Heredity **77** (1996) 535–543 *Received 15 January 1996*

Testing the unique amplification event and the worldwide migration hypothesis of insecticide resistance genes with sequence data

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In the mosquito *Culex pipiens*, over-production of esterases is a common insecticide resistance mechanism. Different alleles at the *esterase A* and *B* loci are known to occur in natural populations, as shown by enzyme electrophoresis and RFLP studies on the *esterase B* locus. Here we analyse the variability of the *esterase A* locus at the nucleotide level in mosquitoes possessing or lacking over-produced esterase A. A surprisingly high level of nucleotide polymorphism is found in coding and noncoding regions, and the extent of polymorphism detected is higher than that previously described for the most polymorphic loci in *Drosophila*. We also show that eight strains from different localities (Africa, America, Asia) that possess the over-produced esterase A2 share the same nucleotide sequence at the *esterase A* locus, strongly supporting the evolutionary scenario of a unique event of amplification of this gene followed by dispersal through migration rather than the occurrence of multiple independent mutational events of the gene.

Keywords: *Culex pipiens*, esterases, evolution, nucleotide polymorphism.

Introduction

The wide use of organic insecticides to control medically important pest species has been a powerful agent of selection in natural populations of many insect species which have developed various degrees of resistance (Georghiou & Lagunes-Tejeda, 1991). In a few species, such as the mosquito *Culex pipiens*, it is possible to identify each gene conferring resistance to organophosphorous insecticides in single individuals.

This mosquito, common in temperate and tropical countries, is subjected to insecticide control in many places. Worldwide surveys of resistance to organophosphorous insecticides have disclosed that only three loci have developed major resistance alleles (Pasteur *et al.*, 1981; Wirth *et al.*, 1990; Georghiou, 1992; Poirié *et al.*, 1992). The first two loci, *Est-2* (or *esterase B*) and *Est-3* (or *esterase A*), code for detoxifying carboxylester hydrolases, and resistant alleles correspond to an esterase over-production (Fournier *et al.*, 1987; Mouchès *et al.*, 1987; Poirié *et al.*, 1992). Five distinct electromorphs have been described so far at the *esterase B* locus and three at the *esterase A* locus (Pasteur *et al.*, 1981, 1984; Raymond *et al.*, 1989; Georghiou, 1992; Poirié *et al.*, 1992; Xu *et al.*, 1994; Vaughan *et al.*, 1995). The over-production of esterase is the result of gene amplification (Mouchès *et al.*, 1986; Raymond *et al.*, 1989; Poirié *et al.*, 1992; Vaughan & Hemingway, 1995) and/or gene regulation (Rooker *et al.*, 1996). In some situations, both *esterase A* and *B* loci are co-amplified, which explains the tight statistical association of some electromorphs, like A2 and B2 (Rooker *et al.*, 1996). The third locus, *Ace*, codes the acetylcholinesterase (insecticide target), and insensitive alleles have been reported in various places (Raymond *et al.*, 1986; Bourguet *et al.*, 1996) but it is not known how many resistant *Ace* alleles have occurred independently.

Restriction maps of the DNA within and around the *esterase B* structural gene can be built, in suscep-

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tible mosquitoes with a single copy of the gene, as well as in mosquitoes with an amplified haplotype (the amplicon is larger than the DNA area mapped). When such maps are compared, large differences are observed. For example, two maps from Californian mosquitoes, one of a nonamplified allele and the other of an amplified allele, have only 21 per cent of their restriction sites in common (Raymond *et al.*, 1991). However, when strains with the B2 electromorph are compared, restriction maps are strictly identical, independently of the geographical origins of the insects considered (Raymond *et al.*, 1991). A similar situation is found for the B1 electromorph: mosquitoes from various localities within the Americas and China possess the same restriction map (Qiao & Raymond, 1995). A large part of the polymorphism detected by restriction enzymes around the *esterase* B structural gene is probably neutral. The similarity of the restriction maps of all B1 (or all B2) haplotypes from diverse and distant geographical areas indicates that all B1 (or all B2) alleles are identical by descent. There are two possible events to explain this: either each allele was first amplified in a particular place, and has then spread, or it has first spread and then been independently amplified in various places.

The first scenario was proposed by Raymond *et al.* (1991) and Qiao & Raymond (1995), based on the argument that the probability of independently amplifying the same allele many times is very low. This is true if the polymorphism of esterase haplotypes in susceptible populations is large (Raymond *et al.*, 1995). In addition, the selective advantage provided by the amplification itself in organophosphorous treated places promotes its spread. The multiple and independent amplification of B2 has been favoured by Hemingway *et al.* (1993) and Ketterman *et al.* (1993), based on variations in the kinetics of the over-produced esterases studied on partially purified enzymes.

Recent data indicate that the RFLP at the 3′ flanking sequence of the *esterase B* gene and the electrophoretic polymorphism at both *esterase A* and *B* loci is large in two susceptible mosquito samples from Europe (Raymond *et al.*, 1995). These results are consistent with the migration hypothesis. So far, no molecular data at the DNA level have been published to test the unique origin and the migration hypothesis for the *esterase A* locus.

We describe here nucleotide variations for the *esterase A* locus in *Culex pipiens* from alleles sampled from various parts of the world. Both nonamplified and amplified *esterase A* alleles are considered, with the aim of comparing the nucleotide variation associated with the widespread A2 variant versus those detected within the nonamplified *esterase A* gene. In addition, the contrasting levels of polymorphism and divergence at synonymous and nonsynonymous sites are analysed to provide some insight into the importance of neutral versus selective forces operating at the *esterase A* locus.

Materials and methods

Mosquito strains

Three types of mosquitoes were used as follows. First, nine strains lacking an over-produced esterase A: BRUGES-A and BRUGES-B, two susceptible isofemale and inbred strains from Belgium (Raymond *et al.*, 1995); MSE, a laboratory strain resistant to organophophorous and carbamate insecticides possessing an insensitive AChE, collected in 1979 near Montpellier, southern France (Raymond *et al.*, 1986; Bourguet *et al.*, 1996); TEM-R, a strain resistant to OP from California (Georghiou & Pasteur, 1978), homozygous for the presence of esterase B1 (Raymond *et al.*, 1993); TRANS-P, a laboratory strain resistant to trans-permethrin from California (Priester & Georghiou, 1978); EVORA, an isofemale strain derived from larvae collected in November 1991 from sewage in downtown Evora, Portugal, and maintained by sib-mating during more than ten generations; EDIT, an isofemale strain derived from a cross between SUTTER-YUBA, a strain from Sutter-Yuba, California (Qiao & Raymond, 1995), and S-LAB (Georghiou *et al.*, 1966), and made homozygous for a single level of amplification of the B1 allele (N. Pasteur *et al.*, unpublished data); ACHE-R, a strain from Cyprus displaying an insensitive acetylcholinesterase, collected in 1987 (Wirth & Georghiou, 1996); KOCHI, a strain resistant to *Bacillus sphaericus*, originating from Kochi, India (Rao *et al.*, 1995). Secondly, eight strains selected for homozygosity for the presence of the highly active A2-B2 esterases: SELAX, the A2-B2 reference strain, derived from a 1984 sample from California (Raymond *et al.*, 1987); BOUAKE, collected in February 1986 from Bouaké, Ivory Coast (Magnin *et al.*, 1988); SUPERCAR collected in March 1994 from Bouaké, Ivory Coast, and provided by F. Chandre (ORSTOM, Montpellier, France); LAHORE, from Pakistan (Beyssat-Arnaouty, 1989); BRAZZA, from Congo (Beyssat-Arnaouty, 1989); BED, a strain collected in January 1991 from Johannesburg, South Africa, and provided by A. J. Cornel (The South African Institute for Medical Research, South Africa); THAI, an

isofemale strain from Bangok, Thailand, collected in 1991; MOOREA, a strain collected in October 1990 in Moorea, French Polynesia (Pasteur *et al.*, 1995). Thirdly, three strains homozygous for the presence of the over-produced esterases A4 and B4 (VIM, from Montpellier, southern France), A5 and B5 (CYPRUS from Cyprus) and A1 (BARRIOL, from Arles, southern France) (Poirié *et al.*, 1992; Chevillon *et al.*, 1995).

Sequencing of esterase

Genomic DNA from single adults was obtained according to Raymond & Marquine (1994). The DNA was suspended in 10 μL of T.E. buffer (Tris HCl, pH 8.0, 10 mM, EDTA 1 mM) and used for PCR without further dilution. PCR primers were designed from the A2 cDNA sequence of the strain PelRR (Vaughan & Hemingway, 1995), to amplify specifically *esterase A* genes. The primer set was 5′-GCAACGGGGGTCGATTACTAC-3′ and 5′-ACTTCATTCGTTCCTGCTCCG-3′. The 100 μL PCR reaction contained 0.25 μL of DNA, 2 μM of each primer, 1.25 mM of each dNTP, 1.5 mM $MgCl_2$ and 2.5 units of Taq polymerase (Goldstar Polymerase, Eurogentec, Louvain-la-Neuve, Belgium) in 1 × enzyme buffer supplied by the manufacturer. The reaction mixture was overlaid with mineral oil and heated in a Crocodile thermocycler (Appligene, Pleasenton, CA, USA) at 95°C for 4 min followed by 30 cycles of 95°C for 1 min, 60°C for 1.50 min, 72°C for 2 min. A final extension step was included at 72°C for 10 min. A 5 μL aliquot was removed for agarose gel electrophoresis and the PCR product was visualized by ethidium bromide staining. PCR products were sequenced following a direct method (Rousset *et al.*, 1992), using individual primers to direct DNA synthesis. The primers used to sequence the introns were, with the position in the PelRR A2 esterase sequence of Vaughan & Hemingway (1995), 5′-GCAACGGGGGTCGATTACTAC-3′ (67–87), 5′-AAACCGTGGACGGAACCGTTG-3′(157–177), 5′-GAGAGGATCTAGTGGGGTGGAG-3′ (336–357), 5′-TACCACATGGTGTCGGACCT-3′ (595–614), 5′-GATTCGGACACCTACAACCACT-3′ (1270–1291). The nucleotide in italics of primer 336–357 corresponds to a A in the PellRR A2 sequence. DNA sequences were manually aligned by minimizing the number of mismatches and gaps assumed in the sequence.

Statistics

The number of synonymous and nonsynonymous sites per codon was estimated following the method of Nei & Gojobori (1986). For computing nucleotide diversity and the number of polymorphic sites, each single or multiple insertion/deletion was considered as one mutation. An unbiased estimate of the *P* value of the exact Fisher test on an R × C contingency table was computed using a Markov chain method. The Markov chain was set to at least 10^6 steps, and 1000 steps of dememorization in order to obtain standard error estimates below 0.005 (see Guo & Thompson (1992) for details). The STRUC program was used for these computations (Raymond & Rousset, 1995).

Results

Sequences and variability of A esterase *genes*

The length of the PCR product obtained from genomic DNA of each strain was around 1800 bp, whereas the corresponding cDNA length is about 1520 bp. The major part of the *esterase A* gene (1755 bp or 84.2 per cent of the gene) was sequenced from strains displaying no over-produced esterase (MSE), and over-produced esterases A1 (BARRIOL), A5 (CYPRUS), A2 (SELAX) and A4 (VIM). Six short introns (named I to VI) were found in the five sequences, respectively at positions 142, 280, 430, 823, 1168 and 1423 of the cDNA A2 esterase sequence (Vaughan & Hemingway, 1995).

The alignments of the five sequences show a high degree of variability either in the exons or in the introns as indicated (Table 1) by the percentage of segregating sites (at least 8.7 per cent and 21.4 per cent, respectively) and the nucleotide diversity per site (at least 0.038 and 0.088, respectively). The polymorphism detected in the coding regions consists only in nucleotide substitutions, whereas introns I, II, V and VI present various levels of polymorphism which involve single or multiple-bp deletion/insertion (Fig. 1).

The level of variability measured by the percentage of polymorphic nucleotide sites is higher in introns than in exons (27.8 per cent vs. 11.3 per cent; Fisher's exact test, $P < 10^{-5}$). Among the six introns, the hypothesis of homogeneity of polymorphic and nonpolymorphic sites is not rejected (Fisher's exact test, $P = 0.11$). The same result is found for the six exons (Fisher's exact test, $P = 0.56$), so that the global nucleotide polymorphism of coding or noncoding regions does not appear to be concentrated in a few areas. There are between seven and 12 differences in amino acid composition between pairs of the five coding sequences (Fig. 2). This high degree of polymorphism in coding sequences is also

Table 1 Nucleotide polymorphism at the *esterase A* locus of *Culex pipiens* from the partial sequences of BARRIOL, SELAX, CYPRUS, VIM and MSE

	Number of bp	% of segregating sites	π	$\pi ns/\pi s$
Intron I	62	24.2	0.145	
Exon II	138	8.7	0.038	0.047
Synonymous	26	38.5	0.15	
Nonsynonymous	112	1.8	0.007	
Intron II	74	21.6	0.088	
Exon III	150	12.7	0.62	0.053
Synonymous	30	53.3	0.226	
Nonsynonymous	120	2.5	0.012	
Intron III	56	21.4	0.093	
Exon IV	393	10.4	0.05	0.056
Synonymous	89	39.3	0.177	
Nonsynonymous	304	2.0	0.01	
Intron IV	57	40.4	0.169	
Exon V	345	13.6	0.068	0.028
Synonymous	64	67.2	0.282	
Nonsynonymous	281	1.4	0.008	
Intron V	55	25.5	0.124	
Exon VI	255	9.8	0.05	0
Synonymous	52	48.1	0.205	
Nonsynonymous	203	0	0	
Intron VI	56	35.6	0.186	
Exon VII	114	12.3	0.053	0.321
Synonymous	20	30.0	0.112	
Nonsynonymous	94	8.5	0.036	
Summary				
Coding	1395	11.3	0.055	0.049
Synonymous	281	48.0	0.205	
Nonsynonymous	1114	2.1	0.01	
Noncoding	360	27.8	0.132	
Total	1755	14.7	0.071	

π refers to the nucleotide diversity per site, πns to the nucleotide diversity per nonsynonymous site and πs to the nucleotide diversity per synonymous site.

measured by the nucleotide diversity per nonsynonymous site, $\pi ns = 0.01$. Within the coding regions, differences are found between synonymous and nonsynonymous sites. Nonsynonymous polymorphism is heterogeneously distributed between exons (Fisher's exact test, $P = 8.6 \times 10^{-4}$, SE $= 1.1 \times 10^{-4}$): 35 per cent are in exon VII and none in exon VI.

Variability of intron IV of non-over-produced esterase A *genes*

We attempted to analyse the polymorphism of the *esterase A* gene in mosquitoes lacking A esterase over-production and in strains displaying over-produced esterase A2 by sequencing only one intron and its partial 3′ flanking region because: (i) the level of variability in introns is far larger than in exons, and (ii) no apparent heterogeneity of nucleotide diversity exists between the different introns. The analysis was focused on intron IV which lacks the insertion/deletion polymorphism present in introns I, II and VI and displays a larger number of polymorphic nucleotide sites than introns III or V. Intron IV and its 3′ flanking region were sequenced from single individuals of the nine strains lacking over-produced esterase A. None of the mosquitoes analysed was heterozygous, which is consistent with the theoretical low heterozygosity of these inbred strains. Both regions present a high polymorphism as each different individual sequenced shows a

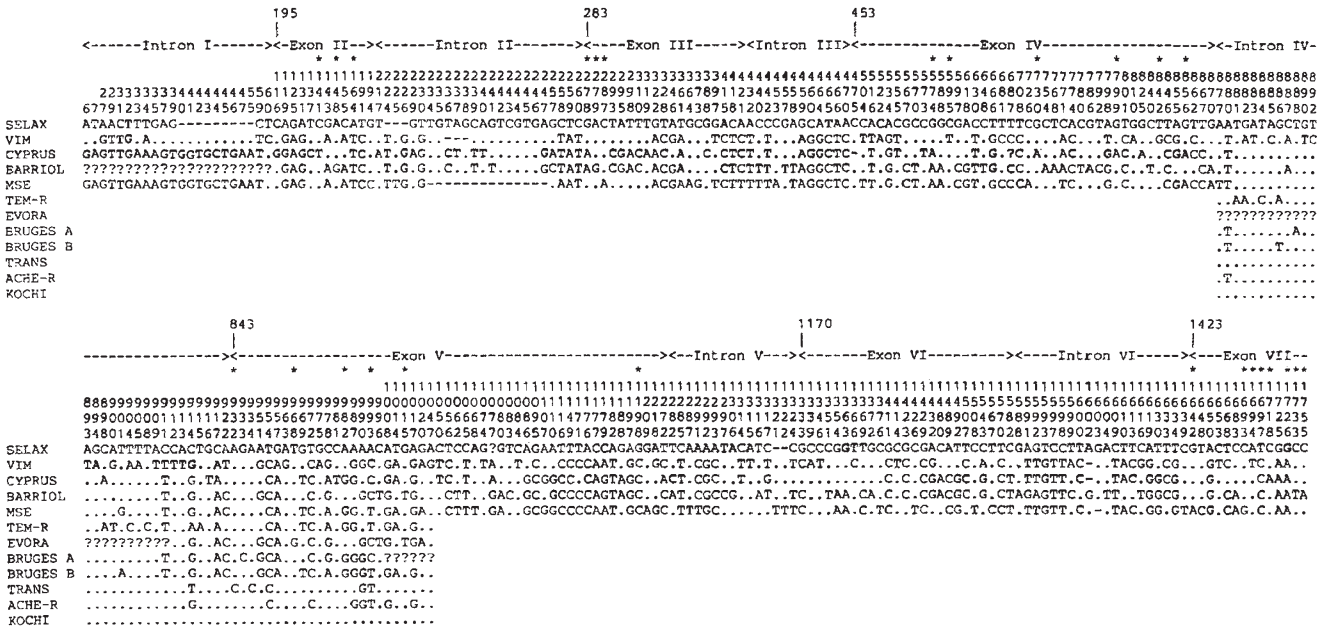

Fig. 1 Variable nucleotides at the *esterase A* locus in *Culex pipiens*. All deletions including single and multiple ones are indicated by a hyphen. The positions of variable sites in the genomic sequence are given by the number above SELAX nucleotides. Position no. 1 corresponds to the 5′ end of intron I (position 142 in Vaughan & Hemingway (1995)). The structure of the locus is given at the top of the figure with the corresponding numbering of the cDNA sequence from Vaughan & Hemingway (1995). Nonsynonymous variable sites are indicated by a star above the nucleotide positions.

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                  1112222222234444444
                  2345237012234451244556
                  8370749261894756745257 4
SELAX             NRDQVPVAVVIVIKVEVQIAAPN
VIM               .S...S..G....N.Q.LT.T..
CYPRUS            .SN.I.IT.I..VN.Q..TET..
BARRIOL           SSS.I.........NLQ..T.TLK
MSE               .S.KI....I..VN.QIET.T..
TEM-R                     ..VN.
EDIT                      ..VN.
EVORA                     .G.NL
BRUGES A                  ..VN?
BRUGES B                  ..VN.
TRANS                     L..N.
ACHE-R                    ...N.
KOCHI                     .....
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Fig. 2 Variable amino acids at the *esterase A* locus in *Culex pipiens*. The positions of variable sites are given by the number above SELAX nucleotides. The first position corresponds to the 5′ end of Exon II.

different allele at this locus, the only exception being two Californian mosquitoes from the strains EDIT and TEM-R. These two laboratory strains containing a single over-produced esterase (B1) were collected 14 years apart. The association of the same *esterase A* allele with the amplified *esterase B1* has been maintained by linkage disequilibrium. In the present study, EDIT and TEM-R cannot be considered as randomly chosen from mosquitoes lacking over-produced esterase A, and thus, in the following, EDIT will not be considered further.

The variability observed in intron IV and its 3′ flanking region in the strains lacking over-produced esterases (Table 2) is of the same order of magnitude as among strains with over-produced esterases A1, A2, A4 and A5. Moreover, in the short coding region sequenced for all strains, no particular site is shared only by mosquitoes possessing an over-produced esterase A. Thus, on this restricted dataset, there is no evidence for a relationship between the nucleotide polymorphism at the *esterase A* locus and the over-production of the corresponding esterase.

Variability of intron IV of A2 esterase *genes*

In contrast, the eight sequences of intron IV and its 3′ flanking region from mosquitoes with over-produced esterases A2 and B2, and originating from various countries (Ivory Coast, Congo, South Africa, Thailand, French Polynesia, USA and Pakistan) are strictly identical, even in the noncoding region.

Table 2 Nucleotide polymorphism at intron IV plus its 3′ flanking region from the sequences of *Culex pipiens* lacking over-produced esterase A and BARRIOL, CYPRUS, SELAX and VIM

	Number of bp	% of segregating sites	π	$\pi ns/\pi s$
Intron IV	57	40	0.169	
Exon V (5′ end)	117	20.5	0.079	0.069
Synonymous	19	94.7	0.301	
Nonsynonymous	98	6.1	0.021	

π refers to the nucleotide diversity per site, πns to the nucleotide diversity per nonsynonymous site and πs to the nucleotide diversity per synonymous site.

Discussion

A high nucleotide polymorphism

The sequences of 84.2 per cent of the *esterase A* structural gene from four laboratory strains displaying over-produced esterases A1, A2, A4 and A5 (BARRIOL, SELAX, VIM and CYPRUS) and from one with no over-produced esterase (MSE) show a high degree of variability at the nucleotide level. This result is not biased by the particular choice of strains displaying over-produced esterases because the partial sequences of the eight strains lacking over-produced esterase confirmed this tendency.

The nucleotide diversity found at the *esterase A* locus is one of the highest thus far described. In *Drosophila pseudoobscura*, the most polymorphic loci are *Adh* (Schaeffer & Miller, 1992), *Xdh* (Riley *et al.*, 1992) and *esterase 5-B* (Veuille & King, 1995), and the nucleotide diversities reported ($\pi = 0.015$, $\pi = 0.012$ and $\pi = 0.012$, respectively) are about five times lower than the value estimated for *esterase A* in *Culex pipiens* ($\pi = 0.071$).

The relatively low number of nonsynonymous compared with synonymous substitutions at the *esterase A* locus in *Culex pipiens* suggests that purifying selection might eliminate a large part of the mutations modifying amino acids sequences. This is true for strains with ($\pi ns/\pi s = 0.049$) or without ($\pi ns/\pi s = 0.099$) over-produced esterase A. This indicates that esterases A might have an important function, which could be insecticide resistance for alleles in mosquitoes displaying over-produced esterases. For strains lacking over-produced esterase, this function is still unknown as is also the case for most esterases (Aldridge, 1993).

The values of nucleotide diversity at nonsynonymous sites ($\pi ns = 0.01$ for over-produced and $\pi ns = 0.028$ for non-over-produced esterases) which are almost twice higher than the values obtained in the three last examples of *Drosophila pseudoobscura* sequences are in agreement with the level of polymorphism observed in allozyme analyses: 18 different electromorphs were observed in a sample of 74 mosquitoes from France and 16 electromorphs in 50 mosquitoes from England (Raymond *et al.*, 1995).

The high level of diversity of the *esterase A* gene indicates either a high mutation rate in this DNA region (local phenomenon) or the existence of an important effective population size in *Culex pipiens* (global phenomenon). The study of nucleotide diversity in other unlinked genes will allow us to discriminate among these possibilities, although ecological data do indicate that population sizes are relatively large (e.g. Bates, 1949; Chevillon, 1994). A test for departure from the neutral hypothesis, e.g. the HKA test which involves the comparison of the nucleotide diversity within and between species (Hudson *et al.*, 1987), would allow us to determine whether selection is responsible for the large number of different alleles found at this locus.

The unique amplification event and the migration hypothesis

The eight strains displaying over-produced esterases A2 and B2 and originating from Africa, North America, Eastern and Western Asia, shared the same allele at the *esterase A* locus. Under the multiple amplification hypothesis, the probability of obtaining eight times the amplification of this allele by randomly sampling within a gametic pool characterized by $\pi = 0.13$ (the value of the nucleotide diversity for intron IV and its 3′ flanking region for all the strains studied here except those displaying A2 esterase) is lower than 10^{-10}. The multiple amplification hypothesis is not supported by the present data, which are consistent with the migration hypothesis of the same amplified A2 allele. In addition, as A2 and B2 structural genes belong to the same amplicon, the A2-B2 association is probably the result of only one amplification event (Rooker *et al.*, 1996).

This migration hypothesis is based on: (i) the existence of a large neutral polymorphism in susceptible mosquitoes, and (ii) the presence of the same amplified allele in populations from distant geographical areas. The first point has been documented at the protein level for both *esterase A* and *B*

loci and indirectly at the DNA level by RFLP studies on the *B* locus region (Raymond *et al.*, 1995). The second point has been addressed at the DNA level only by RFLP studies (Raymond *et al.*, 1991; Qiao & Raymond, 1995). The nucleotide data at the *esterase A* locus of this study are consistent with both points, as the polymorphism found in alleles of non-over-produced esterases is one of the largest thus far described, and all A2 alleles display exactly the same sequence.

In addition, there is direct (Highton & Van Someren, 1970) and indirect (Chevillon *et al.*, 1995; Pasteur *et al.*, 1995) 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 (Curtis & White, 1984). The local invasion of A2-B2 in southern France has been documented: A2-B2 was first found near the international Marseille airport and seaport, and has spread within a few years in all surrounding organophosphorous-treated areas (Rivet *et al.*, 1993).

It is worthy of note that the strain KOCHI displays a sequence similar to those found in the strains with amplified A2, although this strain does not possess over-produced esterases (unpublished data). This can be explained by at least two alternative hypotheses. First, the level of esterase A2 over-production is low and was not detectable by classical allozymic electrophoresis. Secondly, this strain possesses the allele that was shared by the susceptible ancestors prior to the amplification event. This latter hypothesis seems to be the more likely: the B esterase detected by starch gel electrophoresis is different from B2 (which is always associated with A2 when A2 is amplified). Further work is needed on this aspect.

The number of independent amplification events at the *esterase A* and *B* loci could not be estimated easily by just counting the number of over-produced electromorphs. First, because the *esterase A* and *B* loci could be amplified simultaneously, as are A2 and B2 (Rooker *et al.*, 1996) and probably the associated A4 and B4, and A5 and B5 (T. Guillemaud *et al.*, unpublished data), so that only one amplification event is responsible for the presence of the two electromorphs. Secondly, because an esterase over-production is not necessarily the result of gene amplification, as for the over-production of esterase A1 owing to gene regulation (Rooker *et al.*, 1996). Thirdly, because the same electromorph could correspond to distinct alleles, as exemplified by B4 and B5 (Poirié *et al.*, 1992), and B1 and B8 (Vaughan *et al.*, 1995).

The present study illustrates this last point because the electrophoretically indistinguishable esterases A4 (strain VIM) and A5 (strain CYPRUS) originating from France and Cyprus present two different coding and noncoding sequences and thus correspond to two distinct alleles. In addition, there are ten amino acid differences between the A4 and A5 sequences, which is not different from any two sequence pairs drawn at random among pairs of complete sequences (between seven and 12 amino acid differences), indicating that the A4 and A5 sequences are not particularly related. These data confirm the hypothesis that these esterases were different because of their close association with, respectively, esterases B4 and B5 (Poirié *et al.*, 1992).

Taking into account protein and DNA work published so far, the number of independent amplification events at both *esterase A* and *B* loci is between five and ten. The imprecision comes from the fact that a thorough checking has not been performed for all known over-produced esterases. This number corresponds to amplification events which have spread geographically because of the advantage they give in insecticide treated areas, and are therefore at high frequencies and easily detected. A more thorough sampling will probably detect additional events which either are still geographically restricted or at a low frequency, so that the above estimates should be understood as a minimum figure. Each known amplification event has spread geographically, sometimes across continents like A2-B2 (Raymond *et al.*, 1991) and B1 (Qiao & Raymond, 1995), sometimes only in a restricted area like A4-B4 in the western Mediterranean (Poirié *et al.*, 1992), A5-B5 in the eastern Mediterranean (Poirié *et al.*, 1992) and B8 in the northern Carribeans (Vaughan *et al.*, 1995). This relatively low number of independent amplification events, inventoried on a world scale for a pest species with large population sizes, indicates that advantageous mutations (i.e. any molecular events generating a gene amplification at these loci) could be limiting. Once an amplification has occurred, it could apparently spread easily and invade the mosquito genome. Clearly, migration can not be ignored as a driving force in studies and monitoring of insecticide treated populations of the mosquito *Culex pipiens*.

Acknowledgments

We thank C. Bernard and G. Pistre for technical assistance and D. Bourguet and J. Lagnel for helpful

comments and discussions. This work was financed in part by a GDR 1105 du programme Environnement, Vie & Sociétés du CNRS and a CEE grant (No ERBCHRXCT930172). S.R. benefited from a CEE fellowship and T.G. from a MESR fellowship (no. 94137). This is contribution ISEM 96.105 of the Institut des Sciences de l'Evolution (URA CNRS 327).

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