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ORIGINAL ARTICLE

High-throughput genotyping of single-nucleotide polymorphisms in *ace-1* gene of mosquitoes using MALDI-TOF mass spectrometry

Yun Mao[1,†], Feng Tan[2,†], Shuai-Guo Yan[1], Guo-Xing Wu[3], Chuan-Ling Qiao[2], Wen-Xue Zhang[1] and Feng Cui[2]

[1]*College of Life Sciences, Henan Normal University, Xinxiang, Henan Province,* [2]*State Key Laboratory of Integrated Management of Pest Insects & Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing,* [3]*College of Plant Protection, Yunnan Agricultural University, Kunming, China*

Abstract Acetylcholinesterase (AChE) plays a vital role in the nervous system of insects and other animal species and serves as the target for many chemical agents such as organophosphate and carbamate insecticides. The mosquito, *Culex pipiens* complex, a vector of human disease, has evolved to be resistant to insecticides by a limited number of amino acid substitutions in AChE1, which is encoded by the *ace-1* gene. The aims of this study are to identify single nucleotide polymorphism (SNP) sites in the *ace-1* gene of the *C. pipiens* complex and explore an economical high-throughput method to differentiate the genotypes of these sites in mosquitoes collected in the field. We identified 22 SNP sites in exon regions of the *ace-1* gene. Four of them led to non-synonymous mutations, that is, Y163C, G247S, C677S and T682A. We used matrix-assisted laser desorption ionization – time-of-flight mass spectrometry for genotyping at these four sites and another site F416V, which was relevant to insecticide resistance, in 150 mosquitoes collected from 15 field populations. We were able to synchronize analysis of the five SNP sites in each well of a 384-well plate for each individual mosquito, thus decreasing the cost to one-fifth of the routine analysis. Heterozygous genotypes at Y163C and G247S sites were observed in one mosquito. The possible influence of the five SNP sites on the activity or function of the enzyme is discussed based on the predicted tertiary structure of the enzyme.

Key words acetylcholinesterase, genotype, insecticide resistance, MassArray, SNP

Introduction

Acetylcholinesterase (AChE, E.C. 3.1.1.7) plays a vital role in the nervous system of insects by catalyzing the hydrolysis of the neurotransmitter acetylcholine, thus terminating nerve impulses. AChE is the target of organophosphate (OP) and carbamate (CX) insecticides, which irreversibly inhibit activity of the enzyme by covalently phosphorylating or carbamylating the catalytically essential serine residue in the active site, thereby lethally paralyzing insects (Orbett, 1974; Li *et al.*, 2009). A decrease in enzyme sensitivity toward OP or CX insecticide inhibition is one important mechanism by which insects become resistant to such insecticides.

There are two acetylcholinesterases in mosquitoes, AChE1 and AChE2, encoded by *ace*-1 and *ace*-2 genes, respectively (Jiang *et al.*, 2009; Weill *et al.*, 2002). These

Correspondence: Feng Cui, State Key Laboratory of Integrated Management of Pest Insects & Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China. Tel: +86 10 64807218; fax: +86 10 64807099; email: cuif@ioz.ac.cn. Wenxue Zhang, College of Life Sciences, Henan Normal University, Xinxiang, Henan 453007, China. email: wenxuezhang8@sina.com

[†]These authors made equal contributions to this work.

two AChEs differ not only in substrate specificity and inhibitor sensitivity (Bourguet *et al.*, 1996), but also in physiological function. AChE1 plays a key role in insecticide resistance in mosquitoes through mutations in its encoding gene (Weill *et al.*, 2002, 2003). Only three AChE1 variants have been identified from resistant populations of mosquitoes: G119S, F290V and F331W (numbered according to the *Torpedo californica* AChE sequence). The G119S variant has been detected in several mosquito species, including *Anopheles gambiae*, *Anopheles albimanus*, *Culex vishnui*, *Culex pipiens pipiens* and *Culex pipiens quinquefasciatus*, and occurs widely throughout the world (Weill *et al.*, 2003, 2004; Alout *et al.*, 2007; Cui *et al.*, 2006). Position 119 is in the oxyanion hole at the base of the active site gorge of the enzyme. The substitution from glycine (Gly) to serine (Ser) is believed to cause a steric shift and hence enhance the turnover of CX and OP molecules or to block the initial reaction of the enzyme with insecticides (Weill *et al.*, 2004). The F290V variant was discovered in a *C. p. pipiens* population from Cyprus (Wirth & Georghiou, 1996). This site is located at the acyl pocket of the enzyme and affects entrance of insecticide molecules into the active site (Ordentlich *et al.*, 1993; Harel *et al.*, 2000). The F331W variant was observed in a resistant *Culex tritaeniorhynchus* population from East-Asia and is located close to the catalytically crucial histidine residue in the acyl pocket of the enzyme (Nabeshima *et al.*, 2004).

Matrix-assisted laser desorption ionization – time-of-flight mass spectrometry (MALDI-TOF MS) is widely used in many areas. One of its powerful applications is high-throughput genotyping of single nucleotide polymorphisms (SNPs). Based on the addition of a single nucleotide to a genotyping primer complementary to a polymerase chain reaction (PCR) target-sequence, the mass of the primer extension product shows the added nucleotide and accurately reflects nucleotide sequence variation (Haff & Smirnov, 1997; Griffin & Smith, 2000; Sun *et al.*, 2000; Paracchini *et al.*, 2002). Because of its ability to identify multiple polymorphisms in a single reaction, the MALDI-TOF MS is particularly promising as a cost-effective and efficient method for high-throughput genotyping.

Culex pipiens complex is the main vector of filariasis and West Nile virus (Hemingway & Ranson, 2000). In China *C. p. pallens* and *C. p. quinquefasciatus* are prevalent in northern and southern regions, respectively. Large amounts of chemical insecticides have been used in mosquito or agricultural pest control for decades in China (Cui *et al.*, 2007). Insensitive AChE1 was reported the first time in a *C. p. quinquefasciatus* population in 2003 and the G119S variant was determined to be responsible for the insensitivity (Cui *et al.*, 2006).

Table 1 Collection information of 15 field populations of *Culex pipiens* complex sampled in China.

Province	Locality (latitude, longitude)	Date
Henan	Weihe (35°02′N, 113°8′E)	July, 2010
	Yuanyang (35°02′N, 113°58′E)	July, 2010
Shandong	Tai'an (35°38′N, 116°2′E)	July, 2010
Beijing	Beijing (39°54′N, 116°28′E)	July, 2010
Liaoning	Dalian (39°23′ N, 121°58′ E)	July, 2010
Shanxi	Taiyuan (37°27′N, 111°30′E)	August, 2010
Zhejiang	Hangzhou (29°11′N, 118°21′E)	August, 2010
Guangdong	Guangzhou 1 (29°06′N, 113°15′E)	September, 2003
	Guangzhou 2 (29°06′N, 113°15′E)	July, 2005
Jiangsu	Nanjing (31°14′N, 118°22′E)	July, 2009
	Huai'an (32°43′N, 119°36′E)	July, 2009
	Nantong (31°41′N, 120°12′E)	July, 2009
	Wuxi (31°07′N, 119°33′E)	July, 2009
	Lianyungang (34°39′N, 118°24′E)	July, 2009
	Xuzhou (34°15′N, 117°11′E)	July, 2009

In this study we identified several SNP sites in the AChE1 gene and checked their presence in 15 field populations of *C. p. pallens* or *C. p. quinquefasciatus* with an optimized procedure of MALDI-TOF MS. The importance and possible influence of these SNP sites to the activity or function of the enzyme were discussed based on the predicted tertiary structure of the enzyme.

Materials and methods

Mosquito samples

Fifteen field populations of *C. p. pallens* or *C. p. quinquefasciatus* were collected from 13 regions of seven Chinese provinces and Beijing (Table 1) and were raised to the adult stage in the laboratory. After eclosion the adults were frozen at −80°C for later MALDI-TOF MS assay. Two laboratory strains, one *C. p. pallens* strain collected in Beijing in 2008 and one *C. p. quinquefasciatus*

Table 2 Polymerase chain reaction primers and products for *ace-1* transcript and exons cloned from the cDNA library and genome.

Product	Primer	Size (bp)	Note
ORF	F: ATGGAGATCCGAGGCCTAATA	2109	Full length of ORF
	R: TTAAATCTTGAACCGCGTTACTAAA		
Fragment A	F: ATGGAGATCCGAGGCCTAATA	271	Exon 1
	R: CTGTTTGGCCAGAACGCTAA		
Fragment B	F: CACGCACATCCAGCGATTTC	945	Exon 2 and 3
	R: CATGGCAGAAACTTGGATCT		
Fragment C	F: CAGTGTGCGTTGCGCAAATA	1574	Exon 4, 5 and 6
	R: TCGCCAGGCACAAGTAAGAA		
Fragment D	F: GGAGTTGAGTGTAGTGCGAA	281	Exon 7
	R: TTAAATCTTGAACCGCGTTACTAAA		

ORF, open reading frame, cloned from complementary DNA (cDNA) library; fragments A–D were cloned from genomic DNA.
F, forward primer; R, reverse primer.

strain collected in Fuoshan, Guangdong province in 2007, were used for SNP identification by direct sequencing of the AChE1 gene. These two strains were raised in the laboratory for 2 and 3 years, respectively, at $25 \pm 1^{\circ}$C and photoperiod of 14 : 10 L : D. The Fuoshan strain was selected with parathion every 3 months in the laboratory.

DNA and RNA extraction and cDNA synthesis

Genomic DNA from individual mosquitoes was extracted using the cetyl trimethyl ammonium bromide (CTAB) method according to Rogers and Bendich (1988). Total RNA was isolated from 10 mosquitoes using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol supplied with the reagent. About 5μg of total RNA was treated with the TURBO DNA-free kit (Ambion, Austin, TX, USA) to remove genomic DNA contamination, and was reverse-transcripted (RT) into complementary DNA (cDNA) with the SuperScriptTM III first-strand synthesis system for RT-PCR (Invitrogen).

Cloning and sequencing AChE1 cDNAs and exon regions of DNA

The open reading frame (ORF) of AChE1 transcript was amplified from a mosquito cDNA library based on the transcript deposited in GenBank (AJ515147). The exon regions of AChE1 gene were amplified in four fragments (A–D) from the mosquito genome according to the gene structure (NW_001886806). The primers for the ORF and gene fragment amplification and product size are listed in Table 2. The 50 μL PCR reaction was comprised of 50 ng of genomic DNA or 250 ng cDNA, as template, 0.25 μmol/L of each primer, 0.2 mmol/L of each deoxynucleotide triphosphate (dNTP) and 5 units of LA Taq polymerase in 1$\times$ reaction buffer (TaKaRa Bio Inc., Otsu, Shiga, Japan). PCR was performed on a thermocycler (Eppendorf, Westbury, NY, USA) with a denaturing step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s to 2.5 min depending on the product length, and a final step of 7 min at 72°C. PCR products of gene fragments were directly used for sequencing. The amplified ORFs were incorporated into the pGEM-T easy vector (Promega, San Luis Obispo, CA, USA) and transferred into DH5α cells before sequencing.

MALDI-TOF MS

The genotypes of 150 mosquitoes from 15 populations (10 individuals from each population) at five SNP sites of the *ace-1* gene were determined using the MassArray system (Sequenom Inc., San Diego, CA, USA) at BGI Company (Shenzhen, Guangdong, China). This system is based on MALDI-TOF MS. There are seven major steps: (i) PCR amplification of specific fragments of genomic DNA containing the SNP sites; (ii) dNTP degradation using shrimp alkaline phosphatase (SAP); (iii) single base extension; (iv) desalinization with SpectroCLEAN resin; (v) sample dispensation on Nanodispenser SpectroCHIP; (vi) data acquisition; and (vii) data analysis with MassArray typer 4.0 software (all Sequenom Inc.). Primers used for the first step of PCR amplification and single base extension are shown in Table 3. Two negative controls were added, in which water instead of DNA was used as the "template". Eight samples were randomly selected to check the reproducibility of the genotyping.

Before the samples were dispensed on the Nanodispenser SpectroCHIP, all reactions and treatments were done in a 384-well plate. The assay of the five SNP sites for each mosquito was carried out simultaneously in one

Table 3 Primers used for the first step of PCR amplification and single-base extension in the MassArray system.

Single nucleotide polymorphism site	PCR forward primer	PCR reverse primer	Single-base extension primer
Y163C	ACGTTGGATGAAAAGGTGGACGCATGGATG	ACGTTGGATGAGCACACCGGTCCATCTTTC	CGCATGGATGGGCATTCCGT
G247S	ACGTTGGATGACGATCACGTTCTCCTCCGA	ACGTTGGATGCCGTCATGCTGTGGATCTTC	AGTTCCGGAGTAGAAGC
F416V	ACGTTGGATGAACCAAGGATCCGAACGAGC	ACGTTGGATGAGGAAGGCTCCGTCCACAAC	ACGCTGGGTATCTGCGAG
C677S	ACGTTGGATGCCAGCTAACCTCCAAGTAAC	ACGTTGGATGGGATCGATAAGATGTTGAGC	CCGCGCCTAGCGTACCT
T682A	ACGTTGGATGCCGCGTTACTAAAAGTAGTG	ACGTTGGATGCTAGCGTACCTTGCGAAAGC	AGAGTGGATCGATAAGATG

well. The 5 μL PCR amplification system of the first step consisted of 1 × PCR buffer with 2 mmol/L $MgCl_2$, 2 mmol/L $MgCl_2$, 500 μmol/L dNTP mix, 0.1 μmol/L primer mix (10 pieces of primer), 0.5 U hotstarTaq and 1 μL DNA template. The PCR reaction started with a denaturing step at 94°C for 15 s, followed by 45 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 1 min, and a final extension of 3 min at 72°C. After the PCR amplification, 2 μL SAP reaction mix was added in the PCR product: 1.53 μL H_2O, 0.17 μL 10 × SAP buffer, and 0.3 μL SAP enzyme (1.7 U/μL); the mixture was kept at 37°C for 40 min and then incubated at 85°C for 5 min. In the next step of single base extension, 0.62 μL H_2O, 0.2 μL 10 × iPLEX commercial buffer, 0.2 μL iPLEX termination mix, 0.94 μL iPLEX extend primer mix, and 0.04 μL iPLEX enzyme were added in the SAP treatment product. The single base extension reaction started with a denaturing step at 94°C for 30 s, followed by 40 cycles of 94°C for 5 s, 52°C for 5 s, 80°C for 5 s, and a final extension of 3 min at 72°C. During each of the 40 cycles there were five cycles between steps 52°C for 5 s and 80°C for 5 s.

Protein structure prediction

In order to determine the relative positions of the five SNP sites to the conservative or active regions of enzymes, the deduced amino acid sequences of AChE1 from *C. p. quinquefasciatus* were submitted to the I-TASSER online server (http://zhanglab.ccmb.med.umich.edu/I-TASSER) for tertiary-structure modeling. Three parameters were provided along with the predicted models: (i) C-score, a confidence score for estimating the quality of predicted models, typically in the range of –5 to 2 (C-scores of higher values signify a model with a high confidence); (ii) TM-score; and (iii) RMSD. Both TM-score and RMSD are known standards for measuring the accuracy of structure modeling. A TM-score > 0.5 indicates a model of correct topology (Wu *et al.*, 2007; Zhang, 2008).

Results and discussion

SNP sites in the ace-1 *gene*

The ORF of *ace-1* exons contained 2 109 nucleotide pairs and encoded 702 amino acid residues. There was one difference (Y163C) between the cloned AChE1 and the sequence previously reported from strain SR (CAD56155), which was homozygous for the *ace-1* variant G119S (Berticat *et al.*, 2002). The genomic sequences of exon regions were checked in four fragments, with up to 60

individual mosquitoes per fragment, of the Fuoshan strain (*C. p. quinquefasciatus*) or the Beijing strain (*C. p. pallens*) by direct sequencing (Fig. 1). The sequence quality for fragment B of the Beijing strain was of poor quality and data are therefore not reported. We found 22 SNP sites found in the exons, among which four sites led to non-synonymous mutations, that is, Y163C, G247S, C677S and T682A (Table 4). Apart from the G247S variant (a known variant, G119 in the nomenclature of *Torpedo californica* AChE, Weill *et al.*, 2003), the other three non-synonymous variants have not been reported to be involved with insecticide resistance. All the 39 mosquitoes tested from the Fuoshan strain had G247S variant, a known consequence of parathion selection. The two strains showed a large difference in the genotype at the 682 site: 24 out of 32 mosquitoes in Fuoshan strain encoded alanine (Ala) while all 28 mosquitoes of the Beijing strain encoded threonine (Thr). More evidence is needed to verify whether the mutation T682A is related to parathion resistance.

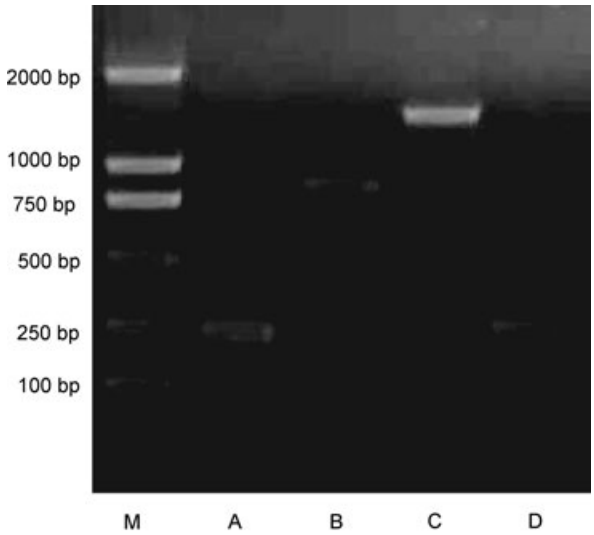

Fig. 1 Electrophoresis of four fragments of the *ace-1* gene cloned from the genome of *Culex pipiens quinquefasciatus*. M, Marker D2000. The sizes of fragments A, B, C, and D are 271 bp, 945 bp, 1574 bp, and 281 bp, respectively.

Table 4 The 22 single nucleotide polymorphism sites of *ace-1* found by direct sequencing in two strains.

Fragment	Position at ORF	Nucleotide variation	Amino acid variation	Number of individual mosquitoes	
				Fuoshan strain	Beijing strain
A	153	G/A	NA	G: 31; A: 0	G: 27; A: 1
B	354	A/G	NA	A: 29; G: 10	
	459	A/G	NA	A: 32; G: 7	
	488	A/G	Y/C	A: 38; G: 1	
	687	G/A	NA	G: 6; A: 33	
	693	G/A	NA	G: 6; A:33	
	739	G/A	G/S	G: 0; A: 39	
	846	T/G	NA	T: 36; G: 3	
C	1003	T/C	NA	T: 28; C: 0	T: 0; C: 3
	1077	G/A	NA	G: 28; A: 0	G: 0; A: 3
	1197	C/T	NA	C: 4; T: 24	C: 0; T: 3
	1236	T/G	NA	T: 28; G: 0	T: 0; G: 3
	1254	C/T	NA	C: 28; T: 0	C: 0; T: 3
	1272	A/T	NA	A: 28; T: 0	A: 0; T: 3
	1290	A/C	NA	A: 28; C: 0	A: 2; C: 1
	1314	G/T	NA	G: 4; T: 24	G: 0; T: 3
	1479	C/A	NA	C: 4; A: 24	C: 3; A: 0
	1548	C/T	NA	C: 4; T: 24	C: 3; T: 0
	1554	G/C	NA	G: 28; C: 0	G: 0; C: 3
	1827	C/A	NA	C: 28; A: 0	C: 0; A: 3
D	2029	T/A	C/S	T: 21; A: 11	T: 23; A: 5
	2044	A/G	T/A	A: 8; G: 24	A: 28; G: 0

NA, synonymous mutation; ORF, open reading frame.

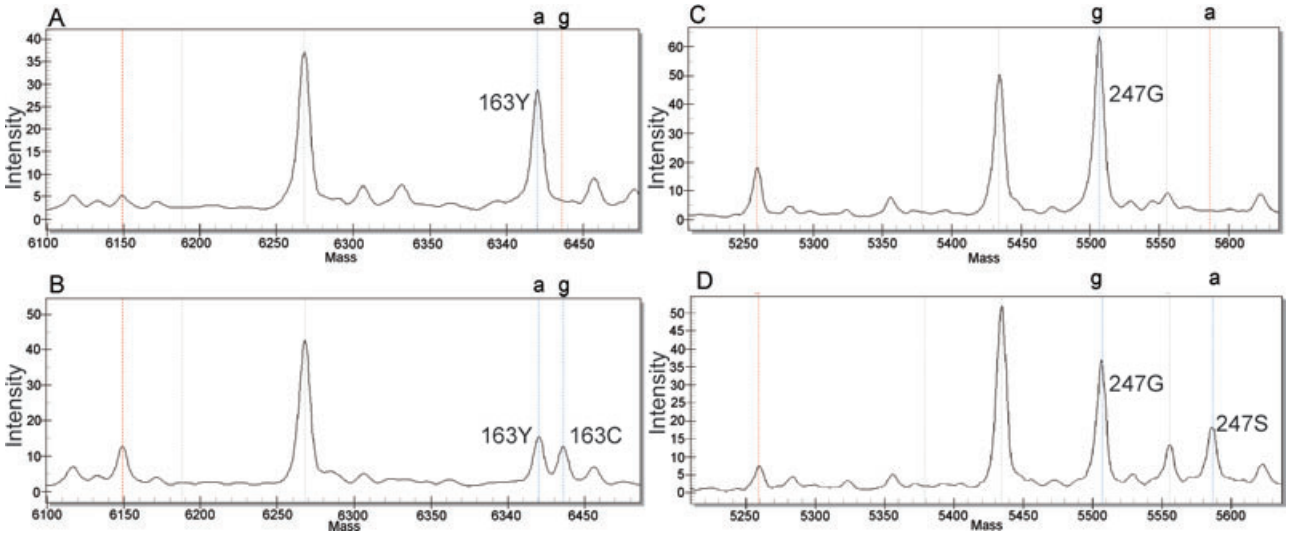

Fig. 2 Matrix-assisted laser desorption ionization – time-of-flight mass spectrometry graphs of single nucleotide polymorphism genotyping at Y163C and G247S sites of the *ace-1* gene in a mosquito from the Lianyungang population.

Genotyping five SNP sites using MALDI-TOF MS

The genotypes of SNP sites Y163C, G247S, C677S, T682A and F416V were assayed with MALDI-TOF MS in the genomic DNA of 150 individual mosquitoes from 15 populations. Although F416V (i.e., F290V in the nomenclature of *Torpedo californica* AChE) was not detected in the two strains by direct sequencing, we

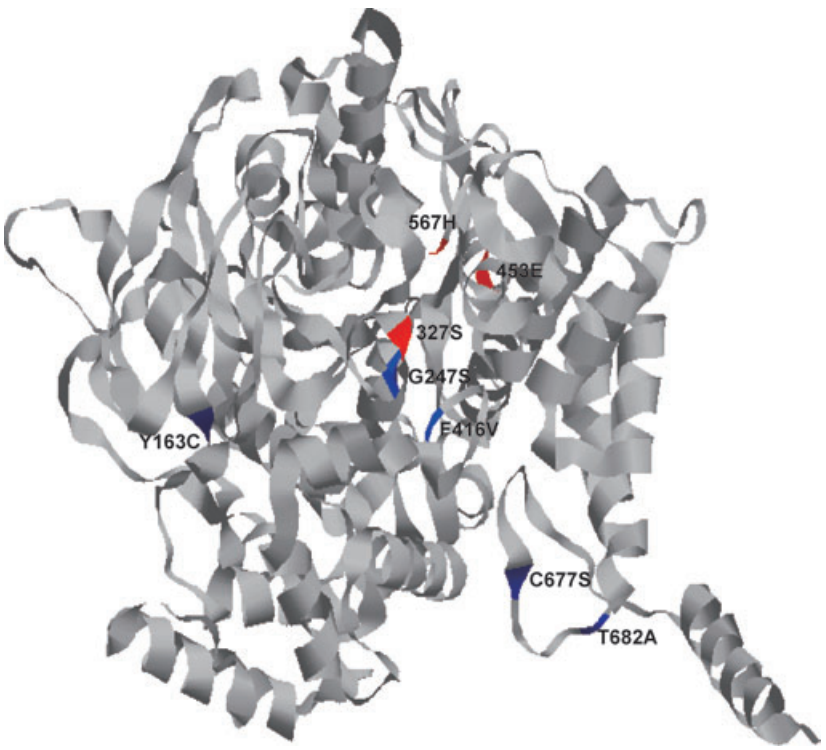

Fig. 3 The predicted tertiary structure of AChE1 from *Culex pipiens quinquefasciatus*. Red, the catalytic triad. Blue, the five SNP sites.

nonetheless analyzed it by MALDI-TOF MS because this variant was proven to afford organophosphate resistance to mosquitoes (Wirth & Georghiou, 1996). The MALDI-TOF MS graphs showed the polymorphism at two SNP sites, Y163C and G247S, in a mosquito from the Lianyungang population. Both sites were heterozygous (Fig. 2).

The MALDI-TOF MS genotyping technique turned out to be high-throughput and cost-effective with reduced reaction volumes and increased sensitivity (Bray *et al.*, 2001). We succeeded in analyzing five SNP sites in each well of a 384-well plate for each mosquito, whereas one SNP site is ordinarily analyzed per well. The five pairs of primers for the amplification of the DNA fragments containing one of the five SNP sites were proven in our study to be compatible to one another in one PCR reaction system, as were the five primers for single-base extension in one extension reaction system. This decreased the experimental cost to one-fifth of the routine analysis and allowed the application of the MALDI-TOF MS in large-scale genotyping of *ace-1* at five SNP sites in this species.

Tertiary positions of the five SNP sites in the enzyme's structure

The tertiary structure of the cloned *C. p. quinquefasciatus* AChE1 was modeled using the I-TASSER online server. The accuracy of the estimated structure was acceptable, as shown by a C-score of -1.97, a TM-score of 0.48 ± 0.15, and a RMSD 12.8 ± 4.2 Å. According to the experimentally determined structures of *T. california* AChE (Sussman *et al.*, 1991) and *Drosophila melanogaster* AChE (Harel *et al.*, 2000), S327, E453 and H567 were the catalytic triad in *C. p. quinquefasciatus* AChE1. The SNP-encoded variants G247S and F416V were located in the oxyanion hole and acyl pocket, respectively, while variants Y163C, C677S and T682A were located far away from the active regions of the enzyme. C677S and T682A were quite close to the C-terminal helix (Fig. 3) and may influence the tilt of the helix, but may not affect catalytic function of the enzyme (Harel *et al.*, 2000). Considering the formation of disulfide bonds between cysteines, the mutations Y163C and C677S may affect the dimerization of two monomers of AChE1 or the stability of the enzyme. Whether the three newly identified SNP sites influence the sensitivity of the enzyme to the inhibition of organophosphate insecticides remains in question and needs further verification.

Conclusion

We genotyped five SNP sites of the *ace-1* gene with MALDI-TOF MS. An optimized experimental system makes the MALDI-TOF MS cost-efficient and high-throughput in the large-scale genotyping of *ace-1* at the five SNP sites in *C. pipiens* complex. Heterozygous genotypes at two SNP sites were observed in one mosquito collected in the field. More experimental evidence is needed to verify the importance of the newly identified SNP sites toward insecticide resistance in mosquitoes.

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Disclosure

The authors declare no conflicts of interests.

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