Insecticide resistance in Chinese populations of the *Culex pipiens* complex through esterase overproduction

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Abstract

In most parts of China, mosquitoes have been subjected to organophosphate (OP) insecticide treatments since the mid-1960s, and resistance gene monitoring in the *Culex pipiens* complex (Diptera: Culicidae) started in only a few locations from the end of the 1980s. Many resistant alleles at the *Ester* locus have been found in field populations, including those commonly found around the world (*Ester*^{B1} and *Ester*^{2}), and those endemic to China (*Ester*^{B6}, *Ester*^{B7}, *Ester*^{8}, and *Ester*^{9}). This situation is atypical, and may represent a complex situation for the evolution of insecticide resistance genes in China. To increase our understanding of the Chinese situation and our ability to manage resistance in the *C. pipiens* complex, a large study was performed. Twenty field populations were sampled from Beijing to Guangzhou. Bioassays with five insecticides (dichlorvos, parathion, chlorpyrifos, 2-sec-butylphenyl methyl carbamate, and propoxur) disclosed resistance levels variable according to the geographic origin, and up to 85-fold for dichlorvos. Six overproduced esterases were identified, including two that have not been previously described. Most of them were found in all samples, although at variable frequencies, suggesting variable selection or a transient situation, e.g., each one was recently restricted to a particular geographic area. The results are discussed in the context of recent alterations to insecticide campaigns, and of the evolution of resistance genes in Chinese *C. pipiens* populations.

Introduction

The *Culex pipiens* complex of mosquitoes (Diptera: Culicidae), common in temperate and tropical countries, is subjected to insecticide control in many places. Worldwide surveys of resistance to organophosphate (OP) insecticides have disclosed that only three loci have developed major resistance alleles (Pasteur & Raymond, 1996; Raymond et al., 2001). Two of the loci, *Est-2* and *Est-3*, code for detoxifying carboxylester hydrolases, and these genes confer OP resistance through overproduced esterases, which is achieved predominantly by gene amplification or by gene upregulation (Rooker et al., 1996; Raymond et al., 1998). The loci are tightly linked, and are always in complete linkage disequilibrium, generally due to the co-amplification of both loci. Thus they are referred to as the *Ester* superlocus. 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), and *Ester*^{B7} (B7) (Raymond et al., 1998, 2001). The third locus, *ace-1*, codes for an acetylcholinesterase (the insecticide target), and alleles that confer insensitivity have been reported in a number of locations (Bourguet et al., 1997; Weill et al., 2003). To date, all resistant alleles at *ace-1* possess the same point mutation at residue 119, changing a glycine to a serine (G119S). The same mutation is found in an insensitive acetylchlinesterase from other mosquito species [*Anopheles gambiae* and *Anopheles albimanus* (Weill et al., 2003, 2004)], although another

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mutation (F331W) has been described in *Culex tritaeniorhynchus* (Nabeshima et al., 2004). Some of these resistant genes are found in restricted areas, e.g., $Ester^{1}$ and $Ester^{4}$ within the western Mediterranean (Poirié et al., 1992; Chevillon et al., 1995; Severini et al., 1997; Ben Cheikh et al., 1998), $Ester^{5}$ within the eastern Mediterranean (Poirié et al., 1992; Severini et al., 1997), and $Ester^{8}$ and $Ester^{9}$ only in China (Qiao et al., 1998; Weill et al., 2001). Other resistance alleles are widespread, such as $Ester^{B1}$ and $Ester^{2}$, which have a worldwide distribution (Raymond et al., 1991, 2001; Qiao & Raymond, 1995).

To devise effective strategies of resistance management, the evolution of insecticide resistance in natural populations should be understood. There are few geographic areas where extensive and longitudinal studies have been performed on resistance genes at the population level. In southern France, more than 30 years of resistance gene evolution are documented. The occurrence, rise, and eventual decrease of each resistance gene (at both *Ester* and *ace-1* loci) have been monitored, allowing the study of the complex interplay between environmental and genetic factors in the evolution of adaptation (Guillemaud et al., 1998; Lenormand et al., 1999; Lenormand & Raymond, 2000; Raymond et al., 2001), and strategies of resistance management (Lenormand & Raymond, 1998). In most parts of China, mosquitoes have been subjected to OP insecticide treatments since the mid-1960s, and resistance gene monitoring in the *C. pipiens* species complex started at the end of the 1980s, but in only a few locations (see Table 1). A large variety of OPs have been used alone or simultaneously, e.g., temephos, malathion, trichlorfon, phoxim, fenitrothion, fenthion, and dichlorvos (Xu et al., 1994). Many resistant alleles at the *Ester* locus have been found in field populations, including those commonly found around the world ($Ester^{B1}$ and $Ester^{2}$), and those endemic to China ($Ester^{B6}$, $Ester^{B7}$, $Ester^{8}$, and $Ester^{9}$; see Table 1 for references). This situation is atypical, and may represent a complex situation for the evolution of insecticide

Table 1 Overview of the geographic distribution of overproduced esterase genes in *Culex pipiens* populations from China

Province or municipality	Locality	Year of sampling	Overproduced esterases								Reference
			B1	A2-B2	B6	B7	A8-B8	A9-B9	NEW1	NEW2	
Beijing	Beijing	1988	+								Georghiou (1992)
	Beijing	1992	+								Qiao & Raymond (1995)
	Beijing	1998	+								Liu & Qiao (2001)
	Beijing	2003	+	+			+	+	+	+	This study
Shandong	Gaomi	1998	+								Liu et al. (2000)
	Qingdao	2003	+	+							This study
Henan	Zhengzhou	2000	+	+			+				Zhang et al. (2003a)
	Zhengzhou	2001	+	+			+	+			Zhang et al. (2003c)
	Zhengzhou	2003	+	+			+	+	+	+	This study
	Shangqiu	2001	+				+	+			Zhang et al. (2003c)
Zhejiang	Several cities	1995	+	+							Wang & Lu (1999)
	Hangzhou	1999	+	+							Li et al. 2001
Hubei	Shashi	1995	+			+	+				Sun & Qiao (2000)
	Wuhan	1995	+			+	+				Sun & Qiao (2000)
	Wuhan	2003	+	+			+	+	+	+	This study
Guangdong	Guangzhou	1994	+	+		+	+				Qiao et al. (1998, 1999)
	Guangzhou	1995	+	+		+	+				Sun & Qiao (2000)
	Guangzhou	2001	+	+			+	+			Zhang et al. (2003b)
	Guangzhou	2003	+	+			+	+	+		This study
	Foshan	1992	+	+	+						Xu et al. (1994)
	Foshan	2001	+	+			+	+			Zhang et al. (2003b)
	Foshan	2003	+	+			+	+	+		This study
	Zhongshan	2001	+				+	+			Zhang et al. (2003b)
Shanghai	Shanghai	1988	+								Georghiou (1992)
	Shanghai	1998		+							Qiao et al. (2003)
Sichuan	Chengdu	1992				+					Xu et al. (1994)
Yunnan	Kunming	1998		+							Liu et al. (2000)
Guangxi	Guilin	1988	+	+							Georghiou (1992)

resistance genes in China. To increase our understanding of the Chinese situation and our ability to manage resistance, field observations should be regularly executed.

This study was conducted in order to address the following points. First, what is the resistance status in Chinese populations of the *C. pipiens* complex with regard to OP and carbamate (CB) insecticides? Second, which resistance genes are involved, and what is their geographic distribution in China? Particular attention is given to the *Ester* locus for its unusual diversity of resistance alleles, and this complex situation will be presented within a general review of what is known about these resistance genes in China. The results are discussed in the context of recent alterations to insecticide campaigns, and of the evolution of resistance genes in Chinese *C. pipiens* populations.

Materials and methods

Mosquito samples and strains

Twenty field populations of the *C. pipiens* complex were collected as egg-rafts, larvae, or pupae, in epigeous breeding sites, from July to September 2003 (Table 2). In the area considered, *Culex pipiens quinquefasciatus* and *Culex pipiens pallens* are present, two subspecies that are not reproductively isolated (Zhao & Lu, 1995, 1996). Subspecies within the *C. pipiens* complex hybridize extensively, and display, where they meet, very large hybrid zones [several hundreds of kilometers (Barr, 1981)]. The same is true for *C. p. pipiens* and *C. p. pallens* (Zhao & Lu, 1996). In addition, both taxa breed true in the lab. The same insecticide resistance genes are found in all subspecies, due to the extensive hybridization (Raymond et al., 1991; Qiao & Raymond, 1995; Pasteur & Raymond, 1996; Callaghan et al., 1998). Thus, no attempt was made to identify the mosquitoes studied to subspecies. Mosquito sampling was carried out mainly in big cities along the north–south railway from Beijing to Guangzhou, except one site (in Qingdao), a seaport (Figure 1). Field larvae (or F0) were taken to the laboratory and one set was used for bioassays, and the other one was reared to adults, then deep frozen and stored in liquid nitrogen for further analyses. When the number of F0 larvae was insufficient, they were bred and bioassays were performed on ensuing generations (maximum F4). Because the resistance genes carry a fitness cost and have a tendency to decrease in frequency in the absence of selection, the resistance levels observed in the F1–F4 generations represent a minimum level for the field populations, as it cannot be excluded that higher LC_{50} values would have been observed in the F0 generation.

Reference strains used were S-LAB, an insecticide-susceptible strain without any known resistance genes (Georghiou et al., 1966); SA2, a resistant strain homozygous for *Ester*^{2}, displaying overproduced esterases A2-B2 (Berticat et al., 2002a); LING, a resistant strain homozygous for

Table 2 Collection sites of *Culex pipiens* sampled in China in 2003

Province or municipality	Locality (latitude, longitude)	Code	Date (day/month)	Type of sites
Guangdong	Guangzhou (23°08′N, 113°15′E)	Karaoke	28/09	Sewage puddle
		Jin1	28/09	Sewage tank
		Jin2	28/09	Well
		Lin	28/09	Sewage
		GongDi	27/09	Cesspool
	Foshan (23°03′N, 113°06′E)	Manda1	30/09	Puddle
		Manda2	30/09	Puddle
		Manda3	30/09	Puddle
		Manda4	30/09	Puddle
Hubei	Wuhan (30°37′N, 114°21′E)	ZhuChang	24/09	Cesspool
		CaiYuan	23/09	Ditch
		LanXi	23/09	Vat
Henan	Zhengzhou (34°48′N, 113°42′E)	Shen	19/09	Water tank
		TaiQiu	19/09	Ditch
Shandong	Qingdao (36°04′N, 120°18′E)	QingDao	15/07	River puddle
Beijing	Beijing (39°54′N, 116°28′E)	BJBJT	10/09	Sewage
		BJTJL	10/09	Ditch
		BJSGZ	11/09	Cesspool
		BJFT	11/09	Sewage
		BJHY	11/09	Sewage

Figure 1 Localities of *Culex pipiens* samples in China in 2003.

Ester[9], displaying over-produced esterases A9-B9 (Weill et al., 2001); and SB1, a resistant strain homozygous for *Ester*^{B1}, displaying overproduced esterase B1.

Insecticide bioassays

Resistance characteristics of larval populations were determined by bioassays on fourth instar larvae, following the method described in Raymond & Marquine (1994). Five insecticides were used in ethanol solutions: three organophosphate (OP) insecticides, dichlorvos, parathion, chlorpyrifos, two carbamates (CB), 2-sec-butylphenyl methyl carbamate (BPMC), and propoxur. All insecticides were produced by Qingdao Insecticide Factory (Qingdao, Shandong, China), except propoxur (Bayer, Leverkusen, Germany). Depending on the number of available larvae, between three and six doses, and between two and five replicates (20 larvae per replicate) per dose were performed with each insecticide. Mortality data were analyzed by the log-probit program of Raymond (1993), based on Finney (1971). This program takes into account eventual natural mortality, and provides LCs and slope for each mortality line, tests parallelism between two or more mortality lines, and computes resistance ratios (RR) with 95% confidence intervals.

Identification of esterase alleles

Esterase phenotypes were established by starch electrophoresis (TME 7.4 buffer system) as described by Pasteur et al. (1981, 1988) using homogenates of thorax and abdomen. Mosquitoes from SB1 and SA2 strains were run as references. Other phenotypes such as A8-B8 and A9-B9 were identified according to their specific migration distance relative to A2-B2 and B1 (Weill et al., 2001).

Genomic identification was required when distinct overproduced esterases displayed similar electrophoretic migrations (e.g., A2-B2 and *New1*, see below). Mosquitoes were cut into two parts: the head and thorax were used to identify the overproduced esterase phenotypes (see above), and the abdomen was used for DNA extraction according to Roger & Bendich (1988). An esterase A polymerase chain reaction (PCR) fragment was acquired using a pair of primers 5′-AAACCGTGGACGGAACCGT TG-3′ (located in exon 2) and 5′-TTCGCTAAACT T TCT TCG TGG-3′ (located in exon 4). The PCR was performed in a 50-µl volume containing 10–100 ng of genomic DNA, 500 pmol of each primer, 100 µM of each dNTP, 1.25 mM $MgCl_2$, 2.5 U of *Taq* polymerase (Eurogentec, Seraing, Belgium) in a 1 × reaction buffer, and run on a PTC100 thermocycler (MJ Research, Inc, Waltham, MA, USA) with a denaturing step at 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 1 min at 72 °C, and a final step of 5 min at 72 °C. Direct sequencing of the PCR fragment was performed, allowing the design of a PCR–restriction fragment length polymorphism (RFLP) test differentiating a new resistance allele (named *New1*, see Results) from *Ester*[2]. Twenty microliters of the 660 bp PCR product was digested by 20 U of *Mbo*II restriction enzyme and digestion products were separated on a 2% (wt/vol) agarose gel stained with ethidium bromide and viewed under UV light. The RFLP profile associated with the allele coding for *New1* shows two distinguishable bands of 400 bp and 140 bp after being digested with *Mbo*II (three bands, i.e., 400 bp, 140 bp, and 110 bp, are produced, but in the 2% agarose gel, the 140 bp and 110 bp bands co-migrate). The RFLP profile associated with *Ester*[2] shows three distinguishable bands of 400 bp, 140 bp, and 80 bp when digested with *Mbo*II (the 30 bp band is not visible). The diagnostic *Mbo*II sites between the alleles coding for *New1* and *Ester*[2] are present in all (30) mosquitoes from each reference strain (SA2, homozygous for *Ester*[2], Berticat et al., 2002a; and a strain homozygous for *New1* (F Cui, M Raymond, A Berthomieu, M Marquine, M Weill & C-L Qiao, unpubl.). The RFLP profile associated with esterase A of *Ester*^{B1} shows three distinguishable bands of 260 bp, 140 bp, and 80 bp (there are two fragments of 140 bp co-migrating). A given resistance allele at *Ester* displays identical RFLP patterns, whatever the geographic origin of the allele (e.g., for *Ester*[2], see Raymond et al., 2001; for *Ester*^{B1} see Qiao & Raymond, 1995). So the above PCR assay, established on the SA2 strain homozygous for an *Ester*[2] allele originating

from California, works for *Ester*^{2} alleles from other geographic areas.

Results

Insecticide resistance status of the populations

Bioassays were performed on 14 populations, either on field-collected individuals or on their laboratory-reared offspring (F1–F4). For all five insecticides, dose–mortality curves were well represented by regression lines ($P>0.05$), with the exception of one sample, ZhuChang with parathion (Table 3) (a more comprehensive table, listing slopes, χ^2, d.f., P-value, etc., is provided as Supplementary Appendix S1). The highest resistance was to dichlorvos, with an 85-fold resistance ratio (RR) in the sample from Guangdong province, followed by parathion, propoxur, and chlorpyrifos and, finally BPMC (with the highest RR being 30-, 14-, 15-, and 5-fold, respectively).

A rather complex pattern of resistance was observed. In Guangdong province, the resistance to dichlorvos varied between 15 and 85, to parathion between 11 and 30, to chlorpyrifos between 5 and 14, to propoxur between 6 and 15, and to BPMC between 2 and 4. In Hubei province, only one collection was available for bioassays, which had a higher resistance to dichlorvos (RR = 19) than those to other insecticides (RR<7). In Henan province, the two populations, Shen and Taiqiu, presented significant differences in OP resistance: Taiqiu was slightly but significantly ($P<0.05$) more resistant than Shen (RR computed with Shen as reference: 4.6-, 1.9-, and 1.7-fold, for dichlorvos,

Table 3 Resistance (bioassays) to five insecticides in populations of *Culex pipiens* from China

	Dichlorvos		Parathion		Chlorpyrifos		BPMC		Propoxur	
Population code	LC_{50} (mg l^{-1}) (95% CI)	RR	LC_{50} (mg l^{-1}) (95% CI)	RR	LC_{50} (mg l^{-1}) (95% CI)	RR	LC_{50} (mg l^{-1}) (95% CI)	RR	LC_{50} (mg l^{-1}) (95% CI)	RR
S-LAB	0.027 (0.020–0.046)	1	0.0004 (0.0003–0.0007)	1	0.0005 (0.0004–0.0006)	1	0.15 (0.13–0.17)	1	0.043 (0.027–0.052)	1
Lin	1.20 (1.07–1.39)	43.7	0.0052 (0.0046–0.0058)	12.8	0.0030 (0.0023–0.0035)	5.5	0.31 (0.28–0.34)	2.1	0.25 (0.23–0.27)	5.8
GongDi	0.89 (0.64–1.08)	32.6	0.012 (0.0089–0.015)	29.9	0.0078 (0.0058–0.0091)	14.4	0.55 (0.51–0.60)	3.8	0.67 (0.58–0.87)	15.6
Manda2	2.35 (1.85–3.03)	85.7	0.0054 (0.0049–0.0059)	13.2	0.0033 (0.0027–0.0037)	6.1	0.32 (0.29–0.34)	2.2	0.29 (0.26–0.31)	6.7
Manda3	1.64 (1.14–2.07)	59.9	0.0069 (0.0057–0.0080)	16.9	0.0073 (0.0069–0.0078)	13.6	0.62 (0.59–0.68)	4.3	0.42 (0.36–0.48)	9.9
Manda4	0.42 (0.37–0.49)	15.3	0.0047 (0.0043–0.0050)	11.4	0.0050 (0.0047–0.0053)	9.2	0.34 (0.27–0.38)	2.3	0.32 (0.29–0.34)	7.4
ZhuChang	0.52 (0.35–1.62)	18.9	0.0025* (0.0016–0.0041)	6.2	0.0018 (0.0017–0.0020)	3.4	0.21 (0.17–0.24)	1.4	0.28 (0.26–0.30)	6.6
Shen	0.15 (0.12–0.17)	5.4	0.0047 (0.0039–0.0054)	11.5	0.0032 (0.0027–0.0036)	5.8	0.16 (0.14–0.18)	1.1	0.30 (0.25–0.35)	6.9
TaiQiu	0.68 (0.53–1.35)	24.8	0.0087 (0.0080–0.0097)	21.4	0.0054 (0.0048–0.0060)	10.0	0.25 (0.23–0.27)	1.7	0.35 (0.33–0.37)	8.2
QingDao	0.12 (0.10–0.14)	4.3	0.0012 (0.0011–0.0013)	2.8	0.0014 (0.0013–0.0016)	2.6	0.16 (0.14–0.18)	1.1	0.15 (0.14–0.17)	3.6
BJBJT	0.20 (0.19–0.22)	7.4	0.0026 (0.0024–0.0029)	6.5	0.0029 (0.0026–0.0034)	5.4	0.46 (0.42–0.51)	3.1	0.16 (0.14–0.18)	3.7
BJTJL	0.15 (0.13–0.17)	5.5	0.0029 (0.0027–0.0031)	7.1	0.0022 (0.0020–0.0025)	4.1	0.42 (0.37–0.46)	2.8	0.16 (0.07–0.21)	3.8
BJSGZ	0.17 (0.15–0.20)	6.1	0.0017 (0.0014–0.0019)	4.0	0.0018 (0.0015–0.0020)	3.2	0.32 (0.29–0.36)	2.2	0.28 (0.26–0.31)	6.6
BJFT	0.16 (0.15–0.18)	5.9	0.0022 (0.0020–0.0025)	5.5	0.0020 (0.0018–0.0022)	3.6	0.31 (0.28–0.34)	2.1	0.18 (0.15–0.21)	4.2
BJHY	0.19 (0.16–0.21)	6.8	0.0029 (0.0026–0.0032)	7.0	0.0034 (0.0028–0.0051)	6.2	0.71 (0.58–1.07)	4.8	0.22 (0.20–0.25)	5.1

*The dose–mortality curve was not well represented by regression line ($P<0.05$).

S-LAB, is the susceptible reference strain; CI, confidence interval; RR, resistance ratio (LC_{50} of the population/LC_{50} of S-LAB).

parathion, and chlorpyrifos, respectively, each 95% CI not including 1). For CB resistance, no difference was observed. In Shandong province, the only sample, Qingdao, showed a very low but significant resistance to all the insecticides (1<RR<5, P<0.05) except to BPMC. In Beijing, all five samples showed a low but significant resistance to the five insecticides (1<RR<7.5, P<0.05). For OP insecticides, the global rank of resistance level of the four provinces and Beijing is Guangdong>Henan>Hubei>Beijing>Shandong. For CB insecticides, all the populations had similar and low resistance levels (RR<10), with the exception of one sample from Guangdong province (Gongdi), which exhibited a surprisingly moderate resistance to propoxur (RR = 15.6), higher than in any other sample (Table 3).

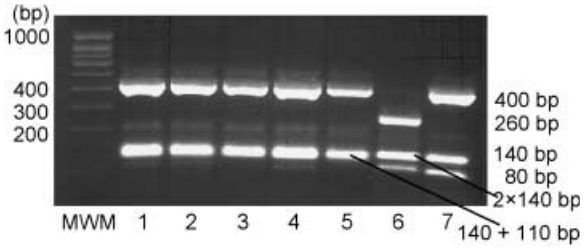

Figure 3 *Mbo*II PCR-RFLP profiles of individuals from populations and standard strains. MWM (molecular weight marker); 1–4: first interpreted as *Ester*^{2}/*Ester*^{9} from the electrophoretic pattern, and in fact heterozygote *Ester*^{9}/*Ester*^{New1}; 5: A9 control from LING strain; 6: A associated with B1 from SB1 strain; 7: A2 control from SA2 strain.

Identification of esterase alleles

A total of 1376 field mosquitoes were analyzed. Starch gel electrophoresis disclosed six different overproduced esterases in the Chinese samples, including four which have been reported previously (B1, A2-B2, A8-B8, and A9-B9; see References in Table 1). Two new patterns were observed repeatedly, both of which displayed one esterase A and one esterase B, and these were further studied. The first one (named *New1* until further characterization) displayed esterases A and B with a migration pattern similar to that of A2-B2, although with a much lower staining intensity (Figure 2). The second (*New2*) displayed an esterase A migrating as A8, and an esterase B migrating as B2. Eleven mosquitoes (0.8%) displayed a pattern with more than two alleles interpreted as the simultaneous presence of A9-B9, A2-B2, and A8-B8, suggesting a composite pattern (Figure 2). Specific studies on this situation will be reported elsewhere. Quantitative variation of esterase activity was apparent (Figure 2), although starch gel electrophoresis is essentially qualitative, and quantification is not reliable. Therefore, quantitative variations were not considered in this study.

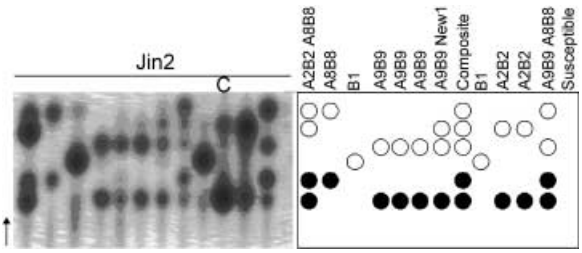

Figure 2 High-activity esterases in single adults from the Jin2 sample analyzed on starch gels. Only part of the gel is shown, the arrow indicates electrophoretic migration of the proteins. A control mosquito (C) from strain SA2 is indicated. Band interpretation is shown in the right panel, with the positions of the known esterase A (black circles) and B (white circles). The composite pattern is interpreted as the simultaneous presence of A9-B9, A2-B2, and A8-B8. An unknown esterase B, present in the third mosquito from the left, on top of B1, is not interpreted. See text for explanations.

In order to ascertain the identity of uncertain protein patterns, DNA identification was performed on 92 mosquitoes, which were analyzed for both their esterase electrophoresis pattern and PCR-RFLP test pattern. All mosquitoes first interpreted as heterozygote *Ester*^{2}/*Ester*^{9} (thus displaying A2, B2, A9, and B9) in starch electrophoresis but with a faiter A2 and B2 staining, showed only the RFLP profile specific for *Ester*^{9} (Figure 3). In addition, direct sequencing of the *New1* PCR fragment (in a strain homozygous for *New1*; F Cui, M Raymond, A Berthomieu, M Marquine, M Weill & C-L Qiao, unpubl.) corresponds exactly (100% similarity of a 660 bp long fragment spanning from exon 2 to exon 4) to the allele sequence of A9 (Genbank accession number AJ302090). This indicates that there exists a new overproduced esterase (NEW1), which is distinct from *Ester*^{2} despite a similar electrophoretic migration as A2-B2 and which has the same RFLP profile and the same (partial) sequence as *Ester*^{9}.

The population frequencies of mosquitoes displaying these six overproduced esterase phenotypes are detailed in Table 4. They are present in all Chinese provinces studied, with the exception of *New2* which is not detected in the Guangdong province. Frequencies varied according to geographic origin. The most prevalent was B1, which was present in all localities, with a high frequency in northern China (up to 84% of mosquitoes displayed it in TaiQiu). In Guangdong province, phenotype A8-B8 prevailed, being present in 33–55% of mosquitoes, followed by B1, A9-B9, and A2-B2. In Hubei province, B1 was the most common allele, followed by A9-B9, and *New2*, and then A2-B2, A8-B8, and *New1*. In Henan province, B1 was prevalent, followed by A2-B2 and A9-B9. In Shandong province, only B1 and

Table 4 Frequency of mosquitoes displaying a given overproduced esterase in field populations of *Culex pipiens* in China

Province or municipality	Population code	n	B1	A2-B2	A8-B8	A9-B9	NEW1	NEW2	SS
Guangdong	Karaoke	62	0.11	0.22	0.38	0.41	0.15	0	0.10
	Jin1	62	0.27	0.11	0.55	0.07	0.02	0	0.18
	Jin2	91	0.15	0.27	0.40	0.45	0.27	0	0.02
	Lin	62	0.12	0.10	0.37	0.29	0.13	0	0.24
	GongDi	19	0.22	0.11	0.37	0.16	0.05	0	0.16
	Manda1	69	0.56	0.17	0.35	0.07	0.03	0	0.12
	Manda2	27	0.33	0.30	0.33	0.11	0.11	0	0.04
	Manda3	62	0.54	0.21	0.39	0.14	0.02	0	0.06
	Manda4	62	0.38	0.32	0.46	0.10	0.05	0	0.05
Hubei	ZhuChang	62	0.52	0.12	0.06	0.18	0.02	0.18	0.16
	CaiYuan	80	0.53	0.09	0.04	0.21	0.01	0.17	0.15
	LanXi	85	0.48	0.02	0.05	0.26	0.09	0.24	0.12
Henan	Shen	102	0.78	0.18	0.07	0.10	0	0.12	0.06
	TaiQiu	92	0.84	0.30	0.08	0.12	0.02	0.04	0.02
Shandong	QingDao	73	0.74	0.04	0	0	0	0	0.23
Beijing	BJBJT	91	0.69	0.07	0.02	0.01	0	0.04	0.25
	BJTJL	62	0.44	0	0	0.02	0	0	0.55
	BJSGZ	62	0.66	0.12	0.10	0.02	0.02	0.04	0.23
	BJFT	62	0.69	0.05	0.03	0	0	0.05	0.31
	BJHY	89	0.81	0.14	0	0.03	0.02	0.02	0.13

n, sample size analyzed; SS, wild (non-overproduced) esterase phenotype; *New1* and *New2*, new esterase patterns detected (see text for explanation).

A2-B2 were detected. In Beijing, the frequency of mosquitoes with B1 was between 44 and 81%, and all other resistance alleles were found in less than 14% of the mosquitoes.

Discussion

It has been nearly 40 years since the OP insecticides were used extensively for agricultural protection and public health in China. In particular, Guangdong, Hubei, Henan, and Shandong were among the provinces where the quantity of insecticides used (mainly OP) exceeded 107 kg annually before 1996 (Hua & Shan, 1996). Unsurprisingly, resistance of mosquitoes to OP insecticides increased rapidly. Two other insecticide families, pyrethroids and CB, were subsequently used to replace OPs partly or completely, particularly in places displaying serious OP resistance. Carbamates were used to control mosquitoes until recently, consistent with the absence of insensitive AChE in China (Qiao et al., 1999). The larval samples in 2003 exhibited various levels of resistance to three OP and two CB insecticides, and all the populations were more resistant to OPs than to CBs, which is in agreement with the longer history of OP application in China.

Overproduced esterases B1, A2-B2, A8-B8, and A9-B9 were found to have invaded nearly all the sampled localities from southern to northern China. B1 was first reported in North America (California, USA) in 1974, and was then found in Latin America (French Guiana, Venezuela, and Puerto Rico) and Asia (China) (Raymond & Pasteur, 1996). A2-B2 is more widespread, as it is present in Asia (Japan, China, Thailand, Pakistan, Sri Lanka, Saudi Arabia, Israel, and Vietnam), Africa (Tanzania, Congo, Nigeria, Burkina Faso, Mali, Ivory Coast, Senegal, South Africa, Egypt, and Tunisia), Europe (Cyprus, Greece, Italy, and France), North America (USA), and the Caribbean (see References in Labbé et al., 2005). Thus B1 and A2-B2 have large geographic distributions, and we now know that migration is responsible for their co-occurrence worldwide (Raymond et al., 1991; Qiao & Raymond, 1995; Guillemaud et al., 1996). In China, B1 was detected in Beijing, Shanghai, and Guangxi in 1988 (Georghiou, 1992), and A2-B2 was recorded in Guangdong in 1992 (Xu et al., 1994). So far, A8-B8 and A9-B9 are endemic to China and were both found for the first time in Guangdong in 1994 (Qiao et al., 1998; Weill et al., 2001). There have been limited population studies of these resistance genes in China; however, from the data available (Table 1), it seems that the distribution ranges of these genes have changed over time. In contrast with 2003, in 1998 A2-B2 was not detected in Beijing, Shandong, and Hubei, A9-B9 was not detected in Beijing and Hubei, and

A8-B8 was not reported from Beijing. In addition, the frequency of each resistance gene seems to have changed within each locality. For example, in Beijing, the frequency of mosquitoes with B1 was ca. 76% in 1998 (Liu & Qiao, 2001), and an average of 66% in 2003; In Zhengzhou, the frequency of mosquitoes with B1, A2-B2, A8-B8, and A9-B9 were 48.9, 46.8, 48.8, and 5.3%, respectively, in 2001 (Zhang et al., 2003c), and 81, 24, 7.5, and 11% on average, respectively, in 2003. Similar changes were noted in Wuhan from 1995 (Sun & Qiao, 2000), in Guangzhou from 1994 (Qiao et al., 1999) and 2001 (Zhang et al., 2003b), and in Foshan from 2001 (Zhang et al., 2003b). These changes reflect a complex pattern of resistance gene evolution, with local increases or decreases of each resistance allele.

New1 and *New2* are two new resistant esterases, both of them being a closely associated esterase A and esterase B. Although they are first detected in the present samples from 2003, they have already a broad geographical distribution in China, particularly *New1* which is present in at least four provinces (Guangdong, Hubei, Henan, and Beijing). This could be the result of their rapid spread over China, despite their probably recent occurrence. An alternative explanation, at least for *New1*, is that it was not previously detected due to its electrophoretic similarity with A2-B2. For *New2*, which has a distinct pattern from all other described overproduced esterases, this explanation is unlikely, and thus its occurrence is probably recent, i.e., around 2001, as it was not detected by Zhang et al. (2003b, 2003c). How *New2*, and perhaps also *New1*, succeeded in expanding over such large areas in a relatively short period of time is an interesting evolutionary question, and assumes that they are associated with a substantial fitness advantage. *New1* and *New2* will be formally described elsewhere, after being isolated, characterized at the molecular level and thoroughly studied for their resistance characteristics.

Six resistance genes have been now recorded at the *Ester* locus in China: $Ester^{2}$, $Ester^{8}$, $Ester^{9}$, $Ester^{B1}$, $Ester^{New1}$, and $Ester^{New2}$ (Table 4). This is the highest diversity of resistance alleles thus far observed at the *Ester* locus in a given area. The various *Ester* resistance alleles do not share the same characteristics concerning their resistance and their cost. Both resistance and cost vary quantitatively according to the gene amplification level, and qualitatively according to each allele (Weill et al., 2000; Pasteur et al., 2001; Berticat et al., 2002b, 2004). As several OP insecticides were used in China against mosquitoes, including temephos, malathion, trichlorfon, phoxim, fenitrothion, fenthion, and dichlorvos, it is possible that each local insecticide usage has selected for a particular resistance gene. Migration extends the geographical range of each of these resistance genes, and they finally mix. As a consequence, the various resistance alleles compete when they are in the same population. This scenario is illustrated by the situation in southern France, where $Ester^{1}$ has been replaced by $Ester^{4}$ over a 10-year-period (Guillemaud et al., 1998), and this event is now followed by the local increase in frequency of $Ester^{2}$ which is starting a genetic invasion (Labbé et al., 2005). A polymorphism of resistance alleles, as observed in China, thus indicates a recent contact between relatively isolated OP treated areas. It is likely that, in the future, one or several of the present alleles will be eliminated as the result of allelic competition. In most places of the world, only few Ester resistance alleles are present. For example, in America, only two resistant alleles, $Ester^{2}$ and $Ester^{B1}$, have been described (for a review, see Labbé et al., 2005). This situation, where a polymorphism exists, could well be instable, leading in a near future to fixation of one of the alleles, such as in tropical Africa where only $Ester^{2}$ is found. *Culex pipiens* from China provides an interesting situation to identify the parameters driving the competition between resistance alleles.

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Supplementary Material

The following supplementary material is available for this article:

Appendix S1. Resistance observed in bioassays to five insecticides in populations of *Culex pipiens* from China.
This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1570-7458.2006.00453.x (This link will take you to the article abstract).

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