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Characterization of novel esterases in insecticide-resistant mosquitoes

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Abstract

In the mosquito *Culex pipiens* complex (Diptera: Culicidae), the amplification of carboxylesterase genes is an important mechanism providing resistance to organophosphate insecticides. Various amplified alleles at the *Ester* locus have been identified over the world. In this study, two newly detected *Ester* alleles, $Ester^{B10}$ and $Ester^{11}$ (including associated $Ester^{A11}$ and $Ester^{B11}$), coding for esterases B10 and A11-B11, respectively, are characterized qualitatively and quantitatively. A high molecular identity is observed both at the nucleotide level and at the deduced amino acid level among the known *Ester* alleles. Real-time quantitative PCR results suggest 2.5-fold amplification of the $Ester^{B10}$ allele, 36.5-fold amplification of the $Ester^{A11}$ allele, and 19.1-fold amplification of the $Ester^{B11}$ allele. The ca. 2-fold difference in amplification level between $Ester^{A11}$ and $Ester^{B11}$ may indicate a new model for the esterase amplification. Bioassays show that these two resistant *Ester* alleles only can confer moderate or low resistance to the tested organophosphate insecticides.

Keywords: Insecticide resistance; Esterases; Gene amplification; Real-time PCR

1. Introduction

Major mechanisms of insecticide resistance involve either an alteration in the rate of insecticide detoxification, or mutation within the target site of the insecticide. Detoxifying enzymes, primarily carboxylesterases (or esterases), glutathione-*S*-transferases and monooxygenases, may be qualitatively or quantitatively changed to confer resistance. In mosquitoes (Diptera: Culicidae) an esterase-based resistance mechanism is the primary mechanism for organophosphorus insecticide (OP) resistance, but does not also confer pyrethroid resistance as it does in some other insect species, such as peach–potato aphids, *Myzus persicae* (Devonshire and Moores, 1982).

Esterase overproduction, which is achieved predominantly by gene amplification or occasionally by gene up-regulation, is a frequent mechanism of resistance to OP insecticides in the members of the *Culex pipiens* complex (Rooker et al., 1996; Raymond et al., 1998). Two esterase loci on chromosome α, *Est-3* (coding esterase A) and *Est-2* (coding esterase B), are involved and amplified, expressing a higher amount of esterases and providing a substantial level of OP resistance. They are highly polymorphic in insecticide susceptible populations of *C. pipiens* complex, up to 16 alleles have been found for each locus (Raymond et al., 1996). But the number of these alleles conferring resistance is limited. These two loci are tightly linked, and are always in complete linkage disequilibrium when amplified, thus they are referred to as the *Ester* superlocus (Lenormand et al., 1998). To date, nine alleles conferring OP resistance have been identified at the *Ester* locus (the corresponding overproduced esterases are named in parentheses): $Ester^{1}$ (A1), $Ester^{2}$ (A2-B2), $Ester^{4}$ (A4-B4), $Ester^{5}$ (A5-B5), $Ester^{8}$ (A8-B8), $Ester^{9}$ (A9-B9), $Ester^{B1}$ (B1), $Ester^{B6}$ (B6), $Ester^{B7}$ (B7) (Raymond et al., 1998, 2001; Buss and Callaghan, 2004). There is evidence that the amplification level varies between these alleles (Weill et al., 2000; Poirié et al., 1992) and for the same allele between strains of different geographical origins (Callaghan et al., 1998).

C. pipiens pallens and *C. pipiens quinquefasciatus* are prevalent in north and south of China, respectively. They have been subjected to OP insecticide treatments since the

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mid 1960s, and have consequently evolved various degrees of resistance toward OPs around the country (Cui et al., 2006a). All the resistant alleles at the *Ester* locus mentioned above except *Ester*^{1}, *Ester*^{4} and *Ester*^{5} have been found among and even within field populations of the *C. pipiens* complex in China, representing a complex situation for the evolution of esterase genes in China (Cui et al., 2006b). Furthermore, in recent resistance field monitoring, two newly observed esterases conferring resistance were detected in many populations of *C. pipiens quinquefasciatus* (Cui et al., 2006b). They were named temporarily New1 and New2, respectively. This paper presents detailed characterizations of these two *Ester* alleles by cloning their DNA and cDNA sequences, quantifying their amplification levels and measuring their implication in insecticide resistance. A comparison among various *Ester* alleles is also performed.

2. Materials and methods

2.1. Mosquito strains and purification

Mosquito strains of *C. pipiens quinquefasciatus* used were: S-LAB, which was OP susceptible and lacked increase of esterase activity (Georghiou et al., 1966); SB1, SA2, MAO2, LING, four resistant strains homozygous for *Ester*^{B1}, *Ester*^{2}, *Ester*^{8} and *Ester*^{9}, respectively (Berticat et al., 2002; Weill et al., 2001); KARA2, a resistant strain, isolated in 2004 from a field sample (KARAOKE) collected in Guangzhou in 2003 (Cui et al., 2006b), homozygous for a new overproduced esterase (first named as New1 here designated as B10); WU, a resistant strain, isolated in 2004 from a field sample (ZHUCHANG) collected in Wuhan (south China) in 2003 (Cui et al., 2006b), homozygous for two overproduced esterases (first named New2 here designated as A11 and B11). For KARA2 and WU, the frequency of the overproduced esterases was increased during four generations by allowing only females displaying the corresponding esterases to reproduce. For each resistant line, homozygous strains were generated by selecting and retaining families in which both parents possessed the alleles coding the corresponding overproduced esterases, as determined by starch gel electrophoresis (TME 7.4 buffer system) (Pasteur et al., 1988).

2.2. Determination of esterase DNA sequences

Genomic DNA from KARA2 and WU strains was extracted from single mosquitoes as described in Roger and Bendich (1988). The complete *Est-3* alleles were amplified with primers Adir (5′ ATGGACGTCGAACACCCGGT 3′) and Arev (5′ CCCTAATAAAGCTTATCTTTGCTG 3′). The complete *Est-2* alleles were amplified with primers Bdir (5′ ATGAGTTTGGAAAGCTTAACCG 3′) and Brev (5′ TCAAAACAGCTCATCATTCACG 3′). The 50 μl PCR mixture contained 20 ng of genomic DNA, 0.25 μM of each primer, 0.2 mM of each dNTP, 2.5 units of Taq polymerase in a 1 × reaction buffer (Expand High Fidelity PCR Kit, Roche). The PCR was run on a thermocycler (Mastercycler gradient, Eppendorf) with a denaturing step at 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, 68 °C for 2–3 min, and a final step of 7 min at 68 °C.

2.3. Determination of esterase cDNAs

Total RNA was isolated from KARA2 and WU strains using a RNeasy Mini kit and treated with DNase I (Qiagen). cDNA was reverse-transcribed from 1 μg of total RNA using MLV reverse transcriptase (Promega). The complete *Est-3* and *Est-2* cDNA was amplified with pairs of primers Adir-Arev and Bdir-Brev, respectively. The 20 μl PCR mixture was comprised of 0.5 mM of each dNTP, 0.625 μM of each primer, 1 μl of diluted template cDNA, 1.5 Units of BD advantage 2 polymerase mix in a 1 × reaction buffer (BD Biosciences Clontech). The template cDNA was initially denatured at 94 °C for 10 min only with water, and then mixed with other contents for PCR, denatured at 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, and a final step of 10 min at 72 °C.

2.4. Estimation of the amplification level of esterase alleles

A real-time PCR method was adopted to estimate the amplification level of esterase alleles. Genomic DNA from KARA2, WU and S-LAB strains was extracted from 10 single mosquitoes of each strain as described in Roger and Bendich (1988), and diluted to 100 ng/μl. Based on the conserved part of the esterase alleles, two primer pairs, Aquantidir 5′ TGGGCAAGCAGATGAAGAAG 3′(in exon 5) and Aquantirev 5′ ATGGTTGTAGGTGTCCGAAT 3′(in exon 6)for the *Est-3* locus, Bquantidir 5′ AGCGGGCTATCGTAATGTCT 3′(in exon 3)and Bquantirev 5′ GGTAGGTCCAAACGGAGTAA 3′ (in exon 3) for the *Est-2* locus, were designed to amplify a 250- and a 230-bp fragment, respectively. *Ace-2*, a single-copy noncholinergic acetylcholinesterase gene not involved in insecticide resistance, was chosen as an internal control for normalizing the DNA levels (Weill et al., 2000). A pair of primers, Acequantidir 5′ GCAGCACCAGTCCAAGG 3′ and Acequantirev 5′ CTTCACGGCCGTTCAAGTAG 3′, was designed in exon 3 of *Ace-2* to amplify a 208-bp fragment (Weill et al., 2000). Standard curves of *Est-3* and *Est-2* loci were produced by subcloning the amplified products into plasmids (Promega). Some 10-fold serial dilutions of stock plasmids were used as quantitative standards to estimate the gene amplification level on genome; the range of standard concentrations was determined empirically.

Real-time PCR was carried out in a 20 μl reaction agent comprised of 1 μl of template DNA or the standard, 10 μl of 2 × SYBR Green PCR Master Mix (Takara), and

0.25 μM of each primer on a Stratagene Mx3000P thermal cycler. The thermal cycling conditions were: 95 °C for 10 s, 40 cycles of 95 °C for 5 s, 58 °C for 20 s and 72 °C for 20 s. To exclude the presence of unspecific products, a melting curve analysis of products was performed routinely after amplification by a high-resolution data collection during an incremental temperature change from 55 to 95 °C with a ramp rate of 0.2 °C/s. PCR products were sequenced to confirm the identify of amplified genes. Three technical replicates were analyzed for each DNA sample and the standard curves. Differences in gene copy number between strains or between loci were analyzed by *t*-test with SPSS 11.0 software. Values were reported as means ± S.E.

2.5. Determination of resistance characteristics of the new esterases to OP insecticides

The resistance characteristics of the new esterases to OP insecticides were determined by performing bioassays on early fourth-instar larvae of KARA2 and WU strains, following the method of Raymond and Marquine (1994). Three OP insecticides were tested in ethanol solutions: chlorpyrifos (Dow Chemical, Midland, MI, USA), fenthion (CIL Cluzeau, Sainte-Foy-La-Grande, France), and temephos (American Cyanamid, Princeton, NJ, USA). The action of a synergist, DEF (S, S, S-tributyl phosphorotrithioate, Interchim, Montluçon, France), an inhibitor of esterases and glutathione-S-transferases, was investigated by exposing larvae to a standard dose (0.08 mg/l) 4 h before the addition of the insecticide solution. In each test, sets of 20 larvae were exposed to different insecticide doses during 24 h. Bioassays on the S-LAB susceptible strain were performed simultaneously. Mortality data were analyzed with the PriProbit program (Sakuma, 1998), testing linearity of dose-mortality response, providing LCs and slope for each mortality line. Resistance ratios (RR) with 95% confidence intervals (CI) were obtained by dividing the LC_{50} of the resistant strain by the LC_{50} of the S-LAB reference strain with the log-probit program of Raymond (1993), based on Finney (1971). Synergism ratios (SR) were computed by dividing the LC_{50} for insecticide alone by the LC_{50} for insecticide plus synergist. To test whether a synergist was more efficient in the resistant than in the susceptible strain, relative synergism ratios (RSR) were compared. The RSR is equal to the RR for insecticide alone divided by the RR for insecticide plus synergist. A $RSR > 1$ indicates that the synergist has a stronger effect in the resistant than in the susceptible strain, that is, that the detoxifying mechanism synergized is enhanced in the resistant strain; a $RSR < 1$ shows that the two strains compared are not different as far as the mechanism inhibited by the synergist is concerned (Poirié et al., 1992).

2.6. Sequence comparisons

The identity of DNA or deduced amino acid sequences between *Est-2* alleles and between *Est-3* alleles was analyzed with the CLUSTALX program. The accession numbers in GenBank of the sequences used in comparison are B1 (M32328), A2 (Z47988), B2 (Z86069), A5 (AY545983), B5 (AY545984), A8 (AJ302089), B8 (EF174325), A9 (AJ302090), B10 (EF174326), A11 (EF174327) and B11 (EF174328).

3. Results and discussion

3.1. Profiles of newly observed esterases in starch gel electrophoresis

The starch gel electrophoresis profile of B10 and its associated esterase A from single adult of purified strain KARA2 is similar to A2-B2, but their coloration is much lighter than A2-B2 of strain SA2 (Fig. 1). The electrophoretic profile of A11-B11 from the purified strain WU was different from that of any known resistant esterase pair (Fig. 1) (Weill et al., 2001) and are now given the designations $Ester^{B10}$ and $Ester^{11}$ within the super locus *Ester*.

3.2. DNA and cDNA sequences of new esterase alleles

The *Est-3* alleles in KARA2 and WU were amplified with the Adir and Arev primers, both giving DNA fragments of 2 kb and cDNA fragments of 1.6 kb. Sequence comparison and alignment with the known *Est-3* alleles showed an identity of 100% to $Est\text{-}3^{9}$ (coding A9) for the *Est-3* of KARA2, ⩾91% at the nucleotide level and ⩾97% at the deduced amino acid level for $Est\text{-}3^{11}$ (coding A11) of WU (Table 1). The homology among all of these *Est-3* alleles was quite high (Table 1). The DNA and cDNA sequences of A11 were registered in GenBank with the accession number EF174327.

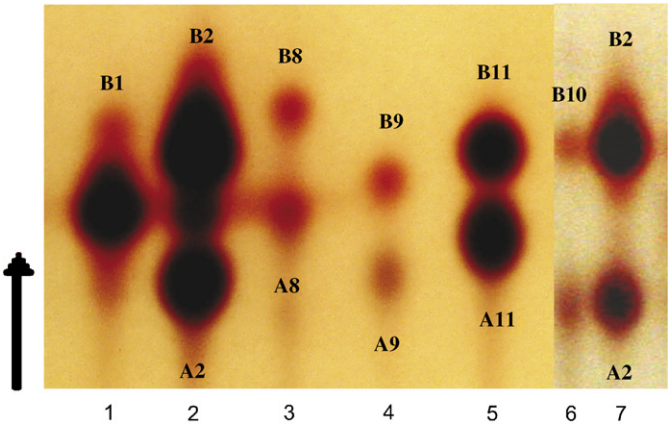

Fig. 1. High-activity of esterases detected in *C. pipiens* complex in China by starch gel electrophoresis. The arrow indicates the electrophoretic migration. Lane 1: resistant strain SB1; Lane 2: resistant strain SA2; lane 3: resistant strain MAO2; lane 4: resistant strain LING; lane 5: resistant strain WU; lane 6: resistant strain KARA2; lane 7: resistant strain SA2. The B1 band in lane 2 was due to contamination.

Table 1
The identity between *Est-3* alleles

	A2 (%)	A5 (%)	A8 (%)	A9 (%)	A11 (%)
A2		97	99	99	98
A5	92		97	97	98
A8	98	91		99	98
A9	98	91	98		97
A11	91	94	91	91	

The data in the upper and lower triangle come from the alignment of deduced amino acid sequences and DNA sequences, respectively.

Table 2
The identity between *Est-2* alleles

	B1 (%)	B2 (%)	B5 (%)	B8 (%)	B10 (%)	B11 (%)
B1		97	98	97	97	98
B2	91		97	99	99	98
B5	95	91		97	98	99
B8	90	95	89		>99	98
B10	90	95	89	>99		98
B11	96	92	96	90	90	

The data in the upper and lower triangle come from the alignment of deduced amino acid sequences and DNA sequences, respectively.

Table 3
The comparison of electric charges and relative molecular weight of the amino acids different between pairs of esterases

Variation of amino acids	Electric charges of amino acids at pH 7.4 (p*I*)		Relative molecular weight of amino acids	
		Total		Total
B10				
R (38)	+3.36 (10.76)		174.4	
A (406)	−1.38 (6.02)	+1.98	89.06	263.46
B8				
Q (38)	−1.75 (5.65)		146.08	
T (406)	−0.87 (6.53)	−2.62	119.18	265.26
B10				
T (62)	−0.87 (6.53)		119.18	
R (256)	+3.36 (10.76)		174.4	
D (461)	−4.43 (2.97)	−1.94	133.6	427.18
B2				
S (62)	−1.72 (5.68)		105.06	
K (256)	+2.34 (9.74)		146.13	
E (461)	−4.18 (3.22)	−3.56	147.08	398.27

The *Est-2* alleles in KARA2 and WU were amplified with the Bdir and Brev primers, displaying DNA fragments of 4 and 2.5 kb, respectively, and cDNA fragments of 1.6 kb. Sequence comparison and alignment with the known *Est-2* alleles showed an identity of ⩾89% at the nucleotide level and ⩾97% at the deduced amino acid level for *Est-2*^{10} (coding B10) of KARA2, ⩾90% at the nucleotide level and ⩾98% at the deduced amino acid level for *Est-2*^{11} (coding B11) of WU (Table 2). The homology among all of these *Est-2* alleles was also quite high (Table 2). Interestingly, like *Ester-2*^{8}, *Ester-2*^{10} had an insertion of 1.5 kb in intron 1 of the DNA sequence. There is no report on the impact of the large insertion in introns of esterases to mosquito insecticide resistance. This insertion could be from recombination or transposition in mosquito genome. *Ester-2*^{10} displayed an extreme identity (>99%) with *Ester-2*^{8} at the deduced amino acid level (only two amino acids changed) although there were obvious differences in electrophoretic migration (Fig. 1). The DNA and cDNA sequences of B10 and B11 were registered in GenBank with accession numbers EF174326 and EF174328, respectively.

Usually the electrophoretic migration ability of a protein is determined mainly by two factors: the quality and quantity of electric charges carried by the protein in a certain electrophoresis buffer, and the molecular weight of the protein. So these two factors were investigated (Table 3) to elucidate a puzzling result: although the deduced amino acid sequence similarity was over 99% for B10 vs. B8 (two amino acid variation) and 95% for B10 vs. B2 (three amino variation), the migration profile of the resistant esterase B10 in starch gel electrophoresis could be easily distinguished from that of B8, but not from B2. When only considering the two changed amino acids between B8 and B10, i.e. R38Q and A406 T, the net charges of the esterase protein will change from −2.62 to +1.98 in the TME buffer system (pH 7.4), so there is not only a quantitative (4.6) but also a qualitative (from negative to positive) difference in electric charge, probably resulting in a slower migration of B10 compared to B8. In contrast, although B10 differs from B2 by three amino acids, i.e. T62S, R256 K and D461E, these changes result in smaller overall difference in net charge (1.62) of the esterase protein (from −3.56 of B2 to −1.94 of B10), which is probably insufficient to distinguish by starch electrophoresis. On the other hand, these are simply the changes from the specific substitutions, which could be minor in the context of the total net charge on the protein as a whole. When the relative molecular weight of the amino acids is considered, it seems not to interfere with the migration profile of these esterases.

3.3. Amplification levels of new esterase alleles

The amplification level of esterase genes in the various resistant mosquito strains was determined with real-time PCR. In KARA2, the *Est-3* is apparently not amplified (mean = 0.9±0.03, $n = 10$, $P = 0.32$) while the amplification level of B10 varied between 1.5 and 6.6 gene copies (mean = 2.5±0.5, $n = 10$, $P < 0.05$) compared with the susceptible strain S-LAB. In WU, the amplification level varied between 31 and 43 gene copies (mean = 36.5±1.5, $n = 10$, $P < 0.05$) for A11 and between 17 and 23 gene

copies (mean = 19.1±0.9, $n = 10$, $P < 0.05$) for B11 compared with the susceptible strain S-LAB. The level of amplification differed significantly ($P < 0.001$) between these two loci in WU, with A11 nearly 2-fold more amplified than B11.

Although there are significant differences in the gene amplification level between *Est-3* and *Est-2* loci for each of the two *Ester* superloci, there was no clear difference in protein expression level as shown by the starch gel electrophoresis (Fig. 1). Transcriptional or translational regulation may play a role in esterase gene expression. A similar phenomenon has been reported in the OP-resistant *C. p. quinquefasciatus* laboratory strain PelRR, where despite linear (1:1) gene co-amplification within the genome, *estβ2*^{1} (i.e. *Est-2*^{2}) transcript levels were 2–30-fold higher than *estα2*^{1} (i.e. *Est-3*^{2}) transcript levels within individual adult mosquitoes (Paton et al., 2000), and on average 3-fold more est$\beta2^{1}$ (i.e. B2) protein was found in a mass homogenate of resistant insects than est$\alpha2^{1}$ (i.e. A2) (Karunaratne, 1994). Functional promoter analysis has shown that the esterase B's promoters were more active than the esterase A's in vitro and that transcription from the *estβ2*^{1} promoter required an initiator sequence approximately 135 bp upstream of the initiating methionine, but did not involve a TATA-box (Hemingway and Karunaratne, 1998; Hawkes and Hemingway, 2002).

As for the mode of esterase gene amplification in genome of *C. pipiens* complex, there have been two models proposed (Guillemaud et al., 1997; Raymond et al., 2001). The first model describes a solo amplification at *Est-2* (Fig. 2a). *Ester*^{B1}, as well as the newly detected allele *Ester*^{B10}, fit well with this model. The other model describes a co-amplification for *Est-3* and *Est-2* with a ratio of 1:1 (Fig. 2b). *Ester*^{2}, *Ester*^{4} and *Ester*^{5}, each comprised of a statistically equal number of copies for *Est-3* and *Est-2* (Table 4), fit well with this second model. However, neither of these models can individually explain the situation with *Ester*^{11}, for which there is a significant difference between the amplification level of *Est-3* and *Est-2* with a ratio of close to 2:1. Consequently a new model for esterase gene amplification should be considered, that is, that *Est-3* and *Est-2* co-amplify with a ratio of 2:1 at the *Ester* super locus (Fig. 2c). This could result from an initial duplication of *Est-3*, then a subsequent amplification encompassing *Est-2* and the duplicated *Est-3* loci.

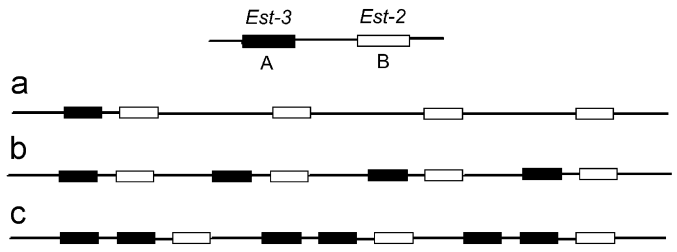

Fig. 2. Models for esterase gene amplification. (a) Only *Est-2* amplifies; (b) *Est-3* and *Est-2* co-amplify with a ratio of 1:1; (c) *Est-3* and *Est-2* co-amplify with a ratio of 2:1.

Table 4
The amplification level of esterase genes in different strains

Strain	Amplification level(±SE)				
	Est-3		*Est-2*		*P*
*TEM-R		0.7±0.1	B1	20.8±0.2	$<2\times10^{-4}$
*SELAX	A2	40.8±7.4	B2	32.4±0.1	0.51
*VIM	A4	5.44±0.6	B4	7.5±0.7	0.24
*CYPRUS	A5	43.3±0.7	B5	60.2±3.3	0.065
KARA2	A9	0.9±0.03	B10	2.5±0.5	0.006
WU	A11	36.5±1.5	B11	19.1±0.9	<0.001
S-LAB	1	1			

*From Guillemaud et al. (1997). Gene amplification levels were calculated using the dot-blot method.

3.4. Resistance to OP insecticides conferred by the new esterases

Resistance to OP insecticides conferred by the esterases in KARA2 or WU was studied with bioassays, using the S-LAB strain as the susceptible reference (Table 5). Linearity of all dose-mortality curves was not rejected ($P > 0.05$) for any of the three strains with the insecticides studied, no matter whether or not the DEF synergist was added, indicating a homogenous tolerance in the strains assayed.

The KARA2 displayed quite low resistance to all OP insecticides tested (RR = 1.6 for chlorpyrifos, RR = 1.3 for fenthion and temephos). Such differences in response to insecticides would be deemed within the 'susceptible' range were they not statistically significant ($P < 0.05$). The addition of DEF to bioassays decreased slightly the resistance level of KARA2 to chlorpyrifos (RR = 1.5, $P < 0.05$, RSR = 1.1), and completely eliminated the resistance to fenthion (RR = 0.4, $P > 0.05$, RSR = 3.3) and temephos (RR = 0.9, $P > 0.05$, RSR = 1.4), indicating that the resistance mechanisms in KARA2 were inhibited by DEF. The WU strain showed a moderate resistance to chlorpyrifos (RR = 7.2) and temephos (RR = 4.6), while a very low resistance to fenthion (RR = 1.6, $P < 0.05$). The addition of DEF completely suppressed the resistance of WU to these three insecticides (RR = 1.1, RSR = 6.5 for chlorpyrifos; RR = 0.8, RSR = 2.0 for fenthion; RR = 0.9, RSR = 5.1 for temephos; $P > 0.05$), indicating that the resistance mechanisms in WU were also inhibited by DEF. Thus, these new found esterase alleles only offered moderate or low resistance to the OP insecticides tested.

Four *Ester* alleles endemic to China have been identified to date, i.e., *Ester*^{8}, *Ester*^{9}, *Ester*^{B10} and *Ester*^{11}, and each have been clearly characterized at a molecular level. The exact nature of another two *Ester* alleles only reported once in China (Xu et al., 1994), *Ester*^{B6} and *Ester*^{B7}, remains unclear. This is the highest diversity of resistance alleles so far observed at the *Ester* locus in a given area, probably reflecting a complex evolution of esterase genes in the context of mosquito control program in China (Cui et al.,

Table 5
Resistance observed in bioassays with various insecticides in the strains KARA2, WU and S-LAB

	LC_{50}(95% CI) (μg/L)	Slope (SE)	χ^2	*P*	RR	SR	RSR
Chlorpyrifos							
KARA2	0.11 (0.10–0.12)	6.49 (0.57)	0.97	0.91	1.6*	–	–
WU	0.48 (0.46–0.50)	6.75 (0.45)	9.84	0.13	7.2*	–	–
S-LAB	0.068 (0.066–0.071)	11.06 (0.98)	2.12	0.71	1.0	–	–
Chlorpyrifos+DEF							
KARA2	0.042 (0.039–0.045)	4.43 (0.45)	1.37	0.85	1.5*	2.7*	1.1
WU	0.031 (0.030–0.033)	4.60 (0.37)	2.58	0.76	1.1	15.6*	6.5
S-LAB	0.027 (0.026–0.029)	9.43 (0.97)	1.82	0.61	1.0	2.5*	–
Fenthion							
KARA2	30.59 (27.53–40.60)	3.41 (0.96)	0.07	0.97	1.3*	–	–
WU	38.51 (33.23–41.70)	4.97 (0.88)	0.95	0.81	1.6*	–	–
S-LAB	23.64 (22.31–25.52)	6.78 (0.83)	0.94	0.81	1.0	–	–
Fenthion+DEF							
KARA2	1.87 (1.72–2.01)	4.92 (0.44)	0.69	0.88	0.4	16.4*	3.3
WU	3.53 (3.34–3.74)	3.93 (0.29)	5.99	0.31	0.8	10.9*	2.0
S-LAB	4.17 (3.67–4.48)	6.95 (1.47)	5.41	0.07	1.0	5.7*	–
Temephos							
KARA2	1.10 (1.04–1.18)	4.03 (0.42)	5.36	0.15	1.3*	–	–
WU	4.01 (3.87–4.15)	8.25 (0.61)	8.39	0.08	4.6*	–	–
S-LAB	0.87 (0.83–0.90)	11.88 (2.15)	0.64	0.73	1.0	–	–
Temephos+DEF							
KARA2	0.38 (0.36–0.39)	8.50 (0.78)	3.49	0.32	0.9	2.9*	1.4
WU	0.37 (0.35–0.38)	11.17 (1.02)	5.48	0.14	0.9	11.0*	5.1
S-LAB	0.43 (0.40–0.45)	11.21 (1.68)	0.01	0.99	1.0	2.0*	–

*Different from 1 at the 5% confidence level.
CI, confidence interval; RR, resistance ratio (LC_{50} of the resistant strain/LC_{50} of S-LAB); SR, synergism ratio (LC_{50} for insecticide alone/LC_{50} for insecticide plus synergist); RSR, relative synergism ration (RR for insecticide alone/RR for insecticide plus synergist).

2006b). This situation, where polymorphism exists, could well be unstable, and it is likely that one or several of the existing alleles will be eliminated as the result of the allelic competition in the future.

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