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IDENTICAL RESTRICTION ENZYME MAPS OF THE AMPLICON CONTAINING THE Estα2[1] and Estβ2[1] INSECTICIDE RESISTANCE GENES IN *CULEX QUINQUEFASCIATUS* COLONIES FROM SAUDI ARABIA, TANZANIA AND SRI LANKA

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Abstract: The esterase Estα2[1], involved in insecticide resistance, has recently been cloned from Sri Lankan *Culex quinquefasciatus*. The Estα2[1] and Estβ2[1] genes are co-amplified on the same stretch of DNA (amplicon). Restriction fragment length polymorphism analysis of the esterases of several *Culex* strains with a range of enzymes showed an invariant restriction fragment map between strains. The results are not at variance with the hypothesis that amplification of Estα2[1] and Estβ2[1] occurred only once and then spread worldwide.

Key words: Amplicon, carboxylesterases, *Culex quinquefasciatus*, gene amplification, insecticide resistance, mosquitoes, restriction enzyme maps.

INTRODUCTION

The mosquito *Culex quinquefasciatus* is a major biting nuisance worldwide and the vector of filariasis in Sri Lanka and other tropical countries. Control of this mosquito is mainly through use of insecticides. Resistance of these mosquitoes to organophosphorous insecticides is due to elevated carboxylesterase activity.[1,2] The underlying molecular mechanism of this elevation is gene amplification.[3,4] Classification of these esterases is according to their preference for the substrates α- and β- naphthyl acetate and to their mobility in native gel electrophoresis (they are numbered in the order of increasing mobility). A superscript is given at the end, once the gene is fully characterised.[4] Sri Lankan *C. quinquefasciatus* populations have elevated Estα2[1] and Estβ2[1] as the major mechanism for organophosphorous insecticide resistance.[5] These two esterase genes occur in the same amplicon in complete linkage disequilibrium.[6]

Estα2[1] and Estβ2[1] have a worldwide distribution. According to the hypothesis put forward by Raymond *et al.*,[7] amplification of Estα2[1] and Estβ2[1] esterases has arisen only once and through a recent migration spread worldwide. However, Estα2[1] and Estβ2[1] carboxylesterases purified from *C. quinquefasciatus* populations

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of Sri Lanka, Tanzania and Saudi Arabia, showed significant differences in their kinetics of interaction with insecticides.[8,9] The elevated isoenzymes of Estβ1, which are also present in *C. quinquefasciatus* and have a worldwide distribution, have been purified from Colombian and Trinidad populations and have shown significant differences in their kinetics of insecticide interaction.[10] Differences at the DNA level have been observed for Estβ1 alleles suggesting a high mutation rate rather than migration is responsible for the occurrence of amplified Estβ1 alleles worldwide.[3] The present work was carried out to analyse Estα2[1] and Estβ2[1] genes at the DNA level, using the same populations for which the kinetic differences were recorded. Digestions carried out with several restriction enzymes allowed us to construct restriction maps for these genes.

METHODS AND MATERIALS

Mosquito strains

Both PelRR and PelSS colonies were derived from a parental colony, established from *C. quinquefasciatus* mosquitoes collected from Peliyagoda, Sri Lanka in 1984. Fenthion treatment had been carried out in the area every two weeks in the *C. quinquefasciatus* larval breeding sites. The organo-phosphate resistant PelRR strain was selected from this colony with temephos. The susceptible PelSS colony was obtained by single family selection for low esterase activity.[11] The strains Dar91, Tanga85 and Muheza were respectively collected from Dar es Salaam, Tanga and Muheza in Tanzania. The Dar91 strain originated from a resistant population selected in the field by chlorpyrifos followed by fenitrothion and colonized in 1991. The Tanga85 strain originated from a chlorpyrifos resistant field population colonized in 1985 and Muheza was collected in 1987. Both these strains were maintained under intermittent chlorpyrifos selection after colonization. SPerm was collected from Jeddah, Saudi Arabia in 1989 where permethrin had been extensively used for more than four years. On colonization, it had a low level of pyrethroid resistance and organophosphate resistance. The levels of these were increased by selection for 20 generations with permethrin and subsequently intermittent selection with malathion and temephos in the laboratory.[12]

Isolation of genomic DNA

The method used was an adaptation of Miller *et al.*[13] About 1g of fourth-instar larvae were ground in liquid N_2. The homogenate was added to 10 vol. of extraction buffer [10 mM Tris/HCl (pH 8.0)/ 0.1 M EDTA/ 0.5% (w/v) SDS/ 20μg/ml pancreatic RNAase]. After incubation for one hour at 37°C, proteinase K was added to a final concentration of 100 μg/ml and the homogenate was incubated at 50°C for three hours. After cooling on ice for 10 min, 0.35 vol. of saturated NaCl was added to

precipitate protein. The homogenate was mixed well and stored on ice for a further 5 min, then centrifuged at 16000 g for 20 min. The supernatant was removed and the DNA precipitated by adding an equal volume of propane-2-ol. The DNA was resuspended in 7.5 ml of 10 mM Tris/HCl (pH 8.0)/ 1 mM EDTA (pH 8.0) (TE) containing 20 µg/ml RNAase and incubated at 37°C for 1 h, then extracted with phenol, phenol/chloroform and finally chloroform. After precipitation with ethanol, the DNA was resuspended in a small volume of TE and stored at 4°C until used for Southern blotting.

Analysis of restriction fragments

PelRR Est$\alpha 2^1$ and Est$\beta 2^1$ esterase cDNA fragments[3,4] were used as probes to detect the haplotype of each esterase. Genomic DNA (10 µg) was digested to completion with *Eco*RI, *Hind*III, *Bam*HI, *Hinc*II, *Eco*RV and *Sal*I in single or double digestions and separated on 0.8% (w/v) agarose gels. The DNA was transferred to charged nylon membranes (Amersham) and hybridized with ^{32}P-labelled probe (specific activity > 2 x 10^6 cpm/µg) at 65°C for 16 h in hybridization buffer (5x Denhardt's solution, 6 x SSC, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, 5% (w/v) polyethylene glycol 8000, 100 µg/ml boiled sheared herring sperm DNA). The final membrane washes were at 65°C in 0.1 x SSC and 0.1% (w/v) SDS for 20 min. Membranes were probed first with the Est$\beta 2^1$ cDNA then stripped and probed with Est$\alpha 2^1$ cDNA. The end of the Est$\beta 2^1$ gene was marked by a Dra 1 site which was characterized by subsequent cloning work.

RESULTS

The restriction maps of the Est$\alpha 2^1$/Est$\beta 2^1$ amplicons for a Sri Lankan (PelRR), three Tanzanian (Dar91, Taga 85 and Muheza) and a Saudi Arabian (SPerm) were produced using a range of different restriction enzymes. DNA prepared by the above method, gave a high quality clean preparation which cut well with the full range of restriction enzymes used. The restriction maps were prepared using the fragment analyser function of DNAstar. The results in Figure 1 show the PelRR restriction map. Maps for all the other resistant strains had identical restriction digest patterns. In contrast, the restriction digest pattern for the insecticide susceptible PelSS strain is distinct from that of the resistant strains (Figure 1). The esterases in the PelSS strain are unamplified, but represent alleles of the same genes in an identical head to head arrangement as those present on the resistance associated amplicon. In the non-amplified esterase, the distance between the two genes (1.7 kb) is slightly less than in the amplicon, where the genes are 2.6 kb apart. The close association of the two genes, allowed the restriction fragment analyses of the two esterases and the intergenic spacer between them to be combined into a single restriction map (Figure 1).

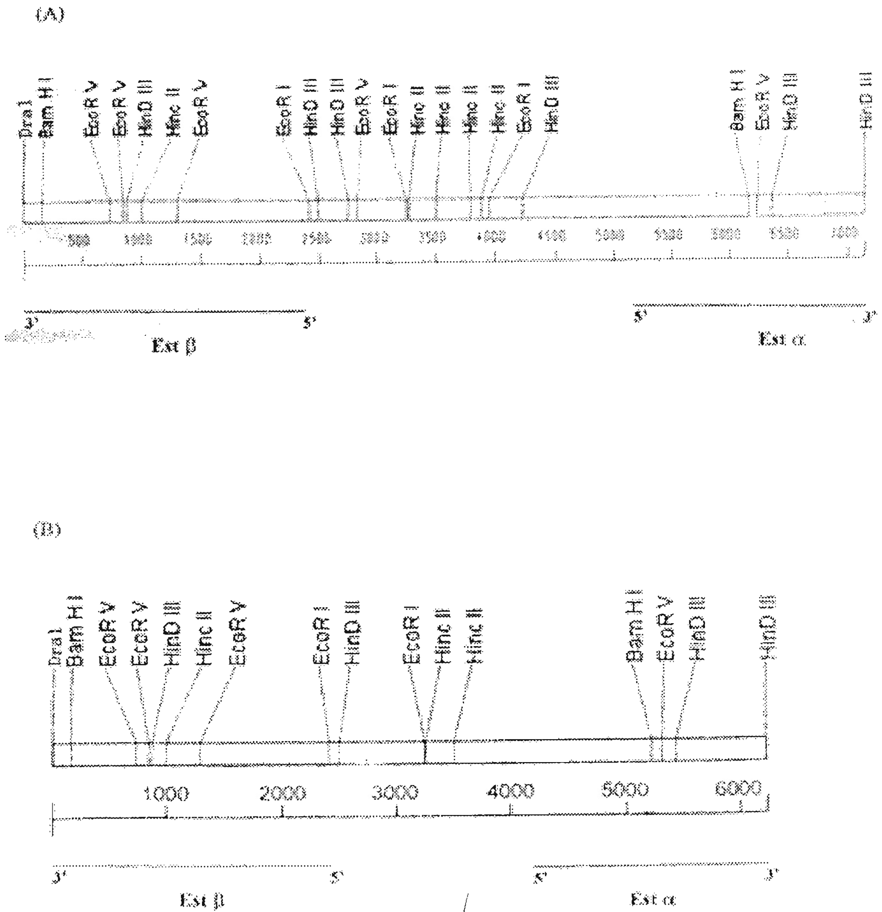

Figure 1: The Pel RR *Culex quinquefasciatus* Estα2ʹ/Estβ2ʹ amplicon restriction retriction map (A) compared to that of the esterases in the non-amplified SS strain (B).

DISCUSSION

A partial cDNA of Estβ1[1] was isolated from the TEMR Californian strain of *C. quinquefasciatus*, and this was used as a probe to detect gene copy number of Estβ1 in different strains. Adults of the resistant TEMR strain with Estβ1[1] had up to 250 times more copies of the gene than the adults of the susceptible strain.[14] Restriction fragment length polymorphism patterns (RFLP), carried out with the same probe, have shown that the structural genes of electrophoretically dissimilar β-naphthyl acetate specific esterases were similar but not identical, whereas their flanking regions varied considerably. However, the flanking sequences of Estβ2 from different geographical locations (Africa, Asia and North America) were identical. On the basis

of these observations, Raymond *et al* [7] proposed the hypothesis that amplification of the Estβ2[1] allele has occurred only once and spread worldwide by migration. It is now known that both Estβ2[1] and Estα2[1] sit on the same amplification unit.[6] Therefore the event causing Estα2[1] amplification must have occurred and spread concurrently with Estβ2[1]. This hypothesis does not, however, account for the different resistance patterns observed in the *Culex* strains which contain only this resistance mechanism. [11,15,16,17] If organophosphates have been the main selecting agents for the Estα2[1]/Estβ2[1] esterase amplification worldwide, this selection has only been operative over the last fifty years at most, and in the majority of countries the time scale would be much shorter than this. Thus, the amplification of the Estα2[1]/ Estβ2[1] unit and its subsequent selection and migration have to be considered as a recent event. This is an extremely short time scale for these amplified genes to spread worldwide. In contrast to the molecular data, which suggest a single origin for resistance, the Estα2[1] and Estβ2[1] esterases purified from five strains which originated in different geographical areas of the world (two of the strains were collected from identical sites to those used in Raymond's study) were kinetically different from each other.[9] Allelic variations have been observed for Estβ1, another important amplified esterase gene of geographically different populations.[3] Using purified enzymes, variation of Estβ1 isozymes has also been shown at the protein level.[10] When considering all these factors, it is curious that the restriction maps of the amplified Estα2[1] and Estβ2[1] genes and the intergenic spacers are identical.

RFLP analysis of present study was carried out using Estα2[1] and Estβ2[1] probes. The results show that Estα2[1] and Estβ2[1] genes found in all five strains are identical on RFLP analysis. This strongly favours the concept that the alleles of Estα2[1] and Estβ2[1] found in *C. quinquefasciatus* populations from different continents are identical. This does not directly agree with the kinetic differences observed at the protein level for these esterases. However, kinetic differences among Estα2[1]s and among Estβ2[1]s are much smaller[8] than among Estβ[1]s which show variations at the DNA level.[3,10] In human serum paraoxonases, kinetic differences between two variants occur due to a single amino acid substitution at a position which is not near the active site.[10] Perhaps the restriction enzymes used have not identified minor mutational changes present among the Estα2[1] and Estβ2[1] genes. It is also possible that the post-transcriptional changes of the same gene product lead to the formation of different forms at the protein level. Sex-, tissue- and age- specific regulation of the genes of esterase 6 and esterase S in *Drosophila* have been studied.[19-21] Extensive polymorphism of the gene product after mRNA processing and subsequent post-translational modifications has been reported for AChE .[22,23] Post-translational proteolytic processing and core-glycosylation have also been studied in rat liver esterases .[24-26]

In contrast to the situation of Estβ1, the present data suggest that numerous different amplicons are not responsible for the occurrence of amplified Estα2[1] and Estβ2[1] alleles worldwide. The frequent appearance of resistant amplified esterase

phenotypes has been observed near international ports suggesting the importance of migration in their spread.[27] Migration must have undoubtedly played an important role in the spread of the amplified resistant genes in the presence of the positive selection pressure of the insecticides. But the whole process may not be as simple as Raymond *et al.* suggested. Mutations and amplifications of the gene loci 'α' and 'β' have occurred on several occasions over a long period of time resulting in different forms of amplified 'α' and 'β' alleles. Clearly the Estα2^1/Estβ2^1 amplicon predominates in *C. quinquefasciatus* populations worldwide, but it is not clear why this should have a selective advantage over the various insect isolates containing different Est α3/Estβ1 amplicons. As these are the only esterase loci which have been amplified numerous times, despite various esterases with the ability to bind pesticides being present in *Culex*, it is likely that there is something in the region of these genes within the genome which predisposes it to amplification.

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