



# Multiple mutations and mutation combinations in the sodium channel of permethrin resistant mosquitoes, *Culex quinquefasciatus*

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A previous study identified 3 nonsynonymous and 6 synonymous mutations in the entire mosquito sodium channel of *Culex quinquefasciatus*, the prevalence of which were strongly correlated with levels of resistance and increased dramatically following insecticide selection. However, it is unclear whether this is unique to this specific resistant population or is a common mechanism in field mosquito populations in response to insecticide pressure. The current study therefore further characterized these mutations and their combinations in other field and permethrin selected *Culex* mosquitoes, finding that the co-existence of all 9 mutations was indeed correlated with the high levels of permethrin resistance in mosquitoes. Comparison of mutation combinations revealed several common mutation combinations presented across different field and permethrin selected populations in response to high levels of insecticide resistance, demonstrating that the co-existence of multiple mutations is a common event in response to insecticide resistance across different *Cx. quinquefasciatus* mosquito populations.

Vector control of mosquitoes with insecticides is an important part of the current global strategy to control mosquito-associated diseases. However, the widespread growth of resistance to insecticides in mosquitoes, especially to pyrethroids, is rapidly becoming a global problem<sup>1</sup>. The voltage gated sodium channels in the insect's nervous system are the primary target of both pyrethroids and DDT, but modifications in the structure of the sodium channels due to point mutations or substitutions resulting from single nucleotide polymorphisms [SNP] results in insensitivity to both these insecticides in the sodium channels via a reduction in or an elimination of the binding affinity of the insecticides to proteins, thus diminishing the toxic effects of the insecticides and resulting in the development of insecticide resistance<sup>2-5</sup>. Among these *kdr* mutations, the substitution of leucine by phenylalanine [L to F], histidine [L to H], or serine [L to S] in the 6<sup>th</sup> segment of domain II (IIS6) has been clearly associated with resistance to pyrethroids and DDT in many insect species, including mosquitoes<sup>6-11</sup>, while other *kdr* mutations appeared to be unique to specific species<sup>3-5</sup>. Systematic *in vitro* site-directed mutagenesis in insect sodium channel genes has revealed multiple regions in the sodium channels that contribute to the binding and action of pyrethroids<sup>12,13</sup>, suggesting that the interactions of multiple mutations may play a role in the response of an insect's sodium channels to insecticides.

A recent analysis by our group on all the naturally occurring mutations, both nonsynonymous and synonymous mutations, and the mutation combinations in the entire *Culex quinquefasciatus* sodium channel of a field parental strain HAmCq<sup>G0</sup> collected from Huntsville, Alabama, USA and its permethrin-selected offspring HAmCq<sup>G8</sup> has revealed the co-existence of multiple sodium channel mutations. We have found that both nonsynonymous and synonymous mutations were observed in resistant mosquitoes and might be important factors contributing to high levels of resistance<sup>14</sup>, with the prevalence of mutations in the resistant mosquito sodium channels increasing dramatically following permethrin-selection. However, it is unclear whether this is unique to this specific resistant population or if it is common to *Cx. quinquefasciatus* field populations subjected to insecticide selection pressure and hence the development of insecticide resistance. The current study therefore sought to further investigate these mutations and their combination in another field mosquito strain MAmCq<sup>G0</sup> of *Cx. quinquefasciatus* collected from Mobile, Alabama, USA and its permethrin-selected offspring MAmCq<sup>G6</sup>.

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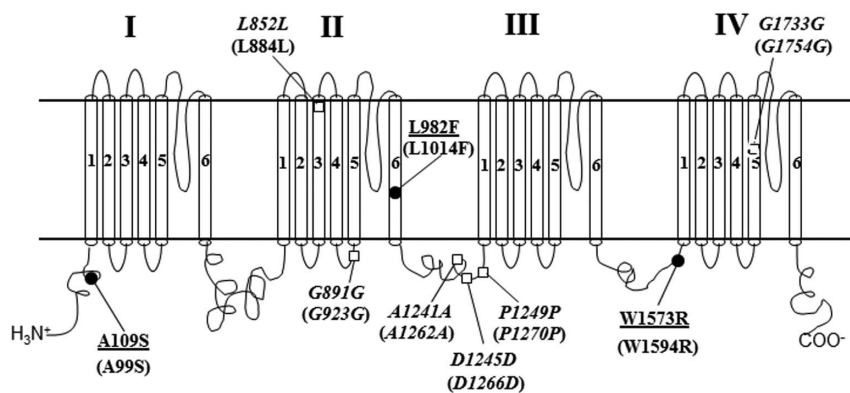
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**Figure 1** | Graphic representation of the locations of synonymous and nonsynonymous mutations in the *Cx. quinquefasciatus* sodium channel. Nonsynonymous mutations are indicated by solid dots and their locations are underlined. Synonymous mutations are indicated by open tetragons and their locations are in italics. Positions of the mutations are numbered according to amino acid sequences of *Cx. quinquefasciatus* (accession numbers: JN695777, JN695778, JN695779); the corresponding positions in the house fly *Vssc1* sodium channel protein are shown in parentheses. The domain locations of the mutations are assigned according to the sodium channel amino acid sequences in house flies<sup>7,22</sup>.

The co-occurrence of both nonsynonymous and synonymous mutations in insecticide-resistant mosquitoes and their inheritance following insecticide selection were characterized and the specific thresholds for the insecticide concentrations at which particular mutations or mutation combinations occur in different mosquito populations or groups were tested. The study provides valuable information confirming that the co-existence of all 9 mutations, both nonsynonymous and synonymous, were indeed presented in resistant mosquitoes across different populations.

## Results

**Nonsynonymous mutations associated with pyrethroid resistance in *Cx. quinquefasciatus*.** We investigated the expression frequency of 3 nonsynonymous ( $A^{109S}$ ,  $L^{982F}$ , and  $W^{1573R}$ ) identified in an earlier study involving a different *Cx. quinquefasciatus* population (Fig. 1) in the sodium channels of the field parental population MAmCq<sup>G0</sup> and its 6<sup>th</sup> generation permethrin-selected highly resistant offspring MAmCq<sup>G6</sup>. The SNPs at the mutation sites were examined in 60 adult individuals from each of the MAmCq<sup>G0</sup> and MAmCq<sup>G6</sup> mosquito populations. All tested individuals in both populations showed expression of the polymorphic T325 allele at the codon  $A^{109}$  (Table 1,<sup>14</sup>), resulting in the substitution alanine to serine ( $A^{109S}$ ). Interestingly, in the susceptible S-Lab population, 65% of the tested individuals expressed the susceptible allele G325, generating a codon encoding alanine, 35% expressed both the G325 and T325 alleles, and none expressed the polymorphic T325 allele (Table 1). A strong correlation between the prevalence of polymorphic allelic expression of A2946T and T4717C at the codons  $L^{982}$  and  $W^{1573}$ , respectively, and the levels of pyrethroid resistance in the *Culex* mosquitoes was identified. While all tested individuals in the susceptible S-Lab population expressed the susceptible alleles A2946 and T4717, producing codons encoding leucine ( $L^{982}$ ) and tryptophan ( $W^{1573}$ ), respectively (Table 1), all tested individuals in the highly resistant MAmCq<sup>G6</sup> population expressed polymorphic allele T2946, producing a substitution codon encoding phenylalanine ( $F^{982}$ ), and 92% also expressed polymorphic allele C4717, generating a substitute codon encoding arginine ( $R^{1573}$ ). The intermediate resistance population, MAmCq<sup>G0</sup>, showed an intermediate level of allelic expression for SNPs of T2946A and T4717C (Table 1). These results suggest that the  $L^{982F}$  and  $W^{1573R}$  mutations are highly likely to be involved in the mosquitoes' elevated levels of pyrethroid resistance, and that individual mosquitoes with these polymorphic alleles are indeed selected by permethrin application. This result confirms our previous findings of sodium channel mutations in a field *Culex* mosquito strain

HAMCq<sup>G0</sup> and its 8<sup>th</sup> generation of permethrin selected offspring HAMCq<sup>G8</sup> reported by Xu et al.<sup>14</sup>

**Expression frequency of synonymous mutations in pyrethroid resistant mosquitoes *Cx. quinquefasciatus*.** We next examined 6 synonymous ( $L^{852}$ ,  $G^{891}$ ,  $A^{1241}$ ,  $D^{1245}$ ,  $P^{1249}$ , and  $G^{1733}$ ) and their correlation with the levels of resistance in *Cx. quinquefasciatus*. The SNP determination also revealed strong correlations between the frequency of polymorphic expression at the 6 synonymous codon sites and the levels of susceptibility and resistance in *Cx. quinquefasciatus* (Table 1). All the synonymous nucleotide polymorphisms, as with the nonsynonymous polymorphisms, showed a strong association between the prevalence of polymorphic codon usage and the evolution of permethrin-selection (Table 1). Non nucleotide substitutions at the synonymous codon sites, besides  $G^{1733G}$ , were detected in S-lab mosquitoes; higher frequencies of the polymorphic expression were detected in MAmCq<sup>G6</sup>; and relatively low frequencies were detected in MAmCq<sup>G0</sup> (Table 1). Only the polymorphisms of A3723G and A5199G at the codons  $A^{1241A}$  and  $G^{1733G}$  showed relatively high frequencies (80% and 95%, respectively) of the polymorphic expression in MAmCq<sup>G0</sup> (Table 1), suggesting that synonymous polymorphisms A3723G at the codon  $A^{1241A}$  and A5199G at the codon  $G^{1733G}$  may evolve in the earliest stage of permethrin selection.

**Correlation of polymorphic allele frequencies with the tolerance of mosquitoes to permethrin.** To examine whether the mutation frequency/occurrence is related to increased levels of resistance or increased levels of tolerance of mosquitoes to certain concentrations of permethrin, and to characterize the permethrin concentration threshold that causes a particular mutation to occur in the mosquitoes and/or the differences in the timing of the occurrence of nonsynonymous and synonymous mutations, we examined the prevalence of each sodium channel mutation and correlated the results with the mosquitoes' tolerance to certain concentrations of permethrin in MAmCq<sup>G0</sup> and its permethrin-selected offspring MAmCq<sup>G6</sup>. We treated mosquito larvae of each population with different concentration of permethrin (Table 2) and assembled them into four groups (1 to 4) of each mosquito strain based on their similar levels of tolerance to permethrin (low to high, respectively). The results showed that all individuals in all tested groups across the field parental and permethrin-selected offspring populations were homozygous for polymorphic allele T325 at the codon  $A^{109S}$  (Fig. 2, Table 3). In addition, with the exception of groups 1 and 2 in MAmCq<sup>G0</sup>, which had the lowest levels of tolerance to permethrin and showed heterozygous individuals for polymorphic allele G5199 at codon  $G^{1733G}$ , all individuals in the tested groups across both

Table 1 | Non-synonymous and synonymous mutations in the sodium channel of *Cx. quinquefasciatus*

Mutation	Strain	n*	Phenotype <sup>†</sup>	Codons <sup>‡</sup> (Frequency [%] ± SE)		
A109S <sup>§</sup>	S-Lab	60	Susceptible	<u>GCA</u> (65 ± 5.0)	<u>G/TCA</u> (35 ± 5.0)	<u>TCA</u> (0)
	MAmCq <sup>G0</sup>	60	10-fold	<u>GCA</u> (0)	<u>G/TCA</u> (0)	<u>TCA</u> (100)
	MAmCq <sup>G6</sup>	60	570-fold	<u>GCA</u> (0)	<u>G/TCA</u> (0)	<u>TCA</u> (100)
L982F <sup>§</sup>	S-Lab	60	Susceptible	<u>TTA</u> (100)	<u>TTA/T</u> (0)	<u>TTT</u> (0)
	MAmCq <sup>G0</sup>	60	10-fold	<u>TTA</u> (22 ± 3.0)	<u>TTA/T</u> (52 ± 6.0)	<u>TTT</u> (26 ± 7.5)
	MAmCq <sup>G6</sup>	60	570-fold	<u>TTA</u> (0)	<u>TTA/T</u> (0)	<u>TTT</u> (100)
W1573R <sup>§</sup>	S-Lab	60	Susceptible	<u>TGG</u> (100)	<u>T/CGG</u> (0)	<u>CGG</u> (0)
	MAmCq <sup>G0</sup>	60	10-fold	<u>TGG</u> (72 ± 10.5)	<u>T/CGG</u> (25 ± 8.5)	<u>CGG</u> (3.0 ± 3.0)
	MAmCq <sup>G6</sup>	60	570-fold	<u>TGG</u> (0)	<u>T/CGG</u> (8 ± 5.5)	<u>CGG</u> (92 ± 6.0)
L852I <sup>#</sup>	S-Lab	60	Susceptible	<u>CTG</u> (100)	<u>CTG/A</u> (0)	<u>CTA</u> (0)
	MAmCq <sup>G0</sup>	60	10-fold resistance	<u>CTG</u> (27 ± 10)	<u>CTG/A</u> (38 ± 7.5)	<u>CTA</u> (35 ± 5)
	MAmCq <sup>G6</sup>	60	570-fold resistance	<u>CTG</u> (0)	<u>CTG/A</u> (6.5 ± 2.8)	<u>CTA</u> (93.5 ± 2.9)
G891G <sup>#</sup>	S-Lab	60	Susceptible	<u>GGC</u> (100)	<u>GGC/A</u> (0)	<u>GGA</u> (0)
	MAmCq <sup>G0</sup>	60	10-fold resistance	<u>GGC</u> (28 ± 10)	<u>GGC/A</u> (42 ± 7.5)	<u>CTA</u> (30 ± 10)
	MAmCq <sup>G6</sup>	60	570-fold	<u>GGC</u> (0)	<u>GGC/A</u> (5 ± 5)	<u>CTA</u> (95 ± 5)
A1241A <sup>#</sup>	S-Lab	60	Susceptible	<u>GCA</u> (100)	<u>GCA/G</u> (0)	<u>GCG</u> (0)
	MAmCq <sup>G0</sup>	60	10-fold resistance	<u>GCA</u> (2 ± 3)	<u>GCA/G</u> (18 ± 2.9)	<u>GCG</u> (80 ± 5.5)
	MAmCq <sup>G6</sup>	60	570-fold	<u>GCA</u> (0)	<u>GCA/G</u> (0)	<u>GCG</u> (100)
D1245D <sup>#</sup>	S-Lab	60	Susceptible	<u>GAC</u> (100)	<u>GAC/T</u> (0)	<u>GAT</u> (0)
	MAmCq <sup>G0</sup>	60	10-fold resistance	<u>GAC</u> (38 ± 7.5)	<u>GAC/T</u> (45 ± 8.5)	<u>GAT</u> (17 ± 5.5)
	MAmCq <sup>G6</sup>	60	570-fold	<u>GAC</u> (0)	<u>GAC/T</u> (8 ± 5.5)	<u>GAT</u> (92 ± 5.5)
P1249P <sup>#</sup>	S-Lab	60	Susceptible	<u>CCG</u> (100)	<u>CCG/A</u> (0)	<u>CCA</u> (0)
	MAmCq <sup>G0</sup>	60	10-fold resistance	<u>CCG</u> (37 ± 5.5)	<u>CCG/A</u> (42 ± 5.5)	<u>CCA</u> (21 ± 5.5)
	MAmCq <sup>G6</sup>	60	570-fold	<u>CCG</u> (0)	<u>CCG/A</u> (5.0 ± 5.0)	<u>CCA</u> (95 ± 5.0)
G1733G <sup>#</sup>	S-Lab	60	Susceptible	<u>GGA</u> (48 ± 12.5)	<u>GGA/G</u> (52 ± 12.5)	<u>GGG</u> (0)
	MAmCq <sup>G0</sup>	60	10-fold resistance	<u>GGA</u> (0)	<u>GGA/G</u> (5 ± 5.0)	<u>GGG</u> (95 ± 5.0)
	MAmCq <sup>G6</sup>	60	570-fold resistance	<u>GGA</u> (0)	<u>GGA/G</u> (0)	<u>GGG</u> (100)

G0 represents the parental insects collected directly from the field; G6 represents the 6<sup>th</sup> generation of permethrin-selected MAmCq<sup>G0</sup> offspring; Values represent mean ± SE for the three replications of frequency (%) analyses for each mutation.

<sup>†</sup>The total number of tested insects (three replicates for each of 10 males and 10 females).

<sup>‡</sup>Data from<sup>31</sup>.

<sup>§</sup>The nucleotide polymorphisms are underlined.

<sup>#</sup>Non-synonymous mutations.

<sup>¶</sup>Synonymous mutations.

the field parental and permethrin-selected offspring populations were homozygous for the mutation, which is consistent with the suggestion that A<sup>109</sup>S and G<sup>1733</sup>G may evolve in the earliest stage of permethrin resistance. A significantly different distribution of the frequency of polymorphisms for the remainder of the 7 nonsynonymous and synonymous mutations was found among different groups of mosquito populations (Figs. 2 and 3). Correlation of the mutation prevalence with the level of tolerance to permethrin revealed the direct relevance of these 7 mutations to permethrin-selection and resistance evolution. Homozygous polymorphic alleles A<sup>2556</sup>, A<sup>2673</sup>, T<sup>2946</sup>, G<sup>3723</sup>, T<sup>3735</sup>, and A<sup>3747</sup> began to appear in group 2 of MAmCq<sup>G0</sup>, with a tolerance to permethrin concentrations between 0.003 and 0.01 ppm (Table 2), suggesting that these polymorphisms

may be responsible for the initiation of moderate levels of permethrin resistance. The most noticeable mutation is the nonsynonymous C<sup>4717</sup>, which emerged starting from group 4 of MAmCq<sup>G0</sup> and exhibited tolerance to permethrin concentrations of more than LC<sub>90</sub> (>0.1 ppm), suggesting that this polymorphism may be the most important for the initiation of high levels of resistance.

**Mutation combinations of the mosquito sodium channel in response to permethrin application.** To investigate the effects of different mutation combinations in mosquitoes' response to permethrin and the specific thresholds of permethrin concentrations at which particular mutations or mutation combinations occur, we examined the frequency of particular synonymous and/or nonsynonymous

Table 2 | Permethrin treatment of field and permethrin-selected *Culex* mosquitoes

Strains	Permethrin Treatments*									
	LC <sub>10</sub> Treatment			LC <sub>50</sub> Treatment			LC <sub>90</sub> Treatment			
	n <sup>‡</sup>	<sup>†</sup> LC <sub>10</sub> PPM	1 <sup>st</sup> Group (collect dead mosquitoes)	n <sup>‡</sup>	<sup>†</sup> LC <sub>50</sub> PPM	2 <sup>nd</sup> Group (collect dead mosquitoes)	n <sup>‡</sup>	<sup>†</sup> LC <sub>90</sub> PPM	3 <sup>rd</sup> Group (collect dead mosquitoes)	4 <sup>th</sup> Group (collect alive mosquitoes)
MAmCq <sup>G0</sup>	~1500	0.003	MAmCq <sup>G0</sup> <LC <sub>10</sub>	~1300	0.01	MAmCq <sup>G0</sup> LC <sub>10-50</sub>	~800	0.1	MAmCq <sup>G0</sup> LC <sub>50-90</sub>	MAmCq <sup>G0</sup> >LC <sub>90</sub>
MAmCq <sup>G6</sup>	~1500	0.3	MAmCq <sup>G6</sup> <LC <sub>10</sub>	~1300	1	MAmCq <sup>G6</sup> LC <sub>10-50</sub>	~800	10	MAmCq <sup>G6</sup> LC <sub>50-90</sub>	MAmCq <sup>G6</sup> >LC <sub>90</sub>

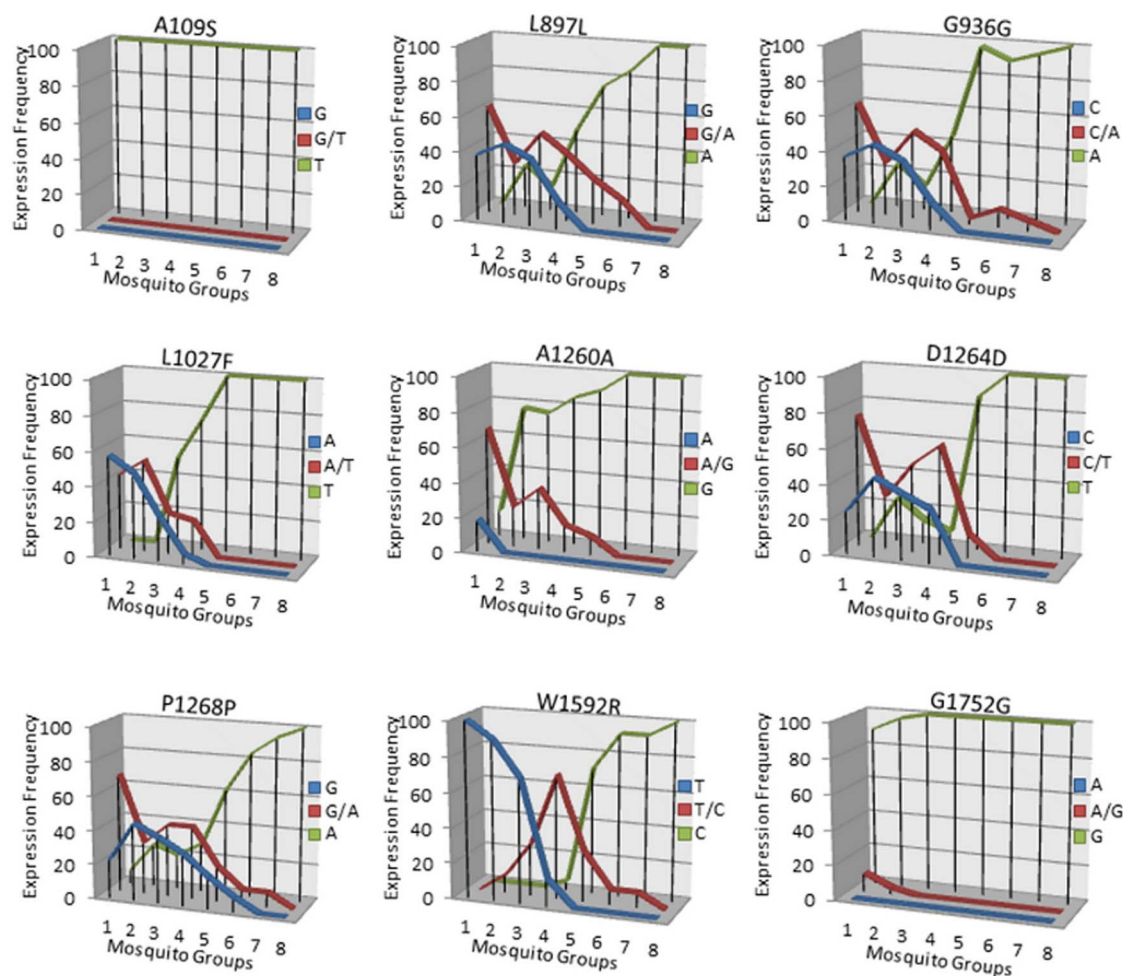
\*Each treatment was performed 3 times.

<sup>†</sup>The concentrations of permethrin administered to these mosquitoes was as identified previously<sup>31</sup>.

<sup>‡</sup>The number of early 4<sup>th</sup> instar larvae used at the beginning of the permethrin treatment with LC<sub>10</sub>.

<sup>§</sup>The mosquitoes surviving permethrin treatment with LC<sub>10</sub> 10 h after treatment.

<sup>¶</sup>The mosquitoes surviving permethrin treatment with LC<sub>50</sub> 10 h after treatment.



**Figure 2 |** Distribution of frequencies of alleles at each of the mutation sites in each of the mosquito groups that are sensitive to or tolerant of different concentrations of permethrin ( $LC_{10}$ ,  $LC_{50}$ , and  $LC_{90}$ ) in MAmCq<sup>G0</sup> field parental populations and their 6<sup>th</sup> generation permethrin-selected offspring, MAmCq<sup>G6</sup>. The frequency of allele expression shown along the Y axis is the percentage of the mosquitoes ( $n = 40$ ) carrying the homozygous or heterozygous allele(s) of the mutation. Mosquito groups are shown along the X axis; 1, 2, 3, and 4 represent the groups in MAmCq<sup>G0</sup> that were dead under  $LC_{10}$  concentration treatment, between  $LC_{10}$  and  $LC_{50}$ , between  $LC_{50}$  and  $LC_{90}$  and alive above  $LC_{90}$ , respectively; and 5, 6, 7, and 8 represent the groups in MAmCq<sup>G6</sup> that are dead under  $LC_{10}$ , between  $LC_{10}$  and  $LC_{50}$ , between  $LC_{50}$  and  $LC_{90}$ , and alive above  $LC_{90}$  concentration treatment, respectively.

mutations that co-occur in the mosquito groups across different populations. The sodium channel mutations were analyzed in a total of 40 individuals, which had all 9 mutations present in their full length sodium channel, in each of the mosquito groups. A total of 31 mutation combinations were identified across the mosquito populations and groups (Table 3, Fig. 3). Category #13 (double homozygous mutations and quintuple heterozygous mutations;  $T^{325}$ ,  $g/a^{2556}$ ,  $c/a^{2673}$ ,  $a/g^{3723}$ ,  $c/t^{3735}$ ,  $g/a^{3747}$ ,  $G^{5199}$ ) was the predominant mutation combination in group 1 (the group with the lowest tolerance to permethrin) of MAmCq<sup>G0</sup>. Categories #14 (triple homozygous mutations and quintuple heterozygous mutations;  $T^{325}$ ,  $g/a^{2556}$ ,  $c/a^{2673}$ ,  $a/t^{2946}$ ,  $G^{3723}$ ,  $c/t^{2735}$ ,  $g/a^{3747}$ ,  $G^{5199}$ ) were the dominant combinations in group 2 of MAmCq<sup>G0</sup>. The difference between categories #13 and #14 was the changes from susceptible homozygous  $A^{2946}$  to heterozygous  $a/t^{2946}$  and from heterozygous  $a/g^{3723}$  to polymorphic homozygous  $G^{3723}$ . A similar transition pattern was identified in the dominant mutation combinations of the consecutive mosquito groups with increased levels of tolerance to permethrin. Category #15, for example, was the predominant mutation combination in groups 3 and 4 (triple homozygous mutations and sextuple heterozygous mutations;  $T^{325}$ ,  $g/a^{2556}$ ,  $c/a^{2673}$ ,  $a/t^{2946}$ ,  $G^{3723}$ ,  $c/t^{2735}$ ,  $g/a^{3747}$ ,  $t/c^{4717}$ ,  $G^{5199}$ ), which showed a single change from heterozygous  $a/t^{2946}$  to polymorphic homozygous  $T^{2946}$  compared

to category #14 in group 2. The occurrence of category #31 (nonuple homozygous mutations,  $T^{325}$ ,  $A^{2556}$ ,  $A^{2673}$ ,  $T^{2946}$ ,  $G^{3723}$ ,  $T^{2735}$ ,  $A^{3747}$ ,  $C^{4717}$  and  $G^{5199}$ ) emerged in the group 4 mosquitoes of MAmCq<sup>G0</sup> with a low frequency of 5%, suggesting that permethrin concentrations at 0.1 ppm may represent the threshold at which the particular #31 nonuple homozygous mutations combination occurs in the field mosquito population MAmCq<sup>G0</sup>.

Comparing mutation combinations in permethrin-selected offspring MAmCq<sup>G6</sup> with those in their field parental mosquitoes MAmCq<sup>G0</sup> revealed a clear shift in the mutation combinations in these populations from the majority being heterozygous mutation combinations, for example categories #14 and #15 in MAmCq<sup>G0</sup>, to the majority being resistant homozygous combinations like category #31 in MAmCq<sup>G6</sup> (Table 3, Fig. 3). Pairwise Goeman's Bayesian scores<sup>15</sup> tested using the AssotesterR software package in R<sup>16,17</sup> revealed the significant correlation between resistance levels of mosquito groups and their SNP combination frequencies (Table 4). A significant ( $P \leq 0.05$ ) transition in the prevalence of the nonuple homozygous mutation combinations (category #31) was observed between the field parental strain and its permethrin-selected offspring (Table 4, Fig. 3). Nevertheless, in place of the combination transition pattern for the predominant mutation combinations

Table 3 | Co-occurrence of the *kdr* mutations in the MAmCq groups with difference levels of tolerance to permethrin

*Mosquito Groups			Polymorphisms at Amino Acid Mutation Sites											
			A109S	L852L	G891G	L982F	A1241A	D1245D	P1249P	W1573R	G1733G			
			G to T	G to A	C to A	A to T	A to G	C to T	G to A	T to C	A to G			
MAmCq <sup>G0</sup>	1	1	10 (7)	T	G	C	A	A	C	G	T	A/G		
		3	7.5 (3.5)	T	G	C	A	A	C	G	T	G		
		4	5 (0)	T	G	C	A	A/G	C	G	T	G		
		8	7.5 (3.5)	T	G	C	A	A/G	C/T	G/A	T	G		
		10	7.5 (3.5)	T	G	C	A	G	C/T	G/A	T	G		
		12	20 (7)	T	G/A	C/A	A	A/G	C/T	G/A	T	G		
		13	35 (7)	T	G/A	C/A	A/T	A/G	C/T	G/A	T	G		
		19	7.5 (3.5)	T	G/A	C/A	A/T	G	C/T	A	T	G		
		2	2	2.5 (3.5)	T	G	C	A	G	C	G	T	A/G	
			4	7.5 (3.5)	T	G	C	A	A/G	C	G	T	G	
	5		12.5 (3.5)	T	G	C	A/T	A/G	C	G	T	G		
	6		12.5 (93.5)	T	G	C	A	G	C	G	T	G		
	9		10 (0)	T	G	C	A/T	G	C	G	T/C	G		
	13		2.5 (3.5)	T	G/A	C/A	A/T	A/G	C/T	G/A	T	G		
	14		27.5 (3.5)	T	G/A	C/A	A/T	G	C/T	G/A	T	G		
	27		25 (0)	T	A	A	A	G	T	A	T	G		
	3		4	12.5 (3.5)	T	G	C	A	A/G	C	G	T	G	
			5	10 (0)	T	G	C	A/T	A/G	C	G	T	G	
		7	15 (7)	T	G	C	T	A/G	C	G	T	G		
		15	17.5 (3.5)	T	G/A	C/A	A/T	G	C/T	G/A	T/C	G		
		17	12.5 (3.5)	T	G/A	C/A	T	G	C/T	G/A	T	G		
		18	12.5 (3.5)	T	G/A	C/A	T	G	C/T	G/A	T/C	G		
		21	10 (7)	T	G/A	C/A	T	G	C/T	A	T	G		
		27	10 (0)	T	A	A	A	G	T	A	T	G		
		4	7	5 (0)	T	G	C	A	A/G	C	G	T	G	
			11	10 (7)	T	G	C	T	G	C	G	T	G	
	15		20 (7)	T	G/A	C/A	A/T	G	C/T	G/A	T/C	G		
	16		15 (0)	T	G/A	A	T	G	C	G	T/C	G		
	20		10 (0)	T	A	A	T	A/G	C/T	G/A	T/C	G		
	22		20 (7)	T	G/A	C/A	T	G	C/T	A	T/C	G		
	23		15 (7)	T	A	A	T	G	C/T	G/A	T/C	G		
	31		5 (0)	T	A	A	T	G	T	A	C	G		
	MAmCq <sup>G6</sup>		1	20	10 (7)	T	A	A	T	A/G	C/T	G/A	T/C	G
				22	2.5 (3.5)	T	G/A	C/A	T	G	C/T	A	T/C	G
		24		10 (0)	T	G/A	A	T	G	T	G/A	T/C	G	
		25		17.5 (3.5)	T	A	A	T	G	T	G	C	G	
		29		12.5 (3.5)	T	G/A	A	T	G	T	A	C	G	
		30		5 (0)	T	A	A	T	G	T	A	T/C	G	
		31		42.5 (10.5)	T	A	A	T	G	T	A	C	G	
		2		24	7.5 (3.5)	T	G/A	A	T	G	T	G/A	T/C	G
25				7.5 (3.5)	T	A	A	T	G	T	G	C	G	
28				15 (7)	T	A	C/A	T	G	T	A	C	G	
29	10 (0)		T	G/A	A	T	G	T	A	C	G			
31	60 (7)		T	A	A	T	G	T	A	C	G			
3	26	7.5 (3.5)	T	A	A	T	G	T	G/A	T/C	G			
	28	5 (0)	T	A	C/A	T	G	T	A	C	G			
	31	87.5 (3.5)	T	A	A	T	G	T	A	C	G			
4	31	100 (0)	T	A	A	T	G	T	A	C	G			

\*Group 1 mosquitoes tolerated permethrin concentration of <LC<sub>10</sub> (i.e., MAmCq<sup>G0</sup><LC<sub>10</sub>, and MAmCq<sup>G6</sup><LC<sub>10</sub>); group 2 mosquitoes tolerated permethrin concentrations of between LC<sub>10</sub>-LC<sub>50</sub> (i.e., MAmCq<sup>G0</sup>LC<sub>10-50</sub>, and MAmCq<sup>G6</sup>LC<sub>10-50</sub>); group 3 mosquitoes tolerated permethrin concentrations between LC<sub>50</sub>-LC<sub>90</sub> (i.e., MAmCq<sup>G0</sup>LC<sub>50-90</sub>, and MAmCq<sup>G6</sup>LC<sub>50-90</sub>); and group 4 mosquitoes tolerated permethrin concentrations >LC<sub>90</sub> (i.e., MAmCq<sup>G0</sup>>LC<sub>90</sub>, and MAmCq<sup>G6</sup>>LC<sub>90</sub>) (Table 2).

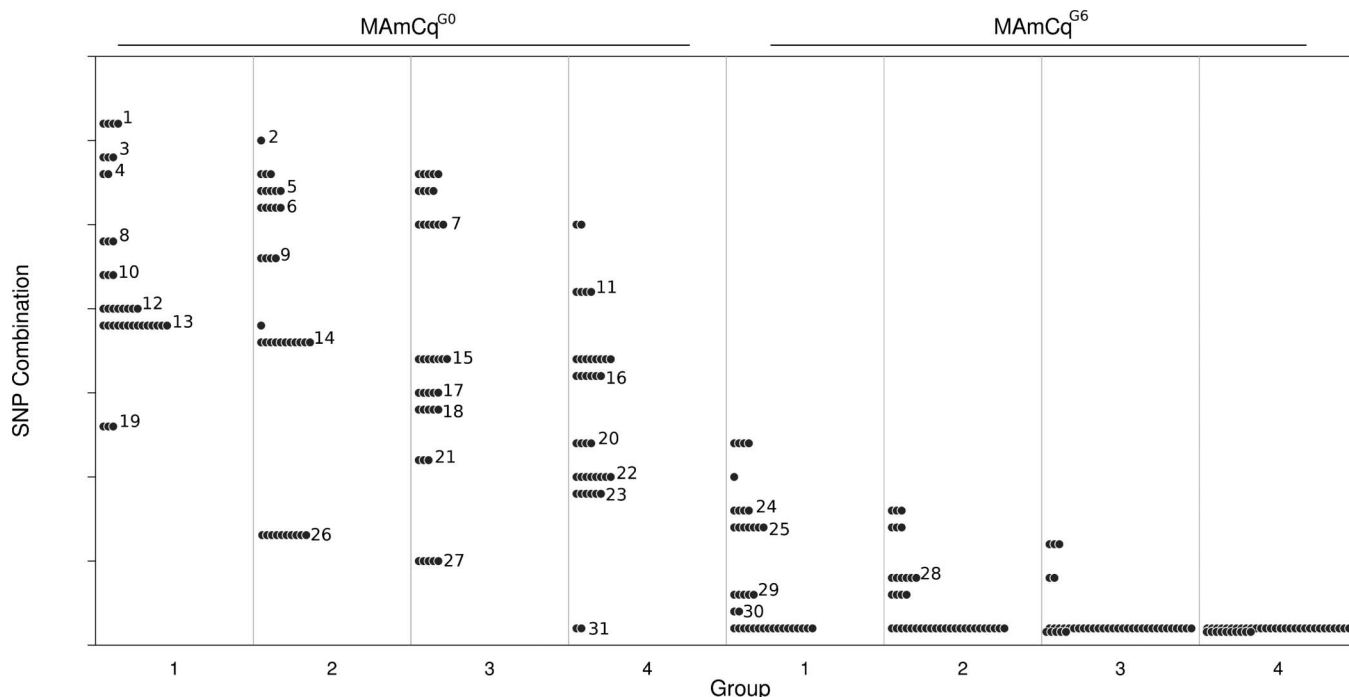
<sup>†</sup>N: The numbers indicate different combinations of the mutations and were assigned by weighing/counting the numbers of the homozygous susceptible alleles, heterozygous, and homozygous resistance alleles in the combination, so the lower numbers indicates higher incidences of homozygous susceptible alleles in the combination and higher numbers indicate higher incidences of heterozygous and homozygous resistance alleles in the combination.

F: the frequency (%) with which each of the mutation combinations occurred in each group. A total of 40 individuals (two replicates for each of 20 4<sup>th</sup> instar larvae) with all ten mutations in their sodium channel cDNAs were analyzed.

identified in the field mosquito population MAmCq<sup>G0</sup>, category #31 (nonuple homozygous mutations) was the predominant mutation combination across all four groups of the permethrin-selected offspring MAmCq<sup>G6</sup>. A significant shift in the prevalence of this mutation combination was also observed in MAmCq<sup>G6</sup>, rising from 42.5% in group 1, the lowest level of tolerance to permethrin treatment, to 100% in group 4, the highest level.

## Discussion

Our previous study of characterizing the mutations and mutation combinations over the entire sodium channel of individual resistant *Culex* mosquitoes HAMCq<sup>G0</sup> and their 8<sup>th</sup> generation permethrin-selected offspring HAMCq<sup>G8</sup>, identifying a total of 9 mutations, 3 of which were nonsynonymous and 6 synonymous<sup>14</sup>. The prevalence of these corresponded closely to the mosquitoes' level of permethrin



**Figure 3 |** Categorical plots of the sodium channel mutation combination patterns in mosquito groups that are sensitive to or tolerant of different concentrations of permethrin in MAmCq<sup>G0</sup> field parental populations and their 6<sup>th</sup> generation permethrin-selected offspring, MAmCq<sup>G6</sup>. The Y axes depict categories of mutation combinations (indicated by the numbers correspond to categories in Table 4) presented in each group (n = 40) of mosquitoes. On the X axes, mosquito groups are shown with the numbers 1–8 representing the same groups of MAmCq<sup>G0</sup> and MAmCq<sup>G6</sup> as in Fig. 2.

selection, permethrin treatment, and resistance to permethrin. However, it is unclear whether these results represent the unique case of this specific resistant population or whether this is a common response in field populations of resistant mosquitoes exposed to insecticide selection pressure. Our current study therefore further investigated all 9 of the mutations<sup>14</sup> and their combinations in individual mosquitoes of a field population of *Cx. quinquefasciatus* mosquitoes MAmCq<sup>G0</sup>, collected from Mobile, Alabama, ~600 km away from the location (Huntsville, Alabama, USA) where the original HAmCq<sup>G0</sup> mosquitoes were collected<sup>14,27</sup>. The *kdr* mutations over the entire mosquito sodium channel were analyzed and the mutation combinations in different mosquito groups categorized in terms of their levels of tolerance to a range of permethrin concentrations within and among the populations of the field parental strains and their permethrin-selected offspring. The current study not only demonstrated that the co-existence of all 9 mutations, both nonsynonymous and synonymous, was presented in the resistant mosquitoes

but also identified common mutation combinations that corresponded to high levels of insecticide resistance among the mosquito populations studied. Interestingly, our results also suggest that the co-existence of multiple mutations is a common feature in insecticide resistant mosquitoes.

Our study found a similar allelic expression pattern of the 9 mutations across the mosquito populations tested to those of our previous finding<sup>14</sup>. A clear shift of mutation combinations was again detected from those with primarily homozygous susceptible alleles, through those with mostly heterozygous alleles, to those with all or nearly all homozygous polymorphic alleles at the mutation sites, corresponding to the increasing tolerance of the mosquito groups to permethrin treatments in both field mosquito populations and their permethrin-selected offspring. Although both HAmCq and MAmCq exhibited their own specific mutation combinations, with a total of 20 mutation combinations identified in the HAmCq mosquitoes (data not shown) and 31 mutation combinations in the MAmCq mosquitoes,

**Table 4 |** Pairwise Goeman’s Bayesian score test values to check for correlations between SNP combination frequencies and permethrin resistance levels

		MAmCq <sup>G0</sup>				MAmCq <sup>G6</sup>			
Group		1	2	3	4	1	2	3	4
MAmCq <sup>G0</sup>	1	-							
	2	120*	-						
	3	22**	90*	-					
	4	760**	450**	200*	-				
MAmCq <sup>G6</sup>	1	2200**	1700**	1300**	600**	-			
	2	2500**	2000**	1600**	800**	30*	-		
	3	2700**	2200**	1800**	300**	60**	8.3*	-	
	4	2800**	2300**	1900**	1000**	90**	18**	1.8**	-

\*P<0.05; \*\*P<0.001

<sup>1</sup>Goeman’s Bayesian score test value based on 500 permutations. Goeman’s Bayesian scores represent a relative value for the comparison of paired samples. The higher the score, the more significant the correlation between resistance level and the SNP combination frequencies for the paired samples.

Table 5 | The 13 common mutation combinations of sodium channels in the mosquito populations of *Cx. quinquefasciatus*

Mutation			Polymorphisms at Amino Acid Mutation Sites								
Combination			A109S	L852L	G891G	L982F	A1241A	D1245D	P1249P	W1573R	G1733G
Category	N <sup>1</sup>	N <sup>2</sup>	G to T	G to A	C to A	A to T	A to G	C to T	G to A	T to C	A to G
A	1	2	T	G	C	A	G	C	G	T	a/g
B	2	6	T	G	C	A	G	C	G	T	G
C	3	8	T	G	C	A	a/g	c/t	g/a	T	G
D	4	10	T	G	C	A	G	c/t	g/a	T	G
E	5	13	T	g/a	c/a	a/t	a/g	c/t	g/a	T	G
F	8	14	T	g/a	c/a	a/t	G	c/t	g/a	T	G
G	9	15	T	g/a	c/a	a/t	G	c/t	g/a	t/c	G
H	13	24	T	g/a	A	T	G	T	g/a	t/c	G
I	14	25	T	A	A	T	G	T	G	C	G
G	15	26	T	A	A	T	G	T	g/a	t/c	G
K	18	29	T	g/a	A	T	G	T	A	C	G
L	19	30	T	A	A	T	G	T	A	t/c	G
M	20	31	T	A	A	T	G	T	A	C	G

N<sup>1</sup>: The numeral indicates the category of mutation combination(s) in the HAmCq mosquitoes identified previously in our study.  
N<sup>2</sup>: The numeral indicates the category of mutation combination(s) in the MAmCq mosquitoes.  
The predominant mutation combinations in mosquito groups of either or both HAmCq and MAmCq mosquito populations are highlighted.

these two *Culex* populations shared 13 categories of mutation combinations (Table 5), the majority of which were the predominant mutation combinations in the mosquito groups in either or both HAmCq and MAmCq mosquito populations in response to certain concentration(s) of permethrin treatments. For example, combination category F - T<sup>325</sup>, g/a<sup>2556</sup>, c/a<sup>2673</sup>, a/t<sup>2946</sup>, G<sup>3723</sup>, c/t<sup>2735</sup>, g/a<sup>3747</sup>, G<sup>5199</sup> (Table 5) - was the predominant mutation combination in group 2 of both the field parental mosquito populations of HAmCq<sup>G0</sup> (category #8) and MAmCq<sup>G0</sup> (category #14). Interestingly, this combination was also the dominant mutation combination in groups 3 and 4 of HAmCq<sup>G0</sup>, whereas combination category G - T<sup>325</sup>, g/a<sup>2556</sup>, c/a<sup>2673</sup>, a/t<sup>2946</sup>, G<sup>3723</sup>, c/t<sup>2735</sup>, g/a<sup>3747</sup>, t/c<sup>4717</sup>, G<sup>5199</sup> - was the dominant combination in groups 3 and 4 of MAmCq<sup>G0</sup>. The only difference between mutation combination categories F and G is a switch from the susceptible homozygous T<sup>4717</sup> to the heterozygous t/c<sup>4717</sup> (Table 5). The first occurrence of combination category F was in the group 2 mosquitoes of both HAmCq<sup>G0</sup> and MAmCq<sup>G0</sup>, both of which have a tolerance to permethrin concentrations of between 0.003 and 0.05 ppm, suggesting that the concentration range of 0.003 to 0.05 ppm represents a threshold, at which the T<sup>325</sup>, g/a<sup>2556</sup>, c/a<sup>2673</sup>, a/t<sup>2946</sup>, G<sup>3723</sup>, c/t<sup>2735</sup>, g/a<sup>3747</sup>, G<sup>5199</sup> mutation combination occurs in field mosquito populations. Mutation combination category M (nonuple homozygous mutations, T<sup>325</sup>, A<sup>2556</sup>, A<sup>2673</sup>, T<sup>2946</sup>, G<sup>3723</sup>, T<sup>2735</sup>, A<sup>3747</sup>, C<sup>4717</sup> and G<sup>5199</sup>) emerged in the group 4 mosquitoes of both HAmCq<sup>G0</sup> and MAmCq<sup>G0</sup> with very low frequencies of 2.5 and 5%, respectively, suggesting that permethrin concentrations between 0.1 and 0.2 ppm may represent the threshold at which the particular individuals with the mutation combination of T<sup>325</sup>, A<sup>2556</sup>, A<sup>2673</sup>, T<sup>2946</sup>, G<sup>3723</sup>, T<sup>2735</sup>, A<sup>3747</sup>, C<sup>4717</sup> and G<sup>5199</sup> could be selected from in field mosquito populations. These results strongly suggest that the same or similar mutation combinations are present in different field populations of *Cx. quinquefasciatus* mosquitoes and are responsible for similar levels of resistance, revealing the importance and common features of these combinations in the development of insecticide resistance in field mosquito populations.

Comparing mutation combinations in the permethrin-selected offspring HAmCq<sup>G8-14</sup> and MAmCq<sup>G6</sup> with those of their field parental mosquitoes HAmCq<sup>G0</sup> and MAmCq<sup>G0</sup> revealed a clear shift from the majority being heterozygous mutation combinations, for example mutation combination categories F and/or G (Table 5), in HAmCq<sup>G0</sup> and MAmCq<sup>G0</sup>, to the majority being homozygous mutation combinations, such as mutation combination category M in both HAmCq<sup>G8-14</sup> and MAmCq<sup>G6</sup>. This clear-cut pattern of mutation

combination was observed following permethrin selection across all the different mosquito populations. Although mutation combination category M was the major mutation combination in all 4 groups of both HAmCq<sup>G8</sup> and MAmCq<sup>G6</sup>, a significant shift in the prevalence of this mutation combination was also observed, rising from 12.5% in group 1 with the lowest level of tolerance to permethrin treatment, to 62.5% in group 4 with the highest level of tolerance, in HAmCq<sup>G8-14</sup> but from 42.5% in group 1 to 100% in group 4 mosquitoes of MAmCq<sup>G6</sup>. The strong correlation between the frequency of the mutation combination and its association with permethrin selection and tolerance to permethrin treatment confirmed that not only are these mutations co-selected by permethrin, but the combination of all 9 mutations is also involved in the high levels of resistance.

Insecticide resistance is generally assumed to be a pre-adaptive phenomenon in which prior to insecticide exposure rare individuals carrying an altered (varied) genome already exist, allowing those carrying the genetic variance to survive insecticide selection<sup>18</sup>. Accordingly, the proportion of individuals carrying the resistance genes, polymorphisms or alleles should increase in a population following selection through inheritance and eventually become predominate in a population subjected to prolonged exposure to insecticides. Indeed, both this study and the previous finding on HAmCq mosquitoes<sup>14</sup> show a clear permethrin selection force favoring individuals carrying the polymorphic alleles. For instance, 0.6 to 1.3% individuals carrying all nine mutations were present in the field populations of both HAmCq<sup>G0</sup> and MAmCq<sup>G0</sup>, but after a few generations of permethrin selection in the laboratory individuals carrying all 9 mutations increased to 34.4% and 72.5% in the populations of HAmCq<sup>G8</sup> and MAmCq<sup>G6</sup>, respectively.

The synergistic effects of the co-existence of insect sodium channel mutations on insecticide resistance have been previously reported by several research groups. Possibly the most notable of these is the co-presence of the methionine (M) to threonine (T) mutation (M918T), termed a *super-kdr* mutation, in the linker connecting IIS4 and IIS5, with the L to F (L1014F) mutation in IIS6 of the sodium channel in *super-kdr* house flies, which exhibit higher levels of resistance to DDT and pyrethroids than *kdr* house flies, where only the L1014F mutation is observed<sup>3,4,7</sup>. Besides the co-existence of the L1014F and M918T mutations in *super-kdr* house flies, the same combination of M-to-T and L-to-F mutations has also been observed in other insect species, namely *Haematobia irritans*<sup>19</sup>, *Thrips tabaci*<sup>20</sup>, and *Myzus persicae*<sup>21</sup>, all of which have been found to exhibit relatively high-level resistance to pyrethroids. However, these three species plus mosqui-



**Table 6 | Oligonucleotide primers\* used for amplifying the sodium channel cDNA fragments and SNP (single nucleotide polymorphism) determination**

Primer name	Function	Primer sequence (5' to 3')	Primer Location (nt)
KDR S16	cDNA fragment 1 and full length amplification	TGTTGGCCATATAGACAATGACCGA	-17 to 8
KDR AS34	cDNA fragment 1 amplification and 5' RACE	GTAATACTGACAATCCTGAACGC	2584 to 1561
PG_KDR S4	cDNA fragment 2 amplification	GCGGTAACACTCTTTCACGGC	2414 to 2435
KDR AS02	cDNA fragment 2 amplification	CCAKCCYTTRAAKGTGGCYACTTG	4411 to 4434
KDR S03	cDNA fragment 3 amplification and 3'RACE	TGAAGCTYGAACACGTGGGG	4370 to 4389
KDR AS09	cDNA fragment 3 and full length amplification	GCTTCTGAATCTGAATCAGAGGGAG	6290 to 6266
C <sub>x</sub> _SNP 2	SNaP determination	GCCACCGTAGTGATAGGAAATT	2923 to 2945
C <sub>x</sub> _SNP 4	SNaP determination	CTCGAGGATATTGACGCTTTTAC	301 to 324
C <sub>x</sub> _SNP 6	SNaP determination	TGAAGGCCATCCGCGGCCCAAG	4693 to 4716
C <sub>x</sub> _SNP 12	SNaP determination	CTTTCGCTGCTCGAGCTCGGTCT	3532 to 2555
C <sub>x</sub> _SNP 13	SNaP determination	TCCATCATGGGCCGAACGATGGG	2649 to 2672
C <sub>x</sub> _SNP 14	SNaP determination	AACTGCTACAAGCGGTTCCCGGC	3699 to 3722
C <sub>x</sub> _SNP 15	SNaP determination	GGTCCCGGCRCTGGCCGGCGA	3713 to 3734
C <sub>x</sub> _SNP 16	SNaP determination	TGGCCGGCGAYGACGACGCGCC	3725 to 3746
C <sub>x</sub> _SNP 18	SNaP determination	ATGTCATCTTCGCCATCTTCGG	5176 to 5198

toes are the only ones where the *super kdr* mutation has been reported. In other insect species, rather than the M-to-T *super kdr* mutation, there is some evidence to suggest that additional sodium channel mutations that co-exist with the L-to-F mutation are associated with high levels of resistance<sup>3-5</sup>. Although the M to T *super kdr* mutation in the linker connecting IIS4 and IIS5 was not identified in the sodium channel sequences of any of the individual mosquitoes in either the current study or the previous study on HAmCq mosquitoes<sup>14</sup>, the synonymous polymorphism of C2673A at codon G<sup>891</sup>G (corresponding to G<sup>23</sup> of the house fly *Vssc1* sodium channel protein) resulting from a single nucleotide polymorphism (SNP) of cytosine to adenine at nt 2673 (C2673A) was found in all the field parental and permethrin-selected mosquito individuals tested. The synonymous codon G<sup>891</sup>G is located in the linker connecting IIS4 and IIS5, five amino acids downstream from the methionine residue (corresponding to the position of the M918T mutation in the house fly *Vssc1* sodium channel protein (Fig. 1, <sup>7,21</sup>). Our results also showed that not only was the C2673A synonymous polymorphism almost always linked with the L<sup>982</sup>F mutation (corresponding to the position of the L1014F mutation in house fly *Vssc1*) in resistant *Culex* mosquitoes, but also that they co-presented together with other mutations in resistant mosquitoes.

As conclusion, our data, taken together with the previous finding on HAmCq mosquitoes<sup>14</sup>, combine to make a strong case linking the incidence of these 9 synonymous and nonsynonymous mutations at the RNA level with the levels of permethrin resistance in *Cx. quinquefasciatus* mosquito populations. Yet, the function of these mutations and their combinations in the sodium channel properties as well as in insecticide resistance remains further characterization. In addition, it is as yet not clear whether mutations that were identified in the *Culex* mosquito sodium channel were post-transcriptional regulated through the RNA editing as that have recently been revealed in the sodium channel of insects<sup>23-26</sup>. Thus, future research should focus on investigating the post transcriptional regulation of the mutations and function and functional interaction of these mutations in the sodium channel in terms of how they may affect the channel's structure and proteins, particularly with regard to its gating properties and the binding configurations of the sodium channel to insecticides. The precise roles of the synonymous mutations in the various sodium channel functions should also be examined in terms of protein structure formation and protein folding<sup>27</sup>, as those identified in other living systems.

## Methods

**Mosquito strains.** Three strains of mosquito *Cx. quinquefasciatus* were studied: MAmCq<sup>60</sup>, the field parental resistant strain collected from Mobile County, Alabama,

USA<sup>28</sup>; MAmCq<sup>66</sup>, the 6<sup>th</sup> generation offspring of laboratory permethrin-selected MAmCq<sup>60</sup>; and S-Lab, an insecticide-susceptible strain.

**Permethrin treatment.** Preliminary concentration ranges for larvae were utilized to generate concentration ranges of LC<sub>10</sub>, LC<sub>50</sub>, and LC<sub>90</sub> for each mosquito strain (Table 2) and then used to treat each of the *Culex* strains, MAmCq<sup>60</sup> and MAmCq<sup>66</sup>, generating 8 larval groups with different levels of resistance to the permethrin insecticide. Briefly, ~1500 4<sup>th</sup> instar larvae of each *Culex* strain were treated with permethrin at their respective LC<sub>10</sub> concentrations. Eight hours after this treatment, the dead mosquitoes were collected as group 1 of each mosquito population (i.e., MAmCq<sup>60</sup>-<LC<sub>10</sub>, or MAmCq<sup>66</sup>-<LC<sub>10</sub>). The surviving mosquitoes were then exposed to permethrin LC<sub>50</sub> concentrations. Eight hours after this treatment, the dead mosquitoes were collected as group 2 of each mosquito population (MAmCq<sup>60</sup>-LC<sub>10-50</sub>, or MAmCq<sup>66</sup>-LC<sub>10-50</sub>). The surviving mosquitoes from the permethrin LC<sub>50</sub> concentration treatment were then exposed to permethrin LC<sub>90</sub> concentrations. Eight hours after treatment, the dead and surviving mosquitoes were separately collected as group 3 (MAmCq<sup>60</sup>-LC<sub>50-90</sub>, or MAmCq<sup>66</sup>-LC<sub>50-90</sub>) and group 4 (MAmCq<sup>60</sup>->LC<sub>90</sub> or MAmCq<sup>66</sup>->LC<sub>90</sub>). Each treatment was repeated 2 times. In this study, the criterion applied was that only individuals that had all 9 mutations could be utilized for the analyses. Data from a total of 40 individual mosquitoes that met this criterion in each of the 8 groups was collected and analyzed.

**Nucleotide polymorphism (SNP) determination for the nucleotide polymorphisms in *Cx. quinquefasciatus*.** SNP determinations utilizing an ABI Prism SNaPshot Multiplex Kit were analyzed on an ABI Prism<sup>®</sup> 3100 Genetic Analyzer using Genemapper software according to the manufacturer's instructions (A&B Applied Biosystems<sup>®</sup>). Total RNAs were extracted from a pool of adult mosquitoes for each of the populations. Two replications were performed for each experiment and a total of 40 individual 4<sup>th</sup> instar larvae were used for each of permethrin treated groups with 20 for each replication. The first strand cDNAs were synthesized from each individual mosquito using the oligo(dT) primer as follows. Three PCR primer pairs, KDR S16 /KDR AS34, PG\_KDR S4/KDR AS02, and KDR S03/KDR AS09 (Table 6) were designed according to the specific sequences of the full length *Culex* sodium channel cDNAs (<sup>14</sup>accession numbers: JN695777, JN695778, and JN695779) to amplify three sodium channel cDNA fragments from each of the individual mosquitoes with polymorphisms. For each PCR reaction, the cDNA template and primer pair were heated to 94°C for 2 min, followed by 40 cycles of PCR reaction (94°C for 45 s, 60°C for 45 s and 72°C for 3 min) and a final extension of 72°C for 10 min. PCR products were then used as the templates for the SNP determination. Each PCR reaction was performed 3 times on the cDNA of each of a total of 40 individual 4<sup>th</sup> instar larvae (20 for each experimental replication) from each of the mosquito groups and for 60 individual 3 day old adults (10 males and 10 females for each experimental replication) from each mosquito population. The PCR products also served as the replication for the SNP determination of each polymorphism. Three replications of the SNP determination were carried out with different preparations of the PCR templates. To confirm that the PCR products used for the SNP determination were, in fact, *kdr* cDNA fragments, PCR products of each mosquito sample were sequenced at least once each. The alleles at the polymorphism site of each mutation were analyzed using Genemapper software according to the manufacturer's instructions and as described by Xu et al.<sup>29,30</sup>. The frequency (prevalence) of polymorphic allelic expression for each of the mutations between and among the groups or populations of the mosquitoes was also measured.

**Data analysis.** The statistically significant difference of the frequency of each of the nucleotide polymorphisms between and among the mosquito samples was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of





variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of  $P \leq 0.05$  was considered statistically significant.

Pairwise Goeman's Bayesian scores<sup>15</sup> were tested for significant correlations between resistance levels and SNP combination frequencies of the paired samples using the AssotesteR package in R<sup>16</sup> based on the recommendations for analyzing multiple SNPs in a given gene<sup>17</sup>. Data were stratified by group within each generation, with SNP combinations representing the cases. A total of 500 permutations were conducted for each pairwise comparison.

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## Author contributions

Performed the experiments: TL LZ QX. Analyzed the data: NL LZ. Contributed reagents/materials/analysis tools: NL KD WRR. Wrote the paper: NL LZ.

## Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

**Competing financial interests:** The authors declare no competing financial interests.

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