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Genetic variation of the mitochondrial genes, CO1 and ND5, in *Aedes aegypti* from various regions of peninsular Malaysia

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Abstract. *Aedes aegypti* is the primary vector of dengue viruses in many parts of the world. In peninsular Malaysia, epidemics of dengue occur more at certain sites particularly in the west central region compared to the eastern region. In this study, we determined the genetic diversity of *Ae. aegypti* collected from 19 localities in 5 regions of peninsular Malaysia based on two mitochondrial DNA genes; CO1 and ND5. 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. Unique low frequencies haplotypes detected in the current sampling was closely related to those previously found in India, Pakistan and Vietnam samples, suggesting recent invasion of these haplotypes through human movement or transportation. This is the first study in Malaysia which serves as an initial preliminary 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.

INTRODUCTION

Aedes aegypti is the main vector of dengue and chikungunya viruses on a global scale (Gubler, 2002, Sam *et al*., 2009; Kamgang *et al*., 2013). Dengue has become a serious public health problem in the tropical and subtropical regions (Gubler, 2012) with over 125 countries are now affected by dengue fever (Wilder-Smith *et al*., 2012; Sam *et al*., 2013). Among the factors leading to the rapid spread of dengue virus includes unplanned urbanization that facilitates the breeding of *Ae. aegypti*, increasing rate of human migration and crowded urban population as well as the expansion of global trades and travels, particularly with the rising trend of low cost air travels. Currently, there is no licensed vaccine or available drugs for treatment of dengue infections, and thus the successful control of dengue transmission relies heavily on effective surveillance and management of the *Aedes* mosquito. In recent years, dengue situation in Malaysia has become increasingly alarming. The total number of dengue cases in Malaysia has increased from 7,103 cases in year 2000 to 46,171 cases in 2010 (Mohd-Zaki *et al*., 2014). The rate at which the number of reported cases increased from year to year rose from 47% between 2012-2013 to 62.1% between 2013-2014 [2012: 20,923 cases (35 deaths); 2013: 40,222 cases (92 death); 2014: 103,610 cases (215 deaths)] (Mudin, 2015).

Aedes aegypti is mainly found in the urban areas (Rudnick, 1965) and it is also known to be an efficient vector for dengue viruses in Malaysia (Sulaiman *et al*., 2002),

There has been a reduction in *Ae. aegypti* population in the 1990's compared to the 1980's, perhaps due to vector control programmes and provision of piped water. In the 1980's the *Aedes* house index ranged from 4.7 to 58.8% (Ho & Vythilingam, 1980), whereas in the 1990's the index ranged from 0.1 to 6.9% (Sulaiman *et al.*, 1996). A more recent report stated that the index ranged from 1.5 to 2% (Mudin, 2015). The control of *Ae. aegypti* remains a challenging task due to its versatility in that it is mainly anthropophagic, its eggs are able to withstand desiccation and it breeds in artificial containers (Beebe *et al.*, 2005). Such characteristics has allowed *Ae. aegypti* to successfully disperse to many urbanized areas where human population is high.

Recent advancement in genomics has contributed to the understanding of biological processes and population dynamics of mosquito vector at the molecular level (Bosio *et al.*, 2005). The genetic relationship of *Ae. aegypti* sampled throughout the world has been characterised since the 1970s (Tabachnick *et al.*, 1979; Tabachnick & Powell, 1979, Myers *et al.*, 1980, Wallis *et al.*, 1984). The focus of earlier population genetic studies on *Ae. aegypti* have ever since changed with more recent studies on placing their emphasis on local patterns of dispersal (Apostol *et al.*, 1994; Apostol *et al.*, 1996; Edman *et al.*, 1998; Scarpassa *et al.*, 2008; Olanratmanee *et al.*, 2013; Rasheed *et al.*, 2013). A variety of molecular markers such as Single Nucleotide Polymorphisms (SNPs), alloenzymes, nuclear DNA and mitochondrial DNA (mtDNA) have been used to characterize the genetic variability and population structure of *Ae. aegypti* (Pashley & Rai 1983, Apostol *et al.*, 1996; Bracco *et al.*, 2007; Urdaneta-Marquez *et al.*, 2008). Among these, the mtDNA has been widely used in population genetic studies of *Ae. aegypti* in different geographic and dengue endemic regions (Gorrochotegui-Escalante *et al.*, 2000). The nature of mtDNA, which is characterized by its maternal inheritance and lack recombination, while having a rapid rate of divergence (Arrivillaga *et al.*, 2002; Bracco *et al.*, 2007) makes it a robust genetic marker for the study of evolutionary relationship. An in-depth understanding of the vector population diversity and dynamics across a wide geographical range is essential for further understanding and predicting the epidemiology of dengue. *Aedes aegypti* is phenotypically polymorphic between populations and it exhibits a wide variation in vector competency for arboviruses (Tabachnick *et al.*, 1985). Studying the genetic variation and patterns of gene flow between different regions could lead to better understanding of the spatial dispersion of important genetic traits such as vector competence and insecticide resistance (Bosio *et al.*, 2005).

Little information is available about the genetic diversity and the nature of gene flow among the populations of *Ae. aegypti* in Malaysia. Here, we utilized the cytochrome oxidase subunit 1 (CO1) and nicotinamide adenine dinucleotide dehydrogenase subunit 5 (ND5) gene sequences of the mtDNA to investigate the intraspecific genetic diversity, phylogenetic relationships and dispersal patterns of *Ae. aegypti* population originating from five regions in peninsular Malaysia. Published sequences of CO1 and ND5 genes of *Ae. aegypti* were used to determine the phylogenetic status of *Ae. aegypti* found in Malaysia. This is the first study that described the population genetics of *Ae. aegypti* in Malaysia based on the mtDNA markers.

MATERIAL AND METHODS

Sample sites and collection method

Mosquitoes were collected from 19 locations within five regions in peninsular Malaysia that comprised the northern, southern, eastern, and western central regions (Fig. 1; Table 1). Collection of specimens were primarily carried out in the state of Selangor and Federal Territory in the western central region of peninsular Malaysia. In the northern region, specimens were collected from Perak, whereas in the eastern region mosquitoes were collected from the states of Pahang and Terengganu. In the southern region, specimens were collected from the state of Malacca.

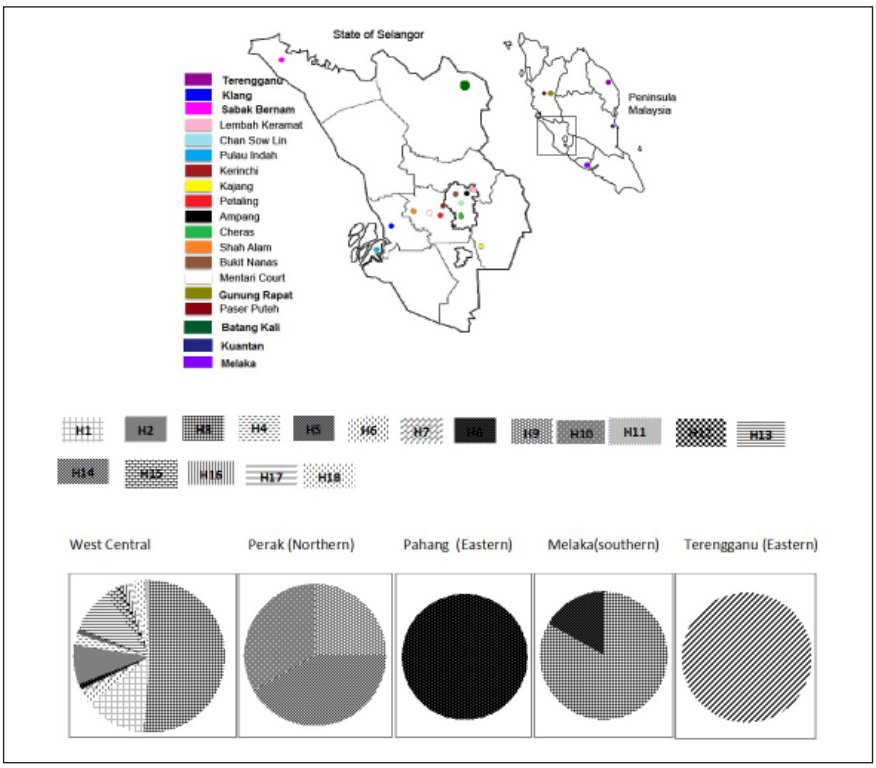

Figure 1. Map showing the sample collection sites and haplotype distribution (H1-H18) of concatenated sequences of CO1 and ND5 for *Ae. aegypti* in each state. All sites from Selangor and Federal Territory Kuala Lumpur were included in west central and Gunung Rapat and Pasir Puteh as Perak (Northern).

Table 1. Collection sites of *Ae. aegypti* from peninsular Malaysia

Code	Locality	State	Region	Coordinates	Sample size
KE	Kerinchi	Federal Territory	West Central	3°6'46"N, 101°39'40"E	9
CH	Cheras	Federal Territory	West Central	3°6'45"N, 101°42'51"E	9
BN	Bukit Nanas	Federal Territory	West Central	3°9'22"N, 101°42'16"E	6
LK	Lembah Keramat	Federal Territory	West Central	3°10'9"N, 101°44'48"E	6
CSL	Chan Sow Lin	Federal Territory	West Central	3°7'40"N, 101°42'55"E	6
KJ	Kajang	Selangor	West Central	2°59'35"N, 101°47'20"E	11
AM	Ampang	Selangor	West Central	3°9'38"N, 101°44'9"E	7
PI	Pulau Indah	Selangor	West Central	2°56'56"N, 101°19'54"E	5
PE	Petaling	Selangor	West Central	3°05'N, 101°39'E	7
SA	Shah Alam	Selangor	West Central	3°5'00"N, 101°32'00"E	5
MC	Mentari Court	Selangor	West Central	3°04'59"N, 101°36'40"E	6
K	Klang	Selangor	West Central	3°2'41"N, 101°26'44"E	4
BK	Batang Kali	Selangor	West Central	3°28'0"N, 101°38'0"E	3
SB	Sabak Bernam	Selangor	West Central	3°40'43"N, 100°59'26"E	4
PP	Pasir Puteh	Perak	Northern	5°49'59"N, 102°24'00"E	8
GR	Gunung Rapat	Perak	Northern	4°34'14"N, 101°7'59"E	4
JA	Jasin	Malacca	Southern	2°13'40"N, 102°25'51"E	12
BM	Bandar Mahkota	Pahang	Eastern	3°49'0"N, 103°19'59"E	3
KT	Kuala Terengganu	Terengganu	Eastern	5°19'46"N, 103°8'13"E	10
Total					125

Mosquitoes were collected either by setting up ovitraps in various locations or by larval surveys. Eight to 10 ovitraps were set per location and were placed at least 200 meters apart. This was to ensure that the progeny were not from a single female. Eggs were hatched into larvae and fed with fish food twice daily. After emergence, the adult mosquitoes were identified morphologically and all *Ae. aegypti* were stored at -20°C prior to DNA extraction. The number of mosquito collected ranged between 5 – 50 mosquitoes per sampling location. Permission was obtained from each state's Health Department to collect samples within the State. Verbal permission was obtained from house owners before setting traps inside and outside the houses.

DNA extraction

Genomic DNA was extracted using Qiagen Dneasy Blood and Tissue Extraction Kit (Qiagen, CA, USA) according to manufacturer's instructions. Extracted DNA was eluted with water and stored in -20°C until required.

PCR amplification and sequencing

Partial sequences of CO1 and ND5 mitochondrial genes 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 min (annealing), 72°C for 1 min (extension) and final extension at 72°C for 5 min.

Agarose gel electrophoresis was carried out to ensure the desired gene was amplified. The 1% agarose gel was stained with Syber Safe dye (Life Techonologies, USA) and single bands of expected sizes indicated the presence of desired gene. Amplified DNA products were excised from agarose gel and purified before they were sequenced at the sequencing facility (Genomics BioSci & Tech. Co., Ltd.). The sequence data obtained for the study were deposited in GenBank (Supplementary Table 1).

Genetic diversity and demographic history

Sequences were edited using BioEdit Sequence Alignment Editor Program (Hall, 1999). The sequences were compared with those published in GenBank database to confirm their identity using Basic Local Alignment Search Tool (BLAST). Indices of sequence statistics including the number of haplotypes, polymorphic sites, haplotype diversity, nucleotide diversity and the average number of nucleotide differences were computed with DnaSP 5.0 (Librado & Rozas, 2009). Neutrality test based on Tajima's D, Fu's and Fu and Li's statistics were conducted with DnaSP (Librado & Rozas 2009) to test for non-neutral evolution and deviation from mutation-drift equilibrium. A pairwise mismatch distribution was plotted using Arlequin v3.1 (Excoffier *et al*., 2006) to examine the demographic expansion of *Ae. aegypti* in peninsular Malaysia. The observed pairwise mismatch distribution generated from sampled sequences was compared with the expected distribution under the sudden demographic expansion model.

Haplotype network and spatial structuring

Haplotype network was constructed using TCS version 1.21 software (Clement *et al*., 2000) based on concatenated sequences of COI and ND5 genes to assess the genealogical

Supplementary Table 1. Haplotypes, location and GenBank accession number for each isolate

CO1-ND5 haplo	Specimen	Locality	CO1 haplo	Accession No.	ND5 haplo	Accession No.
1	AF1	Ampang	C1	KT630395	N2	KT630485
1	AF2	Ampang	C1	KT630396	N2	KT630486
1	AF6	Ampang	C1	KT630397	N2	KT630487
1	AF7	Ampang	C1	KT630398	N2	KT630488
2	AF3	Ampang	C2	KF855369	N2	KP410562
3	AF4	Ampang	C3	KF855370	N1	KF986349
4	AM5	Ampang	C2	KF855371	N1	KF986350
1	BK1	Batang Kali	C1	KT630402	N2	KT630489
1	BK2	Batang Kali	C1	KT630403	N2	KT630491
1	BK3	Batang Kali	C1	KT630404	N2	KT630490
1	BNM1	Bukit Nanas	C1	KT630399	N2	KT630492
1	BNF4	Bukit Nanas	C1	KF855377	N2	KF986337
2	BNF5	Bukit Nanas	C2	KP410555	N2	KF986338
2	BNM6	Bukit Nanas	C2	KT630400	N2	KT630494
3	BNM2	Bukit Nanas	C3	KF855376	N1	KF986339
4	BNM3	Bukit Nanas	C2	KT630401	N1	KT630493
3	SWF3	Chan Sow Lin	C3	KT630405	N1	KT630495
3	SWM4	Chan Sow Lin	C3	KT630406	N1	KT630497
3	SWM5	Chan Sow Lin	C3	KT630407	N1	KT630496
3	SWF2	Chan Sow Lin	C3	KP410556	N1	KF986340
3	SWM7	Chan Sow Lin	C3	KT630408	N1	KT630498
3	SWF10	Chan Sow Lin	C3	KT630409	N1	KT630499
1	CF1	Cheras	C1	KT630410	N2	KT630500
1	CM7	Cheras	C1	KT630414	N2	KT630503
3	CM2	Cheras	C3	KT630411	N1	KT630501
3	CM4	Cheras	C3	KT630413	N1	KT630502
3	CF8	Cheras	C3	KF855386	N1	KF986348
6	CF5	Cheras	C5	KF855384	N2	KP410563
6	CM3	Cheras	C5	KT630412	N2	KT630504
7	CF6	Cheras	C6	KF855385	N2	KF986346
8	CM9	Cheras	C7	KT630415	N2	KT630505
10	GF4	Gunung Rapat	C8	KF855387	N3	KF986352
10	GF5	Gunung Rapat	C8	KP410553	N3	KF986353
10	GF6	Gunung Rapat	C8	KP410554	N3	KF986354
10	GF7	Gunung Rapat	C8	KT630416	N3	KT630506
3	KJM3	Kajang	C3	KT630417	N1	KT630507
3	KJM4	Kajang	C3	KT630424	N1	KT630508
3	KJF5	Kajang	C3	KF855390	N1	KP410567
3	KJF6	Kajang	C3	KF855391	N1	KP410568
3	KJM10	Kajang	C3	KT630418	N1	KT630509
3	KJM1	Kajang	C3	KT630419	N1	KT630510
3	KJF2	Kajang	C3	KF855392	N1	KF986344
3	KJF7	Kajang	C3	KT630420	N1	KT630511

Supplementary Table 1 continue...

Supplementary Table 1 continued...

CO1-ND5 haplo	Specimen	Locality	CO1 haplo	Accession No.	ND5 haplo	Accession No.
3	KJF8	Kajang	C3	KT630421	N1	KT630512
3	KJM9	Kajang	C3	KT630422	N1	KT630513
3	KJM11	Kajang	C3	KT630423	N1	KT630514
3	VF8	Kerinchi	C3	KF855378	N1	KP410569
3	VF9	Kerinchi	C3	KT630425	N1	KT630515
3	VF10	Kerinchi	C3	KF855379	N1	KP410570
3	VF11	Kerinchi	C3	KF855380	N1	KP410571
3	VF12	Kerinchi	C3	KF855380	N1	KP410571
3	VF14	Kerinchi	C3	KT630426	N1	KT630517
3	VF6	Kerinchi	C3	KT630427	N1	KT630516
3	VF7	Kerinchi	C3	KT630428	N1	KT630519
3	VF15	Kerinchi	C3	KT630429	N1	KT630520
2	Klg2	Klang	C2	KT630430	N2	KT630518
2	Klg3	Klang	C2	KT630431	N2	KT630521
2	Klg5	Klang	C2	KT630432	N2	KT630522
11	Klg4	Klang	C9	KT630433	N2	KT630524
8	K2	Kuantan	C7	KT630434	N2	KT630523
8	K3	Kuantan	C7	KT630435	N2	KT630525
8	K4	Kuantan	C7	KT630436	N2	KT630526
3	AUM1	Lembah Keramat	C3	KT630437	N1	KT630527
3	AUM2	Lembah Keramat	C3	KT630438	N1	KT630528
3	AUM3	Lembah Keramat	C3	KT630439	N1	KT630529
3	AUM5	Lembah Keramat	C3	KP410559	N1	KF986334
3	AUM6	Lembah Keramat	C3	KP410560	N1	KF986335
5	AUM4	Lembah Keramat	C4	KT630441	N1	KT630531
3	M1	Melaka	C3	KT630442	N1	KT630532
3	M2	Melaka	C3	KT630446	N1	KT630533
3	M3	Melaka	C3	KT630447	N1	KT630534
3	M5	Melaka	C3	KT630449	N1	KT630536
3	M6	Melaka	C3	KT630450	N1	KT630537
3	M8	Melaka	C3	KT630452	N1	KT630539
3	M9	Melaka	C3	KT630453	N1	KT630540
3	M11	Melaka	C3	KT630444	N1	KT630542
3	M12	Melaka	C3	KT630445	N1	KT630543
3	M7	Melaka	C3	KT630451	N1	KT630538
8	M4	Melaka	C7	KT630448	N2	KT630535
8	M10	Melaka	C7	KT630443	N2	KT630541
12	MCF1	Mentari Court	C10	KF855405	N1	KF986370
13	MCF2	Mentari Court	C10	KF855406	N2	KF986371
13	MCF3	Mentari Court	C10	KF855407	N2	KF986372
13	MCF4	Mentari Court	C10	KT630481	N2	KT630544
13	MCM5	Mentari Court	C10	KT630482	N2	KT630545
13	MCM6	Mentari Court	C10	KT630483	N2	KT630546

Supplementary Table 1 continue...

Supplementary Table 1 continued...

CO1-ND5 haplo	Specimen	Locality	CO1 haplo	Accession No.	ND5 haplo	Accession No.
9	PPM11	Pasir Puteh	C8	KT630454	N3	KT630547
9	PPM12	Pasir Puteh	C8	KT630455	N3	KT630548
9	PPM14	Pasir Puteh	C8	KT630456	N3	KT630549
14	PPF1	Pasir Puteh	C11	KF855393	N3	KF986358
14	PPF2	Pasir Puteh	C11	KF855394	N3	KF986359
14	PPF3	Pasir Puteh	C11	KF855395	N3	KF986360
14	PPF4	Pasir Puteh	C11	KT630457	N3	KT630550
14	PPF5	Pasir Puteh	C11	KT630458	N4	KT630551
3	PTF3	Petaling	C3	KF855398	N1	KF986363
3	PTF5	Petaling	C3	KP410552	N1	KP410566
3	PTM8	Petaling	C3	KT630462	N1	KT630552
3	PTM10	Petaling	C3	KT630460	N1	KT630555
13	PTF4	Petaling	C10	KP675950	N2	KP410565
13	PTF7	Petaling	C10	KT630459	N2	KT630554
13	PTM6	Petaling	C10	KT630461	N2	KT630553
3	PLF2	Pulau Indah	C3	KT630463	N1	KT630556
3	PLF3	Pulau Indah	C3	KF855399	N1	KP410572
3	PLF4	Pulau Indah	C3	KF855400	N1	KP410573
3	PLF6	Pulau Indah	C3	KT630464	N1	KT630557
3	PLF7	Pulau Indah	C3	KT630465	N1	KT630558
16	SB1	Sabak Bernam	C13	KT630466	N2	KT630559
17	SB2	Sabak Bernam	C14	KT630467	N2	KT630560
18	SB3	Sabak Bernam	C2	KT630468	N5	KT630561
18	SB4	Sabak Bernam	C2	KT630469	N5	KT630562
2	SAF2	Shah Alam	C2	KF855402	N2	KF986364
3	SAF1	Shah Alam	C3	KT630470	N1	KT630563
3	SAF3	Shah Alam	C3	KF855403	N1	KF986365
15	SAF4	Shah Alam	C12	KF855404	N2	KF986366
15	SAM5	Shah Alam	C12	KT630471	N2	KT630564
14	TG1	Terengganu	C11	KT630472	N4	KT630565
14	TG2	Terengganu	C11	KT630474	N4	KT630566
14	TG3	Terengganu	C11	KT630475	N4	KT630567
14	TG4	Terengganu	C11	KT630476	N4	KT630568
14	TG5	Terengganu	C11	KT630477	N4	KT630569
14	TG6	Terengganu	C11	KT630478	N4	KT630570
14	TG7	Terengganu	C11	KT630479	N4	KT630571
14	TG8	Terengganu	C11	KT630480	N4	KT630572
14	TG9	Terengganu	C11	KT630481	N4	KT630573
14	TG10	Terengganu	C11	KT630473	N4	KT630574

relationship of *Ae. aegypti* mitochondrial haplotypes. Population structure was assessed using the Spatial Analysis of Shared Alleles (SAShA) method (Kelly *et al*., 2010). Under the assumption of panmixia, this method test for spatial arrangement of the same alleles whether they are more geographically restricted than expected. Index of genetic differentiation (Fst) between populations was calculated using DNAsp 5.0.

Phylogenetic analysis

Phylogenetic relationship was analysed based on individually aligned CO1 and ND5 sequences. Previously published CO1 sequences of *Ae. aegypti* from Brazil, Uganda, Senegal, Thailand, Vietnam, Madagascar, France, Cote d'Ivoire, Cambodia, Guinea, USA, Kenya, Pakistan and India were retrieved from GenBank database and compared with CO1 sequences derived from the present study. All available sequences of ND5 and CO1 were aligned using MEGA 5.2 software (Tamura *et al.*, 2011). Identical sequences from the same country were excluded. The phylogeny of *Ae. aegypti* was estimated using the Bayesian method implemented in BEAST software (Bouckaert *et al.*, 2014). The substitution model, Tamura-Nei with gamma distribution of rate variation among sites (determined using MEGA 5.2), 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.

RESULTS

Genetic diversity

The alignment of the 451 bp CO1 gene sequences showed 19 polymorphic sites (Table 2). 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 3). In total, 14 unique CO1 haplotypes were identified and designated as C1-C14. The ND5 gene was 324 bp in length, with sequence alignment revealing 7 polymorphic sites (Table 2). Nucleotide diversity based on the ND5 gene was π = 0.00742 and haplotype diversity Hd=0.643 (Table 3). Five unique haplotypes of ND5 gene were identified and designated here as N1-N5.

Table 2. Polymorphic regions of concatenated sequences (H1-H18) and matching CO1 haplotypes (C1-C14)

	CO1																					ND5					
	11	68	77	173	197	206	212	218	230	239	305	317	353	356	365	368	374	398	401	482		530	560	563	593	650	716
(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	.	.	.

Table 3. 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

Table 4. Haplotype frequencies in *Ae. aegypti* populations analysed for combined CO1-ND5, CO1 and ND5 genes. The number in parentheses ( ) indicates the number of samples for that particular haplotype

Code	CO1-ND5 Haplotype	CO1 Haplotype	ND5 Haplotype
KE	H3(9)	C3(9)	N1(9)
CH	H1(2); H3(3); H6(2); H7(1); H8(1)	C1(2); C3(3); C5(2); C6(1); C7(1)	N2(2)
BN	H1(2); H2(2); H3(1); H4(1)	C1 (3); C2 (2); C3 (1)	N2(4); N1(2)
LK	H3(5); H5(1)	C3(5); C4(1)	N1(6)
CSL	H3(6)	C3(6)	N1(6)
KJ	H3(11)	C3(11)	N1(11)
AM	H1(4); H2(1); H3(1); H4(1)	C1(4); C2(2); C3(1)	N2(5); N1(2)
PI	H3(5)	C3(5)	N1(5)
PE	H3(4); H13(3)	C10(3)	N1(4); N2(3)
SA	H2(1); H3(2); H15(2)	C2(1); C3(2); C12(2)	N2(3); N1(2)
MC	H12(1); H13(5)	C10(6)	N1(1); N2(5)
K	H2(3); H11(1)	C9(1)	N2(4)
BK	H1(3)	C1(3)	N2(3)
SB	H16(1); H17(1); H18(2)	C2(2); C13(1); C14(1)	N2(2); N5(2)
PP	H9(3); H14(5)	C8(3); C11(5)	N3(7); N4(1)
GR	H10(4)	C8(4)	N3(4)
JA	H3(10); H8(2)	C3(10); C7(2)	N1(11); N2(2)
BM	H8(3)	C7(3)	N2(3)
KT	H14(10)	C11(10)	N4(10)

Analysis based on combined CO1 and ND5 gene sequences were also carried out. A set of concatenated CO1-ND5 sequences of 775 bp were aligned. The alignment of concatenated sequences revealed 26 polymorphic sites, of which 8 were synonymous sites and 18 were non-synonymous sites (Table 2). Analysis of gene diversity showed a nucleotide diversity $\pi = 0.00951$ and haplotype diversity Hd = 0.763 (Table 3).

Eighteen unique CO1-ND5 haplotypes (designated as H1-H18) were identified. Haplotype 3 (H3) represented the most common haplotype at a frequency of 45.6%, followed by other haplotypes: 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.

Haplotype network analysis

The frequencies and a genealogical network of the 18 haplotypes observed are shown in Table 4 and Fig. 2, respectively. The haplotype network showed several distinct groupings, designated here as Group 1 and

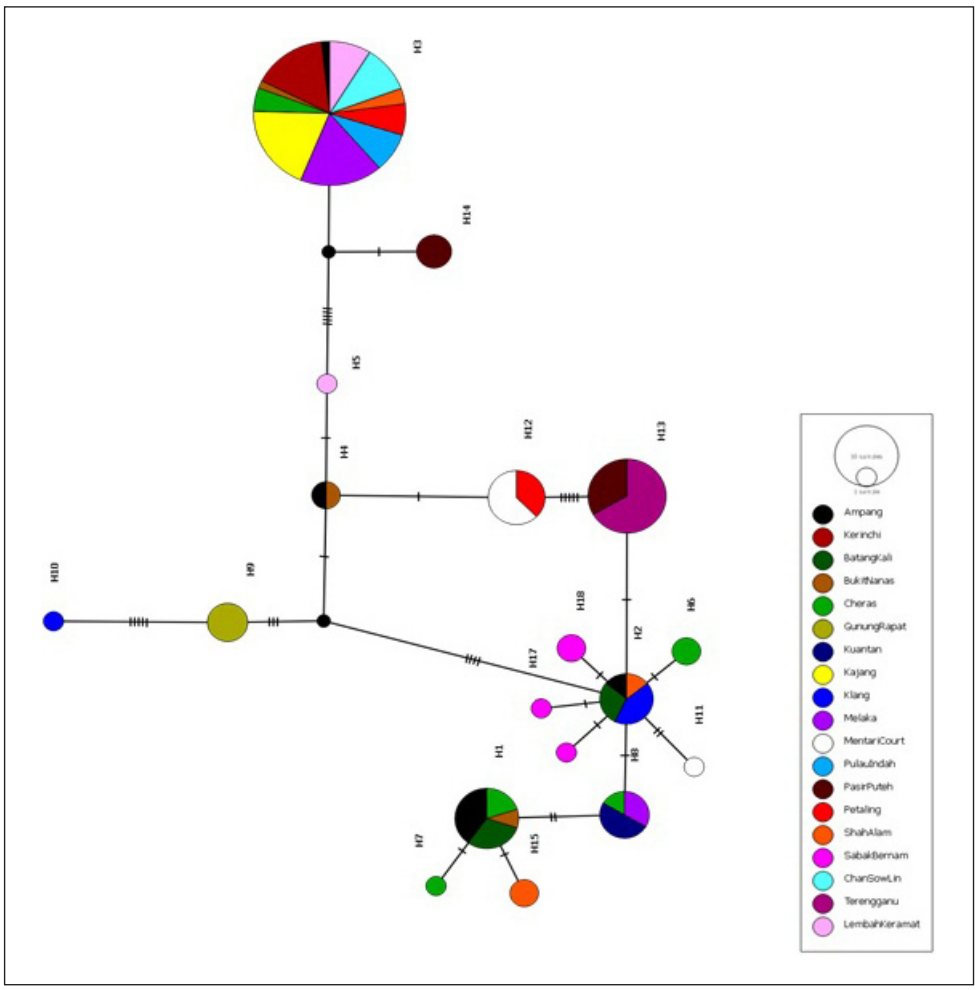

Figure 2. Genealogical network showing the relationship among 18 CO1-ND5 haplotypes of *Ae. aegypti*. Size of circle is proportional to the frequency of a particular haplotype. Each small lines on the line that connects two circles represents a mutational step and black dots represent hypothetical missing intermediates.

2. These distinct groups of haplotypes, separated by 14 mutational steps and are likely to represent at least 2 separate lineages of *Ae. aegypti* in peninsular Malaysia. Group 1 composed of the most prevalent haplotype (H3). Notably, the level of genetic diversity within group 1 is low as it consisted of only two (H3 and H9) very closely related haplotypes. This is probably due to the recent rapid spread of the predominant haplotype H3 across the state of Selangor. On the other hand, Group 2 which consisted of haplotype H1, H2, H6, H7, H8, H12, H13, H14, H15, H16, H17 and H18 showed a more 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 Gunong Rapat (GR) (northern region) sample was separated by 5 mutational steps from H4 and H5 (Fig. 2). Group 2 had higher diversity.

Tests of neutrality

All statistical tests of neutrality were insignificant and thus do not reject the null hypothesis of neutral evolution; 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 3).

Phylogenetic analysis

In general, *Ae. aegypti* CO1 and ND5 haplotypes from peninsular Malaysia were distributed across the phylogenies and clustered with haplotypes from various countries worldwide (Fig. 3 and 4). For CO1, the maximum clade credibility phylogeny revealed two significant clades that were well supported by posterior probability above 0.9 (Fig. 3). Specifically, 3 CO1 haplotypes clustered within Clade 1 and were closely related to *Ae. aegypti* from Vietnam and the Indian subcontinent in various lineages. Clade 2, with the exception of CO1 haplotypes C3, C4, and C14, low frequency CO1 haplotypes (C13, C11, C9, C5 and C8) were more closely related to those originating from India and Pakistan. Haplotypes C2 and C4 were closely related to those previously sampled in Vietnam and Cambodia, whereas C10 and C7 had close relatives to Brazilian *Ae. aegypti* population. The predominant CO1 haplotype (C3) fell within Clade 2 and clustered with haplotype C14 and those from Brazil and France. Both the Senegal sequences formed the basal clade, suggesting the African origin of *Ae. aegypti*.

Similarly, ND5 phylogenetic tree showed two clades (major and minor). Five ND5 haplotypes clustered together with samples from Southeast Asia, Russia, South America and Africa (Fig. 4). The major clade was formed by samples from Southeast Asia, France, Brazil, Guinea and Africa together with haplotypes 1 to 5. Haplotypes N1 (predominant haplotype), N2 (second predominant haplotype) and N3 were seen to be more closely related to those from Brazil.

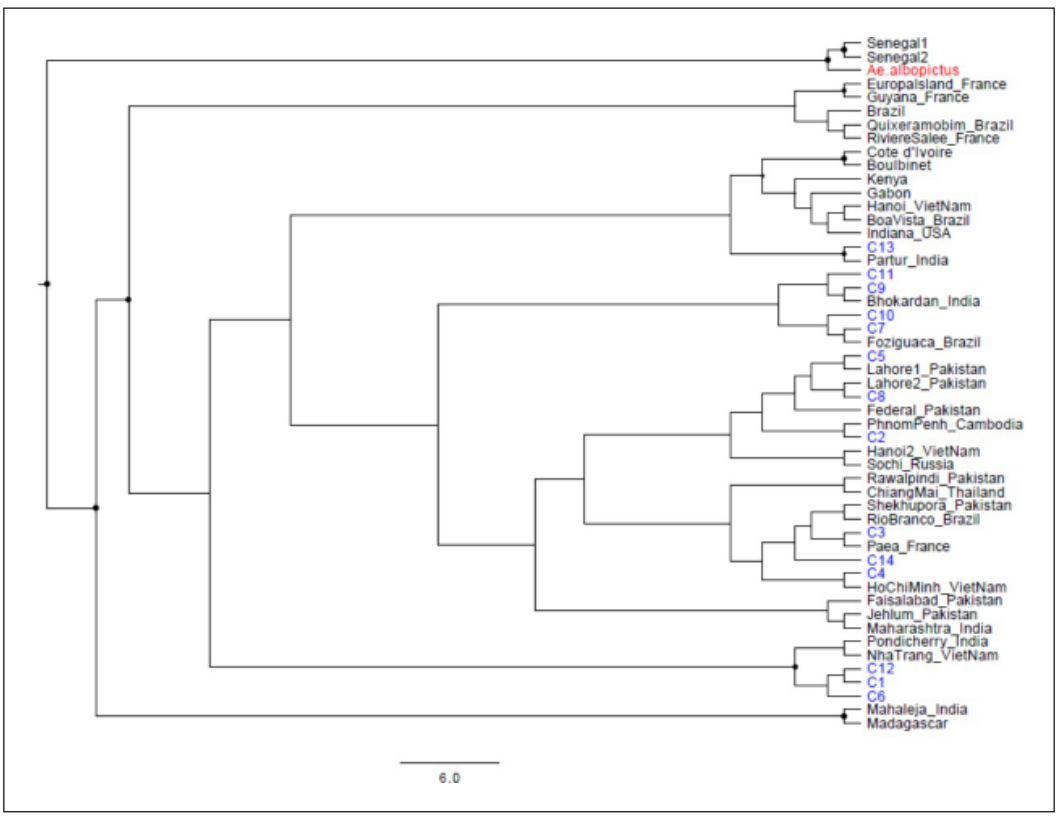

Figure 3. Maximum clade credibility phylogeny of *Ae. aegypti* based on CO1 gene. One CO1 sequence to represent each CO1 haplotype identified in the present study (C1 – C14) was used for comparison with those previously reported. Sequences of CO1 gene of *Ae. aegypti* from different countries were obtained from GenBank database, with accession numbers listed in appendix. Clades supported with posterior probability >0.8 are indicated as black dots on the nodes. *Aedes albopictus* (GenBank:KJ765612.1) was assigned as outgroup.

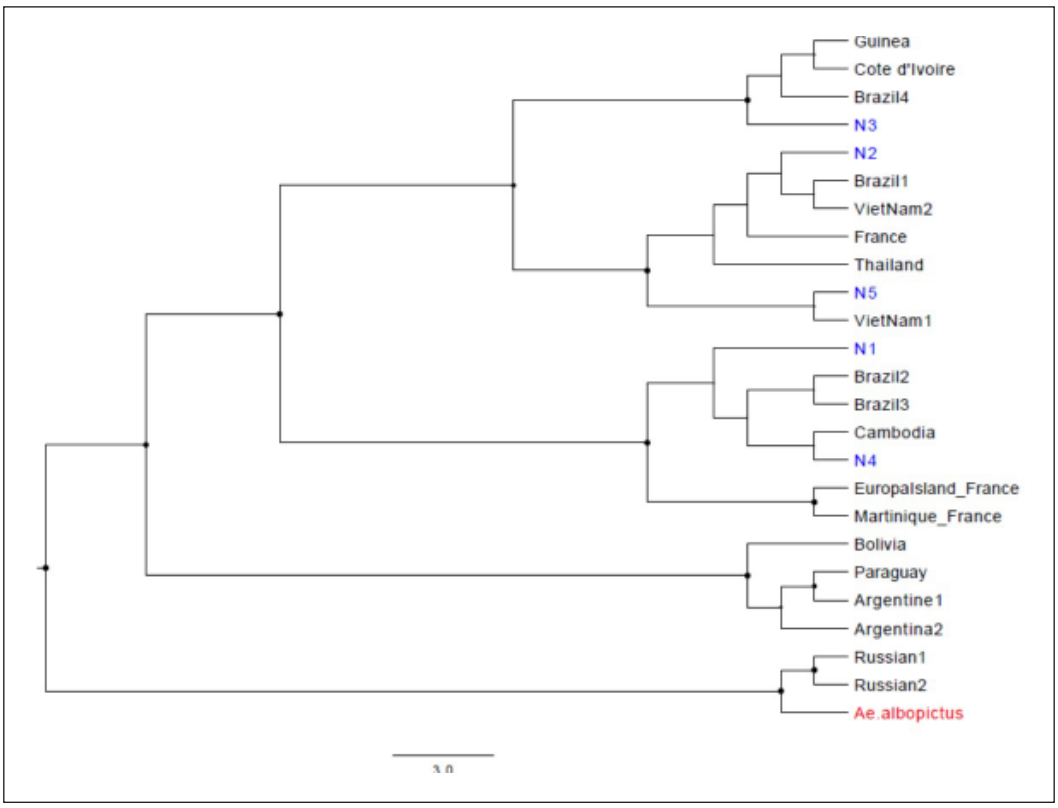

Figure 4. Maximum clade credibility phylogeny of *Ae. aegypti* based on ND5 gene. One ND5 sequence to represent each ND5 haplotype identified in the present study (N1 – N5) was used for comparison with those previously reported. Sequences of ND5 gene of *Ae. aegypti* from different countries were obtained from GenBank database, with accession numbers listed in appendix. Clades supported with posterior probability >0.8 are indicated as black dots on the nodes. *Aedes albopictus* (GenBank:HF566375.1) was assigned as outgroup.

N5 the least frequent haplotype was related to the *Ae. aegypti* from Vietnam, and N4 also low frequency haplotype was related to Cambodia. The minor clade resulted in Southern South America (Bolivia, Paraguay and Argentina) states forming the nodes exclusively. Russian samples formed the basal clade together with the outgroup suggesting mutations.

Analysis of demographic history and population structure

A pairwise mismatch distribution based on the CO1-ND5 concatenated data set revealed a multi-modal distribution profile (Fig. 5), which deviates from expected distribution for a population that has undergone a demographic expansion (Harpending raggedness, r = 0.126, P<0.01). The multi-modal distribution is interpreted as a result of stationary population size, possibly due to genetic drift leading to the existence of population substructure.

The 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 that the spatial distribution of CO1-ND5 haplotypes were significantly different from expectation under panmixia (OM=50.86km compared to EM=109.25km; p-value of OM-EM= 0). This observation suggests that the CO1-ND5 haplotypes were under distributed, which could be due to limitation to gene flow. P value of 0 means that none of the permuted datasets showed a larger divergence than the observed data as shown in Fig. 5. The measure

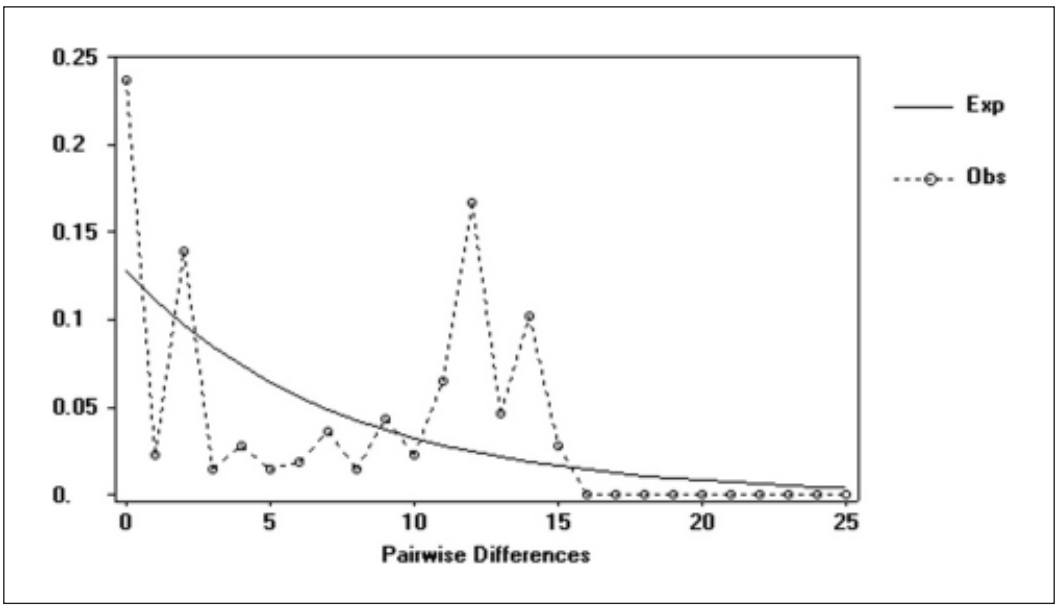

Figure 5. Multi-modal distribution of *Aedes aegypti* CO1-ND5 between haplotypes. The y-axis denotes frequency and x-axis denotes number of segregating sites between pairwise comparisons.

Supplementary Table 2. Fixation index, F_{st} values for pairwise comparison of *Ae. aegypti* populations in peninsular Malaysia as shown above the diagonal. West central region, Melaka (Southern) and Perak (Northern) are located along the west coast of peninsular Malaysia, whereas Terengganu and Kuantan are located in the east coast

	West Central	Kuantan (Eastern)	Melaka (Southern)	Perak (Northern)	Terengganu (Eastern)
West Central	–	0.550	0.125	0.200	0.504
Kuantan (Eastern)		–	0.818	0.631	1.000
Melaka (Southern)			–	0.328	0.504
Perak (Northern)				–	0.490
Terengganu (Eastern)					–

of the extent of genetic differentiation among 5 subpopulations of *Ae. aegypti* in 5 different states based fixation index (F_{st}) revealed moderate to high genetic differentiation ranging between 0.125 to 1.000 (Supplementary Table 2). Genetic differentiation among subpopulations of *Ae. aegypti* along the west central, northern and southern region of peninsular Malaysia (was generally lower (F_{st} ranged between 0.125 - 0.328) compared to that in the east coast (Terengganu and Kuantan, F_{st}=1.000). Pairwise comparisons of genetic differentiation between *Ae. aegypti* subpopulations in the east and the other regions of the peninsular revealed a moderate to high level of genetic differentiation with F_{st} ranging between 0.490 – 0.818.

DISCUSSION

The present study utilized both CO1 and ND5 genes to characterize the genetic variation of *Ae. aegypti* in peninsular Malaysia. Our findings showed that the nucleotide diversity in CO1 gene was greater than that in ND5

gene. This was also reflected by the greater number of haplotypes observed in CO1 gene compared to ND5 gene, possibly due to selective constraint on the rate of mutation in ND5 gene. This observation is consistent with the findings in previous study by Mousson *et al.* (2005), who found higher variability in CO1 gene compared to ND5 or cytochrome b gene.

In general, the genetic diversity of *Ae. aegypti* population in peninsular Malaysia was comparable to studies previously conducted in Thailand and Peru (Bosio & Norris 2005; Costa-da-Silva *et al.*, 2005). Significantly higher indices of genetic diversity of *Ae. aegypti* have also been 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). Differences in genetic diversity of *Ae. aegypti* across different regions or countries could 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*.

Our study provided clear evidence on the population substructure of *Ae. aegypti* in peninsular Malaysia. A predominant *Ae. aegypti* haplotype (H3) was distributed across the central region of peninsular Malaysia. Simultaneously, there were also other sub-populations of *Ae. aegypti* that spread across the same region. The presence of a single predominant haplotype with low diversity is possibly an indication of a bottleneck that could be a result of vector control activities. Although lacking in diversity, *Ae. aegypti* of the predominant haplotype may have been the genetically fittest mosquitoes that spread to many places in the west central region. Places across the west central region are well connected by highways and other land transportation routes, which may play an important role in facilitating the spread of such strain of *Ae. aegypti*.

Our findings revealed several unique haplotypes of *Ae. aegypti* that spread outside the central region of peninsular Malaysia. A unique haplotype (H9) sampled from the state of Perak (Northern region) was closely related to the predominant haplotype in the west central region. However, it is unclear whether haplotype H9 represents the most common haplotype in the northern region. On the other hand, our findings also indicated that 5 out of 8 specimens belonged to haplotype H14 that clustered together with those originating from the state of Terengganu in the eastern region of the peninsular Malaysia. Other unique haplotypes were also found in specimens collected from the same locations where the predominant haplotype H3 were sampled, suggesting the mixing of rare and common haplotypes particularly in the west central region. The presence of unique haplotypes could be due to recent introductions from neighbouring states or overseas, followed by reduced gene flow.

In general, the haplotype network and phylogenetic analysis suggest the presence of two major lineages of *Ae. aegypti* based on the current sampling. Similar observations have also been reported in America (Bracco *et al.*, 2007, Jaimes-Dueñez *et al.*, 2015). It is possible that the differences between populations were resulted of random genetic drift, trait selections or greater selection for resistance in the vector in an outbreak-prone region such as west central region in this case (Mudin, 2915). The east coast states of Kuantan and Terengganu have much less cases of dengue and could that be due to absence of the H3. However, we would be cautious and stress that extensive sampling need to be conducted in these states.

Bayesian phylogenetic inference based on the CO1 and ND5 genes revealed the global relatedness of the haplotypes recovered from peninsular Malaysia. The first lineage (C12, C1 and C6) was more closely related to those originating from India and Vietnam. In the second lineage, since C3 is the predominant haplotype in Malaysia, it can be assumed Brazil, France and Malaysia might have common ancestors. Moreover, C10 and C7 haplotype found in the second lineage is also closely related to Brazil. Unique low frequencies haplotypes was found to be closely related to those from India, Pakistan, Vietnam and Cambodia suggesting a recent invasion of this haplotypes through human movement. Previous phylogeographic study

of *Ae. aegypti* has reported that most Brazilian strains were genetically similar to those from southeast Asia, most probably due to reintroductions after the control programmes had ended (Mousson *et al*., 2005). The findings from the Malaysian *Ae. aegypti* specimens further suggest the possibility of the spread of *Ae. aegypti* beyond the Brazilian region as well as introduction from the African region.

Our findings based on the analysis of spatial arrangement of co-occurring alleles and genetic differentiation revealed that the *Ae. aegypti* population was significantly different from panmixia, indicating the limitation of gene flow possibly due to the various isolating barriers such as geographical, physiological and vector control measures. Alternatively, this observation could be due to the under distribution of *Ae. aegypti* haplotypes, which may be a result of sparse sampling in the present study.

Analysis of genetic differentiation clearly indicates the greater gene flow among subpopulations in the west coast of the peninsular compared to those located in the east coast. It is likely that the well-developed transportation network that linked major towns and cities located along the west coast via the north-south expressway plays an important role in facilitating the mixing of *Ae. aegypti* populations in the west coast region.

The lack of low frequency mutations as displayed by the haplotype network is indicative of a stationary population of *Ae. aegypti* in the west central region. The insignificant neutrality tests based on Tajima's D, Fu and Li's D* and F* statistics, coupled with the multi-modal pairwise mismatch distribution further support the notion that *Ae. aegypti* in peninsular Malaysia have reached a demographic equilibrium or stationary population.

Taken together, findings from the present study revealed the diversity and the existence of a mixture of *Ae. aegypti* strains in the west central region, where dengue is hyper endemic. Although there was a predominant mtDNA haplotype of *Ae. aegypti* that spread across several localities, the degree of its contribution to dengue outbreaks in the area remained unclear. Different populations of *Ae. aegypti* may have distinct susceptibility to arbovirus infections and insecticides. A study conducted by Armstrong & Rico-Hesse (2001) proved deferential arboviral susceptibility of two geographically different *Ae. aegypti*. Further work with increased sampling from wider geographical areas in Malaysia and characterisation using microsatellite markers may be beneficial to shed further light on the population dynamics of *Ae. aegypti* strains and their association with dengue transmission and insecticide resistance. The greater understanding of the local population genetic structure and dynamics of *Ae. aegypti* will lead to a better planning of effective vector control strategies. In particular, focus should be given to the west central region to eliminate *Ae. aegypti* before it starts spreading to the eastern region where frequencies and magnitudes of dengue outbreaks are low at the present time.

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