MOLECULAR IDENTIFICATION AND GENETICS STATUS OF *AEDES* MOSQUITOES IN PENANG

NUR ZAWANI BINTI MUSTAFA KAMAL

UNIVERSITI SAINS MALAYSIA

2016

MOLECULAR IDENTIFICATION AND GENETICS STATUS OF *AEDES* MOSQUITOES IN PENANG

by

NUR ZAWANI BINTI MUSTAFA KAMAL

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

June 2016

ACKNOWLEDGEMENT

Alhamdulillah, thank you Allah, finally, I have completed my master's thesis. However, I should not have done it without the help and support from important people in my life.

First of all, my deepest gratitude and appreciation to my supervisor, Dr. Darlina Md. Naim and my co-supervisor, Dr. Suhaila Abd Hamid for giving me the opportunity to be under their supervision. They have guided me through the journey to complete the project despite their tight schedule.

Million thanks to Lab 308 members who have gone through thick and thin with me. They are my listeners, my advisors, my best friends and are like my family members. Thank you Danial, Kak Adib, Lia, Nurul, Fong, Jam, Lim, Kak Mira for always being my good friends and seniors. Not to forget, the staffs of School of Biological Sciences for helping me throughout my project.

Last but not least, special thanks to my backbone- my family. They are the ones who support and understand me the most, the ones who I can put my trust without doubt, the ones who I can turn to always either sad or happy. Thank you mother, Mdm. Fauziah Tahir, father, Mr. Mustafa Kamal Ahmad, siblings- Along, Achik, Em, Adik and of course, the love of my life, Mr. Fariq Fitri. Thank you once again everyone.

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LIST OF ABBREVIATIONS

ml	Milli liter
µl	Micro liter
TNES Urea	Tris-sodium chloride- EDTA-SDS-Urea
DNA	Deoxyribonucleic acid
M	Molar
NaCl	Sodium chloride
rpm	Revolutions per minute
EDTA	Ethylenediamine tetra-acetic acid
dNTP	Dinucleotide triphosphate
U	Unified atomic mass unit
$MgCl_2$	Magnesium chloride
bp	Base pair

LIST OF SYMBOLS

%	Percentage
°C	Degree of Celcius

PENGENAL PASTIAN MOLEKUL DAN STATUS GENETIK NYAMUK *AEDES* DI PENANG

ABSTRAK

Demam denggi adalah salah satu daripada penyakit bawaan nyamuk yang telah memberi kesan kepada jutaan manusia di seluruh dunia. Kes demam denggi sedang meningkat dengan cepat di seluruh dunia termasuklah di Malaysia. Kajian telah dijalankan terhadap dua vektor utama demam denggi iaitu *Aedes aegypti* dan *Aedes albopictus* untuk membantu dalam merangka strategi terbaik untuk mengawal vektor tersebut. Kajian ini dijalankan untuk mengenalpasti spesies nyamuk di 31 kawasan Pulau Pinang dengan menggunakan penanda sitokrom oksidase subunit 1 (COI) dan untuk memeriksa struktur genetik populasi *Ae. aegypti* dan *Ae. albopictus*. Pengenalpastian menggunakan COI menunjukkan daripada 497 larva nyamuk yang telah berjaya digenotip daripada sejumlah 31 lokasi, 92.35 % adalah daripada genus *Aedes*, 7.04 % genus *Culex* dan 0.6 % genus *Toxorhynchites*. Kajian struktur genetik populasi nyamuk *Aedes* menunjukkan 39 haplotip daripada 106 sampel larva nyamuk *Ae. aegypti* dan 64 haplotip daripada 328 sampel *Ae. albopictus* yang telah dianalisa. Kepelbagaian nukleotid (π) yang rendah dan kepelbagaian haplotip (h) yang tinggi telah direkodkan pada kebanyakan populasi kedua-dua spesies nyamuk. Corak ini mencadangkan berlakunya perkembangan populasi yang boleh membawa kepada mutasi baru. Hasil ini disokong oleh ujian neutraliti; Tajima's *D* dan Fu's *Fs* yang menunjukkan hasil negatif pada kebanyakan populasi kajian. Walau bagaimanapun, analisis taburan *mismatch* menunjukkan hasil yang berbeza untuk *Ae. albopictus* dan *Ae. aegypti* di mana graf taburan bagi *Ae. albopictus* menunjukkan ciri unimodal manakala untuk *Ae. aegypti* menunjukkan ciri bimodal. Ciri unimodal menunjukkan bahawa populasi *Ae. albopictus* telah mengalami pengembangan demografi manakala

taburan bimodal memberikan hipotesis bahawa kadar migrasi *Ae. aegypti* adalah rendah. Pohon filogeni untuk kedua-dua spesies nyamuk menunjukkan nilai bootstrap yang rendah dengan tiada struktur genetik dan hasil ini disokong oleh analisis jaringan *spanning* minima yang menunjukkan percampuran haplotip pada kawasan-kawasan yang berbeza di Pulau Pinang. Hipotesis pembahagian filogeografi yang terhad telah dikuatkan oleh jarak genetik yang rendah dalam dan antara populasi dan nilai *pairwise* F_{ST} yang telah menunjukkan tiada perbezaan bermakna kecuali di Medan Mahsuri. Analisis kepelbagaian molekul (AMOVA) merumuskan bahawa kebanyakan variasi mtDNA berlaku antara sampel di dalam populasi. Oleh itu, hasil kajian memberi kesimpulan bahawa tiada struktur populasi genetik nyamuk *Ae. aegypti* dan *Ae. albopictus* di 31 kawasan di negeri Pulau Pinang. Kajian lanjut perlu dilakukan dengan menggunakan penanda genetik berbeza contohnya penanda mikrosatelit bagi mengkaji struktur populasi genetik nyamuk *Aedes* di Pulau Pinang. Kajian gen ketahanan racun serangga juga perlu dijalankan bagi mengetahui sama ada nyamuk *Aedes* di Pulau Pinang masih boleh dihapuskan menggunakan bahan kimia tertentu ataupun sebaliknya.

MOLECULAR IDENTIFICATION AND GENETICS STATUS OF *AEDES* MOSQUITOES IN PENANG

ABSTRACT

Dengue fever is one of the mosquito-borne diseases affecting millions of people worldwide. The cases of dengue fever are increasing rapidly around the world including in Malaysia. Research has been done towards the two main vectors of dengue fever which are *Aedes aegypti* and *Aedes albopictus* to help in designing the best vector control strategy. The present study was conducted to identify mosquitoes species in 31 locations in Penang using cytochrome oxidase subunit 1 (COI) marker and to examine the population genetics structure of *Ae. aegypti* and *Ae. albopictus.* Identification using COI marker showed that, out of 497 mosquito larvae that were successfully genotyped from 31 locations, 92.35 % were from the genus *Aedes,* 7.04 % from the genus *Culex* and 0.60 % from *Toxorhynchites*. Study on population genetics structure of *Aedes* mosquitoes revealed 39 haplotypes from 106 larvae samples of *Ae. aegypti* and 64 haplotypes out of 328 samples of *Ae*. *albopictus* were analyzed. Low nucleotide (π) and high haplotype diversity (h) was recorded in most of the populations for both mosquito species. This pattern suggested the occurrence of population expansion which can lead to retention of new mutations. The result was supported by neutrality test; Tajima's *D* and Fu's *Fs* which showed negative results for most of the populations studied. However, mismatch distribution analysis showed different results for *Ae. albopictus* and *Ae. aegypti* where the mismatch distribution graph showed unimodal and bimodal characteristics respectively. Unimodal characteristic indicated that the *Ae. albopictus* population has undergone recent demographic expansion while bimodal distribution hypothesized that the migration rate of *Ae. aegypti* is low. The phylogenetic tree for both species showed a low

bootstrap value with no genetic structure and the result was further corroborated by minimum spanning network analysis which showed the mixture of haplotypes in different locations in Penang. The hypothesis of limited phylogeographic partitioning is strengthen by low genetic distance within and between population and pairwise F_{ST} values which showed no significant difference except for some Medan Mahsuri. Analysis of molecular variance (AMOVA) also revealed that most of the mtDNA variation occurred among samples within population. Thus, all of the results concluded that there is no population genetics structure of *Ae. aegypti* and *Ae. albopictus* in 31 locations in Penang. Further research should be done by using other genetic markers such as microsatellite marker to investigate the population genetics structure of *Aedes* mosquitoes in Penang Research on insecticide resistance gene also shoul be carried out to investigate whether *Aedes* mosquitoes in Penang area still can be eliminated by using chemicals or otherwise.

CHAPTER ONE

INTRODUCTION

Mosquitoes, the two winged-flies are from family Culicidae and belong to the order Diptera. From the family, mosquitoes are the most diverse in tropical forest environments (Harbach, 2008). There are altogether 3 500 species of mosquitoes occupy almost every continent. Some species of mosquitoes are very dangerous and caused mosquito-borne diseases worldwide. They severely affected children and adolescents and increase mortality rate. For example, malaria kills more than one million children every year, mostly in sub-Saharan Africa and Japanese encephalitis that has expanded its widespread in the Indian subcontinent and Australasia. 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 of the worldwide (Alshehri, 2013).

Dengue fever caused the highest mortality threat due to viral infection in more than half of the world (Goswami *et al*., 2012). The World Health Organization (WHO) estimates that more than 2.5 billion people (over 40% of the world's population) 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 four viral serotypes (DEN-1, DEN-2, DEN-3 and DEN-4). Dengue is transmitted from viraemic to susceptible humans mainly by bites of *Ae. aegypti* and *Ae. albopictus* mosquitoes (Guha-Sapir & Schimmer, 2005). The symptoms include headaches, bone or joint and

muscular pains, rash and leucopenia. Serious cases will lead to dengue hemorrhagic fever (DHF), which is characterized by high fever, hemorrhagic phenomena, often with hepatomegaly and in severe cases, signs of circulatory failure. The worst case is dengue shock syndrome (DSS) that can lead to death (WHO, 2005).

Ae. 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 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).

Several studies have been conducted on *Ae. aegypti* and *Ae. albopictus* in Malaysia. Yap (1975) investigated the distribution of these species in small towns and villages of Penang Island. The result showed that *Ae. albopictus* was present in most of the locations while *Ae. aegypti* was found only in one location. The current status of these species in Penang is left unknown. A study was conducted by Rahman *et al*. (2013) on temporal and spatial distribution of dengue vector mosquitoes and their habitat patterns in Penang Island, Malaysia. They collected the samples from three areas; rural, suburban and urban areas. The results showed that among the three areas, rural areas produced the highest container index, followed by suburban and urban areas. Other study by Lee and Rohani (2005) investigated transovarial transmission of dengue virus

in both *Ae. aegypti* and *Ae. albopictus*. Results showed that the transovarial transmission of dengue virus in *Ae. albopictus* often occurred prior to the reporting of human cases while transmission in *Ae. aegypti* appeared to maintain or enhance the epidemics.

However, studies on population genetics structure of *Ae. aegypti* and *Ae. albopictus* in Malaysia has not yet being documented. Understanding the population structure of dengue vectors in Penang area is important as characterization of the population genetic structure will help to define the level of population structuring (Riehle *et al*., 2011) and defining the scale on which vector control using area-wide techniques is likely to be most effective. The population structure is relevant to evolution of mosquitoes as thus can help in development and enhancement of dengue control strategies in Malaysia. Understanding the gene flow of the species can help to design application strategies that are less susceptible to mosquito movement (Brelsfoard & Dobson, 2012). The use of cytochrome oxidase subunit 1 (COI) gene marker in this current study is useful in mosquitos' identification through molecular techniques as the COI has been used widely in species identification (Hebert *et al*., 2003). The marker has rapid evolution and can distinguish not only amongst strongly related species but also amongst phylogroups belongings to the same species (Hebert *et al*., 2003). Thus, COI marker was used in this study to achieve the objectives of species identification and genetics study.

Considering the lack of information and documentation of the genetic knowledge of mosquitoes species in Penang, the main objectives of this thesis were:

1. To identify mosquitoes' species in Penang areas by using molecular method based on COI mitochondrial DNA marker.
2. To investigate the population genetics of two common species of *Aedes* mosquitoes in Penang; *Ae. aegypti* and *Ae. albopictus* using COI mitochondrial DNA marker.

CHAPTER TWO

LITERATURE REVIEW

2.1 Dengue Fever Status

Dengue fever cases are increasing worldwide. According to World Health Organization (2011), over 100 million cases of dengue fever were reported annually and 500 000 cases of dengue hemorrhagic fever (DHF) requires hospitalization. It was estimated that 2.5 to 3 billion individuals living mainly in urban areas in tropical and subtropical regions were at risk. The expansion of dengue is expected to increase due to factors such as dynamics of climate change, globalization, travel, trade, socio economics, settlement and also viral evolution (Murray *et al*., 2013). Dengue virus is believed to propagate to Southeast Asia during the second World War as the urbanization after the war has provided suitable conditions for virus transmission (Ooi & Gubler, 2009). The disease has also spread to rural areas despite the thought that it is epidemic to urban areas due to modernization in transport system, piped water supply and solid waste disposal (Bhatia *et al*., 2013),

The first dengue hemorrhagic fever outbreak in Southeast Asia has occurred in Manila, Philippines in 1953/ 1954 (Ooi & Gubler, 2009). Other early outbreak was recorded in Bangkok, Thailand in 1958 (Ooi & Gubler, 2009) and in Malaysia in 1962 (George, 1987). The dengue epidemics cycle that started in Southeast Asia since 1950s have continued until the present time with epidemic being larger in magnitude than before (Ooi & Gubler, 2009). A total number of 386 154 cases of dengue fever was

recorded in 2001 to 2010 in Southeast Asia where Indonesia recorded the highest cases of dengue (104 457) followed by Thailand (76 978), Vietnam (76 364), Philippines (45 409) and Malaysia (37 886) (Shepard *et al*., 2013).

2.1.1 Dengue status in Malaysia

Malaysia is located in the equatorial doldrum area with uniform temperature, high humidity and copious rainfall (Malaysian Meteorological Department, 2013). As a developing country, Malaysia has 28.3 million populations with the population density of 86 persons per square kilometer (Department of Statistics Malaysia, 2010). According to clinical practice guidelines published by Ministry of Health Malaysia in 2010, dengue cases in Malaysia are increasing each year from 1995 to 2007. In recent years, dengue fever cases continue to increase where in 2014 showed the most cases of dengue outbreak in Malaysian history. Statistics showed that up to 23 August 2014, the cumulative number of cases recorded was 65 672, increased by 263% compared to 2013 (MOH, 2014).

Figure 2.1 shows the incidence and fatality rate of dengue fever from 2000 to 2014. The cases of dengue fever showed a drastic increment although the fatality rate decrease. The fatality rate may decrease due to the development in medical field and public awareness on their health and dengue fever risks. However, there are still lack of awareness regarding the need to control the spreading of dengue vector through elimination of *Aedes*' breeding sites.

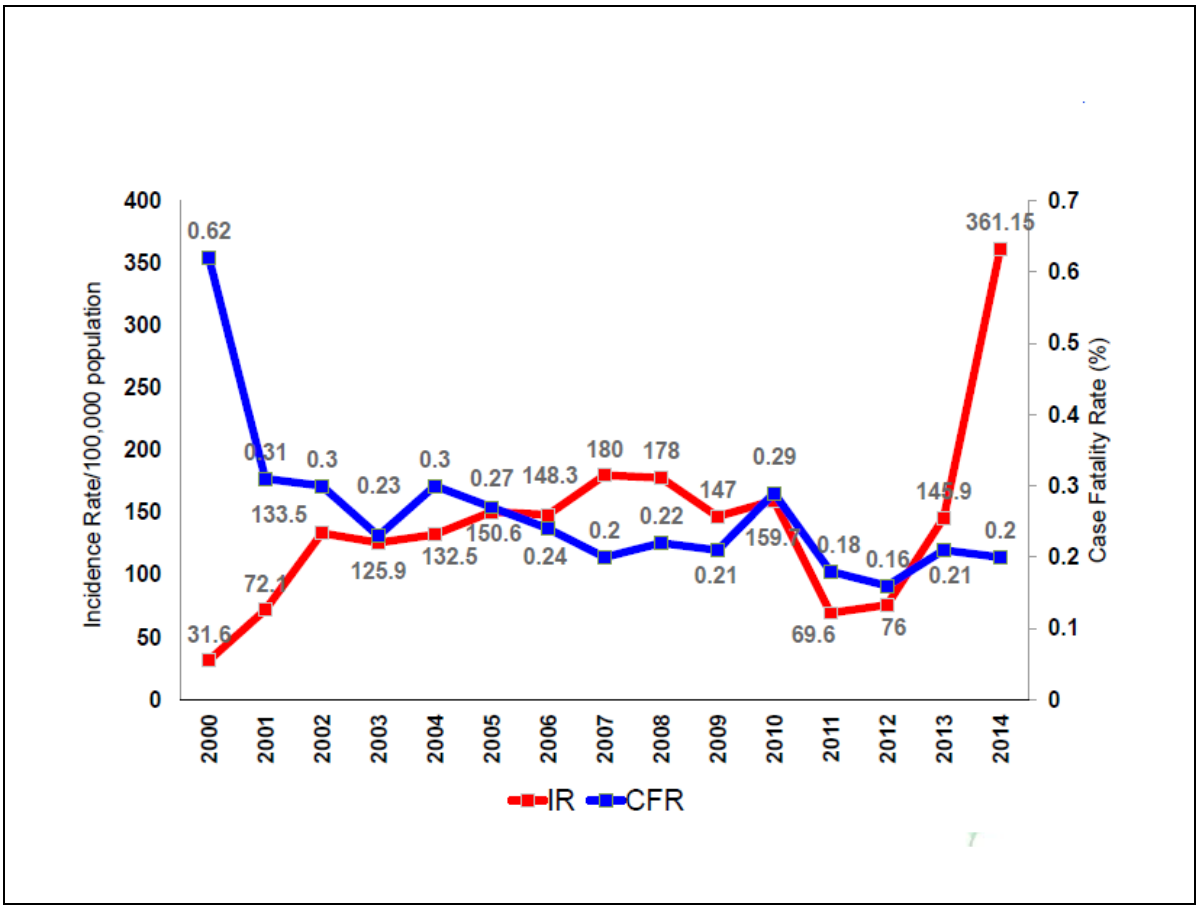

Figure 2.1: Malaysia dengue rate and case fatality rate for year 2000 to 2014.
Source: idengue.remotesensing.gov.my

Malaysia government through Ministry of Health (MOH) has carried out several methods to control dengue vector including campaigns to build public awareness and fogging to kill *Aedes* mosquitoes. Various research also has been conducted through Institute for Medical Research (IMR) on dengue virus and *Aedes* species for example dengue serotype surveillance among dengue patients (Ab-Fatah *et al*., 2015), *Ae. albopictus* breeding preferences (Rozilawati *et al*., 2015) and eco-virological survey of Aedes mosquito larvae (Rohani *et al*., 2014).

2.1.2 Dengue status in Penang

Penang was the first state in Malaysia being affected by dengue fever. The first case of dengue fever was recorded in Penang in early December 1901 which was believed to be introduced in Penang from Singapore in the end of November or early December (Skae, 1902). George (1987) reported the first dengue epidemic occurred in Penang in 1962 before spreading to Selangor in 1974 and other states. This shows that Penang is the pioneer state for dengue fever disease.

Geographically, Penang is located on the northwest coast of the Malaysia peninsula (Tahir *et al*., 2010) with a population of 1.6 million (Department of Statistics Malaysia, 2010). Penang is one of the states that have the densest population per square kilometre (1490 persons) (Department of Statistics Malaysia, 2010). It is a unique state that composed of two parts: the island and the mainland (known as Seberang Perai. The area of Seberang Perai covered an area of approximately 1030 km^2 where the island covers an area of 285 km^2 (Lee & Biswajeet, 2006). In the past few decades, Penang has become a well known industrialized area, shipping route, urbanized and tourism destination in Southeast Asia (Sakari *et al*., 2008) which explained the transmission of dengue fever to Penang from Singapore in early 1900.

Figure 2.2 shows the cases of dengue fever in Penang from 2010 to 2015. The number of cases increased drastically in 2015 compared to the same timeline in previous years. To date, Penang is the fourth state with the highest cases of dengue fever after Selangor, Johor and Kuala Lumpur. Up to the the second week of February, Penang recorded 1026 dengue cases (MOH, 2016). The unique geography of Penang (comprised

of island and mainland) and high number of dengue cases made Penang a suitable location for studies of *Aedes* mosquitoes..

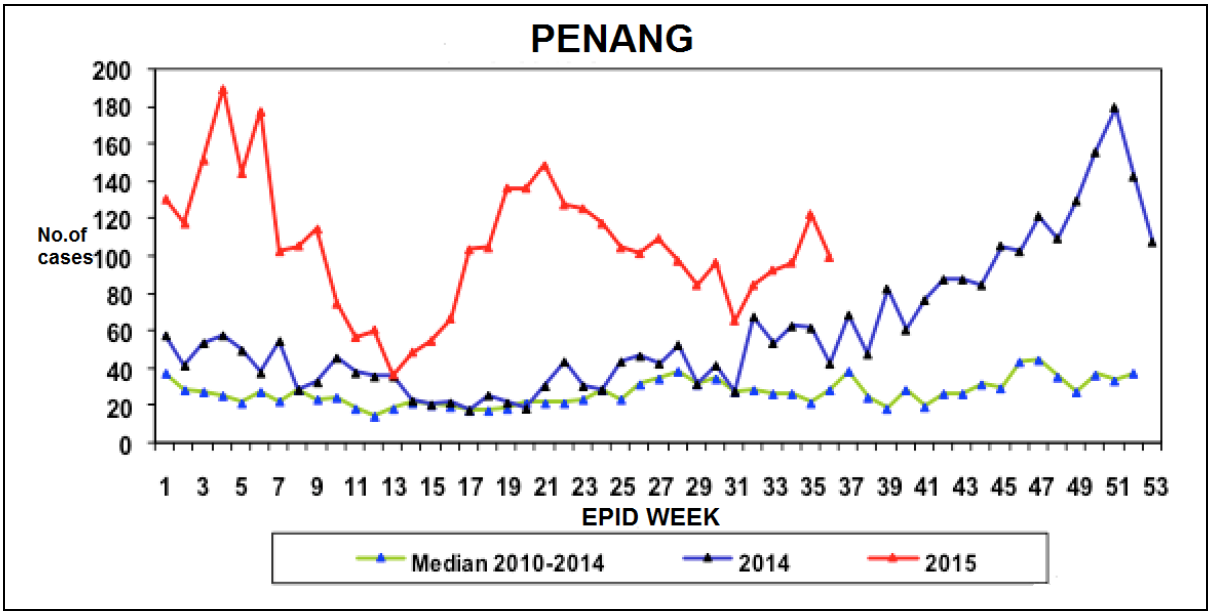

Figure 2.2: Dengue cases in Penang by week from 2010 to 2015.
Source: idengue.remotesensing.gov.my

2.2 Biology of *Aedes* mosquitoes

Dengue virus is a mosquito-borne disease spread by *Aedes* mosquitoes species (WHO, 2011) and the most common vectors for dengue found in Penang are *Ae. aegypti* and *Ae. albopictus*. *Aedes* mosquito comes from Order Diptera, Family Culicidae with Genus *Aedes* (Integrated Taxonomic Information System). Both species of *Ae. aegypti* and *Ae. albopictus* are included in *Stegomyia* (Theobald, 1901), a tropical subgenus with more than 90 species and eight major groups (Leahy & Craig, 1967). While these two species are apparently similar, they are not closely related (Leahy & Craig, 1967). Some differences between the two species include the origin, habitats and morphological characteristics.

2.2.1 Origin

Ae. albopictus and *Ae. aegypti* come from two different origins. *Ae. albopictus* (Skuse) (Diptera: Culicidae) or also known as the Asian tiger mosquito originally came from Southeast Asia (Watson, 1967). Apart from being a vector for dengue fever, it is an epidemiologically important vector for the transmission of other viral pathogens causing chikungunya and yellow fever. *Ae. aegypti* on the other hand originates from Africa and disperse to many areas in the tropics aided by the increasing slave trade in the 16th and 17th century (Lounibos, 2002). Apart from being a vector for dengue virus, *Ae. aegypti* also is one of the vectors for yellow fever (Christopher, 1960). Both species are day feeders (Bloemer, 2009) and to date, co-occur in many regions of the world (Kaplan *et al*., 2010).

2.2.2 Breeding habitat

Ae. aegypti is a tropical and sub-tropical species, thus this mosquito prefers warmer climates (Bloemer, 2009). Rainfall and high humidity environment are advantageous for its activity and propagation (Cheong, 1967). *Ae. aegypti* tends to congregate in urban environment, close to humans, whereas *Ae. albopictus* cluster more in semi-urban and rural habitats (Bloemer, 2009).

According to Bloemer (2009), *Ae. aegypti* and *Ae. albopictus* differ slightly in breeding habitats. *Ae. aegypti* are artificial container breeders such as flowerpots, vases and water storage containers while *Ae. albopictus* prefers to breed in natural container such as in a tree hole. In Malaysia, Cheong (1967) has conducted a study to investigate

specifically on the larval habitats of *Ae. aegypti* in urban areas in Malaysia. The result showed that *Ae. aegypti* preferred to breed mostly in ant traps followed by earthenware jars, storage drums, bathtubs, cans, pots and tires.

Another study has been carried out by Chan *et al*. (1971) on the breeding habitats of *Ae. aegypti* and *Ae. albopictus* in Singapore. Result showed that both species breed mostly in domestic containers but specifically, *Ae. aegypti* breeds in ant traps, earthenware jars, bowls, tanks, tin cans and drums consequently while *Ae. albopictus* was commonly found in earthenware jars, tin cans, ant traps, rubber tires, bowls and drums. In summary, most of the breeding habitats of *Ae. aegypti* were found indoors while only half of *Ae. albopictus* breeding habitats were indoors (Chan *et al*., 1971). However, the results may differ according to the life style of the citizens and the type of housing areas (Chan *et al*., 1971). It is important to investigate about the breeding habitat of the species as it may help in vector control program of vector-borne diseases.

2.2.3 Life cycle

Mosquitoes have four distinctive stages of life cycle, namely the egg, larva, pupae and adult stages (Jones, 2011) (Figure 2.3). Female mosquitoes laid eggs in water or damp surfaces where the eggs will float on them (Becker *et al*., 2010). In general, the eggs of both species are black and oval with a length of 0.5 mm and can withstand desiccation up to one year (Hawley, 1988). After two days, the eggs will hatch and turn into the larval stage (Jones, 2011). Larvae are active feeders where they feed on fine particulate organic matter in the water to live. The larvae need oxygen, thus they must periodically come to the water surface to breathe using siphon (Leslie & James, 2004).

Larvae develop from the first instar to the fourth instar larvae through molting. Their development is temperature dependent, but the process usually took up to five to ten days before the larvae pupate (Hawley, 1988).

The pupae are active but do not feed on anything (Leslie & James, 2004). Thus, the insect can be seen lying on the surface of water where it breathes through its two breathing tubes known as trumpets (Jones, 2011). The pupal stage will last for one to four days before they turn into adults depending on the species and temperature (Jones, 2011). Adult mosquitoes will wait on the surface of the water for its body to dry and harden before being able to fly (Jones, 2011). Adult female mosquitoes are more aggressive than males as they attack humans and animals to develop eggs while male mosquitoes only feed on the nectar of flowers (Jones, 2011).

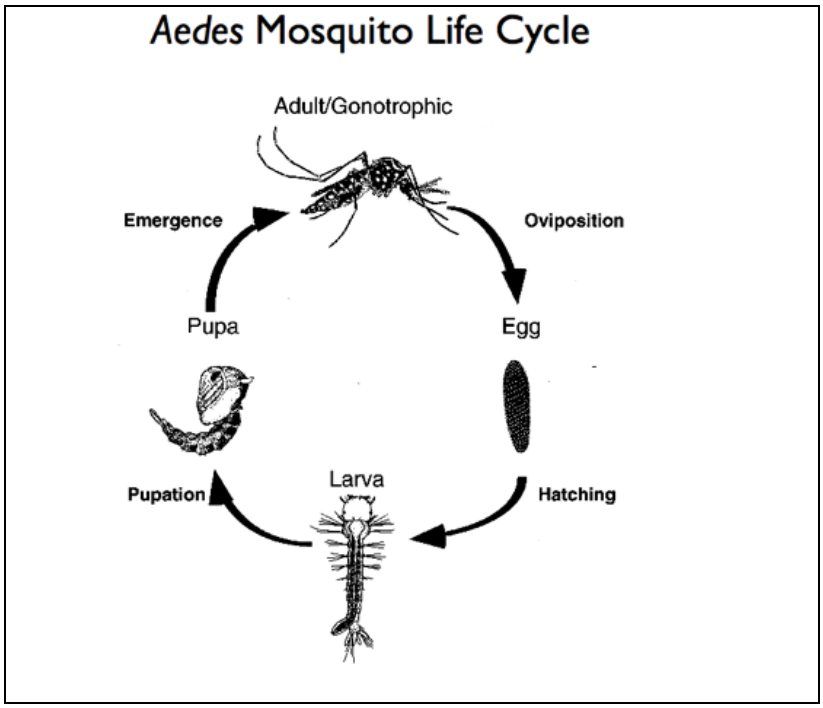

Figure 2.3: Life cycle of mosquitoes
Source: Hopp & Foley (2001)

2.2.4 Morphological characteristics of *Aedes* mosquitoes

Morphological differences of *Ae. aegypti* and *Ae. albopictus* can be seen through three different stages of life cycle, the egg, larvae and adult. Suman *et al.* (2011) investigated the egg morphology of *Ae. aegypti* and *Ae. albopictus* (Figure 2.4).and found that the eggs of *Ae. aegypti* and *Ae. albopictus* to be cigar shaped, shiny jet black with slight dorso-ventral curvature and tapered at ends, however, *Ae. albopictus* eggs are more tapered posteriorly (Suman *et al*., 2011). Eggs of *Ae. aegypti* are significantly longer and broader than those of *Ae. albopictus* (Suman *et al*., 2011).

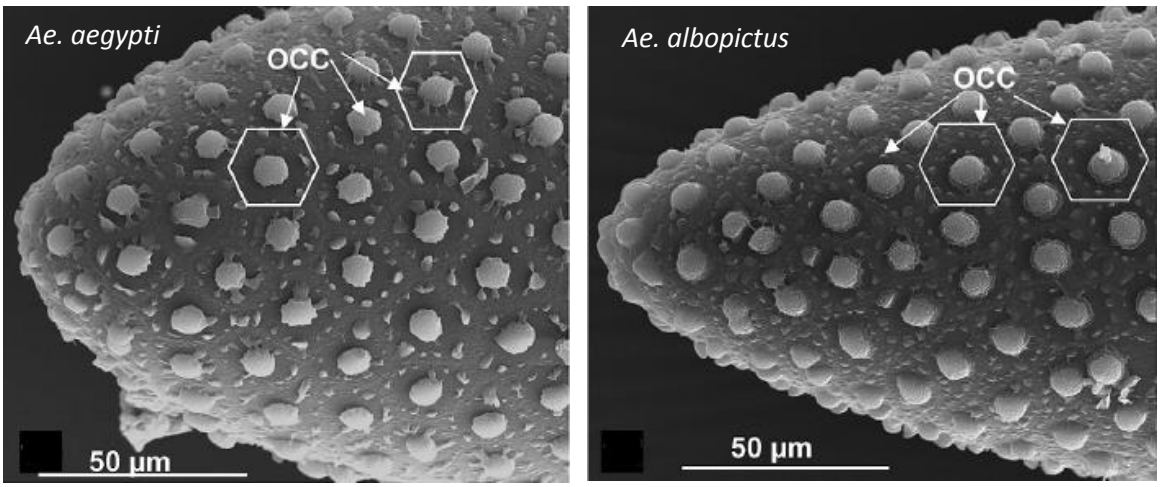

Figure 2.4: Scanning electron micrograph showing posterior region of egg of *Ae. aegypti* and *Ae. albopictus*. OCC, outer chorionic cell
Source: Suman *et al*. (2011)

The larvae of both species also showed different morphology in some parts. Rueda (2004) has identified several pictorial keys to differentiate *Ae. aegypti* and *Ae. albopictus* larvae (Figure 2.5). *Ae. aegypti* larvae has eight to 12 large strong teeth, with well developed lateral denticles while *Ae. albopictus* without lateral denticles. Another difference is the thorn-like structure at the bases of pleural hairs on mesothorax and

meta-thorax of *Ae. aegypti* larvae ended in a single point while for *Ae. albopictus* larvae, they ended in several points. The inner sub-median caudal hair of *Ae. aegypti* has two to four branches while *Ae. albopictus* has single branch or may split into two. In the presnt study, the larvae was not observed morphologically as the larvae is very small and parts of the body may disassociate when preserve in alcohol.

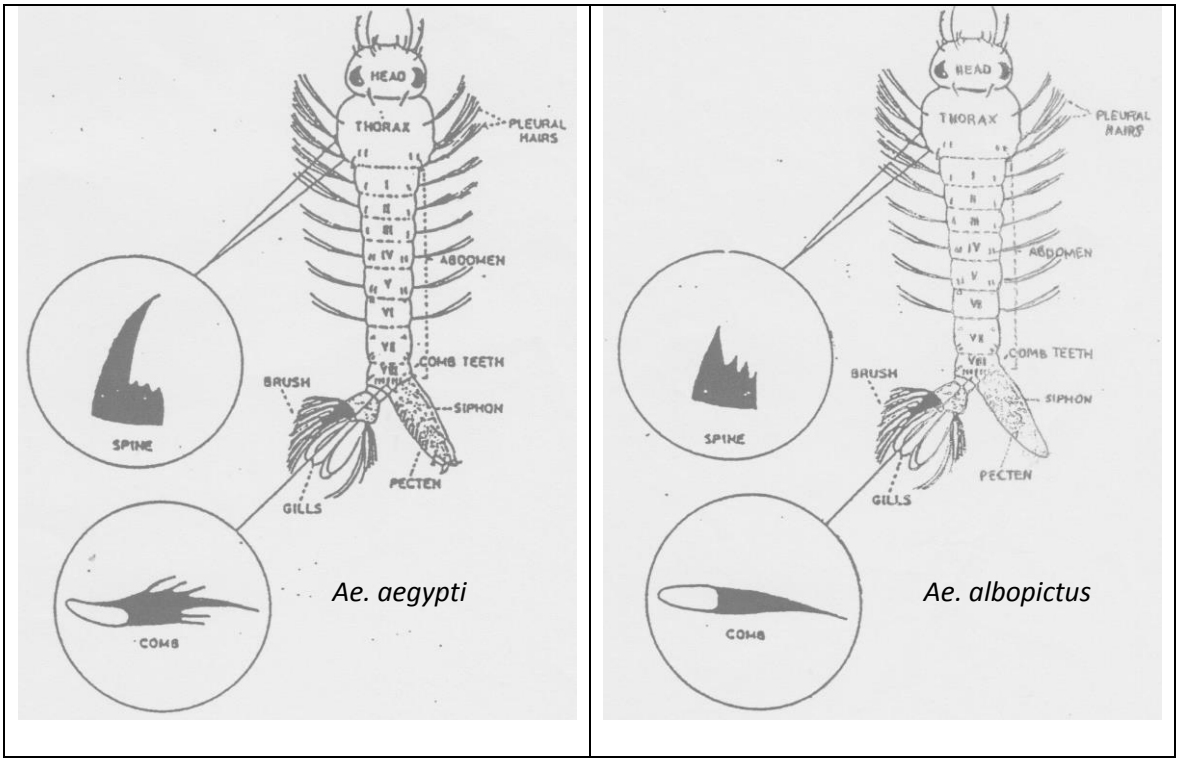

Figure 2.5: Differences in morphological characteristics of *Ae. aegypti* larvae and *Ae. albopictus* larvae shown through their spine and comb shape.
Source: Rueda (2004)

Figure 2.6 shows the morphology of adult *Ae. aegypti* and *Ae. albopictus*. Generally, the adult mosquitoes of subgenus *Stegomyia* are small to medium size, black to dark in colour and highly ornamented with patches, spots, or lines of snow-white scales The proboscis is black with two or more basal white bands on tarsi of at least one pair of legs or one or more tarsal segments completely white (Rueda, 2004). The most obvious body part that can be differentiated between both species is the thorax. *Ae.*

aegypti has black or brown scutum with a pair of submedian-longitudinal white stripes or white lyre-shaped markings but without median-longitudinal white stripe while *Ae. albopictus* has scutum with a narrow median-longitudinal white stripe.

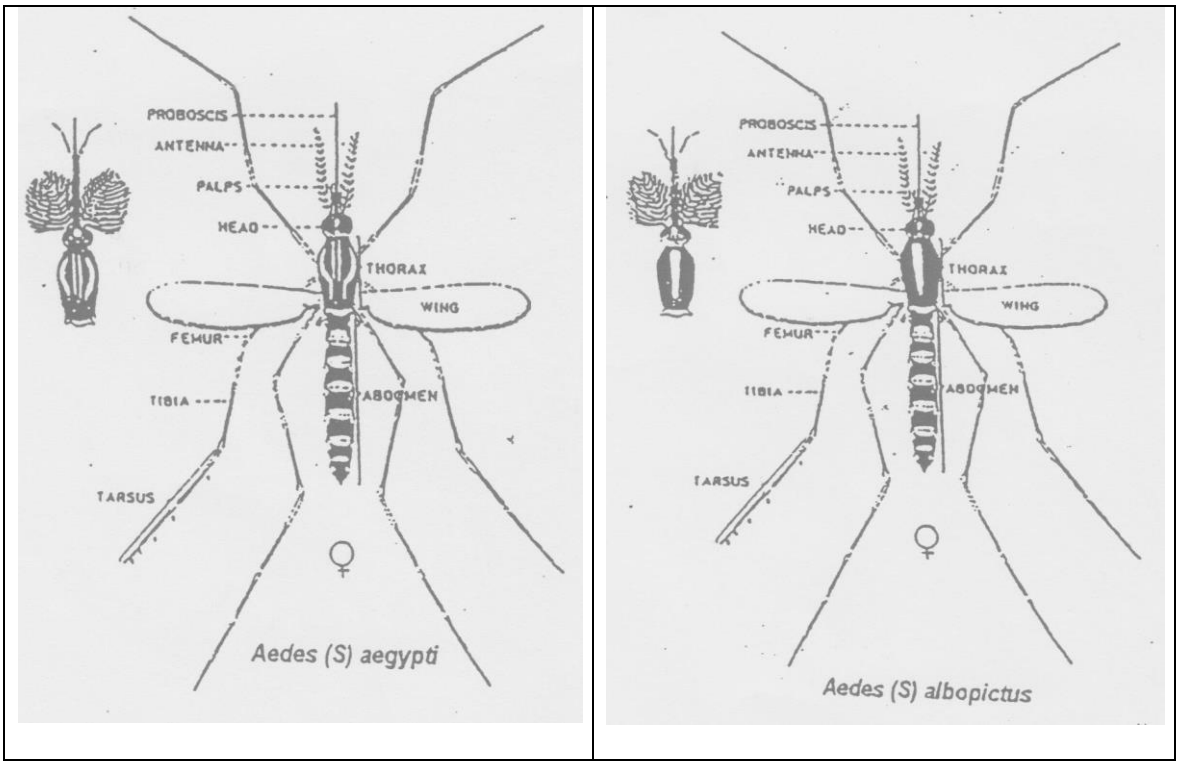

Figure 2.6: Morphological differences between adult *Ae. aegypti* and adult *Ae. albopictus* are shown clearly by the thorax white strip.
Source: Rueda (2004)

2.3 Population genetics study of *Aedes* mosquitoes

Population genetics is a study of evolution. It uses a well developed and ever growing body of theoretical knowledge that allows quantitative predictions (Cavalli-Sforza, 1998). Genetic polymorphisms transmitted in strict mendelian fashion gives useful information where the use of available markers is the key to the analysis (Cavalli-Sforza, 1998). Avise (2000) stated that molecular phylogeny and population genetics study can reveal evidence of past biogeographic events and suggest life history traits that

contribute to shape the distribution of genetic variation among populations. Such studies give information about genetic variation and by applying genetic model, one can make inferences about the biology of organisms (Sunnucks, 2000). 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 & Preet, 2014) especially in Malaysia.

Thus, current study focuses on the population genetics of *Ae. albopictus* and *Ae. aegypti*. These two species have brought considerable interest in multiple studies such as vector competence, insecticide resistance, ecological and evolutionary studies, spatial, temporal and geographical analyses and population genetics study (Kaplan *et al*., 2010). The study of population genetics could provide significant information about dispersal and population dynamics of those species (Gupta & Preet, 2014).

Research on population genetics of *Ae. aegypti* was done in Brazil by Scarpassa *et al*. (2008) using COI marker. The samples were collected from four regions of Brazil and results suggested that the populations probably came from East and West Africa. In some areas, they found moderately high levels of genetic variability and evidence of multiple introductions that enhance the need of constant surveillance by local and regional health authorities for dengue prevention.

Another study by Kamgang *et al*. (2011) used COI marker and NADH dehydrogenase subunit 5 (ND5) to investigate the genetic structure of *Ae. albopictus* in Cameroon. The study suggested that *Ae. albopictus* invasion of Cameroon involved

multiple introductions from tropical sources. More studies were done on the population genetics of these two species in other countries. However, in Malaysia, research on the population genetics of *Ae. aegypti* and *Ae. albopictus* is still not being carried out. 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.

2.4 Mitochondrial markers in population genetics study

The selection of molecular markers is important in population genetics study to understand the history and evolution of the species. The use of genetic markers with appropriate rates of change and therefore, suitable signals, will offer information about almost any population and evolutionary process through the hierarchy of life (Sunnucks, 2000). DNA has been used instead of proteins or other gene products as it carries more information and in principle, the molecular techniques are the same for any segment of DNA and there is a great number of genetic polymorphisms (Cavalli-Sforza, 1998).

Mitochondrial DNA (mtDNA) has been the most widely used marker for population history and diversity (Hurst & Jiggins, 2005; Ballard & Whitlock, 2004; Avise *et al*., 1987). Mitochondrial DNA can be easily amplified from a variety of taxa including insects (Hurst & Jiggins, 2005) and can be extracted even from small or

degraded samples (Waugh, 2007). Mitochondrial DNA is haploid and maternally inherited (Avise, 2004; Wilson *et al*., 1985) with high evolutionary rate and effective population size about one-quarter that of nuclear markers, allowing a chance of recovering the pattern and tempo of recent historical events without an extensive sequencing effort (Hurst & Jiggins, 2005). The maternal inheritance characteristic of mtDNA makes it very sensitive to bottlenecks in population size and population subdivision (Wilson *et al*., 1985). Mitochondrial DNA also known to have low recombination rate, thus the whole molecule can be assumed to have the same genealogical history (Hurst & Jiggins, 2005). Mitochondrial DNA also revealed significant variation among taxa in mtDNA sequence dynamics, gene order and genome size from a diversity of animal groups studied (Harrison, 1989). All of these have made mtDNA as the marker of choice in many population and phylogenetic studies. This current study also use mtDNA as genetic marker or specifically cytochrome oxidase subunit 1 (COI), one of the region in mtDNA.

Cytochrome oxidase subunit 1 (COI) gene is one of the most favored markers for population genetic and phylogeography studies across the animal kingdom (Avise, 1994) and has been widely used in population genetics study of mosquitoes species such as *Culex* and *Aedes* species (Barbosa *et al*., 2014; Pfeiler *et al*., 2013; Raharimalala *et al*., 2012; Kamgang *et al*., 2011) compared to other mitochondrial markers.

Apart from being a tool for population genetics study, COI is also a core fragment for DNA barcoding (Hebert *et al*., 2003). A study on COI gene as marker for species identification has been conducted by Hebert *et al*. (2003) in which the study

showed that the information content of COI is sufficient to enable the placement of organisms in the deepest taxonomic ranks and help in the initial depiction of the species.

2.5 Controlling *Aedes* mosquitoes

In the current situation of dengue fever, it is important to control the spreading of dengue vector; *Ae. albopictus* and *Ae. aegypti*. Various ways have been used in controlling *Aedes* mosquitoes including the use of insecticides and biological control means (Cheong, 1967). Some examples of the insecticide used are temephos, malathion and permethrin (Ponlawat *et al*., 2005). However, effective and sustainable *Aedes* mosquitoes control could not be achieved by reliance on space spraying with insecticides targeted at the adult mosquito (Gubler & Clark, 1996) as insects have the potential to develop insecticide resistance thus making it difficult to comprehend (Cheong, 1967). Ponlawat *et al*. (2005) investigated the insecticide susceptibility of *Ae. aegypti* and *Ae. albopictus* across Thailand. Results showed that *Ae. aegypti* were resistant to permethrin but susceptible to malathion. Temephos resistant was detected in *Ae. aegypti* in most of the sampling sites whereas *Ae. albopictus* showed low levels of resistance to all three insecticides for most of the places.

The use of insecticide also may cause accidental poisoning (Cheong, 1967). Jirakanjanakit *et al*. (2007) has conducted a study on insecticide resistance status in both *Ae. albopictus* and *Ae. aegypti* in Thailand and the results revealed a focal susceptible/resistance profile in the country.

Biological control is an alternative method to the use of insecticides. It is defined as introducing or manipulating organisms to suppress vector populations (Chandra *et al*., 2008). Biological control include uses of predators, fungi, bacteria, protozoa, parasites, sterile males or genetically incompatible specimens (Cheong, 1967). Chandra *et al.* (2008) discussed the use of larvivorous fish as biological agent for mosquito control in urban and semi urban areas as they feed on immature stages of mosquitoes where several parameters were listed. The parameters suggested are fish must be small, hardy and capable of getting about easily in shallow waters among thick weeds where mosquitoes find suitable breeding grounds, drought resistant, live in drinking water tanks and pools without contaminating the water, breed freely and successfully in confined waters and not attractive to other predators.

Research has been carried out to find the best methods to control *Aedes* mosquitoes (Arunachalam *et al*., 2012; Silva *et al*., 2008; Hemingway *et al*., 2006; Gubler & Clark, 1996; Lofgren *et al*., 1970). Silva *et al*. (2008) has investigated the effects of essential oils on *Ae. aegypti* larvae as an alternative to environmental friendly insecticide. The oils were extracted from leaves of *Hyptis fruticosa*, *Hyptis pectinata* and *Lippia gracilis*. They showed potent insecticidal effect against *Ae. aegypti* larvae. Another study by Arunachalam *et al*. (2012) adopted eco-health methods on community-based control of *Ae. aegypti* in Chennai City, India. The approaches include clean-up campaigns, provision of water container covers through community actors and dissemination of dengue information through school children.

However, distributions of *Aedes* mosquitoes were influenced by human and environmental factors (Walker *et al*., 2011) which is differ in each countries. To control *Aedes* mosquitoes, one must understand the mosquitoes itself in term of their evolution and genetics to design the best control strategies for dengue vector. This is the significant reason of the current study of population genetics of *Aedes* mosquitoes in Penang areas.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sampling activities

Mosquito larvae (fourth instar) were collected from 31 locations over four districts in Penang. The coordinate (latitude and longitude) for each sample and date of collection were recorded and listed in Table 3.1 while Figure 3.1 shows the map of all the locations. The four districts were Central Seberang Perai (SPT), North Seberang Perai (SPU), North East (NE) and South West (SW) of Penang. Central Seberang Perai consists of eight sampling locations, SPU three locations, NE 10 locations and SW 10 locations.

Larvae were collected using ovitrap and empty cans from October 2012 to July 2014, where 10 cans per location were left for seven days before being collected. Only third instar and fourth instar larvae were used in this study. The sampling was done in outdoor environment near trees, bushes and drains during daytime in housing areas except for samples from Bagan Dalam and Pokok Sena which were sampled indoor with the consent from the house owner. The sampling was done once in each location. The larvae were preserved in 70% ethanol before DNA extraction. Larvae of *Ae. albopictus* and *Ae. aegypti* can be differentiated morphologically based on the head and the abdomen (siphon, setae, comb scale) (Chung *et al*. 1997). However, due to some difficulties (the larvae is very small and parts of the body may disassociate when preserve in alcohol), polymerase chain reaction (PCR) method was used to determine the species identity.

Table 3.1: Sampling localities, site abbreviations (abbrev.), coordinates (latitude and longitude), and collection date

Localities	Abbrev.	Latitude (N)	Longitude (E)	Collection date
North East				
Pengkalan Quay	WQ	5°24'45	100°20'19	22/07/2014
Gat Lebuh Macallum	GLM	5°24'24	100°20'00	22/07/2014
Hamna	H	5°20'51	100°18'02	02/10/2012
Sungai Nibong Kecil	SNK	5°19'53	100°17'14	16/07/2013
Bukit Jambul	BJ	5°20'14	100°17'01	16/07/2013
Taman Tun Sardon	TTS	5°22'16	100°18'25	11/01/2013
Universiti Sains Malaysia	USM	5°21'26	100°18'26	01/11/2012
Tingkat Sungai Gelugor	TSG	5°22'12	100°18'39	11/01/2013
Tanjung Bungah	TB	5°27'01.	100°17'34	25/10/2012
Batu Feringghi	BF	5°28'24	100°14'56	19/11/2012
South West				
Flat Seri Delima	SD	5°19'36	100°16'34	09/07/2014
Medan Mahsuri	MM	5°19'19	100°16'48	22/07/2014
Taman Sri Gertak Sanggul	GS	5°17'21	100°13'14	09/07/2014
Kampung Jawa	KJ	5°19'41	100°17'42	16/07/2013
Permatang Damar Laut	PDL	5°16'39	100°16'18	10/10/2012
Balik Pulau	BP	5°22'37	100°12'51	25/10/2012
Mayang Pasir	MP	5°19'44	100°17'26	11/01/2013
Sungai Batu	SB	5°17'05	100°14'22	16/07/2013
Teluk Awak	TA	5°27'33	100°12'45	22/07/2014
Batu Maung	BM	5°17'07	100°17'11	16/07/2013
Central Seberang Perai				
Taman Desa Damai	TDD	5°21'47	100°26'27	22/07/2014
Perkampungan Berapit	PB	5°20'54	100°27'31	22/07/2014
Flat Teluk Indah	TI	5°22'33	100°23'48	22/07/2014
Padang Lalang	PL	5°21'17	100°27'19	19/11/2012
Permatang Pauh	PP	5°24'18	100°24'50	19/11/2012
Seberang Jaya	SJ	5°22'54	100°23'56	24/12/2013
Juru	JU	5°20'24	100°25'47	24/12/2013
Macang Bubuk	MB	5°20'48	100°31'25	24/12/2013
North Seberang Perai				
Bagan Dalam	BD	5°25'16	100°22'34	08/10/2012
Pokok Sena	PS	5°29'50	100°27'19	22/10/2013
Kepala Batas	KB	5°31'15	100°26'12	22/10/2013

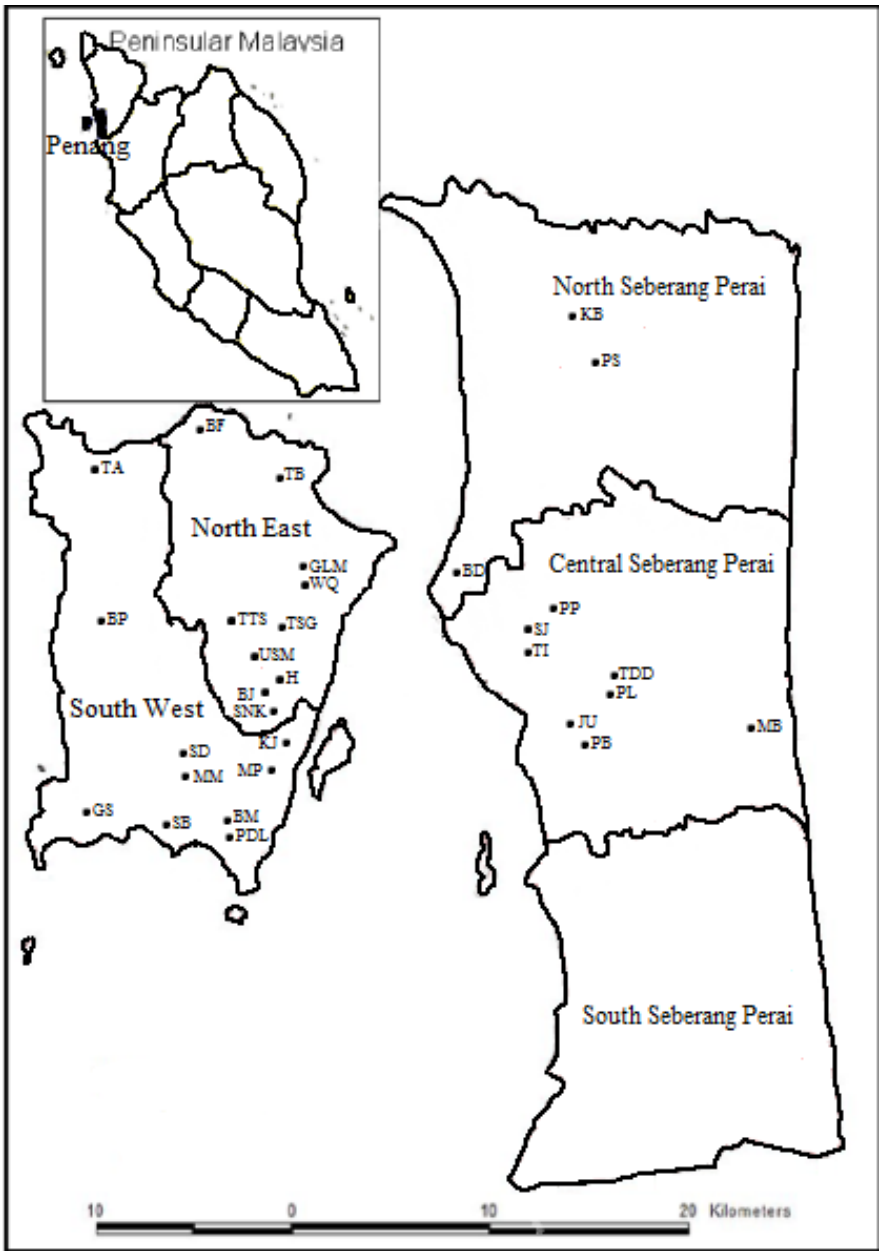

Figure 3.1: Sampling localities of mosquitoes populations analyzed in the present study. See Table 3.1 for sampling site abbreviation.