eradication of *Aedes* mosquito populations. This resulted in reduced levels of genetic variation which have been observed in this current study. Indeed, *Aedes* mosquitoes may retain less genetic variation when subjected to less variation in environmental conditions. Zitko et al. (2011) also conducted the same study of *Ae. albopictus* population in East-Adriatic populations, and they have observed low genetic variation within the population, thus postulated that the observed pattern may be due to the small size of founding populations.

High haplotype diversity, h (>0.5) was recorded within population of both *Ae. aegypti* and *Ae. albopictus* except for Medan Mahsuri where the haplotype diversity of *Ae. albopictus* is below 0.5 (Table 2). High haplotype diversity was partly attributed to the high mutation rate of mitochondrial DNA (Vandewoestijne, 2004). This phenomenon may increase the rate of resistance development as the presence of resistance genes can confer fitness costs to insects (Overgaard, 2006). The high mutation rate was also supported by the minimum spanning network analysis (Figs. 5 and 6) which showed that the haplotype undergone a minimum of one step of mutation before evolving into a new haplotype. The mutation may be correlated with the use of certain insecticides in controlling mosquito-borne diseases (Li et al., 2012). For example, in Malaysia, larvicide and insecticide application such as fogging are the methods used by Malaysian Ministry of Health to control the occurrence of the disease. Thus, the chemical ingredients in the larvicide and insecticides are potentially the factor of mutation for mosquitoes that exposed to the insecticides. Furthermore, Medan Mahsuri is among the highest abundance of *Aedes* mosquitoes found in Penang Island (Nur Aida, 2013) and with this indication, fogging activities using the insecticide was frequently conducted in this area. This may be challenging in controlling *Aedes* population in the future.

Grant and Bowen (1998) classified the genetic variability of populations based on mtDNA markers into four categories; (i) low h and low π (ii) high h and low π (iii) low h and high π (iv) high h and high π . The result of this study fall into the second category (high h and low π) which inferred that the condition was attributed to population expansion after a period of a low effective population size followed by rapid population growth which enhances the

retention of new mutations (Avisé et al., 1984; Rogers and Harpending, 1992; Grant and Bowen, 1998) and the inference support the above statements. On the other hand, only one population of *Ae. albopictus* experienced both low h and π which fall into the first category. The population is Medan Mahsuri (MM) which is located in the South West district of Penang. In this case, we postulated that the population has undergone founder events where a new population is established by a small number of individuals drawn from a large ancestral population (see i.e., Grant and Bowen, 1998; Templeton, 2008). This suggests the recent arrival of *Ae. albopictus* population in Medan Mahsuri. The sampling site of Medan Mahsuri is a terraced housing area and one of the hot-spots regions for dengue fever in 2013 (Ministry of Health Malaysia, 2013). There is a total of five haplotypes that were found in Medan Mahsuri with 15 total number of individuals. The same result (low h and π diversity) was also reported by Surendran et al. (2013) for research on *Anopheles subpictus* in Sri Lanka. They postulated that population bottlenecks arise due to insecticide application as one of the reasons that lead to low nucleotide and haplotype diversity.

4.3. Population demographic expansions

Tests for population expansion (originally derives as a test for selective neutrality) showed negative results of Tajima's D and Fu's F_s for most of the population of both *Ae. aegypti* and *Ae. albopictus* (Table 2). These indicated a qualitative support for population expansion. However, the negative result is not significant except for one population (Medan Mahsuri). This shows that *Aedes* mosquito populations in Penang may have possibly experienced past population growth, but the expansion may have been restricted to separate local areas that resulted in the non-significant negative Fu's F_s and Tajima's D value for most populations studied (Liao et al., 2010). Only Medan Mahsuri showed significant negative results for Tajima's D statistics (Table 2). Tajima's D is sensitive to recent population bottlenecks or population growth which will result in the value to move towards more negative values (Tajima, 1989). *Aedes albopictus* population in Medan Mahsuri may undergo recent population growth, which resulted in the significant negative value of Tajima's D test. This result supports the above hypothesis of low nucleotide and haplotype diversity.

Furthermore, the *Aedes* population tends to increase in areas with a high human population density or rapid population growth (Nazri et al., 2013) because human commute; in this study, from Penang Island to the mainland and vice versa. Medan Mahsuri had a high density of human population (from observation) with improper solid waste disposal especially near the food court and the squatting houses area. A massive infrastructure may create man-made breeding site favored by the *Aedes* mosquitoes (Ghee, 1993).

Table 7

Below diagonal: population divergence between samples (F_{st}) based on 1000 permutations of the sequence dataset implemented in ARLEQUIN version 3.5. Above diagonal: pairwise Tamura-Nei genetic distances (D) among and within 25 populations of *Ae. albopictus* using MEGA X (Kumar et al., 2018). Bold indicates significant at $p < 0.05$.

	WQ	GLM	SNK	TTS	USM	TSG	TB	BF	MM	GS	KJ	PDL	BP	TA	BM	TDD	PB	TI	PL	PP	JU	MB	BD	PS	KB
WQ	0.009	0.002	0.002	0.001	0.004	0.002	0.003	0.002	0.002	0.001	0.003	0.002	0.001	0.002	0.002	0.001	0.002	0.002	0.003	0.004	0.002	0.003	0.003	0.003	0.003
GLM	0.156	0.013	0.003	0.003	0.002	0.013	0.002	0.002	0.003	0.002	0.001	0.012	0.002	0.002	0.003	−0.001	0.002	0.001	0.001	0.002	0.002	0.002	0.001	0.001	0.002
SNK	0.260	0.284	0.003	0.001	0.003	0.001	0.002	0.001	0.002	0.000	0.002	0.001	0.000	0.002	0.001	0.002	0.000	0.001	0.003	0.003	0.003	0.002	0.002	0.002	0.002
TTS	0.250	0.285	0.195	0.002	0.002	0.001	0.001	0.000	0.001	0.000	0.002	0.000	0.000	0.001	0.001	0.002	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.001	0.001
USM	0.423	0.185	0.510	0.364	0.003	0.002	0.000	0.000	0.001	0.002	0.001	0.001	0.001	0.000	0.002	0.002	0.002	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
TSG	0.301	0.273	0.299	0.251	0.413	0.004	0.002	0.001	0.002	0.001	0.002	0.001	0.001	0.002	0.002	0.002	0.002	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
TB	0.312	0.141	0.402	0.246	0.022	0.322	0.004	0.000	0.001	0.001	0.001	0.000	0.001	0.000	0.002	0.001	0.002	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
BF	0.268	0.178	0.317	0.117	0.072	0.271	0.029	0.004	0.001	0.000	0.001	0.000	0.000	0.000	0.001	0.001	0.001	0.000	0.001	0.001	0.000	0.000	0.001	0.000	0.001
MM	0.282	0.291	0.398	0.157	0.036	0.380	0.203	0.136	0.003	0.001	0.003	0.000	0.001	0.000	0.013	0.002	0.002	0.002	0.003	0.002	0.001	0.001	0.002	0.001	0.002
GS	0.078	0.116	0.106	0.031	0.300	0.157	0.171	0.097	0.209	0.006	0.001	0.000	0.000	0.001	0.001	0.001	0.001	0.000	0.001	0.002	0.001	0.001	0.001	0.001	0.001
KJ	0.366	0.090	0.444	0.386	0.158	0.346	0.121	0.211	0.466	0.216	0.003	0.001	0.001	0.002	0.002	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.000	0.000	0.000
PDL	0.280	0.222	0.271	0.037	0.172	0.256	0.070	0.003	0.116	0.071	0.248	0.003	0.000	0.000	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.000	0.001	0.000	0.001
BP	0.177	0.184	0.075	0.016	0.212	0.168	0.119	0.047	0.158	0.022	0.177	0.003	0.005	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001
TA	0.286	0.189	0.375	0.190	0.091	0.281	0.028	0.045	0.071	0.162	0.272	0.067	0.131	0.000	0.003	0.002	0.002	0.001	0.001	0.001	0.000	0.000	0.001	0.000	0.001
BM	0.332	0.262	0.306	0.312	0.428	0.328	0.360	0.286	0.460	0.179	0.323	0.306	0.190	0.389	0.003	0.002	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.001
TDD	0.104	0.046	0.255	0.250	0.231	0.242	0.153	0.165	0.299	0.047	0.099	0.194	0.125	0.200	0.230	0.011	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001
PB	0.221	0.142	0.133	0.287	0.362	0.291	0.248	0.230	0.381	0.101	0.264	0.245	0.090	0.277	0.289	0.121	0.006	0.001	0.002	0.002	0.002	0.001	0.001	0.001	0.001
TI	0.257	0.062	0.290	0.234	0.127	0.247	0.047	0.073	0.348	0.071	0.021	0.086	0.032	0.162	0.236	0.044	0.120	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PL	0.382	0.093	0.499	0.391	0.109	0.374	0.091	0.193	0.421	0.247	0.017	0.241	0.218	0.223	0.366	0.123	0.275	0.022	0.004	0.000	0.001	0.000	0.000	0.000	0.000
PP	0.367	0.118	0.514	0.432	0.164	0.400	0.120	0.213	0.430	0.263	0.145	0.264	0.222	0.222	0.415	0.149	0.324	0.130	0.087	0.003	0.001	0.000	0.001	0.001	0.000
JU	0.246	0.141	0.447	0.312	0.064	0.355	0.022	0.094	0.271	0.204	0.175	0.139	0.157	0.091	0.392	0.160	0.280	0.102	0.134	0.174	0.004	0.000	0.000	0.000	0.000
MB	0.349	0.148	0.442	0.285	0.033	0.346	0.016	0.061	0.272	0.203	0.141	0.096	0.132	0.082	0.378	0.166	0.294	0.054	0.086	0.022	0.063	0.003	0.000	0.000	0.000
BD	0.402	0.155	0.431	0.317	0.065	0.353	0.066	0.146	0.382	0.236	0.019	0.181	0.166	0.200	0.351	0.171	0.291	0.009	0.049	0.130	0.117	0.073	0.003	0.000	0.000
PS	0.370	0.159	0.431	0.276	0.022	0.344	0.022	0.061	0.279	0.208	0.113	0.064	0.110	0.093	0.370	0.167	0.295	0.031	0.090	0.146	0.060	0.011	0.058	0.003	0.000
KB	0.342	0.108	0.392	0.307	0.110	0.309	0.060	0.132	0.384	0.171	0.026	0.161	0.135	0.196	0.300	0.108	0.236	0.034	0.023	0.121	0.119	0.076	0.020	0.058	0.000

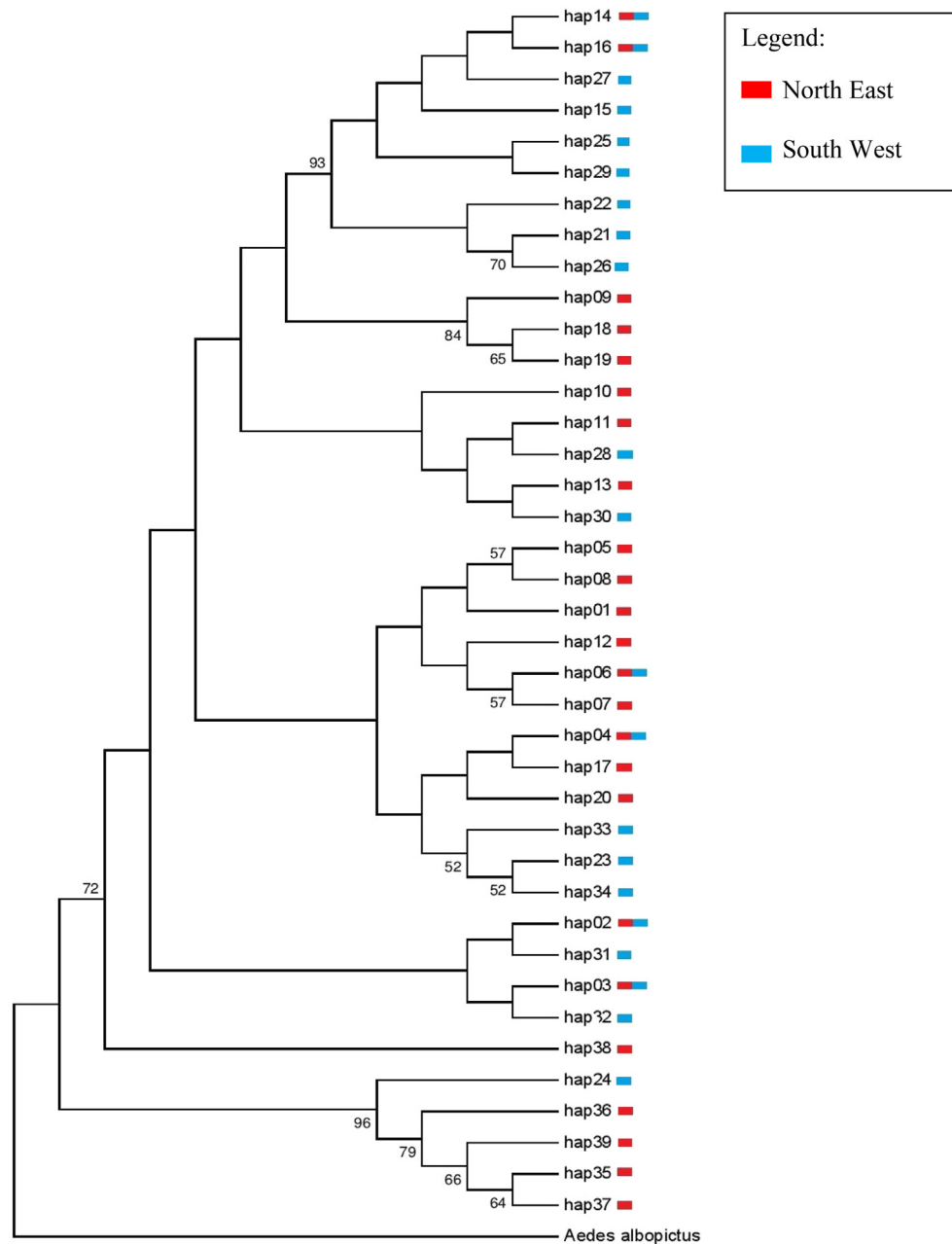


Fig. 3. Maximum likelihood tree showing relationships between COI haplotypes of *Ae. aegypti* from North East and South West of Penang with *Ae. albopictus* as outgroup. Only bootstrap values >50% are displayed.

The result was further corroborated by the mismatch distribution analysis. Graph of mismatch distribution for *Ae. albopictus* showed unimodal characteristics (Fig. 2B). The unimodal characteristics indicate that the population of *Ae. albopictus* in Penang have passed through recent demographic expansions (Lopes et al., 2007). The mechanisms behind this are not fully understood, however, this could be due to the demographic parameters of human that may influence the successful distribution and increasing population size of *Ae. albopictus* species in Penang. Nur Aida et al. (2011) found that *Ae. albopictus* showed increase egg hatch, has a short aquatic life, increased immature survival and fecundity under uncontrolled conditions of temperature and humidity that may contribute to the increment of the population size of the adults. Meanwhile, the mismatch distribution graph of *Ae. aegypti* showed bimodal characteristics (Fig. 2A), differ from other studies on population genetic structure of various animals using mtDNA

markers (Garber et al., 2004; Lopes et al., 2007; Johnson et al., 2007; Kong et al., 2010). The same result was recorded in the study of *Anopheles minimus*, the malaria vector across China, Thailand and Vietnam by Chen et al. (2011). They hypothesized that the bimodal pattern of mismatch distribution shown by the populations probably due to the low migration rate of malaria vector within the studied locations. The hypothesis could also be applied to the population of *Ae. aegypti* in this study, but further research needed, especially the movement pattern and migration rate of the species.

4.4. Genetic convergences

The ML tree of *Ae. aegypti* and *Ae. albopictus* showed no or limited phylogeographic partitioning of haplotypes, as evidenced by the absence of genealogical divergence (Fig. 3 and Fig. 4). The result

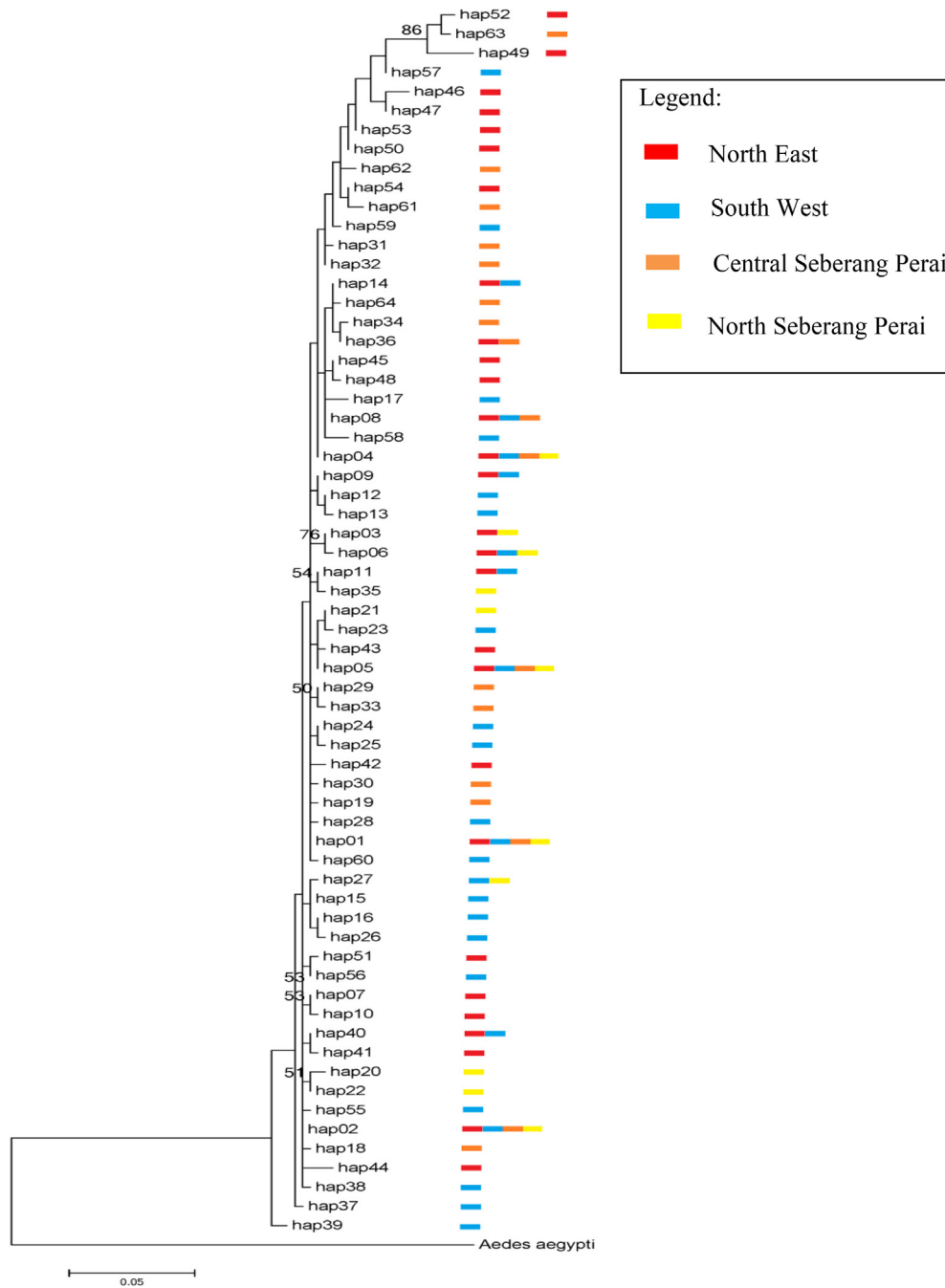


Fig. 4. Maximum Likelihood tree showing relationships between COI haplotypes of *Ae. albopictus* from four regions in Penang (North East, South West, Central Seberang Perai and North Seberang Perai) with *Ae. aegypti* as outgroup. Only bootstrap values >50% are displayed.

is interesting as it shows the flow of haplotypes in different part of the Penang areas which mainly assisted by human movements. The result was further explained by minimum spanning network analysis (Figs. 5 and 6) which showed a mixture of different regions in most of the haplotypes and the mutation steps undergone by the haplotypes which is due to the high mutation rate of mtDNA (Vandewoestijne, 2004). The minimum spanning network clearly showed dominant haplotypes for each species (Hap 2, Hap 3, Hap 4 for *Ae. aegypti* and Hap 1, Hap 2, Hap 4 and Hap 8 for *Ae. albopictus*) where each haplotype was separated by at least one mutational step. Those haplotypes can be found in both regions of Penang Island, suggesting common ancestral haplotype. The presences of shared haplotypes observed in the phylogenetic

tree (Figs. 3 and 4) indicate recent gene flow between populations (Koopman et al., 2007). Horne et al. (2008) also demonstrated that haplotype sharing was resulted from extensive gene flow and migration between distant populations has occurred on a relatively recent evolutionary time scale.

4.5. Population genetic structure

The significant low values of pairwise F_{st} and genetic distance, D (Table 6 and 7) proved the hypothesis of limited phylogeographic partitioning of haplotypes as shown by the absence of genealogical divergence. This was further strengthened by AMOVA where the majority of the total mtDNA sequence variation occurred among

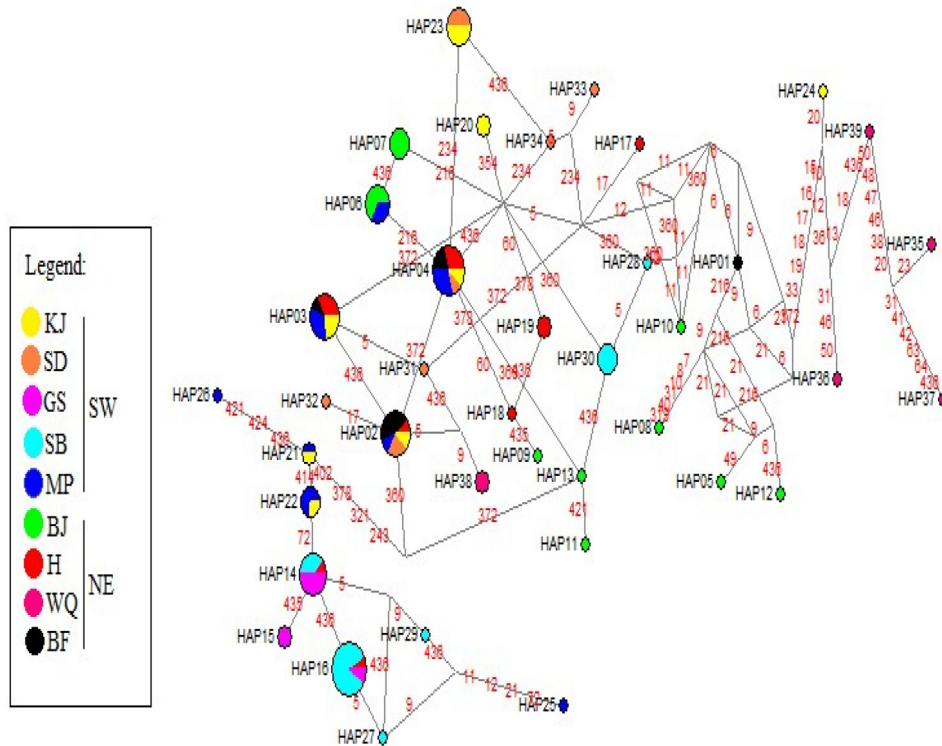


Fig. 5. Minimum spanning network among haplotypes of *Ae. aegypti* in nine locations from the South West (SW) and North East (NE) of Penang Island (KJ = Kampung Jawa, SD = Flat Seri Delima, GS = Taman Sri Gertak Sanggul, SB = Sungai Batu, MP = Mayang Pasir, BJ = Bukit Jambul, H = Flat Hamna, WQ = Pengkalan Weld, BF = Batu Feringhi).

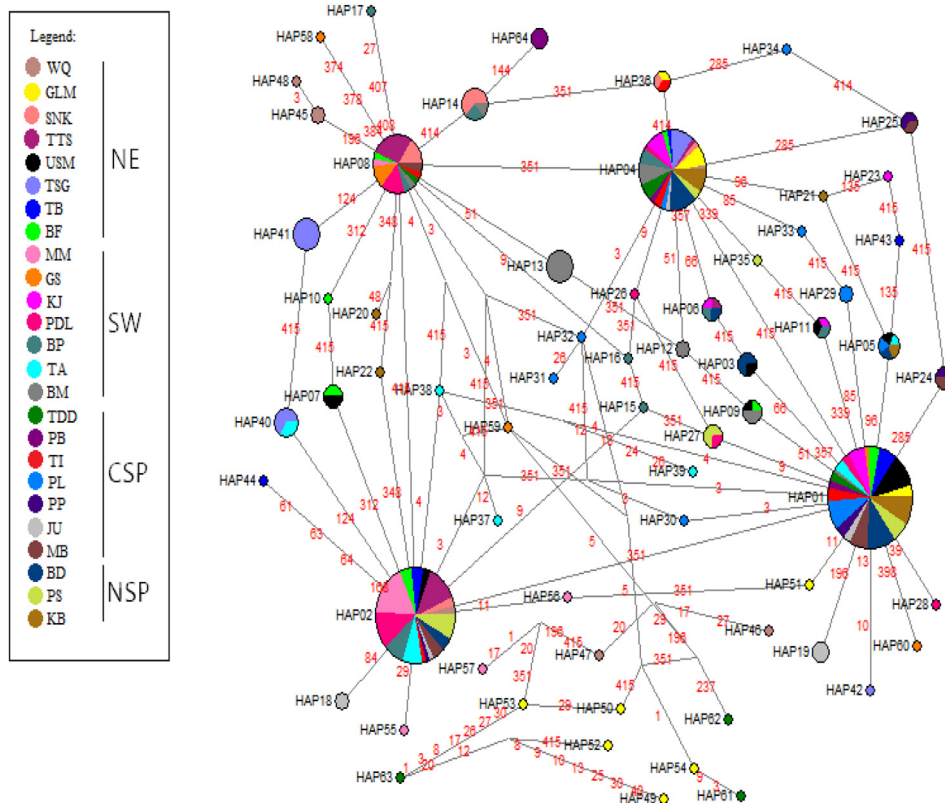


Fig. 6. Minimum spanning network among haplotypes of *Ae. albopictus* from four regions in Penang.

samples within the population and the fixation index (F_{ST}) that showed low values (Table 5). These results stipulate high effective migration and gene flow of both species between regions in Penang. The most appropriate reason for this is that the normal flight ranges of *Aedes* mosquitoes are limited and they have not been observed to fly in strong winds (Novak, 1992). However, Delatte et al. (2013) have reported that the flight ranges may increase when females fail to find a suitable site for oviposition or blood-meals, which likely assisted by the wind.

Movement and migration of mosquitoes may also be assisted by human activities, for example, through the transport of used and waste tires and also from the movement of other water-holding containers (Novak, 1992). Study by Minakawa et al. (2002) revealed that *Anopheles gambiae* larvae were found at the bottom of a wooden fishing boat, and thus implies that a boat may transport *Aedes* mosquito larvae between Penang Island and the mainland (Seberang Perai). The larvae of *Aedes* also could be moved to the island by vehicle transportation across the bridge that connected the mainland and the island as evidenced by the finding of the shared haplotype between Penang island and mainland areas (Figs. 5 and 6). Kay and Farrow (2000) suggested that storm front is the dispersal mechanisms for mosquitoes in Asia. Flood can also be one of the main mediums of transportation for *Aedes* mosquito larvae, whereby in Penang almost every year in some areas were hit by flood (Phuan and Pubalan, 2014) and there are high possibilities that the mosquito's larvae were transported during the event.

5. Conclusion

In this study, all specimens collected were successfully identified using the COI gene. Six species of mosquitoes from three genera namely *Aedes*, *Culex* and *Toxorhynchites* were found in Penang whereby the *Ae. albopictus* can be found in most parts of Penang, including in urban areas, thus postulated that the species begin to replace *Ae. aegypti* and may become the primary vector of dengue virus in Penang. However, further researches are needed to prove this hypothesis. There is no or limited population genetic structure exhibited by both *Ae. aegypti* and *Ae. albopictus* in Penang. Both species showed low genetic diversity with low nucleotide diversity and high haplotype diversity recorded in most of the population. We also found that mutations have occurred rapidly in *Ae. albopictus* and *Ae. aegypti* in Penang and this will be challenging in controlling the populations. There are possibilities that the populations may develop insecticide resistance gene and as a result, controlling them with certain insecticides will become useless and to no avail.

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Author contribution statement

DMN established the theoretical formalism, performed the analytic calculations and numerical simulations. SM contributed to the final version of the manuscript.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2020.01.021>.

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