Journal of the American Mosquito Control Association, 17(4):238–244, 2001
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IDENTIFICATION AND CHARACTERIZATION OF NOVEL ORGANOPHOSPHATE DETOXIFYING ESTERASE ALLELES IN THE GUANGZHOU AREA OF CHINA

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ABSTRACT. In the mosquito *Culex pipiens,* various alleles at the *Ester* locus provide insecticide resistance. These resistance alleles display a heterogeneous geographical distribution, particularly in China, where they are highly diverse. A new resistance allele, *Ester*[9], coding for the overproduced esterases A9 and B9, is characterized and compared to the known resistant allele *Ester*[8] isolated from the same southern China sample (from Guangzhou). Both alleles provide low but significant resistance to chlorpyrifos (relative synergism ratio [RSR] > 3) and temephos (RSR = 1.4), which is consistent with the low level of gene amplification they display (15 copies for *Ester*[9] and 4 copies for *Ester*[8]). The full genomic sequence of the allele coding A8 and A9 is presented, which allowed us to set up a polymerase chain reaction assay to specifically identify these alleles. The peculiar situation in southern China, where numerous resistance alleles coexist, is discussed in comparison with the Mediterranean situation, the only one with a similar diversity of overproduced esterases.

KEY WORDS Gene amplification, insecticide resistance, *Culex pipiens,* esterase

In the members of the *Culex pipiens* L. complex, increased detoxification by esterase overproduction is a frequent mechanism of resistance to organophosphorus (OP) insecticides. Two esterase loci are involved, *Est-2* and *Est-3,* and resistance alleles correspond to an increased production of the enzymes (which bind and inactivate OP insecticides), relative to the basal esterase production of susceptibility alleles (for a review, see Raymond et al. 1998). Various overproduced allozymes have been described. They were named according to the esterase locus involved (A for *Est-3,* B for *Est-2*), and in their order of discovery or report in the literature: A1, A2, and so on, and B1, B2, and so on. For most resistance alleles, esterase overproduction is the result of gene amplification of either 1 locus or the 2 loci (coamplification). The coamplification of the 2 esterase loci (which are separated by an intergenic DNA fragment varying between 2 and 6 kilobases [kb]), explains the tight statistical association of some electromorphs, such as A2 and B2 (Guillemaud et al. 1996, Rooker et al. 1996). Although, strictly speaking, A4, A2, and A1 are coded by alleles at the *Est-3* locus, and B2 and B4 are coded by alleles at the *Est-2* locus, A1, A4-B4, and A2-B2 behave as alleles of a single super locus (named *Ester*) because of the complete linkage disequilibrium between *Est-2* and *Est-3* coamplified alleles. Gene regulation is also present, and is the major mechanism of overproduction of A1 (Rooker et al. 1996).

The evolution of these resistance genes is puzzling. Only 1 resistance allele is found in some large geographic areas, such as *Ester*[2] in southern Asia and in tropical, equatorial, and southern Africa (Raymond et al. 1998). In other situations, several resistance alleles are found in restricted areas, often in the same populations. Do these situations represent 2 different stable states driven by distinct processes? Or do they correspond to transient situations? Long-term field studies in southern France have disclosed that the presence of multiple resistance alleles is unstable, with one of them replacing the other in a 10-year period (Guillemaud et al. 1998). Field studies are lacking in China, and a prerequisite for further studies lies in the characterization of the various resistance alleles present, and the development of molecular tests to identify them.

In this paper, we present and characterize a new resistance allele (*Ester*[9]) from the Guangzhou area in China, and extend the characterization of the previously published *Ester*[8] allele existing in the same geographical area (Qiao et al. 1998). The diverse array of overproduced esterases in the *Culex pipiens* complex from China is discussed in the context of the current knowledge of their evolution.

MATERIALS AND METHODS

Mosquitoes: Several strains were used, including: S-Lab, which is OP susceptible and lacks esterases of high activity (Georghiou et al. 1966); and GUANG, a larval sample collected from a hypogeous breeding site in September 1994 in the suburbs of Guangzhou (south China), and mass-reared in absence of known insecticide exposure. Three strains were derived from GUANG: MAO, with A8 and B8, isolated in 1998 and studied by Qiao et al. (1998); MAO2, with A8 and B8, isolated in 1999 to replace MAO, which was lost accidentally (frozen samples are still available); and LING, with undescribed overproduced esterases (later designat-

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ed as A9 and B9), isolated in 1999. For the last 2 strains, the frequency of each overproduced esterase (A8-B8 and A9-B9, respectively) was 1st increased during 15 generations without insecticide contact, by allowing only females displaying the corresponding esterases to reproduce. Homozygosity of the strains for the alleles coding the corresponding overproduced esterases was achieved by analyzing parents or their offspring, by means of a polymerase chain reaction–restriction fragment length polymorphism (PCR-RLFP) assay (the MP assay; Berticat et al. 2000). All of these strains belong to *Culex pipiens quinquefasciatus* Say (Bourguet et al. 1998).

Detection of highly active esterases and acetylcholinesterases: Highly active esterases were identified in single individual homogenates analyzed by starch electrophoresis with TEM 7.4 buffer systems and detected according to Pasteur et al. (1988). Overproduced esterases from reference strains were run as controls (Guillemaud et al. 1999, Berticat et al. 2000): A1, B1, A2-B2, A4-B4, and A5-B5 (from strains BARRIOL, EDIT, SELAX, SA4, and SA5, respectively). Insensitive acetylcholinesterases were analyzed by means of the TPP test described by Bourguet et al. (1996).

DNA analysis: genomic DNA from MAO2 and LING strains was extracted from single mosquitoes as described in Berticat et al. (2000). The complete MAO2 and LING *Est-3* genes were amplified with the Adir: 5′-ATGGACGTCGAACACCCGGTTG-3′, and Arev: 5′-CCCTAATAAAGCTTATCTT-TGC-3′ primers (Vaughan and Hemingway 1995). Sequences were determined on PCR products with a Big Dye Terminator on a ABI prism 310 sequencer (Perkin Elmer, Boston, MA) with internal primers on both strands (A2D and CREV, see Fig. 2). A specific PCR test for the MAO2 strain was performed with the specific A8dir primer (5′-GGAAGCTTTGGATTTA-3′) mixed with the A2D primer (5′-GCAACGGGGGTCGATTACTA-3′) and the CREV primer (5′-ACTTCATTCGTTCCT-GCTCCG-3′) to obtain the specific A8dir-CREV band of 210 base pairs (bp) and the A2D-CREV control band of 1,866 bp. The 50-μl PCR mix was composed of a standard buffer plus 20 ng of genomic DNA, 12.5 pmol of A8dir and A2D primers and 25 pmol of CREV primer, 100 μM of each deoxynucleoside triphosphate (dNTP), 1.25 mM of $MgCl_2$, and 2.5 units of TAQ polymerase. The PCR program followed a denaturing step at 94°C for 4 min, then 30 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec. A specific PCR test for the LING strain was performed with the specific A9rev primer (5′-GATATTGCTTAAGGTTGTT-3′) mixed with the A2D and CREV primers to obtain the specific A2D-A9rev band of 535 bp and the A2D-CREV control band of 1,866 bp. The 50-μl PCR mix was composed of a standard buffer plus 20 ng of genomic DNA, 12.5 pmol of A9rev and CREV primers and 25 pmol of A2D primer, 100 μM of each dNTP, 1.25 mM of $MgCl_2$, and 2.5 units of TAQ polymerase. The PCR program followed a denaturing step at 94°C for 4 min, then 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec. *Est-2* copy number in each individual was estimated by quantitative PCR, as described in Weill et al. (2000), with a Roche Light Cycler (Roche, Idaho Falls, ID).

Insecticide bioassays: Resistance characteristics of the MAO2 and LING strains were compared by performing bioassays on 4th-stage larvae, following the method of Raymond and Marquine (1994). Three insecticides were used in ethanol solutions: chlorpyrifos (Dow Chemical, Midland, MI), and temephos and malathion (American Cyanamid, Princeton, NJ). The action of a synergist, DEF (*S,S,S*-tributyl phosphorotrithioate, Interchim, Montluçon, France), an inhibitor of esterases and glutathione-*S*-transferases, was investigated by exposing larvae to a standard dose (0.08 mg/liter) 4 h before the addition of the insecticide solution. In each test, sets of 20 larvae were exposed to different insecticide doses during 24 h. The variation of mortality (number of dead mosquitoes) according to the insecticide concentration (which was log transformed) was fitted with a logistic regression as available in GLIM version 4 (Baker 1987). Confidence intervals of insecticide concentrations inducing 50% mortality (or LC_{50} values) were computed with the Fieller macro (Crawley 1993). Resistance ratios (RRs) were estimated by dividing the LC_{50} of the resistant strain by the LC_{50} of the susceptible S-LAB reference strain. The value of the RR of MAO2 or LING relative to 1 was tested as follows. A full model was 1st fitted to the data, DOSE + STRAIN + DOSE:STRAIN, where the colon represents an interaction between a quantitative (DOSE) and a qualitative (STRAIN) variable. Overdispersion was systematically corrected. This model was then simplified by removing the interaction term, and the resulting change in deviance and degree of freedom (df) was used as a test of parallelism. If the parallelism was not rejected, the hypothesis RR = 1 was tested by removing the variable STRAIN from the simplified model, and computing an F from the resulting change in deviance and df. If the parallelism was rejected (so that the interaction term cannot be removed from the full model), the *x*-axis origin was 1st shifted toward the LC_{50} of the susceptible strain, then the estimate of the intercept (and its standard error) of the resistance slope on the *y*-axis was used to test the hypothesis RR = 1 at the LC_{50}. To test whether a synergist was more efficient in the resistant than in the susceptible strain, the relative synergism ratios (RSRs), were compared. The RSR is equal to the RR for insecticide alone divided by the RR for insecticide plus synergist. A RSR > 1 indicates that the synergist has a stronger effect in the resistant than in the susceptible strain, that is, that the detoxifying mechanism synergized is enhanced in the resistant strain; a RSR < 1 shows

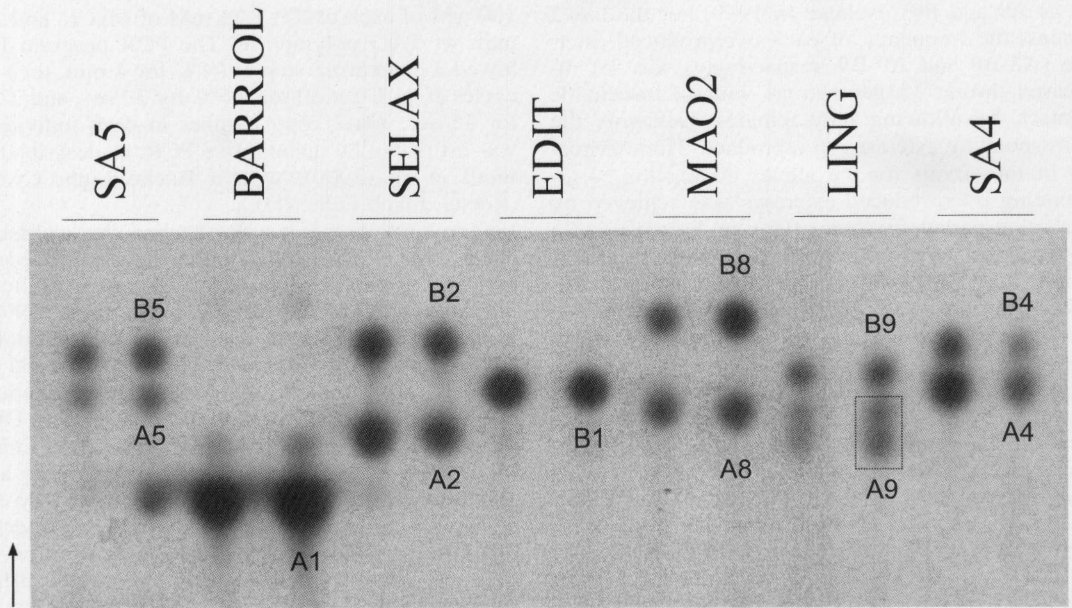

Fig. 1. High-activity esterases of single adults after starch gel electrophoresis (2 individuals per strain, 1 with labels for each esterase). The double spot of A9 is boxed. The arrow indicates the electrophoretic migration. The small A1 spot in 1 of the 2 SA5 individuals is artifactual (gel contamination).

that the 2 strains compared are not different as far as the mechanism inhibited by the synergist is concerned.

RESULTS

Esterase pattern

Starch gel electrophoresis disclosed that the overproduced esterases present in all MAO2 individuals are identical to those of preserved samples of MAO, indicating that the strain MAO2 possessed the overproduced esterases A8 and B8. The LING individuals also carried 2 esterases of high activity, each with an electrophoretic mobility different from all the previously described esterases (Fig. 1). These new esterases will be named A9 (coded by the *Est-3*^{9} allele) and B9 (coded by the *Est-2*^{9} allele). When the super locus *Ester* (= *Est-2* plus *Est-3*) is considered, the new super allele will be designated as *Ester*^{9}.

DNA analysis

The *Est-3* alleles in LING and MAO2 were amplified with the Adir and Arev primers, giving DNA fragments of 1,970 bp and 1,969 bp, respectively. Sequence comparison and alignment with the A2 allele showed a homology of >98.5% at the nucleotide level (Fig. 2). Four nonsynonymous substitutions were found between A2 and A8 alleles, 6 were found between A2 and A9, and 6 were found between A8 and A9. The MP assay proposed by Berticat et al. (2000) to discriminate various amplified *Ester* alleles is based on the variable position of *Hae*III restriction sites. However, *Hae*III restriction sites in the new *Est-3*^{9} allele are identical to those displayed by the *Est-3*^{2} allele (Fig. 2), indicating that the described PCR-RFLP test using this restriction enzyme cannot differentiate them, which was confirmed experimentally (data not shown). To overcome this difficulty, allele-specific primers (A8dir and A9rev) were designed for *Est-3*^{8} and *Est-3*^{9}, in order to propose a PCR test for a straightforward identification of amplified alleles at *Ester* in specimens from China. The primer couples (A8dir and CREV for *Est-3*^{8}, and A2D and A9rev for *Est-3*^{9}, see Fig. 2) specifically amplified the expected DNA fragment (535 bp for LING and 210 bp for MAO2; Fig. 3).

The amplification level of *Est-2* varied between 12.1 and 17.6 gene copies (mean = 15.0, n = 7) in MAO2 and between 3.3 and 6.4 gene copies (mean = 4.2, n = 8) in LING. For comparison, the amplification level in the susceptible reference strain (S-LAB) was estimated at 0.86 (n = 4).

Resistance characteristics

Resistance of MAO2 and LING larvae to OP insecticides was studied by bioassays, by using the S-LAB strain as the susceptible reference (Table 1). All fitted lines explained at least 89% of the deviance, indicating homogeneity of tolerance in the strains assayed. The MAO2 larvae displayed a

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                                          196          405                            614
                                           |            |                               |
        <-------Exon 1------------><Ex 2><I><Exon 3><------Intron 3-------><-

                                  1222223333444455555555555555555555555555666
        26666666777777777788888889344459245855552344578888888888899999999999148
        634567890123456789012345631789074492234519587501234567890123456789373
PEL-R   CGGCCGCAACGGGGGTCGATTACTATTGGCCATTGTGGCCGCACGTGACAACCTTGAGCAATATAC.AT
LING    G------------------------CG----TCG.G----TTCT.-A--------A--------C-G-G
MAO2    -------------------------------TCG------TTCT.C-----TT-----------TTGTG
             A2D                                         A9rev

                  1062            1715               1771
                   |               |                   |
        --Exon 4-->-<Ex 5><I5>-<----Intron 6---><-----------Exon 7-------->

                   11111111111111111111111111111111111111111111111111111111
        7788888999900113334444457777777777777777777788889999999999999999999
        1719999235638280041223022222222333333333578225511111112222222222333
        9380123920842474598344723456789012345679721658345678901234567890123
PEL R   GGTGGCCATGTGAATGGAAAATGTGACCCTTTAGATTTCTTCGTGGCGGAGCAGGAACGAATGAAGT
LING    -CC----TCAA-TTCAA--GTCA---------G----------C-----------------------
MAO2    C-C----TCA-T-T---GGGTC-G--AG----G-----AACTACAA---------------------
                                 A8dir                   CREV
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Fig. 2. Variable nucleotides at the *Est-3* locus. The positions of variable sites in the genomic sequence are given by a number above nucleotides of the *Ester*[2] sequence of the PEL-R strain (Vaughan and Hemingway 1995). Nonsynonymous positions are in bold characters; a period indicates a nucleotide deletion. Position 1 corresponds to the 5′ end of exon 1. The structure of the locus is given at the top of the figure. Boxed areas correspond to the position of *Hae*III restriction sites in 1 or several sequences. The sequences and positions of the 4 internal primers are depicted as shaded areas. Full sequences of *Est-3*[8] and *Est-3*[9] alleles have accession codes AJ302089 and AJ302090, respectively, in the EMBL Nucleotide Sequence database.

moderate but significant ($P < 0.01$) resistance to all OP insecticides tested (RR = 11 for chlorpyrifos, RR = 5.9 for temephos, and RR = 2.4 for malathion). The addition of DEF to bioassays decreased the resistance level of MAO2 for chlorpyrifos (from RR = 11 to 2.7, RSR = 4) and temephos (from RR = 5.9 to 4.3, RSR = 1.4), indicating that DEF inhibits resistance mechanisms in MAO2. For malathion, the addition of DEF slightly increased the resistance level of MAO2. The LING larvae displayed a moderate but significant ($P < 0.01$) resistance to all OP insecticides tested (RR = 6.0 for chlorpyrifos, RR = 5.0 for temephos, and RR = 2.8 for malathion). The addition of DEF to bioassays decreased the resistance level of LING for chlorpyrifos (from RR = 6.0 to 2.0, RSR = 3) and temephos (from RR = 5.0 to 3.6, RSR = 1.4), indicating that DEF inhibits a resistance mechanism in LING. For malathion, the addition of DEF slightly increased the resistance level of LING.

However, the synergist DEF did not completely suppress the resistance of MAO2 or LING, because their temephos or chlorpyrifos RR in the presence of the synergist was significantly ($P < 0.01$) different from 1, indicating the presence of another mechanism conferring a low resistance. All individuals ($n = 29$ for each strain) tested carried sensitive acetylcholinesterase (genotype *Ace.1*[SS]), indicating that target site insensitivity as detected by the TPP assay is not the additional mechanism involved in OP resistance in these strains.

DISCUSSION

Overproduction of the new esterases, A9 and B9, is the result of an amplification at *Ester* (at least *Est-2* is amplified), and is distinct from all other amplifications displaying 2 overproduced esterases (A2-B2, A4-B4, A5-B5, and A8-B8). This corresponds to an independent amplification event, the 7th described in this mosquito complex.

The amplification level associated with the overproduction of A8-B8 in MAO2 is approximately 15, which is associated with a moderate contribution to OP resistance (RSR = 4 for chlorpyrifos). The amplification level associated with the overproduction of A9-B9 in LING is lower (approximately 4), which is consistent with its lower contribution of esterase detoxification to OP resistance (RSR = 3 for chlorpyrifos). These relatively low contributions of A8-B8 and A9-B9 to resistance to chlorpyrifos do not necessarily preclude their higher contribution for resistance to other OP insecticides, and in particular to those that have been used

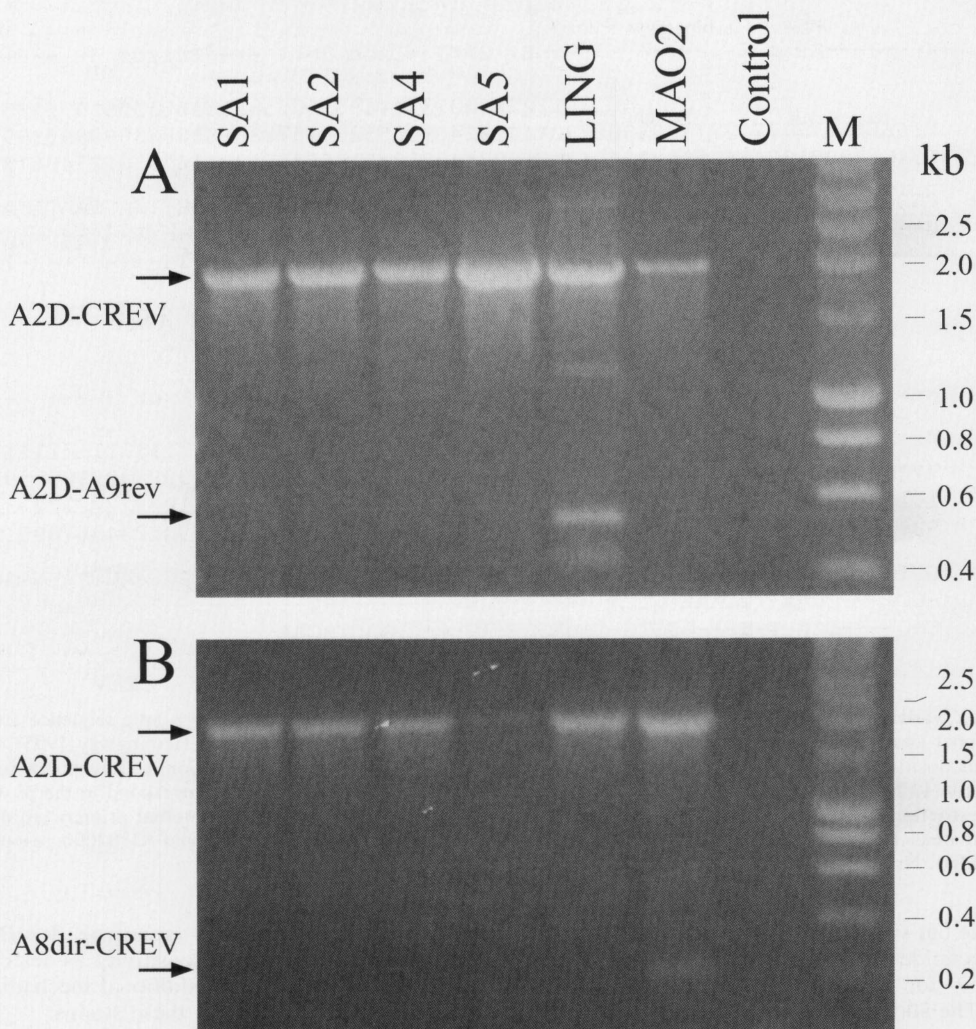

Fig. 3. Allele-specific polymerase chain reaction (PCR) test. The primers A2D and A9rev amplify the expected 535 base pairs (bp) (bottom arrow) only in the LING strain (A), and the primers A8dir and CREV amplify the expected 210 bp (bottom arrow) only in the MAO2 strain (B). The 1,866-bp band (top arrows in A and B), amplified with primers A2D and CREV, represents a positive PCR control. The Control lane represents a negative PCR control (e.g., no DNA added in the PCR).

in southern China against this mosquito (e.g., trichlorfon, phoxim, fenitrothion, fenthion, and dichlorvos in addition to temephos and malathion; see Xu et al. 1994).

The number of overproduced esterases described in China is now quite diverse, including B1, B6, B7, A2-B2, A8-B8, and A9-B9. This is the highest diversity of resistance alleles thus far observed at the *Ester* super locus in a given area. This diversity was found in 3 samples from 1992 (1 near Guangzhou, 1 in the Sichuan area, and 1 in western Beijing) and 1 sample from 1994 made near Guangzhou (Xu et al. 1994, Qiao and Raymond 1995, Qiao et al. 1998, present study). The situation in China is unlike that in the Mediterranean area, where sampling effort has been more extensive. From 1990 to 1996, 209 natural populations sampled in Spain, France, Italy, and Tunisia, totaling more than 11,900 mosquitoes, were analyzed for their overproduced esterases and only 4 resistance alleles were identified (A1, A2-B2, A4-B4, and A5-B5; Chevillon et al. 1995a, 1995b, 1997; Severini et al. 1997; Ben Cheikh et al. 1998; Lenormand and Raymond 2000).

The various *Ester* resistance alleles do not share the same resistance and cost characteristics. Both resistance and cost vary quantitatively according to the gene amplification level, and qualitatively according to particular allele amplified (Guillemaud et al. 1999, Pasteur et al. 2001). As a consequence,

Table 1. Insecticide concentration inducing 50% mortality (LC_{50}) values (and 95% confidence intervals) and slope of the mortality lines observed in bioassays with various insecticides in the strains MAO2, LING, and S-LAB. The percentage of explained deviance (%DV), the resistance ratio (RR), and the relative resistance ratio (RSR) are indicated.

	LC_{50} (mg/L)	(range)	Slope	(SE)	%DV	RR[1]	RSR
Chlorpyrifos							
S-Lab	0.00057	(0.00054–0.00060)	14.8	(1.7)	94.3	1.0	—
MAO2	0.0063	(0.0058–0.0068)	8.3	(0.7)	98.7	11.0**	—
LING	0.0034	(0.0032–0.0036)	13.2	(1.3)	98.2	6.0**	—
Chlorpyrifos + DEF[2]							
S-LAB	0.00031	(0.00029–0.00033)	11.9	(1.3)	97.6	1.0	—
MAO2	0.00085	(0.00078–0.00094)	7.0	(0.8)	94.6	2.7**	4.0
LING	0.00061	(0.00059–0.00065)	11.4	(1.1)	97.6	2.0**	3.0
Temephos							
S-LAB	0.00038	(0.00034–0.00040)	19.7	(4.5)	89.6	1.0	—
MAO2	0.0022	(0.0019–0.0025)	11.7	(2.4)	92.6	5.9**	—
LING	0.0019	(0.0017–0.0021)	8.2	(1.1)	94.6	5.0**	—
Temephos + DEF							
S-LAB	0.00010	(0.00010–0.00011)	10.5	(0.8)	98.2	1.0	—
MAO2	0.00045	(0.00042–0.00047)	10.7	(1.1)	95.8	4.3**	1.4
LING	0.00037	(0.00035–0.00040)	15.2	(1.9)	97.2	3.6**	1.4
Malathion							
S-LAB	0.012	0.011–0.014	7.2	0.8	96.7	1.0	—
MAO2	0.030	0.027–0.032	10.5	1.2	97.5	2.4**	—
LING	0.034	0.032–0.037	10.5	1.1	94.2	2.8**	—
Malathion + DEF							
S-LAB	0.0042	(0.0039–0.0045)	12.6	(1.8)	93.9	1.0	—
MAO2	0.013	(0.012–0.014)	7.5	(0.7)	97.5	3.1**	0.8
LING	0.016	(0.015–0.017)	11.4	(0.9)	98.3	3.8**	0.7

[1] For RR values, asterisks indicate that the test of the null hypothesis RR = 1 is significant at $P < 0.01$.
[2] DEF, *S,S,S*-tributyl phosphorotrithioate.

the various resistance alleles compete when they are in the same population, as shown in southern France, where *Ester*[1] has been replaced by *Ester*[4] over a 10-year period (Guillemaud et al. 1998). Diverse polymorphism of resistance alleles, as observed in southern China, would seem to indicate a recent contact between relatively isolated OP-treated areas. In the future, 1 or several of the existing alleles likely will be eliminated as the result of allelic competition. Most resistance alleles are distinct between China and the Mediterranean (with the exception of *Ester*[2], which is found in both), thus providing 2 independent situations to identify which parameters drive the competition between resistance alleles.

ACKNOWLEDGMENTS

We are very grateful to Benoit Maury for help in biosassays, to S. Lucas for help in the TPP test, and to Nicole Pasteur for helpful comments on the manuscript. This work was financed in part an Academica Sinica/CNRS collaboration. This is contribution 2001.061 of the Institut des Sciences de l'Evolution de Montpellier (UMR CNRS 5554).

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