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Esterase gene amplification in *Culex pipiens*

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Abstract

In the mosquito *Culex pipiens* one of the major resistance mechanisms to organophosphorous pesticides (OPs) is increased detoxification of insecticide. This resistance is the consequence of overproduction of two types of esterases, esterases A and B, coded at two loci, *Est-3* (*A esterase*) and *Est-2* (*B esterase*). We have analysed the genomic structure of these genes in different strains resistant to OPs and have attempted to characterize the different types of mutations leading to the resistant phenotypes.

It is shown that, concerning the more frequent resistant phenotypes, mutations leading to resistance are of two main types. First, overproduction of one A esterase present in Southern France results from a regulatory mechanism. The second type of mutation is gene amplification which involves events that have initially generated the duplication of both the *A* and *B esterase* or only the *B esterase* locus. We report the point that the most frequent esterase overproductions are the results of eight different mutations and that, given the range of distribution of these genotypes, mutation leading to an efficient resistance gene is one of the most limiting factors for the evolution toward resistance in *Culex pipiens*.

Keywords: gene amplification, esterase, resistance.

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Introduction

In insects two main resistance mechanisms to organophosphorous (OP) insecticides have been described. One corresponds to a modification of the target site, acetylcholinesterase (reviewed by Fournier & Mutero, 1994). A second mechanism is an increased detoxification of the insecticide (Oppenoorth, 1985). In the house mosquito *Culex pipiens* such a mechanism has been widely described (Pasteur *et al.*, 1981a,1984), and is the consequence of overproduction of two types of carboxylesterases (E.C. 3.1.1.1), historically named A and B esterases, coded at two loci, *Est-2* (or *B esterase*) and *Est-3* (or *A esterase*). Three pairs of associated overproduced esterases have been observed in natural *Culex pipiens* populations: A2-B2 (Curtis & Pasteur, 1981; Raymond *et al.*, 1987; Villani *et al.*, 1983), A4-B4, and A5-B5 (Poirié *et al.*, 1992). In some cases, overproduction is only found at one locus: B1 (Georghiou *et al.*, 1980), B6, B7 (Xu *et al.*, 1994) and possibly B8 (Vaughan *et al.*, 1995) at the *B esterase* locus and A1 (Pasteur *et al.*, 1981a) at the *A esterase* locus.

The overproduction of all the B esterases investigated so far (B1, B2, B4, B5 and presumably B6, B7 and B8) is due to a tandem amplification of the structural gene (Mouchès *et al.*, 1986; Raymond *et al.*, 1989; Poirié *et al.*, 1992; Xu *et al.*, 1994; Vaughan *et al.*, 1995). The recent cloning of an A esterase gene (Vaughan & Hemingway, 1995) has allowed the investigation of the molecular basis of A esterase overproduction. Restriction enzyme mapping showed that A2 esterase gene lies within 2.2 kb of the B2 esterase gene (Rooker *et al.*, 1996). In mosquitoes with overproduced esterases A2 and B2 the amplification level of *A esterase* was equal to that of *B esterase*, suggesting that the genes were co-amplified. Furthermore, Rooker *et al.* (1996) showed that in one strain with an overproduced A esterase (A1), gene amplification (2-fold at most) could not account for the increased protein level (70-fold (Mouchès *et al.*, 1987)). This indicated that overproduction of A esterases may be the result of two different mechanisms: gene amplification and a regulatory mechanism (Rooker *et al.*, 1996).

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In this paper we characterize the genes coding for known overproduced esterases in the mosquito *Culex pipiens* and the different mechanisms by which esterase overproduction can be achieved.

Results

Estimation of amplification level

We estimated the amplification levels of genes coding for overproduced esterases A5-B5 in CYPRUS, A4-B4 in VIM, A2-B2 in SELAX, B1 in TEM-R and of genes coding for non-overproduced A esterases in TEM-R and non-overproduced A and B esterases in MSE. We used the susceptible reference S-LAB as control with no amplification at either the *esterase A* and *B* loci.

Hybridizations with the *Ace* probe revealed that, in a few cases, there was a non-linear relationship between the quantity of DNA blotted and the radioactive signal (data not shown). Consequently these results could not be used as a control for DNA concentration (see Rooker *et al.*, 1996). As an alternative, we chose a method to estimate the amplification level that was not dependent on variation of hybridization conditions between experiments (see Experimental procedures). For all strains, linear regressions between the radioactive signal of *A* or *B esterase* gene hybridizations and DNA dilution were significant ($P < 10^{-3}$).

Dot-blots were repeated twice (see one example in Fig. 1) and gave the estimates of amplification levels of *A* and *B esterase* loci indicated in Table 1. The amplification levels of *A* and *B esterase* loci were not different ($P > 0.05$) in each strain, with the exception of TEM-R ($P < 2 \times 10^{-4}$). Due to the low number of replicates, this result must be considered as conserva-

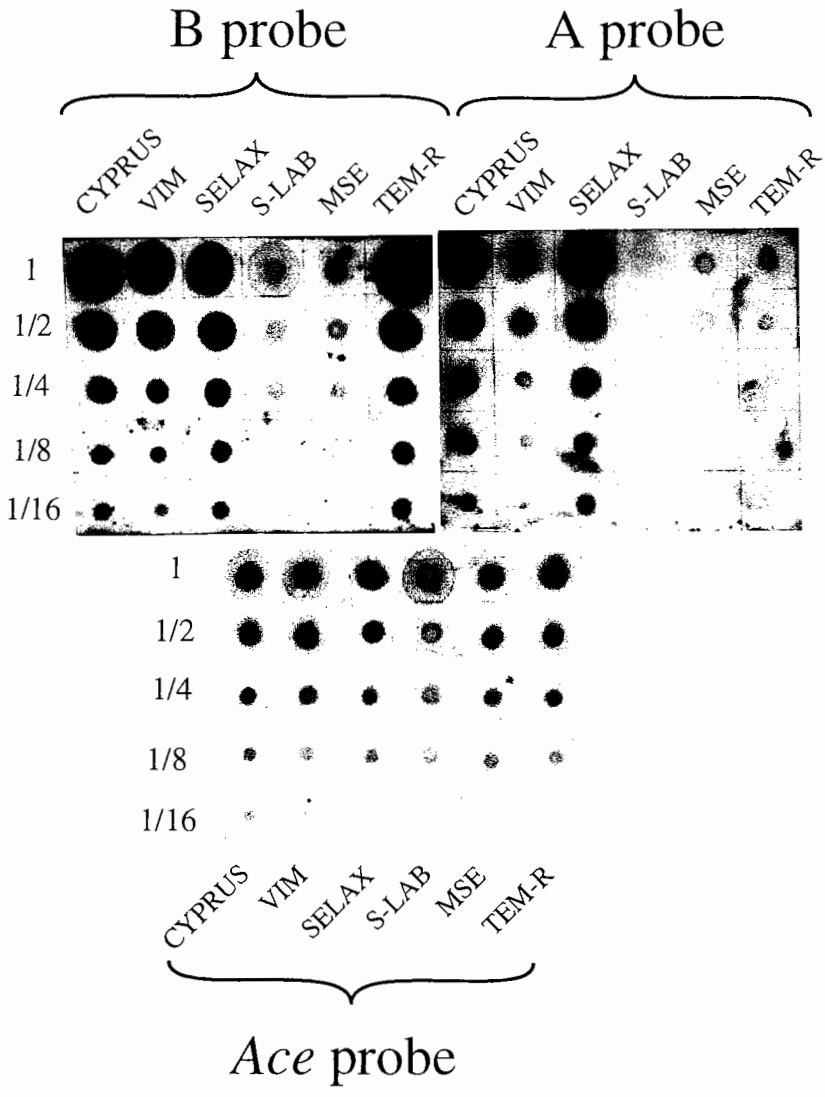

Figure 1. Example of autoradiographies of dot-blots of serially diluted genomic DNA of strains displaying (CYPRUS, SELAX, TEM-R and VIM) or not (S-LAB and MSE) overproduced esterases. The probes used for hybridization are indicated and the ratio (on the right) corresponds to the DNA dilution factors. Analysis was performed on the β-imager measures and not on autoradiographies.

Table 1. Amplification level of *A* and *B esterase* genes for strains displaying overproduced esterases and the reference strain S-LAB. *P* value refers to a two-sided Student *t* test (df = 2) of equality of *A* and *B esterase* gene amplification levels.

	Amplification levels (±SE)					
	Dot blots					Southern blots
	B esterase		*A esterase*	*P* value		*A esterase*
Slope comparison						
Strains						
TEM-R	B1	20.8 ± 0.2	0.7 ± 0.1	$<2 \times 10^{-4}$		0.5
VIM	B4	7.5 ± 0.7	A4 5.44 ± 0.6	0.24	A4	2.5
CYPRUS	B5	60.2 ± 3.3	A5 43.3 ± 0.7	0.065	A5	15
SELAX	B2	32.4 ± 0.1	A2 40.8 ± 7.4	0.51	A2	15
S-LAB		1	1			1
Ace probe comparison						
Strains						
S-LAB		1.12 ± 0.1	1.16 ± 0.1	0.85		

tive. The highest amplification level was found in Cyprus where the *A5* and *B5 esterase* genes were amplified 40–60-fold. In TEM-R the B1 gene was amplified 20-fold whereas the A gene was not amplified. In SELAX the A2 and B2 genes were amplified between 30- and 40-fold, which is not different from previous estimates: 21–40-fold for B2 (Raymond *et al.*, 1989), and 30 (15–60) for A2 and 20 (10–40) for B2 (Rooker *et al.*, 1996). Amplification levels of the *A esterase* locus obtained by the Southern blot method (Fig. 2) are also

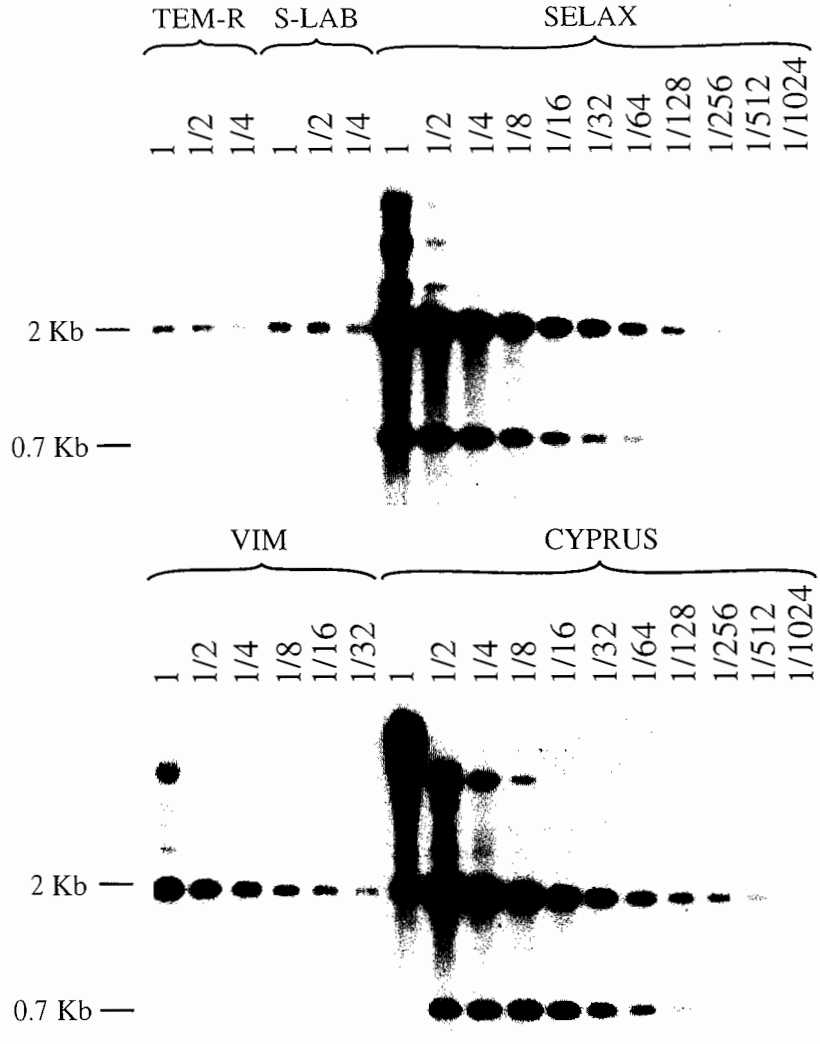

Figure 2. Autoradiography of southern blot of *Acc*I-digested genomic DNA hybridized with the A2 probe of strains displaying (SELAX, VIM and CYPRUS) or not (TEM-R and S-LAB) overproduced A esterase. The ratio indicates the DNA dilution factors.

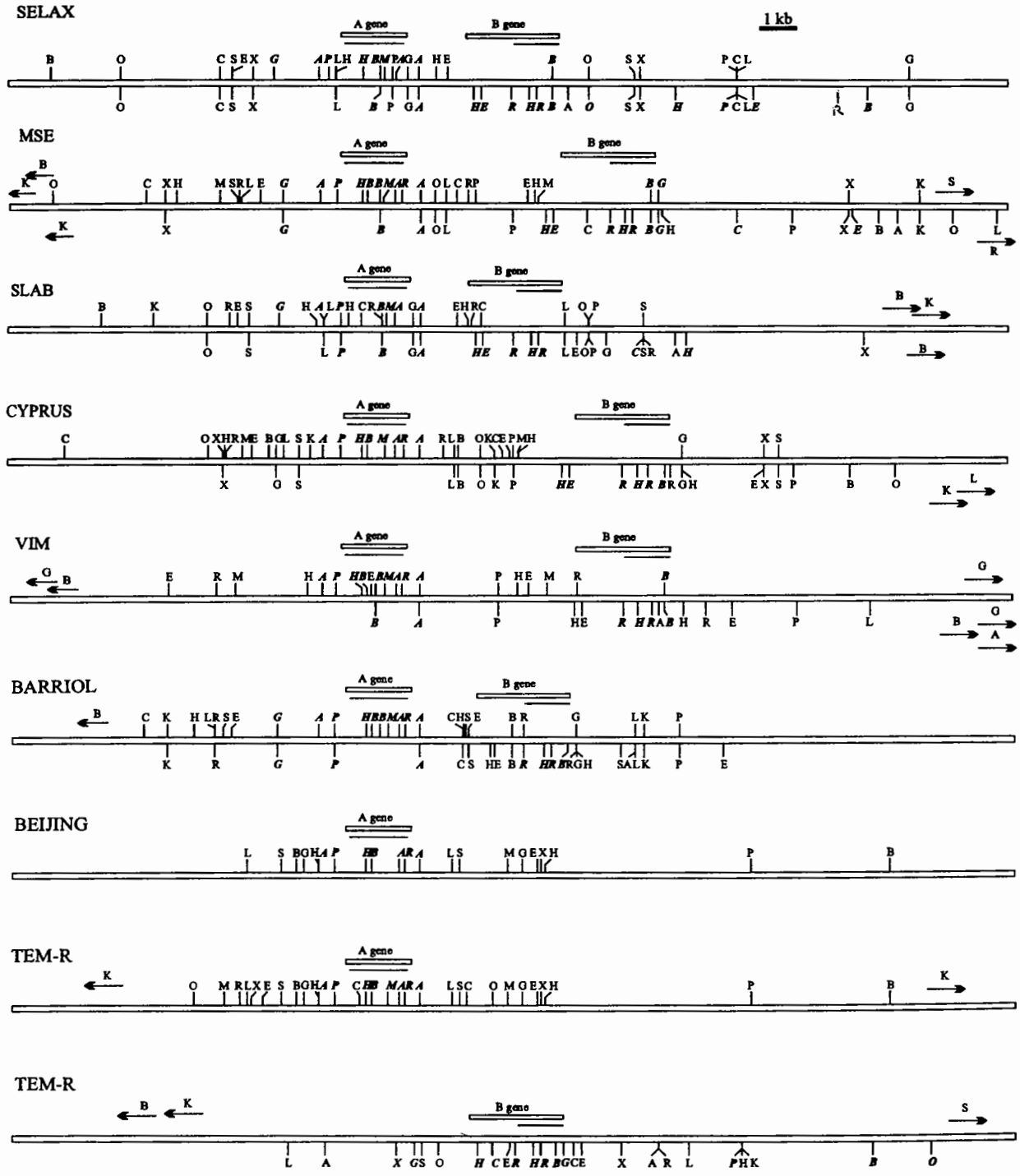

Figure 3. Restriction maps of *A* and *B esterase* genes of strains displaying or not overproduced esterases. For each map, the restriction sites detected by the *A esterase* probe are indicated above and those detected by the *B esterase* probe are below. The position of A and B probes are indicated under the *A* and *B esterase* loci. Homologous restriction sites between two or more maps are in bold and italic characters. Abbreviations: A: *Acc*I; C: *Bcl*I; B: *Bam*HI; G: *Bgl*II; E: *Eco*RI; R: *Eco*RV; H: *Hind*III; K: *Kpn*I; P: *Pst*I; S: *Sac*I; L: *Sal*I; X: *Xba*I; M: *Mun*I; O: *Xho*I. The maps of BARRIOL and SELAX (displaying respectively A1 and A2-B2) are from Rooker *et al.* (1996).

presented in Table 1. It can be seen that they are lower than those obtained with the dot-blot method.

Mapping of the A esterase and B DNA region

Restriction maps of the *A esterase* DNA region were constructed for strains with or without overproduced esterases (Fig. 3), using up to thirteen restriction enzymes. The restriction maps of the B locus area from Poirié *et al.* (1992) of VIM (B4) and CYPRUS (B5) were completed with several additional restriction enzymes. For each strain, the restriction maps using the *A esterase* or *B esterase* (Poirié *et al.*, 1992; Raymond *et al.*, 1991; Qiao & Raymond, 1995) probe were built independently. Homology between restriction sites of the *A esterase* maps across strains was only checked for *Acc*I, *Pst*I, *Hin*dIII, *Bam*HI, *Eco*RV, *Mun*I and *Bgl*II.

For each restriction enzyme the sites detected are the closest to the probe used in hybridization (Fig. 3). Therefore the restriction sites common to the maps of *A* and *B esterase* genes are only those generating fragments large enough to include both probes or those present only once between the probes. Maps of the A and B locus areas overlapped for all the strains indicating that *A esterase* and *B esterase* genes are closely linked in all amplified or non-amplified strains. The only exceptions were strains displaying *B1 esterase* amplification (TEM-R and BEIJING); no overlap could be found between the two maps, indicating that the non-amplified *A esterase* gene and amplified *B esterase* gene are located further than about 20 kb apart.

Comparison of A esterase gene of strains displaying B1

We compared the *A esterase* restriction profiles of four strains displaying overproduced B1 esterase and originating from various geographical areas: EDIT, RICO, BEIJING and TEM-R. The three enzymes used (*Sac*I, *Bam*HI and *Eco*RI) produced the same bands in all strains following hybridization with the 1.8 kb A2 PCR product. In addition, a partial restriction map of the *A esterase* gene region of BEIJING using ten restriction enzymes was the same than that of TEM-R (Fig. 3).

Discussion

Co-amplification of A and B esterase

Two lines of evidence indicate that *A esterase* and *B esterase* genes are co-amplified in CYPRUS (displaying A5 and B5), SELAX (displaying A2 and B2) and VIM (displaying A4 and B4). First, *A esterase* and *B esterase* genes are closely linked in these strains, the intergenic DNA fragment being less than 10 kb. Second, the amplification level for *A* and *B esterase* genes are similar within each strain. This extends the results of Rooker *et al.* (1996) who found a similar situation for the strain SELAX (displaying A2 and B2). Differences in amplification levels were found depending on the method used (dot-blot or Southern blot) as already described by Rooker *et al.* (1996). In both methods, radioactivity levels were directly measured using a β-imager, so that a possible explanation is probably to be found during the extra step of DNA digestion, electrophoresis or transfer on membrane for the Southern method.

We now have evidence from three separate strains that when the A and B esterases are both overproduced, the corresponding structural genes are both amplified within the same amplicon. Hence, the amplification events leading to the overproduction of both A and B esterases must correspond to a unique event for each esterase pair.

In all strains except those with B1, the genomic region of *A* and *B esterase* loci have the same basic structure: in each strain, regardless of whether or not they were amplified, we found a cluster of esterase genes including the two loci in an inverted position, the two 5′ ends being in close proximity. However, the length of the intergenic region was variable among strains. We observed two classes of length: 2.2 kb and 6.3 kb with errors due to the observation scale. These classes did not correspond to amplified versus non-amplified esterases. The *A4* and *B4*, *A5* and *B5 esterases* genes in VIM and CYPRUS respectively, and non-amplified *A* and *B esterase* genes in MSE were found 6.3 kb apart, whereas *A2* and *B2 esterase* genes in SELAX and non amplified *A* and *B esterase* genes in S-LAB were 2.2 kb apart. This implies that the mutational event that has generated the amplicon is not directly related to the difference of intergenic length.

The particular case of the B1 amplicon

An exception to the co-amplification of *A* and *B esterase* genes is found in strains displaying an overproduced B1 esterase. None of the four B1 strains originating from distinct geographical localities displayed an amplified A gene. It is concluded that the *A esterase* locus is not included in the amplicon in strains displaying overproduced B1 esterase.

Despite these findings, restriction mapping of these strains with the A2 probe indicated that they all display the same allele at the A locus. A previous comparison of the A gene between the B1 strains undertaken by Guillemaud *et al.* (1996) showed that sequences of a highly variable intron of the *A esterase* locus were similar between the EDIT and TEM-R strains. Given

that the *A esterase* locus is highly variable in natural populations (Pasteur *et al.*, 1981b; Guillemaud *et al.*, 1996; Raymond *et al.*, 1996; Parker, 1996), this observation indicates that the B1 amplicon and the *A esterase* allele detected in B1 strains are in strong linkage disequilibrium. From the analysis of the structure of other couples of overproduced esterases, we can hypothesize that this genetic linkage disequilibrium is due to a close physical proximity. The maps of *A* and *B esterase* areas obtained from TEM-R are not incompatible with the presence of one B gene at 20 kb apart from the A gene, the two maps sharing the two restriction enzymes *BamH*I and *Kpn*I in their extremity (Fig. 3).

This schema could explain the presence of one of the faint supernumerary bands μ and ν systematically found in hybridization of *Eco*RI digested DNA of strains or individual mosquitoes displaying B1 gene with the B1 probe (Qiao & Raymond, 1995). It could correspond to digestion of the area where the *B* and *A esterase* genes are close together, whereas the published restriction map of B1 (Raymond *et al.*, 1991) corresponds to the amplicon of *B esterase* genes without the *A esterase* structural gene. Fig. 4 summarizes the structure of amplification in strains where A and B genes are co-amplified and in strains where only the B *esterase* gene is amplified. The model clearly shows the functional amplicon, i.e. the DNA fragment originally duplicated, and the apparent amplicon, i.e. the DNA fragment surrounding the probe used. Thus the original breakpoint of amplification is not necessarily at the edge of the (apparent) amplicon established by RFLP studies. Further variation in amplification level could take place by, for example, unequal crossing-over at various places in the (apparent and functional) amplicons.

It is worthy of note that our estimate of the B1 amplification level in TEM-R is very much lower than that of Mouchès *et al.* (1986) or Raymond *et al.* (1989) who found respectively amplification levels of 250 and 150-fold. These differences could be the result of a change in mean copy number of B1 in the TEM-R strain or to the difference in the methods used. Although Raymond *et al.* (1993) did not find any statistically significant decrease in resistance in a strain displaying A2-B2 amplification (SELAX88) between May 1990 and October 1991 and raised without insecticide selection, it remains possible that a decrease in resistance took place during this $1\frac{1}{2}$ years period for a strain displaying B1 (TEM-R88). Indeed, the resistance ratio at LC_{50} (lethal concentration at which 50% of the mosquitoes die) for temephos decreased from 1280- to 674-fold for TEM-R88 and from 32- to 19-fold for SELAX88 (Raymond *et al.*, 1993) between 1990 and 1991, although this last change was marginally not statistically significant. Moreover, they showed that when a strain was not homozygous for esterase gene amplification, the resistance was rapidly lost probably

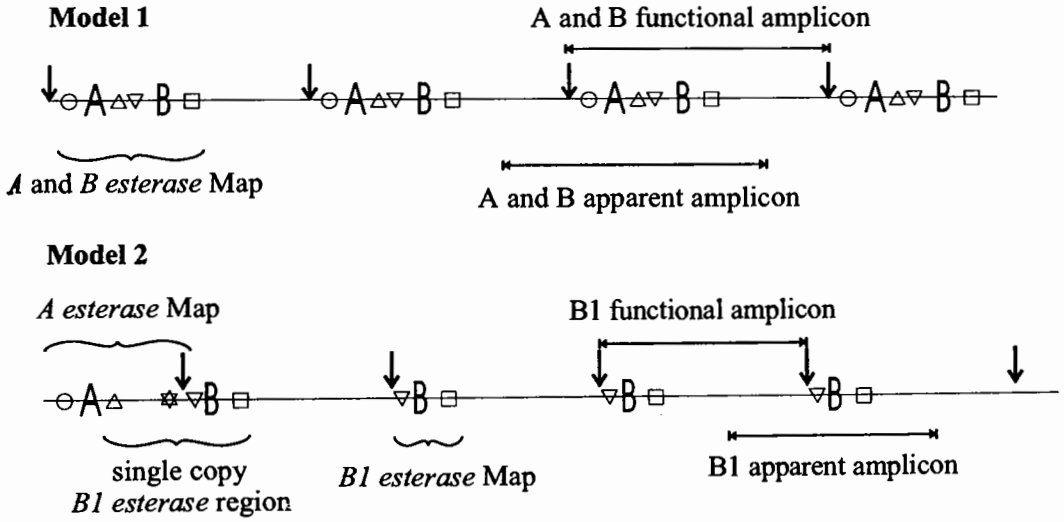

Figure 4. Schematic structures of amplicons displaying *A* and *B esterase* genes (such as A2-B2, A4-B4 and A5-B5) or *B1 esterase* gene only, inferred from restriction maps of this study and results of Qiao & Raymond (1995). Model 1 occurs when the limits of the amplicon (here represented as vertical arrows) encompass both esterase genes. When one of the limits falls between both loci, only one gene is subsequently amplified and is represented by model 2. Circles, triangles, stars and squares represent variation in the flanking DNA regions of the two loci. The single copy *B1 esterase* region in model 2 is supposed to correspond to the initial cluster of esterase genes in B1 strains. This region, displaying a flanking area distinct from that of the B1 amplicon may be responsible for additional bands in B hybridization such as λ and μ bands found in Qiao *et al.* (1995). The amplicon constructed using probes for *Esterase A* and *Esterase B* genes (apparent amplicon) does not necessarily corresponds to the functional amplicon. See text for explanations.

because of the fitness cost related to enzyme overproduction. Given that homozygosity for amplification level has never been checked in any strains, this result may be more general, since differences in esterase gene copy number can produce differences in individual fitnesses. Therefore, lower levels of amplification could be selected for in a strain homozygous for the presence of the amplification (but displaying variable amplification levels) in the absence of insecticide treatment. It is also known that using autoradiography to estimate amplification level, as was done by Mouchès *et al.* (1986) or Raymond *et al.* (1989), leads generally to an overestimation. If we consider the southern blot of *Acc*I digested DNA (Fig. 2), optical density measures would have given amplification levels of about 64-fold for SELAX (versus 15-fold obtained from the β-imager), 128-fold for CYPRUS (versus 15-fold) and 8-fold for VIM (versus 2.5-fold). This reduction in amplification level possibly due to an improvement of the detection method may be compared with the aphid *Myzus persicae*, where the amplification level of esterase E4 in highly resistant clone was estimated by autoradiography around 64 X (Field *et al.*, 1988), whereas only ten to twelve copies were present as revealed by phospho-imager and pulsed field gel electrophoresis analysis (Field *et al.*, 1996).

Evolution of field resistance by enzyme overproduction in Culex pipiens

From the work of Rooker *et al.* (1996), Vaughan *et al.* (1995), Xu *et al.* (1994) and the present study, it is now possible to have an estimation of the independent number of mutational events leading to OP resistance through esterase overproduction at detectable frequency in natural populations. The mutations are of two major classes, i.e. involving gene amplification or gene regulation. A1 esterase overproduction in mosquitoes from Southern France (Pasteur *et al.*, 1981a) is the result of such an increased expression of *A esterase* gene lacking structural amplification (Rooker *et al.*, 1996). Gene amplification in *Culex* mosquitoes involves events that have initially generated the duplication of both the *A* and *B esterase* or only the *B esterase* locus. Each of the three couples of overproduced esterase, A2-B2, A4-B4 and A5-B5, must have resulted from the co-amplification of the two genes as a single event (Rooker *et al.*, 1996; this study). In addition to B1, three different *B esterase* alleles (B6, B7 and possibly B8) are overproduced alone in mosquitoes (Xu *et al.*, 1994; Vaughan *et al.*, 1995), and at least for B1 and possibly for the others it arose from the single amplification of the *B esterase* gene. Therefore, since the 1960s, when OP treatments began against mosquitoes, only seven independent esterase gene amplifications and one mutation (*sensu lato*, i.e. not necessarily one nucleotide change) leading to A esterase overexpression have been reported in natural populations of *Culex pipiens*. Thus, given the geographical distributions and the frequencies of these resistance genes, it is obvious that the mutation leading to an efficient resistance gene corresponds to one of the most limiting factor in the evolution of resistance. It is likely that other overproduced esterases will be reported in the future, as some geographical areas are still not thoroughly studied. The actual figure of eight mutations events is mainly based on partial surveys of the Mediterranean area, Africa, North America, the Caribbean and Asia. Moreover, infrequent descriptions of other overproduced esterases in single individuals from natural populations (e.g. Qiao *et al.* (1995) or Callaghan (1989)) indicate that the range of mutations leading to overproduced esterases is larger than the number of mutations effectively selected in natural populations.

Experimental procedures

Mosquito strains

Mosquito strains of the *Culex pipiens* complex used were: MSE, a strain resistant to OP and carbamate insecticides and possessing an insensitive AChE, collected in 1979 near Montpellier, Southern France (Raymond *et al.*, 1986; Bourguet *et al.*, 1996); S-LAB, a reference susceptible strain from California (Georghiou *et al.*, 1966); TEM-R, an OP resistant strain from California (Georghiou & Pasteur, 1978), with amplified *B1 esterase* (Raymond *et al.*, 1993); BEIJING, a strain collected in 1992 from Beijing, China, also with amplified *B1 esterase* (Qiao & Raymond, 1995); EDIT, a laboratory strain homozygous for B1 following single pair mating, derived from larvae collected in 1988 in California (Qiao & Raymond, 1995); RICO, a natural population displaying B1 esterase, sampled in 1992 in San Juan (Puerto Rico) (Qiao & Raymond, 1995); SELAX, the OP resistant reference strain with amplified A2 and B2, derived from a 1984 sample from California (Raymond *et al.*, 1987); VIM, collected in 1984 in Montpellier, Southern France, with amplified *A4* and *B4 esterases* (Poirié *et al.*, 1992); BARRIOL, a strain from Southern France possessing overproduced A1 esterase (Chevillon *et al.*, 1995); and finally CYPRUS, derived from a strain collected in Cyprus in 1987 with overproduced A5 and B5 esterases (Poirié *et al.*, 1992).

DNA analysis

Restriction mapping. Genomic DNA was extracted from a pool of 100 adults using the method of Raymond *et al.* (1989) and digested with one or two restriction enzymes (*Acc*I, *Bcl*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Xba*I, *Mun*I and *Xho*I) in a total volume of 20 μl. Digested DNA was loaded onto 0.8% agarose gels, the fragments separated by electrophoresis and the whole transferred onto Nylon mem-

branes by Southern blotting using the method of Sambrook *et al.* (1989). The filters were prehybridized and hybridized at 65°C with the ^{32}P-labelled 1.8 kb A2 PCR product (Guillemaud *et al.*, 1996), and washed at high stringency at 65°C (Sambrook *et al.*, 1989). Following autoradiography, filters were stripped of radioactive signal and re-probed with the 1.3 kb *B1 esterase* cDNA probe of Mouchès *et al.* (1990).

Estimation of esterase gene amplification levels. Genomic DNA was extracted as above and treated with RNAse. DNA was quantified using a GenQuant RNA/DNA Calculator (Pharmacia, Cambridge, UK). Gene amplification levels were calculated using the dot-blot and Southern blot methods described by Rooker *et al.* (1996). Serial dilutions of DNA from different mosquito strains were fixed onto a Nylon membrane directly by dot-blotting. The diluted genomic DNA was hybridized with the ^{32}P-labelled 1.8 kb A2 PCR product for *A esterase* detection and the 1.3 kb *B1 esterase* cDNA probe for *B esterase* detection. In addition to this method, *AccI* digestions of serially diluted DNAs from different strains were separated by electrophoresis on agarose gels, blotted and hybridized with the 1.8 kb A2 PCR product, as described previously, in order to estimate the amplification level of *A esterase* genes. Only the same homologous 2 kb band was used for estimation of amplification level and incomplete digestions were not taken into account. The dot-blots were performed in duplicate in order to verify the repeatability of the experiments. In addition, the quantity of DNA blotted was controlled by using an acetylcholinesterase gene probe (Rooker, 1994) known to hybridize with only one locus in *Culex pipiens* (Denis Bourguet, submitted). The quantity of genomic DNA hybridizing with radioactive probes was estimated using a β-imager (Phosphoimager 445 SI, Molecular Dynamics, Sunnivale, Calif., USA) analysis. This method gives a direct estimate of the quantity of β-ray produced by each hybridization.

For each esterase locus, each strain *i* was characterized by the slopes $S(i)$ of the relationship between radioactive signal and DNA dilution. The ratio $S(i)/S(\text{S-LAB})$ is an estimate of amplification level of the locus considered for strain *i*. This method was chosen because the results are obtained from a single membrane, so that differences in hybridization conditions between membranes hybridized with the different probes do not interfere. Esterase gene copy number in S-LAB was estimated using the method described in Rooker *et al.* (1996), i.e. by comparing results of hybridization by *A2* (or *B1*) *esterase* and *Ace* probes.

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References

Bourguet, D., Capela, R. and Raymond, M. (1996) An insensitive acetylcholinesterase in *Culex pipiens* L. mosquitoes from Portugal. *J Econom Entomol* **89**: 1060–1066.

Callaghan, A. (1989) Genetic and biochemical studies of elevated esterase electromorphs in Culex pipiens. Ph.D. thesis, University of London.

Chevillon, C., Pasteur, N., Marquine, M., Heyse, D. and Raymond, M. (1995) Population structure and dynamics of selected genes in the mosquito *Culex pipiens*. *Evolution* **49**: 997–1007.

Curtis, C.F. and Pasteur, N. (1981) Organophosphate resistance in vector populations of *Culex pipiens* L. (Diptera: Culicidae). *Bull Entomol Res* **71**: 153–161.

Field, L.M., Devonshire, A.L. and Forde, B.G. (1988) Molecular evidence that insecticide resistance in peach-potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochem J* **251**: 309–312.

Field, L.M., Devonshire, A.L. and Tyler-Smith, C. (1996) Analysis of amplicons containing the esterase genes responsible for insecticide resistance in the peach-potato aphid *Myzus persicae* (Sulzer). *Biochem J* **313**: 543–547.

Fournier, D. and Mutero, A. (1994) Modification of acetylcholinesterase as a mechanism of resistance to insecticides. *Comp Biochem Physiol* **108C**: 19–31.

Georghiou, G.P., Metcalf, R.L. and Gidden, F.E. (1966) Carbamate resistance in mosquitoes: selection of *Culex pipiens fatigans* Wied for resistance to Baygon. *Bull WHO* **35**: 691–708.

Georghiou, G.P. and Pasteur, N. (1978) Electrophoretic pattern in insecticide-resistant and susceptible mosquitoes. *J Econom Entomol* **7**: 201–205.

Georghiou, G.P., Pasteur, N. and Hawley, M.K. (1980) Linkage relationships between organophosphate resistance and a highly active esterase-B in *Culex quinquefasciatus* from California. *J Econom Entomol* **73**: 301–305.

Guillemaud, T., Rooker, S., Pasteur, N. and Raymond, M. (1996) Testing the unique amplification event and the worldwide migration hypothesis of insecticide resistance genes with sequence data. *Heredity* **77**: 535–543.

Mouchès, C., Magnin, M., Bergé, J.-B., de Silvestri, M., Beyssat, V., Pasteur, N. and Georghiou, G.P. (1987) Overproduction of detoxifying esterases in organophosphate-resistant *Culex* mosquitoes and their presence in other insects. *Proc Natl Acad Sci USA* **84**: 2113–2116.

Mouchès, C., Pasteur, N., Bergé, J.-B., Hyrien, O., Raymond, M., Robert de Saint Vincent, B., de Sylvestri, M. and Georghiou, G.P. (1986) Amplification of an esterase gene is responsible for insecticide resistance in a California *Culex* mosquito. *Science* **233**: 778–780.

Mouchès, C., Pauplin, Y., Agarwal, M., Lemieux, L., Herzog, M., Abandon, M., Beyssat-Arnaouty, V., Hyrien, O., Robert de Saint Vincent, B., Georghiou, G.P. and Pasteur, N. (1990) Characterization of amplification core and esterase B1 gene responsible for insecticide resistance in *Culex*. *Proc Natl Acad Sci USA* **87**: 2574–2578.

Oppenoorth, F.J. (1985) Biochemistry and genetics of insecticide resistance. *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Kerkut, G.A. and Gilbert, L.I., eds), pp. 731–773. Pergamon Press, Oxford.

Parker, P.G.A.N. (1996) The development of biomarkers in freshwater insects for biological monitoring of pollution. Ph.D. thesis, University of Reading.

Pasteur, N., Georghiou, G.P. and Iseki, A. (1984) Variation in organophosphate resistance and esterase activity in *Culex quinquefasciatus* Say from California. *Génét Sél Evol* **16**: 271–284.

Pasteur, N., Iseki, A. and Georghiou, G.P. (1981a) Genetic and biochemical studies of the highly active esterases A′ and B′ associated with organophosphate resistance in mosquitoes of the *Culex pipiens* complex. *Biochem Genet* **19**: 909–919.

Pasteur, N., Sinègre, G. and Gabinaud, A. (1981b) *Est-2* and *Est-3* polymorphism in *Culex pipiens* L. from southern France in relation to organophosphate resistance. *Biochem Genet* **19**: 499–508.

Poirié, M., Raymond, M. and Pasteur, N. (1992) Identification of two distinct amplifications of the esterase B locus in *Culex pipiens* (L.) mosquitoes from Mediterranean countries. *Biochem Genet* **30**: 13–26.

Qiao, C.-L. and Raymond, M. (1995) The same esterase B1 haplotype is amplified in insecticide resistant mosquitoes of the *Culex pipiens* complex from the Americas and China. *Heredity* **74**: 339–345.

Raymond, M., Beyssat-Arnaouty, V., Sivasubramanian, N., Mouchès, C., Georghiou, G.P. and Pasteur, N. (1989) Amplification of various esterase B's responsible for organophosphate resistance in *Culex* mosquitoes. *Biochem Genet* **27**: 417–423.

Raymond, M., Callaghan, A., Fort, P. and Pasteur, N. (1991) Worldwide migration of amplified insecticide resistance genes in mosquitoes. *Nature* **350**: 151–153.

Raymond, M., Fournier, D., Bride, J.-M., Cuany, A., Bergé, J., Magnin, M. and Pasteur, N. (1986) Identification of resistance mechanisms in *Culex pipiens* (Diptera: Culicidae) from southern France: insensitive acetylcholinesterase and detoxifying oxidases. *J Econom Entomol* **79**: 1452–1458.

Raymond, M., Pasteur, N., Georghiou, G.P., Mellon, R.B., Wirth, M.C. and Hawley, M.K. (1987) Detoxification esterases new to California, USA, in organophosphate-resistant *Culex quinquefasciatus* (Diptera: Culicidae). *J Med Entomol* **24**: 24–27.

Raymond, M., Poulin, E., Boiroux, V., Dupont, E. and Pasteur, N. (1993) Stability of insecticide resistance due to amplification of esterase genes in *Culex pipiens*. *Heredity* **70**: 301–307.

Raymond, M., Qiao, C.L. and Callaghan, A. (1996) Esterase polymorphism in insecticide susceptible populations of the mosquito *Culex pipiens*. *Genet Res* **67**: 19–26.

Rooker, S. (1994) Analysis of insect serine esterase genes from medically and agriculturally important pest species. Ph.D. thesis, University of London.

Rooker, S., Guillemaud, T., Bergé, J., Pasteur, N. and Raymond, M. (1996) Coamplification of A and B esterase genes as a single unit in *Culex pipiens* mosquitoes. *Heredity* **77**: 555–561.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Vaughan, A. and Hemingway, J. (1995) Mosquito carboxylesterase Esta2[1] (A2). *J Biol Chem* **270**: 17044–17049.

Vaughan, A., Rodriguez, M. and Hemingway, J. (1995) The independent gene amplification of electrophoretically indistinguishable B esterases from the insecticide-resistant mosquito *Culex quinquefasciatus*. *Biochem J* **305**: 651–658.

Villani, F., White, G.B., Curtis, C.F. and Miles, S.J. (1983) Inheritance and activity of some esterases associated with organophosphate resistance in mosquitoes of the complex of *Culex pipiens* L. (Diptera: Culicidae). *Bull Entomol Res* **73**: 153–170.

Xu, J., Qu, F. and Liu, W. (1994) Diversity of amplified esterase B genes responsible for organophosphate resistance in *Culex quinquefasciatus* from China. *J Med Coll PLA* **9**: 20–23.