Recent Emergence of Insensitive Acetylcholinesterase in Chinese Populations of the Mosquito *Culex pipiens* (Diptera: Culicidae)

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ABSTRACT Organophosphate/carbamate target resistance has emerged in *Culex pipiens* L. (Diptera: Culicidae), the vector of *Wuchereria bancrofti* and West Nile virus (family *Flaviviridae*, genus *Flavivirus*) in China. The insensitive acetylcholinesterase was detected in only one of 20 samples collected on a north-to-south transect. According to previous findings, a unique mutation, G119S in the *ace-1* gene, explained this high insensitivity. Phylogenetic analysis indicates that the mutation G119S recently detected in China results from an independent mutation event. The G119S mutation thus occurred at least three times independently within the *Cx. pipiens* complex, once in the temperate (*Cx. p. pipiens*) and twice in the tropical form (*Cx. p. quinquefasciatus*). Bioassays performed with a purified G119S strain indicated that this substitution was associated with high levels of resistance to chlorpyrifos, fenitrothion, malathion, and parathion, but low levels of resistance to dichlorvos, trichlorfon, and fenthion. Hence, it is possible that in China, dichlorvos, trichlorfon, and fenthion will still achieve effective control even in the presence of the G119S mutation.

KEY WORDS *Culex pipiens*, insecticide resistance, acetylcholinesterase, gene mutation

Insecticides, widely used since the mid-1960s, have elicited numerous cases of resistance in insects. Most resistance mechanisms can be ascribed to two mechanisms: metabolic (alterations of activities of detoxification proteins) and target modifications (mutations in the sodium channel, acetylcholinesterase, and GABA receptor genes) (reviewed by Hemingway et al. 2004). Specific amino acid changes at unique or few critical positions of target proteins explain resistance to various classes of insecticides, such as organochlorines, pyrethroids, organophosphates (OPs), and carbamates. Numerous studies have focused on insect acetylcholinesterase (AChE) because it is the target of OPs and carbamates, two major classes of the worldwide insecticide market. These insecticides inhibit enzyme activity by covalently phosphorylating or carbamylating the catalytic serine residue within the active site gorge (Corbett 1974). AChE insensitivity has been reported for a large number of species (Fournier and Mutéro 1994, Russell et al. 2004).

AChE, encoded by *ace* gene, is one of the most conserved enzymes of higher eukaryotes (Russell et al. 2004). It plays a key role in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. It also has a much less studied role in the development, maturation, and maintenance of vertebrate and invertebrate nervous systems (Grisaru et al. 1999, Ranson et al. 2002, Cousin et al. 2005). In insects, two types of AChE (AChE1 and AChE2, encoded by *ace-1* and *ace-2* gene, respectively), differing in substrate specificity and in some aspects of inhibitor sensitivity, have been characterized (Bourguet et al. 1996b). In higher Diptera insects, such as *Drosophila melanogaster* (Meigen) *Musca domestica* L., and *Bactrocera oleae* (Gmelin), there is only one *ace* gene (*ace-2*), and mutations in *ace-2* gene are responsible for resistance (Mutéro et al. 1994, Walsh et al. 2001, Vontas et al. 2002). In other insects (e.g., *Aphis gossypii* Glover, *Nephotettix cincticeps* (Uhler), *Helicoverpa armigera* (Hübner), *Culex tritaeniorynchus* Giles, *Cx. pipiens*), the gene homologous to the *Drosophila ace-2* was found not to be involved in resistance (Malcolm et al. 1998, Menozzi 2000, Tomita et al. 2000, Mori et al. 2001, Ren et al. 2002). After *ace-1* association with insecticide resistance was found in *Cx. pipiens* (Weill et al. 2002), AChE1 mutations responsible for insensitivity were identified in several insect species (Russell et al. 2004). These two *ace* genes are the result of an ancient duplication event that occurred before the split of nematodes, arthropods, and vertebrates (Weill et al. 2002).

In resistant mosquitoes, the resistance-responsible substitution from glycine (G) to serine (S) at position 119 (G119S, *Torpedo* AChE nomenclature) was selected in *ace-1* gene, at least twice independently in *Cx. pipiens*, once in *Anopheles albimanus* Wiedemann, and once in *Anopheles gambiae* Giles (Weill et al. 2003,

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2004). Another mutation, F331W, has been described in resistant *Cx. tritaeniorhynchus* (Nabeshima et al. 2004). For *Cx. pipiens* complex, the vector of *Wuchereria bancrofti* and West Nile virus (family *Flaviviridae*, genus *Flavivirus*), insensitive AChE1 has been detected in numerous places of Europe, Africa, Asia, and America (Bourguet et al. 1996a, Weill et al. 2003). Although in China mosquitoes have been subjected to a variety of OP insecticide treatments since the mid-1960s, and many resistant alleles at the *Ester* locus (encoding carboxylesterases) were found in field samples of *Cx. pipiens* complex (F.C. et al., unpublished data), insensitive AChE1 has not been reported in this species before. In this article, we 1) describe the presence of insensitive AChE1 in a field sample of *Cx. pipiens* complex in China; 2) identify the resistance-associated mutation in *ace-1* gene and its mutation origin in the light of phylogenies; and 3) investigate the resistance characteristics of a G119S resistant strain to OP insecticides. The emergence of this target resistance in China is discussed in combination with China's background of insecticide application on vector control.

Materials and Methods

Mosquito Samples and Strains. Twenty field samples of *Cx. pipiens* complex were collected as egg rafts, larvae, or pupae in China from July to September 2003: nine in Guangdong province (23°08′ N, 113°15′ E; 23°03′ N, 113°06′ E); three in Hubei province (30°37′ N, 114°21′ E); two in Henan province (34°48′ N, 113°42′E); one in Shandong province (36°04′ N, 120°18′ E); and five in Beijing (39°54′ N, 116°28′ E). One set of larvae was bioassayed with propoxur, a carbamate insecticide, at a 10 ppm final concentration revealing the presence of the G119S mutation. The rest of the collected larvae were reared to adults and then deep frozen and stored in liquid nitrogen for later analyses.

Two strains sharing the same genetic background and only differing by their genotype at the *ace-1* locus were used as references: the insecticide-susceptible strain S-LAB, homozygous for *ace-1*^{S} (Georghiou et al. 1966); and the resistant strain SR, homozygous for *ace-1*^{R} with the G119S mutation (Berticat et al. 2002). Both S-LAB and SR have susceptible alleles at the esterase loci.

Detection of Insensitive Acetylcholinesterase. Larvae from each field sample were bioassayed with a diagnostic dose of propoxur (10 ppm final concentration). Surviving larvae were bred, and their offspring were selected with 10 ppm of propoxur during three subsequent generations. Genotypes at the *ace-1* locus were monitored in the last selected generation.

Insensitive AChE1 presence was assayed using the biochemical Témoin-Propoxur-Propoxur (TPP) test on the head of frozen adults (Bourguet et al. 1996a). Corresponding abdomens were conserved for further molecular analysis, such as sequencing or polymerase chain reaction (PCR)-restriction fragment length polymorphism test (Weill et al. 2004). References for genotypes *ace-1*^{S} *ace-1*^{S}, *ace-1*^{R} *ace-1*^{R}, and *ace-1*^{R} *ace-1*^{S} were individuals from strains S-LAB, SR, and a 1:1 mixture of both, respectively. Fifty-eight adults were tested for each sample.

Sequence Comparisons. Mosquito genomic DNA was extracted from single mosquitoes according to Rogers and Bendich (1988). *ace-1* gene exon 3 was then PCR amplified with primers Ex3dir 5′-CGACTCGGACCCACTGGT-3′ and Ex3rev 5′-GTTCTGATCAAACAGCCCCGC-3′ generating a 518-bp PCR fragment encompassing position 119. The 50-μl PCR reaction contained ≈50 ng of genomic DNA, 0.25 μM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase (Takara Bio, Otsu, Japan) in the manufacturer's reaction buffer. Thirty cycles of amplification were carried out (94°C for 30 s, 57°C for 30 s, and 72°C for 1 min). Sequencing was performed directly on PCR products using the Big Dye terminator kit (Applied Biosystem, Foster City, CA).

The exon 3 sequences of *ace-1* from GONGDI sample (GenBank DQ184675, DQ184676), were aligned with those previously known from strains with various geographical origins (Weill et al. 2003) by using the DNAMAN or CLUSTALX program. A phylogenetic tree was constructed using the distance neighbor joining (DNADIST and NEIGHBOR) and parsimony (DNAPARS) methods of the PHYLIP 3.63 package (http://evolution.genetics.washington.edu/phylip.html). Bootstrap analysis (1,000 replicates) was applied to evaluate the internal support of the tree topology (SEQBOOT and CONSENSE).

Determination of Resistance Characteristics of the G119S Mutation to OP Insecticides. Resistance characteristics of the G119S mutation in AChE1 to OP insecticides were determined by performing bioassays on the early fourth instar of SR strain, following the method of Raymond and Marquine (1994). Nine OP insecticides were tested in ethanol solutions: chlorpyrifos (Dow Chemical, Midland, MI), fenitrothion and fenthion (CIL Cluzeau, Sainte-Foy-La-Grande, France), malathion and temephos (American Cyanamid, Princeton, NJ), dichlorvos and parathion (Qingdao Insecticide Factory, Shandong, China), trichlorfon (Sha Long Da Company, Hubei, China), and phoxim (Da Cheng Company, Shandong, China). In each test, sets of 20 larvae were exposed to different insecticide doses during 24 h. Most bioassays included four doses and four replicates per dose. 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 lethal concentrations (LCs) and slope for each mortality line. Resistance ratios (RRs) with 95% confidence intervals (CI) were obtained by the log-probit program of Raymond (1993), based on Finney (1971).

Results

Identification of Insensitive Acetylcholinesterase. In total, 1,044 field-collected mosquitoes from 18 samples were assayed for the biochemical detection of an

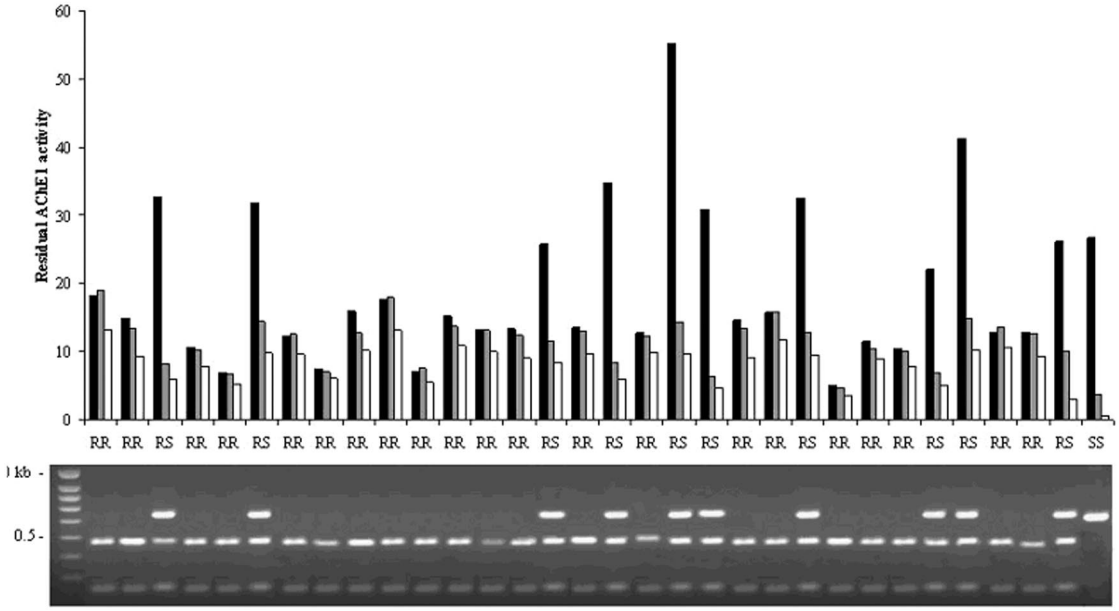

Fig. 1. Detection of insensitive AChE1 genotypes by using TPP (top) and molecular PCR-restriction fragment length polymorphism (bottom) assays. Residual AChE1 activity is measured in absence of propoxur (black) or in presence of 10^{-4} M (gray) or 10^{-2} M (white) propoxur for each individuals of the GONGDI-selected line. The last three individuals are references RR, RS, and SS.

insensitive AChE1. All of them displayed a sensitive AChE1 (corresponding to *ace-1*^{S} *ace-1*^{S} genotype). Unfortunately, adults from GONGDI sample were not conserved in good conditions to perform TPP assays. However, a small ratio of larvae from GONGDI (3.3%) survived 10 ppm propoxur selection, and most offspring (around 95%) could live after selected three generations continually with this high concentration. The TPP test on 29 survivors identified 20 *ace-1*^{R} *ace-1*^{R} and nine *ace-1*^{R} *ace-1*^{S} genotypes in the last selected generation (Fig. 1). These 29 individuals also were tested for the presence of the G119S substitution by using the PCR-restriction fragment length polymorphism test (Weill et al. 2004). All PCR products were cut by Alu I revealing the presence of a serine and results confirmed exactly the TPP genotyping (Fig. 1).

Exon 3 of the *ace-1* gene from three susceptible individuals (GONGDI-S from field sample) and three resistant individuals homozygous (GONGDI-R from propoxur-selected line) were amplified and sequenced. GONGDI-R sequences differ from GONGDI-S at nucleotide position 309 and 355 (position one corresponds to the first nucleotide of the exon 3). The 309 mutation is silent and does not change the amino acid residue, whereas the 355 position is the G119S mutation (i.e., a single point substitution from GGC to AGC), known to confer resistance. Because GONGDI-R differs from the known *Cx. p. quinquefasciatus* resistant allele (C.p.q-R) at three nucleotide positions (69, 75, and 114 in Fig. 2), resistance in GONGDI certainly occurs independently in China. Furthermore, these three mutations are found in GONGDI-S sequences. This hypothesis of GONGDI-R corresponding to an independent evolutionary event is supported by the phylogenetic analysis (Fig. 3), which shows that the resistant alleles are not grouped.

Resistance Characteristics of the G119S Mutation in AChE1 to OP Insecticides. The OP-resistance profile of the mutation G119S was studied with bioassays by

	1 1 1 3 3 3 3 4 4
	6 7 8 1 8 8 0 5 6 9 0 6
	9 5 7 4 0 9 9 5 3 0 6 2
Name	
SLAB	T G A C G G G G C C G G
GONGDI-S	- A - - - - A - - - - T
GONGDI-R	- A - - - - - A - - - T
C.p.q-R	C - - T - - - A - - - T

Fig. 2. Alignment of part of *ace-1* exon 3 sequences of mosquito *Cx. p. quinquefasciatus* from various geographical origins. GONGDI-S (DQ184676) and GONGDI-R (DQ184675) refer to susceptible and resistant individuals of the GONGDI sample, respectively. The GGC to AGC substitution in position 355, leading to G119S mutation is hatched. C.p.q-R refers to the resistant *quinquefasciatus* allele found in BO (Burkina-Faso, AJ512691), DJI (Mali, AJ512698), HARARE (Zimbabwe, AJ512700), MARTINIQUE (Martinique, AJ512706), and RECIFE (Brazil, AJ512711) resistant strains displaying the G119S mutation. The susceptible *Cx. p. quinquefasciatus* sequence shown as reference corresponds to SLAB strain (United States, AJ512712). GONGDI-R sequence differs from C.p.q-R at the three positions (69, 75, and 114).

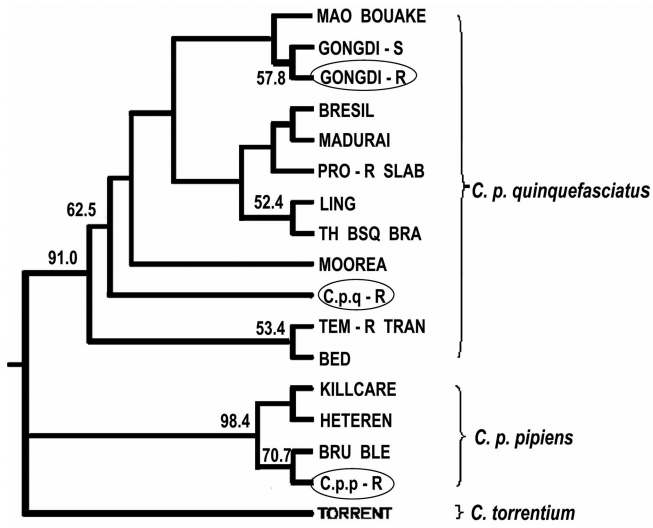

Fig. 3. Phylogenetic tree based on part of the exon 3 sequences of *ace-1* gene. Sequences were aligned, and a bootstrapped rooted tree was constructed according to the distance neighbor joining algorithm. Parsimony analysis using the same data set produced a congruent tree (data not shown). Only bootstrap percentage values >50% are indicated at nodes. *Culex torrentium* (Martini) (AJ512716), the closest known species to the *Cx. pipiens*, is presented as an outgroup. Sequences are named with respect to strains' full or shortened names. C.p.q-R refers to the resistant *quinquefasciatus* allele. Susceptible *Cx. p. quinquefasciatus* strains are cited in Fig. 2 legend. C.p.p-R refers to the resistant *pipiens* allele found in Barriol (France, AJ512688), Espro (Tunisia, AJ512699), Padova (Italy, AJ512708), and Praias (Portugal, AJ512709) strains. Susceptible *Cx. p. pipiens* strains are Bleuet (France, AJ512690), Bruges (Belgium, AJ512695), Heteren (Holland, AJ512701), and Killcare (Australia, AJ512702). GONGDI-S (DQ184676) and GONGDI-R (DQ184675) refer to susceptible and resistant individuals of the GONGDI sample, respectively. The three independent mutational events are framed elliptically.

using the resistant strain SR, homozygous for *ace-1*^{R} with the G119S mutation and displaying no other resistant mechanisms, and the susceptible strain S-LAB as the reference (Table 1). The GONGDI propoxur-selected line was not used for this purpose because it displays resistance conferred by many overproduced

Table 1. Resistance observed in bioassays to nine insecticides in strains SR and S-LAB

Strain	LC_{50} (95% CI) (μg/liter)	Slope (SE)	χ^2	*P* value	RR (95% CI)
Clorpyrifos					
SR	8.41 (8.09–8.79)	8.39 (0.74)	2.64	0.62	122.9 (96.5–156.6)
S-LAB	0.068 (0.066–0.071)	11.16 (0.98)	2.12	0.71	—
Fenitrothion					
SR	261.54 (245.69–277.38)	7.27 (0.77)	1.11	0.29	35.3 (18.4–67.7)
S-LAB	7.42 (7.00–7.72)	9.66 (1.41)	6.74	0.08	—
Fenthion					
SR	169.87 (155.28–184.61)	3.19 (0.27)	7.51	0.06	7.2 (4.7–11.0)
S-LAB	23.64 (22.31–25.52)	6.78 (0.83)	0.94	0.81	—
Malathion					
SR	1576.8 (1474.7–1674.0)	5.01 (0.42)	4.20	0.38	63.5 (50.3–80.2)
S-LAB	24.81 (23.45–26.15)	8.16 (0.83)	1.25	0.53	—
Temephos					
SR	10.93 (9.97–12.18)	2.84 (0.21)	11.03	0.05	12.6 (8.7–18.2)
S-LAB	0.87 (0.83–0.90)	11.88 (2.15)	0.64	0.73	—
Dichlorvos					
SR	63.43 (62.00–64.81)	15.85 (1.24)	3.08	0.38	1.9 (1.4–2.4)
S-LAB	34.10 (32.59–35.41)	11.49 (1.35)	0.31	0.86	—
Parathion					
SR	40.43 (37.33–44.24)	4.05 (0.39)	6.12	0.19	32.3 (24.3–43.1)
S-LAB	1.25 (1.17–1.35)	8.04 (0.74)	0.12	0.99	—
Trichlorfon					
SR	89.86 (85.59–95.84)	7.19 (0.89)	0.87	0.83	2.2 (1.7–2.7)
S-LAB	41.11 (39.55–42.64)	9.39 (0.78)	1.54	0.67	—
Phoxim					
SR	54.35 (42.71–83.50)	1.42 (0.26)	5.10	0.28	15.3 (12.0–19.5)
S-LAB	3.55 (3.37–3.74)	7.10 (0.58)	6.78	0.15	

Resistance ratio is LC_{50} of SR/LC_{50} of S-LAB.

esterases (Cui et al., unpublished data). All dose-mortality responses conformed to linearity ($P > 0.05$) under the Pearson chi-square test. Three levels of resistance were observed (the RR value in parentheses): high resistance to chlorpyrifos (123-fold), fenitrothion (35-fold), malathion (63-fold), and parathion (32-fold); moderate resistance to temephos (13-fold) and phoxim (15-fold); and low resistance to fenthion (seven-fold), dichlorvos (two-fold), and trichlorfon (two-fold).

Discussion

Insensitive AChE1 was found only in one of 20 samples of *Cx. pipiens* collected on a north-to-south transect in China. This insensitivity results from a new G119S mutation event. This mutation has thus occurred at least three times independently within the *Cx. pipiens* complex—once in the temperate (*Cx. p. pipiens*) and twice in the tropical form (*Cx. p. quinquefasciatus*). This situation, combined the same G119S mutation being found in two other mosquito species (*An. gambiae* and *An. albimanus*), suggests that there are few resistance-associated mutations leading to AChE1 insensitivity toward OP or carbamate insecticides in mosquitoes. The glycine residue lies in the oxyanion hole at the base of the active site gorge, substitution by serine causing a steric shift to enhance carbamate and OP turnover, or blocking the initial reaction of AChE1 with insecticides (Weill et al. 2004). The other mutation, F331W, found in *Cx. tritaeniorhynchus* (Nabeshima et al. 2004), is located within the acyl pocket at the base of the gorge itself, at a site that has been implicated in substrate guidance and binding. The change to a bulkier residue may restrict access or binding of the insecticides to the base of the gorge. This relatively low number of independent mutational events, inventoried on a world scale for the pest species with large population sizes, indicates that there is a limitation on the rate of occurrence of new advantageous mutations (Raymond et al. 2001).

The G119S mutation in *Cx. p. pipiens* probably arose firstly in the European continent. The four resistant strains possess the unique exon 3 of AChE1 (Weill et al. 2003) and are derived from France, Tunisia, Italy, and Portugal. They share the closest common progenitor with the susceptible strains from France and Belgium (Fig. 3). Because of lack of data concerning *ace-1*^{S} alleles either in those regions where *pipiens ace-1*^{R} was identified or in other uninvestigated areas, it is not conclusive whether G119S mutation in *Cx. p. pipiens* originated from France. However, the history record on insecticide application and selection of resistant genes in southern France provides some illustrations. OP treatments started in 1968, and *ace-1*^{R} appeared in 1978 and spread quickly, with high frequencies in the treated areas (Lenormand et al. 1998). Furthermore, migration, including passive transportation between distant populations, plays an important role in the spread of resistant genes, such for the island of Corsica where the appearance of *ace-1*^{R} was the result of migration from southern France (Raymond and Marquine 1994).

Appearance of insensitive AChE1 in China seems to be a recent event because the G119S mutation only exists in one of 20 samples investigated in a considerable geographic area. OP treatments were applied from the mid-1960s in China. This is the first report, in this mosquito species, of target resistance to OP insecticides supported by biochemical and molecular evidences. The low frequency and the localized occurrence of insensitive AChE1 suggest a very recent event, which is puzzling considering the ≈40 yr of OP treatments against this species. The bioassay results and the history of treatments may throw light on this point. The G119S mutation can bring forth extremely high resistance to carbamates, such as a 1,600-fold resistance to propoxur (Weill et al. 2002). Very few categories and small quantity of carbamates were applied in China for public health and agricultural purposes in the past. For the OPs, in the laboratory strain SR originating from France, the G119S was associated with high levels of resistance to chlorpyrifos, fenitrothion, malathion, and parathion, but low levels of resistance to dichlorvos, trichlorfon, and fenthion. All these insecticides are used commonly for the vector control in China. However, dichlorvos and trichlorfon are the most prevailing and have the longest history (Liu 1990). There are few advantages the G119S mutation can present under dichlorvos or trichlorfon selection. In Guangdong province, a large amount of malathion and parathion was used, and high resistance of *Cx. pipiens* to them has been reported in Guangzhou since 1997. The G119S mutation is beneficial for mosquitoes' survival under the selection of malathion and parathion. Interestingly, Guangzhou is the unique area where high malathion resistance was found among 83 cities of 15 provinces since 1990s (Cui et al., unpublished review). This finding is coincident with the appearance of AChE resistance only in Guangzhou in this study.

Now, *ace-1*^{R} occurs in China, and it should increase in frequency in treated areas and migrate into the nontreated area, where it is selected against because of its fitness cost (Raymond et al. 2001). A PCR-restriction fragment length polymorphism test has been designed to detect the presence of the G119S mutation in single mosquitoes (Weill et al. 2004), which is a convenient tool in monitoring the developing trend of this particular mutation in field populations. This approach will be beneficial for the vector control practically and for the exploration of the evolution of *ace-1*^{R} under the special context of China

Acknowledgments

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