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Esterases A5-B5 in organophosphate-resistant *Culex pipiens* from Italy

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Abstract. *Culex pipiens* mosquitoes from Lignano city, Udine province, northeast Italy, were found to carry over-produced non-specific esterases A1, A2-B2 and A4-B4 or A5-B5, detected by starch gel electrophoresis, giving multiple resistance to organophosphorus insecticides. In order to differentiate between A4-B4 and A5-B5 esterases, the latter known only from Cyprus whereas the former is widespread in Italy and elsewhere, restriction fragment length polymorphism (RFLP) analysis was performed at the esterase B locus. Both B4 and B5 haplotypes were found. This is the first record of A5-B5 esterase-mediated resistance in continental Europe.

Key words. *Culex pipiens*, chlorpyrifos, temephos, organophosphate resistance, insecticide resistance, RFLP analysis, esterases, Italy.

Introduction

The evolution of insecticide resistance genes in the mosquitoes of the *Culex pipiens* L. complex has been the subject of many studies by different workers in various geographic areas (see review by Pasteur & Raymond, 1996). World-wide surveys of resistance to organophosphorus (OP) insecticides have disclosed only three loci that have developed major resistance alleles (Villani *et al.*, 1983, 1987; Poirié *et al.*, 1992; Georghiou, 1992; Pasteur & Raymond, 1996). Two loci, known as *Est-2* (or *esterase B*) and *Est-3* (or *esterase A*) that code for detoxifying carboxylester hydrolases, have resistant alleles giving esterase over-production (Mouchès *et al.*, 1987; Fournier *et al.*, 1987; Poirié *et al.*, 1992). So far, five distinct electromorphs have been described at the *esterase B* locus and three at the *esterase A* locus (Poirié *et al.*, 1992; Georghiou, 1992; Pasteur & Raymond, 1996). The over-production of esterase is the result of gene amplification (Mouchès *et al.*, 1986; Poirié *et al.*, 1992; Vaughan *et al.*, 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 (close genetic linkage) of some electromorphs, e.g. A2 and B2 (Rooker *et al.*, 1996). The third locus, *Ace*, codes the acetylcholinesterase (insecticide target); insensitive *Ace* alleles have been reported in various localities, e.g. Cuba (Bisset *et al.*, 1991), Italy (Bonning *et al.*, 1991; Villani & Hemingway, 1987) and Portugal (Bourguet *et al.*, 1996), but it is not known if multiple resistant *Ace* alleles occur.

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Some over-produced esterase alleles seem to migrate easily through mosquito populations, becoming geographically widespread in both *Cx pipiens* and *Cx quinquefasciatus* Say, a superspecies pair of allopatric sister species (White, 1979). The best example is probably the associated A2 and B2 (A2-B2) esterases found on four continents, apparently spread intercontinentally via stowaway mosquitoes on aircraft (Curtis & White, 1984) and followed during local invasions (Rivet *et al.*, 1993). Other esterases, e.g. A5-B5 previously described only from Cyprus (Poirié *et al.*, 1992), have a more restricted distribution.

In Italy, A1, A2-B2 and A4-B4 esterases have been reported from various field populations (Villani *et al.*, 1982; Bonning *et al.*, 1991; Marchi & Addis, 1990; Severini *et al.*, 1994), although A2-B2 has probably disappeared from the Lucca area (Severini *et al.*, 1993) where it was first described by Villani *et al.* (1986). This article reports findings in Lignano Sabbiadoro area of northeast Italy, near the Adriatic coast, where *Cx pipiens* field populations showed unusually high resistance to the OP compound temephos in 1989–90 (Zamburlini & Bellantone, 1993). The same location was sampled in 1995 and, from the investigations described below, we attributed organophosphate-resistance to four different factors: A1, A2-B2, A4-B4 and A5-B5 esterases, the last being found for the first time in *Cx pipiens* from continental Europe.

Materials and Methods

Culex pipiens samples were collected during summer 1995 in the sewage station of Lignano city, Udine Province, Friuli-Venezia

Fig. 1. Study localities for *Culex pipiens* in northeastern Italy.

Giulia Region, northeastern Italy (Fig.1). Mosquito control activity is intensive in this area so as not to discourage tourism. Larvicidal applications of OP insecticides are generally alternated with the growth inhibitor diflubenzuron. From collections of *c* 1000 larvae, late third and early fourth instars were used for bioassays according to the W.H.O. (1981) method, whereas the rest were reared to adults which were stored in liquid nitrogen when at least 3 days old.

Batches of twenty larvae were exposed to different OP concentrations of temephos or chlorpyrifos. A long-established laboratory strain ISS (Villani *et al.*, 1982), mantained at Istituto Superiore di Sanità since 1970, was used as the susceptible standard. Mortality was recorded after 24 h exposure, corrected by Abbot's formula, and LD_{50}s were calculated by a computer-assisted program, designed by the technical service of the Istituto Superiore di Sanità.

Over-produced esterases in individual mosquitoes were first identified by means of starch or acrylamide gel electrophoresis, as previously described (Severini *et al.*, 1993). For esterase characterization, the reference strains were Barriol (A1 esterase), SeLax (A2-B2 esterases), VIM (A4-B4 esterases) and Cyprus (A5-B5 esterases), cf. Poirié *et al.* (1992) and Raymond & Marquine (1994). Genotypes of acetylcholinesterase were determined using the TEP test (based on the carbamate propoxur) described by Raymond & Marquine (1994). Genotypes of acetylcholinesterase were determined using the TEP test described by Raymond & Marquine (1994).

DNA identification of esterase genes in single mosquitoes was performed by RFLP as described by Severini *et al.* (1994). The restriction enzymes *Eco*R1, *Bam*H1 and *Bgl*II were used to discriminate esterase A or esterase B haplotypes (Poirié *et al.*, 1992; Guillemaud *et al.*, 1996). The esterase B probe was either the 1.7 kb cDNA fragment or the 2.1 kb genomic fragment obtained with primers designed from the B1 sequence of Mouchès *et al.* (1986). The esterase A probe was the 1.8 kb fragment obtained with primers described in Rooker *et al.* (1996). Mosquitoes with known RFLP pattern were run in each gel as control: strain VIM for A4 and B4 haplotypes, Cyprus for A5 and B5, SeLax for A2 and B2 and Barriol of A1 haplotype (Raymond *et al.*, 1991; Poirié *et al.*, 1992; Rooker *et al.*, 1996; Guillemaud *et al.*, unpubl. data).

Results and Discussion

Culex pipiens larvae from Lignano were found to be highly resistant to chlorpyrifos (203-fold) and temephos (67-fold) (Table 1). The temephos LC_{50} is not significantly different from the value reported from the same area in 1989–90 by Zamburlini & Bellantone (1993), suggesting that the OP resistance observed has stabilized for at least 5–6 years. This high resistance level cannot be readily explained by specific OP resistance genes usually found in European *Cx pipiens* populations, most of which provide only low resistance to temephos (see Table 3 of Severini *et al.*, 1993).

Determination of *Ace* genotypes disclosed the presence of an insensitive acetylcholinesterase in the Lignano population, but its frequency (3/30) was low, which may be explained by relative unfitness in the absence of strong selection pressure (Bonning & Hemingway, 1991). The *Ace* resistance mechanism is therefore unlikely to account for the high resistance observed in *Cx pipiens* at Lignano, unless a novel allele confers high resistance to propoxur used in the TEP test.

Gel electrophoresis revealed that the commonest over-produced esterases were A4-B4 or A5-B5 in about 90% (54/60) of the

Table 1. Larval mortality rates and resistance ratios (RR) of *Culex pipiens* from Lignano with 24 h exposure to chlorpyrifos or temephos, compared to the susceptible reference strain, ISS.

Insecticide/sample	LD_{50} mg/l × 10^{-3}	Slope	χ^2	DF	RR
Temephos					
Lignano Sabbiadoro	30.5	4.55	1.001	2	203
ISS	0.15	1.78	0.286	2	—
Chlorpyrifos					
Lignano Sabbiadoro	28.9	2.46	0.676	1	67
ISS	0.43	1.77	0.335	2	—

Table 2. Over-produced esterases in *Culex pipiens* from Lignano, detected by RFLP or starch gel electrophoresis. *n* = Sample size (some specimens displayed multiple electromorphs).

Electrophoresis				RFLP			
n	A1	A4-B4 or A5-B5	A2-B2	*n*	A4-B4	A5-B5	A2-B2
60	9	54	2	57	4	29	2

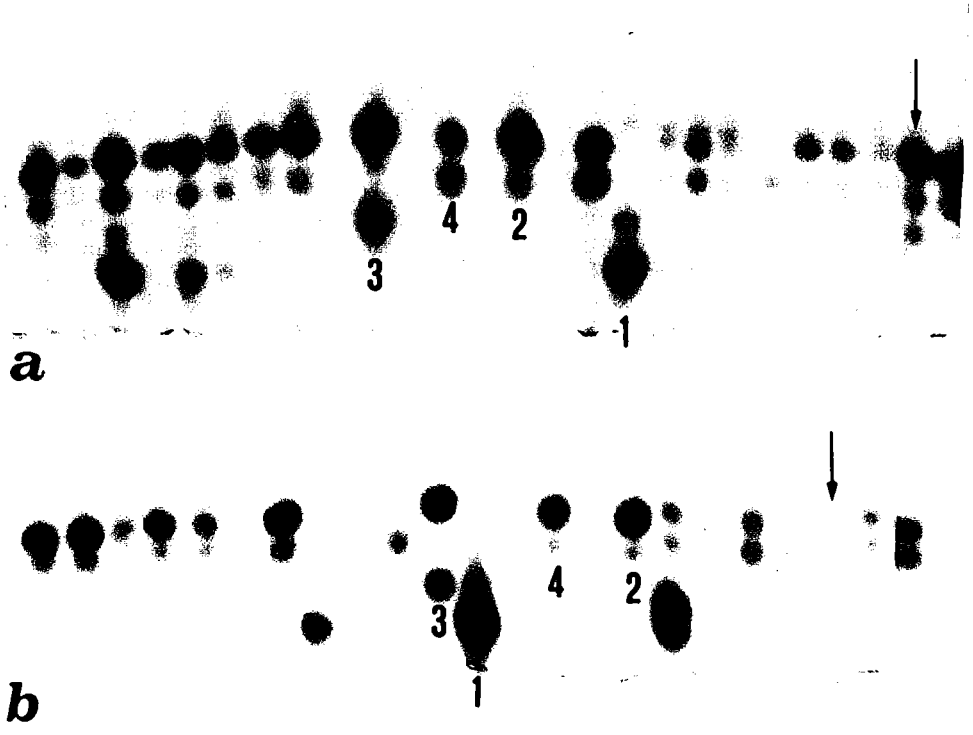

Fig. 2. Starch gel electrophoresis of nonspecific esterases in *Culex pipiens* from Lignano Sabbiadoro. a = A4-B4 or A5-B5 and A2-B2 (arrow); b = A4-B4 or A5-B5 and C (arrow). Reference strains: 1 = Barriol (A1); 2 = Cyprus (A5-B5); 3 = SeLax (A2-B2); 4 = VIM (A4-B4).

mosquitoes analysed among our Lignano samples (Table 2). However, A4-B4 cannot be distinguished from A5-B5 electrophoretically (Poiré *et al.*, 1992). Two mosquitoes displayed an electrophoretic pattern consistent with the presence of both A2-B2 and A4-B4 or A5-B5 (Fig. 2a), and one specimen had an highly active esterase C (Fig. 2b), probably similar to the esterase C1 reported from Corsica in 1988 (Raymond & Marquine, 1994). A1 esterase was found only in nine specimens, indicating a minor role of this esterase in the resistance. The distinction between A4-B4 and A5-B5 is important because they provide very different levels of OP resistance (Poirié *et al.*, 1992). Only RFLP can unambiguously identify the A or B haplotype of these esterases (Poirié *et al.*, 1992; Rooker *et al.*, 1996). Using *Eco*R1, *Bam*H1 or *Bgl*II restriction enzymes for RFLP analysis (Fig. 3), the amplified haplotype of A5-B5 was identified in 51% (29/57) and A4-B4 in 7% (4/57) of mosquitoes from Lignano. Two mosquitoes showed the A2-B2 amplified haplotype.

The arrival of A5-B5 in Italian *Cx pipiens* is probably recent, since A5-B5 was not detected in 1993 at Padova, which is only 120km from Lignano, nor in *Cx pipiens* from other parts of Italy (Severini *et al.*, 1994). The presence of A5-B5 esterases in about half the *Cx pipiens* larvae probably explains their high degree of OP resistance in the Lignano area. By analogy with the low level of OP resistance caused by A2-B2 esterases at Lucca (Villani *et al.*, 1987), the presence of A2-B2 in Lignano *Cx pipiens* apparently provides less OP resistance than A5-B5.

This first record of A5-B5 amplified haplotype in *Cx pipiens* from outside Cyprus, *c* 2000km to the southeast, indicates that

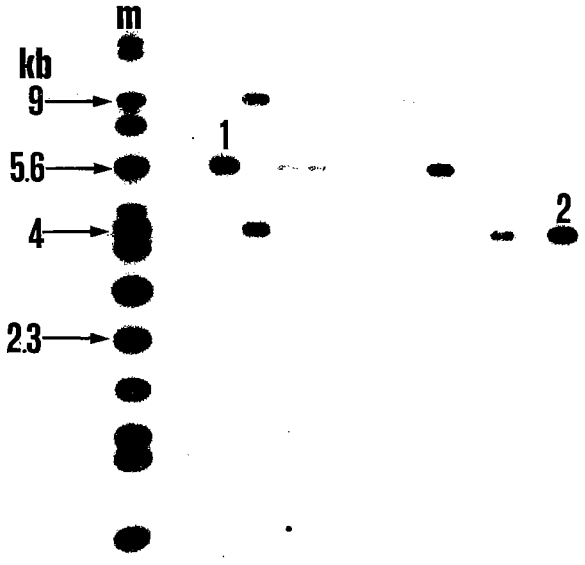

Fig. 3. Southern blot of genomic DNA from single adult *Culex pipiens* mosquitoes from Lignano Sabbiadoro. m = marker, Raoul I (Appligene Inc.); Reference strains: 1 = Cyprus (B5); 2 = VIM (B4).

these esterases may be migrating around the Mediterranean. Alternatively A5-B5 could have arisen *de novo* in *Cx pipiens* by homologous but independent local mutation at Lignano – but, as this has not occurred elsewhere, it seems more parsimonious to assume importation from Cyprus. If so, this resembles the geographic spread of other amplified esterases, such as A2-B2 which are now distributed almost worldwide (Raymond *et al.*, 1991; Rivet *et al.*, 1993), or B1 in America and Asia (Qiao & Raymond, 1995). It is not known why these over-produced esterases migrate so extensively whereas others (A1, A4-B4 or A5-B5) seem to have a more restricted distribution in the Mediterranean area. The limited occurrence of A5-B5 in Italy currently provides a good opportunity to document and study the processes involved in such genetic invasions, and to better understand the migration and interaction of resistant alleles in *Culex pipiens* mosquito populations.

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