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

Ting Li1*, Lee Zhang2*, William R. Reid1, Qiang Xu1†, Ke Dong3 & Nannan Liu1

1Department of Entomology and Plant Pathology, Auburn University, Auburn, AL, USA, 2Genomics Laboratory, Auburn University, Auburn, AL, USA, 3Department of Entomology, Michigan State University, East Lansing, MI, USA.

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. 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. Among these kdr mutations, the substitution of leucine by phenylalanine [L to F], histidine [L to H], or serine [L to S] in the 6th segment of domain II (IIS6) has been clearly associated with resistance to pyrethroids and DDT in many insect species, including mosquitoes, while other kdr mutations appeared to be unique to specific species. 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, 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 collected from Huntsville, Alabama, USA and its permethrin-selected offspring HAmCq 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, 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 of *Cx. quinquefasciatus* collected from Mobile, Alabama, USA and its permethrin-selected offspring MAmCq.
The co-occurrence of both nonsynonymous and synonymous mutations in insecticide-resistant mosquitoes and their inheritance following insecticide selection was 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 (A109S, L982F, and W1573R) identified in an earlier study involving a different Cx. quinquefasciatus population (Fig. 1) in the sodium channels of the field parental population MAmCqG0 and its 6th generation permethrin-selected highly resistant MAmCqG6. The SNPs at the mutation sites were examined in 60 adult individuals from each of the MAmCqG0 and MAmCqG6 mosquito populations. All tested individuals in both populations showed expression of the polymorphic T325 allele at the codon A109S (Table 1), resulting in the substitution alanine to serine (A109S). Interestingly, 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 L982 and W1573, respectively (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 G1733G, were detected in S-lab mosquitoes; higher frequencies of the polymorphic expression were detected in MAmCqG6; and relatively low frequencies were detected in MAmCqG0. The only polymorphisms of A3723G and A5199G at the codons A1241A and G1733G showed relatively high frequencies (80% and 95%, respectively) of the polymorphic expression in MAmCqG0 (Table 1) and showed an intermediate level of allelic expression for SNPs of Culex mosquitoes; higher frequencies of the polymorphic expression were detected in MAmCqG6; and relatively low frequencies were detected in MAmCqG0. This result confirms our previous findings suggesting that synonymous polymorphisms in Culex mosquitoes 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 MAmCqG0 and its permethrin-selected offspring MAmCqG6. 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 A109S (Fig. 2, Table 3). In addition, with the exception of groups 1 and 2 in MAmCqG0, which had the lowest levels of tolerance to permethrin and showed heterozygous individuals for polymorphic allele G5199 at codon G1733G, all individuals in the tested groups across both
The field parental and permethrin-selected offspring populations were homozygous for the mutation, which is consistent with the suggestion that A199S and G275G 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 may be responsible for the initiation of moderate levels of permethrin resistance. The most noticeable mutation is the nonsynonymous C4717, which emerged starting from group 4 of MAmCqG0 and exhibited tolerance to permethrin concentrations of more than LC90 (>0.1 ppm), suggesting that this polymorphism may be the most important for the initiation of high levels of resistance.

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

| Mutation | Strain          | n* | Phenotype† | Gdons‡ [Frequency [%] ± SE] |
|----------|-----------------|----|------------|-----------------------------|
|         |                 |    |            | GCA (65 ± 5.0)              | G/TCA (35 ± 5.0) | TCA (0) |
|         |                 |    |            | GCA (0)                    | G/TCA (0)      | TCA (100) |
|         |                 |    |            | TTA (100)                  | TTA/T (0)     | TTT (0) |
|         |                 |    |            | TTA (22 ± 3.0)             | TTA/T (52 ± 6.0) | TTT (26 ± 7.5) |
|         |                 |    |            | TTA (0)                    | TTA/T (0)     | TTT (100) |
|         |                 |    |            | TGG (72 ± 10.5)            | T/C GG (25 ± 8.5) | CGG (3.0 ± 3.0) |
|         |                 |    |            | TGG (0)                    | T/C GG (8 ± 5.5) | CGG (92 ± 6.0) |
|         |                 |    |            | CTA (0)                    | CTA (38 ± 7.5) | CTA (35 ± 5) |
|         |                 |    |            | CTA (0)                    | CTA (6.5 ± 2.8) | CTA (93.5 ± 2.9) |
|         |                 |    |            | GCC (100)                  | GCC/A (5 ± 5) | GCC/G (95 ± 5) |
|         |                 |    |            | GCC (28 ± 10)              | GCC/A (42 ± 7.5) | GCC/G (30 ± 10) |
|         |                 |    |            | GCC (0)                    | GCC/A (5 ± 5) | GCC/G (95 ± 5) |
|         |                 |    |            | GCC (100)                  | GCC/A (5 ± 5) | GCC/G (95 ± 5) |
|         |                 |    |            | GCC (28 ± 10)              | GCC/A (42 ± 7.5) | GCC/G (30 ± 10) |
|         |                 |    |            | GCC (0)                    | GCC/A (5 ± 5) | GCC/G (95 ± 5) |
| A1241A* |                 |    |            | GCC (100)                  | GCC/A (5 ± 5) | GCC/G (95 ± 5) |
| D1245D* |                 |    |            | GCC (28 ± 10)              | GCC/A (42 ± 7.5) | GCC/G (30 ± 10) |
| P1249P* |                 |    |            | GCC (100)                  | GCC/A (5 ± 5) | GCC/G (95 ± 5) |
| G1733G* |                 |    |            | GCC (48 ± 12.5)            | GCC/G (52 ± 12.5) | GCC/G (95 ± 5) |

*G0 represents the parental insects collected directly from the field; G6 represents the 6th generation of permethrin-selected MAmCqG0 offspring; Values represent mean ± SE for the three replications of frequency [%] analyses for each mutation.
†The number of tested insects [three replicates for each of 10 males and 10 females].
‡Data from31.
§The nucleotide polymorphisms are underlined.
*Non-synonymous mutations.
†Synonymous mutations.

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

| Strains | n* | LC10 PPM | 1st Group (collect dead mosquitoes) | LC50 Treatment | n* | LC50 PPM | 2nd Group (collect dead mosquitoes) | LC90 Treatment | n* | LC90 PPM | 3rd Group (collect dead mosquitoes) | 4th Group (collect alive mosquitoes) |
|---------|----|----------|-------------------------------------|----------------|----|---------|-------------------------------------|----------------|----|---------|-------------------------------------|-------------------------------------|
| MAmCqG0 | 1500 | 0.003 | MAmCqG0, <LC10 | MAmCqG0, LC10–50 | 1300 | 0.01 | MAmCqG0, LC50–90 | MAmCqG0, >LC90 |
| MAmCqG6 | 1500 | 0.3   | MAmCqG6, <LC10 | MAmCqG6, LC10–50 | 1300 | 1    | MAmCqG6, LC50–90 | MAmCqG6, >LC90 |

*Each treatment was performed 3 times.
†The number of early 4th instar larvae used at the beginning of the permethrin treatment with LC10.
‡The number of early 4th instar larvae used at the beginning of the permethrin treatment with LC10.
§The number of early 4th instar larvae used at the beginning of the permethrin treatment with LC10.
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\(^{G0}\). Categories #14 (triple homozygous mutations and quintuple heterozygous mutations; T\(^{325}, g/a^{2556}, c/a^{2673}, a/t^{2946}, G^{3723}, c/t^{3735}, g/a^{3747}, G^{5199}\) were the dominant combinations in group 2 of MAmCq\(^{G0}\). 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\(^{325}, g/a^{2556}, c/a^{2673}, a/t^{2946}, G^{3723}, c/t^{3735}, g/a^{3747}, t/c^{4717}, G^{5199}\), 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\(^{G6}\) 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\(^{G6}\).

Comparing mutation combinations in permethrin-selected offspring MAmCq\(^{G6}\) with those in their field parental mosquitoes MAmCq\(^{G0}\) 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\(^{G0}\), to the majority being resistant homozygous combinations like category #31 in MAmCq\(^{G6}\) (Table 3, Fig. 3). Pairwise Goeman’s Bayesian scores\(^1\) tested using the AssotateR software package in R\(^2\) revealed the significant correlation between resistance levels of mosquito groups and their SNP combination frequencies (Table 4). A significant (P ≤ 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

---

**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\(^{G0}\) field parental populations and their 6\(^{th}\) generation permethrin-selected offspring, MAmCq\(^{G6}\). 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\(^{G0}\) 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\(^{G6}\) 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.
identifying the field mosquito population MAmCqG6, category #31
(nonuple homozygous mutations) was the predominant mutation
combination across all four groups of the permethrin-selected off-
spring MAmCqG6. A significant shift in the prevalence of this muta-
tion combination was also observed in MAmCqG6, 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 HAmCqG6 and their 8th generation permethrin-
selected offspring HAmCqG6, identifying a total of 9 mutations, 3 of
which were nonsynonymous and 6 synonymous. The prevalence of
these corresponded closely to the mosquitoes’ level of permethrin

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

| Polymorphisms at Amino Acid Mutation Sites | A109S | L852L | G891G | L982F | A1241A | D1245D | P1249P | W1573R | G1733G |
|------------------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| MAmCqG6 1 | 1 | 10 (7) | T | G | C | A | A | C | G |
| MAmCqG6 2 | 2 | 2.5 (3.5) | T | G | C | A | G | T | A/G |
| MAmCqG6 3 | 3 | 7.5 (3.5) | T | G | C | A | A/G | C | T |
| MAmCqG6 4 | 4 | 25 (10.5) | T | A | A | T | A/G | C | G |
| MAmCqG6 5 | 5 | 12.5 (3.5) | T | G | C | A | G | T | A/G |
| MAmCqG6 6 | 6 | 12.5 (3.5) | T | G | C | A | A | T | G |
| MAmCqG6 7 | 7 | 10 (0) | T | G | C | A | T | G | A/G |
| MAmCqG6 8 | 8 | 7.5 (3.5) | T | G | C | A | T | G | A/G |
| MAmCqG6 9 | 9 | 10 (0) | T | G | C | A | T | G | A/G |
| MAmCqG6 10 | 10 | 2.5 (3.5) | T | G | C | A | T | G | A/G |
| MAmCqG6 11 | 11 | 7.5 (3.5) | T | G | C | A | T | G | A/G |
| MAmCqG6 12 | 12 | 25 (0) | T | A | A | A | G | T | A/G |
| MAmCqG6 13 | 13 | 12.5 (3.5) | T | G | C | A | T | G | A/G |
| MAmCqG6 14 | 14 | 27.5 (3.5) | T | G | C | A | T | G | A/G |
| MAmCqG6 15 | 15 | 17.5 (3.5) | T | G | C | A | A/T | G | C/T |
| MAmCqG6 16 | 16 | 15 (0) | T | G | C | A | T | G | C/T |
| MAmCqG6 17 | 17 | 12.5 (3.5) | T | G | C | A | T | G | C/T |
| MAmCqG6 18 | 18 | 12.5 (3.5) | T | G | C | A | T | G | C/T |
| MAmCqG6 19 | 19 | 7.5 (3.5) | T | G | C | A | T | G | C/T |
| MAmCqG6 20 | 20 | 10 (0) | T | G | C | A | T | G | C/T |
| MAmCqG6 21 | 21 | 10 (0) | T | G | C | A | T | G | C/T |
| MAmCqG6 22 | 22 | 2.5 (3.5) | T | G | C | A | T | G | C/T |
| MAmCqG6 23 | 23 | 15 (7) | T | A | A | A | T | G | C/T |
| MAmCqG6 24 | 24 | 7.5 (3.5) | T | G | C | A | A/G | C | T |
| MAmCqG6 25 | 25 | 5 (0) | T | G | C | A | A/G | C | T |
| MAmCqG6 26 | 26 | 7.5 (3.5) | T | G | C | A | A/G | C | T |
| MAmCqG6 27 | 27 | 10 (0) | T | A | A | A | G | T | A/G |
| MAmCqG6 28 | 28 | 7.5 (3.5) | T | G | C | A | A/G | C | T |
| MAmCqG6 29 | 29 | 10 (0) | T | G | C | A | A/G | C | T |
| MAmCqG6 30 | 30 | 2.5 (3.5) | T | G | C | A | A/G | C | T |
| MAmCqG6 31 | 31 | 42.5 (10.5) | T | A | A | T | G | T | A/C |
| MAmCqG6 32 | 32 | 7.5 (3.5) | T | G | C | A | A/G | C | T |
| MAmCqG6 33 | 33 | 5 (0) | T | A | A | T | G | T | A/C |
| MAmCqG6 34 | 34 | 2.5 (3.5) | T | G | C | A | A/G | C | T |

* Group 1 mosquitoes tolerated permethrin concentration of ≤LC10 (i.e., MAmCqG6-LC10, and MAmCqG6-LC10); group 2 mosquitoes tolerated permethrin concentrations of between LC10 - LC50 (i.e.,
MAmCqG6-LC10-50, and MAmCqG6-LC10-50); group 3 mosquitoes tolerated permethrin concentrations of between LC50 - LC90 (i.e., MAmCqG6-LC50-90, and MAmCqG6-LC50-90); and group 4 mosquitoes
tolerated permethrin concentrations ≥LC90 (i.e., MAmCqG6-LC90, and MAmCqG6-LC90) (Table 2).

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 4th instar larvae) with all ten mutations in their sodium
channel cDNAs were analyzed.
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 and their combinations in individual mosquitoes of a field population of *Cx. quinquefasciatus* mosquitoes MAmcq<sup>0</sup>, collected from Mobile, Alabama, ~600 km away from the location (Huntsville, Alabama, USA) where the original HAmCq<sup>0</sup> mosquitoes were collected. 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. 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, in the resistant mosquitoes.

![Figure 3](image-url) 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>0</sup> field parental populations and their 6th generation permethrin-selected offspring, MAmcq<sup>6</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>0</sup> and MAmcq<sup>6</sup> as in Fig. 2.

| Table 4 | Pairwise Goeman’s Bayesian score test values to check for correlations between SNP combination frequencies and permethrin resistance levels |
|---------|-----------------------------------|
|         | MAmcq<sup>0</sup> | MAmcq<sup>6</sup> | MAmcq<sup>0</sup> | MAmcq<sup>6</sup> | MAmcq<sup>0</sup> | MAmcq<sup>6</sup> | MAmcq<sup>0</sup> | MAmcq<sup>6</sup> | MAmcq<sup>0</sup> | MAmcq<sup>6</sup> |
|         | 1       | 2       | 3       | 4       | 1       | 2       | 3       | 4       | 1       | 2       | 3       | 4       |
| MAmcq<sup>0</sup> | 1       | -       | -       | -       | -       | -       | -       | -       | -       | -       | -       | -       |
|         | 2       | 120*    | -       | -       | -       | -       | -       | -       | -       | -       | -       | -       |
|         | 3       | 22**    | 90*     | -       | -       | -       | -       | -       | -       | -       | -       | -       |
|         | 4       | 760**   | 450**   | 200*    | -       | -       | -       | -       | -       | -       | -       | -       |
| MAmcq<sup>6</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

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.
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 - T325, g/a2556, c/a2673, a/t2946, G 3723, c/t2735, g/a3747, G 5199 (Table 5) - was the predominant mutation combination in group 2 of both the field parental mosquito populations of HAmCq (category #8) and MAmCq (category #14). Interestingly, this combination was also the dominant mutation combination in groups 3 and 4 of HAmCq, whereas combination category G - T325, g/a2556, c/a2673, a/t2946, G 3723, c/t2735, g/a3747, t/c4717, G 5199 - was the dominant combination in groups 3 and 4 of MAmCq. The only difference between mutation combination categories F and G is a switch from the susceptible homozygous T4717 to the heterozygous t/c4717 (Table 5). The first occurrence of combination category F was in the group 2 mosquitoes of both HAmCq and MAmCq, 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 T325, g/a2556, c/a2673, a/t2946, G 3723, c/t2735, g/a3747, G 5199 combination occurs in field mosquito populations. Mutation combination category M (nonuple homozygous mutations, T223, A2356, A2673, T2946, G2735, T2735, A3747, A4717, C4717 and G4719) emerged in the group 4 mosquitoes of both HAmCq and MAmCq 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 combination mutation of T223, A2356, A2673, T2946, G2735, T2735, A3747, C4717 and G4719 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 and MAmCq with those of their field parental mosquitoes HAmCq and MAmCq revealed a clear shift from the majority being heterozygous mutation combinations, for example mutation combination categories F and/or G (Table 5), in HAmCq and MAmCq, to the majority being homozygous mutation combinations, such as mutation combination category M in both HAmCq and MAmCq. 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 and MAmCq, 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 but from 42.5% in group 1 to 100% in group 4 mosquitoes of MAmCq. 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 mutation 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. Accordingly, the proportion of individuals carrying the resistance genes, polymorphisms or alleles should increase in a population following selection through inheritance and eventually become predominating in a population subjected to prolonged exposure to insecticides. Indeed, both this study and the previous finding on HAmCq mosquitoes 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 and MAmCq, 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 and MAmCq, 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 *house flies*, the same combination of *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. 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*, *Thrips tabaci*, and *Myzus persicae*, all of which have been found to exhibit relatively high-level resistance to pyrethroids. However, these three species plus mosqui-

---

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

| Mutation Combination | Polybasic Acid Acid Sites |
|----------------------|--------------------------|
| A109S | L852L | G891G | N982F | M1241A | 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 |

N1: The numeral indicates the category of mutation combination(s) in the HAmCq mosquitoes.
N2: 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.
channel mutations that co-exist with the L-to-F mutation are assoc-

eated with the sodium channel APRA. In other insect species, the M-to-T

revision revealed in the sodium channel of insects23–26. Thus, future research

synonymous codon G891G is located in the linker connecting IIS4 and

4 amino acids downstream from the methionine residue (corresponding to the position of the M918T mutation in the house mosquito 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 mosquitoes, but also that they co-presented together with other muta-

tions and function and functional interaction of these mutations and properties and the binding configurations of the sodium channel to insecticides. The precise roles of the synonymous mutations in the sodium channel sequences of any of the individual mosquitoes in either the current study or the previous study on HAmCq mosquitoes44, the synonymous polymorphism of C2673A at codon G918G (corresponding to G922 of the house fly Vssc1 sodium channel protein) has been linked 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 mosquitoes44, the synonymous polymorphism of C2673A at codon G918G (corresponding to G922 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 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 mosquitoes44, 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 Culex mosquitoes. 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 insects23–26. 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 folding27, as those identified in other living systems.

Permethrin treatment. Preliminary concentration ranges for larvae were utilized to generate concentration ranges of LC10, LC50, and LC90 for each mosquito strain (Table 2) and then used to treat each of the Culex strains, HAmCqCq and HAmCqCq, generating 8 larval groups with different levels of resistance to the permethrin insecticide. Briefly, ~1500 4th instar larvae of each Culex strain were treated with permethrin at their respective LC10 concentrations. Eight hours after this treatment, the dead mosquitoes were collected as group 1 of each mosquito population (i.e., HAmCqCq<LC10 or HAmCqCq<LC90). The surviving mosquitoes were then exposed to permethrin LC50 concentrations. Eight hours after this treatment, the dead mosquitoes were collected as group 2 of each mosquito population (HAmCqCq<LC50 or HAmCqCq<LC50). The surviving mosquitoes from the permethrin LC50 concentration treatment were then exposed to permethrin LC90 concentrations. Eight hours after treatment, the dead and surviving mosquitoes were separately collected as group 3 (HAmCqCq<LC90 or HAmCqCq<LC90) and group 4 (HAmCqCq<LC90 or HAmCqCq<LC90). 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 3100 Genetic Analyzer using Genemapper software according to the manufacturer’s instructions (A&B Applied Biosystems). 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 4th 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 AS34, and KDR S03/KDR AS34 (Table 6) were designed according to the specific sequences of the full length Culex sodium channel cDNAs (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 2 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 4th 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.23–26. 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

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 | TGGTGCCCATATAGAAGACTGCC  | −17 to 8 |
| KDR AS34    | cDNA fragment 1 amplification and 5' RACE | GATATCTGACAATCCCTGACGTC  | 2584 to 1561 |
| PG_KDR S4   | cDNA fragment 2 amplification | GCCGTAACTACTTCCTCACGGC  | 2414 to 2435 |
| KDR AS02    | cDNA fragment 2 amplification | ACGGGAATAGACCTTGACGGGT  | 4411 to 4434 |
| KDR S03     | cDNA fragment 3 amplification and 3' RACE | CGTAACTTGGACAGTGTCGTT  | 4370 to 4389 |
| KDR AS09    | cDNA fragment 3 and full length amplification | GGCCACGTTAGTGAAGAAATT  | 2923 to 2945 |
| Cx_SNPG2    | SNaP determination | CTGGAGGATTAGACCTTGTTTAC  | 302 to 312 |
| Cx_SNPG3    | SNaP determination | TGAAGGCTACTTGAGCGAGCGG  | 4673 to 4716 |
| Cx_SNPG6    | SNaP determination | CTTTTCGTCTGTAACGTCGTCT  | 3532 to 2555 |
| Cx_SNPG13   | SNaP determination | GCATCCGGAGCGGACGCGGC  | 2649 to 2672 |
| Cx_SNPG14   | SNaP determination | AACTCTAACAGGCAGCTCCGGG  | 3699 to 3722 |
| Cx_SNPG15   | SNaP determination | GGTTCCCGGCCGCTGCGCCGA  | 3713 to 3734 |
| Cx_SNPG16   | SNaP determination | TGCCGCGCGYACGAAACCGCC  | 3725 to 3746 |
| Cx_SNPG18   | SNaP determination | ATGTTCTACCTGCGCCATCTGGG  | 5176 to 5198 |

Methods

Mosquito strains. Three strains of mosquito Cx. quinquefasciatus were studied: HAmCqCq, the field parental resistant strain collected from Mobile County, Alabama, USA25, MAAMqCqCq, the 6th generation offspring of laboratory permethrin-selected MAAMqCq, and S-Lab, an insecticide-susceptible strain.

Three strains of mosquito Cx. quinquefasciatus were studied: HAmCqCq, the field parental resistant strain collected from Mobile County, Alabama, USA23, MAAMqCqCq, the 6th generation offspring of laboratory permethrin-selected MAAMqCq, and S-Lab, an insecticide-susceptible strain.

USA25, MAAMqCqCq, the 6th generation offspring of laboratory permethrin-selected MAAMqCq, and S-Lab, an insecticide-susceptible strain.

USA25, MAAMqCqCq, the 6th generation offspring of laboratory permethrin-selected MAAMqCq, and S-Lab, an insecticide-susceptible strain.

USA25, MAAMqCqCq, the 6th generation offspring of laboratory permethrin-selected MAAMqCq, and S-Lab, an insecticide-susceptible strain.
1. Hemingway, J., Field, L. & Vontas, J. An overview of insecticide resistance. Sci. 298, 96–97 (2002).
2. Soderlund, D. M. & Bloomquist, J. R. Molecular mechanisms of insecticide resistance. In Pesticide Resistance in Arthropods, eds Roush, R. T. & Tabashnik, B. E. (Chapman and Hall, New York), pp 58–96 (1990).
3. Soderlund, D. M. Sodium channels. In Comprehensive Molecular Insect Science, Pharmacology, eds Gilbert, L. I., Iatrou, K. & Gill, S. S. (Elsevier, Pergamon), 5, 1–24 (2005).
4. Dong, K. Insect sodium channels and insecticide resistance. Invert. Neurosci. 7, 17–30 (2007).
5. Davies, T. G. E., Field, L. M., Usherwood, P. N. R. & Williamson, M. S. DDT, pyrethrin, pyrethroids and insect sodium channels. JUBMBSLife 59, 151–162 (2007).
6. Davies, T. G. E., O’Reilly, A. O., Field, L. M., Wallace, B. A. & Williamson, M. S. Knockdown resistance to DDT and pyrethroids: from target-site mutations to molecular modeling. Pest. Manag. Sci. 64, 1126–1130 (2008).
7. Williamson, M. S., Martínez-Torres, D., Hick, C. A. & Devonshire, A. L. Identification of mutations in the house fly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. Mol. Gen. Genet. 252, 51–60 (1996).
8. Martínez-Torres, D. et al. Voltage-dependent Na+ channels in pyrethroid-resistant Culex pipiens L. mosquitoes. Pestic. Sci. 55, 1012–1020 (1999).
9. Xu, Q., Wang, H., Zhang, L. & Liu, N. Kdr allelic variation in pyrethroid resistant mosquitoes, Culex quinquefasciatus (S.). Biochem. Biophys. Res. Commun. 345, 774–780 (2006).
10. Kawada, H. et al. Distribution of a knockdown resistance mutation (L1014S) in Anopheles gambiæ s.s. and Anopheles arabiensis in western and southern Kenya. PLoS ONE 6(9), e24323. doi:10.1371/journal.pone.0024323 (2011).
11. Burton, M. J. et al. Differential resistance of insect sodium channels with kdr mutations to deltamethrin, permethrin and DDT. Insect Biochem. Mol. Biol. 41, 723–732 (2011).
12. Usherwood, P. N. R. et al. Mutations in DIIS5 and the DIIS4-S5 linker of Drosophila melanogaster sodium channel define binding domains for pyrethroids and DDT. FEBS Lett. 581, 5485–5492 (2007).
13. Du, Y. et al. Molecular determinants on the insect sodium channel for the specific action of type II pyrethroid insecticides. Toxicol. Appl. Pharmacol. 234, 266–272 (2009).
14. Xu, Q. et al. Evolutionary adaptation of the amino acid and codon usage of the mosquito sodium channel following insecticide selection in the field mosquitoes. PLoS One 7(10), e47609. doi:10.1371/journal.pone.0047609 (2012).
15. Goeman, J. J., van de Geer, S. A. & van Houwelingen, H. C. Testing against a high dimensional alternative. J. Royal. Statistical. Society 68, 477–493 (2006).
16. Sanchez, G. Assotester: Statistical tests for genetic association studies. R package version 0.1-1. http://CRAN.R-project.org/package=Assotester (2012).
17. Chapman, J. & Whitaker, J. Analysis of multiple SNPs in a candidate gene or region. Genet. Epidemiol. 32, 560–566 (2008).
18. World Health Organization Expert committee on insecticides. WHO Tech. Rpt. Ser. 7th Rpt (1957).
19. Thi Tran, H. T. et al. A G-to-A transition at the fifth position of intron-32 of the dystrophin gene inactivates a splice donor site both in vivo and in vitro. Mol. Genet. Metab. 85, 213–219 (2005).
20. Toda, S. & Morishita, M. Identification of three point mutations on the sodium channel gene in pyrethroid-resistant Tribolium castaneum (Thysanoptera: Thripidae). J. Econ. Entomol. 102, 2296–2300 (2009).
21. Eleftherianos, I., Foster, S. P., Williamson, M. S. & Denholm, I. Characterization of the M918T sodium channel gene mutation associated with strong resistance to pyrethroid insecticides in the peach-potato aphid, Myzus persicae (Sulzer). Bull. Entomol. Res. 98, 183–191 (2008).
22. Inglez, P. J., Adams, P. M., Knipple, D. C. & Soderlund, D. M. Characterization of voltage-sensitive sodium channel gene coding sequences from insecticide-susceptible and knockdown-resistant house fly strains. Insect Biochem. Mol. Biol. 26, 319–326 (1996).
23. Liu, Z., Song, W. & Dong, K. Persistent tetrodotoxin-sensitive sodium current resulting from U-to-C RNA editing of an insect sodium channel. Proc. Nat. Acad. Sci. USA. 101, 11862–11867 (2004).
24. Palladino, M. J., Keegan, L. P., O’Connell, M. A. & Reenan, R. A. A-to-I pre-mRNA editing in Drosophila is primarily involved in adult nervous system function and integrity. Cell 102, 437–449 (2000).
25. Reenan, R. A. Molecular determinants and guided evolution of species-specific RNA editing. Nature 434, 409–413 (2005).
26. Song, W., Liu, Z., Tan, J., Nomura, Y. & Dong, K. RNA editing generates tissue-specific sodium channels with distinct gating properties. J. Biol. Chem. 30, 32554–32561 (2004).
27. Kimchi-Sarfaty, C. et al. A “silent” polymorphism in the MDRI gene changes substrate specificity. Sci. 315, 525–528 (2007).
28. Liu, H., Cupp, E. W., Michier, K. M., Guo, A. & Liu, N. Insecticide resistance and cross-resistance in Alabama and Florida strains of Culex quinquefasciatus. J. Med. Entomol. 41, 408–413 (2004).
29. Xu, Q., Wang, H., Zhang, L. & Liu, N. Sodium channel gene expression associated with pyrethroid resistant house flies and German cockroaches. Gene 379, 62–67 (2006).
30. Xu, Q., Tian, L., Zhang, L. & Liu, N. Sodium channel genes and their differential expression in the mosquito Culex quinquefasciatus. J. Med. Entomol. 47, 1127–1134 (2010).

Acknowledgements
We sincerely thank Drs. C. Kimchi-Sarfaty, M. Gottesman, S. Ambudkar, Z. Sauna, K. L. Fung and Mr. P. Lund for their critical reviews and comments on previous versions of the manuscript. The project described was supported by Award Number R21AI090303 from the National Institute of Allergy and Infectious Diseases, and AAES Hatch/Multistate Grants ALA08-045 and ALA015-1-10026 to N.L.

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.

License: This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivative Works 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/

How to cite this article: Li, T. et al. Multiple mutations and mutation combinations in the sodium channel of permethrin resistant mosquitoes, Culex quinquefasciatus. Sci. Rep. 2, 781; DOI:10.1038/srep00781 (2012).