GENOTYPIC VARIATION OF *AEDES AEGYPTI* AND ITS RELATION TO DENGUE TRANSMISSION

NAVA YUGAVATHY A/P SELVARAJA

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2015

University of Malaya

GENOTYPIC VARIATION OF *AEDES AEGYPTI* AND ITS RELATION TO DENGUE TRANSMISSION

NAVA YUGAVATHY A/P SELVARAJA

DISSERTATION SUBMITTED TO THE FACULTY OF MEDICINE, UNIVERSITY OF MALAYA, FOR THE FULFILLEMENT OF THE DEGREE OF MASTER OF MEDICAL SCIENCE

DEPARTMENT OF PARASITOLOGY
FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2015

University of Malaya

UNIVERSITI MALAYA
ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: NAVA YUGAVATHY A/P SELVARAJA

Registration/Matric No: MGN 130033

Name of Degree: MASTER OF MEDICAL SCIENCE

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

GENOTYPIC VARIATION OF *AEDES AEGYPTI* AND ITS RELATION TO DENGUE TRANSMISSION

Field of Study :

I do solemnly and sincerely declare that:
(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright wether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

Name:

Designation:

University of Malaya

ABSTRACT

Dengue has now become a global public health problem and in Malaysia cases of dengue has increased about 30 folds compared to decades ago. *Aedes aegypti* is the primary vector of dengue and its vectorial capacity differs from one strain to another. In this study, the genetic diversity of *Ae. aegypti* collected from 19 localities in peninsular Malaysia based on two mitochondrial DNA genes; CO1 and ND5 was determined. The total number of haplotypes obtained for CO1 and ND5 gene were 14 (C1-C14) and five (N1-N5), respectively. Haplotype genealogical network constructed using concatenated CO1-ND5 gene sequences revealed 18 haplotypes (H1-H18) that were separated into two distinct lineages. Phylogenetic analysis showed that the first lineage (C11 and C3) was more closely related to those from Brazil and France. Vector competency can be categorized into infection rate and dissemination rate. The presence of haplotypically varied *Ae. aegypti* strains in Klang Valley and its surrounding areas in the state of Selangor where dengue is hyperendemic, suggests the importance to study the degree of its contribution to dengue transmission. Mentari Court, located in Klang Valley, reported high number of dengue cases and was an endemic area in recent years. However, *Ae. aegypti* from Pasir Puteh, located in state of Perak had a unique and specific haplotype. Dengue cases were rare in that location. In the phylogenetic analysis, the mtDNA sequence of Mentari Court and Pasir Puteh sample happens to fall into two different clades, further suggesting the presence of two different lineages of *Ae. aegypti* population. The overall result of this study showed both the population to be susceptible towards DENV type 1 and was able to disseminate the infection by day seven post infection. The percentage of positive organs was higher in Pasir Puteh's *Ae. aegypti* on all three days except on day 10 salivary gland of Mentari Court exhibited a higher rate. However Mentari Court proved to have a better dissemination rate although not significant. This is the first study in Malaysia which serves as an initial preliminary

University of Malaya

phase for a much larger study throughout the country. The understanding of the population diversity of *Ae. aegypti* and its impact on dengue transmission will be essential for planning of effective control programmes to reduce the burden of dengue.

University of Malaya

ABSTRAK

Denggi telah diisytiharkan sebagai ancaman utama kepada kesihatan orang awam dan di Malaysia kes denggi telah meningkat sebanyak 30 kali ganda berbanding dengan sepuluh tahun lalu. *Aedes aegypti* merupakan vektor utama virus denggi dan kompetensi vektor ini berbeza dari satu keturunan ke yang berikutnya. Dalam kajian ini, variasi genetik *Ae. aegypti* dari 19 lokasi di Semenanjung Malaysia dikaji menggunakan dua gen DNA mitokondria; CO1 dan ND5. Jumlah bilangan haplotaip yang diperolehi untuk gen CO1 dan ND5 adalah 14 (C1-C14) dan lima (N1-N5) masing-masing. Pokok filogenetik yang dibina menggunakan rangkaian kombinasi kedua-dua gen, mendedahkan 18 haplotaip (H1-H18) dan kehadiran dua keturunan. Tambahan pula, keturunan pertama (C11 dan C3) lebih berkaitan dengan Brazil dan France. Kompetensi vektor boleh dikategorikan sebagai kadar jangkitan dan kadar penyebaran. Mengkaji tahap sumbangan *Ae. aegypti* kepada denggi di Lembah Klang dan kawasan sekitar negeri Selangor adalah penting. Mentari Court, kawasan perumahan di Lembah Klang, melaporkan jumlah kes denggi yang tinggi dan disenaraikan sebagai kawasan endemik beberapa tahun kebelakangan ini. *Aedes aegypti* dari Pasir Puteh di Ipoh, mempunyai haplotaip yang unik dan khusus dan jumlah kes denggi jarang berlaku di lokasi tersebut. Keputusan keseluruhan kajian ini menunjukkan kedua-dua populasi *Ae. aegypti* yang didedah kepada denggi virus-1 mampu menyebarkan jangkitan pada hari ketujuh. Peratusan organ positif adalah lebih tinggi dalam nyamuk Pasir Puteh untuk semua tempoh yang dikaji kecuali hari ke-10; kelenjar air luir nyamuk Mentari Court menunjukkan kadar positif yang tinggi. Oleh itu, tidak hairan untuk melihat kadar jangkitan denggi yang lebih tinggi dalam nyamuk Pasir Puteh. Walaubagaimanapun, Mentari Court dibukti mempunyai kadar penyebaran yang lebih tinggi. Ini adalah kajian pertama di Malaysia yang boleh berfungsi sebagai penyelidikan fasa awal untuk kajian yang lebih besar. Memahami variasi populasi *Ae. aegypti* dan kesannya terhadap

University of Malaya

penyebaran denggi adalah penting untuk merancang program kawalan wabak denggi di Malaysia.

University of Malaya

ACKNOWLEDGEMENTS

I would like to show my extended gratitude to God the Almighty for giving me the strength, determination, perseverance and some wisdom to explore the scientific research field. There are so many people I would like to thank for making my life a little easier while I was carrying out the project.

First and foremost I would like to thank my immediate supervisor, counselor and mentor Datin Dr. Indra Vythilingam for her brilliant idea on initiating the research. Her extensive help and teaching had brought me to this stage of finalizing my research project. I am truly honored to work with such a great scientist and inspire to be like her one day.

Next I would like to thank Mastura Mohd a former student who did half of the sample collection but was not able to carry out the research project due to some reasons. I would like to thank David Lee from Ngee Ann Polytechnic, Singapore an expert in bioinformatics, who guided me throughout the genetic analysis. Then I would like to thank my good friends Sylvia Joanne, Wong Meng Li and Shii Leong for sharing their valuable knowledge and experience especially on molecular works and statistical analysis. Their presence made my working day more cheerful and bearable. Not forgetting my mother who never failed to encourage me and loving me unconditionally. She was also my inspiration on how to stay strong throughout rain and storm.

Last but not least, my gratitude is extended to the Parasitology Department for their instrument and facility support. This project was funded by University Malaya Research Grants (UMRG) RG372/11HTM and the student grant (PG105 2014A)

University of Malaya

TABLE OF CONTENTS

ORIGINAL LITERARY WORK DECLARATION ii
ABSTRACT iii
ABSTRAK iv
ACKNOWLEDGMENT vi
TABLE OF CONTENTS viii
LIST OF FIGURES x
LIST OF TABLES xiii
LIST OF ABBREVIATION xiv

CHAPTER 1 : INTRODUCTION
1.1 Background 1
1.2 Current global burden of dengue 1
1.3 Dengue situation in Malaysia 3
1.4 Dengue virus 5
1.5 The vector 8
1.6 Distribution of *Aedes* 9
1.7 Morphology of *Aedes* 9
1.8 Biology of *Aedes aegypti* 12
1.9 Objectives of study 15
CHAPTER 2 : LITERATURE REVIEW 16
2.1 Dengue pathophysiology 16
2.2 Interventions 17
2.3 Vector control 18
2.4 Vector study 19
2.5 The genes 20
2.6 Vector competency 21
CHAPTER 3: MATERIALS AND METHODS 24
3.1 Study sites 24
3.2 Collection of mosquitoes from the field 24
3.2.1 Ovitraps 27

University of Malaya

3.2.2 Larval surveys 27
3.3 Mosquito colonisation 28
3.4 DNA extraction 28
3.5 PCR amplification and sequencing 29
3.6 Sequence analysis 30
3.7 Phylogenetic analysis 32
3.8 Dengue virus culture 32
3.9 Infection assay 33
3.10 Dissection of mosquitoes 39
3.10.1 Procedure to obtain midgut 39
3.10.2 Procedure to obtain salivary gland 39
3.11 RNA extraction 40
3.12 Real Time Quantitative PCR amplification of the dengue RNA 40
3.13 Analysis of the quantitated dengue RNA 41
CHAPTER 4: RESULTS 43
4.1 Genetic diversity 43
4.2 Tests of neutrality 47
4.3 Analysis of demographic history and population structure 47
4.4 Analysis of population structure 48
4.5 Haplotype analysis 52
4.6 Phylogenetic analysis 54
4.6.1 CO1 gene 54
4.6.2 ND5 gene 56
4.7 Infection assay 58
4.8 Survival rate 60
4.9 Susceptibility of Mentari Court and Pasir Puteh mosquitoes towards DENV1 60
4.10 Correlation between midgut and salivary gland virus titer 66
CHAPTER 5 : DISCUSSION 68
5.1 Genotypic variation 68
5.2 Infections assay 72
5.3 Limitations and recommendations 76
CHAPTER 6 : CONCLUSION 78
SUPPLEMENTARY 80
REFERENCES 90

University of Malaya

LIST OF FIGURES

Figure 1.1: Global dengue burden. 2

Figure 1.2: Dengue incidence rate and case fatality. 4

Figure 1.3: Dengue cases in all the states of Malaysia. 4

Figure 1.4: Release of dengue-2 virions from infected cells and schematic representation of mature virions. 7

Figure 1.5: Life cycle of dengue virus. 7

Figure 1.6: Physical identification of *Aedes* 11

Figure 1.7: Morphological identification of *Aedes* in larval stage. 11

Figure 3.1: Map of peninsular Malaysia and the state of Selangor. 25

Figure 3.2: Mosquito cup covered with netting. 36

Figure 3.3: Hemotek feeder placed in a feeding chamber. 36

Figure 3.4: Mosquitoes being fed with the hemotek feeder. 37

Figure 3.5: Mosquitoes isolated in paper cups. 37

Figure 3.6: Growth chamber with regulated temperature and humidity. 38

Figure 4.1: Multi-modal distribution of CO1-ND5 between haplotypes. 49

Figure 4.2: Analysis of *Ae. aegypti* CO1-ND5 haplotypes using SAShA. 51

Figure 4.2.1: Haplotype-by-haplotype analysis of CO1-ND5 data sets. 51

Figure 4.3: Genealogical network showing the relationship among 18 CO1-ND5 haplotypes of *Ae. aegypti*. 53

Figure 4.4: Phylogeny tree of *Ae. aegypti* based on CO1 gene. 55

Figure 4.5: Phylogeny tree of *Ae. aegypti* based on ND5 gene. 57

University of Malaya

Figure 4.6: *Ae. aegypti* survival recorded at several intervals. 61

Figure 4.7: Infection rates in midguts and salivary glands. 63

Figure 4.8: Dissemination rate. 64

Figure 4.9: Mean viral copy number in midgut and salivary gland. 65

Figure 4.10: Correlation between midgut and salivary gland viral titer. 67

University of Malaya

LIST OF TABLES

Table 3.1: Localities with the coordinates and methods of collection. 26

Table 4.1: Polymorphic region of the sequences. 45

Table 4.2: Summary statistics of mtDNA gene diversity in *Aedes aegypti.* 46

Table 4.3: Gene flow indices between different populations. 59

University of Malaya

LIST OF ABBREVIATIONS

A.D: Anno Domini

MOH: Malaysian of Health

WP: Wilayah Perseketuan

DENV: Dengue virus

RNA: ribonucleic acid

Ns: non-structural

Kb: kilo base pairs

Nm: nanometer

ER: endoplasmic reticulum

DHF: dengue hemorrhagic fever

DSS: dengue shock syndrome

ADE: antibody enhancement

CD8: cluster of differentiation

FDA: Food and Drug Administration

HIV: Human immunodeficiency virus

HCV: Hepatitis C Virus

SNPs: Single Nucleotide polymorphism

AFLP: Amplified Fragment Length Polymorphism

IR: infection rate

VC: vector competence

DR: dissemination

MIB: midgut infection barrier

MEB: midgut escape barrier

University of Malaya

SIB: salivary gland infection barrier

SEB: salivary gland escape barrier

N: north

E: east

uL: microliter

mL: milliliter

rpm: revolution per minute

EDTA: ethylenediaminetetraacetic acid

PCR: polymerase chain reaction

BLAST: Basic Local Alignment Search Tool

MEGA: Molecular Evolutionary Genetics Analysis

MCMC: Markov Chain Monte Carlo

FBS: fetal bovine serum

MEM: minimum essential medium

cm: centimeter

ffu: foci forming unit

ACL2: Arthropod containment Level 2

Ct: threshold cycle

OM: observed mean

EM: expected mean

qPCR: quantitative real time polymerase chain reaction

University of Malaya

CHAPTER 1: INTRODUCTIONS

1.1 Background

Dengue fever is a vector borne tropical disease caused by dengue virus. Dengue, a spanish word possibly derived from *dinga* in the Swahili phrase *Ka-dinga pepo*, which describes the disease as being caused by an evil spirit. There is no clear time frame on when dengue first emerged as disease causing virus mostly because its symptoms are often not diagnostic. The earliest assumption suggested is from a Chinese medical encyclopedia dating 992 A.D which referred to water poison associated with flying insect (Gubler, 1998). Dengue perhaps originated from Africa and spread out due to globalization and slave trades. After World War 2, certain regions in Southeast Asia became endemic for dengue especially among children. The first documented case was in Penang in 1902 (Skae, 1902) followed by in Manila, Philippines in 1950's (Halstead, 1980). Since then, it became endemic in all countries of Southeast Asia with increasing number being reported each year.

1.2 Current global burden of dengue

More than 100 countries in Southeast Asia, eastern Mediterranean, America, western Pacific and Africa are now dengue endemic regions (Figure 1.1). Incidence rate had increased up to 30 fold in the past 50 years with recent estimate of 390 million people with dengue virus infection in 2012 (WHO, 2012). This figure is three times more than the previous year. True disease burden remains unclear especially in developing countries like India, Indonesia, China and Africa (Bhatt *et al.*, 2013). Both government and its people are affected economically by this disease. Dengue exacts economic burden up to 950 million USD annually in southeast Asia with 2.9 million dengue episodes and 5906 deaths (Shepard *et al.*, 2013).

University of Malaya

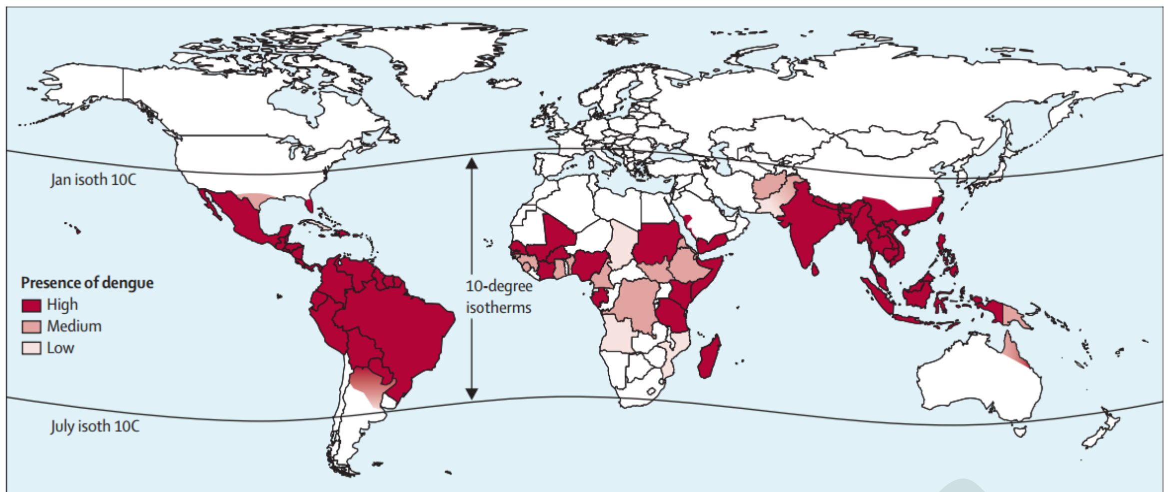


Figure 1.1: Global dengue burden, 2014. Integrated to show relative amount of dengue estimates globally (Guzman & Harris, 2014).

University of Malaya

1.3 Dengue situation in Malaysia

Dengue has been declared as one of the national health threat to the public in Malaysia (Er *et al.*, 2010). All ethnic groups are at risk of infection and all age group are affected with school going children and young adults with higher risk (Mohd-Zaki *et al.*, 2014). Changes in climate factor such as increased rainfall, temperature and relative humidity are the most influential forces of dengue virus transmission in Malaysia (Cheong *et al.*, 2013).

In Malaysia the number of dengue cases has increased sharply at an alarming rate over the past ten years (Mohd-Zaki *et al.*, 2014). The Malaysian Ministry of Health (MOH) is doing all they can to prevent the dengue outbreaks. National Strategic Plan for Dengue reduction by half in 5 years' time which started in April 2009 by the Government clearly failed as the number of cases started increasing from year 2011 and the total number of cases reported in 2014 was 103 753. This is 41% more than the cases reported from the previous year. Although the geographical conditions and climate are almost consistent throughout peninsular Malaysia, certain states are endemic compared to others. Selangor reported the highest number of cases, followed by Federal Territory Kuala Lumpur, Perak and Johor. These are the most populated states in the country. Figures 1.2 and 1.3 below show the dengue incidence and fatality rate from 2000 to 2013 and dengue cases by states from January to Jun 2015 ("iDengue ", 2015).

University of Malaya

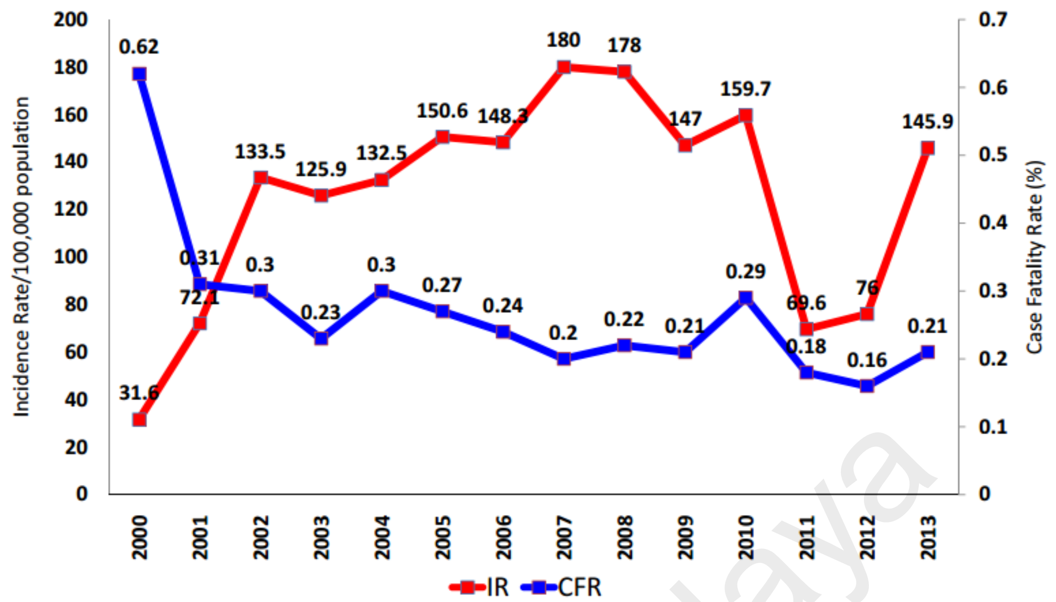


Figure 1.2: Dengue incidence rate and case fatality rate from 2000 to 2013 in Malaysia ("iDengue ", 2015)

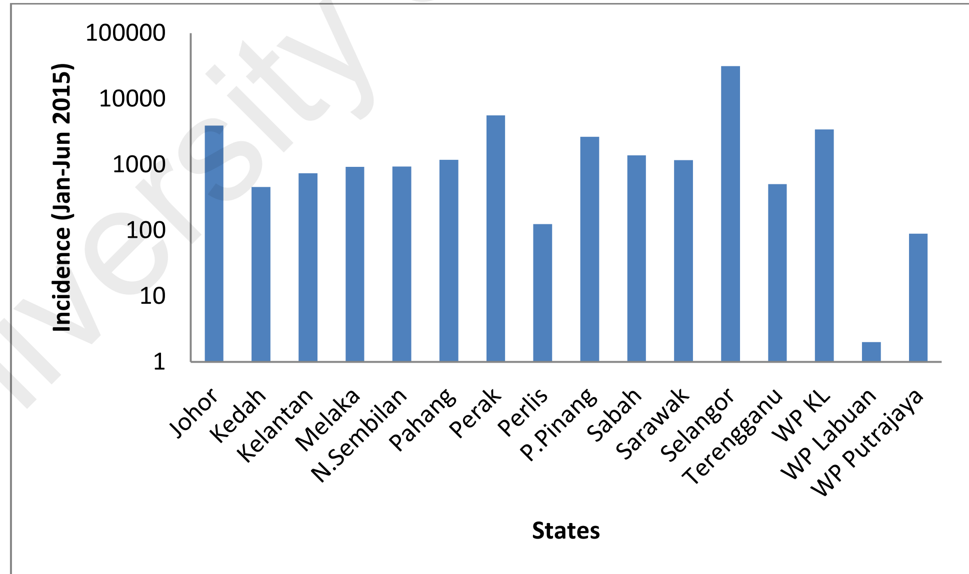


Figure 1.3: Dengue cases from January to June 2015 in all the states in Malaysia ("iDengue ", 2015)

University of Malaya

1.4 Dengue virus

The four serotypes of dengue virus are genetically diverse and share 60-75% identity at the amino acid level. Each serotype DENV1, DENV2, DENV3 and DENV4 has phylogenetically distinct genotypes and clades with 3-6% variation (Guzman & Harris, 2014). Genetic studies of sylvatic dengue strains provide evidence that the four dengue viruses evolved from a common ancestor in subhuman primate populations and that, around 500 years ago, all viruses emerged separately into a human urban transmission cycle (Wang *et al.*, 2000). Mature dengue virions consist of a single-stranded RNA genome surrounded by icosahedral nucleocapsid. A lipid envelope surrounds the capsid and a complete virion is about 50nm in diameter (Figure 1.4). Its genome is about 11kb that codes for three structural proteins, capsid protein C, membrane protein M, envelope protein E; seven nonstructural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5; and short non-coding regions on both the 5' and 3' ends.

The virus enters the definitive host cells via clathrin-dependent receptor-mediated endocytosis (Fragnoud *et al.*, 2012). Although a number of potential receptors have been identified in both vector and human cells, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) serves as a dengue virus attachment factor on dendritic cells (Navarro-Sanchez *et al.*, 2003).

University of Malaya

After virus cell entry (Figure 1.4, 1.5) and uncoating of the nucleocapsid, the RNA molecule is translated as a single polyprotein. During this process, the signal- and stop-transfer sequences of the polyprotein direct its back-and-forth translocation across the endoplasmic reticulum (ER) membrane. The polyprotein is processed co- and post-translationally by cellular and virus-derived proteases into structural and non-structural proteins. The NS proteins initiate replication of viral genome upon protein translation and folding. The C protein will then package the newly synthesized RNA to form nucleocapsid. As the particles travel through ER it matures (Yu *et al.*, 2008). A recent study has shown that the pr peptide remains associated with the virion until the virus is secreted to the extracellular matrix. Upon dissociation of the pr peptide, mature virions are formed that are able to infect new cells (Rodenhuis-Zybert *et al.*, 2010)

University of Malaya

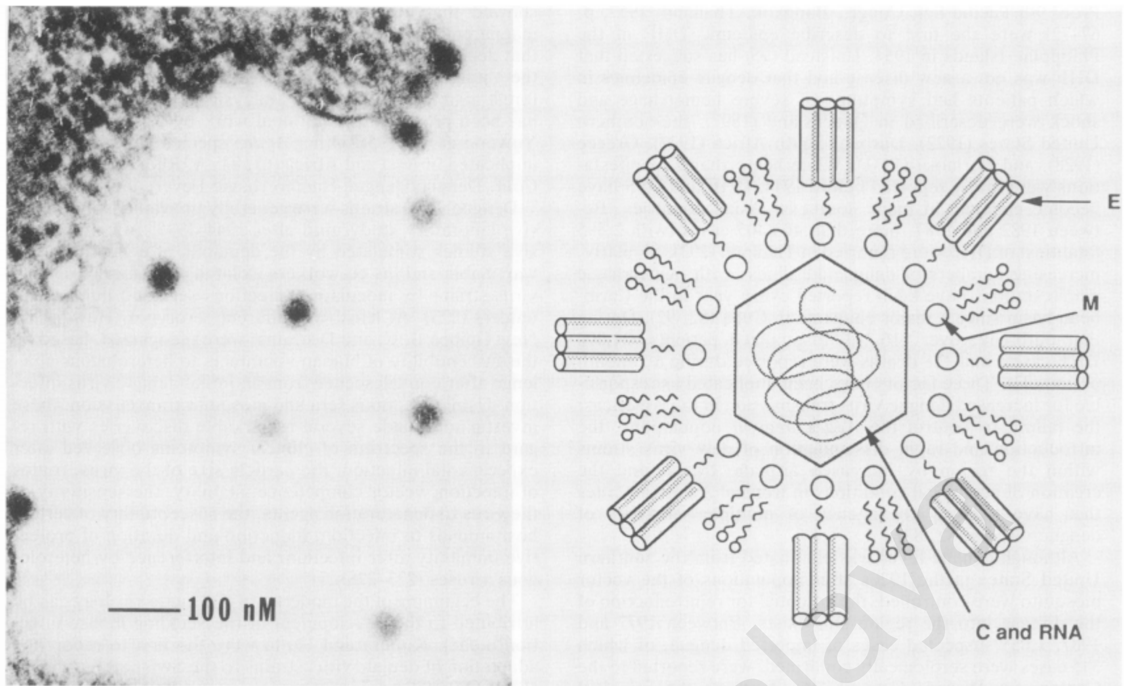


Figure 1.4: Release of dengue-2 virions from infected cells (left) and schematic representation of mature virions (right). E, Envelope protein; M, membrane-associated protein; C, core protein (Henchal & Putnak, 1990).

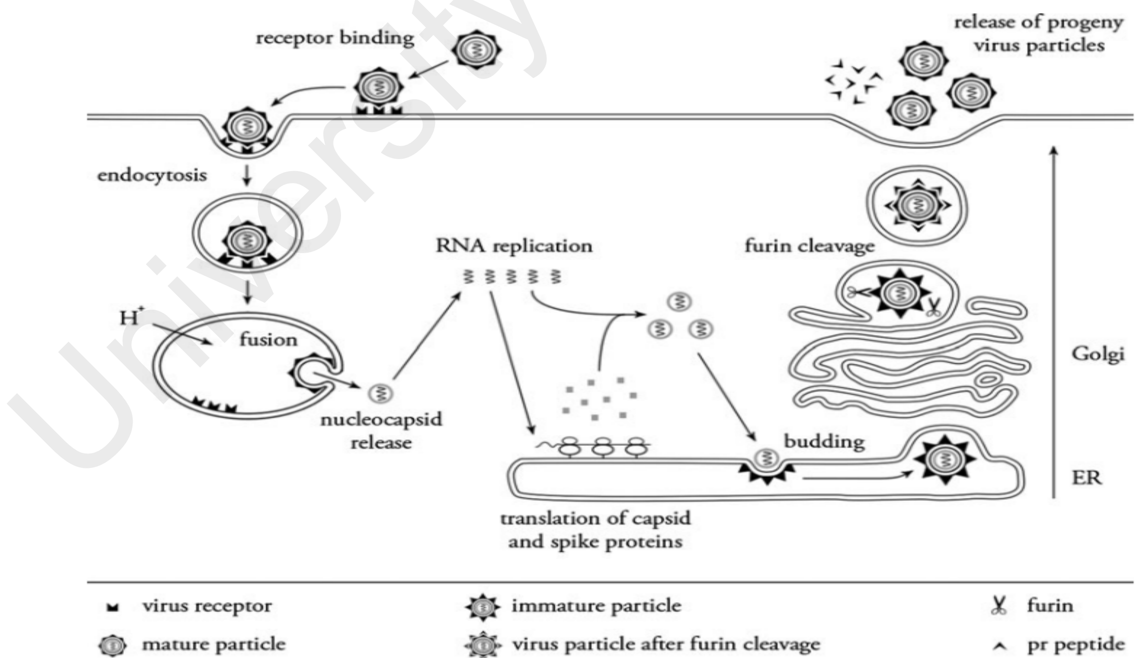


Figure 1.5 : Life cycle of dengue virus (Rodenhuis-Zybert *et al.*, 2010).

University of Malaya

Dengue transmission results from interactions between people, mosquitoes, viruses, and environmental factors. A mosquito carrying the dengue virus is known as dengue vector. The transmission cycle starts with ingestion of viremic blood by mosquitoes. An extrinsic incubation period of 8-10 days is required after feeding on a viremic human for viral replication and internal dissemination in the mosquito before virus appears in the saliva and transmission on refeeding can occur. As the blood meal stimulates oviposition by female mosquitoes, there is an opportunity for transovarial transmission of the virus which increases the risk of infection of the next generation of mosquitoes (Lee & Rohani, 2005) In tropical and subtropical regions, virus transmission between non-human primates and tree-hole breeding mosquitoes is also common (Rudnick, 1978). However, the relationship between the forest cycle and the human dwellings remains unclear. The existence of a completely silent zoonotic transmission cycle might have aided in the emergence of this disease in human population.

1.5 The vector

Mosquitoes are one of the most important vector and pest to man due to their ability to transmit deadly diseases. Almost 3,527 species and subspecies of mosquitoes from 113 genera has been identified in a variety of habitats. They are however absent in Antarctica and few oceanic islands (Manguin & Boëte, 2011). Mosquitoes can be classified into Cullicinae, Anophelinae or Toxorhynchitinae. These subfamilies belong to order Diptera.

Genus *Aedes*, (*Stegomyia)* belongs to family Culicidae and visibly different from the rest due to the black and white strips on its thorax and legs. The two invasive species found in Malaysia are *Aedes aegypti* and *Aedes albopictus*. *Aedes albopictus* is well known for its capacity to host chikungunya virus and dengue virus. Human threat

University of Malaya

follows due to its quick and rapid spread from its native home range Indian Ocean, Islands of western Pacific and East Asia (Hawley, 1988). *Aedes aegypti* on the other hand hosts the yellow fever virus, dengue virus and chikungunya virus.

1.6 Distribution of *Aedes*

Aedes aegypti was introduced to Asia from Africa decades ago and *Ae. albopictus* is native to the continent (Dieng *et al.*, 2010). Since their introduction in Malaysia, *Ae.aegypti* colonized the human dwelling in urban areas and *Ae.albopictus* the rural areas (Rudnick, 1965). The ecology of these two species does not differ much therefore co-existence has been reported in many countries including Malaysia (Chan *et al.*, 1971; Dieng *et al.*, 2010; Kamgang *et al.*, 2013). Currently there have been reports that *Ae. albopictus* is prevalent more widely compared to *Ae. aegypti* (Rozilawati *et al.*, 2007). It is also true that when *Ae. aegypti* is absent in a certain location *Ae. albopictus* will take its place especially in urban areas (Gratz, 2004). However, *Ae. aegypti* is very versatile in that it is mainly anthropophagic, its eggs can withstand desiccation and it breeds in artificial containers (Beebe *et al.*, 2005). Thus, it can be easily dispersed to many areas.

University of Malaya

1.7 Morphology of *Aedes*

Aedes aegypti physical identification in its adult stage includes palpi with white scales, presence of dorso central bristles, scutum with lateral crescent-shaped white marking, scutellum with broad white scales on all lobes and hind tarsus with a basal white band on tarsomeres 1-4, tarsomere 5 is white. In the larval stage, on the eight segment of the abdomen has comb scales with lateral denticles, very small marginal spicules of anal segment; ventral brush with 5 pairs of setae. Inner sub-median caudal hair 2-4 branches

(Huang, 1979). *Aedes albopictus* can be easily distinguished from *Ae. aegypti* by observing the patch of broad flat white scale on the scutellum (Figure 1.6). Fore and mid tarsomere with white bands and hind tarsi with broad white band. In the larval stage, the comb scales on the eight segment does not have lateral denticles (Figure 1.7). Ventral brush with 3- 4 pairs of setae. Inner sub-median caudal hair single or may split into two (Huang, 1972).

University of Malaya

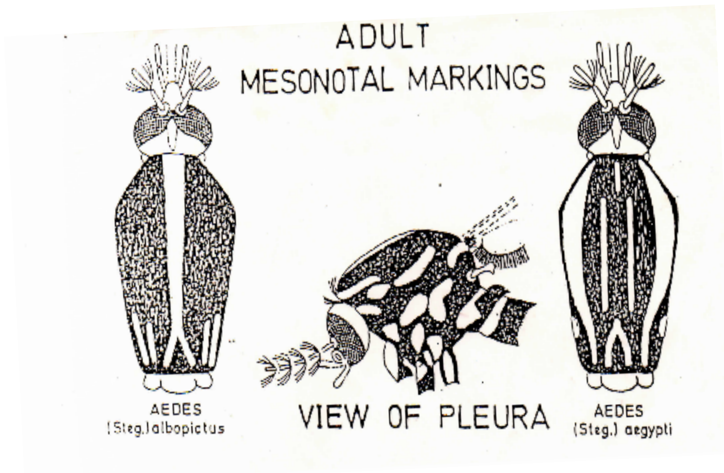


Figure 1.6: Morphological identification of *Ae.aegypti* and *Ae. albopictus* in the adult stage

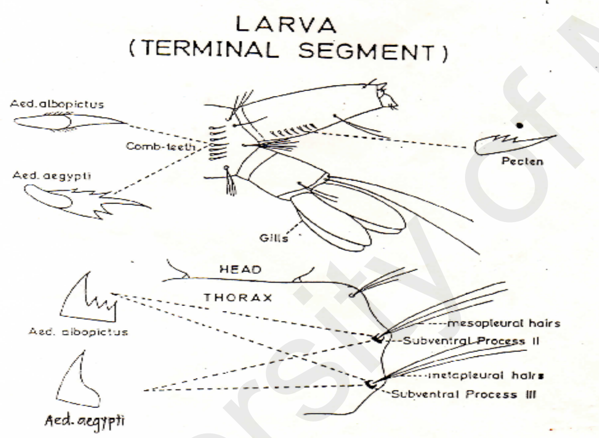


Figure 1.7: Microscopic identification of *Ae.aegypti* and *Ae. albopictus* in the larval stage

University of Malaya

1.8 Biology of *Aedes aegypti*

Aedes aegypti, the primary vector of dengue in Malaysia (Miyagi & Toma, 2000) has four distinct stages in their life cycle namely eggs, larvae, pupae and adult. The first three life stages are in aquatic phase and the last one is an active flying insect. The eggs are oval, elongated, turns black and hard after being deposited. The eggs can withstand desiccation for months and usually hatch in installments. *Aedes aegypti* are container breeders and often practice skip oviposition. Once the eggs hatch, there are four larval instars 1-4 which require about 6 to 10 days maturing into pupa. The development rate depends on the temperature and larval diet. After the 4th instar stage, the larva will become turgid and plump before turning into pupa. At emergence, the pupa is white and eventually turns black and this stage lasts 1- 2 days. Once they emerge into adults, they rest on the water with the help of water surface tension before flying into the environment. In the environment, *Ae. aegypti* generally prefers high humidity and dark places to rest. Male mosquitoes feed on sugar solution from fruits and flowers, and the female after mating will go for a blood meal. The lifespan of the wild mosquitoes can range from 19 days to 54 days or longer (up to 2 months) in laboratory cages (Muir & Kay, 1998).

Macdonald (Macdonald, 1957) has produced evidence to show that *Ae. aegypti* was probably confined to coastal areas in Malaya up to about 1900, and that it has been slowly encroaching into inland areas since that time. Hence, the presence and invasion of this species had also brought dengue into human habitat, making human a definitive host. In Malaysia, dengue was first reported in Georgetown, Penang in 1902 (Skae, 1902). Probably the most important single factor influencing the distribution of *Ae. aegypti* has been man himself. The habits of man with regard to water storage and unwanted containers are reflected in the abundance of the mosquito (Cheong, 1967). Furthermore, the cause of the present day distribution and spread of the species is

University of Malaya

urbanisation, transportation routes, dumping of non-biodegradable containers, tyres and other miscellaneous containers (Monath, 1994; Gubler & Clark, 1995).

The pathogenesis involved in dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) still remains unclear. However, studies have reported it could be due to infection by heterozygous serotypes of dengue. Since the 1960's examination of dengue virus serotypes in Malaysia revealed the presence of all four serotypes. Dengue-4 (DENV4) was the predominant serotype in the late 1960's which was then replaced by dengue-2 (DENV2) by early 1970's. The trend of dominant serotypes later began again in 1986 with dengue-3 (DENV3) becoming the dominant serotype. However by 1996 DENV1 and DENV2 dominated and later only DENV2 was found to be the major isolate (Abubakar & Shafee, 2002). Switching of the predominant dengue virus serotypes in the population might have contributed to the cyclical pattern of dengue virus outbreaks in Malaysia. It is also noted that the cycle of dominance by each serotypes is continuous as studies reported DENV1 dominance in 2005 and DENV2 in 2007. DENV1 and DENV4 was no longer commonly isolated in the year 2009 and the co-circulation of only DENV2 and DENV3 was documented in the country. However, DENV1 and DENV4 were reintroduced in 2010 (Mia *et al.*, 2013).

There are many components to the epidemiological and transmission cycle of dengue viruses from a vector to a definitive host and it could be either genetic or environment. *Aedes aegypti* becomes a potential vector during a viremic blood meal. Due to their ability to host deadly diseases, it became a medically important subject to research. Many studies on *Ae. aegypti* aiming to determine genetic structure and movement patterns of population to infer colonisation history and, vectorial capacity so as to design more effective control campaigns have been conducted in many countries (Bennett *et al.*, 2002; Black *et al.*, 2002). In Malaysia, such studies were conducted on *Ae. albopictus* where they observed a strong genetic variation especially between

University of Malaya

peninsular Malaysia and Borneo population (Black *et al.*, 1988). Analysis on *Culex quinquefasciatus* from residential areas in Malaysia on the other hand, revealed very low genetic diversity (Low *et al.*, 2014). Studies on *Ae. aegypti* have been conducted for instance on epidemio-meteorotropic analytical study in relation to *Ae. aegypti* abundance by (Li *et al.*, 1985), and the results showed that there was a strong correlation between rainfall and dengue cases and the lag time between dengue cases and heavy rainfall was about two to three months. Further studies have been conducted on colonising patterns of *Ae. aegypti* and *Ae. albopictus* using ovitraps and it has been found that mixed breeding occurs and *Ae. aegypti* was found in greater abundance than *Ae. albopictus* in dengue prone areas (Yap, 1975; Chen *et al.*, 2006). However, there are no genotypic studies conducted on this genotypically polymorphic vector. Since Selangor has the most number of dengue cases it is important to gain some knowledge of *Ae. aegypti*'s genotype and its relation to competency rate towards DENV in relation to other states where cases are being reported.

University of Malaya

1.9 Objectives of study

Thus the objectives of the project are:

1. To study the intraspecific genetic diversity, dispersal patterns, and phylogenetic relationships based CO1 and ND5 gene sequences of *Ae. aegypti* from several locations in peninsular Malaysia.
2. To examine the vector competency rate of two different population of *Ae. aegypti* towards DENV1.

University of Malaya

CHAPTER 2: LITERATURE REVIEW

2.1 Dengue pathophysiology

Dengue produces a wide spectrum of illness after an incubation period of 4-8 days. Acute febrile illness, headache, retro-orbital pain, rash, leukopenia, arthralgia and thrombocytopenia (Rothman & Ennis, 1999). Most patients recover after a self-limiting illness while others progress to severe disease. Dengue hemorrhagic fever is an acute vascular permeability syndrome with abnormal hemostasis. It presents with plasma leakage, bleeding tendency, and liver abnormality. Capillary leakage develops quickly within hours during the end of dengue fever symptoms. Hemoconcentration, ascites and pleural effusion indicate intravascular volume loss (Bhamarapravati, 1989). In the absence of intravascular fluid resuscitation, it can progress to dengue shock syndrome. Life-long immunity is acquired against the same serotype through primary infection. However, second heterologous serotype infection will increase the susceptibility to DHF (Halstead, 1980). It was speculated that viral load was causative agent for the intense transformation leading to hemorrhage and plasma leakage. It was then later confirmed both host immunity and viral determinants contributes to DHF pathogenesis.

University of Malaya

2.2 Interventions

The DENV-host interaction due to infection is complicated and unique with sequential consequences of dengue infection, viral immune evasion, host antibody enhancement (ADE), host immune activation and genetic predisposition. The unique interaction complicates dengue vaccine development and provokes many doubts. Possibility in triggering ADE, long-lasting heterotypic immunity by serotypes, ideal animal model to conduct immunisation studies, well established markers and the presence of different transmission pattern in different geographical locations. Despite all these challenges, live attenuated and inactivated viruses, recombinant proteins and DNA vaccines are under development as vaccine candidates (Thomas & Endy, 2011).

The latest finding by Yauch and co-workers (Yauch *et al.*, 2009) on the mechanism by which cross-reactive T cells may contribute to DHF/DSS, revealed that CD8 T cell plays an important role in controlling DENV infection. Immunisation with four dominant epitopes before infection resulted in better DENV clearance and this was mediated by CD8 T cells. The ongoing large phase 3 trial will determine if the vaccine confers protection against all four serotypes or not (Guirakhoo *et al.*, 2006). It is crucial to better understand immune responses to both natural infections and vaccine candidates. Many more challenges has to be solved for instance, optimised neutralisation assay, antibody avidity, antibody-dependent and cell mediated cytotoxicity.

To date the US Food and Drug Administration (FDA) has not approved any drugs against dengue but efforts on developing antiviral drugs that targets viral or host factors are ongoing. The major objective of anti DENV therapy is to prevent patients with DF from developing DHF and DSS. Some obstacles in the drug discovery phase include the absence of good small animal model. Besides that, the length of therapeutic treatment is expected to be less than a week since dengue fever lasts less than a week (Noble *et al.*,

University of Malaya

2010). This is in contrast to the antiviral therapy of chronic diseases like HIV and HCV which require long term treatment. The short period of viremia in DENV patients urges for early diagnosis of DENV infection for a successful development and usage of DENV inhibitors (Guzman & Harris, 2014).

2.3 Vector control

Due to the absence of a vaccine or suitable drugs for control of dengue, vector control remains as a priority in tropical regions. The primary vector mosquito, *Ae. aegypti* is distributed in subtropical and tropical areas of the globe and is now endemic in many countries. In Malaysia, *Ae. aegypti* is found abundantly in human dwelling both indoors and outdoors and remains as principle vector of dengue (Saifur *et al.*, 2012). *Aedes aegypti* is very anthrophagic and lives close to human habitation and does not move very far from its breeding sites and thus is a good vector of dengue (Jansen & Beebe, 2010). Although chemical insecticides were successful in controlling mosquito population in the past decade, development of resistance has hampered their usage. Temephos an organophosphate compound with very low mammalian toxicity has been used as larvicide since 1973 and the efficiency status of this compound was being questioned. A study conducted by (Chen *et al.*, 2005) reported the vector developed a certain degree of resistance and it is not surprising if the compound becomes ineffective in near future. In Singapore, investigation on primiphos-methyl and permethrin revealed development of resistance towards permethrin and primiphos-methyl remain effective for *Ae. aegypti* control as of year 2001 (Ping *et al.*, 2001). However, recent studies in Singapore have confirmed that both *Ae. aegypti* adults and larvae are resistant to pyrethroids and still susceptible to organophosphates (Koou *et al.*, 2014). The futures of all these insecticides are at stake. Recent study conducted in Singapore on deltamethrin treated net concluded limited efficacy due to knockdown resistance mutation observed

University of Malaya

in a few amino acid residue (Pang *et al.*, 2015). The development of resistance towards an insecticide depends on the selection pressure, genetic drift and bottleneck effect of a population. The period of usage and frequency of application on breeding sites and dosage assigned also determines the resistance status (Hudson, 1983). Due to the constant evolution and genetic drift of vector population, continuous efforts are being made to overcome these challenges. New chemical products are in development to improve *Ae. aegypti* control for instance essential oils with larvicidal compounds (Dias & Moraes, 2014). Insecticide-treated curtains and new mosquito traps are some of the endeavors to reduce the vector population and curb dengue (Loroño-Pino *et al.*, 2013).

2.4 Vector study at molecular level

Recent advancement in genomics should improve understanding of biological processes of vector at the molecular level and serve as a stepping stone in designing effective mosquito control strategies. *Aedes aegypti* is phenotypically polymorphic from one population to another and vary in frequencies of molecular and biochemical markers (Tabachnick *et al.*, 1985). Experimenting the genetic variation and gene flow pattern from one region to another is crucial to predict spatial and temporal dispersion of important genetic traits such as vector competence and insecticide resistance. *Aedes aegypti* dispersal occurs through adult flight or transportation of desiccated eggs along commercial routes. Early population genetics work defined genetic relationship throughout the worldwide range of *Ae. aegypti* (Tabachnick *et al.*, 1979; Tabachnick & Powell, 1979; Myers *et al.*, 1980; Wallis *et al.*, 1984) while more recent studies have focused on local patterns of dispersal (Apostol *et al.*, 1994; Apostol *et al.*, 1996; Edman *et al.*, 1998). Many studies focus on genetic variability and the population structure analysis using established molecular markers, which includes SNPs, alloenzymes, nuclear DNA and mtDNA (Pashley & Rai, 1983). Amplified fragment length

University of Malaya

polymorphism (AFLP) and random amplified polymorphic markers are suitable for high-density genome mapping and the use of AFLP in population genetics could lead to serious biased estimates of genetic diversity (Yan *et al.*, 1999). Single nucleotide polymorphisms (SNPs) are the most abundant source of genetic variation among individual organisms however, using SNPs to investigate the genetic diversity and gene flow will require a huge number of samples which eventually require larger budget (Urdaneta-Marquez *et al.*, 2008).

Mitochondrial DNA (mtDNA) has been widely used in population genetic studies of *Ae. aegypti* recently from different geographic and dengue endemic regions. Mitochondrial DNA are maternally inherited, has rapid rate of divergence and also it undergoes limited recombination thus, making it more robust to study evolutionary relationship (Arrivillaga *et al.*, 2002; Bracco *et al.*, 2007). However, such studies are lacking in Malaysia and since dengue seems to be a major problem in the State of Selangor, it would be significant to study the genetic structure of *Ae. aeypti* and compare the vector competency.

University of Malaya

2.5 The genes

Mitochondrial DNA, because of its characteristics, has been widely used in studies of population genetics and evolutionary relationships of many organisms. The most commonly used DNA marker for mosquitoes are Cytochrome oxidase and NADH dehydrogenase.

Mitochondrially encoded cytochrome c oxidase I (CO1) is the component of the respiratory chain that catalyzes the reduction of oxygen to water. CO subunit 1-3 form the functional core of the enzyme complex (Bethesda, 2015a). Many population genetic diversity studies have been conducted using the CO1 gene for instance a study conducted by Beebe and co-workers (Beebe *et al.*, 2005) revealed for the first time the

presence of two haplotype population of *Ae. aegypti* in the mainland of Australia. The CO1 gene has been found to be useful for intraspecific studies of *Anopheles* and in interspecific studies of *Aedes* mosquitoes. (Walton *et al.*, 2000; Cook *et al.*, 2005)

Mitochondrially encoded NADH dehydrogenase 5 (ND5) provides instructions for making a protein called NADH dehydrogenase 5. This protein is part of a large enzyme complex known as complex 1 which is found in mitochondria. Complex 1 is responsible for the electron transport process and it is one of the several enzyme complexes needed for oxidative phosphorylation (Bethesda, 2015b). This gene is one of the most variable genes of mtDNA and was also used in studies of the *Anopheles* and *Aedes* (Besansky *et al.*, 1997; Birungi & Munstermann, 2002; Molina-Cruz *et al.*, 2004).

2.6 Vector competency

The transmission of DENV by *Ae. aegypti* in any geographic area depends on extrinsic factors related to the environment and intrinsic factors associated with virus vector interaction which is also known as vector capability. Many studies have reported wide variation among and within population vector competency (Hardy, 1988). Vector capability can be categorised into (a) the proportion of individual mosquitoes that can be infected in a population considering presence of the virus in the mosquito which is defined as infection rate (IR), (b) the proportion of mosquitoes in which DENV is able to infect, replicate and transmit to salivary gland or head which is defined as vector competence (VC), (c) the vector's ability to transmit DENV where the virus is detected in the saliva is known as disseminated infection rate (DIR) (Gonçalves *et al.*, 2014). Naturally, DENV is ingested through a blood meal and the viral load found in the midgut is dose dependent. However, the ability to overcome the physical barriers in the mosquito depends on the vector's susceptibility.

University of Malaya

After a positive blood meal, there are four different hurdles that the arbovirus needs to overcome before it can become infectious and transmittable. The virus has to cross the midgut epithelium to enter the midgut known as midgut infection barrier (MIB). Once in the midgut it multiplies and has to escape into the hemocole known as midgut escape barrier (MEB). Once in the hemocoel the virus has to multiply in various tissues and invade the salivary gland which is known as the salivary gland infection barrier (SIB). From the salivary gland the virus has to escape the salivary gland escape barrier (SEB) and transmit the virus to the host (Tabachnick, 2013). However, it is not known if all *Ae. aegypti* strains are equally capable to overcome all barriers. Once a vector is productively infected with an arbovirus, it is capable of transmitting the virus for life. A study done by Bosio and co-workers (Bosio *et al.*, 2000) concluded these barriers to infection and dissemination, independent of virus titer, determines vector competence towards DENV and several loci additively manipulates the susceptibility of barriers. Since the barrier vary in prevalence in natural populations, large intraspecific variation of *Ae. aegypti* competency may determine the epidemiology of dengue viruses (Black *et al.*, 2002). The susceptibility of interpopulation through oral infection of dengue among 13 different geographical *Ae. aegypti* population from East and West Africa, Southeast Asia and South Pacific was experimented by Gubler and co-worker (Gubler *et al.*, 1979). It was found populations from South Pacific and Southeast Asia to be more susceptible compared to other regions. This leads to several intrapopulation vector competency studies for instance the susceptibility comparison of Killifi and Nairobi population in Kenya which suggest inefficient transmission ability of DENV-2 by the Nairobi *Ae. aegypti* (Chepkorir *et al.*, 2014). The first study that evaluated vector competency of *Ae. aegypti* towards DENV was performed by Bennett and co-workers (Bennett *et al.*, 2002). The authors reported *Ae. aegypti* population from Mexico and United States exhibits significant variation in their ability to transmit DENV2.

University of Malaya

However, such studies are lacking in Malaysia and it is important to explore the genetically polymorphic vector and its degree of contribution to dengue especially in a dengue endemic location like Klang Valley, Selangor.

According to Failloux *et al* (Failloux *et al.*, 2002) genetic differences among *Ae. aegypti* populations depend on colonisation history and may be associated with DENV susceptibility. This finding leads us to question the different dengue outbreak frequency in different states of Malaysia where the geographical and environmental conditions are similar. Advances and development of genomic studies and research techniques will allow us to decipher transcriptomics and proteomics of *Ae. aegypti* thus improving understanding of biological processes at the molecular level. This eventually will serve as a platform to design new mosquito control strategy. The goal of this study is to uncover evolution history and observe genetic pattern and flow of *Ae. aegypti* population from Klang Valley, Selangor and other states in peninsular Malaysia using two mitochondrial genes. In the presence of distinct genetic haplotypes, the study can be directed to experiment vector competency difference between two distinct populations.

University of Malaya

CHAPTER 3: MATERIALS AND METHODS

3.1 Study sites

Mosquitos were collected from several sites in peninsular Malaysia. Areas in the north, south, east and west were sampled. However, extensive sampling was carried out central west coast of Selangor known as the Klang Valley. Figure 3.1 shows the map of peninsular Malaysia and all the sampling sites. The state, region, global positioning system coordinates and sample sizes have been tabulated (Table 3.1).

3.2 Collection of mosquitoes from the field

Two methods were used for collection of mosquitoes from the field. Ovitraps or larval surveys were carried out depending on the locations. Collection methods are listed (Table 3.1).

University of Malaya

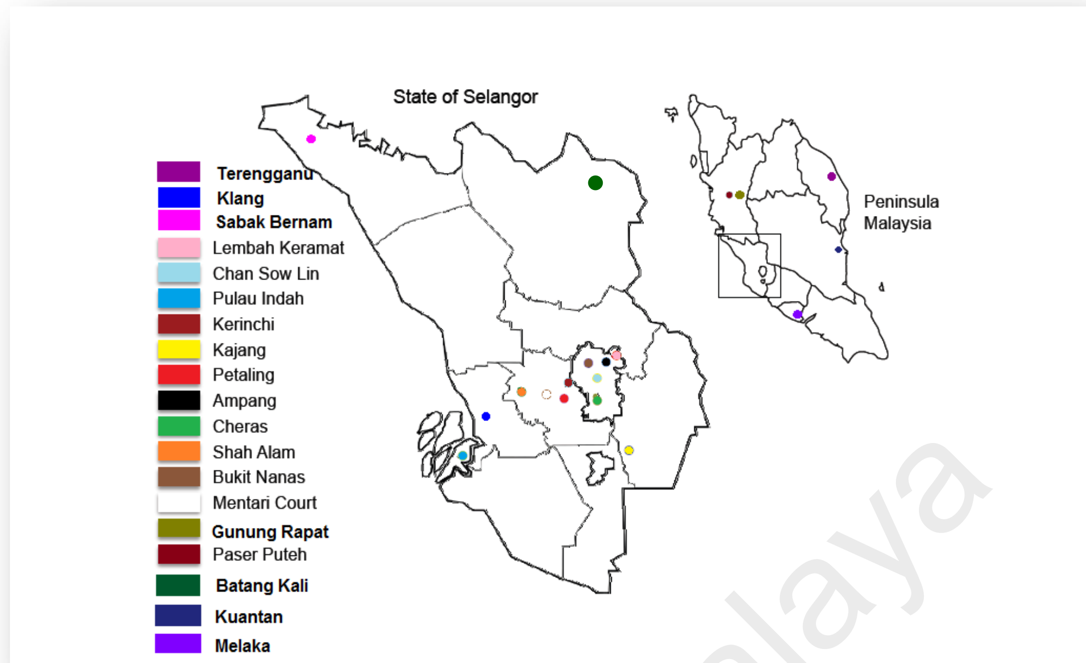


Figure 3.1: Map of peninsular Malaysia and the state of Selangor showing sample collection sites

University of Malaya

Table 3.1: Collection sites, sample size and collection methods of *Ae. aegypti* from peninsular Malaysia.

Locality	State	Region	Coordinates	Sample size	Collection Method
Kerinchi	Federal Territory	West Central	3°6'46"N, 101°39'40"E	9	Ovitrap
Cheras	Federal Territory	West Central	3°6′45″N, 101°42′51″E	9	Larval survey
Bukit Nanas	Federal Territory	West Central	3°9′22″N, 101°42′16″E	6	Larval survey
Lembah Keramat	Federal Territory	West Central	3°10'9"N, 101°44'48"E	6	Ovitrap
Chan Sow Lin	Federal Territory	West Central	3°7′40″N, 101°42′55″E	6	Larval survey
Kajang	Selangor	West Central	2°59′35″N 101°47′20″E	11	Ovitrap
Ampang	Selangor	West Central	3°9′38″N, 101°44′9″E	7	Larval survey
Pulau Indah	Selangor	West Central	2°56′56″N,101°19′54″E	5	Ovitrap
Petaling	Selangor	West Central	3°05′N 101°39′E	7	Ovitrap
Shah Alam	Selangor	West Central	3°5′00″N, 101°32′00″E	5	Ovitrap
Mentari Court	Selangor	West Central	3°04′59″N,101°36′40″E	6	Ovitrap
Klang	Selangor	West Central	3°2’41’’N, 101°26’44’’E	4	Larval survey
Batang Kali	Selangor	West Central	3°28'0"N, 101°38'0" E	3	Larval survey
Sabak Bernam	Selangor	West Central	3°40’43’’N, 100°59’26’’E	4	Larval survey
Pasir Puteh	Perak	Northern	5°49′59″N,102°24′00″E	8	Ovitrap
Gunung Rapat	Perak	Northern	4°34'14"N, 101°7'59"E	4	Ovitrap
Jasin	Malacca	Southern	2°13’40’’N, 102°25’51’’E	12	Larval survey
Bandar Mahkota	Pahang	Eastern	3°49’0’’N, 103°19’59’’E	3	Larval survey
Kuala Terengganu	Terengganu	Eastern	5°19’46’’N, 103°8’13’’E	10	Larval survey
Total				125	

University of Malaya

3.2.1 Ovitraps

Ovitrap is a device which consists of black cylindrical container filled with rain water or hay infused water. A piece of rough brown paper or wooden paddle was partially submerged into the water. Ovitraps were set about 200 meters apart from each other to obtain eggs from different adult mosquitoes. Ten to fifteen containers were placed at each sampling sites; both indoor and outdoor. Indoors, the ovitraps were installed under the sink, beside artificial flower pots, behind curtains, those places that allowed the traps to be hidden from view. Outdoors the traps were set, beside plant vases, inside bushes, under tree shelters and beside drains.

3.2.2 Larval surveys

Larval survey is a method of larvae inspection and collection to identify mosquito breeding sites both indoors and outdoors to. Larval breeding sites such as kitchen and laundry containers, unused toilet flush tanks, flower pots, and water storing barrels were inspected inside the house. Flower pot holders, ground tanks, tyres, cans, and broken domestic appliances were inspected outside the landed houses. Stagnant drain water, mismanaged garbage and broken appliances were inspected in high rise apartment's ground floor. All larvae were collected using Pasteur pipette and transferred into universal bottles that were labeled. These were brought back to the laboratory and allowed to emerge as adults.

University of Malaya

3.3 Mosquito colonization

Eggs obtained from ovitraps were allowed to air dry for about a week at room temperature. The eggs from each trap were placed in individual containers and allowed to hatch. The larvae were fed with tetramin fish food twice daily until they reach pupae stage. Pupae were transferred to individual tubes and allowed to emerge into adults. After emergence the adults were collected and identified and *Ae. aegypti* mosquitoes were stored in -20°C until needed.

Larvae that were obtained from the field were allowed to pupate. The pupae were placed in containers for the emergence of adults. Adult mosquitoes were identified and all *Ae. aegypti* were stored at -20°C until required.

3.4 DNA extraction

Genomic DNA was extracted using Qiagen Dneasy Blood and Tissue Extraction Kit (Qiagen, CA, USA) according to manufacturer's instructions. Whole mosquito was placed into a 1.5 mL centrifuge tube (Axygen, USA) to which 100 µL of ATL Buffer was added. The mosquito was homogenized using a pestle and hand held homogenizer (Kontes Thompson Scientific). The pestle was then rinsed with an additional 80 µL of the ATL Buffer so that all parts of the mosquito were collected in the centrifuge tube. Twenty µL proteinase K was then added to the homogenate to digest protein and remove contamination from nucleases. The mixture was then incubated at 56°C overnight in a thermomixer (Eppendorf, Germany). After incubation, 200 µL of 97% ethanol and Buffer AL was added into the sample and was mixed thoroughly by vortexing to yield a homogenous solution. The homogenized solution was then pipetted into Dneasy Mini Spin column placed in a 2 mL collection tube. The sample was centrifuged at 8000 rpm for 1 minute. The flow-through and the collection tube were

University of Malaya

discarded. The spin column was placed in a fresh two mL collection tube. A volume of 500 µL AW1 Buffer was added into the spin column. The sample was centrifuged for 1 minute at 8000 rpm. Again, the flow-through and the collection tube were discarded. The Dneasy spin column was placed into a fresh 2 mL collection tube and 500 µL of Buffer AW2 was added into it. The tube was centrifuged for 3 minutes at 14000 rpm to dry the Dneasy membrane. The tube was again discarded and the spin column was placed into a clean 1.5 mL centrifuge tube. Sixty µL elution buffer was added directly into the Dneasy membrane and incubated at room temperature for one minute after which it was centrifuged at 8000 rpm for 1 minute. Eluted DNA was stored at -20°C.

3.5 PCR amplification and sequencing

Partial sequences of CO1 and ND5 mitochondrial gene were amplified using conventional PCR. CO1 gene was amplified using primers 5' TAG TTC CTT TAA TAT TAG GAG C-3' and 5' TAA TAT AGC ATA AAT TAT TCC- 3' (Beebe *et al.*, 2005) in a 50 µL reaction volume using 3 mM $MgCl_2$, 1X Green Buffer Go Taq, 0.2 mM dNTP, 2.5 units *Taq* polymerase, 0.3 µM each primer (Promega, Madison, WI) and 5 µL of DNA sample. The optimised amplification profile was initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 1 min (denaturation), 51°C for 1 min (annealing), 72°C for 1 min (extension) and final extension at 72°C for 5 min.

The ND5 gene was amplified using primers 5' TCC TTA GAA TAA AAT CCC GC-3 and 5' GTT TCT GCT TTA GTT CAT TCT TC-3'(Birungi & Munstermann, 2002) in a 50 µL reaction volume using 1.25 mM of $MgCl_2$, 1X Green Buffer Go Taq, 0.2 mM dNTP, 2.5 units of *Taq* polymerase, 1.5 µL of 0.3 µM each primer (Promega, Madison, WI) and 5 µL of DNA sample. The optimised amplification profile was initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 1 min (denaturation), 54°C for 1

University of Malaya

min (annealing), 72°C for 1 min (extension) and final extension at 72°C for 5 min. The PCR product was then subjected to agarose gel electrophoresis.

Agarose gel electrophoresis was carried out to ensure the desired gene was amplified. Here 1% agarose gel was used. 1.0 g of electrophoresis grade agarose powder was added to 100 mL of 1X Tris Borate EDTA (TBE) and heated in microwave oven to dissolve the agarose. The 1% agarose gel was stained with 2 µL of Syber Safe dye (Invitrogen, USA) right after the mixture was heated. The molten agarose was cooled and poured into casting rack with gel comb. After the gel had set, it was transferred to an electrophoresis tank (Major Science Co.Ltd, Taiwan) containing sufficient volume of 1 X TBE buffer for complete submersion. Eight µL of PCR product was loaded into each well. Five µL of 100 base pairs ladder (Bioron, Germany) consisting of ten DNA fragments was loaded into the first well. Electrophoresis was carried out for 50 minutes at 100 volts. Single bands with approximately 450 base pairs for CO1 gene and 330 base pairs for ND5 gene indicated the presence of desired gene. Amplified DNA products were sequenced at the sequencing facility using Sanger Sequencing method (Genomics BioSci & Tech. Co., Ltd.).

3.6 Sequence analysis

Sequences were observed using Chromas Lite 2.1 ("Chromas Lite ", 2012) and edited using BioEdit Sequence Alignment Editor Program. Both the forward and reverse sequence was aligned to produce the consensus sequence. All consensus sequences were aligned using ClustalW in MEGA5.0 with default parameters. The sequences were then BLAST search in GenBank to confirm their identity. The sequence data obtained for the study was deposited in GenBank using sequin, a submission tool. Basic sequence statistics, including the number of haplotypes, polymorphic sites, haplotype

University of Malaya

diversity, nucleotide diversity, average number of nucleotide differences and statistical tests of Tajima's D, Fu and Li were conducted to test non-neutral evolution and deviation from mutation-drift equilibrium with DnaSP 5.0 (Librado & Rozas, 2009). Mismatch distribution or demographic expansion of the mitochondrial gene was tested using Arlequin 3.5 (Excoffier, 2010). The demographic changes were also examined by calculating the Harpending's raggedness index (Harpending *et al.*, 1993) and the sum of squared deviations (SSD) between the observed and expected mismatch for each of the populations were calculated using the methods of Schneider and Excoffier (Schneider & Excoffier, 1999) using Arlequin. This measure quantifies the smoothness of the observed mismatch distribution and a non-significant result indicates an expanding population (Harpending *et al*, 1993). The spatial expansion hypothesis (both raggedness index and SSD) was tested using a parametric bootstrap approach (500 replicates). Spatial analysis of shared alleles (SAShA 2.0) was used to detect geographically restricted alleles by comparing the spatial arrangement of allelic co-occurrence under panmixia. Two files were created for this analysis. A tab-delimited text haplotype by location in which each row corresponds to a haplotype, each column to a location and the numbers in the matrix corresponds to the numbers of that haplotype found in that location. A text file containing the pairwise geographic distances in kilometer between all locations in symmetric form was used. Haplotype network was constructed with concatenated sequence using TCS 1.21 software (Clement M, 2000) to assess the genealogical relationship of *Ae. aegypti* mitochondrial haplotypes. TCS software works with aligned nucleotide sequences saved in NEXUS or PHYLIP format. It collapse sequence into haplotypes and calculates the frequencies of the haplotypes in loaded samples.

University of Malaya

3.7 Phylogenetic analysis

In order to define the global evolutionary history of *Ae. aegypti*, COI haplotypes obtained from the current study and previously published sequences of *Ae. aegypti* from Brazil, Uganda, Senegal, Thailand, Vietnam, Madagascar, France, Cote d'Ivoire, Cambodia, Guinea, USA, Kenya, Pakistan and India were explored by Bayesian inference analysis. ND5 haplotypes on the other hand was compared to Argentina, Paraguay, Bolivia, France, Cambodia, Brazil, Cote d'Ivoire, Guinea, Vietnam, Thailand and Russia. The countries assigned for inference were retrieved from GenBank. Exact identical sequences from the same country were omitted. Phylogenetic relationship of *Ae. aegypti* was estimated using the Bayesian method implemented in BEAST (Drummond *et al*, 2012) software. The substitution model, Tamura-Nei with gamma distribution of rate variation among sites (determined using MEGA 5), a strict clock model and a Bayesian skyline coalescent model were used for this analysis. One hundred million generations of the Markov Chain Monte Carlo (MCMC) chains were run with sampling every 10,000 generations. The first 10 million generations of the run were discarded as burn-in and trees produced from the analysis were annotated to produce the maximum clade credibility tree. The concatenated (CO1-ND5) gene sequence was used to infer phylogenetic relationship between samples collected from different population in the current study using MEGA 5 (Tamura *et al.*, 2011) . The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model.

3.8 Dengue virus culture

Dengue virus serotype 1(DENV1) which was isolated from a patient's sample admitted to the University Malaya Medical Centre, Kuala Lumpur in 2014 dengue outbreak was used in the study. Virus stocks were obtained by inoculating monolayers of C6/36 cells

University of Malaya

at 80% confluency with initial virus inoculum diluted 1:200 in fetal bovine serum (FBS)-free medium. After 1 hour of adsorption at room temperature with gentle rocking, FBS-free medium was replaced with MEM supplemented with 2% FBS and the cells were cultured for up to 7 days at 28°C in 3% CO_2, culture medium containing the viruses were collected and centrifuged at 1000 × *g* for 10 minutes to remove cell debris. Supernatant containing the viruses were sterile-filtered using 0.2 µm syringe filter (Sartorius Stedim Biotech, Germany), aliquot and stored at -80°C .

Virus was quantitated using the formula:

$$\frac{\text{Number of focus}}{d \times V} = \text{pfu/ml}$$

where d is the dilution factor and V is the volume of diluted virus added to the well.

3.9 Infection assay

Aedes aegypti populations used in this study was obtained from Mentari Court, Selangor and Pasir Puteh ,Perak. Larvae were collected from the field and allowed to emerge into adults. Adults identified as *Ae. aegypti* were blood fed on restrained mouse to obtain eggs to start a colony. Both strains of *Ae. aegypti* were newly established in the Insectary of the Parasitology Department, University of Malaya. The colony was maintained at 27°C and relative humidity of 85% with photoperiod 12:12. The larvae were fed on tetramin fish food and each tray contained about 200 larvae. For the study, filial generation three (F3) adults were used.

Filter paper containing eggs were hatched into larvae in pans of water at a density of

University of Malaya

200-250 larvae per liter. The larvae were fed with tetramin fish food twice daily until they reach pupae stage. Pupae were transferred to $45cm^3$ cages. After emergence the adults were maintained at 27°C with relative humidity of 75% and 12-12h light-dark photoperiod. They were fed with 10% sucrose solution incorporated with 0.5% B-complex.

After 5-7 days of emergence, 200 female mosquitoes were separated into four paper cups (50 in each cup). As shown in Figure 3.2, paper cups of height 13 cm and diameter of seven cm were covered with netting on top. A squared window ($4\ cm^2$) shape was cut out on the outer vertical surface of the cup and covered with glove pieces. The glove pieces were cut across to allow filter funnel and aspiration tube to enter. Mosquitoes were starved for 36 hours without sucrose solution before feeding. Two mL of virus stock at $2.5X10^8$ ffu/mL was added to 8 mL of fresh human blood giving a final concentration of $6.25X10^7$ ffu/mL. Female mosquitoes were allowed to feed on the infectious blood meal through the membrane using Hemotek feeding system (Discovery workshop UK). Five aluminum holders were covered with membrane using a rubber ring to keep them in place as shown in Figure 3.3. Two mL of blood was introduced through the ports and sealed using plastic stoppers. The Hemotek feeding system employs an electric heating element to maintain the temperature of the blood meal at 37°C ± 1°C. The same procedure and apparatus were used to feed control mosquitoes, except that these received a non-infectious blood meal. The mosquitoes were allowed to feed for about 30-40 minutes as shown in Figure 3.4. After feeding, the mosquitoes were cold anesthetized by placing them in -20°C for 30 seconds. The cup containing the mosquitoes were immediately placed on ice and transferred to the glove box. Inside the glove box fully engorged females were transferred into new paper cups (10 per cup) using a forceps (Figure 3.5). The mosquitoes were placed inside a lock and lock box and kept in an environmental chamber maintained at 27±1°C; humidity 85%; 12L:12D

University of Malaya

(Figure 3.6). On day zero the mosquitoes were provided plain water by placing a wad of cotton soaked in water (excess removed) on the top of the netting in each cup. From day one onwards 10% sucrose solution with Vitamin B complex was provided instead of water. The cotton pad with sucrose solution was changed every two days to avoid fungal growth. The work was carried out in the arthropod containment level 2 laboratory (ACL2) in the parasitology Department. At 7, 10 and 14 days' post exposure to blood meal about 16-20 live mosquitoes were dissected. The mortality rate was also recorded from days 3-14.

University of Malaya

Figure 3.2: Mosquito cup covered with netting and window tightly covered with glove pieces

Figure 3.3: Hemotek feeder placed in a feeding chamber

University of Malaya

Figure 3.4: Mosquitoes being fed with the hemotek feeder

Figure 3.5: Mosquitoes isolated in paper cups with moist cotton

University of Malaya

Figure 3.6: Growth chamber with regulated temperature and humidity; 12 hL 12H D

University of Malaya

3.10 Dissection of mosquitoes

Mosquitoes were dissected individually to obtain midgut, and salivary gland. As a pre-dissection procedure, glass slides with two drops of saline and 1.4mm Zirconium Beads tube (OPS Diagnostics,Lebanon) with 500uL of MEM growth medium were labelled accordingly. Number of live mosquitoes was recorded and one cup was randomly chosen for dissection. The mosquitoes in the cup were cold anesthetized by placing them in -20'C freezer for 30 seconds and immediately placed the cup into a styrofoam box filled with ice. Prior to dissection, the wings and legs were removed and the body was placed on the glass slide. The abdomen was separated from thorax and placed on a drop of saline.

3.10.1 Procedure to obtain midgut

Abdominal portion on the saline was positioned with the apex of the abdomen facing towards right. The mosquito was held in position by fixing the left dissecting needle in the part that was attached to the thorax. By holding the abdomen with left needle, the right needle was used to gently pull out the stomach with the attached malphigian tubules and ovaries. The midgut was transferred to their respective tubes.

3.10.2 Procedure to obtain salivary gland

Pressure was placed simultaneously on the thorax while the head was pulled away from the thorax. As the head moves away from the thorax the exposed salivary gland was cut with the needle. The glands often trap in the thorax therefore, the tissue of the proboscis and thorax were teased in order to cut the glands. The tri-lobed appearance of salivary gland makes it easy to identify it accurately. The glands were placed in the respective tube.

University of Malaya

3.11 RNA extraction

Prior to RNA extraction, the organs placed in the bead tubes were homogenized using a homogenizer (Bertin Technologies,Lebanon) at a speed of 8000rpm. After homogenization extraction was carried out using QIAGEN VIRAL RNA mini Kit (Hilden, Germany) following manufacturer's protocol. A volume of 565.5 µL buffer AVL and reconstituted buffer AVE and carrier RNA was added into 200 µL of homogenized organ with MEM medium. The solution was vortexed briefly and incubated at 20°C for 10 minute. After incubation, 500 µL of ethanol (96-100%) was added into the sample and vortexed. The homogenous solution was then pipetted into QIAamp Mini Column and centrifuged at 8000 rpm for 1 minute. This step was conducted twice and the tube was discarded. Buffer AW1 was added into the tube and centrifuged at 8000 rpm followed by Buffer AW2 and centrifuged at 14000 rpm. Lastly, RNA was eluted into 1.5mL centrifuge tube with 60µL Buffer AVE.

3.12 Real Time Quantitative PCR amplification of the dengue RNA

The viral RNA used was quantitated using the genesig Real-Time PCR DENV Detection Kit (PrimerDesign Ltd., UK). It is a dengue specific primer and probe mix that can detect through the FAM channel which follows the TaqMan principle was used. During PCR amplification, forward and reverse primers hybridize to the dengue cDNA. A fluorogenic probe was included in the same reaction mixture which consists of a DNA probe labeled with 5'-dye and a 3'- quencher. During amplification, the probe cleaved and the reporter dye and quencher were separated. The increase in fluorescence was then detected by the real-time PCR (Applied Biosystem). The qRT-PCR assay standard plot, ranged from 10 to 10^6 RNA copies, was made by preparing a 10-fold serial dilution of the genesig DENV RNA standard. The genesig DENV RNA standard is a synthetic RNA template with known copy number. The PCR mix was performed in

University of Malaya

a final volume of 20uL containing 10uL of real time master mix, 1uL of probe-primer mix, 4uL of DNAse free water and 5uL of diluted RNA. Amplification profile was carried out according to the genesig manufacturer's protocol: 10 min at 55°C, 8 min at 95°C followed by 50 cycles of denaturation for 10 s at 95°C, annealing for 60 s at 60°C. Raw data was analyzed with StepOne Software v2.2.1 to determine copy number based on the threshold cycles (Ct). The efficiency of the qRT-PCR was measured from the slope of standard curve.

3.13 Analysis of the quantitated dengue RNA

To gain some insight of the dataset, descriptive statistics and graphical displays were used. Differences of infection rate and dissemination rate (calculation shown below) among the Mentari Court and Pasir Puteh vector populations were compared and evaluated using student T-test. Independent T-test was used to analyse the statistical significance of viral copy number in each individual organ on all three days of both populations. P values ≤ 0.05 were considered significant and 95% confidence interval (CI) was used as an estimate of the risk. SPSS software version 22.0 was used for the descriptive statistics (SPSS, 2013). Graph pad 5.0 PRISM, 2015 was used to display the positivity rate of organs for both the vector population as a bar chart. Viral copy number in each organ for overall population has also been shown as a bar chart. Spearman's non-parametric r test was used to test for a statistically significant correlation between viral titer in the midgut and salivary gland.

University of Malaya

Infection rate (IR)

$$\frac{\text{Positive midgut}}{\text{Total mosquito}} \times 100$$

Dissemination Rate (DR)

$$\frac{\text{Positive salivary gland}}{\text{Positive midgut}} \times 100$$

University of Malaya

CHAPTER 4: RESULTS

4.1 Genetic diversity

The alignment of the 451 bp CO1 gene sequences showed 19 polymorphic sites (Table 4.1). Analysis of the CO1 sequences showed a nucleotide diversity π = 0.01082 and haplotype diversity of Hd = 0.757. The average number of nucleotide differences among individual mosquito was 4.759 (Table 4.2). In total, 14 unique CO1 haplotypes were identified and designated as C1-C14. All sequences have been deposited in GenBank (Appendix B).The ND5 gene was 324 bp in length, with sequence alignment revealing seven polymorphic sites. Nucleotide diversity based on the ND5 gene was π=0.00742 and haplotype diversity Hd = 0.643 (Table 4.2). Based on the sequences, five unique haplotypes of ND5 gene were identified, designated here as N1-N5. All sequences have been deposited in GenBank (Appendix B).

Analysis based on combined CO1 and ND5 genes was also carried out. A set of concatenated sequences of 775 bp based on combined CO1 and ND5 genes were aligned. The alignment of the combined CO1-ND5 gene sequences revealed 26 polymorphic sites, of which eight were synonymous sites and 18 were non-synonymous sites. Analysis of gene diversity showed a nucleotide diversity π =0.00951 and haplotype diversity Hd = 0.763 (Table 4.2). Eighteen unique CO1-ND5 haplotypes (designated as H1-H18) were identified. Haplotype 3 (H3) represented the most commonly occurring haplotype at a frequency of 45.6%, followed by H14, H1, H13, H2, H8, H10 and H9, each occurring at 12%, 8.8%, 6.4%,5.6%,4.8%, 3.2%, and 2.4% respectively. Second less observed haplotypes were H4, H6, H15, and H18 at a frequency of 1.6%. The least observed haplotypes include H5, H7, H11, H12, H16, and H17 occurring at 0.8% each from overall sampling figure.

University of Malaya

The polymorphic sites of each sequence and each haplotype are tabulated in reference to C1 isolate that. Nucleotide composition analysis of the concatenated sequence revealed relative values of C; 17.35%, T; 36.05%, A; 33.56%, and G;13.04%.

University of Malaya

Table 4.1: Polymorphic regions of concatenated sequences (H1-H18) and matching CO1 haplotypes (C1-C14)

	CO1																				ND5					
	1	6	7	1	1	2	2	2	2	2	3	3	3	3	3	3	3	3	4	4	5	5	5	5	6	7
	1	8	7	7	9	0	1	1	3	3	0	1	5	5	6	6	7	9	0	8	3	6	6	9	5	1
				3	7	6	2	8	0	9	5	7	3	6	5	8	4	8	1	2	0	0	3	3	0	6
(H1) C1	T	G	G	G	A	A	G	A	C	T	A	G	C	T	A	A	T	C	A	C	C	A	T	C	T	C
(H2) C2	.	.	T	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.
(H3) C3	.	.	T	.	.	G	.	G	T	C	.	A	T	.	.	G	C	T	.	.	T	G	.	.	C	T
(H4) C2	.	.	T	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	.	.	T	G	.	.	C	T
(H5) C4	.	.	T	.	.	.	.	.	T	.	.	.	T	.	.	.	C	.	.	.	T	G	.	.	C	T
(H6) C5	.	.	T	.	.	.	.	.	T	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.
(H7) C6	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
(H8) C7	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.
(H9) C8	.	.	T	.	.	.	A	.	T	C	.	.	T	.	.	.	.	.	G	.	T	G	.	.	.	T
(H10) C8	.	.	T	.	.	.	A	.	T	C	.	.	T	.	.	.	.	.	G	.	T	G	.	T	.	.
(H11) C9	.	.	T	.	.	.	.	.	T	.	.	.	T	C	C	.	.	.	.	.	.	.	.	.	.	.
(H12) C10	.	.	T	.	.	.	.	.	T	.	G	.	T	.	.	.	.	.	.	.	T	G	.	.	C	T
(H13) C10	.	.	T	.	.	.	.	.	T	.	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.
(H14) C11	.	.	T	.	G	G	.	G	T	C	.	A	T	.	.	G	C	T	.	.	T	G	.	.	.	T
(H15) C12	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
(H16) C13	.	A	T	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.
(H17) C14	.	.	T	A	.	.	.	.	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.
(H18) C2	.	.	T	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.

University of Malaya

Table 4.2: Summary statistics of mtDNA gene diversity in *Aedes aegypti*

	CO1	ND5	CO1-ND5	*P*
Nucleotide diversity, π	0.01082	0.00742	0.00951	
Number of haplotypes, H	14	5	18	
Haplotype diversity, Hd	0.757	0.643	0.763	
Average number of nucleotide differences, k	4.759	2.048	6.807	
Tajima's D	0.98744	1.31526	1.20925	Not significant
Fu and Li's D*	0.2642	0.64533	0.72213	Not significant
Fu and Li's F*	1.31526	1.45123	1.09542	Not significant
Fu's Fs	1.333	3.567	1.399	Not significant

P >0.10 ; Not significant

University of Malaya

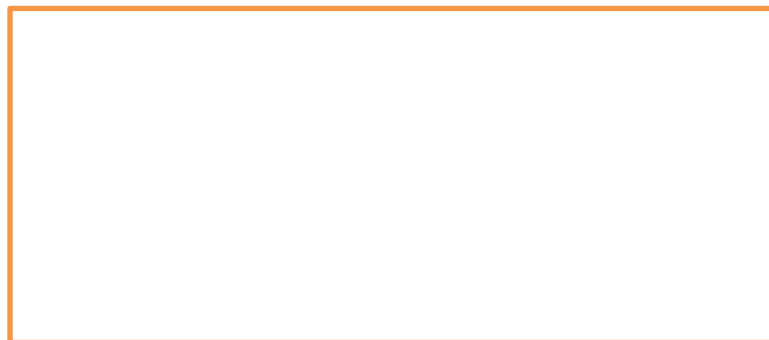
4.2 Tests of neutrality

Tajima's test of neutrality (Tajima, 1989) compares the number of segregating sites per site with the nucleotide diversity. Test of neutrality based on Tajima's D, Fu and Li's D* and F* statistics were performed based on the combined CO1-ND5 data set and also separately for each gene. Neutrality test statistics for all tests were insignificant; Tajima's D (1.20925, $P > 0.10$), Fu and Li's D* (0.72213, $P > 0.10$) and Fu and Li's F* (1.09542, $P > 0.10$) (Table 4.2), and thus do not reject the null hypothesis of neutral evolution. As these tests are also sensitive to demographic changes or selective events, the observation that no departure from neutrality suggests that the variability of CO1 and ND5 gene of *Ae. aegypti* can be explained by the neutral evolution.

University of Malaya

4.3 Analysis of demographic history and population structure

Historical demographic expansions were also utilized by examination of frequency distributions of pairwise differences between sequences. A pairwise mismatch distribution based on the CO1-ND5 concatenated data set revealed a multi-modal distribution profile as shown in Figure 4.1, which deviates from expected distribution for a population that has undergone expansion (Harpening raggedness, r = 0.126, P<0.01). Mean number of pairwise difference computed reveals a relative value of 7.540903 ± 3.544065. Mismatch distribution summarized information about genetic differences between pairs of sample and displayed using a scatter plot with relative frequencies of pairs that differ by zero sites, by one site and so forth. The multimodal distribution and ragged shape suggests a constant population size over a long period of time, and perhaps could be due to the existence of population substructure. In general multimodal mismatch distributions suggest diminishing population sizes or structured size; and a raged distribution indicates that the lineage was widespread (Rogers & Harpending, 1992). The raggedness index under the demographic expansion model for each population was also calculated and found all population had significant raggedness index which suggest the observed distribution deviates from expected distribution for a population that has undergone a demographic expansion.



University of Malaya

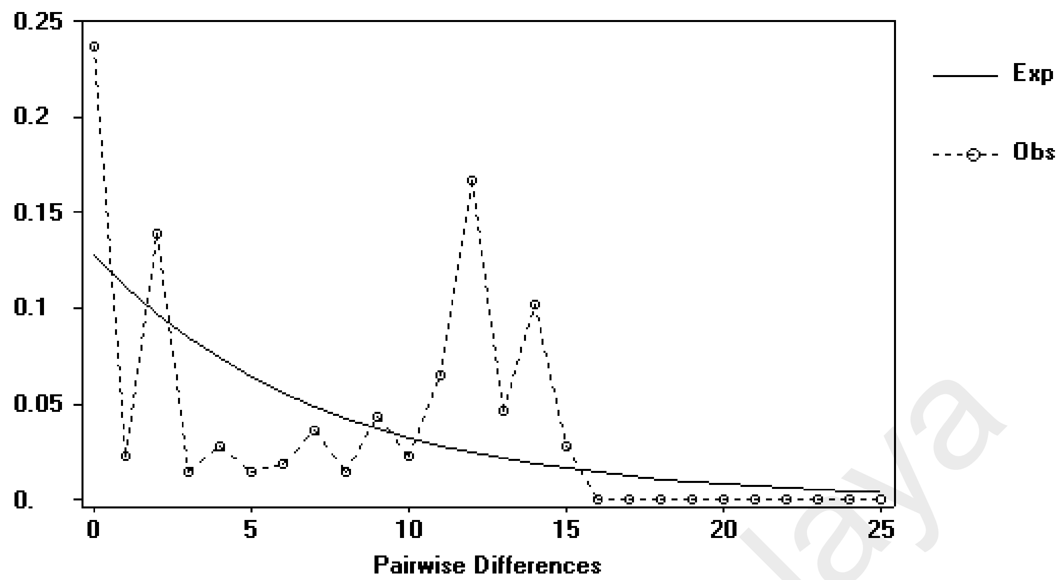


Fig 4.1: Multi-modal distribution of *Ae. aegypti* CO1-ND5 between haplotypes. The y-axis denotes frequency and x-axis denotes number of segregating sites between pairwise comparisons.

University of Malaya

4.4 Analysis of population structure

Evidence of spatial subdivision of *Ae. aegypti* was assessed using the Spatial Analysis of Shared Alleles (SAShA) method to detect for subtle evidence of population structuring. SAShA analysis revealed as shown in figure 4.2, that the spatial distribution of CO1-ND5 haplotypes were significantly different from expectation under panmixia (OM=50.86 km compared to EM=109.25 km, p-value of OM-EM= 0). This observation suggests that the CO1-ND5 haplotypes were under distributed, which could be due limitation to gene flow. With P-value of zero, none of the permuted datasets datasets showed a larger divergence than the observed data (Figure 4.2.1).

University of Malaya

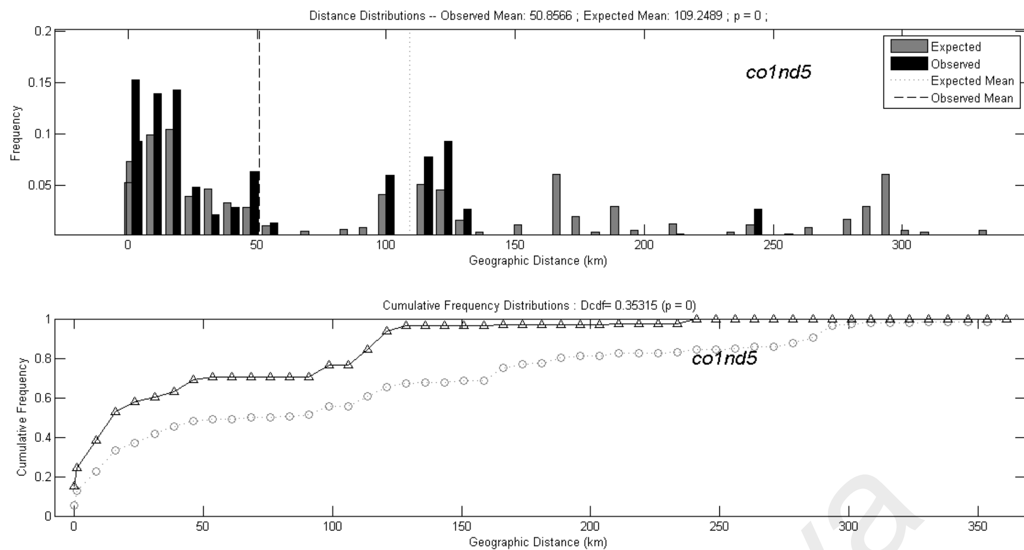


Fig 4.2: Analysis of *Ae. aegypti* CO1-ND5 haplotypes using SAShA reveals that the haplotypes are significantly under distributed

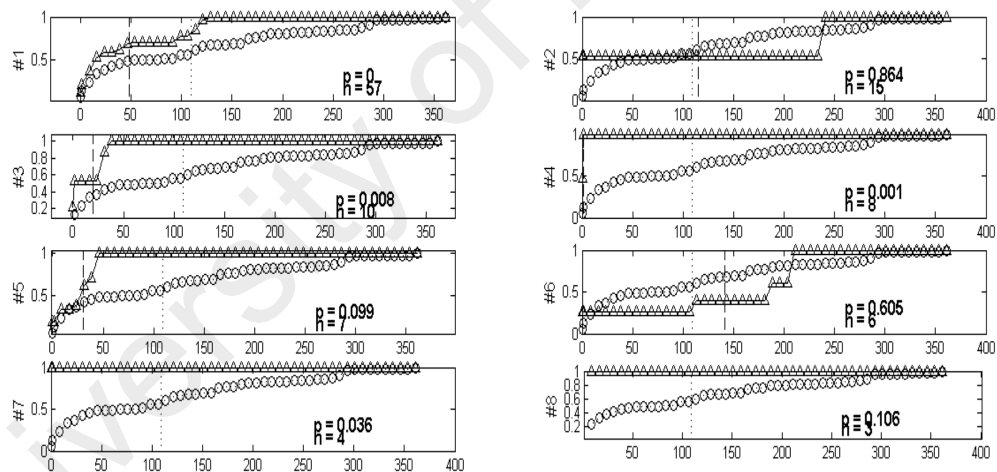


Fig 4.2.1: Haplotype-by-haplotype analysis of CO1-ND5 data sets. The number of occurrences of each haplotype is given (n) as well as the haplotype-specific SAShA statistics. Haplotype number, in order of decreasing frequency, is on the y-axis for each plot. The expected distributions are represented by gray open circles, and observed distributions by dark open triangles.

University of Malaya

4.5 Haplotype analysis

A haplotype network based on the combined CO1-ND5 gene was constructed using the statistical parsimony approach. A total of 18 haplotypes samples in the Malaysian populations of *Ae. aegypti* was assigned. The haplotype network (Figure 4.3) showed several distinct groupings, designated here as group 1, and 2. These distinct groups of haplotypes separated by 14 mutational steps and are likely to represent at least two separate maternal lineages of *Ae. aegypti* in peninsular Malaysia. Group 1 composed of the most prevalent haplotype (H3), which accounted for 45.6% of the total specimen collected. Although appeared as the most prevalent haplotype, the level of diversity within the first clade is low as it is represented by only two (H3 and H9) very closely related unique haplotypes, suggesting a stable population of the predominant haplotype. On the other hand, group 2, which was formed by haplotype H1,H2,H6,H7,H8, H12,H13,H14,H15,H16,H17 and H18 showed a complex evolutionary pattern, with reticulation and several high-frequency haplotypes. Certain haplotypes in group 2 were low frequency derived such as H7, H12, H16, H17 and H18. Haplotype H10 which is exclusive for Gunung Rapat sample was separated by five mutational steps from H4 and H5. Intermediate such as H5 and H11 is low frequency derived haplotypes which occurred only once.

University of Malaya

Figure 4.3: Genealogical network showing the relationship among 18 CO1-ND5 haplotypes of *Ae. aegypti*. Size of circle is proportional to the frequency of a particular haplotype. Each line that connects two circles represents a mutational step and black dots represent hypothetical missing intermediates.

University of Malaya

4.6 Phylogenetic analysis

Phylogenetic analysis was performed based on CO1 and ND5 gene sequence separately given the availability of data in the GenBank database for comparison. The phylogeny of *Ae. aegypti* was inferred using the Bayesian approach implemented in BEAST program (Bouckaert *et al.*, 2014).

4.6.1 CO1 gene

The maximum clade credibility phylogeny revealed two clades (one major, one minor) that are well supported by posterior probability of more than 0.9 (Figure 4.4). In general, 12 CO1 haplotypes clustered within Clade 1 (major clade) and were closely related to *Ae. aegypti* from Southeast Asia and Indian subcontinent in various lineages. In particular, C13, C12, C1, C4, C14, C10 and C6, which represent the low frequency haplotypes except for C1 and C10 were more closely related to the India and Pakistan populations. Haplotypes C7 and C5 were related to two cities in Vietnam and C8 and C9 were related to Brazil population. The minor clade was formed by C11, C3, Brazil and France in which C3 is a predominant haplotype followed by C11. Surprisingly, both the Senegal sequences forms the out group suggesting not to rule out mutation.

University of Malaya

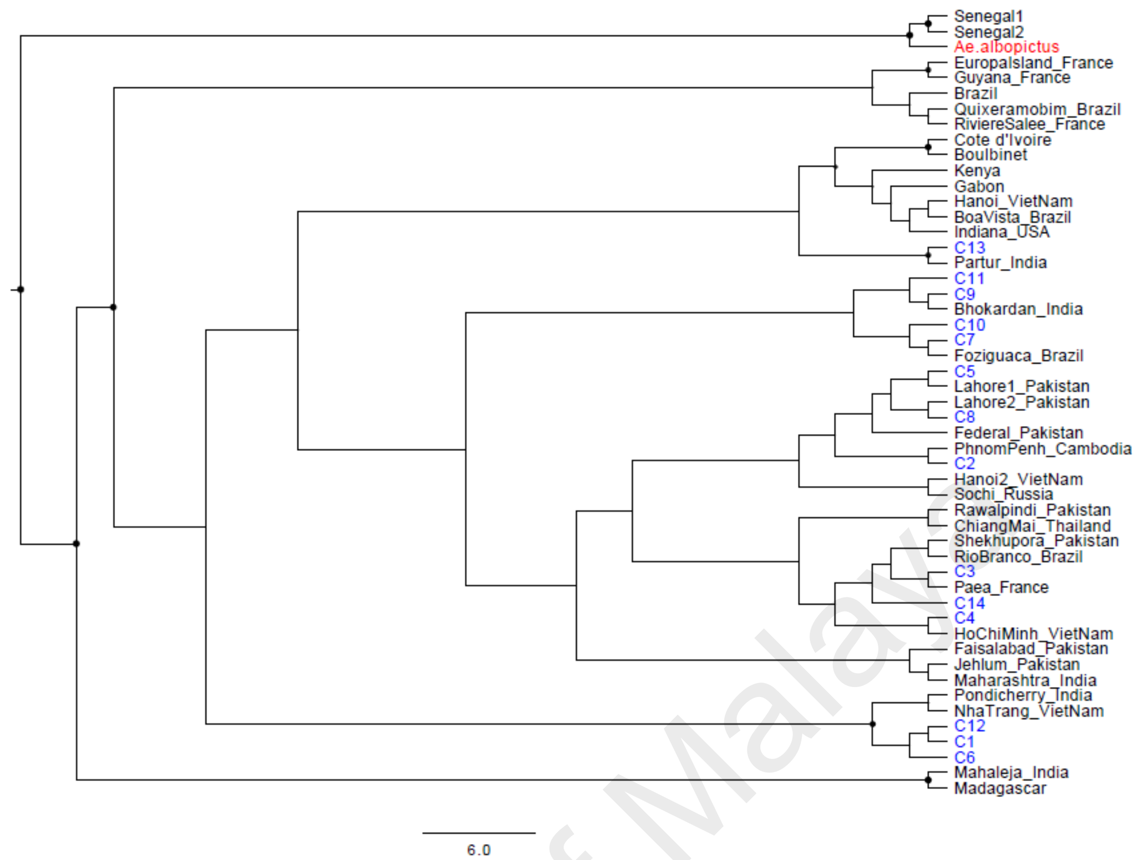


Fig 4.4: Maximum clade credibility phylogeny of *Ae. aegypti* based on CO1 gene. One CO1 sequence to represent each CO1 haplotype identified in the present study (C1 – C11) was used for comparison with those previously reported. Sequences of CO1 gene of *Ae. aegypti* from different countries were obtained from GenBank database, with accession numbers listed in appendix. Clades supported with posterior probability >0.8 are indicated as black dots on the nodes.

University of Malaya

4.6.2 ND5 gene

As a result of Bayesian inference, ND5 phylogenetic tree show two clades (major and minor). Five haplotypes derived from ND5 gene sequence together with samples from Southeast Asia, Russia, South America and Africa was used to generate the tree (Figure 4.5). The major clade is formed by samples from Southeast Asia, Russia, France, Brazil, Guinea and Africa together with haplotypes N1 to N5. Haplotypes N1 (predominant haplotype), N3 and N4 were seen to be more closely related to South America, N5 the least frequent haplotype was related to Vietnam and N2 the second most predominant haplotype was related to Russia. The minor clade resulted in Southern South America states forming the nodes exclusively.

University of Malaya

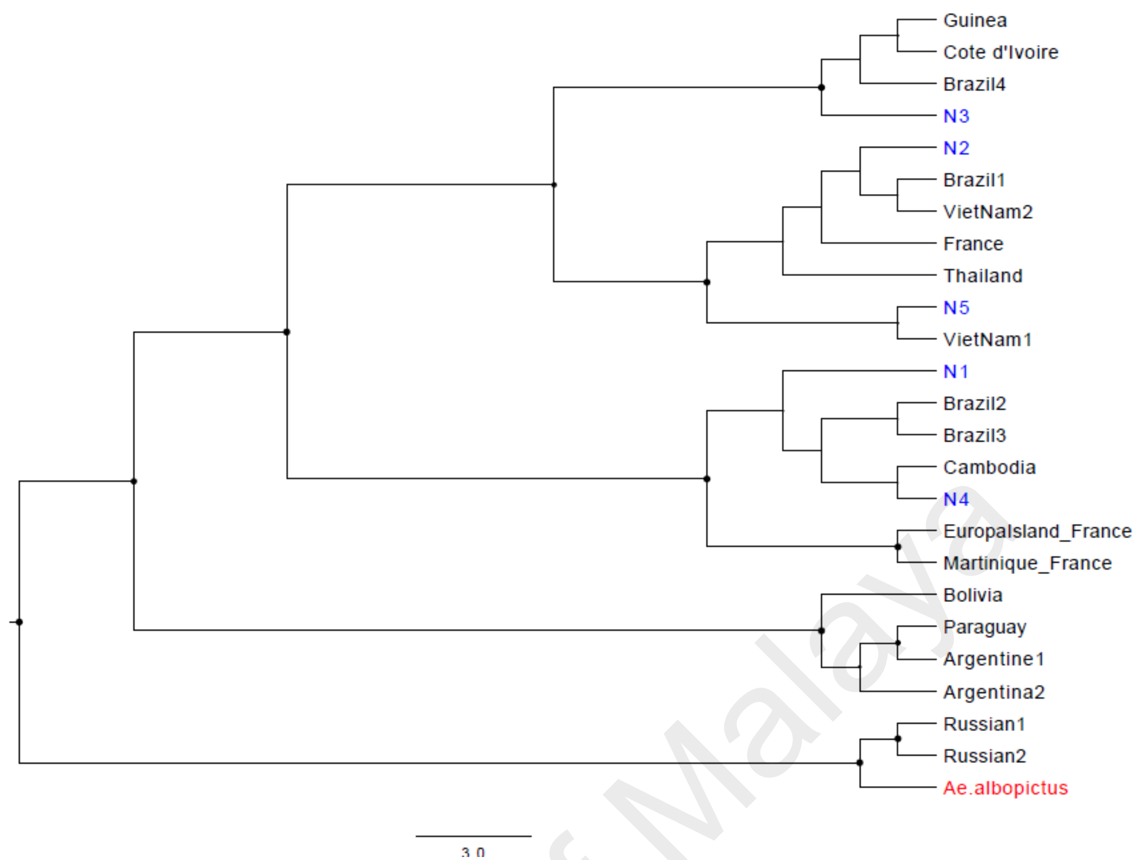


Fig 4.5: Maximum clade credibility phylogeny of *Ae. aegypti* based on ND5 gene. One ND5 sequence to represent each ND5 haplotype identified in the present study (N1 – N5) was used for comparison with those previously reported. Sequences of ND5 gene of *Ae. aegypti* from different countries were obtained from GenBank database, with accession numbers listed in appendix. Clades supported with posterior probability >0.8 are indicated as black dots on the nodes.

University of Malaya

4.7 Selection of sample for infection assay

Two field derived *Ae. aegypti* populations from two different locations were choosen to study the vector competence rate towards DENV1. Mentari Court, a low cost residential area located in Klang Valley, reported high number of dengue cases and is listed as an endemic area in recent years. Pasir Puteh and Gunung Rapat, located in Ipoh a district in the state of Perak were found to have a unique and specific haplotype. It was also noted that the number of dengue cases were rare in that location even in the presence of the vector. In the phylogenetic analysis, the mtDNA sequence of MentariCourt and Pasir Puteh sample happens to fall into two different clades, further suggesting the presence of two different lineages *of Ae. aegypti* population. This concurs with the haplotype network which shows samples from Perak does not fall into the same lineage as Mentari Court. Especially some samples from Pasir Puteh had close relation with the predominant haplotype but not Mentari Court. In addition, pairwise genetic difference was estimated between Mentari Court and Pasir Puteh (Group1) and Mentari Court and Gunung Rapat (Group 2) by calculating Wright's F-statistics (Fst) and gene flow (Nm) using DNAsp. Average number of pairwise nucleotide differences (Kxy), nucleotide substitution per site (Dxy), and net nucleotide substitution per site (Da) between populations were also calculated by DnaSP (Table 4.3). Group 1 showed a higher diversity index which contributed in choosing Pasir Puteh *Ae. aegypti* as a comparative population. Samples from Terengganu was not considered for the vector competence assay because it shares similar haplotye with five P. Puteh samples therefore results can be representative of Terengganu as well.

University of Malaya

Table 4.3: Population genetics indices, pairwise genetic distance and gene flow between populations of Mentari Court compared with Gunung Rapat and Pasir Puteh calculated from nucleotide sequence of mitochondrial CO1-ND5 gene.

Population 1	Population 2	Kxy	Fst	Dxy	Da	Nm
Mentari Court	Pasir Puteh	10.62	0.6946	0.0137	0.0095	0.4
Mentari Court	Gunung Rapat	7.5	0.8888	0.0097	0.0086	0.12

University of Malaya

4.8 Survival rate

A total of 78 infected mosquitoes from Mentari Court and 86 from Pasir Puteh died within a period of the 14 days. The mortality rate of Pasir Puteh was 64% which was slightly higher compared to Mentari Court 61.4%. The survival rate of both populations at different intervals is shown in Figure 4.6. The mortality rate of control (uninfected) mosquitoes was slightly higher for Mentari Court samples by 2.18%.

University of Malaya

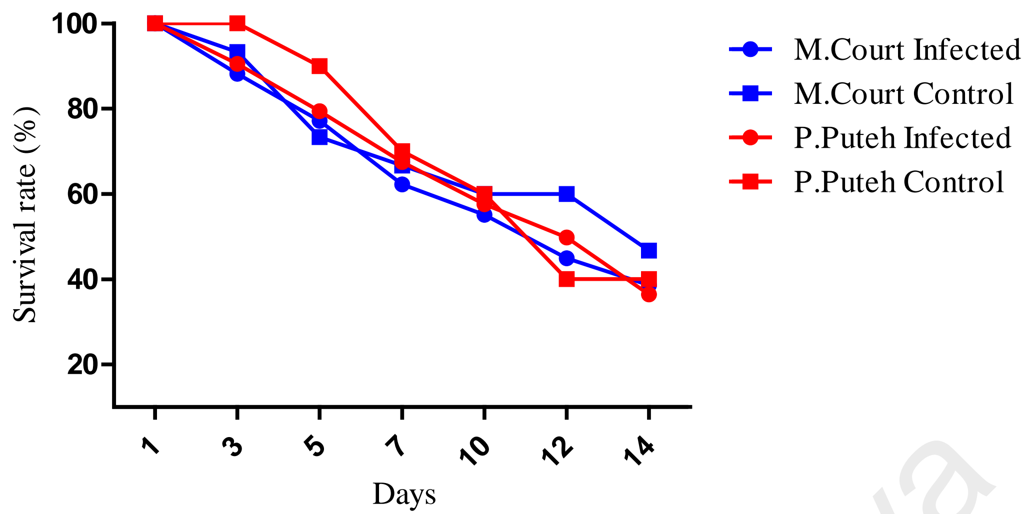


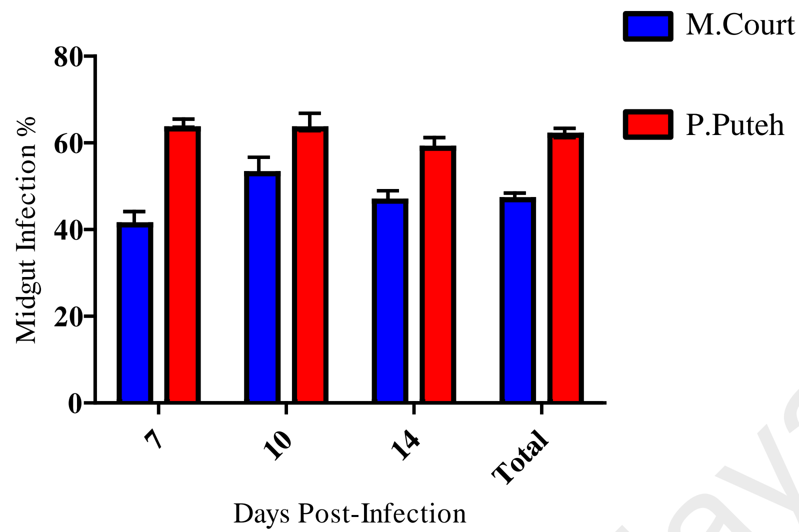
Figure 4.6 : Survival rate of infected and control *Ae. aegypti* from two localities

University of Malaya

4.9 Susceptibility of Mentari Court and Pasir Puteh mosquitoes towards DENV1

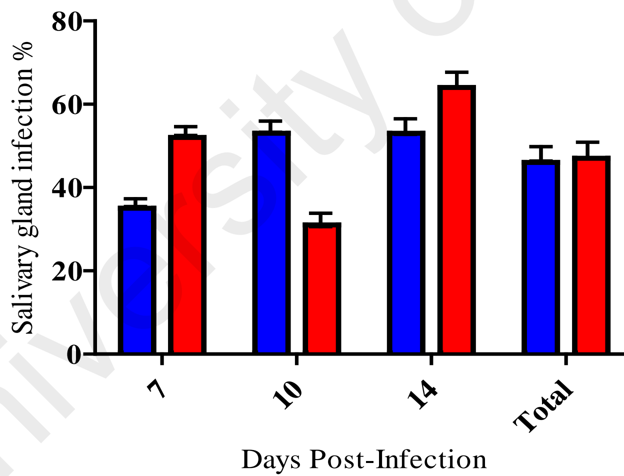
Infection rate (IR) is the proportion of DENV-infected mosquitoes (incidence of virus in the midgut) of the total mosquito sample. In Mentari Court population, a total of 127 *Ae. aegypti* were successful in feeding. However, the mortality rate was high and therefore 15 to 19 samples were dissected at each time point. A total of 49 *Ae. aegypti* was subjected to viral quantitation since 61.4% mortality was recorded. Of these, 26 (53.0%, 95% CI: 39.3-66.3%) mosquitoes had midgut infection and 23 (46.9%, 95% CI: 33.7-60.6%) had salivary gland infected with DENV1. For the Pasir Puteh population 151 *Ae. aegypti* were fully blood fed but mortality observed until day 14 was 64%. Infection rates were measured in a total of 55 mosquitoes. Of these, 35 (63.6%, 95% CI: 50.3-75.1%) had midgut infection and 26 had salivary gland infection (47.3%, 95% CI: 34.6-60.2%).The percent of infected midgut and salivary gland in both locations at all three intervals are shown in Figure 4.7. Pasir Puteh population showed higher percentage positive for all three interval in both the organs except for day 10 in salivary gland. However the dissemination rate calculated was significantly higher in Mentari Court samples for the first two intervals (day 7 and 10). Overall dissemination rate was higher in Mentari Court samples (88.0%) compared to Pasir Puteh (74.3%) and the difference is significant (95% CI:65.6-123.94%, $p<0.001$) as shown in Figure 4.8. Disseminated Rate (DR) is the proportion of DENV-infected mosquito salivary gland of an infected population with virus in the midgut (IR). Therefore, suggesting that Mentari Court mosquitoes are more efficient than Pasir Puteh in the dissemination of dengue-1 virus.

University of Malaya



M.Court (n)	17	17	15	49
P.Puteh (n)	19	19	17	55

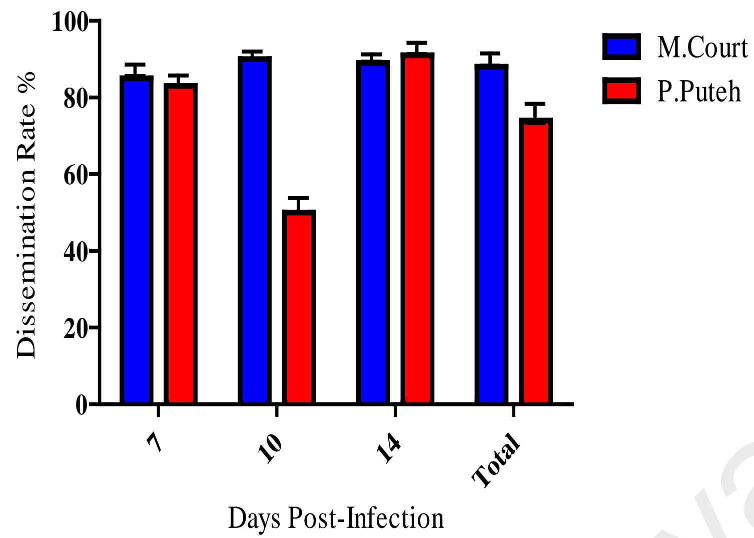
B)



M.Court (n)	17	17	15	49
P.Puteh (n)	19	19	17	55

Figure 4.7: Infection rates in midguts (A) and salivary glands (B) were measured by calculating the percentage of positive organs over total samples. Denominators are shown (n)

University of Malaya

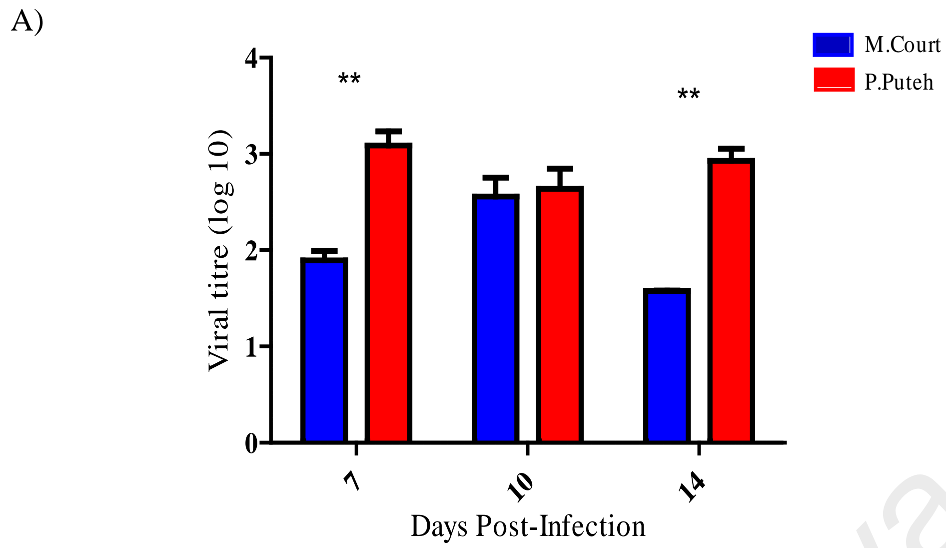


M.Court (n)	7	9	7	23
P.Puteh (n)	12	12	10	34

Figure 4.8: Dissemination rate calculated by percentage of positive salivary gland over total positive midgut. Denominators are shown (n).

University of Malaya

A)



B)

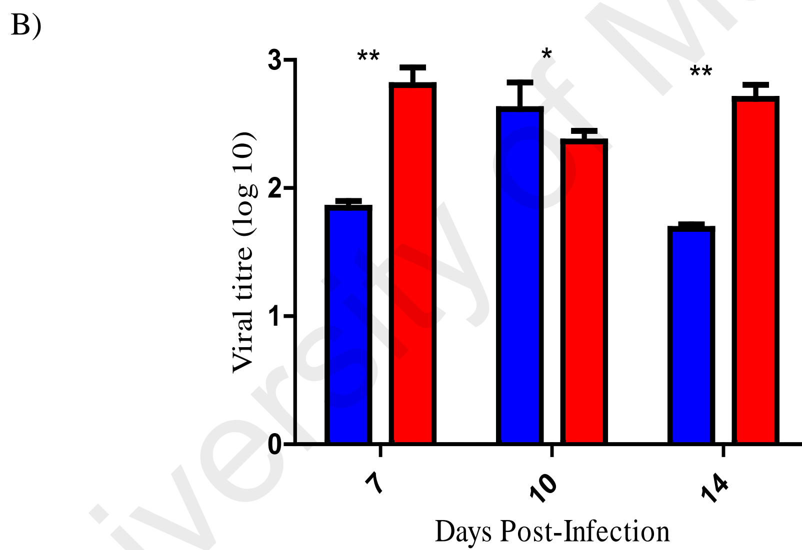


Figure 4.9: The mean viral copy number with standard mean error in midgut (A) and salivary gland (B) is shown. Asterisks indicate significant differences between two locations (*p<0.05, **p<0.01).

University of Malaya

4.10 Comparative dengue virus replication kinetics between *Ae.aegypti* from Mentari Court and Pasir Puteh.

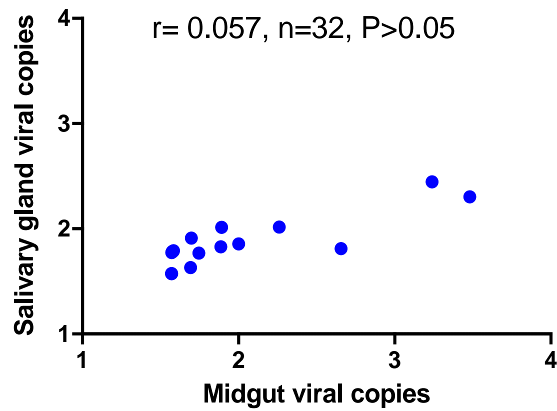
Viral loads for each infected *Ae. Aegypti* midguts and salivary gland from day 7, 10 and 14 post infection for both locations is presented in Figure 4.9. Midguts of *Ae. aegypti* from Pasir Puteh had higher virus replication on all days tested compared to *Ae. aegypti* from Mentari Court and this was significantly different ($P<0.05$) except for day 10. The virus replication in the salivary gland was also higher in the Pasir Puteh population except on day 10, *Ae. aegytpi* from Mentari Court had higher viral titre which is also significant ($P<0.05$).

4.11 Correlation between midgut and salivary gland virus titer

Viral dissemination is associated with midgut viral titer. Overall, viral dissemination did not occur in 11% (3/26) of the Mentari Court mosquitoes and 26% (9/35) in Pasir Puteh mosquitoes. In mosquitoes with dissemination, the midgut viral copy number was plotted against salivary gland viral copy number according to population (Figure 4.10). Samples with low copy number (<50) were excluded from the plot. Spearman's non parametric r test used to test between midgut viral copy number and dissemination shows a positive correlation and significant p value ($r= 0.43$, $p<0.05$) for Pasir Puteh samples. Mentari Court ($r=0.057$, $p>0.05$) shows a very low positive correlation and insignificant p value. Current observation suggests that there is a threshold titer in the infected midgut which may aid and influence viral dissemination to the salivary gland.

University of Malaya

A)



B)

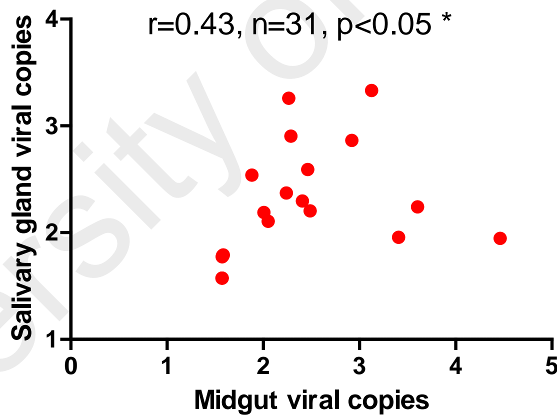


Figure 4.10: Viral titer of midgut versus salivary gland pooled in log 10 values (A) Mentari Court population and (B) Pasir Puteh population. Spearman's non-parametric test is used to test for a correlation between midgut and salivary gland viral titer. Individuals with viral titer <50 were excluded from the plot.

University of Malaya

CHAPTER 5 : DISCUSSION

5.1 Genotypic variation

To our knowledge this is the first study of molecular phylogeography and genetic diversity of *Ae. aegypti* population in Malaysia. The mitochondrial genes have been widely used to understand the genetic variability of mosquito species in various regions throughout the world (Gorrochotegui-Escalante *et al.*, 2000; Mousson *et al.*, 2005; Bracco *et al.*, 2007; Kamgang *et al.*, 2013). Mitochondrial genome is maternally inherited through female eggs and it has more linear and clonal evolution than nuclear DNA (Beebe *et al.*, 2005). Its coding genes also display a more rapid rate of evolution making it useful marker for studying intraspecific population genetic variation. For instance, CO1 gene was found useful for intraspecific studies of *Anopheles* and in interspecific studies of *Aedes* mosquitoes (Beebe *et al.*, 2005). ND5 gene on the other hand, was used for population analysis in *An. gambiae* and *An. arabiensis* (Besansky *et al.*, 1997). In a comparative study, Mousson and co-workers (Mousson *et al.*, 2005) analyzed the variability levels of partial fragments of three mitochondrial genes and concluded ND5 to be the most informative for *Ae. aegypti* compared to *Ae. albopictus*. In our study, we used both the genes to characterize the genetic variation of *Ae. aegypti* within Klang Valley and other regions in peninsular Malaysia.

In this study we observed the genetic diversity in CO1 was greater than that in ND5. This was supported by higher indices in CO1 produced by nucleotide diversity and polymorphic sites. Greater number of haplotypes was also observed in CO1 gene with higher diversity. This perhaps might be due to selective constraint on the rate of mutation in ND5 gene in Malaysian *Ae. aegypti* population. This is in contrast with previous study carried out in Argentina (Llinás & Gardenal, 2012) and a comparative study conducted in *Ae. aegypti* and *Ae. albopictus* (Mousson *et al.*, 2005) which

University of Malaya

suggested ND5 gene to be a better informative site for *Ae. aegypti* compared to CO1 gene.

Genetic studies based on the mitochondrial genes of *Ae. aegypti* populations across different countries and continents showed varying degrees of genetic diversity. We utilized the information of genetic variability in both CO1 and ND5 by combining both genes in a concatenated set of sequences for further analysis. The nucleotide and haplotype diversity of *Ae. aegypti* based on the combined gene sequences (π=0.00951) showed a slightly higher indices compared to a previous study conducted in Argentina, which was based on ND5 gene alone (Llinás & Gardenal, 2012) . This is expected given that more informative sites from the combined sequences are likely to contribute to higher indices of genetic diversity. In general, our findings of genetic diversity indicate that the *Ae. aegypti* population in the present study was comparable to previous studies in Thailand and Peru (Bosio *et al.*, 2005; Costa-da-Silva *et al.*, 2005). Significantly higher indices of genetic diversity were also observed in many studies reported in the Americas (Gorrochotegui-Escalante *et al.*, 2002; Herrera *et al.*, 2006; Bracco *et al.*, 2007; Paduan & Ribolla, 2008; Twerdochlib *et al.*, 2012). Such variability in genetic diversity of *Ae. aegypti* may be due to the differences in vector control programs or strategies, which may result in the increase or loss of diversity due to elimination of certain lineages of *Ae. aegypti*.

Haplotype analysis based on the combined CO1-ND5 sequences revealed 18 unique haplotypes. Haplotype H3 represents the most common haplotype (n=57) and constitutes 45.6% of the specimens analysed. Haplotype H3 was found at 10 closely distributed locations, which include Kajang, Kerinchi, Pulau Indah, Chan Sow Lin, Lembah Keramat, Ampang, Cheras, Shah Alam, Bukit Nanas and Petaling. This reflects the continuing gene flow among these locations, which are well connected by highways and other land transportation routes. Thus, it is possible for species from these regions

University of Malaya

to migrate and colonise the areas quickly. Of particular interest, samples from Melaka (75%) were found to be in haplotype 3, found predominantly in Klang Valley and it might be due to colonisation history. There was a unique haplotype (H9) from the state of Perak and it was closely related to H3, separated by 2 mutational steps within the haplotype network. It is unclear whether haplotype H9 represents the common haplotype in the state of Perak given five out of eight samples was found in H14 together with Terengganu samples. Other unique haplotypes that were only found in single samples includes those from the same locations as haplotype H3, indicating the mixing of rare and common haplotypes in the Klang Valley. The presence of rare unique haplotypes could be due to recent introductions from neighboring states or overseas. Overall the haplotype network suggests the presence of two different lineage of *Ae. aegypti* from the current sampling sites. Similarly this has been reported in America (Bracco *et al.*, 2007; Jaimes-Dueñez *et al.*, 2015). It is possible that the differences between populations are the result of random genetic drift, trait selections or greater selection for resistance in the vector in an outbreak-prone region in this case Klang Valley.

The presence of two maternal lineages in the current sampling sites from Malaysia is further supported by phylogenetic relationship constructed using the Bayesian approach. Bayesian inference analysis of the CO1 and ND5 gene revealed the global relatedness of the sequences recovered from peninsular Malaysia. The first lineage (C11 and C3) is more closely related to Brazil and France. Since C3 is the predominant haplotype in Malaysia, it can be assumed Brazil, France and Malaysia might have common ancestors. Moreover, C9 haplotype found in the second lineage is also closely related to Brazil. The second most commonly occurring haplotype (C2) was also found to be related to Brazil further suggesting the relatedness. Unique haplotypes such as C4, C6, C12, C13 and C14 which occurred at low frequency were more related to India,

University of Malaya

Pakistan and Vietnam samples suggesting recent invasion of this haplotypes through human movement. Surprisingly haplotype C8, combination of Gunung Rapat and Pasir Puteh, Perak samples clustered together with Kenya, USA and Brazil showing their relatedness. As mentioned earlier *Ae. aegypti* was first introduced to Malaysia from Africa. Therefore, the parental genome might be still residing in certain locations in Malaysia and Perak is believed to be one of them. Contradicting samples from Senegal formed minor clade from the root reminding us not to rule out mutations and genetic bottleneck events. Despite low number of haplotypes derived from ND5 gene, the Bayesian approach evolutionary tree concurs with the hypothesis of two maternal lineages in Malaysia. Two clades were observed in which the minor clade was formed by sequences isolated from western South America. The major clade includes all ND5 haplotypes (N1-N5), Brazil, Vietnam, France, Russia, Cambodia, Africa and Thailand. Similar to CO1 phylogenetic tree, the predominant haplotype (N1) seem to be more closely related to France which further strengthen our hypothesis on common ancestor.

Interestingly, our finding based on the analysis of spatial arrangement of co-occurring alleles indicates that the *Ae. aegypti* population was significantly different from panmixia, which probably suggests the limitation of gene flow. Another wide spread deviation from panmaxia is caused by all sorts of isolating barrier; geographical, physiological and vector control measures. Alternatively, this observation could be due to under distribution of *Ae. aegypti* haplotypes, which may be a result of sparse sampling in the present study.

The lack of low frequency derived haplotypes that are closely related to the most common haplotype (H3) as displayed by the haplotype network is an indication of a stable population of *Ae. aegypti* in the Klang Valley. This is supported by the non-significant neutrality tests based on Tajima's D, Fu and Li's D* and F* statistics, suggesting that the *Ae. aegypti* populations are not expanding. Analysis based on the

University of Malaya

pairwise mismatch distribution, which revealed a multi-modal distribution provides further evidence of a stable population of *Ae. aegypti* in the Klang Valley. If populations are subdivided, they can evolve apart, somewhat independently allowing populations to diversify. This is the reason why population structure is a very important part of evolutionary genetics. The low frequency haplotypes found in the Klang Valley might be an initiator of population substructure in the future.

In Malaysia, *Ae. aegypti* is a predominant dengue vector therefore its susceptibility towards the single stranded RNA virus can contribute to its genetic lineage. Adaptation of this virus could alter the genotype of the vector in which we can exclude this limitation as we used mitochondrial DNA to infer out genotypic variation study. However, these differential genotype findings can lead to investigating the susceptibility of virus in distinct *Ae. aegypti* population. According to Failloux *et al* (Failloux *et al.*, 2002) genetic differences among *Ae. aegypti* populations depend on colonization history and may be associated with dengue susceptibility.

The easy nature of dispersal of *Ae. aegypti*, will lead to more mixing of various population and this may either give rise to progeny which are more susceptible to dengue virus or could lead to less susceptible strains. Thus, it is important to study the genotypic variation of *Ae. aegypti* throughout Malaysia and their competency to dengue virus. This will enable a better planned control programme to curb the spread of dengue.

5.2 Infections assay

This study was conducted to test the hypothesis that different population of *Ae. aegypti* may have distinct susceptibility to arbovirus infections (Black *et al.*, 2002). Dengue virus spread and invasion occurs rapidly with the aid of *Ae. aegypti*. There are many factors that contribute to the vectorial capacity of a mosquito including vector

University of Malaya

susceptibility to the virus, length of extrinsic incubation period, mosquito survival, density of susceptible host-vector and proportion of infected mosquitoes that are feeding (Moncayo *et al.*, 2004). We investigated the vector susceptibility by quantitating the presence of DENV1 RNA in midgut and salivary glands at three post infection intervals; day 7, 10 and 14.

To detect and quantitate DENV, quantitative real time polymerase chain reaction (qRT-PCR) has become the method of choice in the past few years (Lanciotti *et al.*, 1992; Vaughn *et al.*, 2000); this method is generally more sensitive and efficient than isolation assays, and can provide a rapid serotype-specific diagnosis. Although there is a relatively good general correlation between the inoculation technique and qRT-PCR using the same host systems to grow DENV, the accuracy of measuring infectious DENV using RNA copy number may vary based on the virus strain or time of infection as the ratio may be significantly different from one another.

The overall result of this study showed both the populations to be susceptible towards DENV1 and are able to disseminate the infection by day seven which is shorter than reported in previous studies (Watts *et al.*, 1986; Chepkorir *et al.*, 2014; Gaye *et al.*, 2014). A shorter extrinsic incubation period for dengue would present many consequences. In nature, *Ae. aegypti* are anthropophagic and have a short gonadotropic cycle. Therefore in the presence of all favorable factors for dengue transmission, for instance, short extrinsic incubation period, frequent feeding and persistently infected salivary gland, *Ae. aegypti* is an efficient DENV vector which explains the epidemic potential of dengue in several locations.

However, the percentage of positive organs was higher in Pasir Puteh mosquitoes on all three days except for day 10 salivary gland of Mentari Court which exhibited a higher rate. But Mentari Court proved to have a better vector competency and dissemination

University of Malaya

rate as a very high rate of DR was recorded. Our findings revealed that the infection rate in both populations were notably higher compared to previous DENV1 infection study carried out on Dakar, Senegal population (0-43.8%)(Gaye *et al.*, 2014) but comparable to DENV2 infection assay on Brazilian population (Gonçalves *et al.*, 2014). Overall result suggests, Pasir Puteh mosquitoes, have a weak midgut infection barrier but a moderately strong midgut escape barrier and salivary gland infection barrier. Mentari Court mosquitoes on the other hand have a moderately strong midgut infection barrier and a weak midgut escape barrier and salivary gland infection barrier. The assumption made on the midgut infection barrier and midgut escape barrier shows an inversely proportional relation. An intrapopulation vector competency study conducted in two urban areas in Kenya (Kilifi and Nairobi) reported similar findings. It was found that Nairobi population has high midgut infection rate but Kilifi population has high dissemination rate (Chepkorir *et al.*, 2014). Geographic and genotypic variation of *Ae. aegypti* populations and its relation to DENV susceptibility has been reported in various studies (Gubler *et al.*, 1979; Knox *et al.*, 2003). An interpopulation study conducted by Moncayo on populations from various geographical locations showed *Ae. aegypti* from Galveston, Texas, were more susceptible than those from Bolivia, but were less susceptible than mosquitoes from Thailand (Moncayo *et al.*, 2004). Our findings concur with these studies because the midgut infection and dissemination rate differed significantly between *Ae. aegypti* population collected from two different locations but exposed to the same conditions in laboratory. Whether the observed pattern of infection and dissemination is related to its genetic inheritance remains unclear. However, certain factors for instance, laboratory conditions, virus titer, blood-virus suspension volume, blood source, feeding time, and post feeding conditions were all constant for both populations. Thus, two different inherited genetic lineages might have contributed to the

University of Malaya

different susceptibility rate of Mentari Court and Pasir Puteh *Ae. aegypti* population towards DENV1.

Genetic inheritance plays an important role in determining the vector capability to host and transmit arbovirus. Genetic studies on vector competence and the loci responsible in vector susceptibility rate were previously mapped by Quantitative Trait Loci. In general, the result suggest transmission of dengue is a quantitative genetic trait under the control of at least three loci (Bosio *et al.*, 2000). To further investigate this suggestion, a genetic marker (67kDa protein) was identified and believed to be a marker for vector competence (Mercado-Curiel *et al.*, 2008).

Besides the efficient dissemination rate recorded in Mentari Court population and the longevity which was slightly higher compared to Pasir Puteh, might have also contributed to the level of endemicity in that location. Nevertheless, mosquito survival and density of susceptible host also plays an important role in dengue transmission. Surprisingly by day 14, positivity rate and viral copy number reduced in both population and similar results have been reported in previous studies performed by Salazar *et al* and Gaye *et al*.(Salazar *et al.*, 2007; Gaye *et al.*, 2014). This could be due to modulation by the metabolic activity in the midgut epithelium which can be investigated by second uninfectious blood meal. If the mosquito failed to re-establish virus production in the midgut after the second blood meal, the reduction can be associated with antiviral response, post-transcriptional or post-translational repression or other self-limiting factor like cell aging and nutrient depletion. Further investigation is important to explain this phenomenon and like several studies (Watts *et al.*, 1986; Lambrechts *et al.*, 2011) environmental determinants of vector pathogen interaction should be considered.

University of Malaya

Absence of dengue outbreak in Pasir Puteh is counter to expectation given that the susceptibility rate in the current study, suggesting presence of other factors that contribute to endemicity. Viremic humans are the most likely source of transmission of DENV (Halstead, 2008). It is often observed that dengue infections occur in clusters; if a member of a particular area is infected, other members are also highly at risk of infection (Neff *et al.*, 1967). This report suggest that population density and physical proximity between households are determinants of outbreaks.

Majority of studies conducted on *Ae. aegypti* competency towards arbovirus was based on DENV2 (Bennett *et al.*, 2002; Knox *et al.*, 2003; Chepkorir *et al.*, 2014; Yixin *et al.*, 2014). Although such studies are lacking on DENV1 and other serotypes, minority also reported, different serotypes did not affect the competency rate within population significantly (Rohani *et al.*, 2009; Lambrechts *et al.*, 2011). For instance both Rohani *et al* and Lambrechts *et al* investigated the effect of temperature on dengue transmission in *Ae. aegypti*. The transmission rate was similar for both serotypes; DENV2 and DENV4 (reported by Rohani *et al.*) and DENV1 and DENV2 (reported by Lambrechts *et al.*) and the vector susceptibility rate were constant for both virus. However Senegal's *Ae. aegypti* population infected with DENV1 and DENV3 revealed DENV1 to be more infectious with better dissemination (Gaye *et al.*, 2014). Additional research is needed to determine whether our present conclusion extends to all DENV serotypes.

5.3 Limitations and recommendations

This study suffers possible limitations due to sparse sampling and in which it might have contributed to low frequency haplotypes. Sample collections have to be extensive, covering whole of Malaysia especially in dengue endemic and dengue free areas. This might help us to uncover the resistant strain if any, in Malaysia. Besides that, comparing dengue susceptibility rate between all other haplotypes is necessary and like several

University of Malaya

other studies (Lambrechts *et al.*, 2011; Whitehorn *et al.*, 2015), it should not be limited to one dengue serotype. Some inconsistencies were observed during infection assay and this can be avoided with larger sample size which again will exceed the allocated budget. Further studies are important to strengthen the dissemination rate hypothesis and this can be done by testing the saliva of the mosquito.

University of Malaya

CHAPTER 6: CONCLUSION

In summary, the application of CO1 and ND5 mitochondrial DNA revealed two different genetic lineages of *Ae. aegypti*. The genetic diversity in CO1 gene is also greater than the ND5 gene and both genes are evolving at a neutral rate. The population study revealed the *Ae. aegypti* population in Malaysia is not expanding and it is stable. The phylogenetic relationship suggests Malaysian haplotypes have close associate with Brazil and France and this might be due to common ancestor. It was also found, the parental genome which originated from Africa to be residing in certain locations in Perak. The genetic flow among this species is also limited and deviates from panmixia which might be due to isolating barrier or sparse sampling. Further work, with increased numbers of specimens sourced from wider geographic areas covering all states in Malaysia and the incorporation of additional markers will help to unravel presence of additional diversity among the *Ae. aegypti* population.

Data from genotypic study on the presence of two lineages of *Ae. aegypti* strain in peninsular Malaysia were analyzed to choose two locations for the vector competence towards dengue assay. Mentari Court is an endemic location in contra to Pasir Puteh and also pairwise genetic differences showed higher indices between these two locations. Therefore infection study was carried out in the *Ae. aegypti* population collected from these locations.

Assuming mosquitoes that have disseminated infection are capable of transmitting the virus, it is not surprising to declare Mentari Court as a dengue endemic location for years. Differences in dengue susceptibility between Mentari Court and Pasir Puteh population may reflect differences in the frequency of alleles at the midgut infection and escape barrier loci which has to be further investigated in future studies. The findings show that both mosquito populations are susceptible to DENV1 with a better infection

University of Malaya

rate in Pasir Puteh population. The results also suggest a lesser dissemination capability and competency of Pasir Puteh population. This could explain why there is no evidence of active dengue transmission in that location. Pasir Puteh mosquitoes might be reservoirs of dengue virus for time being, and its ability to initiate the dengue infection cycle should not be overlooked.

The benefits of this study are to identify the susceptible and resistant *Ae. aegypti* strain towards dengue. Genotypic study might also aid to unravel the genetic pattern and distribution in Malaysia. This is a novel study in Malaysia and therefore should be carried out extensively to aid in vector control and eventually curb dengue. This result can be further used in studies like insecticide resistance among variant genotype, to determine if they exist. In addition, since the mosquito's population varies from one region to another, understanding the local population dynamics can lead to better planning of vector control strategies.

University of Malaya

REFERENCES

Abubakar, S., & Shafee, N. (2002). Outlook of dengue in Malaysia: a century later. *Malaysian Journal of Pathology, 24*(1), 23-28.

Apostol, B. L., Black IV, W. C., Reiter, P., & Miller, B. R. (1996). Population genetics with RAPD-PCR markers: the breeding structure of *Aedes aegypti* in Puerto Rico. *Heredity, 76*(4), 325-334.

Apostol, B. L., Black, W. C., Reiter, P., & Miller, B. R. (1994). Use of randomly amplified polymorphic DNA amplified by polymerase chain reaction markers to estimate the number of *Aedes aegypti* families at oviposition sites in San Juan, Puerto Rico. *The American Journal of Tropical Medicine and Hygiene, 51*(1), 89-97.

Arrivillaga, J., Norris, D., Feliciangeli, M., & Lanzaro, G. (2002). Phylogeography of the neotropical sand fly *Lutzomyia longipalpis* inferred from mitochondrial DNA sequences. *Infection, Genetics and Evolution, 2*(2), 83-95.

Beebe, N., Whelan, P., Van Den Hurk, A., Ritchie, S., & Cooper, R. (2005). Genetic diversity of the dengue vector *Aedes aegypti* in Australia and implications for future surveillance and mainland incursion monitoring. *Communicable Diseases Intelligence*, *29*(3), 299-304

Bennett, K. E., Olson, K. E., de Lourdes Muñoz, M., Fernandez-Salas, I., Farfan-Ale, J. A., Higgs, S., . . . Beaty, B. J. (2002). Variation in vector competence for dengue 2 virus among 24 collections of *Aedes aegypti* from Mexico and the United States. *The American Journal of Tropical Medicine and Hygiene, 67*(1), 85-92.

Besansky, N. J., Lehmann, T., Fahey, G. T., Fontenille, D., Braack, L. E., Hawley, W. A., & Collins, F. H. (1997). Patterns of mitochondrial variation within and between African malaria vectors, *Anopheles gambiae* and *An. arabiensis*, suggest extensive gene flow. *Genetics, 147*(4), 1817-1828.

Bethesda. (2015a). Genetics Home Reference. from http://ghr.nlm.nih.gov/gene/MT-CO1. Assesed on 12th April 2015

Bethesda. (2015b). Genetics Home Reference. from http://ghr.nlm.nih.gov/gene/MT-ND5. Assesed on 12th April 2015

Bhamarapravati, N. (1989). Hemostatic defects in dengue hemorrhagic fever. *Review of Infectious Diseases, 11*(Supplement 4), S826-S829.

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., . . . Sankoh, O. (2013). The global distribution and burden of dengue. *Nature, 496*(7446), 504-507.

Birungi, J., & Munstermann, L. E. (2002). Genetic structure of *Aedes albopictus* (Diptera: Culicidae) populations based on mitochondrial ND5 sequences: evidence for an independent invasion into Brazil and United States. *Annals of the Entomological Society of America, 95*(1), 125-132.

University of Malaya

Black, W., Hawley, W. A., Rai, K. S., & Craig, G. (1988). Breeding structure of a colonizing species: *Aedes albopictus* (Skuse) in peninsular Malaysia and Borneo. *Heredity, 61*, 439-446.

Black, W. C., Bennett, K. E., Gorrochótegui-Escalante, N., Barillas-Mury, C. V., Fernández-Salas, I., de Lourdes Muñoz, M. a., . . . Beaty, B. J. (2002). Flavivirus susceptibility in *Aedes aegypti*. *Archives of Medical Research, 33*(4), 379-388.

Bosio, C. F., Fulton, R. E., Salasek, M. L., Beaty, B. J., & Black, W. C. (2000). Quantitative trait loci that control vector competence for dengue-2 virus in the mosquito *Aedes aegypti*. *Genetics, 156*(2), 687-698.

Bosio, C. F., Harrington, L. C., Jones, J. W., Sithiprasasna, R., Norris, D. E., & Scott, T. W. (2005). Genetic structure of *Aedes aegypti* populations in Thailand using mitochondrial DNA. *The American Journal of Tropical Medicine and Hygiene, 72*(4), 434-442.

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., . . . Drummond, A. J. (2014). BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS Computational Biology, 10*(4), e1003537.

Bracco, J. E., Capurro, M. L., Lourenço-de-Oliveira, R., & Sallum, M. A. M. (2007). Genetic variability of *Aedes aegypti* in the Americas using a mitochondrial gene: evidence of multiple introductions. *Memorias do Instituto Oswaldo Cruz, 102*(5), 573-580.

Chan, K., Ho, B., & Chan, Y. (1971). *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) in Singapore City: 2. Larval habitats*. *Bulletin of the World Health Organization, 44*(5), 629.

Chen, C., Nazni, W., Lee, H., Seleena, B., Mohd Masri, S., Chiang, Y., & Sofian-Azirun, M. (2006). Mixed breeding of *Aedes aegypti* (L.) and *Aedes albopictus* Skuse in four dengue endemic areas in Kuala Lumpur and Selangor, Malaysia. *Tropical Biomedicine, 23*(2), 224-227.

Chen, C., Nazni, W., Lee, H., & Sofian-Azirun, M. (2005). Susceptibility of *Aedes aegypti* and *Aedes albopictus* to temephos in four study sites in Kuala Lumpur City Center and Selangor State, Malaysia. *Tropical Biomedicine, 22*(2), 207-216.

Cheong, W. (1967). Preferred *Aedes aegypti* larval habitats in urban areas. *Bulletin of the World Health Organization, 36*(4), 586.

Cheong, Y. L., Burkart, K., Leitão, P. J., & Lakes, T. (2013). Assessing weather effects on dengue disease in Malaysia. *International Journal of Environmental Research and Public Health, 10*(12), 6319-6334.

Chepkorir, E., Lutomiah, J., Mutisya, J., Mulwa, F., Limbaso, K., Orindi, B., & Sang, R. (2014). Vector competence of *Aedes aegypti* populations from Kilifi and Nairobi for dengue 2 virus and the influence of temperature. *Parasites & Vectors, 7*(1), 1-8.

University of Malaya

. Chromas Lite (Version 2.1). (2012). South Brisbane, Queensland,Australia: Technelysium Pty Ltd.

Clement, M., Posada, D.C. (2000). TCS. A computer program to estimate gene genealogies. *Molecular Ecology, 9*(10) 1657-1659

Cook, S., Diallo, M., Sall, A. A., Cooper, A., & Holmes, E. C. (2005). Mitochondrial markers for molecular identification of Aedes mosquitoes (Diptera: Culicidae) involved in transmission of arboviral disease in West Africa. *Journal of Medical Entomology, 42*(1), 19-28.

Costa-da-Silva, A. L. d., Capurro, M. L., & Bracco, J. E. (2005). Genetic lineages in the yellow fever mosquito *Aedes* (Stegomyia) *aegypti* (Diptera: Culicidae) from Peru. *Memorias do Instituto Oswaldo Cruz, 100*(6), 539-544.

Dias, C. N., & Moraes, D. F. C. (2014). Essential oils and their compounds as *Aedes aegypti* L.(Diptera: Culicidae) larvicides: review. *Parasitology Research, 113*(2), 565-592.

Dieng, H., Saifur, R. G., Hassan, A. A., Salmah, M. C., Boots, M., Satho, T., . . . AbuBakar, S. (2010). Indoor-breeding of *Aedes albopictus* in northern peninsular Malaysia and its potential epidemiological implications. *PloS One, 5*(7), e11790.

Drummond AJ, S. M., Xie D & Rambaut A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7 *Molecular Biology and Evolution, 29: 1969-1973*.

Edman, J., Scott, T., Costero, A., Morrison, A., Harrington, L., & Clark, G. (1998). *Aedes aegypti* (Diptera: Culicidae) movement influenced by availability of oviposition sites. *Journal of Medical Entomology, 35*(4), 578-583.

Er, A., Rosli, M., Asmahani, A., Mohamad Naim, M., & Harsuzilawati, M. (2010). Spatial mapping of dengue incidence: A case study in Hulu Langat District, Selangor, Malaysia. *International Journal of Human and Social Sciences, 5*(6), 3410-3414.

Excoffier, L. and Lischer, H.E.L (2010). Arlequin suite (Version 3.5).

Failloux, A.-B., Vazeille, M., & Rodhain, F. (2002). Geographic genetic variation in populations of the dengue virus vector *Aedes aegypti*. *Journal of Molecular Evolution, 55*(6), 653-663.

Fragnoud, R., Yugueros-Marcos, J., Pachot, A., & Bedin, F. (2012). Isotope Coded Protein Labeling analysis of plasma specimens from acute severe dengue fever patients. *Proteome Science, 10*(1), 60.

Gaye, A., Faye, O., Diagne, C. T., Faye, O., Diallo, D., Weaver, S. C., . . . Diallo, M. (2014). Oral susceptibility of *Aedes aegypti* (Diptera: Culicidae) from Senegal for dengue serotypes 1 and 3 viruses. *Tropical Medicine and International Health, 19*(11), 1355-1359.

University of Malaya

Gonçalves, C. M., Melo, F. F., Bezerra, J. M., Chaves, B. A., Silva, B. M., Silva, L. D., . . . Norris, D. E. (2014). Distinct variation in vector competence among nine field populations of *Aedes aegypti* from a Brazilian dengue-endemic risk city. *Parasites & Vectors, 7*(1), 320.

Gorrochotegui-Escalante, N., Gomez-Machorro, C., Lozano-Fuentes, S., Fernandez-Salas, L., Munoz, M. D. L., Farfan-Ale, J. A., . . . Black, W. C. (2002). Breeding structure of *Aedes aegypti* populations in Mexico varies by region. *The American Journal of Tropical Medicine and Hygiene, 66*(2), 213-222.

Gorrochotegui-Escalante, N., Munoz, M. d. L., Fernandez-Salas, I., Beaty, B. J., & Black, W. (2000). Genetic isolation by distance among *Aedes aegypti* populations along the northeastern coast of Mexico. *The American Journal of Tropical Medicine and Hygiene, 62*(2), 200-209.

Gratz, N. (2004). Critical review of the vector status of *Aedes albopictus*. *Medical and Veterinary Entomology, 18*(3), 215-227.

Gubler, D., Nalim, S., Tan, R., Saipan, H., & Sulianti, S. J. (1979). Variation in susceptibility to oral infection with dengue viruses among geographic strains of *Aedes aegypti*. *The American Journal of Tropical Medicine and Hygiene, 28*(6), 1045-1052.

Gubler, D. J. (1998). Dengue and dengue hemorrhagic fever. *Clinical microbiology reviews, 11*(3), 480-496.

Gubler, D. J., & Clark, G. G. (1995). Dengue/dengue hemorrhagic fever: the emergence of a global health problem. *Emerging Infectious Diseases, 1*(2), 55.

Guirakhoo, F., Kitchener, S., Morrison, D., Forrat, R., McCarthy, K., Nichols, R., . . . Kanesa-Thasan, N. (2006). Live attenuated chimeric yellow fever dengue type 2 (ChimeriVax™-DEN2) vaccine: Phase I clinical trial for safety and immunogenicity: Effect of yellow fever pre-immunity in induction of cross neutralizing antibody responses to all. *Human Vaccines, 2*(2), 60-67.

Guzman, M. G., & Harris, E. (2014). Dengue. *The Lancet*, 385, 453-465

Halstead, S. (1980). Dengue haemorrhagic fever—a public health problem and a field for research. *Bulletin of the World Health Organization, 58*(1), 1.

Halstead, S. B. (2008). Dengue virus-mosquito interactions. *Annual Review of Entomology, 53*, 273-291.

Hardy, J. (1988). Susceptibility and resistance of vector mosquitoes. *The Arboviruses: Epidemiology and Ecology, 1*, 87-126.

Harpending, H. C., Sherry, S. T., Rogers, A. R., & Stoneking, M. (1993). The genetic structure of ancient human populations. *Current Anthropology*, 483-496.

Hawley, W. A. (1988). The biology of *Aedes albopictus*. *Journal of the American Mosquito Control Association. Supplement, 1*, 1-39.

University of Malaya

Henchal, E. A., & Putnak, J. R. (1990). The dengue viruses. *Clinical Microbiology Reviews, 3*(4), 376-396.

Herrera, F., Urdaneta, L., Rivero, J., Zoghbi, N., Ruiz, J., Carrasquel, G., . . . Montoya, A. (2006). Population genetic structure of the dengue mosquito *Aedes aegypti* in Venezuela. *Memorias do Instituto Oswaldo Cruz, 101*(6), 625-633.

Huang, Y.-M. (1972). Contributions to the Mosquito Fauna of Southeast Asia. XIV. The Subgenus Stegomyia of *Aedes* in Southeast Asia I-The Scutellaris Group of Species: DTIC Document.

Huang, Y.-M. (1979). Medical entomology studies-XI. The subgenus Stegomyia of Aedes in the Oriental Region with keys to the species (Diptera: Culicidae): DTIC Document.

Hudson, J. (1983). Susceptibility of *Aedes aegypti* and Culex quinquefasciatus to insecticides in Paramaribo, Suriname, 1979-1981, and experimental selection for resistance. *Cahiers-ORSTOM. Entomologie médicale et parasitologie, 21*(4), 275-279.

iDengue (2015). Retrieved March 2015, from Malaysian Remote Sensing Agency (ARSM), Kementerian Sains, Teknologi & Inovasi (MOSTI). http://idengue.remotesensing.gov.my/

Jaimes-Dueñez, J., Arboleda, S., Triana-Chávez, O., & Gómez-Palacio, A. (2015). Spatio-temporal distribution of *Aedes aegypti* (Diptera: Culicidae) mitochondrial lineages in cities with distinct dengue incidence rates suggests complex population dynamics of the dengue vector in Colombia. *PLoS neglected tropical diseases, 9*(4) e0003553

Jansen, C. C., & Beebe, N. W. (2010). The dengue vector *Aedes aegypti*: what comes next. *Microbes and Infection, 12*(4), 272-279.

Kamgang, B., Ngoagouni, C., Manirakiza, A., Nakouné, E., Paupy, C., & Kazanji, M. (2013). Temporal patterns of abundance of *Aedes aegypti* and *Aedes albopictus* (diptera: Culicidae) and mitochondrial DNA analysis of Ae. albopictus in the Central African Republic. *PLoS neglected tropical diseases, 7*(12), e2590.

Knox, T., Kay, B., Hall, R., & Ryan, P. (2003). Enhanced vector competence of *Aedes aegypti* (Diptera: Culicidae) from the Torres Strait compared with mainland Australia for dengue 2 and 4 viruses. *Journal of Medical Entomology, 40*(6), 950-956.

Koou, S.-Y., Chong, C.-S., Vythilingam, I., Ng, L.-C., & Lee, C.-Y. (2014). Pyrethroid resistance in *Aedes aegypti* larvae (Diptera: Culicidae) from Singapore. *Journal of Medical Entomology, 51*(1), 170-181.

Lambrechts, L., Paaijmans, K. P., Fansiri, T., Carrington, L. B., Kramer, L. D., Thomas, M. B., & Scott, T. W. (2011). Impact of daily temperature fluctuations on dengue virus transmission by *Aedes aegypti*. *Proceedings of the National Academy of Sciences, 108*(18), 7460-7465.

University of Malaya

Lanciotti, R. S., Calisher, C. H., Gubler, D. J., Chang, G.-J., & Vorndam, A. V. (1992). Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *Journal of Clinical Microbiology, 30*(3), 545-551.

Lee, H., & Rohani, A. (2005). Transovarial transmission of dengue virus in *Aedes aegypti* and *Aedes albopictus* in relation to dengue outbreak in an urban area in Malaysia. *Dengue Bulletin, 29*, 106.

Li, C., Lim, T., Han, L., & Fang, R. (1985). Rainfall, abundance of *Aedes aegypti* and dengue infection in Selangor, Malaysia. *The Southeast Asian Journal of Tropical Medicine and Public Health, 16*(4), 560-568.

Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics, 25*(11), 1451-1452.

Llinás, G. A., & Gardenal, C. N. (2012). Phylogeography of *Aedes aegypti* in Argentina: long-distance colonization and rapid restoration of fragmented relicts after a continental control campaign. *Vector-Borne and Zoonotic Diseases, 12*(3), 254-261.

Loroño-Pino, M. A., García-Rejón, J. E., Machain-Williams, C., Gomez-Carro, S., Nuñez-Ayala, G., del Rosario Nájera-Vázquez, M., . . . Lozano-Fuentes, S. (2013). Towards a Casa Segura: a consumer product study of the effect of insecticide-treated curtains on *Aedes aegypti* and dengue virus infections in the home. *The American Journal of Tropical Medicine and Hygiene, 89*(2), 385-397.

Low, V., Lim, P., Chen, C., Lim, Y., Tan, T., Norma-Rashid, Y., . . . Sofian-Azirun, M. (2014). Mitochondrial DNA analyses reveal low genetic diversity in *Culex quinquefasciatus* from residential areas in Malaysia. *Medical and Veterinary Entomology, 28*(2), 157-168.

Macdonald, W. (1957). Interim review of the non-Anopheline mosquitoes. *Malaysian Parasites: I*(16-34), 1.

Manguin, S., & Boëte, C. (2011). *Global impact of mosquito biodiversity, human vector-borne diseases and environmental change*: INTECH Open Access Publisher.

Mercado-Curiel, R. F., Black, W. C., & de L Muñoz, M. (2008). A dengue receptor as possible genetic marker of vector competence in *Aedes aegypti*. *BMC Microbiology, 8*(1), 118.

Mia, M. S., Begum, R. A., Er, A., Abidin, R. D. Z. R. Z., & Pereira, J. J. (2013). Trends of dengue infections in Malaysia, 2000-2010. *Asian Pacific Journal of Tropical Medicine, 6*(6), 462-466.

Miyagi, I., & Toma, T. (2000). The mosquitoes of Southeast Asia. *Mosquitoes and mosquito-borne diseases: biology, surveillance, control, personal and public protection measures. Kuala Lumpur, Malaysia: Academy of Sciences Malaysia. p*, 1-43.

University of Malaya

Mohd-Zaki, A. H., Brett, J., Ismail, E., & L'Azou, M. (2014). Epidemiology of dengue disease in Malaysia (2000–2012): a systematic literature review. *PLoS neglected tropical diseases,8*(11) e3159

Molina-Cruz, A., De Mérida, A. M. P., Mills, K., Rodríguez, F., Schoua, C., Yurrita, M. M., . . . Black, W. C. (2004). Gene flow among *Anopheles albimanus* populations in Central America, South America, and the Caribbean assessed by microsatellites and mitochondrial DNA. *The American Journal of Tropical Medicine and Hygiene, 71*(3), 350-359.

Monath, T. P. (1994). Dengue: the risk to developed and developing countries. *Proceedings of the National Academy of Sciences, 91*(7), 2395-2400.

Moncayo, A. C., Fernandez, Z., Ortiz, D., Diallo, M., Sall, A., Hartman, S., . . . Tesh, R. B. (2004). Dengue emergence and adaptation to peridomestic mosquitoes. *Emerging Infectious Diseases, 10*(10), 1790-1796.

Mousson, L., Dauga, C., Garrigues, T., Schaffner, F., Vazeille, M., & Failloux, A.-B. (2005). Phylogeography of *Aede*s (*Stegomyia) aegypti* (L.) and *Aedes* (*Stegomyia) albopictus* (Skuse)(Diptera: Culicidae) based on mitochondrial DNA variations. *Genetical Research, 86*(01), 1-11.

Muir, L. E., & Kay, B. H. (1998). *Aedes aegypti* survival and dispersal estimated by mark-release-recapture in northern Australia. *The American Journal of Tropical Medicine and Hygiene, 58*(3), 277-282.

Myers, C. w., Warnick, J. e., & Albuquerque, E. x. (1980). Genetics and the origin of a vector population: *Aedes aegypti*, a case study. *Science, 208*, 20.

Navarro-Sanchez, E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier, J. L., . . . Desprès, P. (2003). Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. *EMBO reports, 4*(7), 723-728.

Neff, J. M., Morris, L., Gonzalez-Alcover, R., Coleman, P. H., Lyss, S. B., & Negron, H. (1967). Dengue fever in a Puerto Rican community. *American Journal of Epidemiology, 86*(1), 162-184.

Noble, C. G., Chen, Y.-L., Dong, H., Gu, F., Lim, S. P., Schul, W., . . . Shi, P.-Y. (2010). Strategies for development of dengue virus inhibitors. *Antiviral Research, 85*(3), 450-462.

Paduan, K. D. S., & Ribolla, P. E. M. (2008). Mitochondrial DNA polymorphism and heteroplasmy in populations of *Aedes aegypti* in Brazil. *Journal of Medical Entomology, 45*(1), 59-67.

Pang, S., Chiang, L., Tan, C., Vythilingam, I., Lam-Phua, S., & Ng, L. (2015). Low efficacy of delthamethrin-treated net against Singapore *Aedes aegypti* is associated with kdr-type resistance. *Tropical Biomedicine, 32*(1), 140-150.

University of Malaya

Pashley, D. p., & Rai, K. s. (1983). Comparison of allozyme and morphological relationships in some Aedes (Stegomyia) mosquitoes (Diptera: Culicidae). *Annals of the Entomological Society of America, 76*(3), 388-394.

Ping, L. T., Yatiman, R., & Gek, L. (2001). Susceptibility of adult field strains of *Aedes aegypti* and *Aedes albopictus* in Singapore to pirimiphos-methyl and permethrin. *Journal of the American Mosquito Control Association, 17*(2), 144-146.

Rodenhuis-Zybert, I. A., Wilschut, J., & Smit, J. M. (2010). Dengue virus life cycle: viral and host factors modulating infectivity. *Cellular and Molecular Life Sciences, 67*(16), 2773-2786.

Rogers, A. R., & Harpending, H. (1992). Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution, 9*(3), 552-569.

Rohani, A., Wong, Y., Zamre, I., Lee, H., & Zurainee, M. (2009). The effect of extrinsic incubation temperature on development of dengue serotype 2 and 4 viruses in *Aedes aegypti* (L.). *Southeast Asian Journal of Tropical Medicine and Public Health, 40*(5), 942.

Rothman, A. L., & Ennis, F. A. (1999). Immunopathogenesis of dengue hemorrhagic fever. *Virology, 257*(1), 1-6.

Rozilawati, H., Zairi, J., & Adanan, C. (2007). Seasonal abundance of *Aedes albopictus* in selected urban and suburban areas in Penang, Malaysia. *Tropical Biomedicine, 24*(1), 83-94.

Rudnick, A. (1965). Studies of the ecology of dengue in Malaysia: a preliminary report. *Journal of Medical Entomology, 2*(2), 203-208.

Rudnick, A. (1978). Ecology of dengue virus. *Asian Journal of Infectious Diseases, 2*, 156-160.

Saifur, R. G., Dieng, H., Hassan, A. A., Salmah, M. R. C., Satho, T., Miake, F., & Hamdan, A. (2012). Changing domesticity of *Aedes aegypti* in northern peninsular Malaysia: reproductive consequences and potential epidemiological implications. *PloS One, 7*(2), e30919.

Salazar, M. I., Richardson, J. H., Sánchez-Vargas, I., Olson, K. E., & Beaty, B. J. (2007). Dengue virus type 2: replication and tropisms in orally infected *Aedes aegypti* mosquitoes. *BMC Microbiology, 7*(1), 9.

Schneider, S., & Excoffier, L. (1999). Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. *Genetics, 152*(3), 1079-1089.

Shepard, D. S., Undurraga, E. A., & Halasa, Y. A. (2013). Economic and disease burden of dengue in Southeast Asia. *PLoS Neglected Tropical Diseases, 7*(2), e2055.

University of Malaya

Skae, F. (1902). Dengue fever in Penang. *British Medical Journal, 2*(2185), 1581.

SPSS. (2013). IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

Tabachnick, W., Wallis, G., Aitken, T., Miller, B., Amato, G., Lorenz, L., . . . Beaty, B. J. (1985). Oral infection of *Aedes aegypti* with yellow fever virus: geographic variation and genetic considerations. *The American Journal of Tropical Medicine and Hygiene, 34*(6), 1219-1224.

Tabachnick, W. J. (2013). Nature, nurture and evolution of intra-species variation in mosquito arbovirus transmission competence. *International Journal of Environmental Research and Public Health, 10*(1), 249-277.

Tabachnick, W. J., Munstermann, L. E., & Powell, J. R. (1979). Genetic distinctness of sympatric forms of *Aedes aegypti* in East Africa. *Evolution*, 287-295.

Tabachnick, W. J., & Powell, J. R. (1979). A world-wide survey of genetic variation in the yellow fever mosquito, *Aedes aegypti*. *Genetical Research, 34*(03), 215-229.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics, 123*(3), 585-595.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*(10), 2731-2739.

Thomas, S. J., & Endy, T. P. (2011). Critical issues in dengue vaccine development. *Current Opinion in Infectious Diseases, 24*(5), 442-450.

Twerdochlib, A. L., Bonna, A. C. D., Leite, S. S., Chitolina, R. F., Westphal, B., & Navarro-Silva, M. A. (2012). Genetic variability of a population of *Aedes aegypti* from Paraná, Brazil, using the mitochondrial ND4 gene. *Review Brasileira de Entomology, 56*(2), 249-256.

Urdaneta-Marquez, L., Bosio, C., Herrera, F., Rubio-Palis, Y., Salasek, M., & Black, W. C. (2008). Genetic relationships among *Aedes aegypti* collections in Venezuela as determined by mitochondrial DNA variation and nuclear single nucleotide polymorphisms. *The American Journal of Tropical Medicine and Hygiene, 78*(3), 479-491.

Vaughn, D. W., Green, S., Kalayanarooj, S., Innis, B. L., Nimmannitya, S., Suntayakorn, S., . . . Ennis, F. A. (2000). Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *Journal of Infectious Diseases, 181*(1), 2-9.

Wallis, G. P., Tabachnick, W. J., & Powell, J. R. (1984). Genetic heterogeneity among Caribbean populations of *Aedes aegypti*. *The American Journal of Tropical Medicine and Hygiene, 33*(3), 492-498.

University of Malaya

Walton, C., Handley, J. M., Tun-Lin, W., Collins, F. H., Harbach, R. E., Baimai, V., & Butlin, R. K. (2000). Population structure and population history of Anopheles dirus mosquitoes in Southeast Asia. *Molecular Biology and Evolution, 17*(6), 962-974.

Wang, E., Ni, H., Xu, R., Barrett, A. D., Watowich, S. J., Gubler, D. J., & Weaver, S. C. (2000). Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. *Journal of Virology, 74*(7), 3227-3234.

Watts, D. M., Burke, D. S., Harrison, B. A., Whitmire, R. E., & Nisalak, A. (1986). Effect of temperature on the vector efficiency of *Aedes aegypti* for dengue 2 virus: DTIC Document.

Whitehorn, J., Kien, D. T. H., Nguyen, N. M., Nguyen, H. L., Kyrylos, P. P., Carrington, L. B., . . . Le Thi, D. (2015). Comparative susceptibility of *Aedes albopictus* and *Aedes aegypti* to dengue virus infection after feeding on blood of viremic humans: implications for public health. *Journal of Infectious Diseases*, jiv173.

WHO. (2012). *Global strategy for dengue prevention and control 2012-2020*: World Health Organization.

Yan, G., Romero-Severson, J., Walton, M., Chadee, D., & Severson, D. (1999). Population genetics of the yellow fever mosquito in Trinidad: comparisons of amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers. *Molecular Ecology, 8*(6), 951-963.

Yap, H. (1975). Distribution of *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse) in small towns and villages of Penang Island, Malaysia--an ovitrap survey. *The Southeast Asian Journal of Tropical Medicine and Public Health, 6*(4), 519-524.

Yauch, L. E., Zellweger, R. M., Kotturi, M. F., Qutubuddin, A., Sidney, J., Peters, B., . . . Shresta, S. (2009). A protective role for dengue virus-specific CD8+ T cells. *The Journal of Immunology, 182*(8), 4865-4873.

Yixin, H. Y., Ng, T. S., Frentiu, F. D., Walker, T., van den Hurk, A. F., O'Neill, S. L., . . . McGraw, E. A. (2014). Comparative susceptibility of mosquito populations in North Queensland, Australia to oral infection with dengue virus. *The American Journal of Tropical Medicine and Hygiene, 90*(3), 422-430.

Yu, I.-M., Zhang, W., Holdaway, H. A., Li, L., Kostyuchenko, V. A., Chipman, P. R., . . . Chen, J. (2008). Structure of the immature dengue virus at low pH primes proteolytic maturation. *Science, 319*(5871), 1834-1837.

University of Malaya