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Esterase polymorphism in insecticide susceptible populations of the mosquito *Culex pipiens*

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Summary

Gene amplification involving a particular haplotype has been found at the esterase B locus of mosquitoes from various countries. This similarity has been explained by a unique amplification event followed by migration and selection by organophosphate (OP) insecticides. This assumes that the polymorphism of non-amplified esterase haplotypes is so large that the chance of independent amplification in two distinct populations is negligible. In order to test this assumption, three susceptible populations from northern Europe were sampled and analysed for esterase and haplotype polymorphism. At the protein level, 18 and 16 alleles were found for esterase A and B respectively in one French population ($n = 74$), and 16 and 14 in an English one ($n = 50$). At the DNA level, 24 alleles at the esterase B locus were detected in a sample of 72 mosquitoes from one population, with the use of only one restriction enzyme (*Eco*R V). Restriction maps of two non-amplified haplotypes randomly sampled from a single breeding site in Belgium were built with six restriction enzymes. 60% of all restriction sites were different among the two maps. The huge polymorphism found in northern Europe requires specific explanations for its stability, but it considerably strengthens the hypothesis of migration of amplified haplotypes.

1. Introduction

The large use of organophosphate (OP) insecticides to control populations of the mosquito *Culex pipiens* in various places of the world has brought about dramatic genetic changes. Resistance genes have occurred in all the studied treated populations and are commonly found in high frequencies. One of the most widespread resistance mechanisms is esterase overproduction at two esterase loci. *Est-2* (or esterase B) and *Est-3* (or esterase A), code for detoxifying esterases, and resistance alleles correspond to an esterase overproduction (Fournier *et al.* 1987; Mouchès *et al.* 1987; Poirié, Raymond & Pasteur, 1992). Six distinct electromorphs have been described so far at the *Est-2* locus (named B1, B2, B4, B5, B6 and B7) and four at the *Est-3* locus (A1, A2, A4 and A5) (Georghiou, 1992; Pasteur, Iseki & Georghiou, 1981; Pasteur, Sinègre & Gabinand, 1981; Poirié *et al.*, 1992; Raymond *et al.* 1989; Xu, Qu & Liu, 1994). In the case of esterase B, overproduction corresponds to the amplification of a DNA segment (or amplicon) containing the structural gene (Mouchès *et al.* 1986; Poirié *et al.* 1992; Raymond *et al.* 1989; Vaughan, Rodriguiz & Hemingway, 1995).

A restriction map of the DNA within and around the esterase B gene can be built, in susceptible 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 California, one of a non-amplified allele and the other of an amplified allele, have only 21% of their restriction sites in common (Raymond *et al.* 1991). However, when strains with the B2 elecromorph are compared, restriction maps are strictly identical, independently of their geographical origins (Raymond *et al.* 1991). A similar situation is found for the B1 electromorph: mosquitoes from various parts within the Americas

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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 geographic areas indicates that all B1 (or all B2) alleles are identical by descent. There are two possible events to explain this: either they were first amplified in a particular place, and have then spread, or they have 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 in susceptible populations is large, which is only documented from a small sample study from Portugal (Raymond *et al.* 1991), and from the analysis of a few susceptible mosquitoes from resistant populations in Italy (Severini, Marinucci & Raymond, 1994) and Venezuela (Qiao & Raymond, 1995). In addition, the selective advantage provided by the amplification itself promotes its spread in OP treated places. The multiple and independent amplification of B2 has been favoured by Hemingway *et al.* (1993) and Ketterman *et al.* (1993), based on variation in the kinetics of esterases studied on partially purified enzymes.

An analysis of the polymorphism of susceptible populations could discriminate between these two possibilities. Under the first scenario, the polymorphism at the esterase B locus in non-treated populations should be extensive, and the probability of sampling a non-amplified allele already amplified elsewhere should be very low. The second scenario predicts that the non-amplified A2 and B2 allele (the most common of the B esterase amplification worldwide) are present at a detectable frequency in susceptible populations.

The present study was performed to investigate the extent of polymorphism at the two esterase loci in susceptible populations in order to evaluate the validity of the migration hypothesis. Protein polymorphism at both esterase A and B loci was studied to detect the possible presence of non-amplified A2 and B2. DNA polymorphism around the esterase B locus was also evaluated as the migration hypothesis is based on RFLP data. Two methods were used to evaluate the RFLP polymorphism within a population: either analysing many genes with only one restriction enzyme or analysing two genes taken at random with several enzymes.

2. Material and methods

(i) *Mosquitoes*

One large sample (MERLIN) of larvae and pupae was collected in September 1988 in the Paimpont sewage station located in the Broceliande forest, in Brittany, France. One sample (BRISTOL) of larvae and pupae was collected in June 1991 in Avonmouth near Bristol, England. Adults were allowed to emerge in the laboratory, and were deep frozen when they were at least 3-d-old. A proportion of the MERLIN larvae were used for insecticide bioassays. A third sample (BRUGES) of larvae from Bruges, Belgium, was collected in September 1991 from a manhole near the cathedral. Adults were allowed to emerge in the laboratory, and two randomly chosen isofemale lines were maintained by sib mating during at least 10 generations. The two inbred isofemale lines were designated as BRUGES-A and BRUGES-B.

(ii) *Insecticide bioassays*

Resistance characteristics of MERLIN larvae were analysed by bioassays on fourth-instar larvae in disposable plastic cups holding 99 ml of tap water plus 1 ml of an insecticide solution in alcohol. Three insecticides of analytical grade were used: chlorpyrifos (Dow Chemical, Midland, MI), temephos (American Cyanamid, Princeton, NJ), and fenitrothion (Sumitomo Chemical Co., Japan). In each test, sets of 20 larvae were exposed to different insecticide doses during 24 h. Only discriminating doses killing susceptible larvae were used in order to detect the presence of resistant individuals.

(iii) *Acrylamide gel electrophoresis*

Individual mosquitoes of MERLIN and BRISTOL were cut in two into 0·1 M-TBE, pH 7·5, under a dissecting microscope. The head and thorax was immediately placed in an Eppendorf tube and frozen at −20 °C for DNA studies (see below). Alleles at the Est-2 and Est-3 locus were scored in single mosquito abdomen homogenates using acrylamide gel electrophoresis with Tris–Borate–EDTA buffer (0·1 M, pH 7·5). Each abdomen was homogenized in 6 μl of buffer (TBE 0·1 M, pH 7·5, 20% glycerol) and centrifuged for 30 s at 10000 rev/min. Four μl of each supernatant was loaded onto a 7·5% polyacrylamide gel (Sturdier Equipment). Mosquitoes from the SeLAX reference strain (Wirth *et al.* 1990), possessing A2 and B2 overproduced esterases, were used as marker for the analysis of MERLIN mosquitoes: for each gel, an individual was homogenized in 6 μl and diluted by 17-fold before loading 4 μl. The gel was run at 4 °C at 200 V for 4–5 h, and stained for esterase activity (Pasteur *et al.* 1988). Relative mobilities were estimated from that of A2. According to previous Mendelian inheritance studies on these esterase loci (de Stordeur, 1976), each band corresponded to one allele. In addition, the occurrence of possible epigenetic bands was prevented by the presence of EDTA in the gel buffer (Callaghan *et al.* 1994).

(iv) *RFLP*

Single mosquito genomic DNA was obtained as described in Qiao & Raymond (1995) from the head and thorax (when its abdomen was used for protein studies) or from the whole adult. This produced enough DNA to carry out a digestion with one restriction enzyme, *Eco*R V. Gel migration, transfer, probe labelling (to the 1·3 kb cDNA of B1 esterase), and hybridization were done as described in Raymond *et al.* (1989).

As one conserved *Eco*R V site lies within the area covered by the probe, and another one 400 bp away on the 5′ part (see Raymond *et al.* 1991), two bands per allele are expected. One of these bands should correspond to the 5′ part of the probe and be 400 bp long, so that most heterozygote individuals display only three bands and homozygotes two. The presence of four bands (one being always of 400 bp) was interpreted as either the presence of a duplication or the disappearance of one of the two *Eco*R V sites in one allele.

Genomic DNA from several mosquitoes of BRUGES-A and BRUGES-B was extracted as described in Raymond *et al.* (1989). Restriction maps of the esterase B region were constructed using six restriction enzymes (*Bam*H I, *Bgl* II, *Eco*R I, *Eco*R V and *Pst* I).

(v) *Statistics*

Due to the presence of null alleles, frequencies of alleles detected by protein electrophoresis were estimated by two methods. First by assuming that all homozygotes are true homozygotes, which gives the minimum frequency for the null allele by simple enumeration. Second by assuming Hardy–Weinberg equilibrium and estimating maximum likelihood estimates of allele frequencies using the EM algorithm for incomplete data (Dempster, Laird & Rubin, 1977). Due to the presence of heterozygote deficit (see results), the null allele frequency estimated by the second method is a maximum.

F_{is} was computed according to Weir & Cockerham (1984). Hardy–Weinberg proportions were tested using the probability exact test (Guo & Thompson, 1992) or using the exact *U*-test described by Rousset & Raymond (1995), when the alternative hypothesis is heterozygote deficit. The Genepop software (Raymond & Rousset, 1995) was used for these computations.

3. Results

(i) *Resistance of MERLIN larvae*

No MERLIN larvae survived the discriminating doses of 0·01 or 0·03 ppm of temephos, chlorpyrifos and fenitrothion (60 larvae tested at each dose for each insecticide) and 0·1 ppm of propoxur (40 larvae tested), indicating that larvae resistant to these insecticides were not present (see e.g. Raymond & Marquine, 1994).

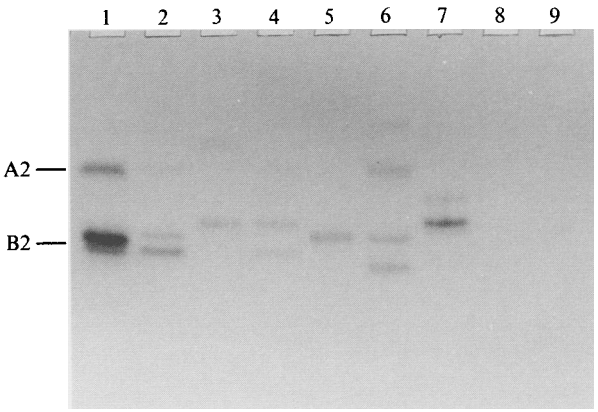

Fig. 1. Acrylamide gel electrophoresis of individual mosquitoes, stained for esterase activity. Well no. 1 is loaded with one SeLAX mosquito diluted 17-fold compared to the others. Wells 2 to 9 are loaded with MERLIN mosquitoes. No esterase activity was apparent for mosquito no. 8 (see text).

(ii) *Protein polymorphism*

Brittany population. A total of 74 mosquitoes from MERLIN were analysed by acrylamide electrophoresis (Fig. 1). Some mosquitoes did not possess active enzymes at one esterase locus, indicating that null alleles were frequent. Seven mosquitoes were probably homozygous for a null allele at both loci, as they did not show any esterase activity (e.g. Fig. 1, well no. 8). However, they could also represent low quality samples, so they were discarded from the analysis. Most individuals displayed one or two bands at each locus, but three mosquitoes displayed three bands for esterase A and one mosquito displayed three bands for esterase B. In all cases, the three bands had the same mobility as one of the bands detected in the other individuals, suggesting that they were not artefactual. For the computation of gene frequencies, one of the three bands taken at random was removed.

Eighteen and 16 alleles were scored for esterase A and B, respectively, including one null allele at each locus. Frequencies were computed first by assuming that the null allele in each locus is present only in null homozygotes. This conservative assumption provided a minimum for the frequency of null alleles, which was 20·9% at the esterase A locus and 13·4% at the esterase B locus. All other allele frequencies were below 16% (Table 1). Frequencies were also computed by assuming Hardy–Weinberg proportions, using the EM algorithm to find maximum likelihood estimates. This assumption provided a maximum for null allele

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Table 1. *Gene frequencies (%) of alleles at the Esterase A and B locus detected by protein electrophoresis*

Allele no.	Esterase A			Esterase B		
		Frequency			Frequency	
	RM	Min.	Max.	RM	Min.	Max.
1	< 0·625	2·2	1·5	1·30	1·5	1·5
2	0·65	2·2	2·2	1·35	3·7	3·0
3	0·70	0·7	0·7	1·40	9·0	6·2
4	0·80	3·7	3·0	1·45	10·4	7·8
5	0·90	4·5	3·0	1·50	15·7	12·5
6	1·00	4·5	3·0	1·55	6·7	5·3
7	1·10	5·2	4·5	1·60	11·9	10·7
8	1·15	3·0	2·3	1·65	6·7	4·6
9	1·20	5·2	3·8	1·70	6·7	4·6
10	1·25	8·2	6·1	1·75	3·0	2·3
11	1·30	9·0	6·9	1·80	2·2	1·5
12	1·35	3·7	2·3	1·85	3·0	3·0
13	1·40	5·2	4·5	1·90	3·0	2·3
14	1·45	11·2	7·1	1·95	1·5	0·8
15	1·50	6·0	3·1	2·15	1·5	0·8
16	1·55	3·7	3·0	Null	13·4	33·2
17	1·6	0·7	0·7			
18	Null	20·9	42·1			

Min., frequencies estimated with the minimum estimate of the null allele frequency. Max., frequencies estimated with the maximum of the null allele frequency. The relative mobility of each allele towards the reference A2 band is indicated (RM).

frequencies, because heterozygote deficit was detected at the DNA level (see below). This maximum was 42·1% and 33·2% for esterase A and B loci respectively. Other allele frequencies were below 13% (Table 1).

No mosquito displayed an esterase band with high activity, indicating that an esterase overproduction resistance mechanism is probably absent from this population. One electromorph had the same mobility as B2, but it was never associated with an A electromorph with the same mobility as A2 (A2 and B2 electromorphs are associated throughout the world), and it was concluded that this similar migration is probably coincidental due to the large number of electromorphs.

In order to ascertain that the level of protein polymorphism obtained in MERLIN are not unique to this population, 50 mosquitoes from an English population (BRISTOL) were also analysed. As in MERLIN, supernumerary bands were found in two mosquitoes, but only at the esterase B locus. A null allele was also present in each esterase locus, with a minimum frequency of 18% and 22% for esterase A and B respectively. Including the null allele, the total number of alleles detected in the BRISTOL sample was 16 for esterase A and 14 for esterase B. As in the MERLIN sample, none of the esterase alleles displayed high activity.

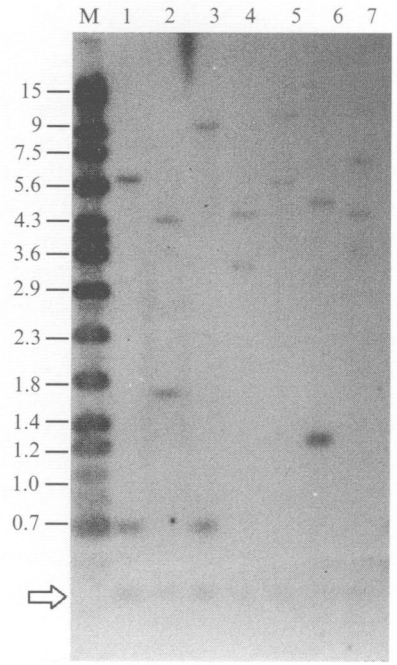

Fig. 2. RFLP on single individuals from MERLIN. Seven wells loaded with mosquitoes are numbered on top. M refers to the size marker (in kb on the left). The common band of 400 bp hybridizing with the 5′ part of the probe is indicated by an arrow.

(iii) *DNA polymorphism on single individuals*

Eighty-two individuals from MERLIN were scored for their genotype at the esterase B locus using *Eco*R V restriction (Fig. 2). None of the bands displayed a high intensity, indicating an absence of the high copy number (more than 40-fold) gene amplification usually found in resistant populations (e.g. Raymond & Qiao, 1995). Two to three bands were observed for most individuals, but 11 individuals displayed four bands. Two explanations are possible. The conserved *Eco*R V site on the 5′ part may have disappeared in some individuals, so that two large bands could belong to the same allele. Alternatively, a gene duplication may have occurred, resulting in two distinct alleles being on a same chromosome, so that three distinct haplotypes are present in a single mosquito. Although this *Eco*R V site seems conserved in Europe (see below), additional work is needed to decide which explanation is true. These 11 mosquitoes were not considered further.

A total of 24 distinct alleles were detected in the remaining 72 mosquitoes (Fig. 2). The most common allele had a frequency of 21%, all the others displayed a frequency below 10% (Table 2).

Hardy–Weinberg proportions were significantly (Probability test, $P < 2 \times 10^{-6}$) rejected, due to a significant deficit of heterozygote ($F_{is} = +0{\cdot}18$, $P < 2 \times 10^{-6}$). The deficit was not due to the removal of the 11 mosquitoes mentioned previously (adding 11 heterozygous mosquitoes possessing genotypes under-represented was insufficient to compensate for the deficit, details not shown).

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Table 2. *Allelic counts and frequencies at the Esterase B locus*

Allele	Count	Frequency
1	30	20·83
2	1	0·69
3	2	1·39
4	5	3·47
5	4	2·78
6	2	1·39
7	2	1·39
8	1	0·69
9	1	0·69
10	10	6·94
11	2	1·39
12	4	2·78
13	6	4·17
14	6	4·17
15	11	7·64
16	14	9·72
17	4	2·78
18	1	0·69
19	11	7·64
20	6	4·17
21	1	0·69
22	11	7·64
23	1	0·69
24	8	5·56
Total	144	100·0

Alleles were detected by RFLP.

Analysis of esterases at the protein and DNA levels on the same mosquito was not successful due to low DNA concentration. Unambiguous results were obtained for only nine BRISTOL and two MERLIN mosquitoes. The two MERLIN mosquitoes displayed the same allele by protein electrophoresis (which could be either an homozygote or an heterozygote with a null allele) although a total of three alleles were detected by RFLP. Six of the nine BRISTOL mosquitoes shared one allele detected by protein electrophoresis (but were not homozygotes), although only three of them have a common allele detected by RFLP.

(iv) *Restriction maps of alleles from BRUGES*

The restriction maps of BRUGES-A and BRUGES-B were established using six restriction enzymes (Fig. 3). 60% (9/15) of the sites are different among the two maps (Fig. 3). These maps were compared to three other maps from European strains available in the literature (Poirié *et al.* 1992; Raymond *et al.*, 1991). Two of these maps correspond to amplified haplotypes (strains VIM from southern France and CYPRUS from Cyprus) and the third one to a non-amplified one (strain MSE from southern France). Four sites, located within the gene, were not variable in this sample (two of these were the two *Eco*R V sites generating the small 400 bp band in Fig. 2). BRUGES-A and BRUGES-B shared two additional sites, but this was true also for the pairs BRUGES-A and VIM, and CYPRUS and MSE.

Discussion

(i) *Extent of polymorphism*

The polymorphism detected in the two samples from Brittany or England, about 14–18 alleles for protein electrophoresis ($n \leqslant 67$) and 24 alleles for RFLP ($n =$

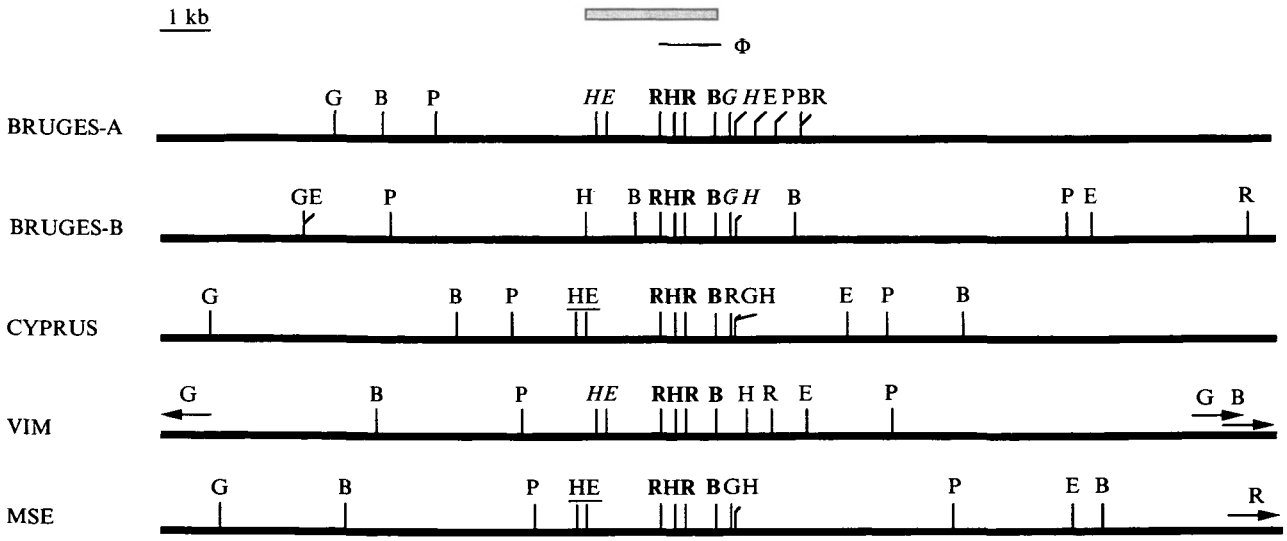

Fig. 3. Restriction maps of the esterase B genes and its flanking sequences from BRUGES-A and BRUGES-B. Other published restriction maps from European strains are presented for comparison: MSE, VIM and CYPRUS (Poirié *et al.* 1992). Restriction enzymes are abbreviated as: B (*Bam*H I), G (*Bgl* II), E (*Eco*R I), R (*Eco*R V), H (*Hin*d III) and P (*Pst* I). Sites outside the map are indicated by an arrow; sites present in all maps are in bold characters; sites present in some, but not all maps are in italics or are underlined. 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 within or flanking the fragment to which the probe hybridized are mapped for each enzyme. The haplotype of VIM and CYPRUS is amplified, the amplicon being larger than the area represented.

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72), is probably an underestimate for the following reasons. Only allelic classes of co-migrating enzymes could be detected by protein electrophoresis. Also, only one allelic class of 'null allele' could be attributed to the absence of enzymatic activity, although there is no reason to think that it is the case. In addition, only one restriction enzyme (*Eco*R V) has been used to characterize the DNA polymorphism. Inspection of the published restriction maps of the esterase B area (Poirié *et al.* 1992; Raymond *et al.* 1991) discloses that at least twelve other restriction enzymes could detect additional polymorphism. Although it is difficult to estimate how many alleles would be detected with 12 restriction enzymes, the use of one additional enzyme should allow us to detect more than 40 alleles, assuming that the second enzyme will reveal similar levels of polymorphism as *Eco*R V. Finally, the precision of the size markers and the size of the gel are limiting factors to score distinct alleles across different gels. As the number of distinct bands identified between two adjacent marker bands was generally only one, a higher number of bands may have been detected by using more precise size markers, a larger gel or different concentrations of agarose. Previous studies by Pasteur & Sinègre (1975) and de Stordeur (1976) of the esterase B locus in a southern France population found fewer alleles (seven and nine respectively), but this difference is probably explained by the fact that they used starch gel electrophoresis.

Esterases are among the most polymorphic proteins thus far described. In insects, 13 alleles were detected in a sample of 127 *Colias eurytheme* (Burns & Johnson, 1967), 14 alleles in a sample of 106 *Hemiargus isola* (Burns & Johnson, 1971), 15 alleles in a sample of 312 *Dacus oleae* (Krimbas & Tsakas, 1971) and 10 alleles at the Esterase 6 locus in a sample of 60 *Drosophila melanogaster* (Labate *et al.* 1989). These numbers are probably underestimated, as indicated by the presence of additional variants detected by thermostability analysis (Labate *et al.* 1989) or sequential electrophoresis (Keith, 1983).

The number of alleles detected by RFLP at the Esterase B locus found in this study is comparable to those found in the *Adh* or Esterase 6 cases (Game & Oakeshott, 1990; Kreitman & Aguadé, 1986; Richmond *et al.* 1990). The two haplotypes isolated from the same breeding site in Bruges, Belgium, did not share any more restriction sites between them than they did with any B esterases from Europe. Although more information is required to quantify the proximity of these haplotypes, this pattern indicates that BRUGES-A and BRUGES-B alleles are separated by a large number of mutation events.

(ii) *Null alleles*

The high frequency of null alleles found at both esterase loci (at least 20·9% and 13·4% for esterase A and B respectively in MERLIN, and 18% and 22% in BRISTOL) is not unique. For example, in *Dacus oleae*, Greek populations displayed a null allele frequency between 10 and 17% (Krimbas & Tsakas, 1971); in *Colias eurytheme* the null allele is the most common (Burns & Johnson, 1967), as in the situation described here. In the mosquito *Culex pipiens*, a minimum frequency of 10·2% of the null allele was obtained by Pasteur & Sinègre (1975) at the Esterase B locus (Est-2 in their paper) in a population from southern France, although this allele was not the most frequent. At an additional esterase locus (Est-1), the frequency of the null was between 34 and 74% (de Stordeur, 1976).

(iii) *Stability of the polymorphism*

The heterozygote deficit could be the result of viability selection, although alternative explanations (e.g. Wahlund effect) cannot be ruled out without further studies. There is evidence of selection for esterase alleles in other insect species, although no general pattern emerge for how selection operates: frequency dependence rather than overdominance (MacIntyre & Wright, 1966), overdominance (Tsakas & Krimbas, 1970), non-systematic overdominance (Kasule & Cook, 1988) and possible mixed selective-neutral situation (Keith, 1983). The physiological role of these esterases is unknown (except for Esterase-6 affecting mating behaviour in *Drosophila melanogaster*), so that the hypothesis that they are involved in parasite resistance as suggested by Hamilton, Axelrod & Tanese (1990) is not out of question.

(iv) *Duplication*

Some mosquitoes displayed three esterase bands, and this could be an indication of the presence of gene duplication within MERLIN and BRISTOL populations. Although alternative explanations can be found, this hypothesis is strengthened by the RFLP analysis which indicates that gene duplication at the esterase B locus is possible for 11 mosquitoes in MERLIN. Esterase gene duplication has been reported in several insect species (e.g. Brady & Richmond, 1992; East, Graham & Whitington, 1990) but not in the mosquito *Culex pipiens*. The present data is insufficient to ascertain the presence of esterase duplication in MERLIN and BRISTOL but, if confirmed, it would be interesting to study if this phenomenon could represent a first step towards the massive gene amplification responsible for OP insecticide resistance.

(v) *The migration hypothesis*

Non-overproduced A2–B2 has not been found in a sample of 148 genes from the susceptible MERLIN population. This means that if their frequency was

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higher than 2·0% in the population, they would have been detected in this sample ($P < 0{\cdot}05$). As exhaustive sampling is practically impossible, their absence cannot be firmly established. It is still possible that non-amplified B2 alleles exist at very low frequencies in every susceptible populations, but how such a situation would be created and maintained requires specific explanation before further considerations. The multiple amplification hypothesis is not supported by the present data.

A unique amplification event prior to extensive migration of esterase B haplotypes seems the most likely hypothesis to explain the existing molecular data. This hypothesis is based on (1) the existence of much neutral polymorphism around the esterase B structural gene in susceptible mosquitoes, and (2) the presence of the same amplified haplotype in populations from distant geographical areas. The second point has been substantially documented (Qiao & Raymond, 1995; Raymond *et al.* 1991; Raymond & Marquine, 1994; Rivet, Marquine & Raymond, 1993; Severini *et al.* 1994; Vaughan *et al.* 1995; Xu *et al.* 1994), and the first point is now supported by the present study of populations from England, Belgium and northern France. 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 man, and the presence of one female with A2–B2 in a plane 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 few years in all surrounding OP-treated areas (Rivet *et al.* 1993).

The polymorphism found around the esterase gene is probably neutral *vis-à-vis* OP insecticides, so that two distinct amplifications, even of the same esterase gene/allele, will probably generate two distinct amplified haplotypes. This situation is illustrated by the B4 and B5 amplified esterases, which have the same electrophoretic mobility and are both found within the mediterranean region. However, their restriction maps are distinct and not particularly related, so that they represent two independent amplification events (Poirié *et al.* 1992). A similar situation has been found recently in Cuba (Vaughan *et al.* 1995), and others will probably be described in the future. These situations do not contradict the migration hypothesis.

Once a haplotype has been amplified and spread in treated populations, some copies may accumulate mutations. This may generate some polymorphism in linkage disequilibrium with the other copies, as described for the B1 amplification (Qiao & Raymond, 1995). If such polymorphism is sufficiently large, it will probably allow us to reconstruct the various historical events of the migration and to trace the origin of the first amplification event for each of the four amplified esterases which are currently spreading among and between OP treated areas around the world.

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