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Insect Biochemistry and Molecular Biology 41 (2011) 29–35

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

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ARTICLE INFO

Article history:
Received 3 July 2010
Received in revised form
14 September 2010
Accepted 17 September 2010

Keywords:
Insecticide resistance
ace-1 duplication
Culex pipiens

ABSTRACT

The status of genes conferring resistance to organophosphate and carbamate insecticides has been examined in *Culex pipiens pipiens* mosquitoes sampled in Algeria. Presence of overproduced esterases was sporadic, but acetylcholinesterase-1 resistant alleles were observed in almost all samples. We focused our study on the AChE1 G119S substitution characterized in almost all samples, mostly at the heterozygous state. A genetic test revealed the presence of *ace-1* duplication associating a susceptible and a resistant *ace-1* copy. Molecular characterization showed a high occurrence of *ace-1* duplication with six distinct duplicated alleles out of four samples. The inferred frequency of duplicated allele suggests that it is replacing the single resistant G119S allele. Finally, we discuss the mechanism at the origin of these duplicated haplotypes and their consequences on the management of insecticide resistance.

1. Introduction

A new adaptive mutation is often associated with negative side effects that may be reduced with time by the selection of compensatory mutations (or modifiers; Fisher, 1958) or by the replacement with less deleterious alleles (Haldane, 1932). Such evolutionary processes have been documented in two studies related to insecticide resistance. The first example is the resistance to diazinon in the blowfly *Lucilia cuprina* (McKenzie and Game, 1987; McKenzie, 1993) and the other is the resistance to organophosphate (OP) and carbamate (CX) insecticides in *Culex pipiens* mosquitoes (Guillemaud et al., 1998).

Due to mosquito control with OP in Southern France, two main resistance mechanisms have been selected in *C. pipiens pipiens* and the polymorphism of the genes involved has been regularly monitored over the last 40 years (reviewed in Raymond et al., 2001; Weill et al., 2005; Labbé et al., 2007b). One resistance mechanism involves the super-locus *Ester*, which codes detoxifying carboxylesterases. Resistant mosquitoes overproduce these enzymes, either through up-regulation or through gene amplification, and several *Ester* alleles have been selected over time, each conferring a different resistance ratio to OP and being associated with a specific fitness cost (Guillemaud et al., 1998; Raymond et al., 2001; Berticat et al., 2002a,b, 2004; Duron et al., 2006; Labbé et al., 2009). Evolution at the *Ester* locus was shown to proceed by allele replacement (Guillemaud et al., 1998; Labbé et al., 2009). The other resistance mechanism involves a modification of the gene *ace-1* coding for the acetylcholinesterase-1 enzyme (AChE1), the target of OP and CX insecticides. The first resistant *ace-1* allele (*ace-1*^{R}, abbreviated R in this paper) resulted from a single amino acid substitution, G119S (Weill et al., 2003). This point mutation confers high resistance to CX and OP, but exhibits strong pleiotropic effects in the absence of insecticide, as revealed by the clinal pattern of its frequency between treated and non-treated areas around Montpellier (Lenormand et al., 1999). The G119S mutation, located in the AChE1 active site prevents inhibition by OP but also interferes with substrate binding, resulting in a reduction of AChE1 activity (Alout et al., 2008). This reduction may account, at least in part, for the reduced fitness observed in mosquitoes homozygous for the G119S AChE1 substitution (Berticat et al., 2002a,b, 2004; Bourguet et al., 2004; Duron et al., 2006). A second *ace-1* allele (or haplotype) was later selected. It originated from a duplication of the *ace-1* gene (named *ace-1*^{D} or D), associating a susceptible and a resistant copy (named D(S) and D(R), respectively), hence producing "permanent heterozygotes", and behaving as a new allele (Labbé et al., 2007a). In Montpellier area, this duplicated haplotype also displays a frequency cline between treated and non-treated areas, indicating a fitness cost relative to the susceptible allele (*ace-1*^{S}, abbreviated S thereafter). However, the D allele has partially replaced the R allele: the *ace-1* duplication seems to be

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doi:10.1016/j.ibmb.2010.09.009

more advantageous by maintaining resistance while restoring part of the fitness of R allele (Lenormand et al., 1998a; Labbé et al., 2007a,b).

Another mutation (F290V, named V here) has recently been identified at the *ace-1* locus of *C. p. pipiens* mosquitoes from Mediterranean countries, and also occurs in duplicated *ace-1* haplotypes (Alout et al., 2007, 2009). This mutation confers distinct levels of insensitivity toward various OP and CX insecticides (Alout and Weill, 2008) but seems also costly; however biochemical analyses revealed that this cost is not associated with AChE1 activity reduction.

In this paper, we have examined the CX and OP resistance status of *Culex p. pipiens* mosquitoes sampled in nine breeding sites from Algeria. Several *Ester* and *ace-1* alleles were observed. Here, we particularly focused on the resistant *ace-1* alleles associated with the G119S mutation only, as data concerning the F290V associated allele are reported elsewhere in relation to other such alleles in the Mediterranean region (Alout et al., 2009). Presence of the G119S mutation was recorded in almost all samples, mostly at the heterozygous state. Crossing experiments revealed the presence of *ace-1* duplication containing a susceptible copy (S) and a resistant (R) copy in all the samples studied, and sequencing of the *ace-1* copies identified six different duplicated haplotypes. The whole data set on CX and OP resistance genes observed in Algerian *C. p. pipiens* is discussed in relation to the management of insecticide resistance.

2. Materials and methods

2.1. Mosquito samples and strains

Culex p. pipiens larvae were collected from nine breeding sites in Algeria (Fig. 1). Samples from Zitoun, Tafna, Smar, Harash and Constantine were collected in 2006, those from Guelma, Douas, Kala and Lac des oiseaux were collected in 2008. All sites sampled were epigeous except the Kala sample, which was hypogeous. Adults emerging from field-collected larvae were stored in liquid nitrogen for further analyses. Four collections (Tafna, Smar, Harash, and Constantine) were allowed to reproduce in the laboratory and their progenies were used for founding strains. Several *C. pipiens* reference strains were used in the experiments: the susceptible strain Slab (Georghiou et al., 1966) lacking any resistant gene; SR and Homoval strains, both lacking overproduced esterase but respectively homozygous for the G119S (Berticat et al., 2002a) and F290V mutations (Alout et al., 2009) at the *ace-1* locus; SA1, SA2, SA4, SB1 and TunB12 strains, which are homozygous for $Ester^{1}$, $Ester^{2}$, $Ester^{4}$, $Ester^{B1}$ and $Ester^{B12}$ alleles, respectively (Berticat et al., 2002a; Ben Cheikh et al., 2008), and all susceptible at the *ace-1* locus.

Fig. 1. *Culex p. pipiens* larvae sampling sites in Algeria.

When necessary, Slab strain was cured of *Wolbachia* infection to avoid cytoplasmic incompatibilities during crosses (Yen and Barr, 1973). To this effect, larvae were reared in water containing tetracycline hydrochloride (20 mg/l, Sigma–Aldrich) for three generations and were used to found the SlabTC strain (Duron et al., 2006).

2.2. Identification of overproduced esterases

Esterase phenotypes were investigated by starch gel electrophoresis in Tris–Maleate buffer-EDTA (pH 7.4) as described by Pasteur et al. (1988). Briefly, α- and β- esterase enzyme from homogenates of individual thorax were separated and revealed using α- and β-naphthyl acetates and Fast Garnett. Allele identification was performed by comparing the electrophoretic mobility of esterases from strains homozygous for the different resistant *Ester* alleles (*nota*: for a same amplified *Ester* allele, the gene amplification level can vary while the sequence remains identical, Weill et al., 2000; Guillemaud et al., 1997). $Ester^{4}$ and $Ester^{5}$ alleles code enzymes with a similar electrophoretic mobility (Poirié et al., 1992); they were discriminated using the PCR-RFLP test developed by Berticat et al. (2000).

2.3. TDP ("Témoin Dichlorvos Propoxur") biochemical test

AChE1 enzymatic phenotypes were analyzed with the TDP test described by Alout et al. (2009). It allows identifying susceptible mosquitoes as well as resistant ones, and discriminates between those carrying the R (G119S) or the V (F290V) mutations at the *ace-1* locus. Enzymatic phenotypes are named according to the AChE1 variants they contain, *i.e.* [SS], [RR], [VV], [RS], [VS], [VR], or [VRS]. Briefly, each mosquito head was homogenized in 400 μl of 0.25 M phosphate buffer containing 1% Triton X-100. The homogenate was centrifuged at 9000 *g* for 3 min, and 100 μl of supernatant were distributed into three wells of a 96-well microtiter plate; 10 μl of ethanol (95%), 10 μl of 5×10^{-5} M dichlorvos, and 10 μl of 10^{-1} M propoxur were added to the first, second and third well, respectively. After 15 min of incubation, 100 μl of acetylthiocholine substrate at a concentration of 1.6 mM were added. Rate of reaction was measured at 412 nm during 15 min with a microtiter plate reader (Biotek, Winooski, VT).

2.4. Detection of ace-1 gene duplications

Two independent analyses were performed. The first was based on crossing experiments as follows. The progenies of Tafna, Smar, Harash and Constantine strains were selected using a 24-h exposure of 1 mg/l propoxur insecticide (a dose that kills only susceptible [SS] individuals) during three generations. Resistant females of each strain were then crossed with susceptible SlabTC males. After fecundation and blood feeding, females of each strain were isolated individually to produce egg rafts and then stored in liquid nitrogen. Each progeny was reared separately and second instar larvae were exposed to 1 mg/l propoxur during 24 h. Females with progenies displaying no mortality were analyzed with the TDP test. If they display a heterozygous phenotype, either [RS] or [VS], then they carry at least one *ace-1* duplicated gene (see Labbé et al., 2007a).

The second analysis was based on a statistical inference. Phenotypic data of each field sample were fitted independently to two distinct models: a 2-alleles model, with the R and S alleles only, and a 3-alleles model, with R, S and the duplicated haplotype D. The presence of such a duplicated haplotype is expected to create an excess of [RS] phenotypes in panmictic populations. This excess can then be used to estimate the frequency of *ace-1* duplication (Lenormand et al., 1998a; Labbé et al., 2007b; Djogbénou et al., 2009). For each sample, expected phenotypic distributions were

computed using allelic frequencies estimated by the 2- and 3-alleles models, assuming *ace-1* locus to be at Hardy–Weinberg equilibrium. The phenotype was considered to be a three-state random variable ([RR], [RS] and [SS]). The log-likelihood of the phenotypic distribution observed in a sample was computed, for each model, as:

$$L_i = \sum_i n_{ij} \ln\left(f_{ij}\right)$$

where n_{ij} and f_{ij} are the observed number and expected frequency of phenotype *i* in population *j*, respectively. It was maximized using the Metropolis algorithm (see Lenormand et al., 1999, 1998b; Lenormand and Raymond, 2000). Two- and 3-alleles model likelihoods of each sample were compared using *F*-tests: by construction the 3-alleles model has a higher likelihood, but the presence of the duplication was considered validated when this likelihood was significantly higher than that of the 2-alleles model. The support limits for the frequency of the resistant and the duplicated haplotype (R and D, respectively) were also estimated.

2.5. *ace-1* sequencing

Total DNA was extracted using a CTAB protocol (Rogers and Bendich, 1988). In this study, we focused only on G119S associated alleles, then part of *ace-1* gene comprising the end of exon 2, the following intron 2 and exon 3 was amplified using specific primers: Intron2dir 5′-GCG CGA GCA TAT CCA TAG CAC T-3′ and CpEx3rev 5′-GAC TTG CGA CAC GGT ACT GCA-3′, generating a 588 bp fragment. PCR was run for 30 cycles (94 °C for 30 s, 52°C for 30 s, and 72°C for 30 s). PCR products were purified with the QIAquick gel extraction kit (QIAGEN). Sequences of *ace-1* G119S from homozygous [RR] field mosquitoes were performed directly on purified PCR products. For each strain (Tafna, Smar, Harash and Constantine), sequences of duplicated haplotypes were acquired from four [RS] females that were shown to contain the duplication in the crossing experiments (16 females sequenced in total). PCR products were cloned to separate the different copies, using the TOPO®TA Cloning Kit (Invitrogen), according to the manufacturer's instructions. Clones were screened to discriminate susceptible and G119S resistant copies by PCR using the same primers followed by AluI digestion (Weill et al., 2004). For each female, 6 susceptible and 6 resistant clones were sequenced, to avoid Taq polymerase misreading, on an ABI Prism 310 sequencer using the Big Dye terminator kit (Applied Biosystems).

2.6. Sequence analysis

Sequences were aligned using the Multalin software (http://prodes.toulouse.inra.fr/multalin/multalin.html; Corpet, 1988). The similarity between the various sequences was assessed with ClustalW (Neighbor-Joining method, v1.83, http://www.ddbj.nig.ac.jp/search/clustalw-e.html, Thomopson et al., 1994). Deduced amino acid sequences were obtained with ClustalW to determine whether the mutations identified were synonymous or non-synonymous. We investigated the position of each non-synonymous mutation in a model of *C. pipiens* AChE1 3D structure (Weill et al., 2004), using PyMOL 0.99rc6 (Delano Scientific LLC).

3. Results

3.1. Resistance gene status of field samples

Mosquitoes were sampled in nine breeding sites of Algeria in a 75 km-wide area along the Mediterranean coast, extending from the western to the eastern borders (Fig. 1). They were analyzed for the presence of overproduced esterases and AChE1 substitutions.

A total of four resistant *Ester* phenotypes ([$Ester^1$], [$Ester^2$], [$Ester^4$] and [$Ester^{B12}$]) were observed in six of the nine samples (Table 1), with the frequency of carriers not exceeding 36% and no mosquito displaying simultaneously two overproduced enzymes. Identification of $Ester^4$ allele was confirmed by PCR-RFLP in four individuals from Tafna and Zitoun and in two individuals from Kala (see Materials and Methods). Overall, $Ester^1$ and $Ester^{B12}$ carriers are relatively rare, whereas $Ester^4$ and $Ester^2$ carriers have variable frequencies and both can reach 21% in some populations.

Insensitive AChE1 was observed in almost all samples, and the two substitutions responsible for resistance, G119S and F290V were present (Table 2). The F290V mutation is very rare in the central and the eastern part of the country with only one carrier in three samples, while near the west border (Tafna and Zitoun) higher frequencies were observed. Detailed results concerning this mutation are reported elsewhere (Alout et al., 2009). In contrast, the G119S mutation was found in almost all samples. The [RS] phenotype was the most abundant with frequencies above 60% in four samples (Smar, Harash, Guelma and Kala). The [RR] phenotype was rare or absent, except in Kala where its frequency reached 17%.

3.2. Evidence for *ace-1* duplication

The high frequency of [RS] phenotypes in some samples suggested that the *ace-1* locus might be duplicated in Algerian populations, as found in many other countries (Lenormand et al., 1998a; Labbé et al., 2007a). In order to test this hypothesis, we searched evidence for duplicated haplotypes by crossing experiments, following the procedure used by Labbé et al. (2007a). Several heterozygous [RS] females from the strains derived from Tafna, Smar, Harash and Constantine field samples were crossed with a susceptible SlabTC male and displayed a progeny all resisting the propoxur exposure (see Materials and Methods). This result shows that at least one duplicated *ace-1* haplotype (D) associating a susceptible copy D(S) with a G119S D(R) copy is present in the four strains.

The presence of a duplicated haplotype (D) in field samples is likely to explain the high frequency of [RS] phenotypes observed in some populations (Table 2). The statistical inference designed to test this assumption compares the observed phenotypic frequencies with those expected either in absence or in presence of a duplicated haplotype (excluding Tafna and Zitoun in which [VS] and [VRS] enzymatic phenotypes had a frequency of ~12%, which precludes a robust analysis, Table 2). Although this method is not as accurate as direct observation of the different genotypes, it has been shown to correctly estimate the duplication frequency in field samples where this frequency was independently estimated using

Table 1
Frequency of the various overproduced esterase phenotypes in the populations sampled. *N* is the number of individuals analyzed. The frequency of each phenotype is given, together with the corresponding number of individuals indicated in brackets.

Sample name	*N*	Observed overproduced esterase phenotypes				
		None	[$Ester^1$]	[$Ester^2$]	[$Ester^4$]	[$Ester^{B12}$]
Tafna	38	0.75 (29)	0	0	0.21 (8)	0.04 (2)
Zitoun	50	0.64 (32)	0	0.12 (6)	0.20 (18)	0.04 (2)
Smar	32	1.00 (32)	0	0	0	0
Harash	50	0.96 (48)	0.04 (2)	0	0	0
Constantine	17	1.00 (17)	0	0	0	0
Guelma	29	0.97 (28)	0	0.03 (1)	0	0
Lac des oiseaux	48	0.90 (43)	0	0.10 (5)	0	0
Douas	58	1.00 (58)	0	0	0	0
Kala	29	0.72 (21)	0	0.21 (6)	0.07 (2)	0

Table 2
Frequency of AChE1 enzymatic phenotypes in Algerian field samples (adapted from Alout et al., 2009, where only the F290V mutation data were published). N is the number of individuals analyzed. S, R and V represent the AChE1 susceptible, the G119S and the F290V mutations, respectively. Frequency of each phenotype is indicated, together with the corresponding number of individuals indicated in brackets.

Sample name	N	AChE1 phenotypes (TDP test)						
		[SS]	[RS]	[RR]	[VV]	[VS]	[VR]	[VRS]
Tafna	38	0.46 (17)	0.25 (10)	0.04 (1)	0	0.12 (5)	0	0.12 (5)
Zitoun	50	0.88 (44)	0	0	0	0.04 (2)	0	0.08 (4)
Smar	32	0.34 (11)	0.66 (21)	0	0	0	0	0
Harash	50	0.24 (12)	0.66 (33)	0.08 (4)	0	0.02 (1)	0	0
Constantine	17	0.71 (12)	0.29 (5)	0	0	0	0	0
Guelma	29	0.21 (6)	0.72 (21)	0.03 (1)	0	0.03 (1)	0	0
Lac des oiseaux	48	0.75 (36)	0.23 (11)	0	0	0.02 (1)	0	0
Douas	58	1 (58)	0	0	0	0	0	0
Kala	29	0.21 (6)	0.62 (18)	0.17 (5)	0	0	0	0
Total number	351	202	119	11	0	10	0	9

crosses (Lenormand et al., 1998a). We found that observed phenotypic frequencies is best explained by the presence of a D allele in three samples: Smar and Harash (where a D allele was also detected by crossing experiments) and Guelma. The frequency of the D allele is predicted to be 41% in Smar, 21% in Harash and 32% in Guelma (Table 3). The presence of the D allele cannot be confirmed from the phenotypic frequencies in other samples, as the differences between the models with and without D are not statistically significant. However, the *ace-1* duplication was shown to be present when sequencing resistant mosquitoes in Constantine. This discrepancy between statistical inference and direct sequencing is probably due to a yet too low frequency of the duplicated haplotype in this locality.

3.3. Characterization of duplicated ace-1 haplotypes

A fragment of the *ace-1* gene encompassing the G119S mutation was amplified in four [RS] females per strain analyzed carrying the duplication detected by crossing experiments. Susceptible D(S) and resistant D(R) copies were cloned and sequenced (Table 4). All D(R) copies were identical to the single R sequence present in homozygous resistant [RR] insects of each sample (when present) and to the *C. p. pipiens* subspecies sequence previously described (Weill et al., 2003). The R sequence is also identical to the D(R) copy identified in duplicated *C. p. pipiens* haplotypes from France (Labbé et al., 2007a), indicating a single evolutionary origin for the G119S mutation in this subspecies. D(S) sequences were numbered according to Labbé et al. (2007a) nomenclature, and since all were different from those already described, numbering began at D_7(S). In the four females of the Tafna strain, only one susceptible sequence (D_7(S)) was identified. In females from Smar, two different D(S) sequences were observed; one differs from the D(R) sequence by the G119S mutation only and was named D_8(S), the other was a new sequence and was named D_9(S). In Harash sample, we found also two different D(S) sequences: one identical to the D_9(S) sequence, the other new and named D_{10}(S). Finally in females from Constantine, there were two distinct D(S) sequences highly different from one another and from the others; they were named D_{11}(S) and D_{12}(S). Thus the four Algerian samples tested contained 6 new duplicated haplotypes (D_7 to D_{12}), with only one of them being observed in two locations (D_9 in Smar and Harash). They all differ by at least 6 synonymous mutations except in the D7S copy from Tafna and D10S from Harash where the C95R and R104K substitutions were identified. They are located on a flexible loop at the periphery of the enzyme (at some distance from the active site) and are therefore unlikely to interfere with the catalytic properties (data not shown).

Sequence of duplicated *ace-1* D haplotypes from Algeria were compared with those described by Labbé et al. (2007a) from different parts of the world by constructing a genetic distance tree of D(R) and D(S) sequences (Fig. 2). Samples from Martinique, Cuba and Philippines (named Mart, Cuba and Pal, respectively) belong to the subspecies *Culex p. quinquefasciatus*, whereas all the others, from southern France and Algeria, belong to the *Culex p. pipiens* subspecies. The *ace-1* sequences observed in *C. p. pipiens* differ by an 8bp deletion in the intron-2 sequence as compared to *C. p. quinquefasciatus* (Labbé et al., 2007a). Thus, as expected, *ace-1* sequences found in Algeria cluster with those of French *C. p. pipiens*. In France and Algeria, all D(R) sequences are identical, but all D(S) sequences differed and their differentiation does not display any particular geographic pattern.

4. Discussion

In this study, we identified two mechanisms responsible for resistance to organophosphate insecticides in the *C. p. pipiens* mosquitoes from Algeria: overproduction of detoxifying esterases encoded by the *Ester* superlocus and insensitive

Table 3
Theoretical allele frequencies in presence or absence of a duplicated haplotype (D). pS, pR and pD are the estimated frequencies of the susceptible, the resistant G119S and the duplicated alleles, respectively (pS = 1 − pR − pD). *F*-test statistics comparing the likelihoods of the two models (see material and methods), and the corresponding *p*-value for each sample are indicated. Presence of duplication is assumed from the phenotypic data when the likelihood of the 3-alleles model is significantly higher than that of the 2-alleles model (bolded *p*-value).

Sample name	Without D		With D					*F*-test value	*p*- value	
	pR	pS	PR	Support limits	PD	Support limits	pS			
Harash	0.41	0.59	0.28	(0.16–0.42)	0.21	(0.08–0.47)	0.51	6.97	**0.008**	**
Smar	0.33	0.67	0	(0–0.24)	0.41	(0.18–0.56)	0.59	10.71	**0.001**	***
Lac des oiseaux	0.11	0.89	0	(0–0.17)	0.12	(0–0.2)	0.88	1.43	0.232	NS
Kala	0.48	0.52	0.42	(0.25–0.58)	0.13	(0–0.51)	0.45	1.73	0.189	NS
Douas	0.25	0.75	0.25	(0.18–0.33)	0	(0–0.03)	0.75	0	1	NS
Guelma	0.4	0.6	0.19	(0.04–0.38)	0.32	(0.04–0.38)	0.49	8.6	**0.003**	**
Constantine	0.15	0.85	0	(0–0.27)	0.16	(0–0.3)	0.84	0.87	0.352	NS

Table 4
Sequence alignment of partial *ace-1* copies of *C. p. pipiens* duplicated haplotypes. The *C. p. pipiens* G119S allele sequence (Weill et al., 2003) is used as the reference sequence (R copy). Polymorphism is indicated for the D(R) and D(S) copies of each duplicated haplotype *ace-1*^{D}. The positions are numbered from the first nucleotide of intron 2 and of exon 3 and the dash (–) indicates identity and star (*) indicates deletion. The mutation at position 361 (highlighted) is the only mutation conferring resistance (G119S).

	Intron 2															Exon 3																		
	1	2	3	3	3	3	3	3	3	3	4	4	4		8	1	1	1	2	2	2	3	3	3	3	3	3	3	3	3	4	4	4
	5	7	1	2	3	4	6	7	8	9	1	2	5	9	1	5	3	5	5	6	1	3	4	5	8	4	6	4	1	3	9	2	0	6
R	T	A	C	C	C		T	T	G	C	A	A	A	C	G	G	A	A	C	T	G	A	G	G	G	C	T	T	A	C	T	T	G	C
D(R)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
From France																																		
D_2(S)Maurin	–	–	–	–	–	C	–	C	–	–	–	–	–	T	A	–	T	G	–	–	–	C	–	–	–	–	C	–	G	–	C	–	–	–
D_3(S)Biface	–	–	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	C	–	–	–	C	–	–	C	–	C	–	G	A	C	C	–	T
D_6(S)Dumont	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	T	G	–		–	C	–	–	–	–	C	–	G	–	C	–	–	–
From Algeria																																		
D_7(S)Tafna	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	A	–	–	–	A	A	C	A	C	C	G	–	C	C	–	T
D_8(S)Smar	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G		–	–	–	–
D_9(S)Smar/Harash	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	A	–	A	–	–	–	–	–	C	C	G	–	C	C	A	–
D_{10}(S)Harash	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	C	–	–	–	–	C	C	G	–	C	C	A	–
D_{11}(S)Constantine																–			–		–	C	–	–	–	–	C	C	G	–	C	C	–	T
D_{12}(S)Constantine	–	*	G	G	G	G	C	A	T	T	T	C	–			–			–		–	C	–	–	–	–	C	C	G	–	C	C	–	T

acetylcholinesterase encoded by the *ace-1* gene. Both genes displayed several resistance alleles in eight of the nine studied localities, and the frequency of susceptible individuals, harbouring susceptible alleles on both loci varied between 21% (in Kala and Guelma) and 100% (in Douas).

Six of the nine samples display no or low frequency (≤10%) of resistant *Ester* allele carriers, suggesting that they appeared recently, most probably by migration (Raymond et al., 1991). In the Western region (Tafna and Zitoun), the frequency of overproduced esterases carriers reached 25–36%, the predominant allele being *Ester*^{4}. Close to the Eastern border (Kala), their frequency reached 28%, with *Ester*^{2} being predominant. As evolution of insecticide resistance through esterase overproduction appears to proceed by allele replacement rather than by selection of modifiers (Guillemaud et al., 1998; Labbé et al., 2005), this suggests that the less frequent alleles may be in the process of being replaced by *Ester*^{2} in the Eastern region and by *Ester*^{4} in the Western region. Indeed, the various *Ester* resistant alleles do not share the same characteristics: both resistance and fitness cost vary quantitatively according to the gene amplification level, and qualitatively according to each allele (Weill et al., 2000; Pasteur et al., 2001; Berticat et al., 2002a,b, 2004; Labbé et al., 2009). Resistance allele frequencies will depend in part on the local intensity and on the nature of insecticide treatments, and in the other part on the fitness of resistant individuals. At selection equilibrium, the most frequent allele would be the most advantageous in the local conditions (i.e. the one providing the best local fitness). For example, in Montpellier area (South of France), the first resistant allele to appear in 1972, *Ester*^{1}, has been replaced by the less costly *Ester*^{4} allele, after a change in insecticide treatment in 1984 (changing OP insecticides and reducing quantity; see Guillemaud et al., 1998). *Ester*^{2} appeared later (1991) and seems to be taking over both *Ester*^{1} and *Ester*^{4}, without any change in insecticide treatment, (Labbé et al., 2005, 2009).

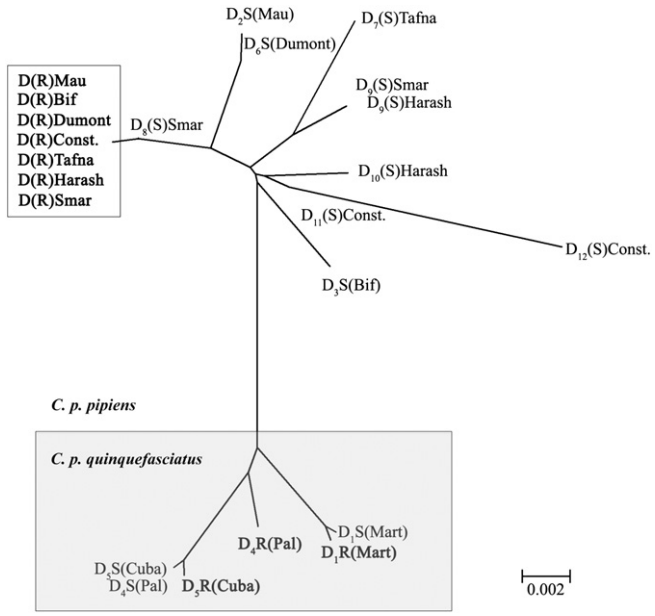

Fig. 2. Genetic distance tree of *ace-1* sequences from *C. p. pipiens* carrying a duplicated haplotype. Sequences of intron 2 and exon 3 were used and G119S mutation was not included in order to consider only "resistance neutral" mutations. D(R) sequences are in bold characters and those of *C. p. pipiens* are included in a square. Samples of *C. p. quinquefasciatus* are framed in grey.

The second resistance mechanism, the insensitive AChE1, was present in almost all samples, and two different mutations (G119S and F290V) were found. Due to the fitness cost associated with the G119S mutation in non-treated areas (Raymond et al., 2001; Berticat et al., 2002a,b, 2004; Duron et al., 2006), its presence indicates that the selection pressure of OP or CX insecticides is (or has been in the recent past) relatively strong in Algeria. In the Zitoun sample, no individual harbouring the G119S AChE1 was detected, whereas the F290V AChE1 was observed in 12% of the analyzed mosquitoes. The selection of the F290V AChE1 may have been favoured relatively to the G119S AChE1 because it provides higher resistance to specific insecticides (Alout and Weill, 2008; Alout et al., 2009).

Distinct OP insecticides are used in each city independently in Algeria, including methomyl, chlorpyrifos, malathion, parathion-methyl, pyrimiphos-methyl, fenitrothion and dichlorvos; however we failed to obtain more information on local insecticide application procedures. We nevertheless suggest that each local insecticide treatment (nature and quantity) has selected for a particular resistance gene or allele and that the local environmental conditions are probably critical to explain the present distribution of resistance alleles, as stressed out by Labbé et al. (2005). The Algerian situation is in sharp contrast from that observed in Tunisia: mostly one, sometime two resistant alleles co-exist in most Algerian populations, while many distinct resistant *Ester* and *ace-1* alleles co-exist in each Tunisian samples studied so far (Ben Cheikh

et al., 2009). In Tunisia, a large variety of insecticides is also used (at least chlorpyrifos, temephos, fenitrothion, pyrimiphos-methyl and other insecticide classes such as pyrethroids and *Bacillus* toxins [Ben Cheikh et al., 2008, 2009]). The difference between the Algerian and the Tunisian situation may be due to the fact that only a single insecticide is used in each Algerian city (or district), compared to a mixture of several insecticides applied at the same time in the Tunisian regions.

Using a genetic test and molecular analyses on four strains derived from field samples, we characterized six distinct duplicated haplotypes containing the G119S mutation (Table 4, Fig. 2). All of them harbour the same D(R) copy (identical to the R copy present in homozygous [RR] individuals), but it is associated with distinct D(S) copies, which are different from those already described in France (see Labbé et al., 2007a,b). Moreover, a statistical inference from the *ace-1* allele frequencies indicated that the presence of the duplicated *ace-1* gene was the most probable hypothesis to explain the excess of [RS] heterozygous *ace-1* phenotypes at least in three samples (Guelma, Smar and Harash). The estimated frequency of individuals carrying this D allele (estimated between 21 and 41%) is in sharp contrast with the very low frequency of homozygous [RR] mosquitoes (0–8%). This is consistent with a better advantage of the duplicated D allele over the non-duplicated R allele in the environmental conditions of the studied sites: in treated areas, the selection pressure favours the resistant *ace-1* copy of a duplication because the enzyme coded by the susceptible copy is inhibited, while in non-treated areas, the susceptible *ace-1* copy most probably restores at least partially the fitness cost associated with the resistant copy (Bourguet et al., 1996; Labbé et al., 2007a,b).

Several mechanisms of duplication have been proposed (for more details see Labbé et al., 2007a). Among the most probable, one involves duplication of the R allele followed by a S119G reversion on one of them, restoring a susceptible S copy; this mechanism would thus result in high similarity between D(S), D(R) and the single R copies. Another mechanism involves an unequal crossing-over in a heterozygous [RS] individuals resulting in D(R) and D(S) copies not more similar than the single R and S alleles sampled randomly in the population. Secondary events of recombination may then occur between a duplicated haplotype and a single *ace-1* copy or between two duplicated haplotypes, shuffling *ace-1* copies in duplicated haplotypes and increasing their variety (Labbé et al., 2007a). In Smar sample, the D_8(S) copy differs from the R copy only by the G119S mutation, and may thus result from the first mechanism. For the other duplications, the differences between the D(S) and the D(R) copies would more likely result from the second mechanism.

Interestingly, all duplications share the same resistant D(R) copy, identical also to the single resistant R allele in *C. p. pipiens* subspecies (Weill et al., 2003; Labbé et al., 2007a). Then, it is impossible to infer the number of independent duplication events (named duplication *sensu stricto* by Labbé et al., 2007a) leading to the nine duplicated haplotypes observed in Algerian and French *C. p. pipiens*. Indeed, either multiple duplications *sensu stricto* of *ace-1* gene or a single duplication event followed by multiple secondary recombinations could explain the observed variety of the duplicated haplotypes. We know that at least two duplications *sensu stricto* probably appeared independently in *C. p. pipiens* from southern France (Labbé et al., 2007b). It is then also probable that more than one duplication event occurred in Algeria.

Together with the characterization of five other D haplotypes associated with the F290V mutation in the Mediterranean region (Alout et al., 2009), this study reveals a high occurrence of *ace-1* duplications in *C. p. pipiens*. We know from recent studies on human genomes (Korbel et al., 2008) that the number of genes with intraspecific copy number variation can be quite high (10^{-4}–10^{-6} per generation) in contrast to previous studies based on interspecific comparisons that estimated the rate of duplication to be of the same magnitude as the rate of mutation per nucleotide site (2×10^{-8} per generation; Lynch and Conery, 2000; Gu et al., 2002). Our study of a single gene in *C. pipiens* appears to confirm this high intraspecific rate of duplication.

In conclusion, our survey demonstrated the presence of several insecticide resistant alleles in *C. p. pipiens* mosquitoes in Algeria. Sequence analysis revealed a high occurrence of *ace-1* duplications in *C. p. pipiens,* at least in the Mediterranean region. Although, we could not determine the duplication rate of the *ace-1* gene, it seems to be very high. The *ace-1* allele frequencies suggest that the duplicated alleles are less costly than the single R allele. They are thus predicted to invade the OP-treated areas. Unfortunately, the cost associated with the non-duplicated R allele constitutes an asset to manage insecticide resistance since stopping insecticide treatments (or changing nature of insecticides) is probably sufficient to decrease its frequency (Lenormand et al., 1999); by solving the trade-off between fitness cost and resistance, *ace-1* duplications might reduce the chances of eliminating AChE1 resistance mechanisms and stress the importance to develop new tools for vector control.

Acknowledgement

We are very grateful to C. Bernard, and S. Unal for technical assistance and Benharzallah Naouel (Université Mohamed Khider, Biskra, Algeria) and B. Alout and A. Benamira for collecting mosquito in Constantine and in the eastern border of Algeria, respectively. This work was financed in part by the ANR Morevol Sante-Environnement (*Ministère délégué à la Recherche*) and by Comité de Coopération Marseille Provence Méditerranée supervised by Robert Bismuth. All sequence data were obtained on the Environmental Genomic Platform of the IFR Montpellier-Environnement-Biodiversité. Contribution 2010. 109 of the Institut des Sciences de l'Evolution de Montpellier (UMR CNRS 5554).

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