Filariasis vector in China: insecticide resistance and population structure of mosquito *Culex pipiens* complex

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Abstract: Seven field populations of mosquito *Culex pipiens* complex (Diptera: Culicidae) were collected from four provinces of China. The resistance status of larvae to dichlorvos, parathion, chlorpyrifos, fenobucarb (BPMC) and propoxur were determined by bioassays, disclosing that they were more resistant to organophosphate (moderate or low resistance) than to carbamate (low or no significant resistance) insecticides. Starch gel electrophoresis confirmed the presence and distribution of overproduced esterases B1, A2-B2, A8-B8 and A9-B9, the frequencies of which varied according to their regional origins. Electrophoretic polymorphism at four putatively neutral loci (*got-1, got-2, pgi* and *pgm*) showed that the overall genetic differentiation found across all populations was significantly large (Fst = 0.28, $P < 10^{-4}$), and genetic exchange was slightly restricted by distance isolation ($P = 0.018$).

Keywords: *Culex pipiens*; organophosphate; carbamate; overproduced esterases; population structure

1 INTRODUCTION

Resistance to chemical insecticides is widespread among a large number of insect pest species.[1–3] The mosquito *Culex pipiens* L., the vector of *Wuchereria bancrofti* Cobbold and West Nile virus,[4] is one of the few insect species in which insecticide resistance, in particular to organophosphate products, has been studied in the wild at the gene level.[5] Three loci were proven to have developed major resistance alleles.[6,7] The first two loci, *est-2* and *est-3*, code for detoxifying carboxylester hydrolases and confer OP resistance through overproduced esterases, which is achieved predominantly by gene amplification or by gene upregulation.[8,9] The third locus, *ace-1*, codes for acetylcholinesterase (the insecticide target), and insensitive alleles have been reported in multiple locations.[10,11]

In most parts of China, mosquitoes have been subjected to organophosphate (OP) insecticide treatments since the mid-1960s, and resistance gene monitoring in the *C. pipiens* complex started in only a few locations from the end of the 1980s (unpublished data). A large variety of OPs were used alone or simultaneously, e.g. temephos, malathion, trichlorfon, phoxim, fenitrothion, fenthion and dichlorvos,[12] and many resistant alleles of esterases have been found in natural populations (unpublished data). An in-depth understanding of the mechanisms that control the evolution of insecticide resistance in natural populations is one of the keys that will make it possible to devise methods of resistance management.[13] One of the first requirements is a periodic monitoring of resistance genes over time and their geographic distribution. In this study, seven field populations of *C. pipiens* complex were collected from four provinces of China where no or few investigations of resistance mechanisms and population genetic structure in this mosquito species have been carried out, in order to address the following points. What is the resistance status with regard to OP and carbamate (CB) insecticides in these populations? What kinds of overproduced carboxylesterase are responsible for resistance in each population and how do they distribute? What role does migration (passive or positive) play in the dispersal of resistance genes? The investigation of target resistance (insensitive acetylcholinesterase) will not be taken into account in this study owing to the completely different experimental measures from overproduced carboxylesterases.

2 MATERIALS AND METHODS

2.1 Mosquito samples

Culex pipiens complex were collected as larvae, pupae or adults in seven localities of China between August 2003 and June 2004 (Table 1; Fig. 1). Field larvae (or F0) were taken to the laboratory; one set was used for bioassays, the other one was reared to adults, then deep frozen and stored in liquid nitrogen for

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

Province	Locality (latitude, longitude)	Code	Date	Development stage	Type of site
Liaoning	Huludao (40°48′N, 120°48′E)	HLD	16/9/03	Larvae and pupae	Ditch
Zhejiang	Hangzhou (30°14′N, 120°09′E)	HZ	7/6/04	Adults	Residential area
	Fuyang (30°04′N, 119°57′E)	FY	29/8/03	Adults	Residential area
Guangxi	Huanjiang (24°48′N, 108°12′E)	HJ	1/6/04	Larvae and pupae	Puddle
	Nanning (22°48′N, 108°20′E)	NN	8/6/04	Larvae and pupae	Vat
Hainan	Haikou (20°02′N, 110°20′E	HK	29/4/04	Larvae and pupae	Puddle
	Sanya (18°12′N, 109°30′E)	SY	27/4/04	Larvae and pupae	Sewage

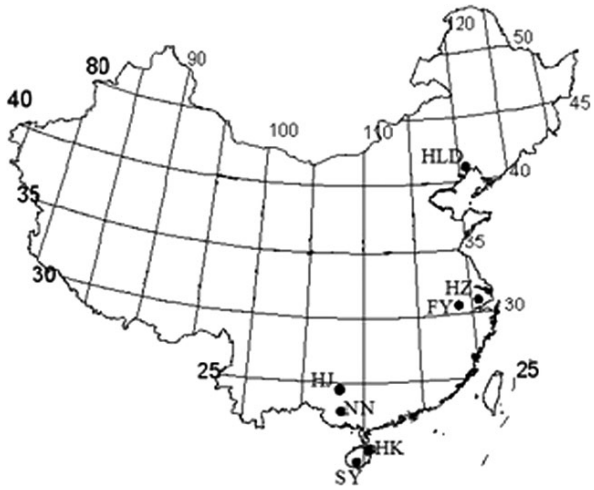

Figure 1. Localities of the seven populations of *Culex pipiens* collected in China.

further analyses. When the number of F0 larvae was insufficient, they were bred and bioassays were performed on ensuing generations (maximum F4).

2.2 Insecticide bioassays

Resistance characteristics of larval populations were determined by bioassays on fourth-instar larvae, following the method described by Raymond and Marquine.[14] Five insecticides were used in ethanol solutions: three OP insecticides, dichlorvos, parathion and chlorpyrifos, and two CB insecticides, fenobucarb [2-*sec*-butylphenyl methylcarbamate (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 five doses and three replications (20 larvae per replicate) per dose were used with each insecticide. Bioassays were also carried out on S-LAB, an insecticide-susceptible strain without any known resistance genes.[15] Mortality data were analysed by the log-probit program of Raymond,[16] based on Finney.[17] This program takes into account an eventual natural mortality and provides LC values and slope for each mortality line, tests parallelism between two or more mortality lines and computes resistance ratios (RRs) with 95% confidence intervals.

2.3 Resistance esterase identification

The presence or absence of highly active esterases was determined on single mosquito homogenates by starch gel electrophoresis (TME 7.4 buffer system) as described by Pasteur *et al*.[18] Two laboratory strains of known resistance were used as controls: esterase B1 strain SB1, esterase A2-B2 strain SA2.[19] Other phenotypes such as A8-B8 and A9-B9 were identified according to their migration distance contrasted with A2-B2 and B1.[20]

2.4 Genotype determination of neutral genes

Genotypes of four putatively neutral gene loci were determined by starch electrophoresis (TME 7.4 buffer system) on adult mosquito homogenates:[18] *got-1* and *got-2* (two glutamate-oxaloacetate transaminases, EC 2.6.1.1), *pgi* (phosphogluco-isomerase, EC 5.3.1.9) and *pgm* (phosphoglucomutase, EC 2.7.5.1). SA2 and SB1 were used as reference standard. For each enzyme the electromorph band of the reference strains was designated '100' and other electromorphs (alleles) were numbered according to their relative electrophoretic mobility, i.e. 110, 120, etc., for faster bands, and 90, 80, etc., for slower bands.

2.5 Statistics

Conformity with Hardy–Weinberg (HW) expectations was tested for the proportions of genotypes at each locus, using the exact U-score test in the presence of the alternative hypothesis of heterozygote deficiency.[21] A global test across samples and/or loci was also performed.[21] Genotypic associations between each pair of loci in each population were tested using the probability test described by Raymond and Rousset.[22] For each locus pair, global tests (Fisher's method) were performed across all populations. Departure from HW was measured using the Fis estimator proposed by Weir and Cockerham.[23] Genotypic differentiation between populations or groups of populations was tested by computing an unbiased estimate of the *P* value of a log-likelihood (G) based exact test.[24] Population differentiation was measured using the Fst estimator.[23] Isolation by distance was analysed as described by Rousset,[25] i.e. computing the relationship between pairwise estimates of Fst/(1 − Fst) and logarithm of geographic distance. A possible positive relationship was tested with a Mantel test, using the Spearman rank correlation coefficient statistic. Geographical distances between samples were taken as the

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shortest measurement on a map. Computations were performed with the Genepop version 3.4 software,[26] and multiple testing was done using the Bonferroni method.[27]

3 RESULTS

3.1 Insecticide resistance status of the populations

Bioassays were performed either on field individuals (F0) or on their laboratory offspring (F1–F4). For all five insecticides, dose–mortality curves were well represented by regression lines ($P > 0.05$) with one exception, HZ to dichlorvos (Table 2). The resistance levels to the two types of insecticide among populations were varied. Most of them exhibited a low but significant resistance (RR < 10, $P < 0.05$), while a moderate resistance ($10 \leq$ RR < 21) was found only in SY to dichlorvos and parathion, and in HLD to parathion and chlorpyrifos. The resistance was not significant ($P > 0.05$) in NN to dichlorvos, in FY, SY, HJ and HLD to BPMC and in HJ to propoxur

Table 2. Resistance observed in bioassays to five insecticides in populations of *Culex pipiens* from China[a]

Insecticide and population	*N*	LC_{50} (95% CI) (mg L^{-1})	Slope (SE)	χ^{2b}	RR (95%CI)	G
Dichlorvos						
S-LAB	240	0.027 (0.020–0.046)	1.81 (0.49)	3.27	1	
HLD	240	0.188 (0.164–0.215)	4.65 (0.75)	0.0011	6.9 (4.7–10.0)	F1
HZ	360	0.107 (0.070–0.159)	3.25 (0.64)	8.39*	3.9 (2.2–6.9)	F1
FY	300	0.119 (0.108–0.132)	5.65 (0.67)	4.84	4.3 (3.0–6.3)	F4
HJ	240	0.051 (0.035–0.061)	3.62 (0.76)	3.36	1.8 (1.2–3.0)	F1
NN	300	0.033 (0.003–0.051)	2.09 (0.74)	3.93	1.2 (0.6–2.4)	F0
HK	300	0.228 (0.187–0.258)	4.69 (0.82)	1.17	8.3 (5.5–12.5)	F2
SY	300	0.563 (0.515–0.620)	6.18 (0.73)	2.65	20.5 (14.2–29.6)	F2
Parathion						
S-LAB	240	0.0004 (0.00031–0.00065)	1.97 (0.41)	2.46	1	
HLD	360	0.0049 (0.0045–0.0053)	6.13 (0.67)	2.33	11.9 (8.3–17.1)	F2
HZ	300	0.0031 (0.0028–0.0034)	6.53 (0.89)	1.94	7.6 (5.2–11.0)	F1
FY	300	0.0015 (0.0013–0.0017)	3.29 (0.37)	1.34	3.6 (2.5–5.0)	F4
HJ	300	0.0013 (0.0012–0.0014)	5.44 (0.62)	2.28	3.2 (2.2–4.6)	F1
NN	240	0.0016 (0.0014–0.0018)	5.91 (0.87)	1.99	3.8 (2.5–5.9)	F0
HK	240	0.0027 (0.0021–0.0032)	3.52 (0.73)	3.69	6.6 (4.4–9.8)	F2
SY	240	0.0041 (0.0038–0.0044)	7.92 (1.31)	0.96	10.0 (7.0–14.3)	F2
Chlorpyrifos						
S-LAB	240	0.00054 (0.00045–0.00062)	4.20 (0.75)	5.36	1	
HLD	300	0.0055 (0.0050–0.0063)	5.09 (0.79)	3.27	10.2 (7.2–14.5)	F2
HZ	300	0.00085 (0.00074–0.00097)	3.84 (0.59)	2.04	1.6 (1.1–2.2)	F1
FY	240	0.0012 (0.0010–0.0013)	4.71 (0.66)	0.39	2.1 (1.5–3.1)	F4
HJ	360	0.00017 (0.00002–0.00030)	1.60 (0.47)	2.56	0.3 (0.2–0.6)	F1
NN	300	0.00081 (0.00071–0.00091)	4.54 (0.78)	2.05	1.5 (1.1–2.1)	F0
HK	300	0.0014 (0.0010–0.0017)	2.57 (0.49)	0.18	2.5 (1.7–3.7)	F2
SY	300	0.0026 (0.0022–0.0028)	4.67 (0.71)	0.19	4.7 (3.3–6.7)	F2
Fenobucarb						
S-LAB	240	0.15 (0.13–0.17)	3.79 (0.60)	0.49	1	
HLD	300	0.16 (0.13–0.19)	3.29 (0.52)	0.59	1.1 (0.8–1.6)	F2
HZ	300	0.23 (0.16–0.28)	2.95 (0.72)	2.75	1.6 (1.1–2.3)	F1
FY	300	0.17 (0.15–0.19)	4.26 (0.48)	2.24	1.2 (0.9–1.6)	F4
HJ	300	0.11 (0.10–0.12)	4.18 (0.52)	0.22	0.7 (0.5–1.0)	F1
NN	240	0.08 (0.06–0.10)	3.87 (0.91)	0.56	0.6 (0.4–0.8)	F1
HK	300	0.25 (0.23–0.28)	6.34 (0.91)	0.32	1.7 (1.2–2.5)	F2
SY	300	0.25 (0.14–0.30)	3.75 (0.94)	0.11	1.7 (1.0–2.9)	F2
Propoxur						
S-LAB	240	0.043 (0.027–0.052)	3.54 (0.90)	1.17	1	
HLD	240	0.098 (0.078–0.113)	4.32 (0.72)	0.0002	2.3 (1.4–3.8)	F1
HZ	300	0.105 (0.090–0.121)	4.25 (0.53)	3.38	2.5 (1.5–3.9)	F1
FY	300	0.205 (0.186–0.224)	5.28 (0.56)	0.06	4.8 (3.1–7.4)	F4
HJ	240	0.047 (0.005–0.072)	2.01 (0.73)	3.59	1.1 (0.5–2.2)	F1
NN	300	0.095 (0.087–0.106)	6.71 (0.94)	0.62	2.2 (1.4–3.6)	F1
HK	300	0.158 (0.137–0.177)	5.19 (0.67)	1.72	3.7 (2.3–5.9)	F2
SY	360	0.166 (0.146–0.185)	4.11 (0.43)	5.18	3.9 (2.5–6.0)	F2

[a] N = number of larvae tested; CI = confidence interval; RR = resistance ratio (LC_{50} of population/LC_{50} of S-LAB); G = generation considered in bioassays.

[b] Chi-square testing linearity of dose–mortality response (*$P < 0.05$).

(the 95% CI of RR included the value 1). In two cases the populations seemed more susceptible than the control strain S-LAB, i.e. HJ to chlorpyrifos and NN to fenobucarb (RR < 1, and its 95% CI did not include the value 1). To summarise, these populations were more resistant to OP (moderate or low resistance) than to CB (low or no significant resistance) insecticides.

3.2 Identification of resistant esterases

A total of 467 mosquitoes were analysed by starch gel electrophoresis, confirming the presence of overproduced esterases B1, A2-B2, A8-B8 and A9-B9. Their frequencies in each population are detailed in Table 3. The most prevalent were B1 and A2-B2, which were present in almost all localities, although at different frequencies. A8-B8 was only found in Hainan (SY and HK) and Guangxi (NN and HJ) province, and A9-B9 only in one sample (HJ) from Guangxi with a very low frequency (3.3%). A large proportion of susceptible individuals also existed in some populations, such as 50% in HK. In Guangxi's samples (NN and HJ), although all four amplified esterases were detected, their colourations with substrates α- and β-naphthyl acetate were much lighter than in other samples, meaning that the amplification levels of esterase genes were very low, corresponding to the low resistance level to OP insecticides. An uncertain phenotype of esterase B, the migration position of which was a little higher than that of B8, was identified in HZ population with a frequency of 50%. Specific studies for it will be reported elsewhere.

Table 3. Frequency (%)[a] of mosquitoes displaying a given overproduced esterase in field populations of *Culex pipiens* from China[b]

Province	Population	*N*	B1	A2-B2	A8-B8	A9-B9	SS
Liaoning	HLD	107	75.7	0.9	0	0	24.3
Zhejiang	HZ	60	58.3	15.0	0	0	23.3
	FY	60	68.3	1.7	0	0	30.0
Guangxi	HJ	60	18.3	28.3	21.7	3.3	43.3
	NN	60	33.3	21.7	8.3	0	41.7
Hainan	HK	60	0	40.0	11.7	0	50.0
	SY	60	11.7	75.0	6.7	0	18.3

[a] The sum of phenotypic frequency in each population is not necessarily equal to 1, as some individuals are heterozygous with two overproduced esterases.
[b] *N* = sample size analyzed; SS = esterase non-overproduced phenotype.

3.3 Genetic structure of populations

The genetic structure was investigated in the seven populations. A total of 450 mosquitoes were analysed for the four putatively neutral loci (*got-1, got-2, pgi* and *pgm*). All of them were polymorphic. Overall, 1734 genotypes were available for statistical analysis. Frequencies of each allele in each sample are presented in Table 4. Genotypic associations were tested at each pair of loci in each sample. Random association was rejected ($P < 0.05$) in four out of 28 tests, but none remained significant when taking into account the multiple tests. A global test across populations for each locus pair revealed no pairs with significant values ($P > 0.05$). Significant departure from HW equilibrium, on account of heterozygote deficiency, was observed in three out of 21 cases (Table 4). Two remained significant when the number of tests

Table 4. Allelic frequencies observed at four putative allozyme loci for seven mosquito populations[a]

Population locus	HLD	HZ	FY	HJ	NN	HK	SY	All
got-1	(60)	(58)	(60)	(60)	(60)	(60)	(90)	
100	0.33	0.97	0.81	1.00	0.98	1.00	0.99	
120	0.66	0.03	0.19	0	0.03	0	0.01	
140	0.02	0	0	0	0	0	0	
Fis	−0.26	+0.49	−0.12		**+0.66**			−0.10
got-2	(60)	(60)	(60)	(60)	(60)	(60)	(90)	
80	0.35	0.18	0.56	0.88	0.91	0.73	0.69	
100	0.65	0.83	0.44	0.12	0.09	0.27	0.31	
Fis	+0.13	+0.14	+0.10	+0.04	−0.09	+0.07	−0.23	+0.01
pgi	(60)	(57)	(60)	(60)	(60)	(60)	(60)	
90	0.04	0.40	0.03	0	0	0.01	0	
100	0.96	0.60	0.93	1.00	1.00	0.99	1.00	
110	0	0	0.04	0	0	0	0	
Fis	−0.04	**+0.50**	−0.05					+0.31
pgm	(60)	(59)	(60)	(60)	(60)	(60)	(60)	
60	0.01	0	0	0	0	0	0	
80	0.25	0.12	0.40	0.08	0.03	0.15	0.03	
100	0.58	0.88	0.52	0.92	0.94	0.80	0.83	
120	0.17	0	0.08	0	0.03	0.05	0.09	
140	0	0	0	0	0	0	0.05	
Fis	−0.03	−0.13	−0.17	+0.14	**+0.26**	−0.19	−0.13	−0.08

[a] Number of mosquitoes analysed in parentheses. Bold Fis values indicate a significant ($P < 0.05$) departure from HW owing to heterozygote deficiency.

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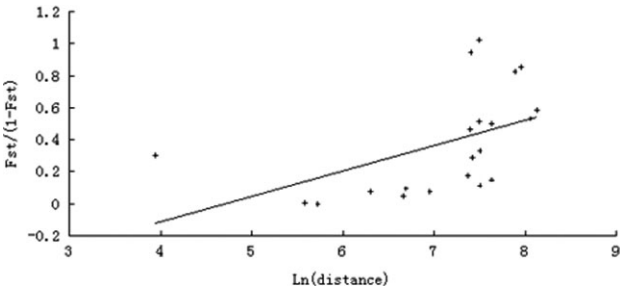

Figure 2. Relationship between pairwise Fst/(1 − Fst) values and logarithm of geographic distances (km) for all samples.

performed was taken into account. For all loci and samples, no significant ($P > 0.05$) heterozygote excess or deficiency was found.

The overall genotypic differentiation found across all populations was significantly large (Fst = 0.28, $P < 10^{-4}$). This genetic variation was partially explained by distance, as a slightly significant ($P = 0.018$) increase in differentiation was found with distance, with a slope of 0.16 (Fig. 2).

4 DISCUSSION AND CONCLUSIONS

The present study showed that the seven Chinese *Culex pipiens* populations are more resistant to OP than to CB insecticides, which is consistent with the history of insecticide applications in China. It has been nearly 40 years since the OP insecticides were first introduced into China, and they have been used extensively for agricultural protection and public health in most parts of the country.[28] Unsurprisingly, the resistance of mosquitoes to OPs has increased rapidly. Pyrethroid insecticides were then used partly or completely to replace OPs in places displaying serious OP resistance. CBs have been used to control mosquitoes only in recent years, accounting for the absence of high CB resistance in this mosquito species in China (unpublished data).

The populations studied here exhibit moderate or low resistance to OPs, which reflects the combined effect of amplified esterase types, amplification levels, local selection pressure and genetic exchange among populations. In most samples there are only two types of amplified esterase, B1 and A2-B2 or A2-B2 and A8-B8, which is in contrast with some locations of China where six types of esterase coexist in a population sample with high resistance to OP insecticides.[10] In Guangxi's two samples, even though three or four types of esterase were found, their amplification levels were very low, not even able to confer a moderate resistance. The selective pressure in most of the localities is not very high, viewed from the large proportion of susceptible individuals in the populations. The susceptible individuals are more competitive than those with costly resistant alleles in non-treated or lightly treated areas.[7] Statistical analyses of neutral genes have confirmed that there is a large genetic differentiation among populations, and genetic exchange is restricted by distance isolation. Hence, the spread of A8-B8 and A9-B9 from Hainan, Guangxi province, to Zhejiang, Liaoning province, is apparently precluded by their relatively recent appearance in combination with the large distance (1600–3300 km), which requires several generations of positive migration. As seen in Table 3, the moving trend of B1 is opposite to that of A2-B2: B1 appears to be moving northwards, while A2-B2 is moving southwards. The broad spread of B1 and A2-B2 is probably due to passive transportation from one treated area to another. There is direct[29] and indirect[30] evidence of large-scale migration of this mosquito by passive transportation by humans, and the presence of one female with A2-B2 in an aircraft has been established.[31] Thus, future spreading of A8-B8 or A9-B9 by passive migration cannot be excluded if sanitary regulations are not enforced.

To date, nine distinct overproduced esterases have been identified over the world: A1, A2-B2, A4-B4, A5-B5, A8-B8, A9-B9, B1, B6 and B7.[7,9] Their distribution ranges are: A1 and A4-B4 within the western Mediterranean;[32–35] A5-B5 within the eastern Mediterranean;[32,34] B1 in North America, Latin America and Asia;[5] A2-B2 in Asia, Africa, Europe, North America and the Caribbean;[36] A8-B8, A9-B9, B6 and B7 only in China.[12,20,37] In an extensive investigation of Chinese *C. pipiens* field populations recently (unpublished data), there were six overproduced esterases conferring OP resistance, i.e. B1, A2-B2, A8-B8 and A9-B9 plus two new phenotypes (temporarily called New1 and New2) which probably do not exist in the localities studied here. This is the highest resistance allele diversity observed thus far at the esterase loci in a given area. It is likely that, in the future, one or several of the existing alleles will be eliminated as a result of allelic competition, as illustrated by the situation in southern France, where A1 has been replaced by A4-B4 over a 10 year period,[38] and then followed by the local occurrence of A2-B2 which has started a genetic invasion.[36] Regularly monitoring the dynamics of these resistant alleles in field populations is helpful in predicting the trend of their future changes, thereby improving resistance management.

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