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Distribution and dynamics of esterase alleles in *Culex pipiens* complex in China

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ABSTRACT

To investigate insecticide resistance and dynamic changes of carboxylesterase polymorphism in mosquitoes with time in the *Culex pipiens* complex (Diptera: Culicidae), nine field mosquito populations were collected in China. The resistance levels of fourth-instar larvae to organophosphate (dichlorvos, parathion, and chlorpyrifos), carbamate (fenobucarb and propoxur), and pyrethroid (permethrin, deltamethrin and tetramethrin) insecticides were determined by bioassay. Larvae had more resistance to organophosphate insecticides than to carbamate insecticides. A low but significant resistance was observed for carbamate insecticides. The resistance to pyrethroid insecticides varied from sensitive to high. Starch gel electrophoresis revealed the presence of the overproduced esterases B1, A2B2, A8B8, A9B9, B10 and A11B11. The frequency of each overproduced esterases varied depending on its regional localities. Compared with published surveys, the *C. pipiens* complex, which exhibited a high polymorphism of applied esterase alleles in China, showed dynamic evolution over time under local specific insecticide selection. The results are discussed in the context of recent alterations to insecticide campaigns, and in the evolution of resistance genes in Chinese *C. pipiens* populations.

Introduction

Synthetic insecticides have been widely used to protect crops from infestation and prevent the spread of vector-borne diseases in humans for a long time. The use of synthetic insecticides has become an important selective process of field insect populations. As a result, insecticide resistance has been observed in many pests, such as house fly (Rinkevich et al., 2007; Memmi, 2010; Fang et al., 2011), cotton bollworm (Fitt, 2008; Zalucki et al., 2009), and mosquitoes (Weill et al., 2003; Russell and Kay, 2004; Enayati and Hemingway, 2010). The mosquito (genus *Culex*), which is the major vector of filariasis, Japanese encephalitis, and West Nile virus, has gradually evolved insecticide resistance (Hemingway and Ranson, 2000).

Worldwide surveys of insecticide resistance indicate that the major mechanism of insecticide resistance involves an alteration in the rate of insecticide detoxification or mutation in the target site of the insecticide. As one of the dominative detoxifying enzymes of mosquitoes (Diptera: Culicidae), carboxylesterases (or esterases) play an essential role in the resistance to insecticides. The mechanism has been clarified by overproduction of nonspecific carboxylesterases (Rooker et al., 1996; Raymond et al., 1998; Cui et al., 2006a). Twelve alleles conferring insecticide resistance have been identified in the *Culex pipiens* complex: *Ester*^{1} (A1), *Ester*^{2} (A2B2), *Ester*^{4} (A4B4), *Ester*^{5} (A5B5), *Ester*^{8} (A8B8), *Ester*^{9} (A9B9), *Ester*^{B1} (B1), *Ester*^{B6} (B6), *Ester*^{B7} (B7), *Ester*^{B10} (B10), *Ester*^{11} (A11B11), and *Ester*^{12} (B12) (Qiao et al., 1998, 1999; Raymond et al., 1998, 2001; Cui et al., 2007b; Ben Cheikh et al., 2008). The level of amplification varies in the aforementioned alleles, and even for the same allele existing in different geographical regions. In fact, some resistance alleles are distributed worldwide, e.g., the *Ester*^{2} has been found in Africa, Asia, North and South America, and Europe (Raymond et al., 1991, 2001), and *Ester*^{B1} is present in China and North America (Qiao and Raymond, 1995). Other resistance alleles are found in restricted geographic areas. For example, *Ester*^{1} and *Ester*^{4} are distributed in the western Mediterranean (Poirie et al., 1992; Chevillon et al., 1995; Severini et al., 1997; Ben Cheikh et al., 1998), while *Ester*^{5} is distributed in the eastern Mediterranean (Poirie et al., 1992; Severini et al., 1997). Additionally, *Ester*^{8}, *Ester*^{9}, *Ester*^{10} and *Ester*^{11} are only observed in China (Qiao et al., 1998; Weill et al., 2001; Cui et al., 2006a, 2007a, 2007b). *Ester*^{12} is only identified in Tunisia (Ben Cheikh et al., 2008).

To predict the resistance spectra of mosquitoes and develop rapid and sensitive biochemical diagnostic assays detecting mosquito resistance, understanding resistance mechanisms of esterase is essential. But, extensive and longitudinal studies on the dynamics of insecticide resistance alleles in natural populations have been done in very few geographic areas. In southern France, the resistance gene evolution of over 40 years is recorded (Guillemaud et al., 1998; Labbe et al., 2007). The dynamics of each resistance gene have been monitored,

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furthering the understanding of the complex interplay between environmental and genetic factors in the evolution of adaptation, and strategies of resistance management (Guillemaud et al., 1998; Lenormand et al., 1999; Lenormand and Raymond, 2000; Raymond et al., 2001; Labbe et al., 2007). In China, however, monitoring resistance genes has only studied sporadically in a few locations since the 1980s, even though mosquitoes had been treated by organophosphate insecticides (OPs) since the mid-1960s (Cui et al., 2006b, 2007a). In our previous study, many resistance alleles at the *Ester* superlocus were identified in field populations, including those commonly observed globally (*Ester*^{1} and *Ester*^{2}), and those endemic to China (*Ester*^{8}, *Ester*^{9}, *Ester*^{10} and *Ester*^{11}) (Cui et al., 2006a). Additionally, a large number of resistance alleles at the *Ester* locus co-exist in different locations. So, field surveys should be regularly carried out in China to improve our understanding of this atypical and complex situation for the evolution of insecticide resistance genes, and our ability of resistance management.

In this study, we report the results of a recent investigation of the field *C. pipiens* populations in China, including the resistance levels with regard to OPs, carbamate insecticides (CBs), pyrethroid insecticides (PYs), and the involved resistance *Ester* alleles. The results for the evolution and the management of insecticide resistance in *C. pipiens* populations are discussed and compared with those in published surveys.

Materials and methods

Mosquitoes

Four field *C. pipiens* complex populations were collected as larvae, pupae or adult at different locations in Beijing, China (Table 1). Field larvae were split into two lots upon arrival at the laboratory. The first was used for bioassays and the second was developed to the adult stage, then deep frozen and stored in liquid nitrogen for further analyses.

In addition, four lab standard strains were used as controls. S-Lab was an insecticide-susceptible strain without any known resistance genes (Georghiou et al., 1966). SB1, SA2, and LING were, insecticide-resistant strains homozygous for *Ester*^{1}, *Ester*^{2}, and *Ester*^{9}, respectively, and overproducing esterases B1, A2B2, and A9B9, respectively (Weill et al., 2001; Berticat et al., 2002).

Insecticide bioassays

Resistance characteristics of larval populations were determined by bioassays on the fourth-instar larvae as described previously (Marquine and Raymond, 1994). Seven insecticides were used in ethanol solutions: two OP insecticides (dichlorvos and parathion), two CB insecticides (fenobucarb and propoxur), and three PY insecticides (deltamethrin, permethrin and tetramethrin). All insecticides were produced by Qingdao Insecticide Factory (Shandong, China), except for propoxur (Bayer, Leverkusen, Germany). According to the number of larvae available, five doses and three replicates (20 larvae per replicate) per dose were treated with each insecticide. Mortality data were analyzed by the log-probit program (Raymond, 1993), based on a prior iteration (Finney, 1971). This program takes into account an eventual natural mortality, provides LC_{50} values and slope for each mortality lines, tests parallelism between two or more mortality lines, and computes resistance ratios (RR) with 95% confidence intervals (Cui et al., 2007a).

Table 1
Collection sites of *Culex pipiens* complex sampled in China.

Province or municipality	Locality (latitude, longitude)	Code	Date (year, day/month)	Type of sites
Guangdong	Guangzhou (23°08′N, 113°15′E)	MM	2007, 28/06	Sewage
		HZQ	2007, 08/07	Sewage ditch
	Foshan (23°03′N, 113°06′E)	DTY	2007, 06/07	Sewage ditch
Hubei	Wuhan (30°37′N, 114°21′E)	JY	2007, 11/09	Ditch
	Sashi (30°16′N, 112°17′E)	LXX	2007, 11/09	Sewage puddle
Henan	Xinxiang (35°18′N, 113°52′E)	XXH	2006, 03/09	River
		HSD	2006, 03/09	Puddle
Shandong	Zibo(36°48′N, 118°03′E)	ZC	2006, 08/09	Sewage puddle
Beijing	Beijing (39°54′N, 116°28′E)	CY	2007, 30/10	Sewage ditch

Identification of esterase alleles

Each adult mosquito was cut into head–thorax and abdomen portions. The head–thorax of each mosquito was homogenized and exposed to highly active esterases with the starch gel electrophoresis in Tris–EDTA buffer system (Pasteur et al., 1988). SB1, SA2 and LING strain mosquitoes were used as reference in each gel. The abdomen of each mosquito was stored in liquid nitrogen for DNA extraction. More than 50 individuals were analyzed for each population.

Molecular identification was only performed on uncertain cases. DNA extraction was performed as described previously (Roger and Bendich, 1988). Polymerase chain reaction (PCR) for esterase fragment was performed using a pair of primers 5′-ATGCTCAACCGCC GAAACCG-3′ and 5′-CACGGACCAATTGTTCAGCAC-3′ for the *Est-3* locus or 5′-CGGTTGGGATGGRSARGGTGG-3′ and 5′-ATGCGGTAGTG GTTGTARAACTC-3′ for the *Est-2* locus. PCR was conducted 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 $MgCl_2$, and 2.5 U of *Taq* polymerase (Takara) in a 10× reaction buffer, and was run on a PTC100 thermocycler (MJ Research, Waltham, MA, USA) with a denaturing step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 56 °C for 30 s, and 45 s at 72 °C, and a final step of 10 min at 72 °C. The PCR fragments were sequenced and analyzed by the BLAST program of the NCBI.

Results

Insecticide resistance status of the populations

Using S-Lab as control, resistance levels of the nine populations of *C. pipiens* from China to OP, CB and PY insecticides were studied by bioassay. The results are summarized in Table 2. For all seven insecticides, dose-mortality curves fit a linear regression model ($P > 0.05$), except for two samples: HZQ with propoxur and LXX with tetramethrin. The resistance to permethrin was highest with 88-fold resistance ratio (RR) in the sample from Guangdong province, followed by dichlorvos, parathion, fenobucarb, tetramethrin, propoxur, and deltamethrin (the highest RR was 4-fold).

Table 2 also showed that there was a rather complex pattern of insecticide resistance for the *C. pipiens* complex in China. In Guangdong province, the resistance to permethrin varied between 4 and 88 and to parathion varied between 5 and 11, while a low but significant resistance to other insecticides (RR from 2 to 8) was displayed without the RR of MM to deltmethrin (RR = 1.1). In Hubei province, the two populations, JY and LXX, displayed a low but significant resistance to propoxur, fenobucarb, tetramethrin, and deltmethrin (RR from 2 to 8), and moderate but significant resistance to dichlorvos and permethrin (RR from 10 to 16). Additionally, JY clearly showed more resistance to parathion than LXX did (RR from 12 and 8). In Henan province, the two populations presented significant difference in OP resistance: HSD showed more resistance than XXH (RR was computed with XXH as reference: 2.3-fold for parathion and 2.6-fold for dichlorvos). For CB and PY resistance, no difference was observed: both populations displayed a low but significant resistance to CB insecticide, and they nearly did not show any resistance to PY

Table 2
Resistance observed in bioassays to seven insecticides in populations of *Culex pipiens* complex from China.

Population code	Parathion		Dichlorvos		Propoxur		Fenobucarb		Deltamethrin		Permethrin		Tetramethrin	
	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	LC_{50}(mg L^{-1}) (95%CI)	RR	LC_{50}(mg L^{-1}) (95%CI)	RR
S-Lab	0.0006 (0.0005–0.0007)	1	0.106 (0.091–0.126)	1	0.052 (0.046–0.060)	1	0.065 (0.060–0.071)	1	0.0023 (0.0021–0.0026)	1	0.007 (0.006–0.009)	1	0.188 (0.133–0.268)	1
MM	0.0030 (0.0027–0.0034)	4.9	0.546 (0.478–0.643)	5.1	0.153 (0.138–0.171)	2.9	0.208 (0.179–0.236)	3.2	0.0021 (0.0017–0.0026)	1.1	0.028 (0.021–0.034)	4.2	0.646 (0.568–0.719)	3.4
HZQ	0.0030 (0.0025–0.0035)	4.8	0.715 (0.656–0.770)	6.7	0.204 (0.118–0.349)	3.9	0.275 (0.240-p.305)	4.2	0.0043 (0.0040–0.0056)	2.5	0.069 (0.054–0.092)	10.6	0.427 (0.376–0.476)	2.3
DTY	0.0071 (0.0061–0.0080)	11.4	0.873 (0.770–0.988)	8.2	0.255 (0.173–0.299)	4.9	0.206 (0.165–0.260)	1.9	0.0078 (0.0053–0.0159)	4.0	0.573 (0.443–0.833)	88.4	1.150 (0.53–1.37)	6.1
JY	0.0072 (0.0065–0.0083)	11.6	1.714 (1.517–1.931)	16.1	0.284 (0.253–0.312)	5.4	0.521 (0.485–0.563)	8.0	0.0045 (0.0038–0.0052)	2.3	0.094 (0.028–0.327)	14.6	1.037 (0.840–1.181)	5.5
LXX	0.0048 (0.0042–0.0054)	7.8	1.090 (0.964–1.263)	10.2	0.205 (0.180–0.234)	3.9	0.231 (0.113–0.230)	3.5	0.0049 (0.0042–0.00549)	2.5	0.083 (0.067–0.102)	12.8	1.341 (0.547–3.330)	7.1
HSD	0.0073 (0.0068–0.0078)	11.7	1.152 (1.036–1.356)	10.9	0.254 (0.234–0.274)	4.9	0.327 (0.299–0.355)	5.0	0.0007 (0.0006–0.0009)	0.3	0.009 (0.008–0.010)	1.4	0.305 (0.277–0.334)	1.6
XXH	0.0031 (0.0028–0.0034)	5.0	0.444 (0.398–0.486)	4.1	0.173 (0.155–0.188)	3.3	0.382 (0.350–0.415)	5.9	0.0007 (0.0005–0.0009)	0.3	0.005 (0.005–0.007)	0.8	0.30940 (0.275–0.348)	1.7
ZC	0.0117 (0.0109–0.0128)	18.8	3.436 (2.898–5.485)	32.3	0.202 (0.164–0.228)	3.9	0.335 (0.311–0.358)	5.1	0.0063 (0.0053–0.0071)	3.2	0.094 (0.057–0.141)	14.4	0.858 (0.745–0.968)	4.6
CY	0.0034 (0.0029–0.0038)	5.4	2.571 (2.179–3.070)	24.2	0.276 (0.254–0.297)	5.3	0.430 (0.395–0.462)	6.6	0.0024 (0.0018–0.0031)	1.3	0.102 (0.074–0.140)	15.7	0.694 (0.593–0.816)	3.7

CI: Confidence interval.
RR: Resistance ratio (LC_{50} of population/LC_{50} of S-Lab).

insecticide, as indicated by RR values lower than 2. In Shandong province, only one collection was available for bioassays. It had a high resistance to dichlorvos (RR = 32), a moderate but significant resistance to permethrin (RR = 14) and a low but significant resistance to other insecticides. In Beijing, also only one collection was available for bioassays, which exhibited a higher resistance to dichlorvos (RR = 24) and to permethrin (RR = 16) than that to other insecticides (RR < 7).

In summary, the level of resistance to OP insecticides according to location was Shandong > Beijing > Hubei > Henan > Guangdong. All the populations had similar resistance to CB insecticides, which was low but significant (2 < RR < 10). For PY insecticides, a rather complex pattern of insecticide resistance was observed, with the exception of Henan province, where the mosquitoes were sensitive to the tested insecticides.

Identification of esterase alleles

Overall, 621 field mosquitoes were analyzed for identification of esterase alleles (four individuals from DTY are presented in Fig. 1 as an example). Starch gel electrophoresis disclosed six known overproduced esterases in these field samples, i.e. esterase B1, A2B2, A8B8, A9B9, B10, and A11B11. DNA identification confirmed some uncertain profiles, but no other resistant esterase alleles were found in this study. The population frequencies of mosquitoes of these six overproduced esterase phenotypes are listed in Table 3. Frequencies varied according to geographic origin. The most prevalent was B1, which was present in all localities, especially in Shandong province, where the phenotype was displayed in all mosquitoes. In Henan province, only three overproduced esterases phenotypes were detected: the most common phonotype was B1 with 81% mosquitoes in HSD and 75% mosquitoes in XXH, followed by A2B2 and A8B8. In Hubei province, B1 was the most common allele, present in 58 or 96% of mosquitoes, followed by A9B9, A2B2 and A11B11. B10 was not found for this location. In Guangdong province, B1 and A8B8 prevailed (up to 67% of mosquitoes displayed it in MM), followed by A2B2, with A9B9, B10, and A11B11 being the least abundant. In Beijing, four esterase phenotypes were found: the most common was B1, and all other resistance alleles were found in < 11% of the mosquitoes. In addition, most populations had four resistant esterases co-existing. There were few individuals heterozygous for esterase alleles, while composite pattern was not found.

Dynamics of esterase allele frequency

As seen in Table 3, we compared the result from this work with published surveys of the population frequencies of overproduced esterase phenotypes in different geographic regions. The results about the average esterase frequencies in the different years are shown in Figs. 2–5.

In Guangdong province, only three esterase genotypes were observed in 1994 (Qiao et al., 1999), while there were six esterase genotypes in 2007. The average frequencies of esterase B1 and A2B2 were slowly increased in the past (Fig. 2). As for A8B8, the average frequency fluctuated between 35% and 57% from 1994 to 2007 (Qiao et al., 1999; Sun and Qiao, 2000; Zhang et al., 2003b; Cui et al., 2006a). Furthermore, the esterase A9B9 was first observed in 1995 (Qiao et al., 1999) whose average frequency dramatically declined from 57% to 8%. In addition, very few mosquitoes displayed the B10 and A11B11.

In Hubei province, there were only three surveys about the frequency of esterases (Sun and Qiao, 2000; Cui et al., 2006a). The average frequencies of the individual mosquitoes' esterase genotypes were summarized in Fig. 3. The average frequency of esterase B1 has slowly increased from 47% to 77%. However, the average frequency of esterase

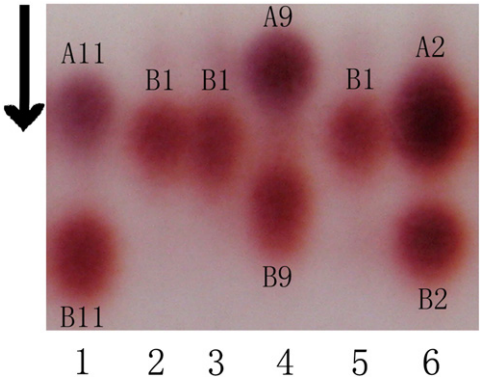

Fig 1. High-activity esterases in single adults from DTY sample analyzed on starch gel electrophoresis. Only part of gel is shown, the arrow indicates the electrophoretic migration of protein. Lane 1: individual 1, which expressed A11–B11. Lane 2: the control of $Ester^{B1}$. Lane 3: individual 2, which expressed B1. Lane 5: individual 5, which expressed B1. Lane 4: individual 4, which expressed A9–B9. Lane 6: control of $Ester^{2}$.

Table 3
Frequency of overproduced esterase in field populations of *Culex pipiens* complex in China.

Province or municipality	Year of sampling	Locality	Code	N	Esterase frequency[a]								Reference
					B1	A2B2	B7	A8B8	A9B9	B10	A11B11	SS	
Guangdong	1994	Guangzhou		119	0.05	0.10	0.45	0.35				0.18	Qiao et al. (1999)
	1995	Guangzhou		89	0.13	0.16		0.57	0.57				Sun and Qiao (2000)
	2001	Guangzhou	GZ	103	0.21	0.34		0.53	0.63				Zhang et al. (2003b))
		Foshan		98	0.26	0.41		0.47	0.31				Zhang et al. (2003b)
		Zhongshan	ZS	88	0.04	0		0.53	0.47				Zhang et al. (2003b)
	2003	Guangzhou	Karaoke	62	0.11	0.22	0	0.38	0.41	0.15	0	0.10	Cui et al. (2006a)
			Jin1	62	0.27	0.11	0	0.55	0.07	0.02	0	0.18	Cui et al. (2006a)
			Jin2	91	0.15	0.27	0	0.40	0.45	0.27	0	0.02	Cui et al. (2006a)
			Lin	62	0.12	0.10	0	0.37	0.29	0.13	0	0.24	Cui et al. (2006a)
			GongDi	19	0.22	0.11	0	0.37	0.16	0.05	0	0.16	Cui et al. (2006a)
		Foshan	Manda1	69	0.56	0.17	0	0.35	0.07	0.03	0	0.12	Cui et al. (2006a)
			Manda2	27	0.33	0.30	0	0.33	0.11	0.11	0	0.04	Cui et al. (2006a)
			Manda3	62	0.54	0.21	0	0.39	0.14	0.02	0	0.06	Cui et al. (2006a)
			Manda4	62	0.38	0.32	0	0.46	0.10	0.05	0	0.05	Cui et al. (2006a)
	2007	Guangzhou	MM	60	0.12	0.27	0	0.67	0	0	0	0.27	This study
			HZQ	89	0.44	0.26	0	0.15	0.17	0.01	0.01	0.12	This study
		Foshan	DTY	61	0.61	0.16	0	0.43	0.07	0.02	0.02	0.02	This study
Hubei	1995	Wuhan		67	0.42	0.09	0.09	0.33					Sun and Qiao (2000)
		Shashi		84	0.51	0.03	0.04	0.55					Sun and Qiao (2000)
	2003	Wuhan	ZhuChang	62	0.52	0.12	0	0.06	0.18	0.02	0.18	0.16	Cui et al. (2006a)
			CaiYuan	80	0.53	0.09	0	0.04	0.21	0.01	0.17	0.15	Cui et al. (2006a)
			LanXi	85	0.48	0.02	0	0.05	0.26	0.09	0.24	0.12	Cui et al. (2006a)
	2007	Wuhan	JY	91	0.58	0.13	0	0	0.10	0	0.36	0.04	This study
		Shashi	LXX	49	0.96	0.02	0	0.02	0	0	0.06	0.04	This study
Henan	2001	Zhengzhou	ZZ	50	0.20	0		0.66	0.16				Zhang et al. (2003a)
		Shangqiu	SQ	94	0.49	0.47		0.49	0.05				Zhang et al. (2003a)
	2003	Zhengzhou	Shen	102	0.78	0.18	0	0.07	0.10	0	0.12	0.06	Cui et al. (2006a)
			TaiQiu	92	0.84	0.30	0	0.08	0.12	0.02	0.04	0.02	Cui et al. (2006a)
	2006	Xinxiang	XXH	60	0.75	0.12	0	0.03	0	0	0	0.20	This study
			HSD	57	0.81	0.12	0	0	0	0	0	0.11	This study
	2010	Weihe	WHE	35	0.83	0	0	0	0.09	0.06	0.03	0.06	Liu et al. (2011)
		Yuanyang	YUY	41	0.88	0.2	0	0.05	0	0	0	0.17	Liu et al. (2011)
Shangdong	1998	Gaomi		81	0.82	0							Liu and Qiao (2001)
	2003	Qingdao	QingDao	73	0.74	0.04	0	0	0	0	0	0.23	Cui et al. (2006a)
	2007	Zichuan		61	1.00	0	0	0	0	0	0	0	This study
	2010	Taian	TAA	45	0.98	0.2	0	0.05	0	0	0	0.25	Liu et al. (2011)
Beijing	1998	Beijing	BJ	82	0.76	0							Liu and Qiao (2001)
	2001	Beijing	BJ	42	0.79	0		0.07	0				Zhang et al. (2003a)
	2003	Beijing	BJBJT	91	0.69	0.07	0	0.02	0.01	0	0.04	0.25	Cui et al. (2006a)
			BJTJL	62	0.44	0	0	0	0.02	0	0	0.55	Cui et al. (2006a)
			BJSGZ	62	0.66	0.12	0	0.10	0.02	0.02	0.04	0.23	Cui et al. (2006a)
			BJFT	62	0.69	0.05	0	0.03	0	0	0.05	0.31	Cui et al. (2006a)
			BJHY	89	0.81	0.14	0	0	0.03	0.02	0.02	0.13	Cui et al. (2006a)
	2006	Beijing	YMY	60	0.75	0.10	0	0	0.01	0	0.06	0.23	Yan et al. (2008)
			TZ	58	0.68	0.10	0	0	0.05	0	0.17	0.17	Yan et al. (2008)
			CY	90	0.58	0.04	0	0.02	0	0	0.01	0.26	Yan et al. (2008)
			SH	117	0.64	0.05	0	0	0.03	0	0	0.21	Yan et al. (2008)
	2007	Beijing	CY	93	0.65	0.03	0	0.01	0.11	0	0	0.27	This study
	2010	Beijing	BJI	36	0.72	0.03	0	0	0	0	0	0.25	Liu et al. (2011)
Liaoning	2003	Huludao	HLD	107	0.76	0.01	0	0	0	0	0	0.24	Cui et al. (2007a)
	2010	Pulandian	LPU	43	0.56	0.14	0	0	0	0	0	0.33	Liu et al. (2011)

N: Number of individuals analyzed.
SS: Esterase non-overproduced phenotype.
[a] The sum of phenotypic frequency in each population is not necessarily equal to 1, as some individuals are heterozygous with two overproduced esterases.

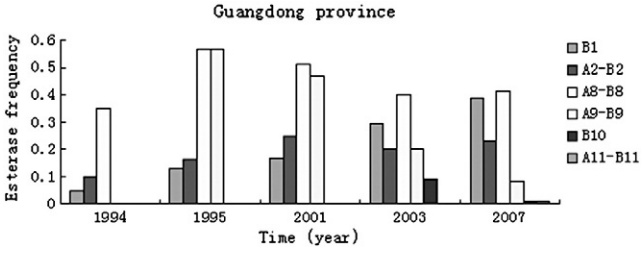

Fig. 2. Dynamics of overproduced esterase alleles in field populations of *Culex pipiens* complex from Guangdong province.

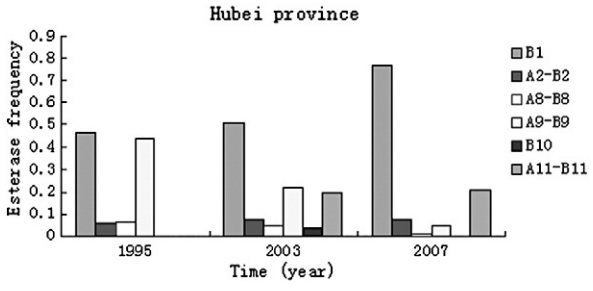

Fig .3. Dynamics of overproduced esterase alleles in field populations of *Culex pipiens* complex from Hubei province.

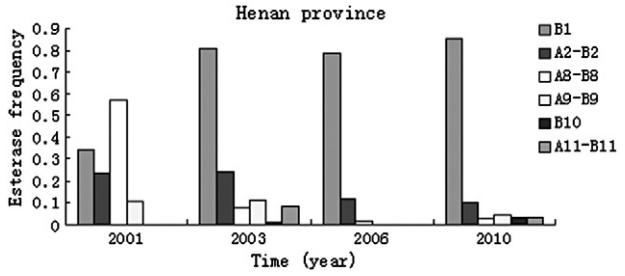

Fig. 4. Dynamics of overproduced esterase alleles in field populations of *Culex pipiens* complex from Henan province.

A9B9 has precipitously decreased from 44% to 5%. As for the other esterases, which were displayed in few individual mosquitoes, there was a limited change about the frequencies of esterase genotypes.

In Henan province, there were four surveys about the frequency of esterases (Zhang et al., 2003a; Cui et al., 2006a; Liu et al., 2011). The average frequency of esterase B1 was 85.5% in 2010 and was 79.5% in both 2003 and 2006, which were higher than those in 2001 (Fig. 4). This contrasted with the average frequency of esterase A2B2 and A8B8. There was almost no change about the frequencies of the other esterase genotypes.

In Shandong province, three were also four surveys about the frequency of esterases (Liu and Qiao, 2001; Cui et al., 2006a; Liu et al., 2011), and only three esterase genotypes were observed (Table 3). The most prevalent genotype was B1, followed by A2B2 that was only displayed in 2003 and 2010, and A8B8 was only displayed in 2003 with a frequency of was 5% (Cui et al., 2006a; Liu et al., 2011).

In Beijing, the results of six surveys about the population frequencies of overproduced esterase phenotypes were summarized in Fig. 5 (Liu and Qiao, 2001; Zhang et al., 2003a; Cui et al., 2006a; Yan et al., 2008; Liu et al., 2011). The most common allele was B1, whose average frequency fluctuated between 65% and 79% from 1998 to 2010. Very little fluctuation was observed on the average frequencies of all other esterase genotypes, except in 2010 when only two esterase genotypes were observed.

In Liaoning province, only two surveys about the frequency of esterases have been documented (Cui et al., 2007a; Liu et al., 2011), and only two esterase genotypes were observed (Table 3). The frequency of mosquitoes with B1 was between 56 and 76%, and A2–B2 were found in <14% of the mosquitoes (Cui et al., 2007a; Liu et al., 2011).

These changes reflect a complex pattern of resistance gene evolution under local selective pressure of insecticides.

Discussion

In this study, nine field populations of *C. pipiens* were surveyed for their susceptibilities to three different classes of insecticides. Compared to prior surveys, these various levels of insecticide resistance indicate that the insecticide resistances of different geographic regions are potentially correlated with the selection pressures from variable histories and intensities of insecticide application in China. It has been over 40 years since OP insecticides were first introduced into China, and they have been used extensively in most parts of the country (Hua and Shan, 1996; Cui et al., 2006a). However, CBs and PYs have replaced OPs partially or completely in some regions of China, especially in places displaying serious OP resistance. Additionally, the bioassay results also indicated that these various levels of insecticide resistance may reflect different insecticide selection pressures exerted on field mosquito populations. Indeed, the mosquitoes of ZC were mostly collected from the Shan Dong Da Cheng Pesticides (previously known as Shan Dong Pesticides Factory), so that the parathion and dichlorvos resistances of ZC were higher than the others. In Guangdong province, DTY field mosquitoes, which were collected from a sewage ditch where permethrin was used, had an 88-fold resistance ratio to permethrin. Further studies involving social scientists, chemical ecologists, and environment biologists will be needed to document the amount, frequency, and diversity of insecticides used in these areas to explore in greater detail the putative selective pressures leading to the selection of insecticide resistance.

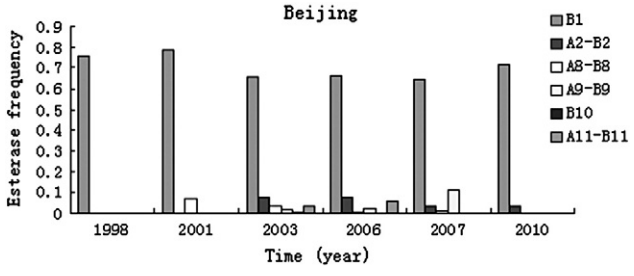

Fig. 5. Dynamics of overproduced esterase alleles in field populations of *Culex pipiens* complex from Beijing.

One of the main findings of this study was the confirmation of six esterase alleles in China, including $Ester^{B1}$, $Ester^{2}$, $Ester^{8}$, $Ester^{9}$, $Ester^{10}$, and $Ester^{11}$ (Table 3). However, different esterase alleles were observed in different geographic regions. For example, only one or two esterase alleles were identified in Shandong and Liaoning provinces, while all six esterase alleles were confirmed in the other provinces or municipalities in surveys conducted in other years. This may due to the low number of samples, particularly in Shandong and Liaoning provinces where only one sample was studied in each survey. In general, the sample sizes that were used in research studies were not large enough to produce sufficiently precise reliability coefficients, which in turn could cause imprecise estimates of the esterase frequency in larger areas. In addition, from the data available (Table 3), it seems that the distribution ranges of these alleles has changed over time. In China, the most common esterase allele was $Ester^{B1}$, which was identified with fluctuating frequencies in all populations in the past. The average frequency of overproduced esterase in China slowly increased from 1994 to 2010, the exception of 1998. In 1998, only one esterase allele $Ester^{B1}$, which average frequency was 79%, was observed in China (Liu and Qiao, 2001). Except for the surveys in 1998 and 2010, the frequencies of $Ester^{8}$ and $Ester^{9}$ declined, and the frequencies of $Ester^{2}$, $Ester^{10}$ and $Ester^{11}$ displayed almost no change. This may be the result of allelic competition as illustrated by the situation in southern France, where $Ester^{1}$ was replaced by $Ester^{4}$ over a 10-year period (Guillemaud et al., 1998; Labbe et al., 2007). It is likely that one or several of the existing alleles will be eliminated in the different geographic regions in the future. Hence, more populations and continuous monitoring in the future are required to explain and prove the dynamic changes of the resistant alleles in China.

In conclusion, the surveyed *C. pipiens* complex populations in China showed variable resistance levels to OP, CB, and PY insecticides, and a high polymorphism in resistant esterase alleles was observed in these populations. The data indicate a dynamic evolution over time under local specific insecticide selection. These results identify competition between resistant esterase alleles, and should be helpful in predicting their future changes, thereby improving resistance management.

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