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SHORT NOTE

Polymorphisms and fluctuations in copy number of amplified esterase genes in *Culex pipiens* mosquitoes

A. Callaghan,[1] T. Guillemaud,[2] N. Makate[1] and M. Raymond[2]

[1]*Division of Zoology, School of Animal and Microbial Sciences, University of Reading, Whiteknights, Reading, UK, and* [2]*Laboratoire Genetique et Environnement, Institut des Sciences de l'Evolution (CNRS, UMR 5554), Universite Montpellier II, Montpellier, France*

Abstract

In *Culex pipiens* mosquitoes, A2 esterase alleles are co-amplified with B2 esterase alleles in response to selection with organophosphate insecticides. In this study the amplified A2 and B2 sequences were compared between twelve strains from four continents by restriction mapping. The restriction maps were almost identical in each strain throughout 22 kb surrounding the genes, suggesting that this represents a constant core sequence. A polymorphism was found in two strains collected from Egypt and Kenya in the mid 1980s. This polymorphism was present in all copies of the amplicon, which suggests that a mechanism of sequence homogenization was operating, i.e. concerted evolution. These two strains were almost certainly descendants from the same population and migration probably occurred along the River Nile. Although the maps were almost identical in each strain, dot blotting demonstrated that amplification levels differed by up to 13-fold between strains. Thus the presence of the A2-B2 haplotype cannot be used to indicate the level of amplification or any particular degree of resistance.

Keywords: *Culex pipiens*, esterases, amplification, insecticide resistance, concerted evolution.

Received 11 September 1997; accepted 13 January 1998. Correspondence: Dr A. Callaghan, Division of Zoology, School of Animal and Microbial Sciences, University of Reading, Whiteknights, P.O. Box 228, Reading RG6 6AJ, UK. e-mail: a.callaghan@reading.ac.uk.

Introduction

In the mosquito *Culex pipiens* organophosphate resistance is achieved by the overproduction of non-specific carboxylesterases that detoxify the insecticide by sequestration (Cuany *et al.*, 1993). Two closely linked loci (Est-3 and Est-2) code for these esterases (named A and B esterases respectively) and the protein products from both are overproduced. This overproduction either results from the amplification of one (the B gene) or both (A and B) structural genes (Mouchès *et al.*, 1986; Raymond *et al.*, 1989; Poirié *et al.*, 1992; Vaughan & Hemingway, 1995; Rooker *et al.*, 1996; Guillemaud *et al.*, 1997) or by regulation of gene expression (the A1 allele) (Rooker *et al.*, 1996).

The worldwide distribution of *Culex pipiens* mosquitoes with amplified B2 esterase alleles has been shown to result from extensive migration (Raymond *et al.*, 1991, 1996; Guillemaud *et al.*, 1996). These genes are amplified in response to selection with organophosphate insecticides (OPs). The mutation which caused the initial amplification of B2 was a unique event. Subsequent selection pressures may have resulted in increases or decreases in copy number through processes such as unequal sister chromatid exchange. However, the level of the B2 allele amplification in different strains has never been measured to see if this is the case.

Recently it was suggested that the gene coding for A2 esterases, which is always found overproduced in strains with an amplified B2 allele, is also amplified (Vaughan & Hemingway, 1995; Rooker *et al.*, 1996). Restriction mapping of the A2 allele in a strain from California (SELAX) placed it within 2.2 kb of the B2 allele, which led the authors to hypothesize that A2 and B2 alleles were co-amplified (Rooker *et al.*, 1996). This has since been proved by Makate (1997) who cloned the B2 allele from the same strain and showed that the A2 allele was also present in the same section of amplified DNA and by Vaughan *et al.* (1997) in a parallel study.

The aim of this study was twofold. The first aim was to see whether there were differences in A2-B2 amplicon copy number between strains collected from different geographical areas. If there were differences it would show that there is a mechanism operating to increase and decrease the level of amplification. The strains used in the analysis included those collected from the field more than 10 years ago. It was thought that a comparison between these and more recently collected strains might give information about changes in copy number over time. The second aim was to see if the co-amplification of the A2 and B2 alleles was widespread. Sequences in and around the A2 and B2 alleles were mapped using RFLP techniques. Previous work by Raymond *et al.* (1991, 1996) demonstrated that the B2 allele had spread worldwide by migration since the restriction maps collected from strains throughout four continents were identical. Similar maps of the A2 allele were constructed. If the A2 allele was co-amplified with the B2 allele in each strain, then all the A2 allele maps should also be identical.

Results and Discussion

Restriction maps

Twelve strains of *C. pipiens* were included in the analysis of the amplified A2 and B2 esterase alleles by restriction mapping (Table 1). These strains were chosen to cover North America, North and South Africa, Asia and Europe and represented collections from the 1980s in addition to collections from the early 1990s. Three of the strains (SELAX, BOUAKE and NAIROBI) had been maintained in the laboratory for many generations. The restriction maps of the A2 and B2 alleles in all twelve strains were extremely similar (Fig. 1). The original comparison of six A2-B2 strains (four of which are also used in this study) only looked at the fragments that hybridized with the B1 allele probe (Raymond *et al.*, 1991). The addition of fragments that hybridize to the 1.8 kb A2 allele probe has extended the restriction map approximately 5 kb upstream of the previous maps. The fact that these maps are identical over this 22 kb region strongly suggests that this section of the amplified unit containing A2 and B2 alleles (amplicon) is conserved within as well as between strains. This is also the case with the amplified B1 allele (the first example of esterase gene amplification in *C. pipiens*) where the amplified DNA fragment has a conserved core of 25 kb (Mouchès *et al.*, 1990). These results provide additional evidence to support the hypothesis that the A2-B2 allele amplification has spread worldwide by migration (Raymond *et al.*, 1991).

A polymorphism was found in the region between the A2 and B2 alleles (*Xba*I digests) in the strains SOFT (from Egypt) and NAIROBI (from Kenya) which were collected in the mid-1980s by two separate groups of workers. This mutation was not found in the other African strains SAYADA (collected 1990), BOUAKE (collected 1986) or BED (collected 1991). The *Xba*I polymorphism was present in every amplicon copy in SOFT and NAIROBI since there were no faint bands of the same length as those found in *Xba*I digests of the other strains. The fact that all amplicons share this mutation strongly suggests that a mechanism for concerted evolution such as gene conversion or unequal crossing-over (reviewed by Li, 1997) is taking place in these strains. These results support the hypothesis that the amplified esterase genes in these two strains have a very recent common ancestor. On a world-wide scale these strains were collected from areas that are geographically close. Although SAYADA (Tunisia) was geographically closer to SOFT (Egypt), the two strains with the mutation were linked by the River Nile, which may have facilitated migration. In France, *Culex* strains with the A2-B2 amplification were shown to migrate up the Rhone river (Rivet *et al.*, 1993). Whether

Table 1. Esterase A2 gene copy numbers in *C. pipiens* strains. Amplification levels are estimates in comparison to a single copy number in the susceptible strain SLab.

Strain	Amplification level	Origin	Date collected	Reference
SELAX	40	California	1984	Raymond *et al.*, 1987
BOUAKE	26	Ivory Coast	1986	Magnin *et al.*, 1988
BED	11	Johannesburg	1991	Guillemaud *et al.*, 1996
THAI	3	Bangok	1991	Guillemaud *et al.*, 1996
MOOREA	17	French Polynesia	1991	Pasteur *et al.*, 1995
LAHORE	19	Pakistan	1985	Beyssat-Arnaouty, 1989
SAYADA	22	Tunisia	1990	Cheikh & Pasteur, 1993
MEKONG	14	Vietnam	1990	This study
SOFT	–	Egypt	1986	Beyssat-Arnaouty *et al.*, 1989
NOUA	18	Mauritania	1992	This study
NAIROBI	14	Kenya	1985	This study
TRINITE	19	Martinique	1990	Yebakima *et al.*, 1995

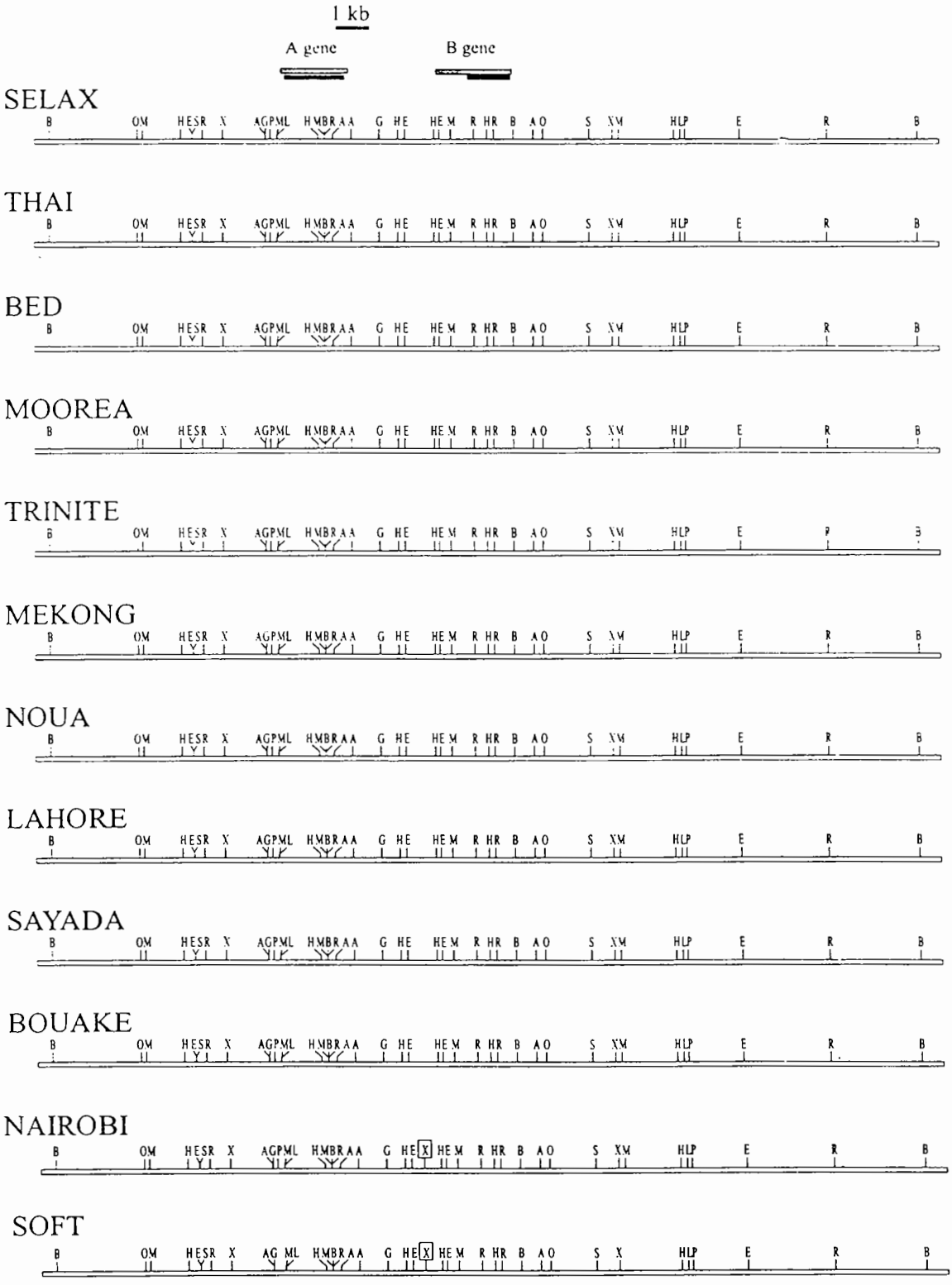

Figure 1. Restriction maps of the A2-B2 esterase amplicon in twelve strains of *C. pipiens*. The maps were constructed using A2 and B1 gene fragments as DNA probes (the black lines below the genes). The boxed restriction site represents an *Xba*I additional site. The letters represent the following restriction enzymes: (A) *Acc*I, (B) *Bam*HI, (G) *Bgl*II, (E) *Eco*RI, (R) *Eco*RV, (H) *Hind*III, (M) *Mun*I, (P) *Pst*I, (S) *Sac*I, (L) *Sal*I, (X) *Xba*I, and (O) *Xho*I.

or not a similar migration took place between Nairobi and Egypt is difficult to establish. Although mosquitoes can migrate up to 1 km a day, it seems unlikely that migration by flight alone would account for this without the help of passive transport as in the case of Polynesian mosquitoes that have been shown to migrate extensively by aircraft between islands (Pasteur *et al.*, 1995). This polymorphism has not migrated as far as South Africa, since the 1991 collection had the 'normal' *Xba*I band.

Levels of amplification

Amplification levels in eleven of the twelve A2-B2 strains were calculated by comparison to those in a standard laboratory OP-susceptible strain SLAB without esterase gene amplification (Georghiou *et al.*, 1966; Guillemaud *et al.*, 1997) (Table 1). The analysis of gene amplification levels showed that the amplification level is not stable in the field with levels ranging from 3- to 22-fold increasing to 40-fold following laboratory selection, some values being distant by more than 2 standard units, indicating significant differences. In order to see whether there had been a general change in the level of amplification in the field over the past 20 years, differences in the mean amplification level in the 1980s including 1990 (21.97) and after 1990 (12.03) was tested using a Mann-Whitney test ($U=4$, $n1=4$, $n2=6$, $P=0.058791$). This demonstrated a downward trend which was marginally non-significant. However, since the first group included the laboratory-selected strains, it may suggest that in fact there is no significant difference in amplification levels between these two groups. This is perhaps not surprising, since, although the populations existed at around the same time, they were probably exposed to different selection pressures under different control programmes. Therefore we would not necessarily expect there to be any correlation between amplification level and decade. However, what the results do demonstrate is that there must be an independent mechanism operating in the field, such as unequal crossing-over, that has caused fluctuations in amplicon copy number, subsequent to the intial mutation that caused the amplification. Selection by OPs and fitness costs may increase or decrease the frequency of individuals in a population with high amplicon copy numbers. However, at least one of the older strains (SELAX) was selected with OPs over a number of generations during the past 10 years of its maintenance in the laboratory and therefore may not represent the situation in the field in the 1980s.

Up until now, resistant strains with amplified esterases have often been characterized by levels of resistance (e.g. by their LC50) and by the level of amplification (Poirié *et al.*, 1992; Guillemaud *et al.*, 1997) as though these characteristics were constant over time. The resistance gene alleles themselves have also been so characterized, for example, the B1 gene amplification was often cited in the literature as having 250 copies of the amplicon (Mouchès *et al.*, 1990; Fournier *et al.*, 1987) without reference to a particular strain or to the date the strain was analysed. In the same way, the A5-B5 and A2-B2 alleles were quoted as though their presence always gave a particular resistance ratio (Raymond & Marquine, 1994; Rivet *et al.*, 1993). Clearly there are differences of around 10-fold in amplification levels between strains with the A2-B2 haplotype which could translate into differences in resistance. Caution should be used in assuming the resistance status of a strain based solely on the presence of any particular haplotype. The amplification of the B1 allele in the TEM-R strain has reduced from 250 copies down to twenty copies during a period of 10 years without selection (Guillemaud *et al.*, 1997). However, it is possible that this decrease is attributable to an improvement in the techniques used to estimated copy number.

This work is the first study to measure the variability in esterase gene amplification levels in *C. pipiens* strains homozygous for the same amplified loci. The fact that variation exists gives some insight into the dynamics of the amplification phenomenom. There is strong evidence to show that in *C. pipiens* amplified esterases are integrated into the chromosome (Nancé *et al.*, 1990). Therefore variations in copy number are not due to the loss or gain of extrachromosomal elements as is often found with amplified DNA in cell lines (Schmike, 1984; Ford & Fried, 1988). In *Myzus persicae*, amplified esterase gene copy number can also vary. There is evidence to show that fluctuations in the copy number of this gene which is integrated into the chromosome are related to unequal sister chromatid exchange (Field & Devonshire, 1997).

Despite the differences in amplification level between the strains, the RFLP data were identical. Therefore loss or gain in copy number does not appear to affect the core sequence. If the amplification level was altered by a mechanism of unequal crossing-over within the detectable 22 kb core sequence, we may expect to find additional faint bands of lower level amplification that represent novel combinations. A few additional bands of low level amplification were detected in some of the digests, but in all cases they could have been caused by partial digestions, especially since they were more often found in overloaded gels. Therefore, if unequal crossing over is responsible for fluctuations in gene copy number, it is either occurring outside of the 22 kb core or, alternatively, the cross-over point is identical on each chromatid, i.e. at

exactly the same basepair. It is currently not possible to choose between these two hypotheses.

The results from this study prove that the co-amplification of the A2 and B2 alleles is widespread. This increases the likelihood that the original mutation that caused the amplification of these two alleles was a single event. The homogeneity of the *Xba*I polymorphism in the SOFT and NAIROBI strains (the latter having fourteen copies) suggests concerted evolution is occurring either by gene conversion or unequal crossing over. The latter is probably the more likely, bearing in mind the differences in esterase gene copy numbers. These two strains must either share a common ancestor, or are derived one from the other and have most probably migrated along the River Nile. Thus polymorphisms in the A2-B2 amplicon have begun to help us to reconstruct migration routes of the mutation in *C. pipiens* mosquitoes.

Experimental procedures

Strains

The strains used in this chapter are described in Table 1. The strains were collected from the field and selected in the laboratory for homozygosity for the presence of the highly active A2-B2 esterases, frozen as adults and stored in liquid nitrogen. SELAX, BOUAKE and NAIROBI were all reared in the laboratory for several years following selection with insecticides. Adults of these strains were frozen and stored in liquid nitrogen as above.

DNA extraction and RFLP analysis

Genomic DNA was extracted from whole adult mosquitoes using the method of Raymond *et al*. (1989). DNA was digested with one or two restriction enzymes (*Acc*I, *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Mun*I, *Pst*I, *Sac*I, *Sal*I, *Xba*I and *Xho*I). The DNA fragments were separated by agarose gel electrophoresis, blotted onto nylon filters (HybondN+) after the method of Southern (1975) and visualized by hybridization to ^{32}P-labelled 1.8 kb A2 or 1.3 kb B1 probes (Rooker *et al*., 1996; Mouches *et al*., 1986). Filters were washed at high stringency.

Dot blotting

Genomic DNA was extracted as above and treated with RNAse. DNA was quantified using GenQuant RNA/DNA Calculator (Pharmacia). Gene amplification levels were calculated using a dot blot method modified from Guillemaud *et al*. (1997). 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. 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 (Ace) known to hybridize with only one locus in *C. pipiens* (Malcolm *et al*., 1998). The quantity of genomic DNA hybridizing with radioactive probes was estimated using a β-imager analysis (Phosphoimager 445 SI, Molecular Dynamics). For each locus hybridized, each strain (i) was characterized by the slopes S (i) of the relationship between radioactive signal and DNA dilution. The ration S(i)/S(SLab) is an estimate of amplification level of the locus considered for strain i. However, for the Ace probe, the ratios S(i)/S(SLab) were different due to small differences in DNA concentration. Therefore to take this into account, the true amplification level was obtained by computing [S(i)/S(SLab)]A2 probe/ [S(i)/S(SLab)] Ace probe.

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