The same esterase B1 haplotype is amplified in insecticide-resistant mosquitoes of the *Culex pipiens* complex from the Americas and China

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In *Culex pipiens*, overproduction of nonspecific esterases is a common mechanism of resistance to organophosphate insecticides. The esterases are attributed to closely linked loci named *A* and *B*, and overproduction of all esterases B is due to gene amplification. In order to determine if the esterase B1 identified by electrophoretic studies in *Culex pipiens* mosquitoes from different countries is overproduced due to the amplification of the same DNA haplotype, the amplified region encompassing the structural esterase *B1* gene was characterized by restriction mapping and RFLP. The same amplified haplotype was found in mosquitoes with an esterase B1 protein, independently of their geographical origin: French Guiana, Venezuela, Puerto Rico, California and China. Large variations in amplification levels were observed. It is concluded that B1 amplification has a unique origin, either in America or in Asia, and has subsequently spread by migration. This migration is more limited than that of A2–B2 esterases, since B1 is confined to the Americas, the Carribean and part of China, whereas the A2–B2 distribution now includes the Americas, the Carribean, Asia, Africa, the Pacific islands and Europe.

Keywords: *Culex pipiens*, gene amplification, insecticide resistance, migration, RFLP.

Introduction

In mosquitoes of the *Culex pipiens* complex, increased esterase detoxification by two types of esterases (A and B) is a frequent mechanism of resistance to organophosphorous insecticides (OP). Eight esterases of high activity have been observed in natural populations: esterase A1 in Mediterranean countries, esterase B1 in North America, and three groups of associated esterases: A2 and B2 in numerous countries of three continents, A4 and B4 in the eastern Mediterranean, and A5 and B5 in Cyprus (Pasteur & Georghiou 1989; Pasteur *et al.*, 1981; Raymond *et al.*, 1991; Poirié *et al.*, 1992; Severini *et al.*, 1993; Raymond & Marquine 1994; Chevillon *et al.*, 1995). The large increase in esterase activity observed in OP-resistant mosquitoes is due to the overproduction of the enzymes, resulting from the amplification of a DNA sequence containing the corresponding structural gene in the case of esterases B (Mouchès *et al.*, 1986, 1990; Raymond *et al.*, 1991; Poirié *et al.*, 1992).

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The identification of overproduced esterase proteins in natural populations relies on their electrophoretic characteristics (mobility and substrate preference, α- and β-naphthyl acetates for esterases A and B, respectively), and the linkage of alleles of the *A* and *B* esterase genes. All esterases B2 identified by these criteria have been shown to correspond to the amplification of the same haplotype, which has never been found in a mosquito lacking an amplified esterase B (Raymond *et al.*, 1991, and unpublished data). But these criteria cannot discriminate between the overproduced esterases B4 and B5, which have the same electrophoretic mobility in standard conditions (but are not identical) and are also associated with electrophoretically similar esterases A (A4 and A5, Poirié *et al.*, 1992). Although electrophoretic studies have revealed the presence of esterase B1 in many localities of the U.S.A. (Beyssat-Arnaouty *et al.*, 1989; Pasteur & Georghiou, 1989), Central America and Asia (Georghiou, 1992), the esterase *B1* haplotype has only been characterized in the Californian reference TEM-R strain (Mouchès *et al.*, 1990, Raymond *et al.*, 1991). The present study was thus undertaken to determine whether electrophoretically identical

esterases B1 from different countries correspond to the same amplified haplotype.

Materials and methods

Mosquitoes

The mosquito populations included three laboratory strains (TEM-R, EDIT and BEIJING), and three field samples (CAYENNE, PUNTA and RICO). TEM-R is the esterase B1 reference strain derived from a Californian population collected in 1974 (Ranasinghe & Georghiou, 1979, Georghiou *et al.*, 1980), and recently made homozygous for the presence of overproduced esterase B1 (Raymond *et al.*, 1993). EDIT derives from crosses between the SUTTER-YUBA strain (derived from larvae collected in 1988 in California by G. P. Georghiou, University of California, Riverside) and the susceptible S-LAB reference strain (Georghiou *et al.*, 1966); it was founded with the offspring of one female SUTTER-YUBA× S-LAB crossed with one S-LAB male, and selected by single pair-crosses between adults with esterase B1 until homozygosity of esterase *B1* was attained. All mosquitoes of the EDIT strain thus contain an esterase *B1* gene amplification identical by descent on each homologous chromosome. BEIJING is derived from larvae collected in July 1992 west of Beijing (China), and subsequently selected with temephos every generation. PUNTA, CAYENNE and RICO are three field samples collected as larvae in March 1991 in Punta del ~~E~~ (Venezuela), in June 1991 in Cayenne (French Guiana), and in February 1992 in San Juan (Puerto Rico), respectively. Adults from each strain or field collection were stored in liquid nitrogen until the present study.

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Starch gel electrophoresis

Presence or absence of highly active esterases was determined in single mosquito homogenates, using starch gel electrophoresis with Tris-Maleate-EDTA buffer systems (Pasteur *et al.*, 1988). Each gel contained mosquito extracts from the following reference strains: BARRIOL with esterase A1 (Pasteur & Raymond, unpublished), VIM with esterases A4 and B4 (Poirié *et al.*, 1992), TEM-R with esterase B1 (see above), and SELAX with esterases A2 and B2 (Wirth *et al.*, 1990).

Restriction maps

Genomic DNA was extracted from a pool of 100 adults according to Raymond *et al.* (1989), digested with one or several 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, *Xho*I), run on agarose gels, transferred onto a Nylon membrane according to Sambrook *et al.* (1989), prehybridized and hybridized with the 1.3 kb B1 cDNA probe of Mouchès *et al.* (1990).

RFLP on single mosquitoes

Single mosquito genomic DNA was obtained using a simplification of a method provided by C. Malcolm (QMW, University of London, England). Each mosquito was homogenized in 150 μL of buffer (1 per cent SDS, 50 mM Tris HCl pH 8.0, 25 mM NaCl, 25 mM EDTA, pH 8.0) using a polypropylene pestle, and incubated at 65°C for 30 min. One hundred μL of a 3 M K-acetate solution were added into each homogenate, which was left one hour on ice and then centrifuged for 10 min at 10000 *g*. DNA was precipitated by adding two volumes of ethanol to each supernatant. This produced enough DNA to carry out a digestion with one restriction enzyme (*Eco*RI) which differentiates all known amplified haplotypes (Raymond *et al.*, 1991; Poirié *et al.*, 1992). Gel migration, transfer, probe labelling (the 1.3 kb cDNA of B1 esterase), and hybridization were done as described in Raymond *et al.* (1989). After migration, a picture of each gel was obtained to control for the amount of DNA loaded in each lane, and to help the quantitative interpretation of the results. *Eco*RI digests were also performed on 1–5 μg DNA from mosquitoes with known haplotypes and were run on each gel as a control: *B2* (strain SELAX) and *B1* (strain TEM-R).

Results

Protein identification

A highly active esterase of the same substrate preference and electrophoretic mobility as esterase B1 in TEM-R mosquitoes was detected in lab or field mosquitoes (Table 1) from China (BEIJING), South America (PUNTA and CAYENNE), the Caribbean (RICO), and North America (EDIT). In addition, we observed esterases A2–B2 in CAYENNE mosquitoes, and two new esterases of high activity, preferentially hydrolysing α-naphthyl acetate. One esterase had an electrophoretic mobility very similar but not identical to A2, and was present in mosquitoes from South America (CAYENNE and PUNTA) which also possessed esterase B1; it will be provisionally designated as A6 until further characterized. The other was found in mosquitoes from South America (CAYENNE and PUNTA) and the Caribbean (RICO), and was identical to esterase C2 (which probably

belongs to a separate locus) observed in a recent study of Martinique populations (Yébakima *et al.*, 1994).

Esterase B haplotype characterization

Restriction maps of the amplified DNA region hybridizing with esterase B1 cDNA were built using 13 restriction enzymes for the TEM-R, EDIT, BEIJING and RICO samples, where esterase B1 protein was the only overproduced esterase B and was present in all (TEM-R and EDIT) or most mosquitoes (Table 1).

The same restriction map was obtained with all samples (Fig. 1), but variations in the number of gene copies were observed between samples. Considering the amount of DNA loaded in each well and the radioactivity of the DNA fragments hybridizing with the esterase B1 cDNA probe, it can be concluded that the number of gene copies was much higher in TEM-R than in the other samples (data not shown). These variations are difficult to interpret for BEIJING and RICO mosquitoes which were not homozygous for the presence of esterase B1, but they clearly indicate

Table 1 Origin of the mosquito samples or strains used, and the identification of their overproduced or highly active A or B esterase proteins

| Mosquitoes	Country	A or B overproduced esterase	References
Reference strains			
SELAX	USA, CA	A2–B2	Wirth *et al.*, 1990
TEM-R	USA, CA	B1	Georghiou *et al.*, 1980
VIM	France	A4–B4	Poirié *et al.*, 1992
BARRIOL	France	A1	Pasteur *et al.*, unpublished
Strains			
BEIJING	China	B1	This study
EDIT	USA, CA	B1	This study
Field samples			
RICO	Puerto-Rico	B1	This study
CAYENNE	French Guiana	B1, A2–B2, C2, A6	This study
PUNTA	Venezuela	B1, C2, A6	This study
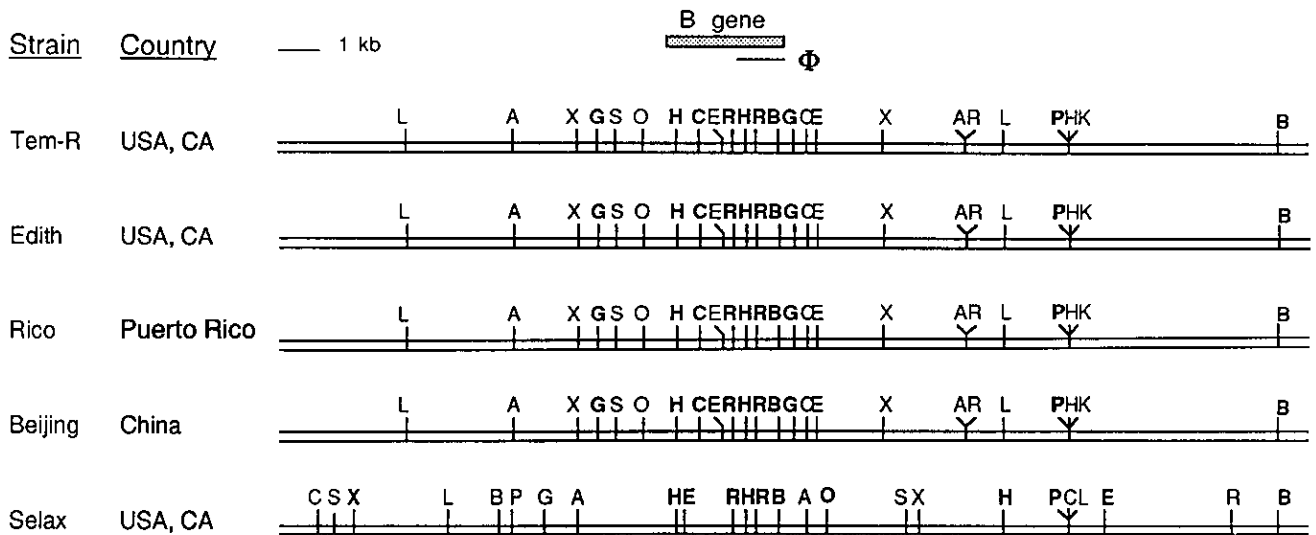
Fig. 1 Restriction maps of the esterase *B* genes and their flanking sequences from the B1 reference strain (TEM-R), three strains or wild samples possessing the B1 overproduced protein (EDIT, RICO and BEIJING) and the B2 reference strain (SELAX). Restriction enzymes are abreviated as: A (*Acc*I), B (*Bam*HI), C (*Bcl*I), 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) and O (*Xho*I). The localization of the esterase *B* gene is indicated by the shaded box and the 1.3 kb (Φ) cDNA probe by a line below. Only the sites flanking the fragment to which the probe hybridized are mapped for each enzyme.

distinct levels of gene amplification in EDIT and TEM-R mosquitoes which are both homozygous for the presence of esterase B1.

Esterase B haplotype characterization in single mosquitoes

Because of the small amount of genomic DNA extracted from a single mosquito, only one restriction enzyme (*Eco*RI) was used to characterize esterase B haplotypes. With this endonuclease, the esterase *B1* haplotype yields an amplified fragment of 2.1 kb, whereas the esterase *B2* haplotype yields a fragment of 9 kb (see Fig. 1 and Raymond *et al.*, 1991).

Six out of 15 PUNTA (Venezuela) mosquitoes (Fig. 2) displayed an amplified 2.1 kb band characteristic of the esterase *B1* haplotype, as expected from the electrophoretic identification of esterase B1 protein which disclosed 30 out of 58 mosquitoes with B1 (Fisher exact test on contingency table, $P > 0.35$). No mosquito had an amplified 9 kb band typical of the esterase *B2* haplotype, which also is in agreement with electrophoretic data (Table 1), and one had an amplified 1.3 kb band (Fig. 2, mosquito no. 9) corresponding to none of the amplified haplotypes described so far. The nine mosquitoes lacking an amplified band (Fig. 2, lanes nos 2–6, 8, 10, 13 and 15) displayed two bands of various sizes from which at least eight alleles could be identified; none of these mosquitoes had the same genotype.

Thirteen out of 16 CAYENNE (French Guiana) mosquitoes (Fig. 3) displayed an amplified 2.1 kb band

characteristic of the esterase *B1* haplotype, four had an amplified 9 kb band that may correspond to the esterase *B2* haplotype (Fig. 3, nos 5, 8, 13 and 15) and one (Fig. 3, no. 3) had no amplified band.

Two additional observations were made from the single mosquito study. The first concerns the number of copies of the esterase *B1* haplotype. This number was clearly lower in PUNTA and CAYENNE than in TEM-R mosquitoes: although about 10 times less DNA was loaded in TEM-R wells, the radioactivity of the TEM-R band was equivalent to or higher than PUNTA and CAYENNE B1 bands. In addition, large differences in copy number were observed between mosquitoes of the same population (e.g. lanes nos 16 and 14 of Fig. 3 displayed the same intensity of the 2.1 kb band, but lane no. 14 was loaded with five times more DNA than lane no. 16).

The second observation concerns two DNA fragments of 3 and 5.6 kb (μ and ν in Figs 2 and 3) which hybridized with esterase B1 cDNA in several (four of the six PUNTA, and nine of the 13 CAYENNE) mosquitoes with the amplified 2.1 kb fragment. When comparison within each lane can be made (avoiding variations due to differences in DNA amount), these two bands displayed a much lower radioactivity than the 2.1 kb band. They were also observed in TEM-R, EDIT and RICO but not in BEIJING mosquitoes, where they seemed to be replaced by three other bands (of the same low intensity as μ and ν) of 2.6, 3.2 and 4.2 kb. The exact nature of these bands is unknown, and they could represent (1) a polymorphism within the B1 amplification or (2) incomplete digestion
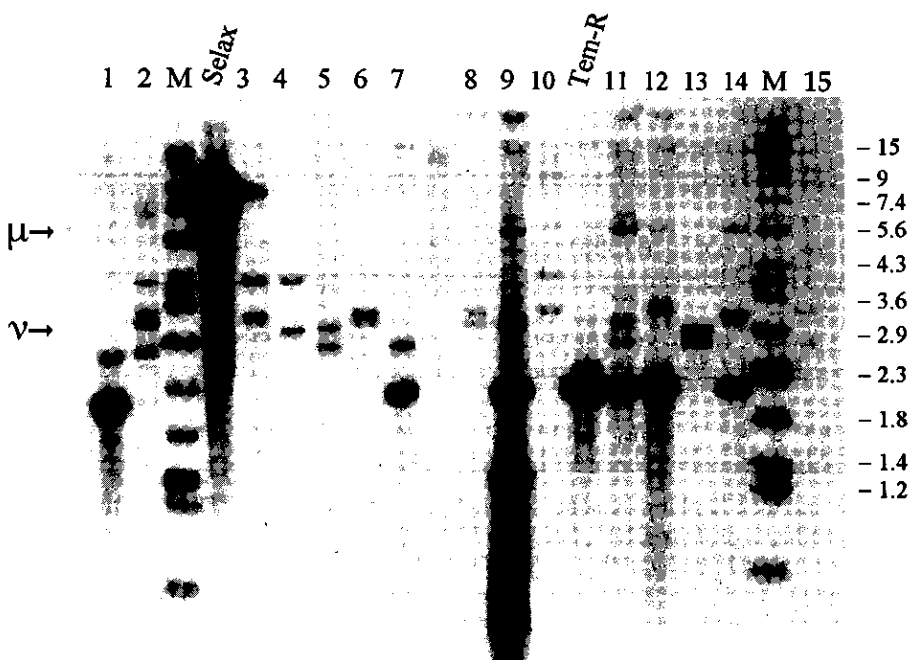

Fig. 2 RFLP on single mosquitoes from the PUNTA field sample. Sixteen wells loaded with field mosquitoes are numbered on top. M refers to the size marker (in kb on the right). DNAs from SELAX and TEM-R reference strains were loaded as controls. Single copy bands (μ and ν) associated with B1 amplification are indicated. Supernumerary bands in individuals nos 2 and 3 are considered to be artefactual: digestion was not complete for individual nos 2, and the upper 9 kb band in individual no. 3 is probably a contamination from the SELAX well.
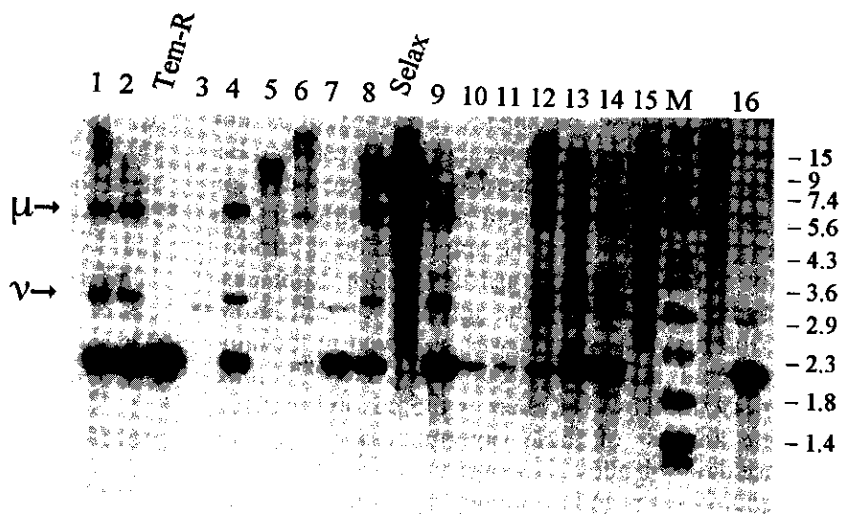
Fig. 3 RFLP on single mosquitoes from the CAYENNE field sample. Sixteen wells loaded with field mosquitoes are numbered on top. M refers to the size marker (in kb on the right). DNAs from SELAX and TEM-R reference strains were loaded as controls. Single copy bands (μ and ν) associated with B1 amplification are indicated.

products. In single mosquitoes the intensity of these bands is about the same as that of the nonamplified and polymorphic alleles, as can be seen in Fig. 2 where the DNA content in each lane is approximately the same, except in the TEM-R lane which contains less DNA.

Discussion

The present study shows that mosquitoes with an over-produced esterase B1 from China (BEIJING), North America (EDIT and TEM-R), and the Caribbean (RICO) have an identical amplified haplotype with respect to their restriction sites with 13 endonucleases. The analysis of single mosquitoes from South America (CAYENNE and PUNTA) revealed the presence of the *Eco*RI 2.1 kb fragment typical of the esterase *B1* haplotype in many mosquitoes. Although a more detailed study of South American populations is needed, it seems that the same esterase *B1* haplotype is also amplified in *Culex pipiens* populations from these countries.

Previous studies (Raymond *et al.*, 1991, and unpublished data) have shown an extremely large polymorphism of the region encompassing the esterase *B* gene when it is not amplified. A similar observation was noted in mosquitoes from South America: at least eight alleles could be deduced from the nine PUNTA mosquitoes lacking an amplification (Fig. 2). As Raymond *et al.* (1991) pointed out, such a large polymorphism makes it very unlikely that the same haplotype could become amplified in two different geographical regions. Thus, as for esterase B2, we can

conclude that amplification of the esterase *B1* haplotype derives from a unique event which occurred in a specific population. The existence of variations in the number of esterase *B1* copies between mosquitoes of the same geographical area (e.g. California or French Guiana) or of different geographical areas, indicates that this unique event was followed by other events which have increased the number of gene copies within each amplification. Previous studies by Pasteur *et al.* (1984) that looked at resistance levels and esterase B1 activity in mosquitoes homozygous for the presence of different esterase *B1* alleles (i.e. what we would call today different 'amplified alleles'), indicated that, in California in 1979, there were at least four levels of esterase B1 amplification. In contrast to the first amplification event, those that follow and increase the number of copies are therefore very common, and they most probably can occur repeatedly in the same or in different populations. As a consequence, the overall B1 amplification organization may differ in mosquitoes from distinct geographical areas. These differences thus probably reflect the different histories of copy proliferation from the initial event which was at the origin of the multiplication of the *B1* haplotype.

The nature of the supernumerary bands is unknown. Their occurrence in association with amplified fragments (e.g. μ and ν bands in North and South America) suggests that they are closely linked to the B1 amplification, and it is possible that they correspond to a polymorphism within the amplification or to incomplete digestion products. The presence of the supernumerary bands μ and ν in strains or samples from the

Americas and the Carribean, and their absence from China further suggests that *B1* alleles from the Americas are more closely related to one another than they are to Chinese *B1* alleles. Presently, it is not possible to infer the geographical origin of esterase B1 amplification (e.g. the Americas, the Carribean or Asia), either from the present study (restriction site polymorphism within the B1 amplification is insufficient for a phylogenic reconstruction), or from past field studies, because of the absence of data from China (the first B1 esterase was observed in mosquitoes collected in 1974 in California, Georghiou & Pasteur, 1978). However, there is the possibility that a complete analysis of these supernumerary bands or sequence data will allow a reconstruction of the various B1 migration paths and eventually trace the geographical origin of the initial amplification event.

The migration hypothesis (Raymond *et al.*, 1991; this study) is based on (1) the existence of a large neutral polymorphism around the esterase *B* gene in susceptible mosquitoes, and on (2) the presence of the same amplified haplotype (characterized mainly by neutral polymorphisms) in populations from distant geographical areas. This hypothesis has been recently questioned (Hemingway *et al.*, 1993; Ketterman *et al.*, 1993) on the basis of biochemical evidence, but alternative explanations for the similarity of restriction maps have not been proposed. We consider that data on protein quantitative parameters (K_m, k_a, V_m) cannot be used to undermine a hypothesis based on qualitative genetic data.

Four amplification events have been detected so far, each one with a very distinct haplotype (Mouchès *et al.*, 1986; Raymond *et al.*, 1989; 1991; Poirié *et al.*, 1992). Two of them, B1 and B2, have migrated extensively and reached at least three continents (Raymond *et al.*, 1991; Rivet *et al.*, 1993; this study) from a still unknown geographical origin. The other two are present only in Mediterranean countries (Poirié *et al.*, 1992; Raymond & Marquine, 1994). An understanding of the present situation may help to predict the evolution of resistance genes in the wild and facilitate resistance management. Biochemical studies on isolated strains have been useful to identify and characterize resistance genes, but only population studies and the use of adequate molecular techniques are able to solve key issues on the evolution of insecticide resistance.

Acknowledgements

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