Molecular Insight into the Knockdown Resistance (*kdr*) in the Voltage Gated Sodium Channel (*vgsc*) Gene of the Main Dengue Vector, *Aedes aegypti* (Diptera: Culicidae) and the Discovery of Novel Regional Specific Point Mutation A1007G in Malaysia.

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Abstract

Background: Characterization of the insecticide resistance mechanism imparts the society with the information on the evolutionary process involved in the adaptation of *Aedes aegypti* mosquito to environmental changes. Investigating the phenotypic status of the target mosquitoes, their resistance level as well as elucidating the genotypic profile provides information about the involvement of insecticide resistance mechanism, in terms of portraying the evolution of resistance in the field, to eventually managing vector control programs. In this current study, we investigated the quantification responses for the phenotypic and genotypic resistance of *Ae*. *aegypti* population from different states in Malaysia.

Methods: We tested insecticide susceptibility status of adult *Ae*. *aegypti* from populations of States of Penang, Selangor and Kelantan (Peninsular Malaysia) against permethrin 0.25% and pirimiphos-methyl 0.25% through WHO bioassay kit. Permethrin-resistant and permethrin susceptible samples were then genotyped for domains II and III in the voltage gated sodium channel (*vgsc*) gene using allele specific PCR (AS-PCR) for the presence of diagnostic single nucleotide mutations. AS-PCR results were then validated in sequencing these two domains to identify any possible additional point mutations.

Results: Adult WHO bioassay revealed that populations of *Ae*. *aegypti* from these three states were highly resistant towards Permethrin 0.25% and Pirimiphos-methyl 0.25%. Genotyping results showed that three knockdown (*kdr*) mutations (i.e. S989P, V1016G and F1534C) were associated with pyrethroid resistance in these populations. We also report for the first time the presence of the A1007G mutation in Malaysian populations of *Ae*. *aegypti*.

Conclusions: This study brings an insight on the occurrence and association of point mutations with insecticide resistance in Malaysian populations of *Ae. aegypti*. The results reveal the widespread of several *kdr* mutations in the field with the consequence to compromise the use of pyrethroid insecticides in vector control programmes. Knowledge on the distribution of target site resistance throughout Malaysia is vital to ensure the success of the insecticide-based vector control program.

Keywords: *Aedes aegypti*; *kdr* resistance; A1007G; pyrethroid; insecticide resistance; Malaysia

Background

The mosquito *Aedes aegypti* is a primary vector for some arboviral diseases such as chikungunya [1], dengue [2], Zika [3, 4] and yellow fever [5] which have gained attention worldwide due to their fast spreading trend [6, 7, 8, 9, 10]. This species is notoriously recognized in transmitting dengue fever and dengue haemorrhagic fever especially in tropical and sub-tropical countries due to their successful adaptation from feeding on animals in natural forest ecosystems to preferably feeding on humans in anthropogenic modified habitats [11, 12]. In Malaysia, dengue has become a major threat in public health with 93,344 cumulative dengue cases and 137 deaths reported in 2019, an increase of 81.8% and 69.1% respectively from the previous year [13, 14, 15]. Although tetravalent dengue vaccine has recently been introduced and portrays good efficacy profile during clinical trial, there are still some restrictions which need to be solved before commercialized in large scale. [16].

Various approaches have been conducted to control populations size and distribution of *Ae. aegypti*; most of them rely on the insecticide-based intervention targeting the immature and imago stages of this species [17]. In Malaysia, pyrethroid and organophosphate are routinely used during the vector control programs conducted by the Ministry of Health, and also by the private pest

control operators and the local communities [18, 19]. Such excessive use of these insecticide classes for a long period, led to over dependence and improper usage of these insecticides to control the mosquitoes, eventually causing resistance in this vector.

In Malaysia, three common knockdown (*kdr*) mutations within *Ae. aegypti* voltage gated sodium channel gene (*vgsc*) are known to be associated with pyrethroid resistance. The non-synonymous mutations S989P, V1016G and F1534C correspond to a substitution of amino acid serine to proline, valine to glycine and phenylalanine to cysteine, respectively, within domains II and III. These mutations were previously shown to be widely distributed across Malaysia [20, 21, 22]. Other studies have reported that these mutations are widely established across Southeast Asia, including Indonesia, Thailand, Singapore, Myanmar and Vietnam [23, 24, 25, 26, 27]. Currently, a total of 11 non-synonymous mutations have been detected in the *vgsc* gene of pyrethroid-resistant *Ae. aegypti* population worldwide; five of them (V410L, S989P, I1011M/V, V1016G/I and F1534C) have been *in vitro* functionally characterized in the expression system of *Xenopus oocytes* and reported to confer *kdr* resistance in *Ae. aegypti* [28, 29, 30, 31, 32, 33, 34, 35, 36].

Inappropriate usage and over-exposure to insecticides that share the same mode of action can lead to the selection of several types of insecticide resistance in the mosquitoes: 1) the modification of the mosquito cuticle, leading to reduced penetration of the insecticide into the insect's exoskeleton, 2) the presence of the single nucleotide polymorphism (SNP) resulting in the modification of nucleotide in the target gene and consequently changing the amino acid of the target sites in mosquito, 3) increasing in the enzymatic activity in detoxifying the insecticides; or 4) changes in the mosquito behaviour enabling them to survive in the toxic environment [37].

The two resistance mechanisms that are commonly associated with pyrethroid resistance within insects are 1) increased metabolic detoxification activity [38] and 2) the insensitivity of the

target site such as sodium channel gene, *Ace-1* gene and GABA receptor [39]. Although, organophosphate and pyrethroid have different modes of action, both insecticides target the nervous system of the insect which eventually leads to its death [40]. Several point mutations have been functionally identified in the *vgsc* gene to reduce its sensitivity by preventing the binding of the insecticide to the target gene, making the insect experience rapid nerve firing and paralysis and consequently leading to the knockdown resistance (*kdr*) [28, 29, 33, 35].

Characterizing target site resistance mechanism is an essential key to improve the management strategies of the vector control. Hence, the aim of this study is to further elucidate the insecticide resistance mechanism associated within Malaysian *Ae. aegypti* population phenotypically and genotypically.

Methods

Sampling and rearing of mosquitoes

Six populations of *Aedes aegypti* were collected from three states in Malaysia; Penang, Selangor and Kelantan in 2017 by placing the ovitraps at residential areas for five days. The sites were selected based on the number of reported dengue cases in *idengue* website which is the Malaysian national dengue database (Ministry of Health) and were routinely sprayed with insecticides particularly permethrin and pirimiphos-methyl. We selected two sites within each state (total = six sites): Sungai Dua (SD) and Balik Pulau (BP) in Penang, Alam Budiman (AB) and TUDM, Subang (TDM) in Selangor, and Pauh, Panji (PNJ) and Flat Buluh Kubu (FLT) in Kelantan. The ovitraps were randomly placed at the potential breeding sources and less exposed to direct sunlight. The traps were collected after five days and brought back to the insectary for culturing purposes.

The eggs of *Aedes* mosquitoes from all the localities were hatched and upon emergence of first instar larvae, they were fed with the larval food containing grounded cat biscuit, beef liver powder, milk powder and yeast with a ratio of 2:1:1:1. The adult mosquitoes were morphologically identified to the species level based on the pattern on the thorax. The adult *Ae. aegypti* were supplied with 10% sucrose solution. All larvae and adults were maintained at room temperature of

$28 \pm 2\,^{\circ}C$ with a relative humidity of $75 \pm 10\%$. The local VCRU strain which has never been exposed to any insecticides was used as the reference strain.

WHO adult bioassay

The adult mosquito bioassay was performed according to the World Health Organization (WHO) protocol [41]. Twenty female mosquitoes of *Ae. aegypti* of three to five days old were tested against permethrin 0.25% (Type I pyrethroid) and pirimiphos-methyl 0.25% (organophosphate) for one hour and replicated five times. During the 60 minutes exposure time, the knocked-down individuals were scored every five minutes interval. Subsequently, they were supplied with 10% sucrose solution ad libitum and maintained under insectary conditions. After 24 hours exposure, the whole bodies of the surviving mosquitoes were transferred and kept in -80℃ and those of the dead samples were preserved in silica gel inside microcentrifuge tubes for *kdr* genotyping.

Synergist bioassay

The synergism assay was conducted as a first screening to investigate the potential role of superfamily enzyme mixed function oxidases and esterase in the insecticide metabolic detoxification. Piperonyl butoxide (PBO) was used before the exposure to permethrin insecticide because it is known as a common synergist for pyrethroid [42] and inhibit enzyme from the family of esterases and mixed function oxidases which are mainly associated with metabolic resistance caused by pyrethroid, organophosphate and carbamate exposure. The test was performed on the individuals from all sampling sites because the percentage mortality was less than 90% mortality from the previous WHO bioassay test. Adult female mosquitoes were exposed to PBO 4% one hour before the exposure to Permethrin 0.25%. The mortality of the individual mosquitoes was

recorded after 24 hours. To compare the results obtained between the two treatments, we either use only the insecticide or a combination of synergist and insecticide.

Genotyping of *kdr* mutations in voltage gated sodium channel (*vgsc*) gene in *Aedes aegypti*

Extraction of genomic DNA (gDNA)

The gDNA of 20 dead and alive samples from all localities were extracted from legs and wings following Livak protocol [43]. The body of survived mosquitoes were kept in -80 ℃ for the quantification of metabolic resistance by real-time PCR. The DNA concentration and purity were measured using a Nanodrop spectrophotometer at 260 nm. The gDNA samples were stored in the -20℃ for the downstream application.

Detection of mutation V1016G by using allele specific PCR (AS-PCR) in *Aedes aegypti*

To determine the presence of point mutations V1016G and F1534C conferring pyrethroid resistance, 20 female mosquitoes from each resistant and susceptible field population were randomly chosen for this genotyping using AS-PCR. From a total of 100 female mosquitoes, 20 resistant individuals and 20 susceptible individuals were genotyped using AS-PCR protocol as previously described by Stenhouse et al. [24]. V1016G mutation was genotyped in 20 resistant and susceptible individuals that were exposed to permethrin previously. Each reaction used consisted of a total volume of 15 μl of 1.25mM $MgCl_2$, 1× PCR buffer (Thermo Scientific, USA), 0.3 μM forward primer (Gly1016f), 0.2 μM for each reverse primer (Gly1016r or Val1016r), 200 μM dNTP mixture (Promega, USA), 1.25 units DreamTaq Hot Start DNA polymerase (Thermo Scientific, USA) and 25-100 ng of gDNA. The amplification was carried out using Bio-rad MyCycler^{TM} Thermal Cycle (Hercules, California, USA). The thermal cycling condition was: 94℃ for 2 min (initial denaturation); 35 cycles of 94℃ for 30 sec (denaturation), 58℃ for 30 sec

(annealing), 72℃ for 30 sec (extension) and 72℃ for 2 min (final extension). The PCR products were loaded checked onto 3% agarose gel. The sizes of the two amplified fragments are 60 base pairs [bp] for valine and 80 bp for glycine.

Genotyping of F1534C by using allele specific (AS-PCR) in *Aedes aegypti*

For the amplification of F1534C in domain III segment 6 (DIII S6), this *kdr* mutation was genotyped by AS-PCR as described by Yanola et al. [44]. The PCR reaction was performed in final volume of 15 μl with a final concentration of 0.5 mM $MgCl_2$, 1.5× PCR buffer (Thermo Scientific, USA), 0.3 μM for each forward primer (F1534-f and C1534-f), 0.23 μM reverse primer (CP-r), 0.2mM dNTP mixture (Promega, USA), 1.25 units DreamTaq Hot Start DNA polymerase (Thermo Scientific, USA) and 25-100 ng of gDNA. The reaction was run at 95 ℃ for 2 min (initial denaturation); 35 cycles of 95℃ for 30 sec (denaturation), 56℃ for 30 sec (annealing), 72℃ for 45 sec (extension) and 72℃ for 2 min (final extension). The products were run in 3% agarose gel.

Validating polymorphism sites of the voltage gated sodium channel (*vgsc*) gene in *Aedes aegypti* by DNA sequencing

Some of the AS-PCR products were sequenced in order to verify the accuracy of results obtained from the AS-PCR assay and, also, to detect any possible novel mutation. The regions of DIIS6 and DIIIS6 in the *vgsc* gene (where the V1016G and F1534C mutations occur) were separately amplified (by PCR) using the protocol described in Stenhouse et al. [24] and Yanola et al. [44]. Each reaction was performed in 20 μl final volume consisting of 1.5 mM $MgCl_2$, 0.2 μM each for forward and reverse primers for each fragment, 0.4 mM dNTP, 1× PCR buffer and 1.0 units of GoTaq G2 Flexi DNA Polymerase (Promega, USA). PCR amplification began with 3 min at 95 ℃ (heat activation step), 35 cycles of 95 ℃ for 30 sec (denaturation), 57 ℃ for 30 sec (annealing), 72

℃ for 30 sec (extension) and 72 ℃ for 3 min (final extension). PCR products were sent to Integrated DNA Technologies, Inc. for sequencing.

Chromatograms were first edited using MEGA 6 Molecular Evolutionary Genetic Analysis software, version 6.06 [45]. The sequences determined in this study have been deposited in GenBank (GenBank accession nos. MT237357-MT237435). Then, all nucleotide sequences of the haplotype for domains II and III were aligned (no indels needed). Our phylogenetic matrix comprises 46 individuals and 491 and 346 nucleotide positions for domain II and domain III respectively. We inferred the relationships among the individuals using a maximum parsimony method of phylogenetic reconstruction as implemented in PAUP* v4b10 [46]. Susceptible *Musca domestica* (GenBank accession number U38813.1) was selected as the outgroup to root the tree. The haplotype TCS network was built using the PopART [47,48] software to determine the correspondence/relation between haplotype and the resistance/susceptible phenotypes.

Results

WHO adult bioassay

The diagnostic dosage for *Aedes* mosquito was used for permethrin and the tentative dosage for *Anopheles* was used for pirimiphos-methyl. Prior to the exposure towards permethrin and pirimiphos-methyl, WHO bioassays on the field strains revealed phenotypic resistance in populations from Selangor, Penang and Kelantan with percentage of mortality less than 90% (Figure 1). *Aedes aegypti* from all six populations were highly resistant towards both insecticides with percentage mortality, after 24 hours exposure, varying from 0% to only 18% and 3% to 58% for Permethrin 0.25% and Pirimiphos-methyl 0.25%, respectively. Full susceptibility was observed in *Ae. aegypti* from the VCRU laboratory strain, after the exposure towards both insecticides (Figure 1).

High resistance towards permethrin was observed in all six populations. The lowest percentage of mortality was observed in the populations from Pauh, Panji (PNJ) and Flat Buluh Kubu (FLT), Kelantan with 0% and 1% mortality, respectively, indicating that these two populations developed very strong resistance and the need of additional assessment regarding the

genetic mechanisms and distribution of such resistance. *Aedes aegypti* from TUDM, Subang (TDM) shows the highest mortality compared to other populations with 18%, although such percentage of mortality is still quite low. Widespread resistant towards Pirimiphos-methyl 0.25% was also observed in all populations with the highest resistance level recorded in Alam Budiman, Selangor (AB) with 3% mortality. Meanwhile, the population from Balik Pulau, Penang (BP) exhibited the highest mortality against this insecticide with 58% mortality.

The overall low percentage mortality observed in all six populations against these two insecticides indicate that they acquired strong resistant towards these insecticides and suggest these insecticides might not be effective for the control of mosquito populations. Resistance ratio (RR) cannot be determined in this study because there were no knocked down mosquitoes during the one-hour exposure towards permethrin and pirimiphos-methyl and this probably reflects that the populations are highly resistant towards both insecticides.

Synergism assay with piperonyl butoxide (PBO)

Percentage mortality of all populations except Pauh, Panji, (PNJ) after subjected to one-hour pre-exposure to 4% PBO before being exposed to permethrin, displays an increase in the mortality ranging from 2% to 69% (Figure 1). Pre-exposure to the synergist instigates a partial recovery in population from TUDM, Subang (TDM) and Balik Pulau (BP) with mortality of 29% and 69% respectively after being exposed to permethrin. However, synergist with PBO significantly increased the susceptibility towards permethrin only in the population from Balik Pulau (BP) (X^2 = 7.244, df= 1, P= 0.007). After exposure to PBO, a slight recovery of susceptibility was observed from Flat Buluh Kubu (FLT) (from 0% before to 2% after), Alam Budiman (AB) (from 3% before to 7% after) and Sungai Dua (SD) (from 12% to 19%). No impact of pre-exposure to synergist

PBO was observed in the population from Panji (Kelantan), 0% mortality before and after exposure to synergist suggesting that target site resistance might play a major role in this population.

Detection of *kdr* mutation in the *vgsc* gene of Malaysian population *Aedes aegypti*

167 samples including dead and alive mosquitoes from all populations were genotyped in order to determine the presence of S989P, V1016G and F1534C in DII and DIII and the allelic frequencies. Results of the genotyping are shown in Tables 2 (S989P), 3 (V1016G) and 4 (F1534C).

To detect other possible point mutations in the *vgsc* gene of the Malaysian population *Ae. aegypti*, the partial *vgsc* gene was sequenced. The length of the first fragment is 491 bp, spanning codons 989, 1011 and 1016; the same synonymous mutation was detected at the nucleotide positions 33 and 132: A to G. Sequence comparison with the pyrethroid susceptible *Musca domestica* (GenBank accession number U38813.1) and *Ae. aegypti* China strain (GenBank accession number MF794972.1) revealed six nucleotide substitutions, four of them non-synonymous resulting in four amino acid changes in the domains II and III of the *vgsc* gene in the permethrin resistant and susceptible samples from the six populations in Malaysia.

Several non-synonymous mutations in domain II, have been detected at codon 989 and 1016. Samples from Sungai Dua (SD), Balik Pulau (BP), Alam Budiman (AB) and TUDM, Subang (TDM) were heterozygous and homozygous for a double amino acid change. This amino acid change occurs at codon position 989, a change of wildtype amino acid serine (TCC) to proline (CCC), due to T/C substitution at nucleotide position 52. This mutation was specifically detected in populations from Penang, Selangor and in one sample from Flat Buluh Kubu, Kelantan (FLT) with the allelic frequency ranging from 0.1 to 1.0 (Table 2).

At codon 1016, a polymorphism was detected in populations from Penang and Selangor (but not in those of Kelantan). A mutation from valine to glycine at codon position 1016 was identified within these populations. A mutation of wildtype GTA to mutant allele GGA was detected from those populations with allelic frequency ranging from 5% to 55% (Table 3). Among 167 samples genotyped, a total of 46 samples were partially sequenced in domain II to valid the results obtained from AS-PCR for point mutation V1016G. Eighteen samples showed discrepancy where heterozygous allele (V/G) (from AS-PCR) turned out to be homozygous wild type allele (V/V) after Sanger sequencing. This might be due to the presence of two consecutive alternative mutations in domain II segment 6 leading to genotyping error, hence resulting in the false positive results (Ishak et al., 2015). In addition, the amplification of the non-specific band happens due to the mismatch of the single base in the gene, hence it is unable to prevent the non-specific amplification during the PCR extension (Singh et al., 2009).

A novel non-synonymous substitution has been discovered at codon position 1007, a mutation from alanine (GCC) to glycine (GGC), happens due to changes of nucleotide C to G at position 1007 in the sequence. Our results show that only permethrin resistant samples from Pauh, Panji (PNJ) and Flat Buluh Kubu (FLT) have this novel amino acid substitution, with the percentage of 1007G allele were 85% and 90%, respectively. Interestingly, the populations that possess this mutation will not co-occur with other point mutations either S989P or V1016G in domain II and could only be found in samples from Kelantan state. Our results indicate this point mutation alone in domain II might be responsible in conferring the high resistance in the phenotypes since there is no other point mutation in domain II that coexist after genotyping using direct sequencing (Figure 2). Result from the direct sequencing reveals populations from Kelantan that possess this novel mutation will coexist with another point mutation in domain III which is

the F1534C mutation (Figures 2 and 3). All samples from these localities were a mixture of heterozygous and homozygous for the double substitution mutations. These populations were either heterozygous or homozygous mutant to codon 1007 and shares another amino acid change at codon 1534 and are homozygous mutant at this position (Table 8). This might explain why the population from Kelantan were highly resistant towards permethrin.

In domain III, a change from phenylalanine (TTC) to cysteine (TTG) at codon 1534 was detected in all six populations with allelic frequency ranging from 0.028 to 0.975 and populations from Kelantan showing the highest allele frequency for the permethrin-resistant samples; more than 90%. We also recorded that the mutant allele, 1534C are common in the susceptible samples from Penang and TUDM, Subang (TDM) (Table 4 and Figure 4).

Association between *kdr* mutation at domain II and III with pyrethroid resistance

To assess the correlation with the resistance phenotype, a total of 167 resistant and susceptible mosquitoes from all populations were then genotyped at domains II and III. To ascertain the impact of *kdr* mutations at different codons; 989, 1007, 1016 and 1534 of the *vgsc* gene towards pyrethroid resistance, the S989P, A1007G, V1016G and F1534C mutations were analysed separately for their associations with the permethrin resistance.

The S989P mutation in domain II is not significantly associated with the pyrethroid resistance in all populations (Fisher's exact test, $p>0.05$) and we presume the populations were not at Hardy-Weinberg equilibrium due to low samples size tested for genotyping at codon 989 (Table 2). Fisher's exact test was conducted to compare the differences in 1016G of the allelic frequency between the resistant and susceptible phenotype from each locality. At codon 1016, population from TUDM, Subang (TDM) was significantly associated with the permethrin resistance [Odds

Ratio (OR): 3.696, $p<0.05$] and population from Sungai Dua, Penang (SD) was slightly correlated with the permethrin resistance [OR: 0.29, $p<0.05$] (Table 3). In most of the localities, the differences in the allelic frequencies between alive and dead mosquitoes were not significantly correlated ($p > 0.05$). More heterozygote mosquitoes survived after the exposure towards permethrin (Figure 4).

We cannot determine the correlation between novel mutation, A1007G with the permethrin resistance in population from Kelantan due to the low number of susceptible samples obtained after WHO bioassay. Despite that, we found an extremely high 1007G allele frequency ranging from 85% to 90% (Table 5). This probably explained why the mortality of the population from Kelantan were exceptionally low (Table 6).

The frequency of the 1534C allele in domain III was significantly different between alive and dead mosquitoes from all six populations (X^2=51.26, df= 1, p<0.001). The 1534C mutant allele was highly significant associated in the population from Balik Pulau (BP) and TUDM, Subang (TDM) [Odds Ratio (OR): 3.318, $p<0.05$ and OR: 16.852, $p<0.05$] (Table 2). There is no significant correlation observed between 1534C genotype and permethrin resistance in Sungai Dua (SD) [OR: 1.069, $p>0.05$]. From the Fisher's exacts test, we found that Alam Budiman (AB), Pauh, Panji (PNJ) and Flat Bulu Kubu (FLT) were not in the Hardy-Weinberg equilibrium ($p>0.05$) and we speculate that this might be due to the deficit of the heterozygote allele.

Distribution of triple-loci and quadruple-loci of the genotypic combination in the domain II and III of the *vgsc* gene in *Ae. aegypti*

In a total of 46 samples genotyped in domain II and III, we found 13 different combination patterns of substitutions in Malaysian populations of *Ae. aegypti* with nine types of triple-locus and four

type of quadruple-locus combination observed within those populations. The combination of the triple-locus genotype detected in each population (Selangor and Penang) ranging from one single type to four different types. Meanwhile, two to three types of combination for quadruple-locus genotyped was observed in the population from Kelantan (Table 6). Triple-locus wild type homozygote, S989+V1016+F1534 (Type 1) and quadruple-locus wild type homozygote, S989+A1007+V1016+F1534 (Type 10) was found in four susceptible samples from Selangor and one susceptible sample from Kelantan respectively. Most of the locus genotyped were a combination of two to three amino acid substitution. We noticed triple-loci *kdr* genotypes (Type 3,4,5,6) resulted in the phenotypic resistant in the samples from Selangor and Penang. The presence of single mutation, S989+1016G+F1534 (Type 2) were seen in the population from Selangor only. Interestingly, the combination of the triple mutant, 989P+1016G+1534C happens to be present in one susceptible sample from Penang. This individual which possesses this genotype combination survived after 24 hours exposure period, suggesting the heterozygous allele at codon 1534 might confer lack resistance phenotypically in this sample. Four combination of quadruple-locus genotype was particularly present in population from Kelantan only with three combinations of locus; 989P+A1007+1016G+F1534; S989+1007G+V1016+1534C; S989+1007A/G+1534C (Type 11, 12 and 13) were detected in permethrin-resistant samples and one locus, S989+A1007+V1016+F1534 (Type 10) found only in permethrin-susceptible sample. Those loci consist of the new mutation with a high number resulting in 15 individuals (Kelantan population) surviving after the exposure of the permethrin.

Haplotype distribution and polymorphism analysis of the *vgsc* gene fragment in Malaysian population of *Ae. aegypti*

Seven haplotypes were identified with five haplotypes present in domain II and two haplotypes was observed in domain III. These haplotype variations produced four amino acid substitution. In the coding region of domain II, we found five polymorphic sites resulting in three non-synonymous changes and two synonymous changes. Meanwhile in the coding region of domain III, one polymorphic site at codon 1534 could be observed leading to the two haplotypes created in this *vgsc* fragment. In general, the *vgcs* gene exhibits a low polymorphism level for all six populations in Malaysia with a low number of the mutational steps between the haplotypes in domain II and III as shown in the TCS network (Figure 5). From the TCS network analysis in domain II, four resistant haplotypes were observed with one singleton haplotype 4 (H4) and haplotype 5 (H5) which is a combination of the new mutation and synonymous change were detected in Kelantan samples only.

There are five different haplotypes with two major haplotype that are established from all six populations *Ae. aegypti* in Malaysia. Haplotype 1 consists of populations from Sungai Dua (resistant and susceptible), Balik Pulau (resistant and susceptible), Alam Budiman (resistant), TUDM, Subang (susceptible) and Flat Buluh Kubu (resistant) and this is a major haplotype in domain II, found in 15 individuals out of 47 individuals. Only two susceptible samples from Sungai Dua and Balik Pulau (Penang) harbor these mutations. These populations have mutations towards S989P and V1016G which is mostly are genotypically homozygous resistance towards both point mutations. Meanwhile, haplotype 2 consists of populations from Alam Budiman (resistant), TUDM, Subang (resistant and susceptible) and Balik Pulau (susceptible). In this haplotype, all samples from those populations have mutation V1016G but inhibit mutation S989P. They are homozygous resistant towards V1016G but homozygous susceptible towards S989P. One susceptible sample from TUDM, Subang (Selangor) and Balik Pulau (Penang) fortuitously exhibit

this haplotype in the *vgsc* polymorphism of domain II. For haplotype 3, Alam Budiman (susceptible), TUDM, Subang (resistant and susceptible) and Flat Buluh Kubu (susceptible) were found. All samples in this haplotype are homozygous susceptible towards S989P and V1016G. In haplotype 4, only several samples population from Pauh, Panji and Flat Buluh Kubu (resistant) is found. All samples are homozygous susceptible for S989P and V1016G. Surprisingly, we did find a novel regional mutation in domain II segment 6 which is A1007G. This haplotype was homozygous mutant and heterozygous towards A1007G. This resistant haplotype (H4) which has the new mutation might have evolved from the wild type homozygote (haplotype 3) through one mutational step. Most of the resistant populations from Flat Buluh Kubu and Pauh, Panji belong to Haplotype 5 (H5). This haplotype derived from haplotype 4 through one mutational step, it possesses mutation A1007G and also causes synonymous changes at nucleotide position 132.

There are only two haplotypes that were found to be associated with the mutation F1534C in domain III of the *vgsc* gene. There is one major haplotype that is associated with this mutation which is haplotype 1 (H1). This haplotype consists of samples from Pauh, Panji (resistant), Flat Buluh Kubu (resistant), Alam Budiman (resistant), TUDM, Subang (resistant), Sungai Dua (resistant and susceptible) and Balik Pulau (susceptible). We observed homozygous resistant allele (1534C) within this haplotype. We suspect this resistant haplotype might have evolved from the susceptible homozygote through one mutational step. In haplotype 2 (H2), samples Alam Budiman (resistant and susceptible), TUDM, Subang (resistant and susceptible) and Balik Pulau (Resistant) were detected. All samples in this haplotype have homozygous susceptible allele, F1534.

There is no significant difference in the Tajima's D estimation in the *vgsc* fragment for both domain II and III demonstrating a low number of the polymorphisms in the *vgsc* within those populations (Table 7). The presence of the predominant haplotypes in both domain II and III gave

us an idea that there is selection pressure in the *vgsc* gene fragment in both domain II and III which is in agreement with the existence of the *kdr* mutation in this Malaysian population of *Ae*. *aegypti*. Maximum parsimony phylogenetic tree analysis of the *vgsc* gene display an association between the pyrethroid resistance and the single nucleotide polymorphism of domain II and III in the *vgsc* gene respectively (Figure 6A and 6B). The existence of polymorphism in exon 15 to 16 domain II and exon 23 to 25 of domain III of *vgsc* gene potentially correlated with the permethrin resistance. Reconstruction of the maximum parsimony tree revealed the haplotype pattern within those domains clustered according to the phenotype of the mosquito samples.

Discussion

The development of insecticide resistance worldwide has become worrisome problem since a few decades ago [10]. Malaysia is one of the countries that is confronted with this scenario [49]. The present study reveals that the susceptibility status and the distribution of the *kdr* allele in the Malaysian main dengue vector, *Aedes aegypti* in Malaysia. This study focuses on the significant role of target site mutation in conferring pyrethroid resistance in this species.

Resistance profiles of *Aedes aegypti*

Results of the susceptibility bioassay revealed that *Ae*. *aegypti* populations from Penang, Kelantan and Selangor exhibit high resistance towards the insecticides from the class of pyrethroid (Type I: permethrin) and organophosphate (pirimiphos-methyl) with the percentage mortality after 24-hour exposure less than 90% confirming these populations are resistant as shown recently [20, 21, 22]. Our results contradicted a study conducted in 2011 and also previous studies [20,21,22] revealing that *Ae*. *aegypti* populations from Penang were at that time fully susceptible towards pirimiphos-methyl and malathion [50]. One explanation could be that the Penang populations recently

developed resistance against these insecticides compromising the application of this insecticide during vector control programmes. Phenotypic pyrethroid resistance is still increasing in Malaysian population of *Ae*. *aegypti* as a consequence of the wide usage of pyrethroid for decades. To understand the genetic basis of resistance mechanisms and their distribution in Malaysia, those populations were subjected to additional investigation.

Currently, pyrethroid and organophosphate are the major classes of insecticides that are widely used to eliminate the *Aedes* mosquitoes during the dengue outbreak and these classes of insecticides have been routinely switched by the Ministry of Health Malaysia throughout the control programmes, and not only that, the industry of pest control also has been using the same classes of insecticides for the same purpose. This action probably resulted in the selection pressure within the natural populations due to the high exposure towards the insecticides during the control measures hence have the ability in adapting to the changes of the harsh environment [51]. High occurrence of permethrin resistance in *Ae*. *aegypti* is frequently reported in many countries from Southeast Asia including Singapore, Indonesia, Cambodia, Laos, Thailand, Myanmar and Vietnam [23, 26, 27, 52, 53, 54, 55, 56, 57]. The increasing occurrence of the pyrethroid resistance is alarming to governments and private sector involved in vector management.

Development of resistance towards pyrethroid insecticide in Malaysia is notably observed since the year 2001 and it is gradually increasing yearly. Although the authorities practice insecticide rotation, the use of insecticides from the same class and having the same mode of action causes the resistance problem to not be solved. Future studies such as dose-response study and intensity bioassay should be conducted in local populations to determine the strength of the resistance level among these populations, thus can assist in managing insecticide resistance.

Resistance is usually the combination of two or more mechanisms. Pyrethroid resistance in *Ae*. *aegypti* is often associated with target site resistance and metabolic resistance [38, 39]. A few studies conducted in Malaysia reveal that several non-synonymous mutations (S989P, V1016G and F1534C) are present in the *kdr* gene of *Ae*. *aegypti* and none of them were reported to occur in *Ae*. *albopictus*. First report on the occurrence of the point mutation in the *kdr* gene in Malaysian population of *Ae*. *aegypti* was detected in 2010 whereby the authors found mutation V1016G and F1534C associated with the pyrethroid resistance in *Ae*. *aegypti* populations from Penang, Kuala Lumpur, Kota Bharu and Johor Bharu [20]. In year 2018, Rasli et al. [21] have reported the co-occurrence of point mutation, S989P alongside with the reported mutation V1016G in the permethrin-resistant field strains from Kedah and Johor. Subsequently, Leong et al. [22] found the presence of those mutations in the populations from Selangor associated with pyrethroid and DDT resistance. Our present study is in line with the reported point mutations which is associated with pyrethroid resistance in the Malaysian population of *Ae*. *aegypti*.

In addition, we unexpectedly discovered a potential novel substitution mutation, A1007G from Kelantan population, which occurs as a single mutation in domain II without the association of other mutations, S989P and V1016G, but co-occur with the point mutation in domain III, F1534C. We observed a significant correlation between V1016G and F1534C genotypes with the pyrethroid resistance in several localities within these states in Malaysia which shows that those genotypes could possibly attribute towards the pyrethroid type II resistance. In addition, high frequency of the *kdr* mutations; S989P, A1007G, V1016G and F1534C was detected amongst the six populations in Malaysia, demonstrating target site resistance is partially associated with the resistance towards permethrin. The widespread of these point mutations of S989P, V1016G and F1534C in the *kdr* gene of the Malaysian population *Ae*. *aegypti* might explain the large-scale

usage of the insecticide from the class of pyrethroid which had been extensively used by the government and the private sector to control the population of mosquito vectors and especially *Aedes* species in Malaysia.

A novel mutation in Malaysian *Aedes aegypti* population

The mutation A1007G was found in the population from Kelantan with a high frequency was observed (f≥85%). Direct neurophysiological analysis has not yet been proven, and this step is crucial in seeking evidence whether this new mutation is responsible in causing the pyrethroid resistance within the permethrin-resistant population. We hypothesize this particular mutation play a similar function as the other *kdr* mutations since this mutation is located within one of the four specific amino acid residues in the P-region segments, DIIS6 [58]. Changes of nucleotide C to G at position 1007 in domain II leading to the amino acid substitution from alanine to glycine presumably give rise to the alteration of the target site in the sodium channel, hence reducing its sensitivity towards permethrin. Modification in the insect voltage gated sodium channel which normally regulates sodium ion within the gene, makes the channel less functional, hence delaying the closing of the channel [59, 60]. The previous studies from other research groups are able to detect other regional mutations in the Asian continent. First report in Vietnam found point mutation L982W in domain II is associated with the DDT and pyrethroid cross resistance in the resistant population of *Ae*. *aegypti* [28]. In 2009, a research group from Taiwan concluded that a high resistant fold in their permethrin resistant strain is conferred by the association of a novel mutation, D1763Y with the other substitution mutation, V1016G [30]. These co-existing mutations conceivably causes synergist effect in the knockdown resistance towards permethrin. The sensitivity of the *vgsc* gene towards permethrin resistant is reduced by 190-fold. The presence of the point mutation, T1520I and F1534L in the domain III of the *kdr* gene in the Indian *Ae*. *aegypti*

population might be partly responsible towards the pyrethroid resistance [34, 61]. These new mutations have yet to be functionally confirmed in the oocytes' system. Similarly, our study also has a limitation in validating this new mutation. Due to facilities constraint, we are unable to conduct the functional validation of our potential novel substitution mutation, A1007G express in the *Xenopus oocytes*. By conducting the experiment, we might possibly know the conformational occurrence of this new mutation in conferring pyrethroid resistance. However, high occurrence of the 1007G allele frequency together with the 1534C allele in that populations provide us an information that the co-occurrence of this new mutation with F1534C could be one of the contributing factors in the pyrethroid resistance in the Kelantan population.

To our current understanding, Lien et al. [62] discovered this new mutation in pyrethroid resistant population from Vietnam which is in line with our study. However, they did not further elucidate the role of this particular mutation. An attempt was made by a research group from United States of America to investigate this mutation in the American population of *Ae*. *aegypti* [63]. Unfortunately, this mutation could not be detected from any samples, suggesting *kdr* allele A1007G, is a part of other geographic mutations similar to V410L [36], G923V, L982W [28], I1011V/M [29], T1520I [34], F1534L [61], D1763Y [30].

Low genetic diversity was observed in the *vgsc* gene fragment spanning point mutation S989P, A1007G, V1016G and F1534C from all localities, indicate these genes are under selection pressure which might support that knockdown resistance play a role in the pyrethroid resistance within Malaysian population of *A. aegypti*. This selection might be due to low polymorphism of the *vgsc* gene fragment with low number of mutational steps between haplotypes. The similar pattern was also observed in the previous report by Ishak et al. [20], stating that F1534C mutation is under selection pressure across Malaysia, thus resulting in the reduction of genetic diversity

within domain III of the *vgsc* gene. However, this scenario was not detected in Malaysian population of *Ae. albopictus* since to date, there is no mutation discovered within the Malaysian strain. Currently in China, Zhou et al. [64] detected allele 1016G and 1532T might evolved from the common susceptible *Ae*. *albopictus*. In the present study, we found two *kdr* mutations commonly classified under the same haplotype within domain II of the *vgsc* gene. This could be an alarming observation that could affect the authorities in controlling the population of *Ae*. *aegypti* in Malaysia due to survival adaptation of the target mosquitoes towards the same class of insecticide used in the control program.

Role of metabolic resistance in *Aedes aegypti*

A partial recovery of the susceptibility in Balik Pulau, Penang (BP) and a slight recovery of the susceptibility in the other locations after the pre-exposure towards PBO suggest that metabolic resistance could be involved in conferring permethrin resistance in the Malaysian population of *Ae*. *aegypti*. PBO is widely known as the most frequent synergist used with the combination of the pyrethroid insecticide in controlling the resistant mosquitoes [65]. In general, synergist like PBO can act as an enzyme inhibitor in the metabolic enzyme defence system and acts by binding the PBO metabolites to the enzyme from superfamily group of monooxygenases P45O and non-specific esterases, hence, resulting in the detoxification of enzyme to oxidize. Thus, the effectiveness of the pyrethroid will increase against the pyrethroid resistant mosquitoes [42]. In the present study, the pre-exposure towards PBO might be considered as an ineffective synergist against the resistant *Ae*. *aegypti* mosquitoes as it is unable to restore full susceptibility after the exposure towards permethrin. There might be other possible involvement of the resistance mechanism such as modification of the mosquitoes' cuticle within the Malaysian population, as

the enhancement of the metabolic enzyme system can lead to the reduced penetration of the insecticides towards the cuticular insects [66, 67].

Conclusions

By elucidating the resistance mechanism involved in the Malaysian strain of *Aedes aegypti*, we can figure out the geographical distribution of the mutations involved alongside with their frequencies. The present study reflects the high occurrence of the reported mutations and the arising of new point mutations in the *vgsc* gene within the Malaysian permethrin-resistant strain. Henceforth, surveillance and monitoring of these mutations in the *vgsc* gene should be conducted regularly in order to detect any possible involvement of new point mutations and also the frequency level. This act can instigate an insightful-decision making factor on the proper usage of insecticide against the target vector which is a part of the integrated resistance management that enables the authorities to control the widespread of the resistance within the target mosquito population in Malaysia. Understanding the resistance mechanism involved in the mosquito population will enlighten the authorities to have better planning in the management of the vector control program.

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Author's contribution

MAMA, IHI, ISJ and NAUAB performed the mosquito collection. MAMA performed bioassay, molecular genotyping, data tabulation and analysis and wrote the first draft of manuscript. IHI conceived the study design, analysis of data and improvising the manuscript. MFFW, SL and GA perform data analysis and further improved the content of the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent of publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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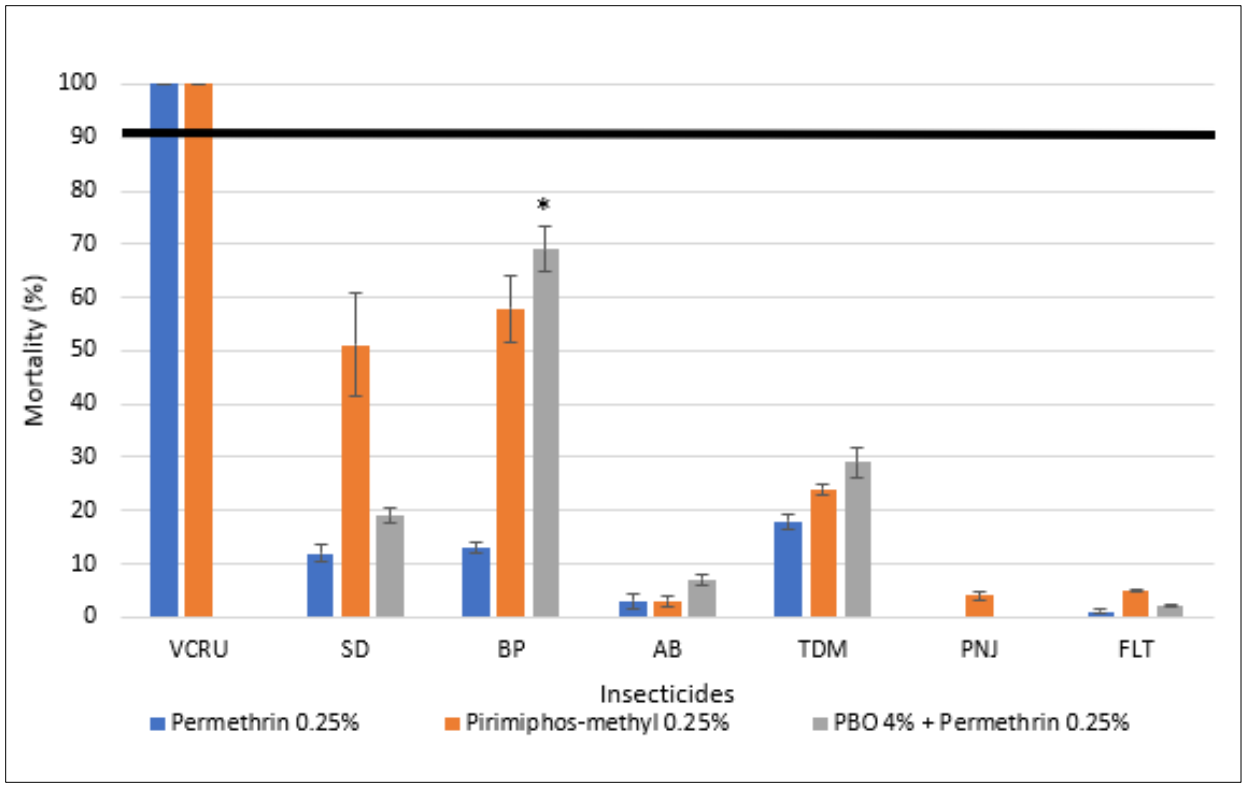

Fig. 1 Percentage mortality of female *Aedes aegypti* from various locations across Malaysia towards two classes of insecticides and a synergist. Error bars represent standard deviation. Black horizontal line indicates resistant level (mortality less than 90% is considered phenotypically resistant; WHO, 2016). Statistically significant difference for the exposure with and without PBO is indicated by *P<0.05.

```
Musca domestica              972   MHSFMIVFRV LCGEWIESMW DCMYVGDVSC IPFFLATVVI    1011
Aedes aegypti China          979   MHSFMIVFRV LCGEWIESMW DCMLVGDVSC IPFFLATVVI    1018
AB_PYR_R_DII                       MHSFMIVFRV LCGEWIEPMW DCMLVGDVSC IPFFLATVVI
AB_PYR_S_DII                       MHSFMIVFRV LCGEWIESMW DCMLVGDVSC IPFFLATVVI
TDM_PYR_R_DII                      MHSFMIVFRV LCGEWIESMW DCMLVGDVSC IPFFLATVVI
TDM_PYR_S_DII                      MHSFMIVFRV LCGEWIESMW DCMLVGDVSC IPFFLATVVI
SD_PYR_R_DII                       MHSFMIVFRV LCGEWIEPMW DCMLVGDVSC IPFFLATVVI
SD_PYR_S_DII                       MHSFMIVFRV LCGEWIEPMW DCMLVGDVSC IPFFLATVVI
BP_PYR_R_DII                       MHSFMIVFRV LCGEWIEPMW DCMLVGDVSC IPFFLATVVI
BP_PYR_S_DII                       MHSFMIVFRV LCGEWIEPMW DCMLVGDVSC IPFFLATVVI
PNJ_PYR_R_DII                      MHSFMIVFRV LCGEWIESMW DCMLVGDVSC IPFFLGTVVI
FLT_PYR_R_DII                      MHSFMIVFRV LCGEWIESMW DCMLVGDVSC IPFFLGTVVI
FLT_PYR_S_DII                      MHSFMIVFRV LCGEWIESMW DCMLVGDVSC IPFFLATVVI
                                   ********** ******* ** *** ****** ***** ****


Musca domestica                    GNLVVLNLFL ALLLSNFGSS SLSAPTADND TNKIAEAFNR IARFKNWVKR NIADCF      1068
Aedes aegypti China                GNLVVLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL      1074
AB_PYR_R_DII                       GNLVGLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
AB_PYR_S_DII                       GNLVVLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
TDM_PYR_R_DII                      GNLVGLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
TDM_PYR_S_DII                      GNLVGLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
SD_PYR_R_DII                       GNLVGLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
SD_PYR_S_DII                       GNLVGLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
BP_PYR_R_DII                       GNLVGLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
BP_PYR_S_DII                       GNLVGLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
PNJ_PYR_R_DII                      GNLVVLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
FLT_PYR_R_DII                      GNLVVLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
FLT_PYR_S_DII                      GNLVVLNLFL ALLLSNFGSS SLSAPTADNE TNKIAEAFNR ISRFSNWIKS NIANAL
                                   **** ***** ********** *********  ********** * ** ** *  ***
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Fig. 2 Alignment of the amino acid sequences for the partially amplified domain II, segment 6 of the voltage gated sodium channel gene in the susceptible *Musca domestica* (GenBank accession number U38813.1) and *Aedes aegypti* from China (GenBank accession number AY663385.1) compared to the permethrin-resistant and permethrin-susceptible strains from six localities in Malaysia. The highlighted letters show the positions of mutation S989P, A1007G and V1016G. Letter 'R' and 'S' in the sample name represents resistant and susceptible phenotypes respectively. Asterisk (*) represents identical amino acid. Letters highlighted with grey are the conservative substitution.

```
Musca domestica              PNRNACKSEN YTWENSAMNF DHVGNAYLCL FQVATFKGWI      1500
Ae. aegypti susceptible      PDVNACVAEN YTWENSPMNF DHVGKAYLCL FQVATFKGWI      40
AB_PYR_R_DIII                PDVNACVAEN YTWENSPMNF DHVGKAYLCL FQVATFKGWI
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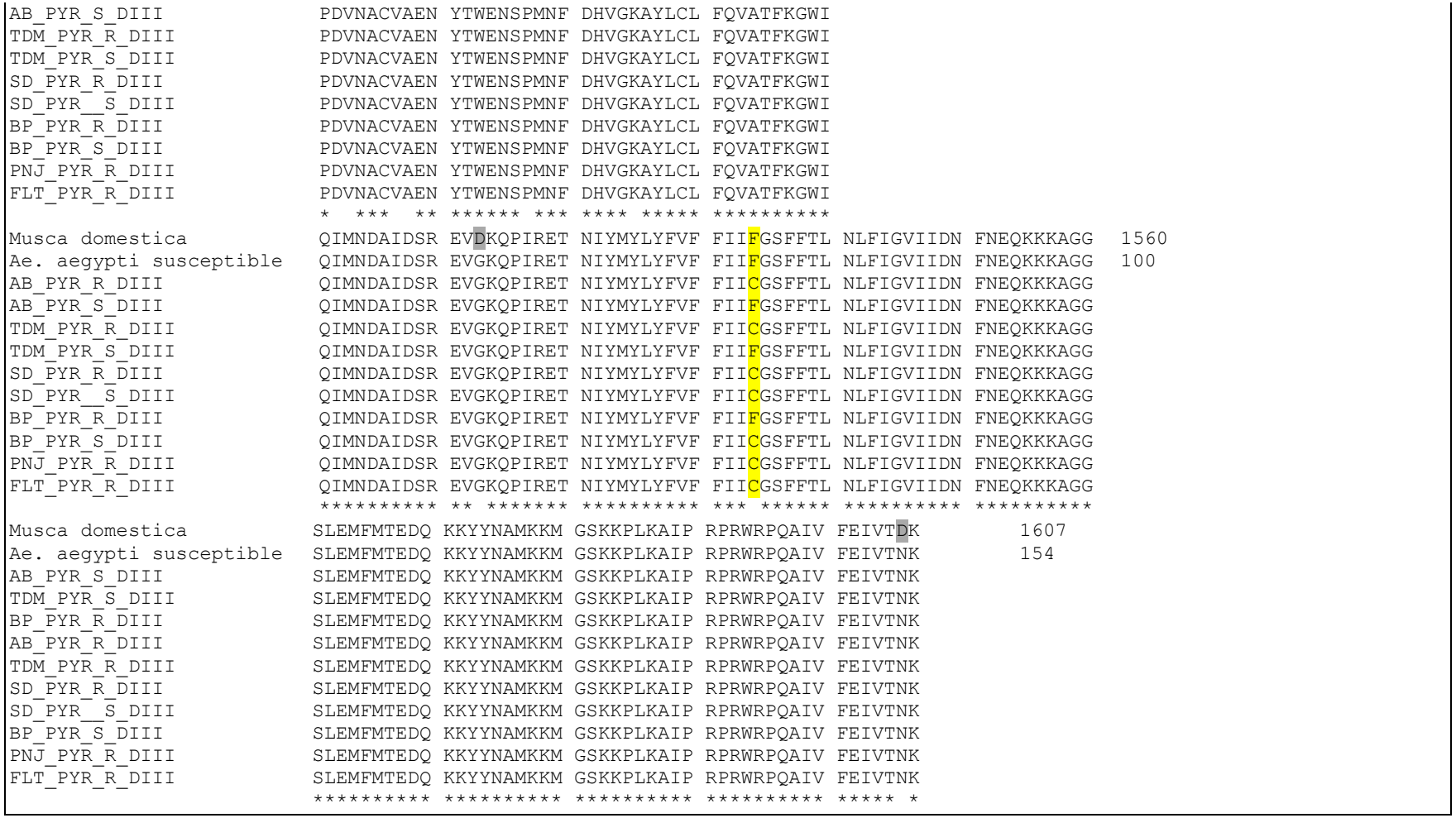

Fig. 3 Amino acid alignment sequences of the partially amplified domain III, segment 6 of the voltage gated sodium channel gene in the susceptible *Musca domestica* (GenBank accession number U38813.1) and Aedes aegypti (GenBank accession number MF794972.1) compared to the permethrin-resistant and permethrin-susceptible strains from six localities in Malaysia. Letter 'R' and 'S' represents resistant and susceptible phenotype respectively. The highlighted letter shows the position of F1534C in domain III segment 6. Asterisks (*) represent identical amino acid. Letters highlighted with grey are the conservative substitution.

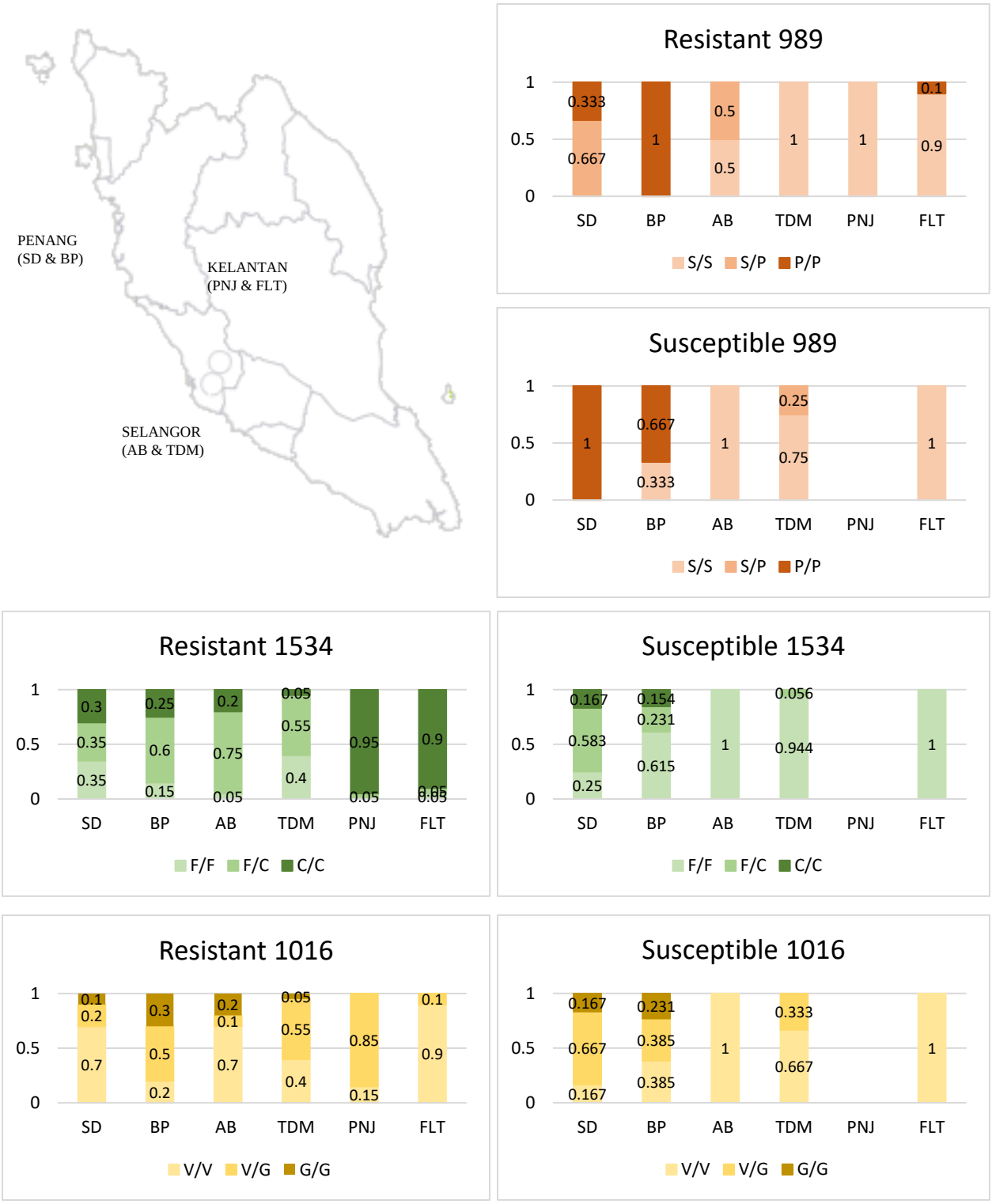

Fig. 4 Illustration map of Malaysia; three states in Malaysia where the resistance study was conducted and also the distribution of *kdr* allele at codon 989, 1016 and 1534 in the phenotype resistant and susceptible of field population of Malaysian *Ae. aegypti*.

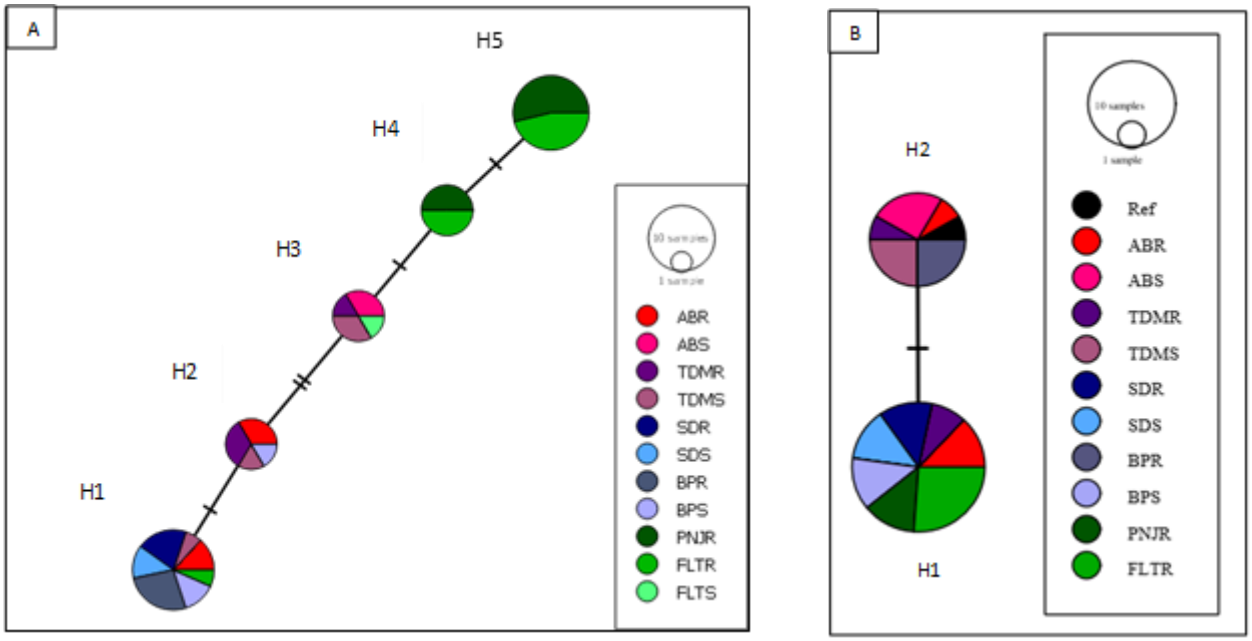

Fig. 5 TCS network for the *vgsc* haplotype of fragment domain II (a) and III (b) between permethrin-resistant and permethrin susceptible samples in all six population of *Ae*. *aegypti* in Malaysia.
ABR, Alam Budiman resistant; ABS, Alam Budiman susceptible; TDMR, TUDM resistant; TDMS, susceptible; SDR, Sg. Dua resistant; SDS, Sg. Dua susceptible; BPR, Balik Pulau resistant; BPS, Balik Pulau susceptible; PNJR, Panji resistant; FLTR, Flat Buluh Kubu resistant; FLTS, Flat Buluh Kubu susceptible

Table 7 shows the summary statistics of the polymorphism at the *vgsc* fragment of domain II and III in the Malaysian population of *Ae. aegypti*.

Domain	N	PS	π	D	Φ_{ST}
DII	47	5	9.42961e+06	3.3123e+09^{ns}	0.77635
DIII	34	1	0.0016929	1.3518^{ns}	0.73996

N, number of samples; PS, number of polymorphic sites; π, nucleotide diversity; D, Tajima's D (P<0.05); Φ_{ST}, AMOVA; ns, not significant

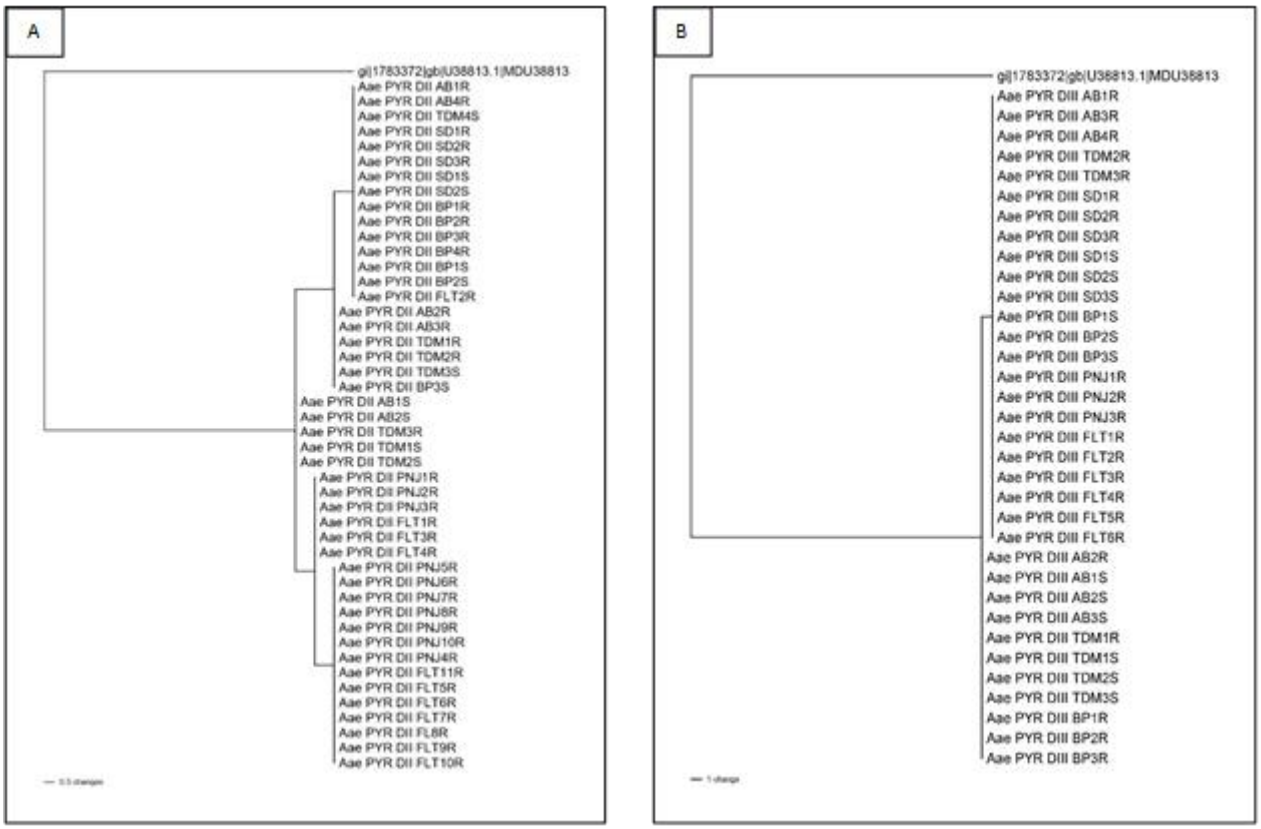

Fig. 6 Maximum parsimony phylogenetic tree of the *vgsc* fragment spanning exon 15 to exon 16 in domain II (a) and exon 23 to exon 25 in domain III (b) shows a correlation between haplotype and phenotype of resistance and susceptible samples. Susceptible *Musca domestica* (GenBank accession number U38813.1) was used as an outgroup.

Table 1 Sequence of oligonucleotides used to amplify the specific regions of the voltage gated sodium channel (*vgsc*) gene in *Aedes aegypti.*

Method	Primer name	Primer sequence (5'-3')	Product size	Region in sodium channel	Reference
AS-PCR	Gly1016f	ACCGACAAATTGTTTCCC		DIIS6	Stenhouse et al., 2013
	Val1016r	GCGGGCAGCAAGGCTAAGAAAAGGTTAATTA	60 bp		
	Gly1016r	GCGGGCAGGGCGGCGGGGGCGGGGCCAGCAAGGCTAAGAAAAGGTTAACTC	80 bp		
	F1534-f	GCGGGCTCTACTTTGTGTTCTTCATCATATT	93 bp	DIIIS6	Yanola et al., 2011
	C1534-f	GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTTGTGTTCTTCATCATGTG	113 bp		
	CP-r	TCTGCTCGTTGAAGTTGTCGAT			
Direct sequencing	IIP_F	GGTGGAACTTCACCGACTTC	581 bp	DIIP-DIIS6	Yanola et al., 2011
	IIS6_R	GGACGCAATCTGGCTTGTTA			
	IIIS6_F	GCTGTCGCACGAGATCATT	635 bp	IIIS4-IIIS6	
	IIIS6_R	GTTGAACCCGATGAACAACA			

Table 2 Correlation of genotype S989P with the resistance phenotype towards permethrin for the field strain *Aedes aegypti*.

States	Location	Phenotype Status	n	S989P							
				Genotype			Alleles		Frequency P allele	Odd ratio (OR)	Fisher's exact test (p-value)
				S/S	S/P	P/P	TCC	CCC			
Penang	Sungai Dua (SD)	Resistant	3	0	2	1	2	4	0.667	0	0.333
		Susceptible	2	0	0	2	0	4	1		
	Balik Pulau (BP)	Resistant	4	0	0	4	0	8	1	∞	0.165
		Susceptible	3	1	0	2	2	4	0.667		
Selangor	Alam Budiman (AB)	Resistant	4	2	2	0	6	2	0.25	∞	0.424
		Susceptible	2	2	0	0	4	0	0		
	TUDM (TDM)	Resistant	3	3	0	0	6	0	0	0	0.571
		Susceptible	4	3	1	0	7	1	0.125		
Kelantan	Pauh, Panji (PNJ)	Resistant	10	10	0	0	20	0	0	N/A	N/A
		Susceptible	-	-	-	-	-	-	-		
	Flat Buluh Kubu (FLT)	Resistant	10	9	0	1	18	2	0.1	∞	0.823
		Susceptible	1	1	0	0	2	0	0		

"-" No individuals died during adult bioassay, thus the genotype and the allelic frequency cannot be determined.

Table 3 Results on the association of V1016G allele with the insecticide resistance phenotype.

States	Location	Phenotype Status	n	V1016G							
				Genotype			Alleles		Frequency G allele	Odd ratio (OR)	Fisher's exact test (p-value)
				V/V	V/G	G/G	GTA	GGA			
Penang	Sungai Dua (SD)	Resistant	20	14	3	3	31	9	0.225	0.29	0.03
		Susceptible	12	2	8	2	12	12	0.5		
	Balik Pulau (BP)	Resistant	20	4	10	6	18	22	0.55	1.667	0.450
		Susceptible	13	5	5	3	15	11	0.538		
Selangor	Alam Budiman (AB)	Resistant	20	14	2	4	30	10	0.25	∞	0.315
		Susceptible	3	3	0	0	6	0	0		
	TUDM (TDM)	Resistant	20	7	9	4	23	17	0.425	3.696	0.0234
		Susceptible	18	12	6	0	30	6	0.167		
Kelantan	Pauh, Panji (PNJ)	Resistant	20	10	10	0	30	10	0.25	∞	0.999
		Susceptible	0	-	-	-	-	-	-		
	Flat Buluh Kubu (FLT)	Resistant	20	18	2	0	38	2	0.05	∞	0.999
		Susceptible	1	1	0	0	2	0	0		

"-" No individuals died during adult bioassay, thus the genotype and the allelic frequency cannot be determined.

Table 4 shows the Results on the association of F1534C allele with the insecticide resistance phenotype.

States	Location	Phenotype Status	n	F1534C							
				Genotype			Alleles		Frequency C allele	Odd ratio (OR)	Fisher's exact test (p-value)
				F/F	F/C	C/C	TTC	TGC			
Penang	Sungai Dua (SD)	Resistant	20	7	7	6	21	19	0.475	1.069	0.999
		Susceptible	12	3	7	2	13	11	0.458		
	Balik Pulau (BP)	Resistant	20	3	12	5	18	22	0.55	3.318	0.042
		Susceptible	13	8	3	2	19	7	0.269		
Selangor	Alam Budiman (AB)	Resistant	20	1	15	4	17	23	0.575	∞	0.022
		Susceptible	3	3	0	0	6	0	0		
	TUDM (TDM)	Resistant	20	8	11	1	27	13	0.325	16.852	0.001
		Susceptible	18	17	1	0	35	1	0.028		
Kelantan	Pauh, Panji (PNJ)	Resistant	20	0	1	19	1	39	0.975	∞	0.999
		Susceptible	0	-	-	-	-	-	-		
	Flat Buluh Kubu (FLT)	Resistant	20	1	1	18	3	37	0.925	∞	0.02
		Susceptible	1	1	0	0	2	0	0		

"-" No individuals died during adult bioassay, thus the genotype and the allelic frequency cannot be determined.

Table 5 Association of potential novel mutation A1007G allele with the insecticide resistance phenotype.

States	Location	Phenotype Status	n	A1007G							
				Genotype			Alleles		Frequency G allele	Odd ratio (OR)	Fisher's exact test (p-value)
				A/A	A/G	G/G	GCC	GGC			
Kelantan	Pauh, Panji (PNJ)	Resistant	10	0	3	7	3	17	0.85	N/A	0.999
		Susceptible	-	-	-	-	-	-	-		
	Flat Buluh Kubu (FLT)	Resistant	10	1	0	9	2	18	0.90	∞	0.026
		Susceptible	1	1	0	0	2	0	0		

"-" No individuals died during adult bioassay, thus the genotype and the allelic frequency cannot be determined.

Table 6 Triple loci and quadruple loci of *kdr* genotype combination (S989P, A1007G, V1016G and F1534C) with the pyrethroid resistance in *Ae. aegypti*.

Type	Genotype	Selangor		Penang		Kelantan		Combined		Mortality
		R	S	R	S	R	S	R	S	
1	SVF/SVF	0	4	0	0	0	0	0	4	1.00
2	S**G**F/S**G**F	3	1	0	0	0	0	3	1	0.25
3	S**G**F/S**GC**	1	0	0	0	0	0	1	0	0.00
4	S**GC**/S**GC**	1	0	0	0	0	0	1	0	0.00
5	S**G**F/**PG**F	0	1	1	0	0	0	1	1	0.50
6	S**G**F/**PGC**	2	0	1	0	0	0	3	0	0.00
7	**PG**F/**PG**F	0	0	4	4	0	0	4	4	0.50
8	**PG**F/**PGC**	0	0	0	1	0	0	0	1	1.00
9	**PGC**/**PGC**	0	0	1	0	0	0	1	0	0.00
10	SAVF/SAVF	0	0	0	0	0	1	0	1	1.00
11	**PAG**F/**PAG**F	0	0	0	0	1	0	1	0	0.00
12	S**GVC**/S**GVC**	0	0	0	0	15	0	15	0	0.00
13	SAV**C**/S**GVC**	0	0	0	0	4	0	4	0	0.00

Table 8 Additive effect of the *vgsc* gene fragment at domain II and III in *Ae*. *aegypti* population collected from Pauh, Panji (PNJ) and Flat Buluh Kubu (FLT), Kelantan.

Samples	Phenotype	Loci			
		989	**1007**	**1016**	**1534**
PNJ 1	Resistant	S/S	A/**G**	V/V	**C**/**C**
PNJ 2	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
PNJ 3	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
PNJ 4	Resistant	S/S	A/**G**	V/V	**C**/**C**
PNJ 5	Resistant	S/S	G/**G**	V/V	**C**/**C**
PNJ 6	Resistant	S/S	A/**G**	V/V	**C**/**C**
PNJ 7	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
PNJ 8	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
PNJ 9	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
PNJ 10	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
FLT 1	Resistant	**P**/**P**	A/A	**G**/**G**	F/F
FLT 2	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
FLT 3	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
FLT 4	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
FLT 5	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
FLT 6	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
FLT 7	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
FLT 8	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
FLT 9	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
FLT 10	Resistant	S/S	**G**/**G**	V/V	**C**/**C**
FLT 11	Susceptible	S/S	A/A	V/V	F/F

Bold letter shows a mutant amino acid.