Heterogeneous genetic invasions of three insecticide resistance mutations in Indo-Pacific populations of *Aedes aegypti* (L.)

Running title: Resistance by invasion in Indo-Pacific *Ae. aegypti*

Nancy M. Endersby-Harshman[1*•], Thomas L. Schmidt[1*], Jessica Chung[1], Anthony van Rooyen[2], Andrew R. Weeks[1,2], Ary A. Hoffmann[1]

•corresponding author (nancye@unimelb.edu.au)

[1]Pest and Environmental Adaptation Research Group, School of BioSciences, Bio21 Institute, 30 Flemington Rd, Parkville, The University of Melbourne, Victoria 3010, Australia

[2]cesar Pty Ltd, 293 Royal Parade, Parkville Victoria 3052, Australia

*Equal first authors

Abstract

Nations throughout the Indo-Pacific region use pyrethroid insecticides to control *Aedes aegypti*, the mosquito vector of dengue, often without knowledge of pyrethroid resistance status of the pest or origin of resistance. Two mutations in the sodium channel gene (*Vssc*) of *Ae. aegypti* modify ion channel function and cause target-site resistance to pyrethroid insecticides, with a third mutation having a potential additive effect. Of 27 possible genotypes involving these mutations, some allelic combinations are never seen while others predominate. Here, five common combinations in *Ae. aegypti* from the Indo-Pacific region are described and tracked historically through genomic analyses. We tested the hypothesis that resistance allele combinations evolved *de novo* in populations, versus the alternative that dispersal of *Ae. aegypti* facilitated genetic invasions of allele combinations between populations. We used genome-wide SNP markers to compare mosquitoes with identical and different resistance profiles at three analytical levels: autosome, individual, and population.

These comparisons indicated a higher level of genomic similarity among mosquitoes of the same resistance allele profile, both within and between locations. Similarities were most pronounced when analyses used only SNPs located within the autosomal arm containing the *Vssc* gene (3q). We also identified 19 F_{ST} outlier SNPs flanking the *Vssc* gene that showed structuring by resistance profile and not by population. Spatial patterns of similarity were heterogeneous for different resistance profiles. These findings indicate that genetic invasions have contributed to the widespread occurrence of *Vssc* allele combinations in *Ae*. *aegypti* in the Indo-Pacific region, pointing to undocumented mosquito invasions between countries.

Key words: insecticide resistance, voltage sensitive sodium channel (*Vssc*), single nucleotide polymorphism (SNP), genetic invasion, invasive species, genetic hitchhiking

Introduction

Target-site or knockdown resistance (*kdr*) due to mutations in the sodium channel gene is one of the main mechanisms that compromises control of the dengue vector, *Aedes aegypti*, with pyrethroid insecticides (Smith, Kasai, & Scott, 2016). These *kdr* mutations have been detected widely in pest insects, following the first discovery of the L1014F mutation in the housefly, *Musca domestica* (Williamson, Denholm, Bell, & Devonshire, 1993), which conferred resistance to DDT. The term '*kdr*' now covers a range of mutations in different locations in the voltage-sensitive sodium channel (*Vssc*), with some being found across insect taxa and others being taxon-specific. To aid comparison of mutation sites between taxa, the numbering of codons is usually kept consistent with the codon numbers of the homologous region of the sodium channel gene of the housefly. Although multiple mutations (synonymous and non-synonymous) have been identified in the sodium channel of *Ae*. *aegypti*, only those non-synonymous mutations found in four positions (codons 1534, 1016, 1011 and 410) have been shown, in electrophysiological assays, to influence the function of the channel so that the toxic action of pyrethroid insecticides is diminished (Du et al., 2013; Haddi et al., 2017).

Sodium channel mutations at codons 1016 and 1534 have been known for many years in *Ae*. *aegypti* and occur within the pyrethroid receptor sites in Domains II (S6) and III (S6) of the protein molecule (Du et al., 2013). Mutation I1011M, which also occurs in Domain II (S6), has been found in *Ae*. *aegypti*, but only in Latin America and the Caribbean (Brengues et al., 2003; K. Saavedra-Rodriguez et al., 2007). The mutation at codon 410 (V410L) in Domain I (S6) has been discovered more recently in *Ae*. *aegypti*, but only from Brazil, and is thought to be involved in the gating function of the channel rather than affecting insecticide binding (Haddi et al., 2017). In the Indo-Pacific region, two resistance mutations are found, namely F1534C and V1016G. A third mutation, S989P, which is often in perfect linkage with V1016G, is not known to reduce the sensitivity of the sodium channel based on results of Du *et al*. (2013), but appears to confer some additive pyrethroid resistance in the homozygous state in combination with 1016G in *Ae*. *aegypti* from Yogyakarta, Indonesia (Wuliandari et al., 2015). An additional mutation (D1794Y), which appears to have a similar effect to S989P when found in

conjunction with V1016G, is known from *Ae*. *aegypti* in Taiwan (Chang, Huang, Chang, Wu, & Dai, 2012; Chang et al., 2009; Lin, Tsen, Tien, & Luo, 2013).

Smith *et al*. (2016) summarised the findings of multiple studies on the combinations of mutations that lead to pyrethroid resistance as follows: the V1016G mutation found alone confers resistance to Type I (those without an α-cyano group) and Type II pyrethroids (those with an α-cyano-3-phenoxybenzyl group) whereas F1534C alone confers resistance only to pyrethroids of Type I and to DDT (Du, Nomura, Zhorov, & Dong, 2016). The degree of resistance induced by V1016G alone is four times that of F1534C alone when expressed in *Xenopus* oocytes (Hirata et al., 2014). S989P alone in *Xenopus* oocyte trials has not been shown to confer resistance (Hirata et al., 2014).

Target-site resistance to pyrethroids is an autosomal, incompletely recessive trait controlled by a single gene (Chang et al., 2012) which has important implications for the resistance status of the heterozygote. Chang *et al*. (2012) expected the heterozygote at each site to be close to susceptible and this is the case for S989P+V1016G or F1534C (Plernsub, Saingamsook, Yanola, Lumjuan, Tippawangkosol, Sukontason, et al., 2016). However, Plernsub *et al*. (2016) found some enhancement of resistance in the triple heterozygote (S989P/V1016G/F1534C) which showed resistance intermediate between a 1534C homozygous mutant strain and a 989P/1016G homozygous mutant strain in Thailand. Ishak (2015) demonstrated a similar effect in the absence of S989P in *Ae*. *aegypti* in Malaysia and concluded that V1016G and F1534C heterozygotes occurring in the same individual have an additive effect on deltamethrin resistance of the mosquito.

Crosses performed by Plernsub *et al*. (2016) between a 1016GG/989PP and a 1534CC strain revealed that combinations of alleles are co-inherited; a haplotype donated by one parent maintains strong linkage patterns between the combination of the mutation sites which limit genotypes found in offspring of crosses and in the

population in general. Several studies have noted that mutant homozygote 1016G is often found in conjunction with a wildtype homozygote at F1534 and *vice versa* (Ishak et al., 2015; Hitoshi Kawada et al., 2014; Stenhouse et al., 2013). It appears that certain haplotypes of the three mutation sites predominate in a population and there is little evidence of crossing over to disrupt the phase patterns found.

Management of *Ae*. *aegypti* as a vector of dengue and other arboviruses requires knowledge of its insecticide resistance status and the likelihood of this status changing over time (Moyes et al., 2017; Plernsub, Saingamsook, Yanola, Lumjuan, Tippawangkosol, Walton, et al., 2016). Mosquito populations may become resistant to insecticides by local selection on new mutations or following the introgression of resistant genotypes into a new region which we refer to here as a "genetic invasion". Local selection pressures from Type I and Type II pyrethroid insecticides are expected to vary and select for specific genotypes, as was observed in *Ae*. *aegypti* from Yucatan State, Mexico (Karla Saavedra-Rodriguez et al., 2015). In the absence of strong local selection, resistance alleles are unlikely to increase in a population. *Vssc* resistance alleles have not been detected in *Ae*. *aegypti* in northern Australia (Endersby-Harshman et al., 2017) suggesting that they are either not present or occur at very low frequency. If the latter case is correct, then the absence of local selection has prevented resistance alleles from increasing to a detectable frequency in this location. Resistance generated through local selection in one location may spread to others as mosquitoes disperse, resulting in the same resistance mutations occurring in unrelated populations.

Broad-scale geographic variation in the incidence of *Vssc* mutations occurs in *Ae*. *aegypti*; for example, in Southeast Asia the V1016G mutation is abundant, and in South America the V1016I mutation occurs at the same site; V1016G is clearly causative of pyrethroid resistance while V1016I has an ambiguous status (Smith et al., 2016). At a finer geographic scale, the occurrence and frequency of the 1016, 1534 and 989 mutations can also vary (Li et al., 2015; Wuliandari et al., 2015). However, it is not clear if this variation reflects movement

patterns of mosquitoes (genetic invasion) or ongoing mutation and selection. Evidence of resistance mutations spreading as mosquitoes move has been found in Ghana where mutation F1534C appeared, as a first record in Africa, associated with an intron phylogeny from Southeast Asia and South America (H. Kawada et al., 2016).

To resolve the importance of local mutation/selection and the movement of resistance alleles between populations, the distribution of resistant alleles can be compared to high resolution patterns of genetic differentiation among populations (Yang et al., 2019). Genomic approaches using single nucleotide polymorphism (SNP) markers have been highly effective at identifying differentiation among *Ae*. *aegypti* populations both at broad geographic scales (J. E. Brown et al., 2014; Gloria-Soria et al., 2018; Rašić, Filipović, Weeks, & Hoffmann, 2014; Thomas L. Schmidt et al., 2019) and at local scales (Jasper, Schmidt, Ahmad, Sinkins, & Hoffmann, 2019; T. L. Schmidt, Filipovic, Hoffmann, & Rasic, 2018). Use of genomic markers provides an opportunity to test whether geographic patterns in resistance alleles match those established among populations, which would provide support for the movement of resistance alleles rather than *de novo* evolution of resistance. Additionally, as the recently developed AaegL5 genome assembly (Matthews et al., 2018) allows most SNPs to be assigned to autosomes, we can investigate whether SNPs from the same region as the *Vssc* gene show patterns of differentiation distinct from other SNPs.

Here we report on a comparison of the distribution of resistance alleles and population genomic structure of *Ae*. *aegypti* in the Indo-Pacific region. Our study aims to (1) determine the geographical distribution of *Vssc* mutations at codons 989, 1016 and 1534 in *Ae*. *aegypti* from throughout the region; (2) investigate genomic differences between *Ae*. *aegypti* with similar and dissimilar *Vssc* profiles; and (3) infer the possible processes leading to the *Vssc* distribution patterns found in mosquitoes throughout the Indo-Pacific.

Methods

Insect collection

Samples of *Ae. aegypti* were collected from the field from 43 locations covering 11 countries in the Indo-Pacific region from April 2015 to February 2018 (Table S1). Mosquitoes were collected as adults or larvae from water containers and, in the case of larvae, were reared to late instar stages or adults before being preserved and stored in either >70% ethanol or RNAlater® (AMBION, Inc., Austin, Texas, USA). Related individuals identified from genomic analysis (see below) were excluded from analyses.

DNA extraction

DNA was extracted from adult mosquitoes or late instar larvae using the DNeasy® Blood and Tissue kit (QIAGEN Sciences, Maryland, USA) according to the instructions of the manufacturer. Two final elutions of DNA were made with the first being used for construction of genomic libraries (see below) and the second being used for screening of *Vssc* mutations after being diluted 1:10 with water.

Screening of Vssc *mutations*

The amino acid positions relating to the mutation sites in this study are labelled as S989P, V1016G and F1534C (Figure 1) according to the sequence of the most abundant splice variant of the house fly, *Musca domestica*, *Vssc* (GenBank accession nos. AAB47604 and AAB47605) (Kasai et al., 2014). These mutation sites are equivalent to those in other studies labelled as S996P, V1023G and F1565C based on the *Vssc* homologue in *Ae*. *aegypti*, the AaNav protein (GenBank accession no. EU399181) (Du et al., 2013).

V1016G

_____**GTA**_____
_____**GTA**_____Wildtype homozygote (**TT**)

_____**GGA**_____
_____**GGA**_____Mutant homozygote (**GG**)

_____**GTA**_____
_____**GGA**_____Heterozygote (**TG**)

F1534C

_____**TTC**_____
_____**TTC**_____Wildtype homozygote (**TT**)

_____**TGC**_____
_____**TGC**_____Mutant homozygote (**GG**)

_____**TTC**_____
_____**TGC**_____Heterozygote (**TG**)

S989P

_____**TCC**_____
_____**TCC**_____Wildtype homozygote (**TT**)

_____**CCC**_____
_____**CCC**_____Mutant homozygote (**CC**)

_____**TCC**_____
_____**CCC**_____Heterozygote (**TC**)

Figure 1. Key to *Vssc* mutations in *Aedes aegypti*. Codons are numbered according to the homologous sodium channel gene in the house fly, *Musca domestica*.

Custom TaqMan® SNP Genotyping Assays (Life Technologies, California, USA) were developed for each of the three target site mutations based on sequence information from Wuliandari *et al*. (2015) as follows:

(i) Mutation site 989 is located in the P-region which links the membrane spanning segments S5 and S6 in Domain II of the *Vssc/para* gene. Forward primer sequence is 5'-TTCATGATCGTGTTCCGGGTATT-3'. Reverse primer sequence is 5'-ACGTCACCCACAAGCATACAAT-3'. Probe sequence is CCCACATGGATTCGAT for the wildtype allele and CCACATGGGTTCGAT for the mutant. The SNP assay differentiates between the **T**CC (Serine) wildtype state and the **C**CC (Proline) mutant allele. An amplicon of 53 bp is produced.

(ii) Mutation site 1016 is located in the first codon of exon 21 of the *Vssc/para* gene (domain II, subunit 6). The SNP assay differentiates between the G**T**A (Valine) wildtype state and the G**G**A (Glycine) mutant allele. Forward primer sequence is 5'- CGTGCTAACCGACAAATTGTTTCC -3'. Reverse primer sequence is 5'- ATGAACCGAAATTGGACAAAAGCAA -3'. Probe sequence is AGAAAAGGTTAAGTACCTGTGCG for the wildtype allele and AAGGTTAAGTCCCTGTGCG for the mutant. An amplicon of 52 bp is produced.

(iii) Mutation site 1534 is located in the 24th codon of exon 31 in the *Vssc/para* gene (domain III, subunit 6). The SNP assay differentiates between the T**T**C (Phenylalanine) wildtype state and T**G**C (Cysteine) mutant allele. Forward primer sequence is 5'- TCTACATGTACCTCTACTTTGTGTTCTTCA -3'. Reverse primer sequence is 5'- GATGATGACACCGATGAACAGATTC -3'. Probe sequence is AACGACCCGAAGATGA for the wildtype allele and ACGACCCGCAGATGA for the mutant. An amplicon of 52 bp is produced.

Probes for the wildtype allele in each assay were labelled with VIC reporter dye in conjunction with a Minor Groove Binder (MGB) and a non-fluorescent quencher (NFQ). Probes for the mutant allele were labelled with FAM reporter dye, MGB and NFQ. Three replicates of each TaqMan® assay were run on a LightCycler® II 480 (Roche, Basel, Switzerland) real time PCR machine in a 384-well format. The PCR Master Mix contained 40x TaqMan® assay as described above (0.174 µL), 2x KAPA Fast PCR Probe Force qPCR Master Mix (KAPABIOSYSTEMS, Cape Town, South Africa) (3.5 µL), ddH_2O and genomic DNA as prepared above (2 µL). Conditions for the PCR run were pre-incubation of 3 min at 98°C (ramp rate 4.8°C/ s) followed by 40 cycles of amplification at 95°C for 10 s (2.5°C/ s ramp rate) and 60°C for 20 s (2.5°C/ s ramp rate) (Acquisition mode: single) with a final cooling step of 37°C for 1 min (2.5°C/ s ramp rate). Endpoint genotyping was conducted using the Roche LightCycler® 480 Software Version 1.5.1.62.

Testing for genetic invasions

To look for indications that resistance alleles have spread across populations via genetic invasion, we investigated genetic differentiation between individuals in the same population, between individuals in different populations, and between populations. These analyses were motivated by the expectation that, if resistance alleles had been spread by genetic invasion, other alleles from the invasive population would be introduced to the invaded population at the same time. This could lead to weaker genetic differentiation between individuals and populations that share a history of genetic invasion. These patterns should be strongest among alleles at SNPs on the same autosome arm as the *Vssc* gene (q arm of autosome 3; hereafter denoted 3q), compared with those at SNPs from other genomic regions, due to linkage. Considering the slow recombination rate of *Ae. aegypti* (Bennett et al., 2005; S. E. Brown, Severson, Smith, & Knudson, 2001), patterns across the same autosomal arm may remain detectable even if gene flow ceased many generations ago or involved only a small number of invasive migrants.

At the individual level, for populations in which a given resistance profile has become established via genetic invasion from a single source population, non-wildtype individuals with identical *Vssc* resistance profiles will have attained these profiles from the same invasive ancestors, and thus these individuals will have alleles in the 3q region that are identical by descent via this invasion. Genetic distances among these individuals should thus be smaller than distances between these individuals and those of different resistance profiles, including wildtype individuals, when considering SNPs in the 3q region. However, if the genetic invasion was via multiple source populations, we could expect equal or even greater genetic distances among these individuals. Similarly, for a population in which resistance evolved independently, we would expect equal genetic distances. For non-3q SNPs, similar patterns to 3q SNPs may be expected if the invasion is ongoing, but once gene flow ceases, we would expect to see effects similar to those under independent evolution.

We can make similar predictions for comparisons across populations. If a given non-wildtype *Vssc* resistance profile is found in a pair of populations as a result of population *i* genetically invading population *j*, then individuals in population *i* with that profile should be more genetically similar to identically-profiled individuals in population *j* than to individuals in population *j* with other resistance profiles. Again, these effects should be greater for SNPs in the 3q region than for non-3q SNPs. This investigation of relative genetic distances between individual pairs will help reveal which populations, if any, have shared histories of genetic invasion, and thus to what extent genetic invasion is responsible for the current distribution of pyrethroid resistance throughout the Indo-Pacific.

We also investigated SNPs with high F_{ST} in the 3q region, providing a third analytical level complementary to individual and population-level analyses. This analysis aimed to locate F_{ST} outlier SNPs proximate to and in strong linkage with the *Vssc* gene, and to investigate whether alleles at these SNPs showed structuring by population (indicating independent resistance evolution) or by *Vssc* genotype (indicating genetic invasion).

SNP genotyping and filtering

Tests for genetic invasion were conducted on a SNP dataset comprising 135 *Ae. aegypti* across ten of the populations screened for pyrethroid resistance. Vietnam was omitted from these analyses due to accidental loss of samples. We also omitted populations lacking resistance mutations. All *Ae. aegypti* were genotyped at genome-wide single nucleotide polymorphisms (SNPs) using the double-digest RAD sequencing (ddRADseq) methodology of Rašić *et al.* (2014) and the bioinformatics pipeline Stacks (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). The dataset was pre-filtered to ensure no first-order relatives were included. Similar methods have been described elsewhere (e.g. Thomas L. Schmidt et al. (2019)) and are detailed here in the Supplementary Information.

For pairwise analyses among populations, we ensured that genetic differentiation measurements would not be biased by uneven sample sizes by randomly sampling a number of genotypes from each population equal to the minimum population sample size. For large SNP datasets, five genotypes can be sufficient for estimating genetic differentiation (Willing, Dreyer, & van Oosterhout, 2012). For pairwise analyses among individuals uneven sample size was not relevant and we used all 135 *Ae. aegypti*. For detection of F_{ST} outlier SNPs we sampled eight genotypes from each population, first removing individuals in order to preserve maximum variation in *Vssc* genotypes in that population (Figure 2), then removing them in order of missing data.

The three datasets were processed in Stacks and VCFtools (Danecek et al., 2011) with the following filters: SNPs must be biallelic, be present in 75% of mosquitoes in each population, have a minor allele count of > 2 (following Linck and Battey (2019)), and have less than 5% missing data. Filtered datasets were partitioned into two subsets, one containing SNPs located on the q region of autosome 3 (3q), the other containing SNPs found on autosome 1, autosome 2, and the p arm of autosome 3 (non-3q).

Comparisons between individuals

We investigated whether mosquitoes of the same resistance profile were more genetically similar to each other than to other mosquitoes from the same collection location. We assigned mosquitoes with the A, C, D, G and H genotypes to a resistance profile group "Profile GTC" (one or two copies of either Haplotype H1 or H4; Figure 2), and assigned mosquitoes with the B, C, E, and H genotypes to a second group "Profile TGT" (one or two copies of Haplotype H2; Figure 2). Note the nomenclature refers to the mutations in order of degree of resistance conferred from highest to lowest, i.e. 1016/1534/989. Mosquitoes with the C and H genotypes belonged to both Profile GTC and Profile TGT. Analyses indicated that mosquitoes with H1 and H4 haplotypes had similar genetic distance patterns (see Results), so they were treated as a single profile. We assigned mosquitoes with the F genotype to a group representing the non-resistant "Profile TTT" (two copies of

Haplotype H3; Figure 2). We used the R packages vcfR (Knaus & Grünwald, 2017) and adegenet (Jombart, 2008) to calculate Euclidean genetic distances between all individual pairs, using the 3q and non-3q SNP datasets.

For comparisons within populations, we selected each of the Profile GTC mosquitoes from a population and calculated its mean distance to other Profile GTC mosquitoes of the same population, and its mean distance to mosquitoes not of Profile GTC. Differences between the means were investigated using paired t-tests. These analyses were also run across populations, so that Profile GTC mosquitoes from population *i* were compared with Profile GTC and non-Profile GTC mosquitoes from population *j*. We also performed paired t-tests on all pairs of genetic distance scores for each population *i* and population *j*, and a paired t-test on all pairs of scores within a Profile and corrected for multiple comparisons using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). We omitted all analyses for which fewer than two mosquitoes were available for any calculation. The above analyses were repeated for Profile TGT mosquitoes.

We calculated relative genetic distances for all comparisons within and between populations, by dividing the mean genetic distance to mosquitoes of the same resistance profile by the mean genetic distance to mosquitoes not of that profile. These parameters were expressed as 95% confidence intervals, with relative distances < 1 indicating smaller genetic distances between mosquitoes of the same resistance profile and relative distance = 1 being the null expectation.

F_{ST} outlier detection

We used the program BayeScan v2.1 (Foll & Gaggiotti, 2008) to detect SNPs within the 3q region showing strong allele frequency divergence among populations. We ran the MCMC algorithm on the dataset of 135 *Ae.*

aegypti for 500,000 iterations following a burn-in period of 50,000 and 20 pilot runs of 5,000 iterations. Every 10th iteration was sampled, and F_{ST} outliers were called conservatively with the neutral model having prior odds 100 times higher than the model with selection. SNPs identified as F_{ST} outliers were examined in a principal component analysis (PCA) using the R package LEA (Frichot & François, 2015), to determine whether genetic variation among individuals at these SNPs was best predicted by population of origin or *Vssc* resistance profile.

Comparison between populations

We investigated whether populations with similar frequencies of the three resistant haplotypes (H1, H2, and H4; see Figure 2) tended to be more genetically similar to each other than to other populations, considering their geographical location. We used the frequencies of the three haplotypes in each population to generate a distance matrix of Euclidean dissimilarities using the R package vegan ((Oksanen et al., 2019), function 'vegdist'). We used the R packages vcfR (Knaus & Grünwald, 2017) and hierfstat (Goudet, 2005) to estimate pairwise F_{ST} between populations at the 3q and non-3q regions, from which we calculated linear genetic distances (Rousset, 1997) using $\frac{F_{ST}}{1-F_{ST}}$. Linear genetic distances were used as dependent variables in distance-based redundancy analyses (dbRDAs), which used *Vssc* haplotype frequencies and ln-transformed geographical distance as independent variables.

As dbRDA accepts distance matrices for dependent variables only, we transformed both independent variables into principal components (PCs) using the vegan function 'pcnm'. We ran an initial dbRDA of the PCs of geographical distance on the genetic distance estimates and retained PCs that were significant at 95% confidence for inclusion in the final models. We used all PCs of *Vssc* haplotype frequencies in the final models. All dbRDAs were built with the vegan function 'capscale'.

Results

Vssc *mutations*

Variation was identified in the *Vssc* alleles and genotype combinations of *Ae*. *aegypti* throughout the Indo-Pacific region. Eight of the possible 27 combinations of genotypes of the three sodium channel mutation sites were identified in the samples of *Ae*. *aegypti* and five of these were present in high numbers (Table 1). Mutation sites 1016 and 989 were perfectly linked within our sample with respect to genetic state (except in a small number of individuals from Taiwan) and were in negative linkage disequilibrium with site 1534. If we consider each possible state, namely wildtype homozygote, mutant homozygote and heterozygote, only three putative haplotypes are required to construct each of the observed combinations (excluding those from Taiwan) (Figure 2a). One additional haplotype is required to construct the two extra genotypes from Taiwan (Figure 2b).

Table 1. Frequency of *Vssc kdr* genotypes identified in *Ae*. *aegypti* from specific countries in the Indo-Pacific region

Genotype code:			**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1016/1534/989:			**GGTTCC**	**TTGGTT**	**TGTGTC**	**TGTTTC**	**TTTGTT**	**TTTTTT**	**GGTTTC**	**TGTGTT**
Country	**n**	**Profile:**	**GTC**	**TGT**	**GTC, TGT**	**GTC**	**TGT**	**TTT**	**GTC**	**TGT**
Taiwan	20		0.05	0.60	0.10				0.15	0.10
Sri Lanka	24			0.17		0.04	0.63	0.17		
Indonesia (Bali)	67		1.00							
Singapore	29		0.21	0.24	0.55					
Malaysia	30			0.40	0.60					
Thailand	40		0.25	0.50	0.25					
Vietnam	97			0.11			0.47	0.41		
Vanuatu	20		1.00							
New Caledonia	24			0.04			0.33	0.63		
Fiji	24			0.71			0.21	0.08		
Kiribati	20		0.10	0.45	0.25	0.05	0.15			
TOTAL	**395**		**0.27**	**0.23**	**0.13**	**0.01**	**0.19**	**0.15**	**0.01**	**0.01**

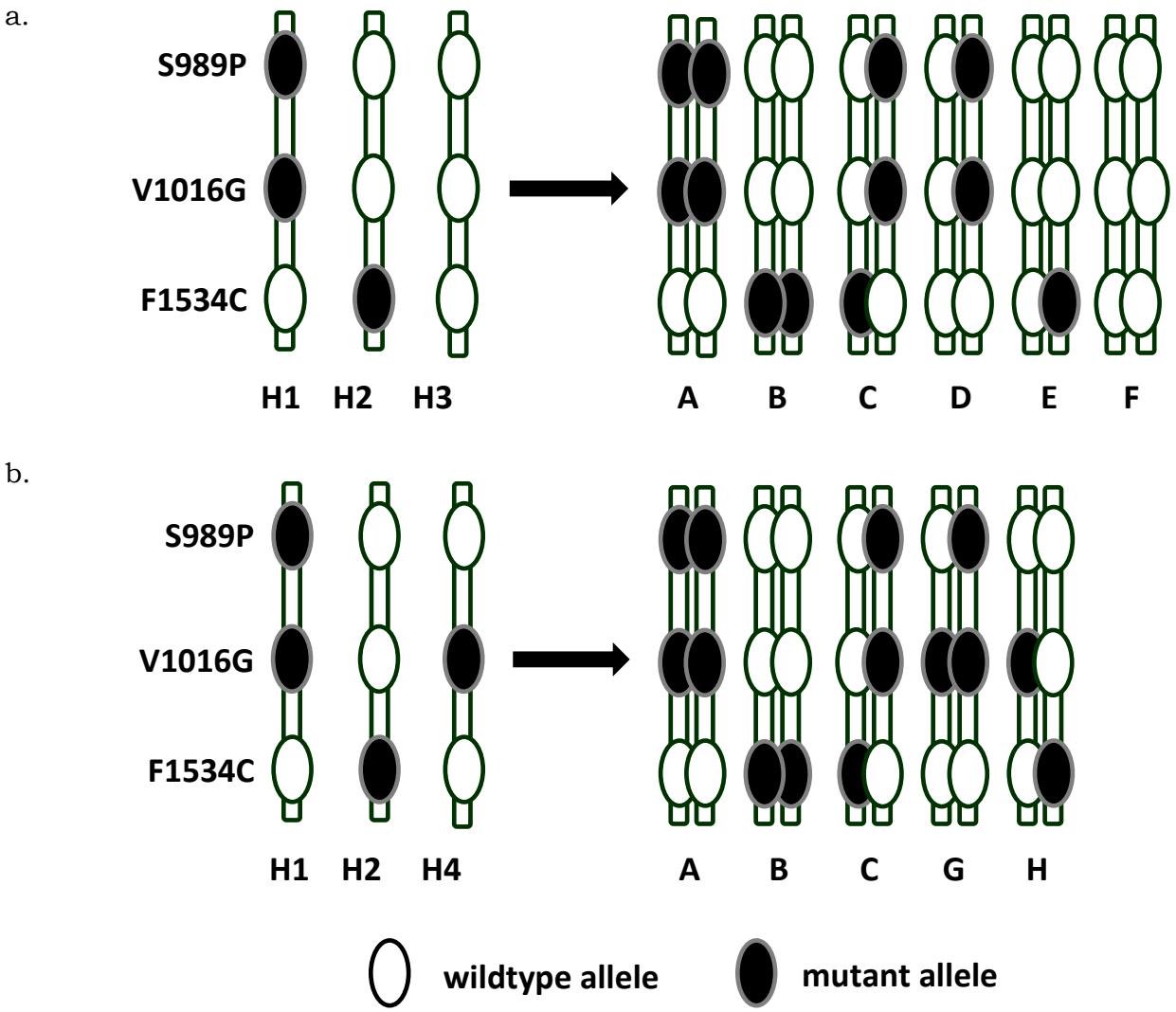

Figure 2 a). Putative haplotypes and phase of common mutant *Vssc kdr* genotypes of *Aedes aegypti* found in the Indo-Pacific region (excluding Taiwan sample), b) Putative haplotypes required to construct genotypes found in Taiwan sample. (Note that H1 = Profile GTC and H2 = Profile TGT).

Patterns of *Vssc* mutations were varied across the geographic dataset. From one to five mutation combinations (genotypes) were found at each geographic location with the most combinations (five) being identified from the remote Republic of Kiribati and from Taiwan (Table 1). Mosquitoes from Bali, Indonesia and the Republic of Vanuatu showed only one genotype pattern (homozygous mutant for 1016G and 989P, but wildtype for F1534 – designated as genotype A). Mosquitoes from Vietnam, New Caledonia and Fiji only showed mutations at the 1534 site (homozygous and heterozygous, genotypes B and E) and the sample also contained some completely wildtype individuals (genotype F). *Aedes aegypti* from Singapore, Thailand and

Malaysia showed three main genotype combinations, namely A, B and C (C = heterozygous at each of the three sites) (Figure 3).

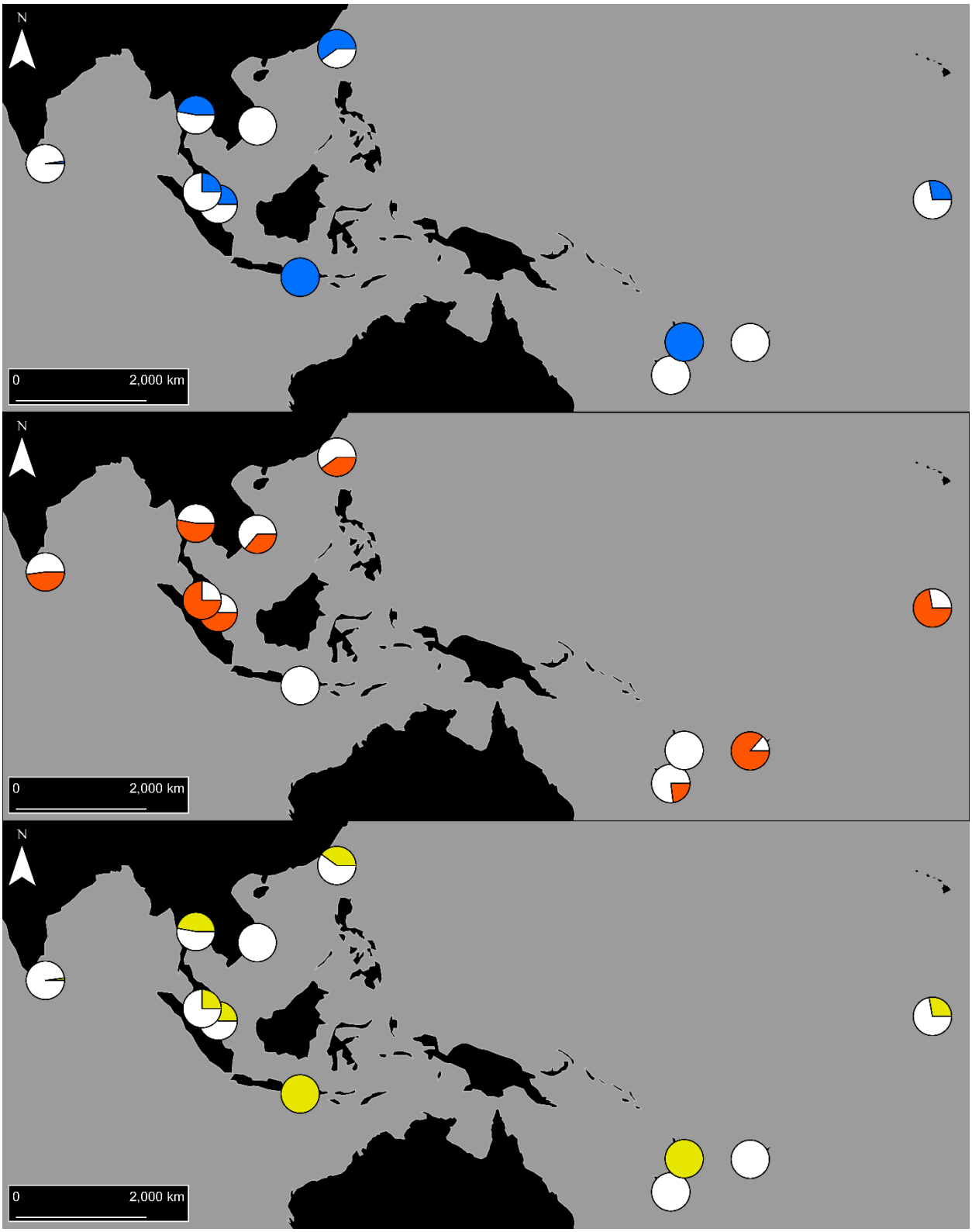

Figure 3. Distribution and frequency of *Vssc kdr* mutations in *Aedes aegypti* from the Indo-Pacific (Blue = 1016G, Orange = 1534C, Yellow = 989P)

SNP genotyping and filtering

After filtering the dataset of 135 *Ae. aegypti* used in analysis of individuals, we retained 9,854 SNPs from the 3q region and 40,120 SNPs from the non-3q region. The dataset of 80 *Ae. aegypti* used for F_{ST} outlier detection contained 10,171 3q SNPs. The dataset of 80 *Ae. aegypti* used for population analysis contained 9,342 3q SNPs and 38,538 non-3q SNPs.

Comparisons between individuals

We observed different divergence patterns among mosquitoes with Profile GTC and Profile TGT resistance, both within and between populations. We also found different patterns of divergence when using SNPs from 3q and non-3q regions. Figures 4-6 illustrate paired t-test results, showing two-tailed significance of t-statistics at 95% confidence after Benjamini-Hochberg correction, and the 95% confidence intervals of relative genetic distances. For all analyses involving Taiwan, there was no change to the significance or directionality of results whether haplotype H4 was treated as identical to H1, or treated as different and excluded, so we treated it as identical to H1.

For Profile GTC mosquitoes, SNPs in the 3q region had relative genetic distances of < 1 for every pairwise comparison within (Figure 4) and between (Figure 5) populations, except for the within-population comparison of Taiwan, for which only two mosquitoes not of Profile GTC were available. Comparison of all populations showed 3q relative genetic distances among Profile GTC mosquitoes were ~4 – 5% smaller overall (Figure 5), which is similar to the difference seen within populations (Figure 4). The consistent directionality in relative genetic distance indicates that the 3q region has broad similarities among Profile GTC mosquitoes across populations.

Profile GTC analyses using non-3q SNPs showed fewer relative distances < 1, and for those < 1 the effect sizes were less pronounced (Figures 4, 5). Within-population analyses were mostly non-significant, and while many

significant interactions were observed between specific population pairs, non-3q genetic distances between Profile GTC mosquitoes were only ~1% smaller overall.

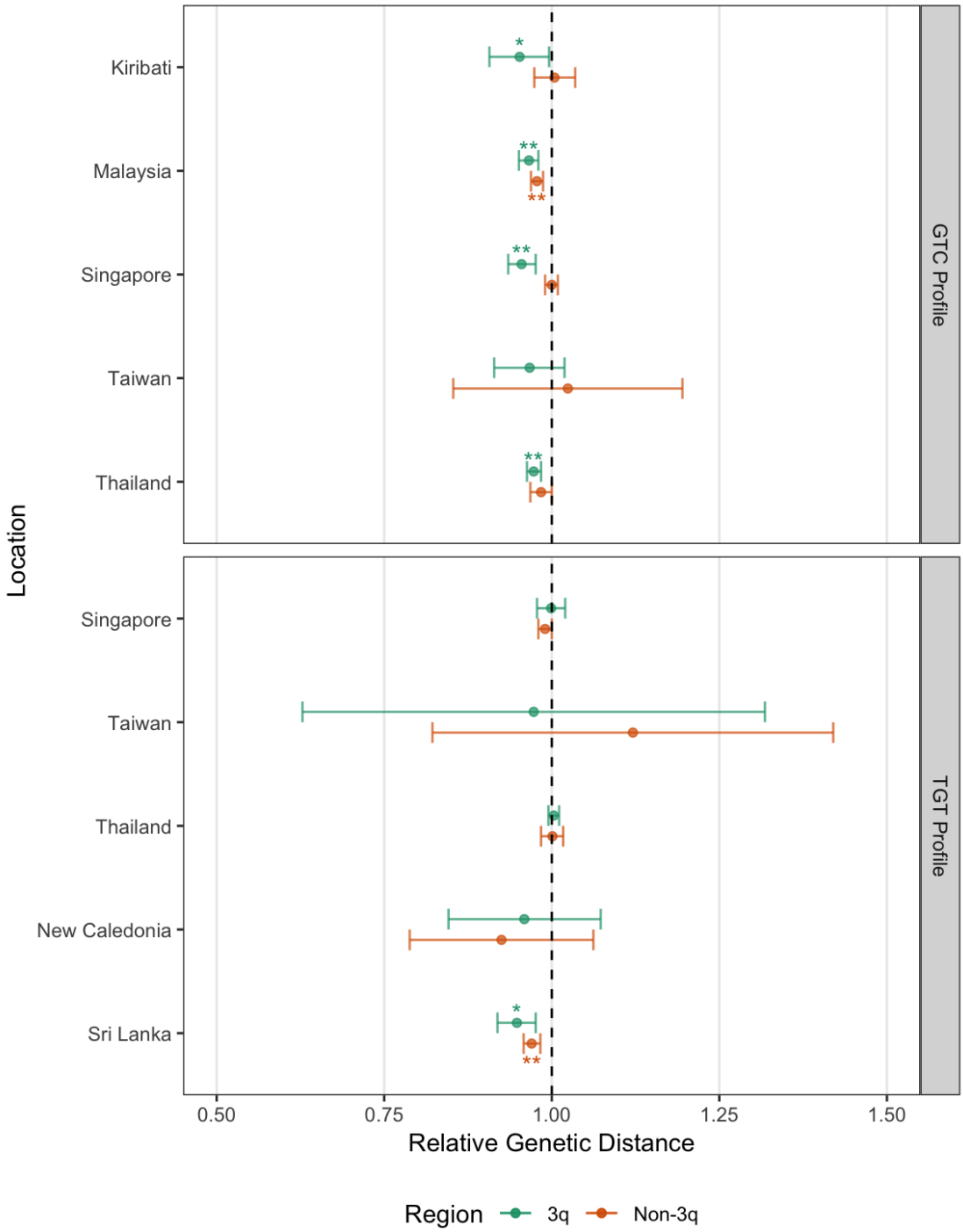

Figure 4. Relative genetic distance of profiles GTC and TGT from within-population paired t-tests for *Aedes aegypti* mosquitoes for SNPs in the 3q region (green bar) and those outside this region (orange bar). The confidence range indicates the lower and upper bounds of relative genetic distance, with a null expectation of 1. Smaller relative distances indicate smaller genetic distances between mosquitoes of Profile GTC than between mosquitoes of Profile GTC and other resistance profiles and similarly for Profile TGT.

Population *j* : Mixed Vssc profiles [n(GTC), n(non-GTC)]

Population *i* : [n(GTC)]	Kiribati [4, 5]	Malaysia [9, 9]	Singapore [11, 4]	Taiwan [6, 2]	Thailand [12, 6]	Total
Bali [18]	*** 0.934, 0.945	*** 0.950, 0.956	*** 0.949, 0.961	*** 0.934, 0.942	*** 0.972, 0.979	*** 0.949, 0.956
Kiribati [4]		*** 0.968, 0.980	** 0.952, 0.973	** 0.925, 0.969	*** 0.973, 0.982	*** 0.958, 0.973
Malaysia [9]	*** 0.934, 0.952		*** 0.950, 0.976	*** 0.927, 0.948	*** 0.963, 0.980	*** 0.947, 0.960
Singapore [11]	*** 0.939, 0.950	*** 0.956, 0.968		*** 0.927, 0.945	*** 0.969, 0.983	*** 0.949, 0.960
Taiwan [6]	*** 0.933, 0.950	*** 0.950, 0.971	*** 0.947, 0.974		* 0.966, 0.995	*** 0.953, 0.968
Thailand [12]	*** 0.934, 0.946	*** 0.953, 0.962	*** 0.954, 0.971	*** 0.921, 0.931		*** 0.941, 0.952
Vanuatu [14]	*** 0.918, 0.942	*** 0.964, 0.972	*** 0.967, 0.981	*** 0.933, 0.948	* 0.986, 0.998	*** 0.955, 0.967
Total [74]	*** 0.936, 0.942	*** 0.958, 0.963	*** 0.959, 0.967	*** 0.933, 0.939	*** 0.976, 0.982	*** 0.953, 0.957

Population *j* : Mixed Vssc profiles [n(GTC), n(non-GTC)]

Population *i* : [n(GTC)]	Kiribati [4, 5]	Malaysia [9, 9]	Singapore [11, 4]	Taiwan [6, 2]	Thailand [12, 6]	Total
Bali [18]	0.995, 1.001	*** 0.987, 0.992	0.992, 1.000	*** 0.985, 0.990	*** 0.978, 0.986	*** 0.989, 0.992
Kiribati [4]		0.991, 1.004	0.985, 1.003	0.984, 1.001	*** 0.979, 0.984	*** 0.988, 0.995
Malaysia [9]	* 0.991, 1.000		* 0.989, 0.999	* 0.990, 0.999	*** 0.972, 0.978	*** 0.986, 0.993
Singapore [11]	0.992, 1.000	*** 0.982, 0.992		*** 0.981, 0.990	*** 0.977, 0.986	*** 0.985, 0.990
Taiwan [6]	* 0.995, 0.999	* 0.987, 0.997	** 0.991, 0.997		*** 0.974, 0.987	*** 0.988, 0.994
Thailand [12]	*** 0.989, 0.994	** 0.987, 0.995	* 0.993, 0.999	0.992, 1.000		*** 0.992, 0.996
Vanuatu [14]	0.996, 1.002	** 0.995, 0.999	*** 0.992, 0.997	0.996, 1.003	*** 0.979, 0.983	*** 0.992, 0.996
Total [74]	*** 0.995, 0.998	*** 0.990, 0.993	*** 0.994, 0.997	*** 0.990, 0.994	*** 0.979, 0.982	*** 0.990, 0.992

Figure 5. Results of between-population paired t-tests for *Aedes aegypti* mosquitoes of Profile GTC, for SNPs in the 3q region (upper matrix) and those outside this region (lower matrix). The lower and upper bounds of relative genetic distance are indicated in each cell, with a null expectation of 1. Asterisks indicate significance at 95% confidence after Benjamini-Hochberg correction, with the following key: P < 0.05 (*), P < 0.01 (**), P < 0.001 (***).White cells indicate that 95% confidence intervals overlap 1 and cell colour increases in intensity as the distance from 1 becomes greater. Smaller relative distances indicate smaller genetic distances between mosquitoes of Profile GTC than between mosquitoes of Profile GTC and other resistance profiles.

Population *j* : Mixed Vssc profiles [n(TGT), n(non-TGT)]

Population *i* : [n(TGT)]	New Caledonia [5, 8]	Singapore [13, 2]	Sri Lanka [8, 3]	Taiwan [6, 2]	Thailand [13, 5]	Total
Fiji [10]	* 0.982, 0.998	0.990, 1.003	* 0.984, 0.998	** 1.008, 1.027	0.996, 1.004	0.995, 1.003
Kiribati [8]	** 0.971, 0.987	0.995, 1.012	0.980, 0.999	* 1.003, 1.027	* 0.985, 0.997	0.990, 1.001
Malaysia [18]	*** 0.975, 0.982	0.995, 1.016	0.989, 1.005	*** 1.015, 1.037	0.998, 1.011	0.998, 1.007
New Caledonia [5]		0.983, 1.016	0.976, 0.991	1.002, 1.048	0.997, 1.012	0.994, 1.012
Singapore [13]	*** 0.976, 0.986		** 0.982, 0.995	*** 1.013, 1.028	0.992, 1.010	0.993, 1.003
Sri Lanka [8]	*** 0.974, 0.987	0.991, 1.019		* 1.005, 1.044	0.992, 1.005	0.994, 1.010
Taiwan [6]	** 0.972, 0.987	0.975, 1.022	0.979, 1.003		0.990, 1.019	0.986, 1.000
Thailand [13]	*** 0.969, 0.979	0.989, 1.004	*** 0.970, 0.985	* 1.006, 1.032		** 0.985, 0.998
Total [81]	*** 0.978, 0.982	0.997, 1.005	*** 0.986, 0.992	*** 1.017, 1.026	0.998, 1.004	* 0.996, 1.000

Population *j* : Mixed Vssc profiles [n(TGT), n(non-TGT)]

Population *i* : [n(TGT)]	New Caledonia [5, 8]	Singapore [13, 2]	Sri Lanka [8, 3]	Taiwan [6, 2]	Thailand [13, 5]	Total
Fiji [10]	*** 1.010, 1.021	* 0.991, 0.999	0.995, 1.004	*** 1.008, 1.014	*** 0.993, 0.997	* 1.000, 1.006
Kiribati [8]	*** 1.010, 1.020	* 0.991, 0.999	1.000, 1.008	* 1.003, 1.014	0.994, 1.000	* 1.001, 1.007
Malaysia [18]	*** 1.009, 1.014	*** 0.985, 0.994	0.997, 1.004	*** 1.014, 1.023	*** 0.988, 0.992	0.999, 1.005
New Caledonia [5]		* 0.991, 0.998	0.995, 1.013	* 1.003, 1.016	0.993, 1.001	0.998, 1.005
Singapore [13]	*** 1.006, 1.012		0.999, 1.007	*** 1.010, 1.016	*** 0.988, 0.994	** 1.001, 1.007
Sri Lanka [8]	*** 1.010, 1.012	** 0.976, 0.991		** 1.005, 1.014	*** 0.981, 0.990	0.992, 1.002
Taiwan [6]	*** 1.012, 1.016	0.993, 1.007	0.998, 1.010		** 0.992, 0.998	1.000, 1.007
Thailand [13]	*** 1.012, 1.018	*** 0.986, 0.995	0.995, 1.002	*** 1.008, 1.016		* 1.001, 1.007
Total [81]	*** 1.011, 1.014	*** 0.990, 0.994	1.000, 1.003	*** 1.011, 1.015	*** 0.991, 0.994	*** 1.001, 1.004

Figure 6. Results of between-population paired t-tests for *Aedes aegypti* mosquitoes of Profile TGT, for SNPs in the 3q region (upper matrix) and those outside this region (lower matrix). The lower and upper bounds of relative genetic distance are indicated in each cell, with a null expectation of 1. Asterisks indicate significance at 95% confidence after Benjamini-Hochberg correction, with the following key, P < 0.05 (*), P < 0.01 (**), P < 0.001 (***).White cells indicate that 95% confidence intervals overlap 1 and cell colour increases in intensity as the distance from 1 increases (purple cells in a direction <1 and pink cells in a direction >1). Smaller relative distances indicate smaller genetic distances between mosquitoes of Profile TGT than between mosquitoes of Profile TGT and other resistance profiles.

Results for Profile TGT mosquitoes showed greater heterogeneity than those for Profile GTC, and there were marked differences between elements of the 3q and non-3q datasets (Figures 4, 6). While some between-population comparisons (New Caledonia, Sri Lanka) followed similar patterns to Profile GTC mosquitoes, in which relative distances < 1 were more common in the 3q dataset than the non-3q dataset, for other comparisons (Singapore, Thailand) these patterns were reversed. Likewise, some comparisons showed relative distances > 1, particularly those involving Taiwan. Nevertheless, comparison of all populations showed 3q genetic distances among Profile TGT mosquitoes were overall significantly smaller, though the effect size was only ~0.2% (Figure 6). When using non-3q SNPs, these genetic distances were ~0.2% larger.

Comparison between populations

Initial dbRDAs showed that one of the eight PCs of geographical distance was statistically significant for both 3q and non-3q genetic distances (both $P < 0.02$), so this was used as a conditional variable in the final dbRDAs. After conditioning, the three PCs of *Vssc* haplotype frequencies were significantly associated with genetic distances at 95% confidence for both 3q and non-3q SNPs. However, the association was stronger for 3q SNPs ($F_3 = 1.764$, $P = 0.018$) than for non-3q ($F_3 = 1.653$, $P = 0.050$) SNPs.

F_{ST} outlier detection

We identified 23 outlier SNPs in the 3q region, with F_{ST} ranging from 0.361 – 0.585. Nineteen of these SNPs were distributed throughout a 7.3 Mbp region (3:313,503,264 – 320,758,694) containing the *Vssc* gene (3:315,926,360 – 316,405,638), with three of these SNPs located within a *Vssc* intron region. PCA on these 19 SNPs indicated that genetic variation in this region aligned much more closely with variation in *Vssc* resistance profile than with variation in population of origin (Figure 7). This was most clearly observed in populations containing multiple resistance profiles (e.g. Sri Lanka, Thailand, Singapore), for which individuals showed great variation in principal component scores.

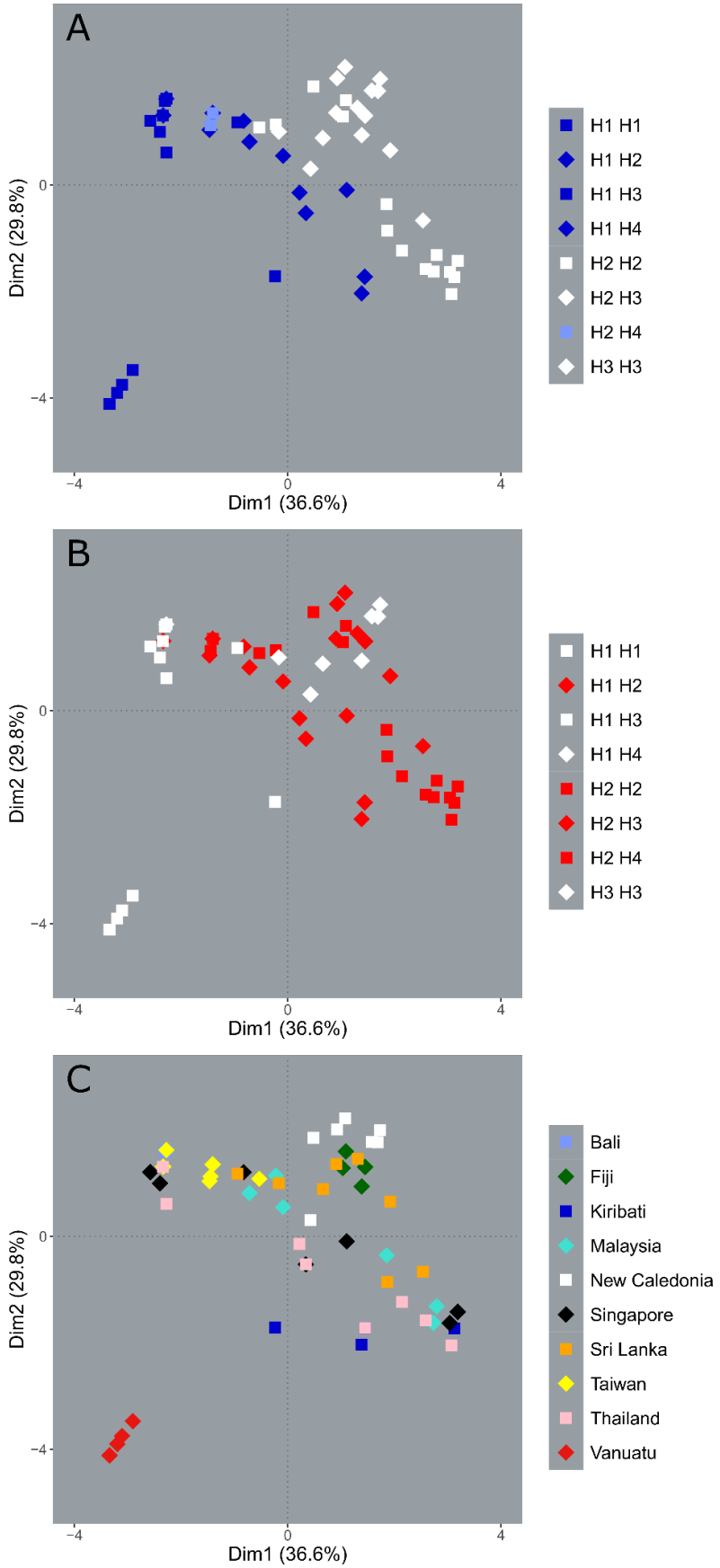

Figure 7. PCA of variation at 19 F_{ST} outlier SNPs surrounding the *Vssc* gene region of *Aedes aegypti*. Individuals in each subplot are coloured by: (A) Resistance Profile (GTC and non-GTC), (B) Resistance Profile (TGT and non-TGT), and (C) population of origin. The light blue colour seen in (A) reflects the uncertain categorisation of Haplotype 4 from Taiwan.

Discussion

This paper has outlined the geographical distributions of three sodium channel mutations found in *Ae*. *aegypti* from the Indo-Pacific and, by investigating variation in genetic structure at the autosomal, individual, and population levels, has demonstrated that these mutations have likely attained their present regional distributions via genetic invasion. This assesses an important question about how target-site resistance arises in mosquito populations, which can occur through both genetic invasion and local *de novo* mutation. Overall, these patterns provide strong evidence that both *Vssc* genotypes (mutations at codons 1016 and 1534, Profiles GTC and TGT respectively) have spread throughout the region by human transportation of mosquitoes, though heterogeneous results for the two resistance profiles suggest dissimilar histories of evolution and invasion. Our results indicate that strong linkage within the *Vssc* gene region restricts which of the 27 possible genotypes can be produced, which will help inform control strategies suited to local conditions as resistance status varies greatly among these genotypes. In deriving these results, we also present a detailed geographical summary of pyrethroid resistance in the Indo-Pacific, a critically important region for dengue control.

The negative linkage disequilibrium between *Vssc* mutations at codons 1016 and 1534 as well as the perfect linkage between mutation state at codons 1016 and 989 means that there was no possibility of finding an individual mosquito homozygous for all three mutations. The triple homozygous mutation (989P+1016G+1534C) has been found to enhance resistance to Type I and Type II pyrethroids to a very high level in *Xenopus* oocyte experiments when created artificially in the laboratory (Hirata et al., 2014). Hirata *et al* (2014) caution that a single crossing over event could result in an individual with the triple mutation, however, this homozygous combination of mutations has been found only rarely in *Ae*. *aegypti* from the field (Ishak et al., 2015; Hitoshi Kawada et al., 2014; Sayono et al., 2016) and its resistance status in that context is not yet clear. Our proposal of inheritance by linked haplotypes with no recombination between mutation sites 1016 and 1534 may explain why the triple mutant does not often arise, even though a heterozygote

1016/1534/989 is common. It is also possible that the triple mutant has a high fitness cost (Hirata et al., 2014; Plernsub, Saingamsook, Yanola, Lumjuan, Tippawangkosol, Sukontason, et al., 2016). A low number of *Ae. aegypti* collected from Saudi Arabia have been identified as being triple mutant heterozygotes with all three mutations on the same chromosome (unlike the combination of our haplotypes H1 and H2, Fig. 2a) and these individuals were susceptible to deltamethrin (Al Nazawi, Aqili, Alzahrani, McCall, & Weetman, 2017).

In the absence of recombination, the presence of only three *Vssc* haplotypes (H1, H2, H3) explains why 21 of the 27 genotypes do not occur in the region (excluding Taiwan). The addition of a fourth haplotype (H4) in the sample from Taiwan enables formation of the two extra genotypes (GG/TT/TC, TG/TG/TT) found there. If H4 were combined with H3, another of the 27 possible genotypes could be constructed, but individuals of this genotype (TG/TT/TT) were not observed. The new haplotype in Taiwan (G/T/T) may be a result of recombination, with the G/T/C haplotype found elsewhere in the world. It may be that this haplotype was introduced to Taiwan and then recombined to produce G/T/T. Alternatively, G/T/C and G/T/T could both have evolved in Taiwan, before the G/T/C haplotype spread elsewhere, although it is then not clear why G/T/T has failed to spread. Our analyses at the autosomal and individual levels appear to indicate that recombination following invasion is the most parsimonious explanation of the distribution of H4, however, small sample sizes preclude us from confirming this.

Our analyses of resistance Profiles GTC and TGT indicate that genetic invasion has likely played a significant role in establishing the current distributions of their associated haplotypes. We conclude this from evidence across three analytical strata: the autosome, the individual, and the population. At the autosomal level, we detected a high-F_{ST} region of 7.3 Mbp (~2.4 cM (Matthews et al., 2018)) flanking the *Vssc* gene, at which the 19 outlier SNPs showed structuring according to resistance profile rather than population. As alleles at these SNPs should not be conferring any selective advantage related to resistance, we can conclude that their

structuring is likely a result of linked selection with SNPs conferring resistance. Thus we would expect to see these SNPs structured according to population if resistance mutations have arisen *de novo* in populations, and according to resistance profile if alleles have been transported between populations by genetic invasion. At the individual and population levels, evidence for genetic invasion was shown by the significantly smaller genetic distances between individuals of the same resistance profile and between populations with similar proportions of resistance alleles, and that these results were stronger for SNPs in the 3q autosomal region than for other SNPs. While the role of human transportation in establishing geographical distributions of *Aedes* mosquitoes is already well recognised (Tatem, Hay, & Rogers, 2006), these results indicate that dispersal along transportation networks has also helped to establish current distributions of pyrethroid resistance mutations. These results stand in contrast to a similar investigation of pyrethroid resistance in Australian red-legged earth mite (*Halotydeus destructor*) populations, which showed that the present distribution of resistance had been attained by multiple *de novo* mutations (Yang et al., 2019).

Although these signs of genetic invasion were observed for both resistance profiles, the evidence and associated interpretation was much clearer for Profile GTC than Profile TGT. PCA of outlier SNPs showed Profile GTC separating convincingly from non-GTC individuals, and pairwise comparison of individuals showed consistent, significant directionality in almost all categories, with stronger directionality and significance when considering 3q SNPs alone These findings are consistent with the mutation at codon 1016 and possibly also 989 having evolved only once in the Indo-Pacific region. Alternatively, these mutations may have been introduced to the region by genetic invasion from an external source. Following their emergence in the region, the mutations have attained their current distribution through a series of genetic invasions. Determining the population(s) in which these mutations originated will require further investigation.

Results for Profile TGT are resistant to such a direct interpretation. Overall, there are still clear signs of genetic invasion, specifically from the PCA of outlier SNPs, from the more heterogeneous yet present directionality in pairwise comparisons, and from the more heterogeneous yet stronger directionality for 3q SNPs. However, this presents no strong evidence of the mutation at codon 1534 arising only once, though this might nevertheless remain a more parsimonious explanation than one of multiple *de novo* mutations. One hypothesis is that genetic invasions of the codon 1534 mutation occurred many generations before the codon 1016 and 989 mutations, and over time the signal at the individual level has degraded in the populations invaded first, while the signal remains at the 7.3 Mbp region flanking the *Vssc* gene region. Consistent with this is another hypothesis that resistance has been lost in some of the regions where Profile TGT is present, such as in Fiji and New Caledonia, wherein the mutation at codon 1534 reverts to the wildtype. This is consistent with the higher observed frequency of wildtype or wildtype heterozygotes in these areas. Additionally, the higher frequency of wildtype heterozygotes among our sample of Profile TGT mosquitoes may itself be a sufficient explanation for the inconsistent directionality and significance of some of the individual-level comparisons, relative to those of Profile GTC.

Due to uneven sample sizes, our data do not allow identification of the original source of the *Vssc* haplotypes that are now spread throughout the Indo-Pacific, but populations of the same species studied by others have shown evidence of selective sweeps affecting this gene around codons 1534 (Ishak et al., 2015) and 1016 (Wuliandari et al., 2015). Similar evidence of selection has been found in *Ae*. *aegypti* from South America around codon 1016 (K. Saavedra-Rodriguez et al., 2007), but segregation of South American mosquitoes from those of the Indo-Pacific region has long been recognized (Smith et al., 2016), centring around the different and predominant mutation, V1016I. Furthermore, Saavedra-Rodriguez *et al*. (2007) show high levels of recombination between codons 1011 and 1016 in *Ae*. *aegypti* from South America, and evidence for local selection of pyrethroid resistance has been obtained in *Ae*. *aegypti* from Mexico (Karla Saavedra-Rodriguez et al., 2015). V1016G appears to have arisen before S989P (Li et al., 2015), as S989P is never (Hitoshi Kawada et al., 2014) or rarely (Wuliandari et al., 2015) found alone. A different type of study is needed to pinpoint the

historical centre of selection for the resistance haplotypes we have found in the Indo-Pacific region. Nevertheless, our approach of looking at the *Vssc* mutations in the context of genetic population structure helps indicate the extent of movement of resistance alleles in *Ae*. *aegypti* in the Indo-Pacific and provides little evidence for independent evolution of resistance in different populations throughout the region.

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Data Accessibility Statement

Resistance genotype data are available within the manuscript. The aligned .bam sequence files will be made available through NCBI Genbank at the time of publication.

Author Contributions

NMEH, TLS, AAH designed the study. NMEH, TLS, JC performed research and analysed data. AvR contributed assay design. NMEH, TLS, AAH, JC and ARW wrote the paper.

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