A sex-linked Ace gene, not linked to insensitive acetylcholinesterase-mediated insecticide resistance in Culex pipiens

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A sex-linked *Ace* gene, not linked to insensitive acetylcholinesterase-mediated insecticide resistance in *Culex pipiens**

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Abstract

An acetylcholinesterase (AChE) gene, *Ace.x*, showing 93% identity of deduced amino acid sequence to *Anopheles stephensi Ace* has been cloned from a *Culex pipiens* strain homozygous for insensitive AChE (iAChE) mediated insecticide resistance. DNA sequence of genomic DNA clones identified exons 2–5. RFLP of six clones indicated four possible alleles. Linkage analysis located *Ace.x* to chromosome I, less than 0.8 centimorgans from the sex locus, whereas the locus conferring resistance was 2.0 centimorgans from *plum-eye* on chromosome II. *Ace.1* coding for AChE1, which is associated with resistance, is therefore autosomal. We propose that *Ace.x* is the recently postulated *Ace.2* coding for the biochemically distinct AChE2, which is not associated with resistance.

Keywords: acetylcholinesterase, sex-linked gene, *Culex pipiens*, insecticide resistance.

Introduction

AChE (EC 3.1.1.7) catalyses the hydrolysis of the neurotransmitter acetylcholine (ACh) and thus is a key enzyme at cholinergic synapses in the insect central nervous system (see Toutant, 1989). Its inhibition by organophosphates and carbamates causes a desensitization of the ACh receptor leading to a blockage of nerve impulse transmission (Eldefrawi, 1976; Eldefrawi *et al.*, 1982).

Culex pipiens is a complex of species and/or geographical, or physiological forms, with a worldwide distribution (Harbach *et al.*, 1985; Miller *et al.*, 1996). In tropical and subtropical regions they are major vectors of filariasis and arboviruses and in many temperate regions a serious biting nuisance. *C. pipiens* has been subject to extensive control measures using insecticides and has evolved multiple resistance mechanisms. Important are two conferring resistance to organophosphates: one involves overproduction of esterases conferring an increased ability to detoxify the insecticide, the other is mediated by an acetylcholinesterase (AChE) insensitive to insecticide inhibition (Bourguet *et al*., 1996a; Raymond & Pasteur, 1996). Propoxur-insensitive AChE in *C. pipiens* was first reported in a strain (MSE) selected from a resistant population collected in Southern France (Raymond *et al*., 1985). Genetic analysis of this strain attributed most of the insecticide resistance to a single gene, which from the previous studies coded for an insensitive AChE (Raymond *et al*., 1985, 1987).

Insensitive AChEs have been detected in more than twenty-five insect species (Fournier & Mutéro, 1994). In order to identify the mutation(s) conferring insecticide insensitivity, genes coding AChE (*Ace*) have been cloned and sequenced in some insect species including: *Drosophila melanogaster* (Hall & Spierer, 1986), *Anopheles stephensi* (Hall & Malcolm, 1991), *Aedes aegypti* (Anthony *et al.*, 1995), *Leptinotarsa decemlineata* (Zhu & Clark, 1995) and *Musca domestica* (Williamson & Devonshire, pers. comm.).

Although vertebrates have two cholinesterases, AChE and butyrylcholinesterase (BuChE, EC 3.1.1.8),

*The results of this paper were determined separately by two laboratories (1 + 2) and combined into a single paper for clarity.

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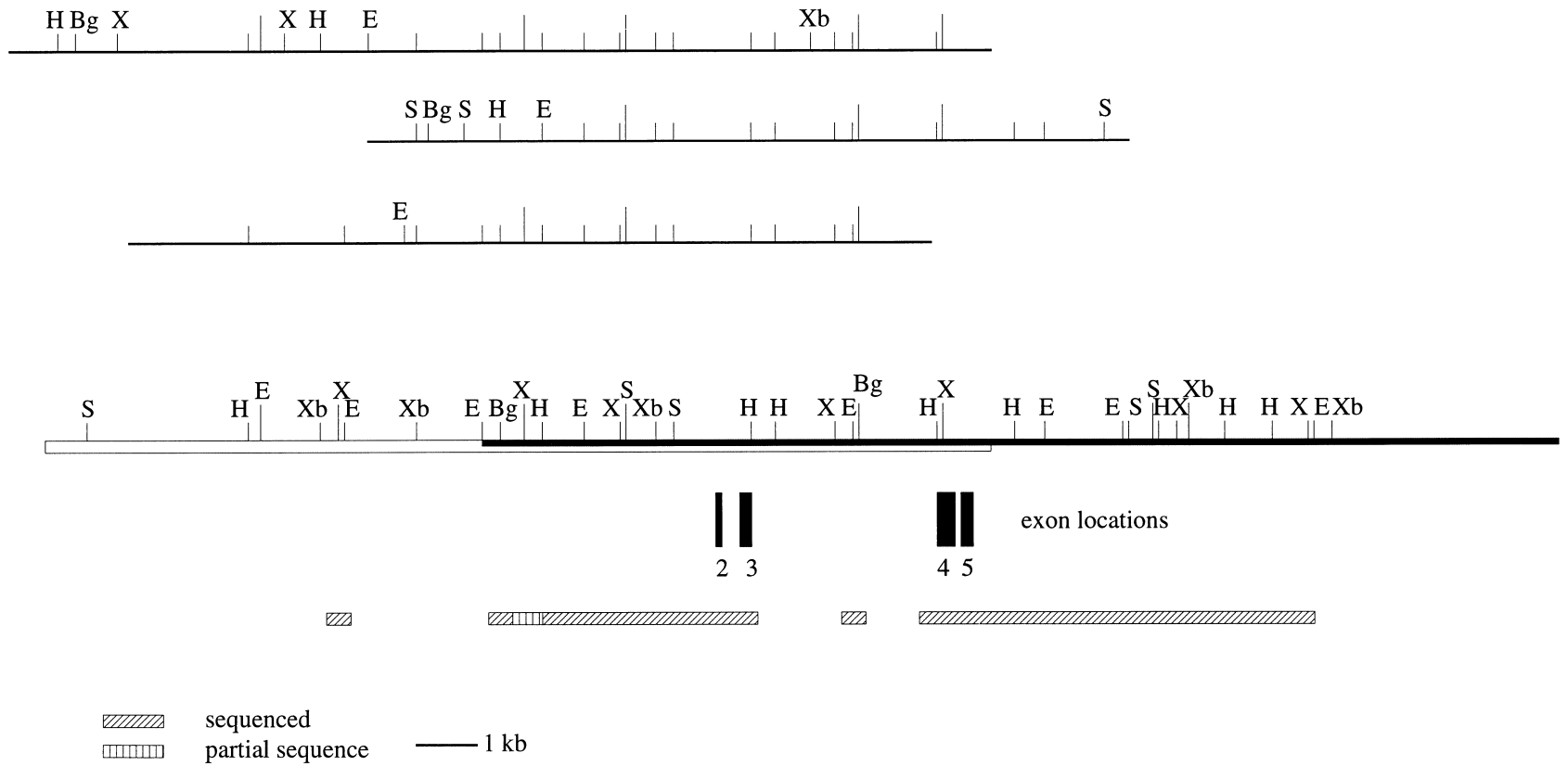


Figure 1. Maps of *Culex pipiens Ace.x* clones showing restriction fragment length polymorphism compared with the contiguous map of overlapping clones 2, 4 and 5. Clone 4 is shorter than clone 5, but otherwise appears identical. The partial *ace.x* gene structure and the extent of DNA sequencing is also shown. The short segment indicated as partial sequence contains two GC-rich regions producing secondary structures which interfere with sequencing. (Bg, *Bgl* II; E, *Eco* RI; H, *Hind* III; S, *Sal* I; Xb, *Xba* I; X, *Xho* I.)

only one was reported in arthropods (Toutant, 1989). However, two AChEs, AChE1 and AChE2, have recently been described in *C. pipiens* (Bourguet *et al.*, 1996a). These enzymes differ in their substrate specificity, inhibitor sensitivity and electrophoretic migration pattern, but display identical sedimentation profiles. Two cholinesterases, with different properties, are also present in gnathostome vertebrates and in amphioxus and are the product of distinct genes (Massoulié *et al.*, 1993; Sutherland *et al.*, 1997). In vertebrates an alternative splicing of the mRNA produced by one of the two cholinesterase genes gives two peptides, but they have identical catalytic properties. It is unlikely that *Culex pipiens* AChE1 and AChE2 proteins derive from post-translational modifications or an alternative splicing of the mRNA of a single gene, so that AChE1 and AChE2 are thought to be the product of two distinct genes: *Ace.1* and *Ace.2* (Bourguet *et al.*, 1996a).

This paper presents the initial characterization of an acetylcholinesterase gene, provisionally named *Ace.x* in *Culex pipiens*, and the analysis of its inheritance in relation to insecticide resistance due to insensitive AChE1. DNA sequence from intact open reading frames corresponding to exons 2–5 was obtained, showing 93% identity of deduced amino acid sequence to *Anopheles stephensi Ace*. *Ace.x* was found to be tightly linked to the sex locus (linkage group I), whereas *Ace.1* is located on linkage group III. We suggest that *Ace.x* is the putative second gene *Ace.2* proposed by Bourguet *et al.* (1996a).

Results

Sequence analysis of Ace.x

A homologous AChE gene probe (FG) was used to screen an unamplified MSE genomic DNA library from which six clones were isolated and fully characterized. A 2.2 kb *Hin*d III-*Xho* I fragment (Fig. 1), containing regions of homology to the FG probe, was subcloned from clone 5. This was sequenced and found to contain intact open reading frames, which by comparison to the *An. stephensi* AChE gene, corresponded to exons 2 and 3 (Fig. 2a). The sequence analysis was repeated and extended with clone 2 by shot-gun subcloning of fragments generated with *Hind* III, *Xho* I, *Eco RI* and *Sal* I into M13, pUC18 and pUC19. The sequence obtained encompassed open reading frames corresponding to exons 2–5 (Fig. 2b). The overlapping sequence with clone 5 was identical. Despite extensive sequencing of the regions flanking exons 2 and 5 (Fig. 1), no further exons were identified, presumably indicating that introns 1 and 5 are very large.

A comparison of the predicted amino acid sequence for exons 2–5 with other Dipteran AChEs is shown in Fig. 3. A high degree of AChE sequence identity (~93%) can be observed between the mosquitoes. This is reflected in the results of a phylogenetic tree based on the available AChE sequence data (including unpublished data for *An. albimanus*, and *An. gambiae*), which shows that the mosquitoes form a monophyletic group within the Diptera (Fig. 4). *An. stephensi* and *An. gambiae*, which both belong to the subgenus *Cellia* (Gillies & De Meillon, 1968; Rao, 1984), are distinct from *An. albimanus*, which belongs to the subgenus *Nyssorhynchus* (Pan American Health Organisation, 1996). *C. pipiens* and *Ae. aegypti* are separate from the *Anopheles* although the bootstrap value suggests less confidence in this distinction.

Given the possibility of two *Ace* genes in *C. pipiens* (Bourguet *et al.*, 1996a), and the observation of restriction fragment length polymorphism (RFLP) in the isolated clones (Fig. 1) from a strain homozygous for insecticide-insensitive AChE, it was necessary to investigate the relationship between the clones. All six clones were used to provide templates for PCR amplification of fragments encompassing part of exon 2 through to part of exon 3. The fragments were subsequently analysed by direct sequencing and restriction digests. Clone 3 was single and double digested with *Hind* III and *Xho* I, and shot-gun subcloned into pUC19 and pUC18 and the subclones partially sequenced. All of the sequences obtained from the regions in which the restriction maps were fully conserved were identical to corresponding sequence already obtained from clones 2 and 5. Cross-hybridization between fragments of different clones showed cross homology even within the less conserved 5′ regions. The clones therefore appear to contain alleles of the *Ace.x* locus. The sequence analysis was not extensive enough to establish if there were any differences at all in the coding regions between the clones.

Inheritance of Ace.x

*RFLP on genomic DNA. Eco*RI digestion of genomic DNA revealed 3 fragments (4.7, 3.4 and 2.9 kb) hybridizing with the *Ace.x* probe (Fig. 6). These were named respectively as *A*, *B* and *C*. Females of the susceptible strain S-lab (S) had only *C*, and those from the resistant strain MSE (M) had only *A*. MSE males had *A* and *B* fragments. The inheritance of these fragments was studied by analysing the male and female offspring of the cross S × M and backcrosses S × (S × M), (S × M) × M and M × (S × M) (Fig. 5).

The results (Table 1) were in agreement with a mendelian segregation of three alleles at the same

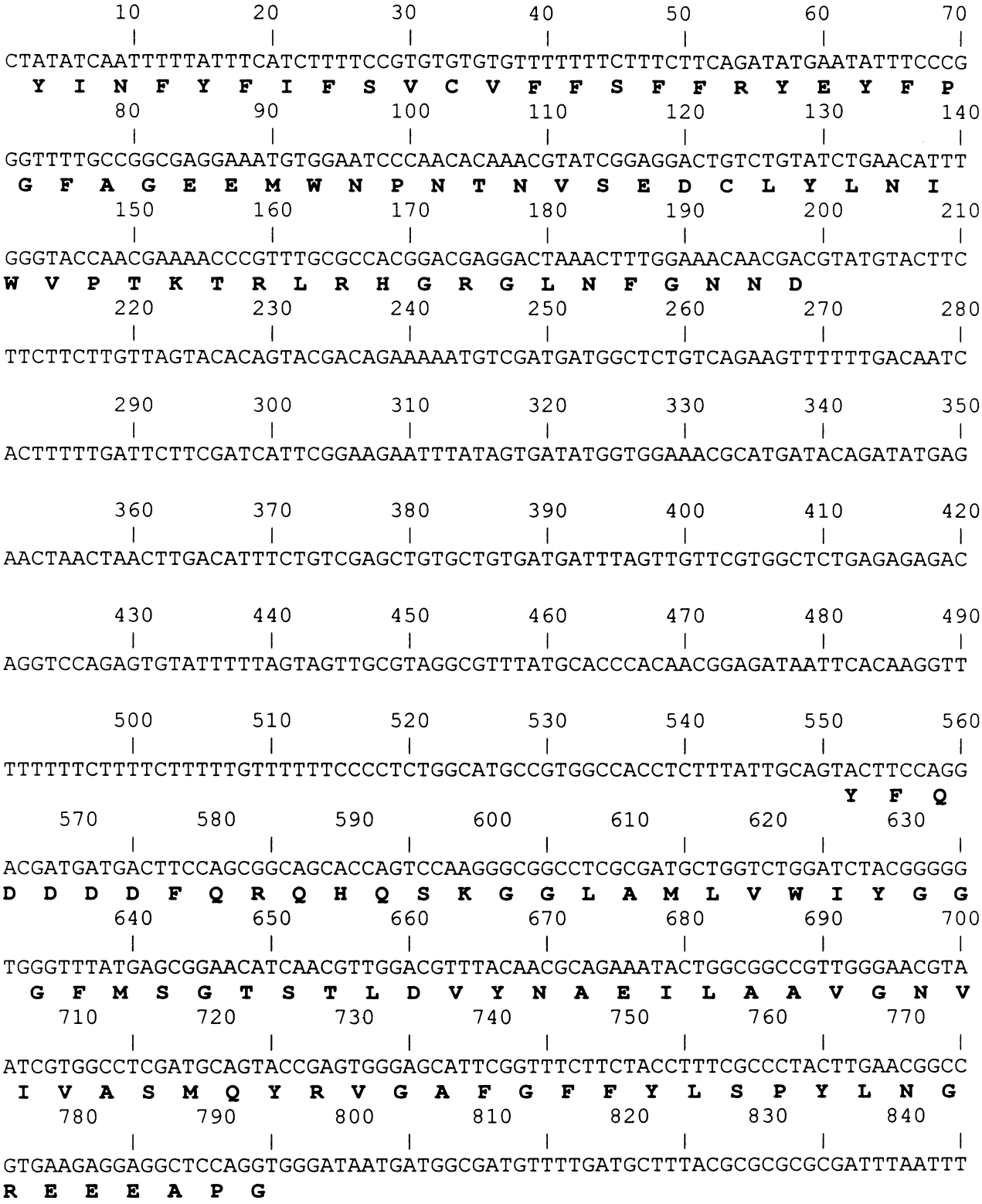


Figure 2(a). DNA sequence and inferred amino acid sequence of *Culex pipiens* AChE gene: exons 2 and 3.

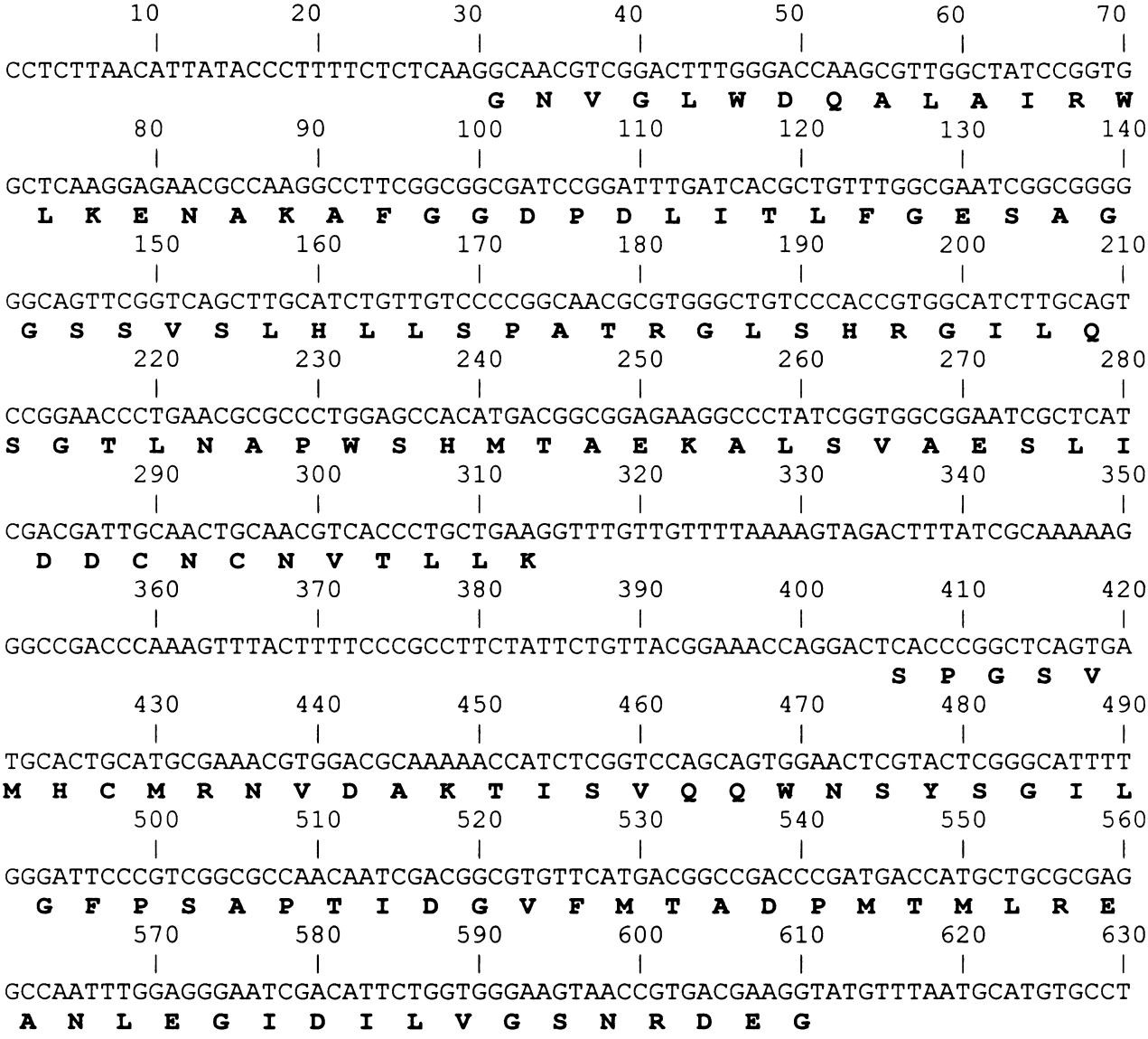
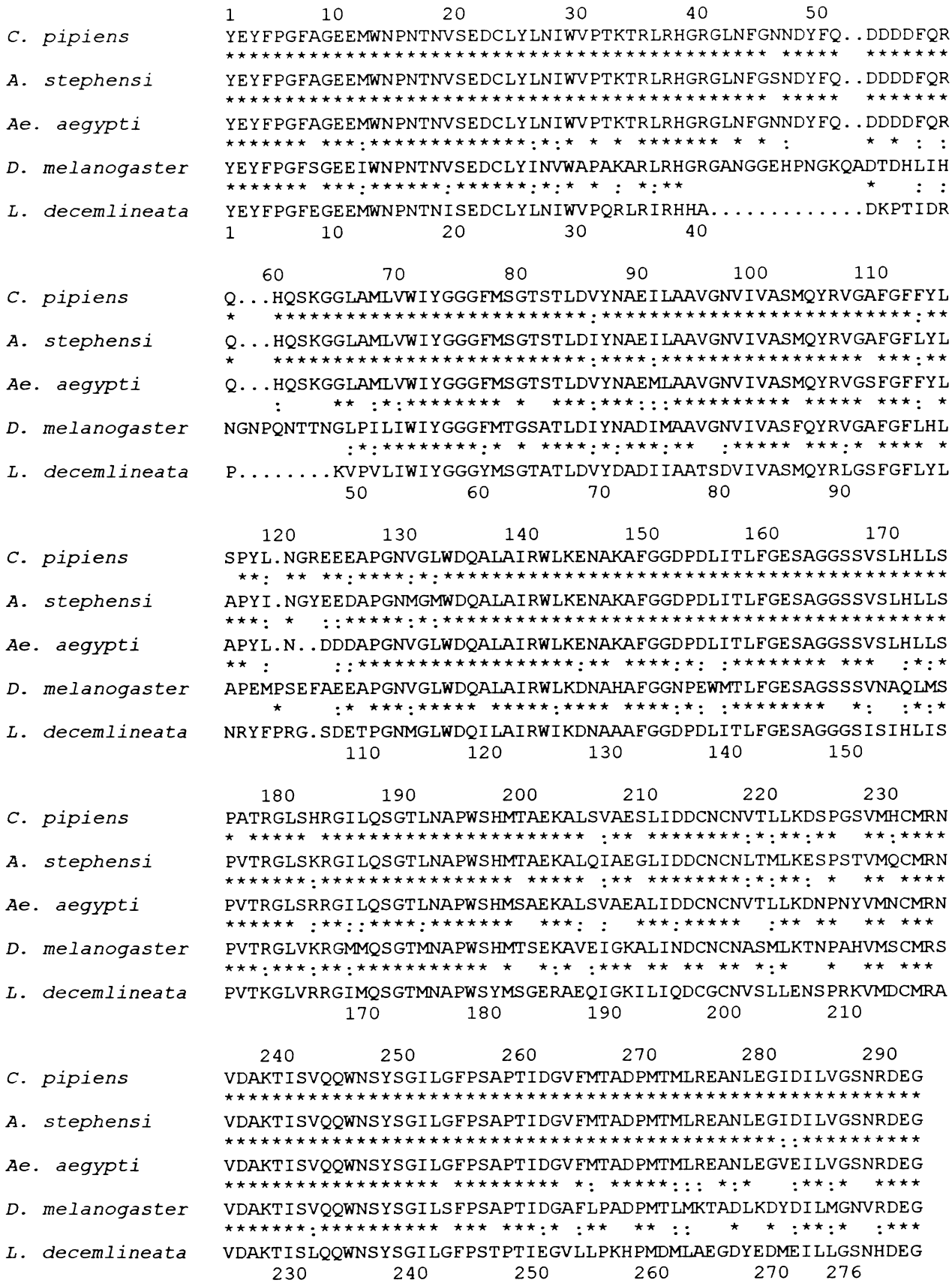


Figure 2(b). DNA sequence and inferred amino acid sequence of *Culex pipiens* AChE gene: exons 4 and 5.

locus. However, all males studied were heterozygous, either *AB* or *BC*, depending on the cross analysed (Table 1), whereas females never carried the *B* allele, being homozygous (*AA* or *CC*) or heterozygous *AC*. These observations are consistent with a tight linkage of *Ace.x* to the sex locus. In *C. pipiens*, sex is determined by two alleles *M* and *m*, so that males are *Mm* and females *mm* (Gilchrist & Haldane, 1947, in

Table 1. *Ace.x* genotypes of offspring of cross S × M and backcrosses S × (S × M), (S × M) × M and M × (S × M) determined by RFLPs on genomic DNA of single mosquitoes using a nucleotide *Ace.x* probe (see Fig. 2).

	Offspring genotypes						
	Parental genotypes		Females			Males	
Crosses	Females	Males	*AA*	*AC*	*CC*	*AB*	*BC*
S × M	*CC*	*AB*	0	18	0	0	19
S × (S × M)	*CC*	*BC*	0	0	33	0	18
(S × M) × M	*AC*	*AB*	18	15	0	18	18
M × (S × M)	*AA*	*BC*	0	19	0	20	0


1 10 20 30 40 50
C. pipiens YEYFPGFAGEEMWNPNTNVSEDCLYLNIWVPTKTRLRHGRGLNFGNNDYFQ..DDDDFQR
A. stephensi YEYFPGFAGEEMWNPNTNVSEDCLYLNIWVPTKTRLRHGRGLNFGSNDYFQ..DDDDFQR
Ae. aegypti YEYFPGFAGEEMWNPNTNVSEDCLYLNIWVPTKTRLRHGRGLNFGNNDYFQ..DDDDFQR
D. melanogaster YEYFPGFSGEEIWNPNTNVSEDCLYINVWAPAKARLRHGRGANGGEHPNGKQADTDHLIH
L. decemlineata YEYFPGFEGEEMWNPNTNISEDCLYLNIWVPQRLRIRHHA.....DKPTIDR
1 10 20 30 40
60 70 80 90 100 110
C. pipiens Q...HQSKGGLAMLVWIYGGGFMSGTSTLDVYNAEILAAVGNVIVASMQYRVGAFGFFYL
A. stephensi Q...HQSKGGLAMLVWIYGGGFMSGTSTLDIYNAEILAAVGNVIVASMQYRVGAFGFLYL
Ae. aegypti Q...HQSKGGLAMLVWIYGGGFMSGTSTLDVYNAEMLAAVGNVIVASMQYRVGSFGFFYL
D. melanogaster NGNPQNTTNGLPILIWIYGGGFMTGSATLDIYNADIMAAVGNVIVASFQYRVGAFGFLHL
L. decemlineata P.....KVPVLIWIYGGGYMSGTATLDVYDADIIAATSDVIVASMQYRLGSFGFLYL
50 60 70 80 90
120 130 140 150 160 170
C. pipiens SPYL.NGREEEAPGNVGLWDQALAIRWLKENAKAFGGDPDLITLFGESAGGSSVSLHLLS
A. stephensi APYI.NGYEEDAPGNMGMWDQALAIRWLKENAKAFGGDPDLITLFGESAGGSSVSLHLLS
Ae. aegypti APYL.N..DDDAPGNVGLWDQALAIRWLKENAKAFGGDPDLITLFGESAGGSSVSLHLLS
D. melanogaster APEMPSEFAEEAPGNVGLWDQALAIRWLKDNAHAFGGNPEWMTLFGESAGSSSVNAQLMS
L. decemlineata NRYFPRG.SDETPGNMGLWDQILAIRWIKDNAAAFGGDPDLITLFGESAGGGSISIHLIS
110 120 130 140 150
180 190 200 210 220 230
C. pipiens PATRGLSHRGILQSGTLNAPWSHMTAEKALSVAESLIDDCNCNVTLLKDSPGSVMHCMRN
A. stephensi PVTRGLSKRGILQSGTLNAPWSHMTAEKALQIAEGLIDDCNCNLTMLKESPSTVMQCMRN
Ae. aegypti PVTRGLSRRGILQSGTLNAPWSHMSAEKALSVAEALIDDCNCNVTLLKDNPNYVMNCMRN
D. melanogaster PVTRGLVKRGMMQSGTMNAPWSHMTSEKAVEIGKALINDCNCNASMLKTNPAHVMSCMRS
L. decemlineata PVTKGLVRRGIMQSGTMNAPWSYMSGERAEQIGKILIQDCGCNVSLLENSPRKVMDCMRA
170 180 190 200 210
240 250 260 270 280 290
C. pipiens VDAKTISVQQWNSYSGILGFPSAPTIDGVFMTADPMTMLREANLEGIDILVGSNRDEG
A. stephensi VDAKTISVQQWNSYSGILGFPSAPTIDGVFMTADPMTMLREANLEGIDILVGSNRDEG
Ae. aegypti VDAKTISVQQWNSYSGILGFPSAPTIDGVFMTADPMTMLREANLEGVEILVGSNRDEG
D. melanogaster VDAKTISVQQWNSYSGILSFPSAPTIDGAFLPADPMTLMKTADLKDYDILMGNVRDEG
L. decemlineata VDAKTISLQQWNSYSGILGFPSTPTIEGVLLPKHPMDMLAEGDYEDMEILLGSNHDEG
230 240 250 260 270 276

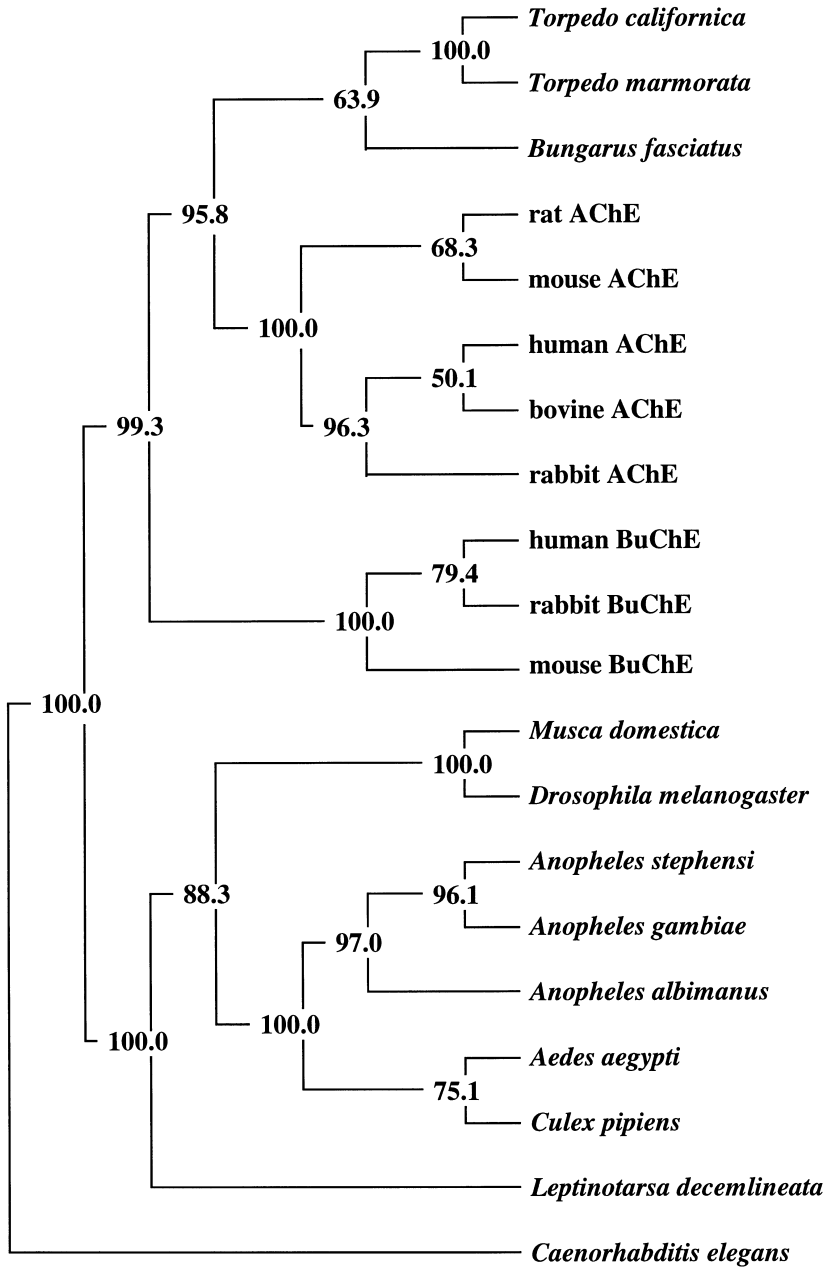


Figure 4. Phylogenetic tree based on inferred amino acid sequences from published insect AChE gene sequences (ESTHER database; Cousin *et al*., 1996) and unpublished sequence for *An. gambiae* (Banks *et al*.) and *An. albimanus* (Hall *et al*.). Only sequences corresponding to exons 2–5 (with reference to *An. stephensi Ace*) are included.

Clements, 1992). Thus, the offspring of the S × M cross between *mC/mC* females and *mA/MB* males gave *mC/mA* females and *mC/MB* males. The backcross (S × M) × M between *mC/mA* females and *mA/MB* males gave *mA/mA* and *mA/mC* females, plus *mA/MB* and *mc/MB* males. The backcross of S × (S × M) between *mC/mC* females and *mC/MB* males gave *mC/mC* females and *mC/MB* males. Finally, the backcross M × (S × M) between *mA/mA* females and *mC/MB* males gave *mC/mA* females and *mA/MB* males. No recombinants between *Ace.x* and the sex locus were detected (n = 196), indicating that the two loci are at less than 1.5 units of recombination at the 0.05 confidence level.

Figure 3. Alignment of inferred amino acid sequences from published insect AChE gene sequences. Only sequences corresponding to exons 2–5 (with reference to *An. stephensi Ace)* are included (Anthony *et al.*, 1995; Hall & Malcolm, 1991; Hall & Spierer, 1986; Malcolm & Hall, 1990; Zhu *et al.*, 1996).

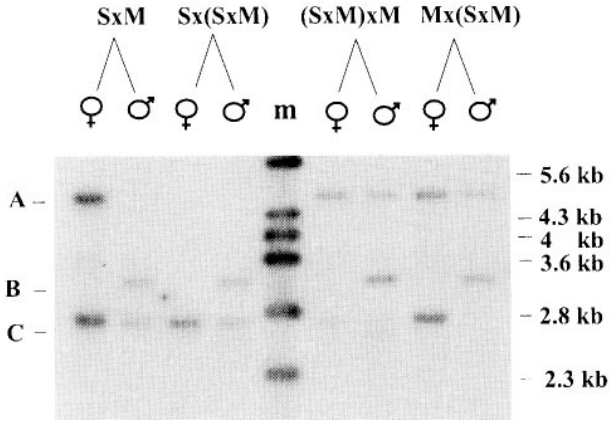


Figure 5. RFLP profiles of males and females of cross S × M and backcrosses S × (S × M), (S × M) × M and M × (S × M) obtained on genomic DNAs using a 700 bp *Ace.x* probe. m = marker.

RFLP on PCR products. The 700 bp fragments of *Ace.x* amplified from MSE and S-lab DNAs, then digested with *Sca* I, are shown in Figs 5 and 6. Both strains produced 230 bp fragments, but in S-lab the remaining 470 bp fragment was cut into 350 bp and 120 bp. This polymorphism is described here as allele D (470 and 230 bp), characteristic of MSE, and allele E (350, 230 and 120 bp), characteristic of S-lab.

Male and female offspring of cross S × M and three backcrosses were analysed to determine their genotypes (Figs 6 and 7 and Table 2). As expected, all offspring from S × M displayed four bands (i.e. 470, 350, 230 and 120 bp) corresponding to the heterozygous *DE* genotype. If *Ace.x* is autosomally inherited, the backcrosses are expected to produce 50% homozygotes (*DD* or *EE* depending on the backcross considered) and 50% heterozygotes *DE* in both sexes. This was the case for backcross (S × M) × M with heterozygous female parents, but not for the other two where the male parent was heterozygous, thus confirming that *Ace.x* is close to the sex locus. No recombinants between *Ace.x* and the sex locus were detected (n = 165), giving a genetic distance between the two loci below 1.8 units of recombination at the 0.05 confidence level.

Both methods therefore indicate that the *Ace.x* gene is tightly linked to the sex locus; taken together no

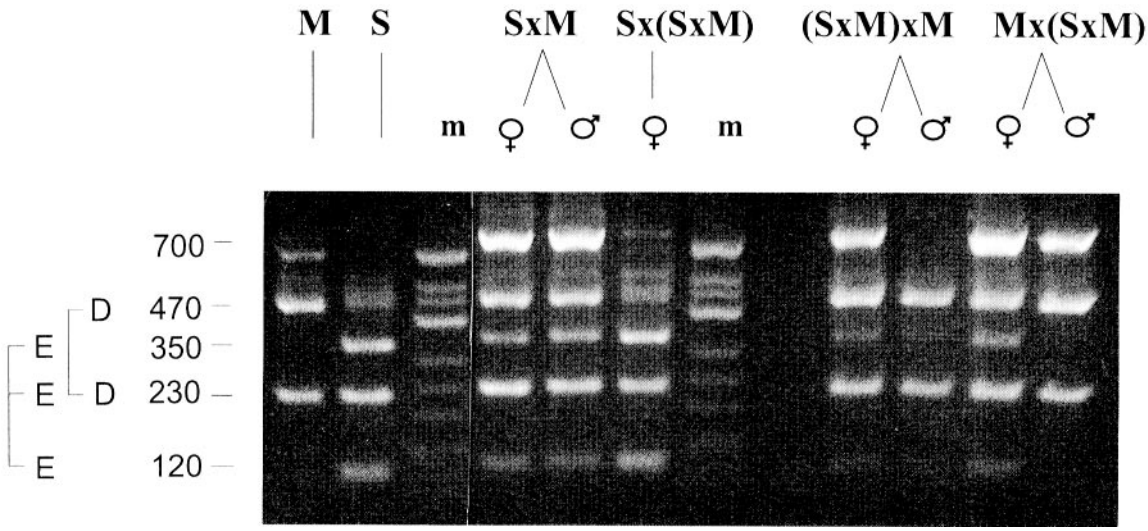


Figure 6. RFLP profiles of males and females of cross S × M and backcrosses S × (S × M), (S × M) × M and M × (S × M) obtained on a 700 bp PCR product of *Ace.x* probe. m = marker.

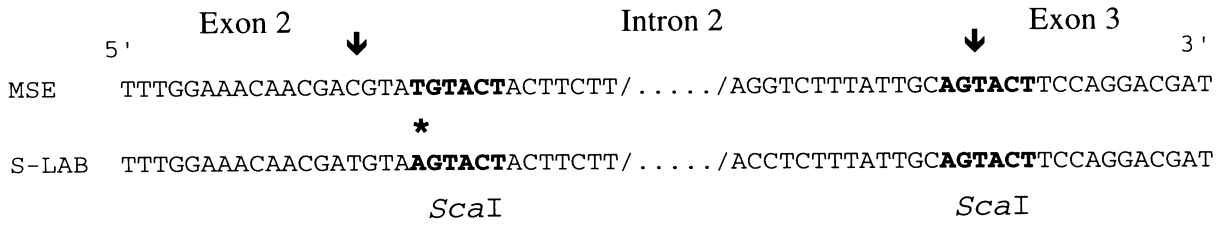


Figure 7. *Sca*I restriction sites on the 700 bp fragment. Mosquitoes from the S-LAB strain possesses two restriction sites whereas only one is present on individuals from the MSE strain. This difference was due to a single pair base mutation (·). Thus, the *Sca* I restriction site (located at the beginning of the intron 2), allows the discrimination between alleles of each strain. Arrows indicate the intron/exon junctions.

Table 2. *Ace.x* genotypes of offspring of cross S × M and backcrosses S × (S × M), (S × M) × M and M × (S × M) determined by RFLPs on a 700 bp PCR product of *Ace.x* (see Fig. 3).

	Offspring genotypes							
	Parental genotypes		Females			Males		
Crosses	Females	Males	*EE*	*ED*	*DD*	*EE*	*ED*	*DD*
S × M	*EE*	*DD*	0	15	0	0	15	0
S × (S × M)	*EE*	*ED*	40	0	0	0	39	0
(S × M) × M	*ED*	*EE*	0	10	9	0	13	15
M × (S × M)	*DD*	*ED*	0	42	0	0	0	44

Table 3. *Ace.1* genotypes of offspring of cross S × M and backcrosses S × (S × M) and M × (S × M) determined by characterizing the inhibition characteristics of AChE enzyme in single mosquitoes. *R* and *S* alleles correspond to $Ace.1^R$ and $Ace.1^S$ respectively.

	Offspring genotypes							
	Parental genotypes		Females			Males		
Crosses	Females	Males	*SS*	*RS*	*RR*	*SS*	*RS*	*RR*
S × M	*SS*	*RR*	0	96	0	0	96	0
S × (S × M)	*SS*	*RS*	31	33	0	32	32	0
M × (S × M)	*RR*	*RS*	0	29	35	0	31	33

recombinants were detected from 361 individuals giving a genetic distance below 0.8 centimorgans at the 0.05 confidence level.

Inheritance of the Ace.1 *gene*

Ace.1 inheritance was investigated by analysing the inhibition properties of the AChE1 enzyme in the offspring of various crosses (Table 3). As expected, all mosquitoes from the S strain were $Ace.1^{SS}$ and all those from the M strain $Ace.1^{RR}$. All mosquitoes of the F1 (S × M) offspring were $Ace.1^{RS}$ heterozygous, and segregation of the *Ace.1* alleles in offspring of backcrosses S × (S × M) and M × (S × M) was consistent with an autosomal localization of *Ace.1*. In particular, there were no difference in the proportions of males and females carrying the same genotype ($P > 0.8$), as well as in the expected proportion of $Ace.1^{RS}$ genotypes (Table 3).

To further confirm that insecticide resistance conferred by $Ace.1^R$ is not sex-linked, the sex ratio in offspring of backcross S × (S × M) was studied among mosquitoes which had survived different insecticide exposures (Table 4). The insecticide doses used completely inhibit sensitive AChE1, so that no $Ace.1^{SS}$ individuals survive, $Ace.1^{RS}$ mosquitoes are partly resistant and $Ace.1^{RR}$ individuals are unaffected. If males and females from backcross S × (S × M) have different *Ace.1* genotypes, a sex-ratio bias is expected after insecticide treatments. Compared with the control, no sex ratio bias was observed in mosquitoes which had survived exposure to propoxur doses inducing a mortality of 29% and 95% or to a chlorpyrifos dose inducing 74% mortality (Table 4).

Finally, the linkage relationships of *Ace.1* with *plum eye* was investigated. This gene is a marker of one of the two autosomes localized in linkage group III of *C. pipiens*. The strain YPL, which is homozygous for the sensitive AChE1 allele and plum eye, was used in crosses with MSE, which is homozygous for insensitive AChE1 and wild-type eye. The eye phenotype of offspring from the backcross Y × (Y × M), reared in absence of insecticide (control), or exposed during 24 h to 0.025 ppm of chlorpyrifos, were compared. In the control, the proportion of eye phenotypes was not different from 1:1 ($P > 0.5$). Chlorpyrifos gave a mortality rate of 65 ± 3%. As this insecticide concentration kills all susceptible $Ace.1^{SS}$ mosquitoes, survivors were only heterozygous ($Ace.1^{RS}$). Among them, 254 had wild-type eyes, indicating a $plAce.1^S/+$ $Ace.1^R$

Table 4. Larval mortality and sex ratio of offspring of S × (S × M) cross in the presence of various concentrations of two insecticides: propoxur (a carbamate) and chlorpyrifos (an organophosphate). For each concentration, larval mortality was estimated after 24 h of insecticide exposure and sex-ratio were calculated on adults. Differences between the number of surviving larvae and of adults are due to delayed mortality. Fisher's exact tests were performed to evaluate sex ratio biases.

	Dose[a]	% Mortality (*n*)[b]	Sex ratio[c] (*n*)[d]	*P*
Propoxur	0	0 (280)	1.17 (276)	–
	2	29 (320)	1.21 (203)	0.93
	10	95 (1200)	1.39 (55)	0.65
Chlorpyrifos	0	0 (120)	0.88 (119)	–
	0.02	74 (360)	0.79 (68)	0.76

[a] Insecticide concentration in mg/l. [b] Number of larvae tested. [c] Ratio males/females. [d] Number of adult emerged.

Table 5. Linkage relationship between chlorpyrifos resistance (*Ace.1*^{R}) and eye phenotype controlled by the *plum-eye* locus, observed in the offsprings of backcross Y × (Y × M).

	Phenotypes		
	Wild-type	Plum	Total
Control	103	106	209
Selected[a]	254	5	259

[a] 0.025 ppm chlorpyrifos.

genotype, and five displayed a *plum-eye* phenotype indicating a *plAce.1*^{R}/*plAce.1*^{S} genotype (Table 5). The gene coding chlorpyrifos resistance (i.e. *Ace.1*) is therefore significantly ($P < 0.0001$) associated with the *plum-eye* locus located on the third linkage group. The proportion of recombinant survivors gives an estimate of the genetic distance between the *plum-eye* and *Ace.1* loci of 2 centimorgans.

Discussion

Since other mosquito AChE genes (Malcolm & Hall, 1990; Hall & Malcolm, 1991; Malcolm *et al*., 1992; Hall *et al*. and Banks *et al.*, unpubl.) contain small intron, we decided to use genomic DNA clones for the analysis of the *C. pipiens* gene. However, except for intron 4, the gene apparently contains large introns and is thus more similar to that of *D. melanogaster* (Fournier *et al*., 1989). Nevertheless, this approach revealed evidence of heterozygosity at an AChE gene when homozygosity was expected; i.e. an observation that would have been overlooked by analysis of cDNA alone.

The inferred amino acid sequence from *Ace.x* shows a high level of identity with the other dipteran AChEs (Fig. 3) confirming that the gene codes for an AChE. The partial gene organisation implied from comparison to other dipteran AChE genes gives intron exon boundaries identical to the *An. stephensi* AChE gene including the additional intron between exons 3 and 4 (Malcolm & Hall, 1990; Hall & Malcolm, 1991), which is absent in *D. melanogaster* (Fournier *et al.*, 1989).

Despite the RFLP found in the six genomic clones, DNA sequence and cross-hybridization data supported the view that the clones contained alleles of the same gene. This is consistent with the strategy used for screening the genomic DNA library, which employed a homologous probe containing an intron. The polymorphism suggests that this AChE gene has not been subjected to strong selection, whereas resistance to organophosphates in this strain was selected to fixation. This can be compared with results from studies on the flanking regions of certain non-specific esterase genes conferring organophosphate resistance in this species, which show no RFLP despite a widespread geographical distribution (Raymond *et al*., 1991; Raymond & Pasteur, 1996).

The results of the various crosses used in the linkage analysis show that *Ace.x* is tightly linked to the sex locus (at less than 0.8 centimorgans) whereas *Ace.1* is associated with *plum-eye* (at less than 2 centimorgans). The sex determining locus belongs to linkage group I which corresponds to the smallest pair of chromosomes I (Bhalla *et al.*, 1974). *Plum-eye* locus belongs to linkage group III (Guptavanij & Barr, 1979), which corresponds to the medium size chromosome II (Heyse *et al.*, 1996). These results show unambiguously that *Ace.x* and *Ace.1*, the gene involved in insecticide resistance due to modification(s) of AChE1, are distinct. The absence of any difference between males and females at the *Ace.1* locus was previously observed in natural population studies (Raymond & Marquine, 1994), and under laboratory conditions in crosses involving several resistant strains (Bourguet & Raymond, unpublished results). Thus, since *Ace.x* is not *Ace.1*, we propose that it is *Ace.2*, and codes for the AChE2 protein recently identified by Bourguet *et al.* (1996a). Unfortunately, no variant of the AChE2 enzyme is presently known (i.e. AChE2 possess the same biochemical properties in all strains studied of *C. pipiens*, unpublished data) so that it is not possible to provide conclusive evidence that this protein is coded by a sex-linked gene.

The occurrence of two cholinesterases is now documented in several species. In vertebrates the situation is as follows. Gnathostome species possess two related cholinesterases, acetylcholinesterase and butyrylcholinesterase, differing by their substrate specificity (Massoulié *et al.*, 1993). These two cholinesterases are encoded by distinct genes with approximately 50% homology at the amino acid level (Taylor, 1991). If the distinction between AChE and BuChE is usually clear in birds and mammals, these two enzymes are more similar in bony and cartilaginous fishes (e.g. Toutant *et al.*, 1985). The situation is different in agnathan vertebrates, as only a single cholinesterase was identified in the hagfish *Myxine glutinosa* and the lamprey *Petromyzon marinus* (references in Sutherland *et al.*, 1997). These observations have been interpreted as the possible occurrence of a gene duplication in an ancestor of the elasmobranchs accompanied by subsequent divergent evolution producing AChE and BuChE in the jawed vertebrates (Chatonnet & Lockridge, 1989). Recently, Sutherland *et al.* (1997) have reported the presence of two cholinesterase activities and genes in the cephalocordate amphioxus *Branchiostoma floridae* and *B. lanceolatum*. Phylogenetic analysis suggests that these two

genes are a result of a duplication event in the lineage of the amphioxus (Sutherland *et al.*, 1997), although this scenario has a relative low (76%) bootstrap support.

In invertebrates the presence of two cholinesterases has been reported in the nematode *Steinernelma carpocapsae* (Arpagaus *et al.*, 1992), the medicinal leech *Hirudo medicinalis* (Talesa *et al.*, 1995a), the snail *Helix pomatia* (Talesa *et al.*, 1995b) and the oyster *Crassostrea giga* (Bocquené *et al.*, 1997). Moreover, the nematode *Caenorhabditis elegans* possesses three genes encoding three different cholinesterases with specific kinetic and pharmacological properties (Arpagaus *et al.*, 1992).

The presence of two AChEs in *C. pipiens* is of special interest since it was thought that insects only possess one such enzyme. This raises the question as to whether it is a general feature in mosquito genera or is restricted to *C. pipiens*. The *Ace* genes of *An. stephensi, An. albimanus, An. gambiae* and *A. aegypti* (Hall & Malcolm, 1991; Anthony *et al.*, 1995; Hall *et al.*, unpubl.; Banks *et al.*, unpubl.) have strong nucleotide and deduced amino acid sequence homologies with the *Ace.x* gene of *C. pipiens*. The relatedness of the genes is entirely consistent with the relatedness of the species (Fig. 4) and suggests that they correspond to the same gene. If that is the case, the *Ace* genes presently cloned from all five mosquito species do not code for the AChE involved in the synapse junction and hence in insecticide resistance. For *An. albimanus* this hypothesis is supported by the absence of differences in the deduced amino-acid sequence of an *Ace* from a strain homozygous for insecticide insensitive AChE, compared to a cloned *Ace* from a susceptible strain (Hall *et al*., unpublished data). The presence of a valine residue at position 86 in *Ace.x*, which is also present *in A. aegypti Ace* (Anthony *et al*., 1995), may also be worth noting (Fig. 3). This is isoleucine in *D. melanogaster*, but a change to a valine in this species is associated with a low level of insecticide insensitivity (Fournier *et al*., 1992; Mutéro *et al*., 1994). The presence of a valine may have a role in the insecticide insensitivity of AChE2 (Bourguet *et al.*, 1996a).

An alternative proposal is that *C. pipiens* is a special case and that *Ace.2* is the consequence of a recent duplication and transposition. Amplification of non-specific esterase genes in organophosphate-resistant strains of *C. pipiens* is a well-established phenomenon (e.g. Pasteur & Raymond, 1996) and recently evidence for a duplication of the *Ace.1* locus has been reported (Bourguet *et al.*, 1996b). Furthermore, biochemical and pharmacological studies of the acetylcholinesterase activity of several mosquito species (including *A. aegypti* and *A. stephensi*) did not reveal the presence of two acetylcholinesterase enzymes (Bourguet *et al.*, 1997).

Experimental procedures

Mosquito strains

Three strains of *Culex pipiens* s.l. were used: MSE (M) which is homozygous for an insecticide insensitive AChE1 (Bourguet *et al*., 1996c; Raymond *et al*., 1987); S-LAB (S) which is a standard insecticide susceptible strain (Georghiou *et al*., 1966) and YPL (Y) which is homozygous for the linkage group III marker *plum-eye* and is insecticide susceptible (Guptavanij & Barr, 1979).

Cloning and DNA sequence of Ace.x

Unless otherwise stated, all molecular biology procedures followed standard protocols described in Sambrook *et al*. (1989) or manufacturer's instructions. Genomic DNA from the MSE strain was extracted following the procedure described by Raymond *et al*. (1989). Extraction of other mosquito DNA samples followed a modified version of the procedure described by Cockburn & Seawright (1988). The DNA was prepared from individual insects, or batches of not more than five. Insects were placed in 50 ml of extraction buffer (1% SDS, 50 mM Tris HCl pH 8.0, 25 mM NaCl, 25 mM EDTA), soaked for 1–5 min and then homogenized. A further 100 μl of extraction buffer was added and the tubes incubated at 68°C for 15 min. 100 μl of 3 M potassium acetate (pH 7.2) was added, the tubes were inverted several times and left on ice for 5 min and then centrifuged at high speed for 3 min. The supernatant was removed and the DNA/RNA precipitated with 2 volumes of ice cold 100% ethanol. Where necessary, RNA was removed by resuspending the DNA/RNA pellet in 0.5 μg/ml pancreatic RNase in 10 mM Tris-HCl (pH 7.5).

PCRs of genomic DNA using the degenerate primers F and G were run as previously described (Malcolm *et al.*, 1992). The primers were as follows: F 5′-GA(AG)GA(AG)ATGTG-GAA(TC)CC (17 mer); G 5′-ACCTA(GTA)AT(GA)CC(CTGA)CCA (14 mer). PCRs using specific primers are described below.

A genomic DNA library was prepared in the replacement bacteriophage vector λDash® II and the bacterial strains P2392 and LE392 supplied by the manufacturer (Stratagene). 10 μg of MSE genomic DNA was partially digested with *Sau*3A. Aliquots of the digest were removed after 5, 15 and 25 min and the digest stopped by rapid phenol/chloroform extraction and ethanol precipitation. The digested DNA was then treated with calf intestinal alkaline phosphatase. The alkaline phosphatase was inactivated and the samples were once again phenol/chloroform extracted and the DNA ethanol precipitated. The digests were checked on electrophoresis and the aliquot containing fragments within the optimum size range of 18–23 kb was used to set up ligations with *Bam* HI *Hind* III digested λDash® II. The library was screened unamplified by plating out batches of 25,000 recombinants using Nunc 24 × 24 cm tissue culture dishes and preparing plaque lifts with Hybond-N™ (Amersham International). DNA probes were labelled with [α-^{32}P]dCTP (specific activity 3000 Ci/mmol, ICN Pharmaceuticals Inc.) using the Pharmacia Ready-To-Go™ DNA labelling kit. Maxi-preparations of DNA from isloated λDash® II clones were obtained following the protocol described by Perbal (1988).

The Pharmacia T7 Sequencing™ Kit was used for DNA sequencing of both single- and double-stranded DNA templates. Multiple sequence alignment was performed by the program of Corpet (1988) using Dayhoff replacement values.

The phylogenetic tree was prepared using sequences obtained from the ESTHER database (Cousin *et al*., 1996). Heuristic searches of the most parsimonious trees were carried out on the data with both the PROTPARS program of the PHYLIP 3.57 package (Felsenstein, 1995) and PAUP version 3.1.1 (Swofford, 1993), performed with the zero-length branches collapse option. Only potentially informative sites were used for phylogenetic reconstruction, i.e. those with at least two amino acid states shared by more than one sequence. Single-site gaps were treated as a new state. To evaluate the internal support of the parsimony tree, bootstrap values were calculated from 1000 replicates of heuristic searches on the whole data set using the combination of the three programs SEQBOOT, PROTPARS and CONSENSE of the former package.

Inheritance of Ace.x

F1 crosses and backcrosses were obtained by mass-crossing adults as follows (noting the female parent first): S × M: S-LAB × MSE; S × (S × M): S-LAB × (S-LAB × MSE); (S × M) × M: (S-LAB × MSE) × MSE; M × (S × M): MSE × (S-LAB × MSE); Y × (Y × M): YPL × (YPL × MSE).

Inheritance of *Ace.x* was studied by RFLPs on PCR products and genomic DNAs. In both cases the two following steps were undertaken: a search of an endonuclease giving distinct restriction patterns in MSE and S-LAB mosquitoes, and an analysis of the inheritance of these patterns.

RFLP on PCR products. For both strains, genomic DNA extraction of 100 mosquitoes was performed as described by Raymond *et al.* (1989). A 700 bp fragment (which includes part of exon 2, intron 2, and part of exon 3) of the *Ace.x* gene was amplified using the oligonucleotide primers F1457 (5′-GAGGAGATGTGGAATCCCAA) and B1246 (5′-TGGAGCCTCCTCTTCACGGC). Amplifications were performed in a 50 μl volume, containing 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.1‰ (w/v) Tween 20, 1.25 mM $MgCl_2$, 250 μM of each dNTP, 100 ng of each primer, 10–100 ng of adult mosquito DNA and 2.5 units of *Taq* polymerase (Eurogentec). The tubes were then quickly transferred to the thermal cycler (Thermocycler Crocodile II, Appligene). After 5 min at 93°C, reactions were cycled thirty-five times through the following temperature profile: 93°C for 1 min, 52°C for 1 min and 72°C for 90 s. The tubes were finally incubated at 72°C for 10 min. 100 μl PCR products from each strain were purified (Geneclean II Kit, Bio 101 Inc.) and resuspended in 20 μl H_2O. Purified PCR products were sequenced following the procedure of Rousset *et al.* (1992) with the primers used for amplification (i.e. F1457 and B1246). A *Sca* I restriction site that discriminates M from S mosquitoes was found in the intron part of the 700 bp fragment (Fig. 3). S mosquitoes possessed two *Sca*I restriction sites but only one is shared with M. Thus, *Sca*I allows an analysis of *Ace.x* inheritance. This was performed on single mosquito genomic DNA obtained following Qiao & Raymond (1995). The 700 bp fragment of *Ace.x* of each individual was amplified as described above. Aliquots of 10 μl of each amplification product were digested with *Sca*I and loaded onto a 1.5% (w/v) agarose gel with TBE buffer.

RFLP on genomic DNA. For each strain, genomic DNA extraction of 100 mosquitoes was performed according to Raymond *et al.* (1989). DNA was digested with several enzymes (*Sal* I, *Sac* I, *Hin*d III, *Bam* HI, *Eco* RI, *Eco* RV, *Pst* I, *Bgl* II and *Xba* I), run on 0.8% (w/v) agarose gels, and transferred onto a Nylon membrane according to Sambrook *et al.* (1989). Hybridizations were performed by random priming as described in Raymond *et al.* (1989). The probe used for hybridization was the 700 bp fragment of *Ace.x* amplified by PCR (see above). *Eco*RI gave a good discrimination between MSE and S-LAB alleles, thus allowing the analysis of their inheritance which was performed using genomic DNA of single mosquitoes, extracted according to the method of Qiao & Raymond (1995) and digested by *Eco*RI. Gel migration, transfer, probe labelling and hybridization were as above.

Inheritance of Ace.1

Based on biochemical properties of AChE1 protein, two alleles have been described (Bourguet *et al.*, 1996d): $Ace.1^S$ coding an insecticide sensitive AChE1 and $Ace.1^R$ coding a modified (i.e. insecticide resistant) AChE1. S-LAB and MSE mosquitoes are homozygous $Ace.1^{SS}$ and $Ace.1^{RR}$, respectively. Therefore inheritance of *Ace.1* was indirectly investigated by determining the *Ace.1* genotype of single individuals from different crosses according to Bourguet *et al.* (1996d). Briefly, single mosquitoes homogenates in phosphate buffer were centrifuged. Supernatants were used to estimate AChE activity (according to Ellman *et al.*, 1961) in the absence of insecticide (A0) and in the presence of 10^{-4} M (A1) and 10^{-2} M (A2) propoxur (Bayer, Leverkusen, Germany). Activities were measured on a Spectramax 250 (Molecular Devices) at 412 nm over a period of 15 min. Absence of A2 activity (i.e. absence of insensitive AChE1) corresponds to $Ace.1^{SS}$ genotypes. If A2 is not null, then A0 and A1 are compared: identical activity (i.e. indicating an absence of sensitive AChE1) corresponds to $Ace.1^{RR}$ genotypes, whereas a lower A1 activity (i.e. indicating the presence of sensitive AChE1) is characteristic of $Ace.1^{RS}$ genotypes.

Insecticide resistance

Insecticide bioassays were performed in plastic cups on fourth instars as described in Raymond & Marquine (1994). In each test, sets of twenty larvae were exposed to insecticide doses lethal for $Ace.1^{SS}$ but not for $Ace.1^{RR}$. These doses were: 2 and 10 mg /l of propoxur and 0.2 mg /l of chlorpyrifos (Interchim, Montluçon, France) in presence of PB (piperonyl butoxide: FLUKA AG, St Quentin, France; 5 mg/l of PB applied 4 h before the addition of chlorpyrifos). PB inhibits the MFO (mixed function oxidase) eventually involved in chlorpyrifos resistance (see Raymond *et al.*, 1987). To standardize the bioassays, the final concentration of solvent (alcohol) was adjusted to 1%. Controls, where larvae experienced the same conditions except the presence of the insecticide, were run at the same time. As sex determination is not easy on larvae, survivors of 24 h exposure were rinsed in water and reared to adults.

Ace.1 *chromosomal location*

Plum-eye phenotype (*pl*) can be distinguished in late fourth-instars and young pupae. In homozygotes *pl/pl*, eye colour varies from light to deep purple and the ocelli of the pupae are much smaller and paler than those of wild type $+/+$. Heterozygotes $pl/+$ have wild-type eyes, as *pl* is recessive. To analyse the association between *Ace.1* and *plum-eye* loci, fourth-instars from the Y $\times$ (Y $\times$ M) backcross were randomly separated into two sets: one (220 larvae) was used as control and the other (850 larvae) was exposed during 24 h to 0.025 ppm chlorpyrifos, a concentration killing all $Ace.1^{SS}$ so that all survivors are $Ace.1^{RS}$. All the late fourth-instars or young pupae were examined visually to determine their eye phenotype.

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