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Species identification and genetic diversity of *Aedes* in Penang (Malaysia) based on Cytochrome Oxidase Subunit I

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Abstract. *Kamal MZM, Dangnga MS, Irmayani, Md. Naim D. 2020. Species identification and genetic diversity of* Aedes *in Penang (Malaysia) based on Cytochrome Oxidase Subunit I. Nusantara Bioscience 12: 6-12.* Cases of dengue fever are expanding globally and recently it was identified as being notable in remote/rural regions in South-East Asia in spite of the previous belief that it is outbreak to urban areas, especially in Malaysia. In an effort to constraint, the extent of the virus, the most important thing is to know the distribution of the vector that causes the disease. This research aims to recognize all species of mosquitoes distributed in Penang utilizing DNA barcode method in which the mtDNA cytochrome oxidase subunit 1 (COI) as a gene marker. Approximately 497 larvae of mosquitoes were obtained from four regions in Penang, Malaysia namely Central Seberang Perai, North Seberang Perai, North East, and South West of Penang. All samples were extracted and PCR amplified. Sequences were employed in BLAST of GenBank and aligned with MUSCLE. The ABGD analysis was performed to partition all the samples and substitution saturation analysis was employed in DAMBE. Phylogenetic relationships among nucleotides were reconstructed utilizing Maximum Likelihood (ML) and Neighbor-Joining (NJ) calculated following Kimura 2-parameter. Intra and interspecific genetic variation among population were also conducted based on Tamura-Nei parameter and all analyses were employed in MEGA version 6.0. As a result, COI gene has successfully recognized all larvae obtained in this study. We found a total of six species (including outgroup sequence) as revealed by BLASTn, ABGD and phylogenetic analysis. Genetic distance test has further supported the results. This study revealed that there is no visible population structure and all species have a uniform distribution throughout all sampling locations in Penang. We also found that *Ae. albopictus* has occupied most areas in Penang, and this includes urban locations, hence, suggest that *Ae. albopictus* probably succeed *Ae. aegypti* as the main vector for dengue virus in Penang.

Keywords: *Aedes*, COI, dengue, mtDNA, phylogenetics

INTRODUCTION

Mosquitoes belong to family Culicidae are the two-winged flies from order Diptera. In tropical forest habitat, the species are the most varies in which approximately 3500 species were recorded (Harbach 2008). Several species of this insect are life-threatening in which it can cause mosquito-borne diseases and it can be a very difficult epidemic to control as it may infect both adolescents and adults as well as exacerbate the rate of mortality. To make this problem worse, in sub-Saharan Africa, more than a million children were killed due to malaria every year. Additionally, Japanese encephalitis (JE) has diversified its predominant distribution in subcontinent of Australasia and India. Nowadays, in Southeast Asia, dengue fever has broadened its diversity for many years, and this is also an alarming disease caused by mosquitoes in Southeast Asia (Tolle 2009). Consequently, dengue fever has been considered as the greatest diseases of mosquito-borne viral that effects human being and become the major source of death for children around the world (Alshehri 2013).

Every year, approximately more than two billion people (more than 40% of populations in the world) have a high tendency to be affected by dengue fever in which worldwide infections rate are within the range of 50-100 million people yearly (WHO 2005). Consequently, this causes the outbreak to become a major threat to humans (Goswami et al. 2012). There are four viral serotypes that caused dengue hemorrhagic fever and dengue fever namely DEN-1, DEN-2, DEN-3, and DEN-4. These serotypes were transferred from viraemic to receptive humans through the bite of *Aedes albopictus* and *Aedes aegypti* (Guha-Sapir and Schimmer 2005). Once infected, symptoms that will be experienced by patients include rash, leucopenia, neuralgia as well as fibrous and bone/joint pains. The dengue hemorrhagic fever will be the consequence of a severe case in which the patients will experience hemorrhagic fever with hepatomegaly. Additionally, patients can also experience circulatory malfunction. The most severe case of viraemic transmission is the dengue shock syndrome that will cause death to the patient (WHO 2005).

Currently, in order to investigate the mechanism of disease transmission and enhance the disease control system, populations of mosquito were being examined based on behavior traits and molecular characteristics utilizing several genetic markers (i.e. nuclear and mitochondrial DNA target regions) (Herrera et al. 2006; Brelsfoard and Dobson 2012). However, study on mosquitoes' distribution in Malaysia, especially in Penang was very scarce. Penang is a state in Malaysia that has

recorded high numbers of patients infected with dengue virus (MoH 2014). *Aedes aegypti* and *Aedes albopictus* are dengue vectors mostly distributed in Penang and in August 2014, there are approximately 1300 dengue fever cases has been recorded in more than five locations of dengue outbreak (MoH 2014). This number has increased by more than double the number that has recorded in the previous year. Based on these very disturbing facts, this current research aims to recognize all species of mosquitoes found in Penang employing the most utilized mitochondrial DNA gene namely cytochrome oxidase subunit I (COI). This is very crucial as a starting effort in controlling the spreads of the virus effectively. Additionally, very few studies have been carried out relating to the distribution of mosquitoes in Penang.

MATERIALS AND METHODS

Study area and sample collections

All mosquito samples in a form of instar (4th instar) were obtained from four zones in Penang namely North Seberang Perai (NSP), Central Seberang Perai (CSP), South West (SW) and North East (NE) (Figure 1). The samples were gathered applying ovitrap and empty containers and/or cans during the year 2013-2014. There are ten ovitrap were set off 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 to 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, ascribed to the size of larvae that is very tiny and some larvae have been broken during preservation in alcohol, identification of mosquitoes was conducted based on polymerase chain reaction (PCR).

DNA isolation

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

PCR-amplified samples and DNA sequencing

The target position of mtDNA COI was intensified using PCR and the reaction compound contained 1.2 dNTP mix (1.6 mM/µl), 5.0µl of 10x buffer, 0.3µl of 2U DNA polymerase i-Taq+ (Intron, Korea), 5.0µl Magnesium Chloride (20 mM/µl), 1.5µl of each reverse and forward primers, 2.5µl of genomic DNA templates and distilled ddH_2O in 50µl of final volume. Plausible contamination was detected using negative control in all samples. The PCR condition comprises of 3 min initial denaturation at 96ºC followed by 35X (95ºC for 46 seconds, 45.5ºC for 50 seconds, 70ºC for a min with final elongation at 70ºC for 10 min), conducted in the BIORAD (USA) thermal cycler. All specimens were subject for electrophoresis process in 1.8% agarose gel consists 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'-GGAGGATTTGGAAATTGATTAGTTC-3' and C1-N-2191-5' CCCGGTAAAATTAAAATATAAACTTC-3'. All samples were subjected to purification process following iNtRON Biotechnology (Korea). Approximately 30µl of the PCR products that have a clean band chose and sent out to NHK Bioscience (Korea) for sequencing process.

Figure 1. Locations of samples collected from four zones in Penang, Malaysia

Genetic diversity and species identification

The amplified samples were employed in the GenBank database based on the BLAST algorithm with all available respective genes of 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 subsidence automatically and implemented in the Collapse version 1.2 (Provan et al. 2005). All sequences then were rearranged utilizing MUSCLE software (Edgar 2004) with default parameters and all aligned haplotypes were then synchronized in MEGA version 6.0 (Tamura et al. 2013). 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.

Each sample was determined for correct identification and employed in the ABGD (Automatic Barcode Gap Discovery) procedure (Puillandre et al. 2012) in an effort to analyze all samples accurately. ABGD is an automatic procedure that is based on genetic distance method to determine a pause/gap for barcode that will partition a putative species in the dataset by confirming that intra and interspecific genetic distance do not overlap. In this current research, the values for the prior *P* (prior maximum divergence of intraspecific diversity) have been setup varies from 0.001 to 0.1 and $X = 1.0$. The whole data set will be interpreted as one species if the *P*-value was set too high (Puillandre et al. 2012). The genetic divergence examination was quantified based on Jukes-Cantor 69 (Jukes and Cantor 1969) analysis and performed at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html.

Genetic divergence and phylogenetic analysis

The efficacy of phylogenetic details in all sequences analyzed in this study was examined based on substitution saturation test and implemented in DAMBE (Xia 2013). Then, 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 version 6.0 (Tamura et al. 2013). Only samples that have a unique haplotype will be included in the analysis and represent the sampling locations. MEGA version 6.0 (Tamura et al. 2013) also employed to examine the DNA evolution's finest fit model. In this current study, the best model was Kimura 2-parameter (Kimura 1980) for both *Ae. albopictus* and *Ae. aegypti*. The significance of all phylogenetics 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 were calculated following Tamura-Nei (Tamura and Nei 1993) distance and employed in MEGA version 6.0 (Tamura et al. 2013).

RESULTS AND DISCUSSION

Mosquito distribution

Approximately 497 mosquito larvae were obtained from four districts/zones in Penang that consist of n=161 (North East), n=165 (South West), n=114 (Central Seberang Perai) and n=57 (North Seberang Perai) (Table 1).

Molecular identification of mosquitoes

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). Of the 497 mosquito larvae collected, *Aedes* (92.35%) was the most genus found in Penang, in which *Ae. albopictus* mostly disperse (67.2%), followed by 25.2% account for *Ae. aegypti*. Genus *Toxorhynchites* (<1%) and *Culex* (7.04%) was also found in the studied area.

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 method that relied on the morphological attributes. There is no doubt that 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 worldwide (see e.g. characteristics Engdahl et al. 2014; Talbalaghi and Shaikevich 2011). The reliability of COI region as the best barcode for mosquitoes analyzed in this study was further supported by the ABGD analysis. The species' numbers defined by the ABGD ranged with the change prior thresholds from 0.001 to 0.100 prior intraspecific divergences (*P*) for COI (Figure 2). The lowest threshold values varied from 0.001 to 0.002 and grouped nearly all haplotypes as a different species (27 species). Threshold values ranged from 0.004 to 0.100 partitioned only six species. This further supports the results by BLASTn analysis (Table 1) in which demonstrated that six mosquito species (including one outgroup sequence retrieved from GenBank) has been identified in the present study.

Table 1. Distribution of mosquitoes in Penang, Malaysia inferred by BLASTn analysis with maximum identity for each genus

Localities	No. of *Ae. albopictus*	Maximum identity	No. of *Ae. aegypti*	Maximum identity	Others	Maximum identity	Total
North East	113	99%	48	99%	0	99%	161
South West	101	99%	64	99%	0	99%	165
North Seberang Perai	52	99%	5	99%	0	99%	57
Central Seberang Perai	68	99%	9	99%	16 (*Culex gelidus*)	99%	16
					18 (*Culex pipiens*)	99%	18
					3 (*Toxorhynchites* sp.)	99%	80
Total	334		125		37		497

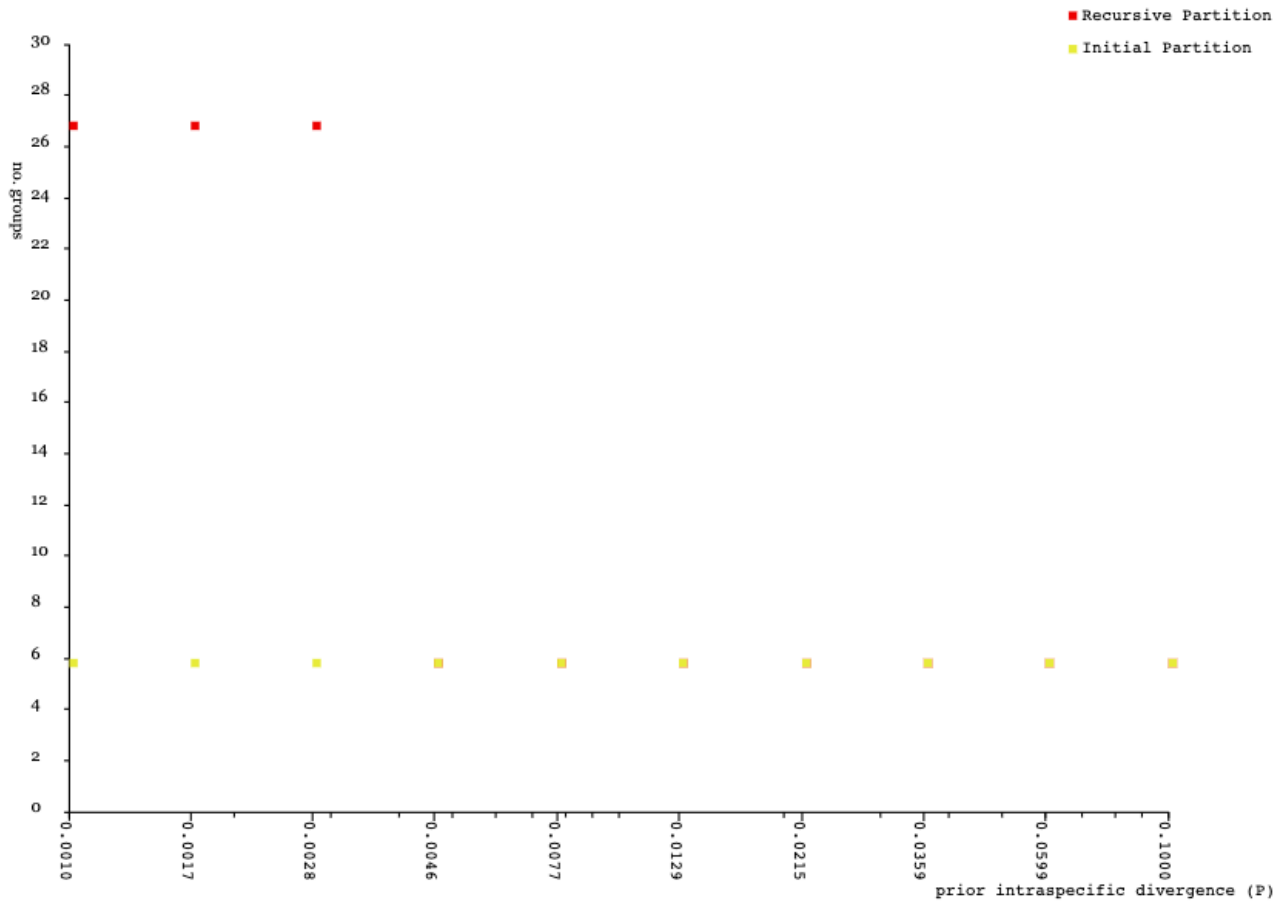

Figure 2. Distribution of COI pairwise based on Jukes-Cantor 69 (JC69) distance values for all species implemented in ABGD, presenting the DNA barcode gap among the assumed highest conspecific deviation and the lowest congeneric deviation

Observation of mosquitoes' molecular properties in this study revealed that *Aedes* comprises more than 90% of the total samples. A different species of mosquitoes requires distinct reproduction area (Laird 1988), however, several species able to permit their breeding ground selection (Service 1993). In this case, some mosquitoes (for e.g. *Culex* and *Aedes*) able to reproduce in diverse environments and habitat. Nonetheless, there are also species that precisely choose their reproduction ground, for example, mosquitoes that live in a hole of tree. This study revealed the selection of breeding places in *Aedes* and *Culex* notwithstanding that the number of the later was less than 8.0% in Penang (Table 1). The output from ABGD analysis has resulted in the same grouping as shown by ML phylogenetic tree model of all species. Thus, the six species clusters (including an outgroup sequence) that were demarcated by the ABGD (Figure 2) approach via the high prior threshold for COI marker are supported by the outcome of ML analysis (Figure 4).

Nucleotide composition

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 recorded 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%.

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) have 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).

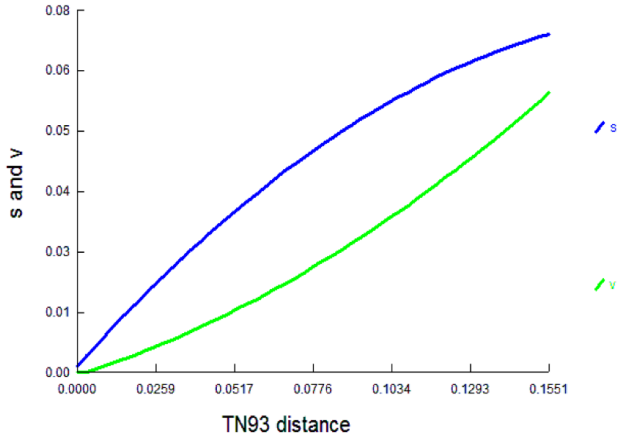

Figure 3. Substitution saturation graph showing the transition (s) rate higher than transversion (v) rate. No substitution saturation detected in this graph

Table 2. Intra and interpopulation pairwise genetic distance of *Ae. aegypti* calculated utilizing Tamura-Nei (Tamura and Nei 1993) model and implemented in MEGA 6.0.

	NE	SW	NSP	CSP
NE	0.004			
SW	0.013	0.001		
NSP	0.011	0.009	0.002	
CSP	0.007	0.012	0.002	0.003

Table 3. Intra and interpopulation pairwise genetic distance of *Ae. albopictus* calculated utilizing Tamura-Nei (Tamura and Nei 1993) model and implemented in MEGA 6.0.

	NE	SW	NSP	CSP
NE	0.002			
SW	0.012	0.001		
NSP	0.008	0.002	0.003	
CSP	0.005	0.011	0.007	0.005

The number of substitutions for *Ae. aegypti* is 13.67; 6.81 transversions (Tv) and 6.86 transitions (Ti). Meanwhile, the number of substitutions for *Ae. albopictus* was low compared to *Ae. aegypti* with 6.68; 3.64 transversions (Tv) and 3.04 transitions (Ti). However, both results demonstrated that the transversion percentage was high when compared to the transition percentage for both species. This is not the situation as reported by previously published research that demonstrated higher rate of transition than the transversion in mitochondrial genome of insects (Graur and Li 2000; Wakeley 1996). Due to the results, saturation for substitution possibly altered the sequence of amino acid, consequently, will influence the reliability of reconstruction of phylogenetic tree (Xia 2008). This may be solved by utilizing the third codon for phylogenetic tree reconstructions because the genetic code's redundancy has a tendency to mask the mutation at the third location, therefore, the location did not target for selection (Barry 1942). In order to prove the latter statement, the substitution saturation test was conducted utilizing only the third codon. The result can be seen in Figure 2, in which demonstrating that the third codon location was free from substitution saturated. Based on the results, the third codon location was used to reconstructed phylogenetic trees.

Genetic distance and phylogenetic analysis

Genetic distance utilizing Tamura-Nei parameter demonstrated a minimum genetic deviation within population (running from 0.001-0.005) when compare to genetic divergence between population (ranging from 0.002-0.013) for *Ae. aegypti* (Table 2). This pattern was also exhibited by *Ae. albopictus* in which the genetic deviation for intrapopulation (running from 0.001-0.004) was low compared to between population (ranging from 0.002-0.012) (Table 3). The ML and NJ tree for both species exhibited the same structure, hence only ML tree was presented. The pattern exhibited by ML tree was similar to the pattern shows by the tree in *Ae. aegypti*, in which no evidence of population structure can be concluded for both species of *Aedes* from Penang and this result was substantiated with a high bootstrap value.

The values of genetic distance (*D*) that is significantly low for both species (Tables 2 and 3) were also corroborated by the phylogenetic tree (Figure 4) in which no obvious cluster from all the districts/zones in Penang and this was supported by a high bootstrap value. This phenomenon was probably due to the strong and effectual migration event as well as gene flow between districts in Penang. Novak (1992) recorded that *Aedes* cannot fly out from the ranges which are very sparse, and they are also not been spotted flying in a strong wind. Nevertheless, the flight distance possibly expands if females unsuccessful to search for an appropriate location for reproduction and/or blood-meals, which feasibly facilitated by winds (Delatte et al. 2013). Human activities (such as used/wasted tires and containers transportation) were also proved to become one of the methods for mosquitoes and/or larval migration and movement (Novak 1992). Minakawa et al. (2002) has reported that mosquito larvae (*Anopheles gambiae*) were discovered have attached at the boat rear, and this implicates that the larvae of *Aedes* may possibly be transported from Seberang Perai (mainland), in which the boat act as an intermediary tool. The fact that no visible population structure and all species have a uniform distribution throughout all sampling locations in Penang suggests that *Ae. albopictus* start to take place the roles of *Ae. aegypti* as the predominant vector for dengue virus in Penang. Despite that, additional research on population genetics of *Aedes* will be required in order to further support this postulation.

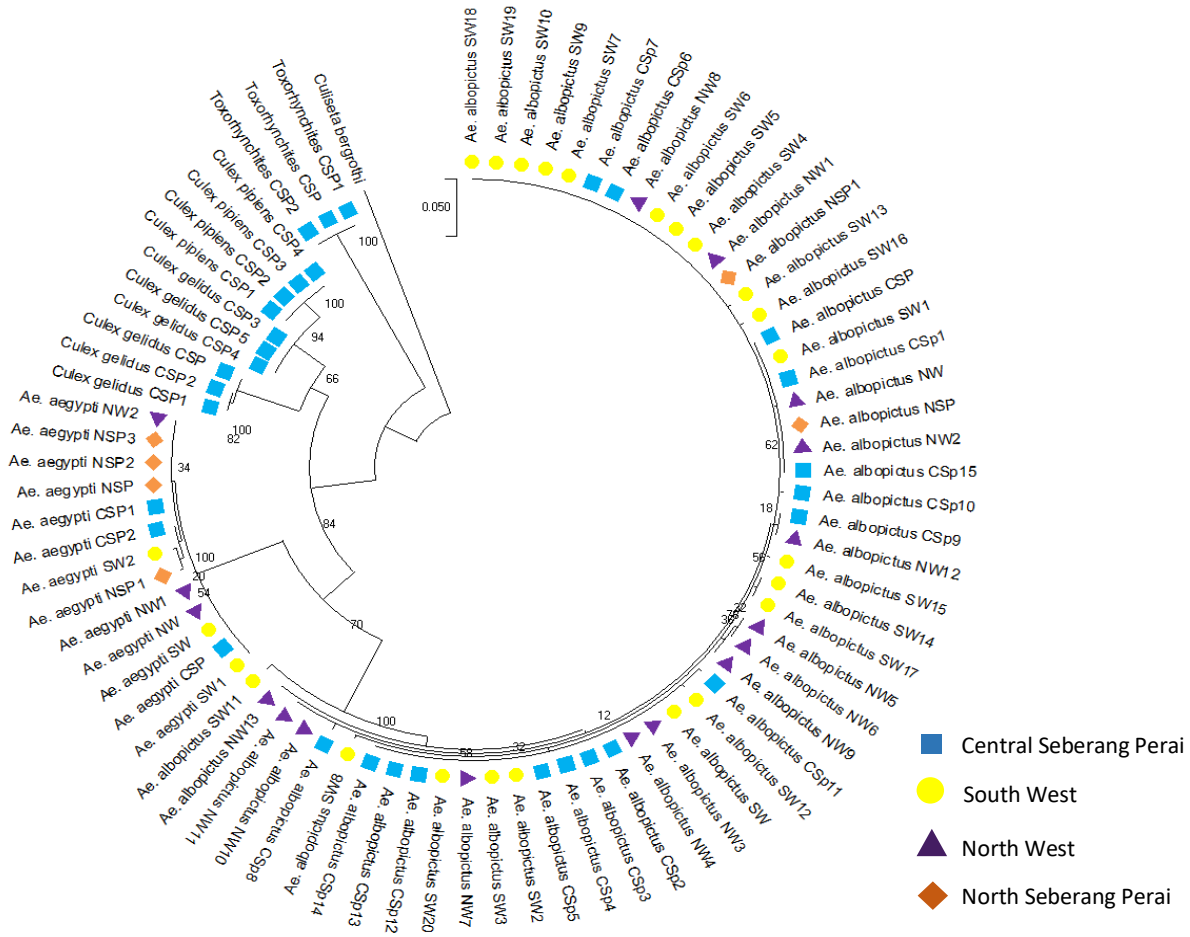

Figure 4. The history of evolutionary for all samples analyzed and was deduced utilizing the ML (Maximum Likelihood) procedure and Kimura 2-parameter (Kimura 1980), indicates that there is no obvious pattern of population distribution across Penang, Malaysia

In conclusion, as presented in this research, all mosquitoes were successfully collected and recognized based on COI gene marker. There are five different species representing three genera were recorded in Penang; *Toxorhynchites*, *Culex* and *Aedes*. This current research was also divulged that *Ae. albopictus* is the most commonly found mosquitoes and widely distributed, especially in urban area of Penang. This hypothesizes that *Ae. albopictus* starting to take place the role of *Ae. aegypti* as the principal dengue vector in Penang. Nevertheless, additional data and research are desired to support this theory. The COI has proven to be the best marker for species partitioning and recognition in which all specimens analyzed in this study demonstrated high identification match (99%) with conspecific segments from GenBank.

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