Point Mutations Associated with Organophosphate and Carbamate Resistance in Chinese Strains of *Culex pipiens quinquefasciatus* (Diptera: Culicidae)

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Abstract

Acetylcholinesterase resistance has been well documented in many insects, including several mosquito species. We tested the resistance of five wild, Chinese strains of the mosquito *Culex pipiens quinquefasciatus* to two kinds of pesticides, dichlorvos and propoxur. An acetylcholinesterase gene (*ace1*) was cloned and sequenced from a pooled sample of mosquitoes from these five strains and the amino acids of five positions were found to vary (V185M, G247S, A328S, A391T, and T682A). Analysis of the correlation between mutation frequencies and resistance levels (LC50) suggests that two point mutations, G247S (r² = 0.732, P = 0.065) and A328S (r² = 0.891, P = 0.016), are associated with resistance to propoxur but not to dichlorvos. Although the V185M mutation was not associated with either dichlorvos or propoxur resistance, its RS genotype frequency was correlated with propoxur resistance (r² = 0.815, P = 0.036). And the HWE test showed the A328S mutation is linked with V185M, also with G247S mutation. This suggested that these three mutations may contribute synergistically to propoxur resistance. The T682A mutation was negatively correlated with propoxur (r² = 0.788, P = 0.045) resistance. Knowledge of these mutations may help design strategies for managing pesticide resistance in wild mosquito populations.

Introduction

Acetylcholinesterase (*AChE, EC 3.1.1.7*) is a key enzyme in the nervous system of both vertebrates and invertebrates that terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine (*ACh*) released from the presynaptic membrane [1]. The inhibition of *AChE* by organophosphate and carbamate insecticides leads to the desensitization of the *ACh* receptor, thereby blocking nerve signal transmission. Organophosphates and carbamates have structures analogous to *ACh* and inhibit *AChE* competitively at the active site. Hydrolysis of these pesticide compounds retards the reactivation of the enzyme or inactivates it [2]. The extensive use of organophosphate and carbamate insecticides has resulted in the development of high levels of resistance to them among insects [3,4,5,6].

*Ace1* is the key *AChE* gene in insects. Several studies have found evidence that a point mutation in the *ace1* gene is associated with resistance to organophosphate and carbamate pesticides. This point mutation changes the structure of *AChE* making it insensitive to these insecticides. The first report of this mutation conferring insecticide resistance was in the two-spotted spider mite in 1964 [7]. Subsequent studies have demonstrated that many insect species have developed resistance to organophosphate and carbamate pesticides through decreased sensitivity of *AChE* [8], including many mosquito species, such as *Anopheles gambiae* [9], *Cx. pipiens* [10,11], *Cx. pipiens quinquefasciatus* [12], *Cx. tritaeniorhynchus* and *Cx. vishnui* [13]. However, so far, only three *ace1* mutations, G1198S, F331W and P290V (T. californica numbering) [13,14,15,16], have been confirmed to be involved in such resistance in mosquito species. Determining the mutations that confer resistance to specific pesticides is important to designing effective strategies for managing pesticide resistance. *Cx. pipiens quinquefasciatus* is the main mosquito species in urban environments in southern China and one of the most studied in terms of insecticide resistance. We here report the results of an investigation of mutations in the *ace1* gene in five wild Chinese populations of *Cx. pipiens quinquefasciatus*. Knowledge of these mutations may have practical benefits for reducing pesticide resistance in this species.

Results

Resistance of the Five Mosquito Populations to Dichlorvos and Propoxur

LC50 values of the five different populations ranged from 0.266 to 1.67 ppm for dichlorvos, and from 0.279 to 1.27 ppm for propoxur (Table 1). The HC strain had the lowest LC50 and was...
Table 1. Levels of dichlorvos and propoxur resistance in five populations of Cx. pipiens quinquefasciatus.

| Population | Insecticide | LC50 and LC90 (ppm) (95% CL) | Regression Equation | Standard Deviation | Slope | Confidence Limits |
|------------|-------------|------------------------------|---------------------|-------------------|-------|------------------|
| LA         | Dichlorvos  | 0.095 (0.094, 0.122)        | Y = 0.040 + 2.827x  | 2.827             | 0.197 | 1.955, 3.672    |
|            | Propoxur    | 0.115 (0.095, 0.150)       | Y = 0.040 + 2.827x  | 2.827             | 0.197 | 1.955, 3.672    |
| GN         | Dichlorvos  | 1.189 (1.050, 1.521)       | Y = 0.212 + 2.827x  | 2.827             | 0.197 | 1.955, 3.672    |
|            | Propoxur    | 1.266 (1.073, 1.595)       | Y = 0.212 + 2.827x  | 2.827             | 0.197 | 1.955, 3.672    |
| HP         | Dichlorvos  | 0.750 (0.661, 0.853)       | Y = 0.306 + 2.827x  | 2.827             | 0.197 | 1.955, 3.672    |
|            | Propoxur    | 0.266 (0.224, 0.309)       | Y = 0.212 + 2.827x  | 2.827             | 0.197 | 1.955, 3.672    |
| HC         | Dichlorvos  | 0.266 (0.224, 0.309)       | Y = 0.212 + 2.827x  | 2.827             | 0.197 | 1.955, 3.672    |
|            | Propoxur    | 0.266 (0.224, 0.309)       | Y = 0.212 + 2.827x  | 2.827             | 0.197 | 1.955, 3.672    |
| QB         | Dichlorvos  | 1.240 (1.051, 1.464)       | Y = 0.174 + 2.827x  | 2.827             | 0.197 | 1.955, 3.672    |
|            | Propoxur    | 0.598 (0.559, 0.639)       | Y = 0.174 + 2.827x  | 2.827             | 0.197 | 1.955, 3.672    |
| SF         | Dichlorvos  | 1.672 (1.520, 1.822)       | Y = 0.687 + 3.076x  | 3.076             | 0.197 | 1.955, 3.672    |
|            | Propoxur    | 0.785 (0.738, 0.837)       | Y = 0.687 + 3.076x  | 3.076             | 0.197 | 1.955, 3.672    |

Identification of Ace1 Mutations

To identify mutations in the ace1 gene, the cDNA of a pooled sample of mosquitoes from each of the five populations was cloned and sequenced. Five mutations (V185M, A328S, A391T, and T682A) in the pooled ace1 gene were identified (Figure 1), and the sequence was deposited in GenBank under the accession number KF680946. Note that this identification of 5 mutations does not imply all occur in the same ace1 gene. The V185M mutation was GTG to ATG, the G247S mutation was GGC to AGC, the A328S mutation was GCC to TCC, the A391T mutation was GCC to ACC, and the T682A mutation was ACA to GCA.

Polymorphism of the Ace1 Gene in Natural Population

1. Determination of the allele frequencies. The allele frequencies of each mutation were determined by specific PCR amplification using the primers Cx-ace2-F, Cx-ace2-R and Cx-ace3-F, Cx-ace3-R on the cDNA obtained from individual mosquitoes. Genotypes of each mosquito in each population was determined by sequencing, and mutation frequencies (R%) computed (Table 2). We can see from Table 2 that the V185M, A328S and T682A mutations were present at different frequencies in all five strains. However, the A391T mutation was only found in the HP and QB strains, and the G247S mutation was found in all but the HC strain.

2. Hardy–Weinberg Equilibrium (HWE) test and genetic linkage analysis of the mutations. The results of GENEPOP software analysis of HWE and genetic linkage of the ace1 gene were shown in Tables 2 and 3. The HWE test indicates the QB and GN populations have a heterozygote deficit with respect to the laboratory strain (P< 0.05), and the HP population a heterozygote excess with respect to the A391T mutation (P<0.05). Mutations in all other populations did not deviate from the HWE and none of the five mutations deviated from the HWE across all populations (P> 0.05).

Results of linkage disequilibrium analysis of the five mutations are shown in Table 3. Evidence of linkage disequilibrium was found for V185M with respect to the A328S and A391T mutations (P<0.05). The G247S and A328S mutations' linkage disequilibrium P-value was 0.0821, only slightly above 0.05. This suggests that these two mutations might exist in the same gene. Our sequencing data indicated that these two mutations do indeed occur in the same ace1 gene in some mosquitoes. But the conclusion had to be confirmed by more data. All other gene polymorphism was randomly distributed.

Correlation of Resistance with Mutation Frequencies

The correlation between resistance to dichlorvos and propoxur and the frequencies of four mutations (V185M, G247S, A328S, T682A) are shown in Figure 2 and Table 4. The four mutations' frequencies were all not significantly correlated with dichlorvos resistance. Although the frequency of the V185M mutation was the most susceptible to both dichlorvos and propoxur. The SF strain had an LC50 to dichlorvos of 1.67 ppm and was 17.6 times more resistant to dichlorvos than the laboratory strain (LC50 0.095 ppm). The GN strain had an LC50 to propoxur of 1.27 ppm and was 11.0 times more resistant to propoxur than the laboratory strain (LC50 0.115 ppm). The HP strain was 7.89 times more resistant to dichlorvos, and 4.62 times more resistant to propoxur, than the laboratory strain. The QB strain was 13.1 times more resistant to dichlorvos, and 5.20 times more resistant to propoxur than the laboratory strain.
uncorrelated with propoxur resistance (Figure 2 A), its RS genotype frequency was ($r^2 = 0.815$, $P = 0.036$) (Figure 2 B). The correlation between the frequency of the G247S mutation and propoxur resistance was close to significance ($r^2 = 0.732$, $P = 0.065$), and there was a significant linear relationship between the frequency of the A328S mutation and propoxur resistance ($r^2 = 0.891$, $P = 0.016$) (Figure 2 C, D). The frequency of the T682A mutation was negatively correlated with propoxur ($r^2 = 0.788$, $P = 0.045$) resistance (Figure 2 E).

3D Models of Mutations and Structural changes at the Catalytic Site

A 3D model was made of the Cx. pipiens quinquefasciatus ace1 gene sequence allowing the location and structure of four mutations to be visualized (Figure 3). The V185M and A391T mutations are distant from the active site of the enzyme-catalytic triad (S327, H567, E453; S200, H440, E327 in T. californica) (Figure 3A, B). The other two mutations, G247S and A328S, are close to the catalytic site (Figure 3C, D) and could therefore potentially affect the binding between AChE and its substrates (Ach: ZINC3079336 and propoxur: ZINC1590885). Figure 3E-H illustrates the change in amino acids and H-bonds associated with the G247S and A328S mutations. These two substitutions change the amino acids present at catalytic sites removing the two H-bonds (S327(8)Oc-O3, S327(8)Oc-O4) between AChE and Ach (Figure 3E, F) and reducing the three H-bonds between AChE and propoxur (G247(4)-O13, S327(8)Oc-O11, H567(14)-NH27) to one (S327(8)Oc-NH27) (Figure 3G, H). Hence, these two mutations could have a major effect on the catalytic activity of the AChE enzyme.

Discussion

The indiscriminate use of insecticides over more than half a century has resulted in high levels of insecticide resistance in many mosquito species [13,17,18]. We tested the resistance of five Chinese Cx. pipiens quinquefasciatus populations to dichlorvos and propoxur. Our results show that, compared to a laboratory strain, these five populations displayed a 2.80- to 17.6-fold resistance to dichlorvos and 2.43- to 11.0-fold resistance to propoxur. The frequent use of these insecticides has created an intense selection pressure for traits that confer resistance to them, such as changes in behavior, epidermal structure, metabolic enzymes and target site mutations. Resistance may be conferred by any one, or more than one of these mechanisms. Osta et al (2012) found that the dramatic reduction in the frequency of the G119S (T. californica numbering) mutation in Culex pipiens mosquitoes was probably due to the increased use of pyrethroids over organophosphate insecticides [19]. Therefore, alternating between different kinds of insecticides is one way of minimizing the development of resistance to any one kind.

We used cloning and sequencing to identify five point mutations in the ace1 gene of Chinese Cx. pipiens quinquefasciatus. HWE tests suggest that these five mutations do not deviate from the HWE across all populations. However, the tests also indicated that the QB and GN populations were deficient in heterozygotes with respect to the T682A mutation and that HP population had an excess of heterozygotes with respect to the A391T mutation ($P < 0.05$). Further work will be required to determine the reasons for these departures from the HWE. Linkage disequilibrium analysis indicated significant linkage between the V185M mutation and the A328S and A391T mutations. Although linkage between other mutations was statistically insignificant, that between the G247S and A328S mutations was nearly so ($P = 0.0021$). Our sequencing...
Table 2. Mutation frequencies of five ace1 gene mutations and HWE test in five populations of *Cx. pipiens quinquefasciatus*.

| Mutations | Strains | Numbers | Mutation frequency (R %) | P-value of HWE | HWE across strains |
|-----------|---------|---------|--------------------------|----------------|--------------------|
|           |         |         |                          | deficit | excess | $\chi^2$ | P     |
| V185M     | GN      | 36      | 25.0                     | 1.00    | 0.06   | 9.84    | 0.45  |
|           | HP      | 33      | 6.10                     | 1.00    | 0.91   |         |       |
|           | HC      | 30      | 16.7                     | 0.15    | 0.99   |         |       |
|           | QB      | 31      | 11.3                     | 1.00    | 0.68   |         |       |
|           | SF      | 30      | 23.3                     | 0.50    | 0.84   |         |       |
| G247S     | GN      | 36      | 18.1                     | 0.73    | 0.70   | 0.00    | 1.00  |
|           | HP      | 33      | 1.50                     | No$^1$  | No     |         |       |
|           | HC      | 30      | 0.00                     | No     | No     |         |       |
|           | QB      | 30      | 11.7                     | 1.00    | 0.67   |         |       |
|           | SF      | 30      | 5.00                     | 1.00    | 0.95   |         |       |
| A328S     | GN      | 36      | 47.2                     | 0.83    | 0.39   | 1.77    | 1.00  |
|           | HP      | 33      | 19.7                     | 0.77    | 0.66   |         |       |
|           | HC      | 34      | 2.90                     | 0.73    | 0.53   |         |       |
|           | QB      | 30      | 11.7                     | 1.00    | 0.67   |         |       |
|           | SF      | 30      | 16.7                     | 1.00    | 0.41   |         |       |
| A391T     | GN      | 15      | 0.00                     | No     | No     | 7.79    | 0.10  |
|           | HP      | 22      | 47.7                     | 1.00    | 0.02   |         |       |
|           | HC      | 13      | 0.00                     | No     | No     |         |       |
|           | QB      | 23      | 54.3                     | 0.84    | 0.45   |         |       |
|           | SF      | 22      | 0.00                     | No     | No     |         |       |
| T682A     | GN      | 35      | 18.6                     | 0.01    | 1.00   | 18.0    | 0.06  |
|           | HP      | 32      | 51.6                     | 0.73    | 0.53   |         |       |
|           | HC      | 36      | 48.6                     | 0.90    | 0.28   |         |       |
|           | QB      | 33      | 39.4                     | 0.03    | 1.00   |         |       |
|           | SF      | 31      | 24.2                     | 0.89    | 0.44   |         |       |

$^1$No is no information, the reasons are because the site is homozygous for one mutation in this sample or because there is a single heterozygote.

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results suggest that these two mutations occur within the same ace1 gene in some mosquitoes but further work is required to confirm this hypothesis.

These results are the first report of the V185M mutation in *Cx. pipiens quinquefasciatus*. Although there was no apparent correlation between the frequency of this mutation and resistance to dichlorvos and propoxur, the frequency of its RS genotype was significantly correlated with propoxur resistance ($r^2 = 0.815$, $P = 0.036$). Although the 3D model indicates that V185M is located far from the active site, the positive linear relationship between its RS genotype frequency and propoxur resistance, and its apparent linkage with the A328S mutation suggest that it may be involved in propoxur resistance. Of course, we cannot rule out the possibility that insecticide resistance involves multiple duplication of the ace1 gene. Further research needs be required to determine how this might affect the catalytic center.

Our results (Figure 2, Table 4) suggest that the G247S mutation is not associated with propoxur resistance and that the A328S mutation is. The G247S mutation corresponds to G119S in *T. californica* which has been associated with insecticide resistance in mosquitoes by several authors [20,21]. The G119 position is part of the oxyanion hole (G118, G119, and A201 in *T. californica*), close to the catalytic Serine (S200) where a G to S substitution would reduce accessibility to inhibitors and substrate by steric hindrance. S119 is close enough to the catalytic residues to alter the presentation of inhibitors and substrates. This could be the reason this mutation confers resistance to some insecticides [22,23]. Although the correlation between the frequency of the G247S

| Locus pair         | $\chi^2$ | df | P-Value |
|--------------------|----------|----|---------|
| V185M & G247S      | 11.237   | 8  | 0.1887  |
| V185M & A328S      | 23.804   | 10 | 0.0081  |
| G247S & A328S      | 13.988   | 8  | 0.0821  |
| V185M & A391T      | 7.5840   | 2  | 0.0226  |
| G247S & A391T      | 3.4992   | 2  | 0.1738  |
| A328S & A391T      | 5.8691   | 4  | 0.2091  |
| V185M & T682A      | 4.8208   | 10 | 0.9028  |
| G247S & T682A      | 2.4273   | 6  | 0.8765  |
| A328S & T682A      | 14.160   | 10 | 0.1658  |
| A391T & T682A      | 1.1946   | 4  | 0.8790  |
| V185M & T682A      | 7.5840   | 2  | 0.0226  |
| G247S & T682A      | 3.4992   | 2  | 0.1738  |
| A328S & T682A      | 5.8691   | 4  | 0.2091  |
| A391T & T682A      | 14.160   | 10 | 0.1658  |
| V185M & T682A      | 2.4273   | 6  | 0.8765  |
| G247S & T682A      | 1.1946   | 4  | 0.8790  |
| A328S & T682A      | 7.5840   | 2  | 0.0226  |
| A391T & T682A      | 3.4992   | 2  | 0.1738  |
| V185M & T682A      | 5.8691   | 4  | 0.2091  |
| G247S & T682A      | 14.160   | 10 | 0.1658  |
| A391T & T682A      | 1.1946   | 4  | 0.8790  |

Figure 2. Linear regression of the relationship between resistance levels (LC50) and mutation ratios. Resistance levels to propoxur are plotted against the ratios of V185M (A), the RS ratio of V185M (B), G247S (C), A328S (D), and T682A (E). doi:10.1371/journal.pone.0095260.g002
mutation and propoxur resistance was not statistically significant (P = 0.065), numerous prior publications have reported such an association [9,10,12] and noted that this mutation is often combined with other mutations in resistant strains. Therefore, we suspect that G247S probably is involved in propoxur resistance. We may have failed to detect a significant correlation between the frequency of this mutation and resistance because of its low frequency in our sample, which could be because most mosquitoes carrying it were heterozygotes. Furthermore, the resistance conferred by this mutation may be nearly recessive under certain bioassay conditions [24].

The A328 position corresponds to the A201 position in T. californica, which is located within the active gorge of the enzyme, close to the catalytic site, and is a part of the oxyanion hole. Li et al (2009) also found the A328S mutation in Cx. pipiens pallens and made a three-dimensional model of AChE to visualize this mutation. However, they did not demonstrate a relationship between the A328S mutation and resistance [25]. Khajehali et al (2009) also found the A328S mutation in Cx. pipiens quinquefasciatus and Cx. pipiens pallens, which is located within the active gorge of the enzyme, and that the frequency of this mutation was around 0.500. The genetic linkage analysis indicates a linkage between this mutation and V185M, however, in view of the small sample size further work is required to confirm this. The three-dimensional model revealed that the A391 mutation is distant from the active site. This indicates that this mutation is unlikely to affect catalytic activity and is probably not involved in dichlorvos and propoxur resistance. How this mutation developed and its function, if any, in pesticide resistance requires further investigation.

Our results provide the first evidence of the T682A mutation in Cx. pipiens quinquefasciatus. The frequency of this mutation was negatively correlated with propoxur resistance (r² = 0.788, P = 0.045). Fournier et al (1988) found that AChE in Drosophila melanogaster was composed of two, non-covalently associated, polypeptides of 55 and 16 kDa. AChE is an amphipathic protein linked to the membrane of neuronal cholinergic synapses via a glycolipid anchor at the C-terminal end of the 55 kDa polypeptide [31]. Nabeshima et al (2004) found an I697M replacement near the C-terminus (Ile701) in Culex tritaeniorhynchus, but considered that this was unlikely to be the cause of AChE insensitivity [32]. Our results also indicate that the T682A mutation is near the C-terminus of AChE, and that the frequency of this mutation is negatively correlated with propoxur resistance. Despite its negative correlation with resistance, it’s possible that this mutation may change the C-terminus structure of AChE thereby reducing its attachment to the membrane and the stability of enzyme. We don’t know whether this mutation works in combination with the other four mutations or not, or if its apparent negative relationship with resistance is related to fitness costs.

In conclusion, we found five ace1 gene mutations in Cx. pipiens quinquefasciatus that are correlated with propoxur, but not dichlorvos resistance. The V185M mutation was first confirmed in Cx. pipiens quinquefasciatus and may be involved in propoxur resistance. The allele frequencies of the G247S and A328S mutations were positively correlated with resistance. Therefore, the G247S and A328S mutations are also likely to confer propoxur resistance. The A391T mutation appears unrelated to dichlorvos and propoxur resistance and the T682A mutation appears negatively correlated with resistance to propoxur. Identifying the mutations that confer resistance to specific insecticides can inform the choice of insecticides for a given insect population, thereby reducing the development of resistance and improving the efficacy of control.

### Table 4. The analysis results of correlation between propoxur LC50 and mutation frequencies.

| Mutations | Insecticide | R (95% CL) | R² | P | Significance |
|-----------|-------------|-----------|----|---|-------------|
| V185M     | Propoxur    | 0.647(−0.549,0.974) | 0.419 | 0.238 | No          |
| V185M (R5%)| Propoxur    | 0.903(0.101,0.994) | 0.815 | 0.036 | Yes         |
| G247S     | Propoxur    | 0.855(−0.110,0.990) | 0.732 | 0.065 | No          |
| A328S     | Propoxur    | 0.944(0.366,0.996) | 0.891 | 0.016 | Yes         |
| T682A     | Propoxur    | −0.887(−0.993,−0.023) | 0.788 | 0.045 | Yes         |

1CL = confidence limits.

2r² > 0.05.

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Figure 3. Three dimensional model of the AChE of *Culex pipiens quinquefasciatus* based on the structure of *T. californica* (PDB: 3ZV7). The four mutations are shown as red, green and blue van der Waals spheres and the catalytic triad (S327, H567, E453; S200, H440, E327 in *T. californica*) is shown in yellow. A–D illustrates the four mutations. A shows the V185 and A391 positions and B the M185 and T391 mutations. C and D show the G247S and A328S mutations, and the catalytic triad. E–H shows changes in the enzyme–substrate complex; Ach (E, F) and propoxur (G, H) are shown in green and the H-bond as yellow dotted lines. Amino acids are marked with numbers. E1–14 (wild-type enzyme) are W212, G245, G246, G247, Y249, Y258, E326, S327, F416, Y456, F457, H567, G568, I571 respectively; The two H-bonds were composed of S327 Ogamma and O3, S327 Ogamma and O4. F1–14 (G247S/A328S mutant) are W212, F244, G245, G246, S250, G251, T252, L255, Y258, S327, Y456, H567, G568, I571 respectively; G1–16 (wild-type enzyme) are W212, G245, G246, G247, Y249, Y258, E326, S327, W360, F416, Y456, F457, F527, H567, G568, I571 respectively; The three H-bonds were composed of G247(4) NH and O13, S327(8) Ogamma and O11, H567(14) and NH27. H1–17 (G247S/A328S mutant) are W212, F244, G245, G246, S247, Y249, L255, Y258, E326, S327, W360, F416, Y456, F457, F527, H567, I571 respectively. The only H-bond was composed of S327(10) Ogamma and NH27.

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Materials and Methods

Statement of Ethical Approval

No ethical approval was required as no regulated animals were used in this study. Pre-permission (May–September 2012) was granted for observation, collection and field research on mosquitoes in Guangdong and Hainan Provinces, which was conducted as part of the Infective Diseases Prevention and Cure Project of the China National Ministry of Public Health (No.2008ZX10004 and No.2012ZX10004219). All field studies on Cx. pipiens quinquefasciatus were authorized by Guangdong and Hainan Provincial CDC Committees for Animal Welfare and Animal Ethics (address: 176 Xingang West Road, Guangzhou, Guangdong province, and 44 Haifu Road, Haikou, Hainan province, P. R. China).

Mosquito Strains

Specimens of Cx. pipiens quinquefasciatus were collected from five different field sites; Guangzhou Nansha (E113°29′29.35″, N22°48′4.13′′) and Haikou Poxiang (E110°19′33.79″, N19°59′55.07″) in May 2012, and Haikou Changliu (E110°11′50.36″, N20°0′50.25″), Qionghai Boao (E110°34′57.13″, N19°09′42.07″) and Sanya Fenghuang (E109°26′54.38″, N18°18′2.91″) in September 2012. The susceptible strain had been reared in an insectarium for more than 10 years without exposure to any insecticides.

Bioassay

Bioassays were conducted by putting thirty late 3rd or early 4th instar larvae into pans containing 200 ml water. Measured quantities of insecticides were added to each pan using an automatic pipette according to the methods specified by the WHO [33]. Larval mortality was recorded 24 h after each treatment. No food was offered to larvae during bioassays. Larvae were maintained in the laboratory under a 14L:10D photoperiod, 75% relative humidity and temperature of 26±1°C during bioassays. Bioassays of each insecticide were repeated three times. Statistical analyses were performed using SPSS software version 13.

Extraction of RNA and cDNA Synthesis

Total RNA was extracted from specimens from each population with Trizol reagent (GIB) following the manufacturer’s protocol and cDNA synthesized from the total RNA using cDNA synthesis kit (TaKaRa). The cDNA was stored at −20°C.

PCR Amplification

Gene specific primers based on the published insecticide resistant sequence of the Cx. pipiens quinquefasciatus ace1 gene (GenBank Accession No.:CQ753634.1, this includes a G119S mutation related to propoxur resistance) were designed in NCBI-Primer-BLAST and used to amplify the ace1 gene of each population. The ace1 gene is 2109 bp and is divided into three sections (Figure 4). The primers used are shown in Table 5.

Cloning and Sequencing of PCR Products

PCR products were purified using a universal DNA purification kit (TIANGEN) and the purified products were ligated into the pEASY-T1 vector (TRANSGEN). The recombinant plasmids were cloned into Trans1-T1 competent cells (TRANSGEN). The microbials were spread on LB solid medium (including ampicillin, X-gal, IPTG) and cultured overnight. White clones were selected, placed in LB liquid medium and cultured to turbidity. Positive clones were identified by PCR using M13 forward and reverse primers and sequenced by Sangon Biotech [25]. Based on the discovery of clones, the genotype of individual mosquitoes was determined for each amino acid position by specific PCR amplification and sequencing. In this procedure, a single mosquito’s RNA was extracted and reverse transcribed to cDNA, followed by cDNA synthesis, as shown in Table 5.

Table 5. The primers used to amplify the Cx. pipiens quinquefasciatus ace1 gene.

| Primers   | 5′→3′ Sequence | length (bp) | PCR parameters |
|-----------|----------------|-------------|----------------|
| Cx-ace1-F | ATGGAGATCCGAGGCTTAAT | 420         | 94°C,5 min; 94°C,30 s; 62°C,30 s; 72°C,1 min,35 cycles; 72°C,7 min. |
| Cx-ace1-R | GCCCTTGTCGCCGCTATAT | 111         | 94°C,5 min; 94°C,30 s; 62°C,30 s; 72°C,1 min,35 cycles; 72°C,7 min. |
| Cx-ace2-F | CGGACCCACTGGTCATAACG | 932         | 94°C,5 min; 94°C,30 s; 65°C,30 s; 72°C,1 min,35 cycles; 72°C,7 min. |
| Cx-ace2-R | ACCCTCTCGTGTGTGCTTG | 932         | 94°C,5 min; 94°C,30 s; 65°C,30 s; 72°C,1 min,35 cycles; 72°C,7 min. |
| Cx-ace3-F | CGCTCAAGAAAACCGGA | 795         | 94°C,5 min; 94°C,30 s; 55°C,30 s; 72°C,1 min,35 cycles; 72°C,7 min. |
| Cx-ace3-R | TTAATCTTGGAACCCGCT | 795         | 94°C,5 min; 94°C,30 s; 55°C,30 s; 72°C,1 min,35 cycles; 72°C,7 min. |
then amplified by specific PCR before sequencing. Calculated mutation frequencies were based on the sequencing results.


d Hardy–Weinberg Equilibrium (HWE) Test and Genetic Linkage Analysis of the Mutations

The Hardy–Weinberg equilibrium (HWE) describes the theoretical frequency of two alleles of a single locus in the absence of mutation and selection after one generation of random mating in an indefinitely large population with discrete generations [34]. We used GENEPOP software to analyze the HWE and genetic linkage of mutations.

Correlation of Pesticide Resistance with the Allele Frequency of Different Mutations in the Five Mosquito Populations

The resistance (LC50) of the five populations to propoxur and dichlorvos was determined by bioassay and the allele frequencies of the various mutations were determined by gene specific amplification and sequencing as described above. The LC50 of a laboratory strain that had not been exposed to either pesticide was also determined to serve as a control. Correlations between resistance and mutation frequency were analyzed using Graphpad Prism 5.

Three-dimensional (3D) Modeling

The ace1 gene sequence of Cx. quinquefasciatus was translated into an amino acid sequence of AChE1. The protein was then modeled against the 3D structure of T. californica AChE (PDB accession no. 3ZV7) using the SWISS-MODEL homology modeling server (http://swissmodel.expasy.org/) [35,36,37] and molecular docking using the LibDock utility in Discovery Studio 2.5 [38].

Author Contributions

Conceived and designed the experiments: MHZ CXL YD XYG YMZ DX TYZ. Performed the experiments: MHZ XR ZMW TY XJZ. Analyzed the data: MHZ CXL GW HDZ. Contributed reagents/materials/analysis tools: MHZ CXL. Wrote the paper: MHZ.

References

1. Mutero A, Pralavorio M, Bride JM, Fournier D (1994) Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. Proceedings of the national academy of sciences 91: 5922–5926.

2. Kono Y, Tomita T (2006) Amino acid substitutions conferring insecticide insensitivity in Ace-paralogous acetylcholinesterase. Pesticide biochemistry and physiology 85: 123–132.

3. Hemingway J, Giovannoli GP (1983) Studies on the acetylcholinesterase of Anopheles albimanus resistant and susceptible to organophosphate and carbamate insecticides. Pesticide biochemistry and physiology 19: 167–171.

4. Kwon DH, Im JS, Ahn JJ, Lee JH, Marshall Clark J, et al. (2010) Acetylcholinesterase point mutations putatively associated with monooctrotopes resistance in the two-spotted spider mite. Pesticide biochemistry and physiology 96: 36–42.

5. Hemingway J (1982) Genes of organophosphate and carbamate resistance in Anopheles atroparvus [Diptera: Culicidae]. Journal of economic entomology 75: 1055–1058.

6. Chandre F, Darriet F, Dosanné JMC, Riviere F, Pasteur N, et al. (1997) Distribution of organophosphate and carbamate resistance in Culex pipiens quinquefasciatus [Diptera: Culicidae] in West Africa. Journal of medical entomology 34: 664–671.

7. Swissaert HR (1986) Cholinesterase inhibition in spider mites susceptible and resistant to organophosphate. Science 183: 129–131.

8. Fournier D (2005) Mutations of acetylcholinesterase which confer insecticide resistance in insect populations. Chemico-Biological Interactions 157: 257–261.

9. Weill M, Fort P, Berthomieu A, Dubois MP, Pasteur N, et al. (2002) A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non–homologous to the ace gene Drosophila. Proceedings of the Royal Society of London Series B: Biological Sciences 269: 2007–2016.

10. Weill M, Lutaffa G, Mogensen K, Chandra F, Berthomieu A, et al. (2003) Comparative genomics: Insecticide resistance in mosquito vectors. Nature 423: 136–137.

11. Abou H, Berthomieu A, Hadjiyasul A, Weill M (2007) A new amino-acid substitution in acetylcholinesterase 1 confers insecticide resistance to Culex pipiens mosquitoes from Cyprus. Insect biochemistry and molecular biology 37: 41–47.

12. Djojberou L, Akôgbeto M, Chandre F (2008) Presence of insensitive acetylcholinesterase in wild populations of Cx.quinquefasciatus from Benin. Acta Tropica 107: 272–274.

13. Abou H, Berthomieu A, Cui F, Tan Y, Berticat C, et al. (2007) Different amino-acid substitutions confer insecticide resistance through acetylcholinesterase 1 insensitivity in Culex vishnui and Culex tritaeniorhynchus [Diptera: Culicidae] from China. Journal of medical entomology 44: 463–469.

14. Ben Cheikh R, Berticat C, Berthomieu A, Pasteur N, Ben Cheikh H, et al. (2009) Genes conferring resistance to organophosphorous insecticides in Culex pipiens [Diptera: Culicidae] from Tunisia. Journal of medical entomology 46: 523–530.

15. Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, et al. (2004) The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. Insect molecular biology 13: 1–7.

16. Abou H, Lâbâ P, Berthomieu A, Pasteur N, Weill M (2009) Multiple duplications of the rare ace-1 mutation F290V in Culex pipiens natural populations. Insect biochemistry and molecular biology 39: 894–901.

17. Suman DS, Tikar SN, Parashar BD, Prakash S (2010) Development of insecticide resistance in Culex quinquefasciatus mosquito from different locations in India. Journal of Pesticide Science 35: 27–32.
35. Arnold K, Bordoli L, Kopp J, Schwede T (2005) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22: 195–201.
36. Kiefer F, Arnold K, Kunzli M, Bordoli L, Schwede T (2009) The SWISS-MODEL Repository and associated resources. Nucleic Acids Research 37: D387–D392.
37. Peitsch MC (1995) Protein modeling by E-mail. Bio/Technology 13: 658–660.
38. Accelrys Inc (2009) Discovery Studio 2.5 Guide. San Diego. http://www.accelrys.com.
39. Li CX (2005) Study on the genes responsible for organophosphate and carbamate resistance in Culex pipiens pallens. Beijing Institute of Microbiology and Epidemiology.