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A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the *ace* gene in *Drosophila*

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Acetylcholinesterase (AChE) is the target of two major insecticide families, organophosphates (OPs) and carbamates. AChE insensitivity is a frequent resistance mechanism in insects and responsible mutations in the *ace* gene were identified in two Diptera, *Drosophila melanogaster* and *Musca domestica*. However, for other insects, the *ace* gene cloned by homology with *Drosophila* does not code for the insensitive AChE in resistant individuals, indicating the existence of a second *ace* locus. We identified two AChE loci in the genome of *Anopheles gambiae*, one (*ace-1*) being a new locus and the other (*ace-2*) being homologous to the gene previously described in *Drosophila*. The gene *ace-1* has no obvious homologue in the *Drosophila* genome and was found in 15 mosquito species investigated. In *An. gambiae*, *ace-1* and *ace-2* display 53% similarity at the amino acid level and an overall phylogeny indicates that they probably diverged before the differentiation of insects. Thus, both genes are likely to be present in the majority of insects and the absence of *ace-1* in *Drosophila* is probably due to a secondary loss. In one mosquito (*Culex pipiens*), *ace-1* was found to be tightly linked with insecticide resistance and probably encodes the AChE OP target. These results have important implications for the design of new insecticides, as the target AChE is thus encoded by distinct genes in different insect groups, even within the Diptera: *ace-2* in at least the Drosophilidae and Muscidae and *ace-1* in at least the Culicidae. Evolutionary scenarios leading to such a peculiar situation are discussed.

Keywords: evolution; acetylcholinesterase; insect; insecticide resistance

1. INTRODUCTION

Acetylcholinesterase (AChE, enzyme commission nomenclature EC 3.1.1.7) terminates synaptic transmission at cholinergic synapses in the central nervous system of insects, by rapid hydrolysis of the neurotransmitter acetylcholine (Toutant 1989). Numerous studies have focused on insect AChE because it is the target of organophosphates (OPs) and carbamates, two major classes of pesticides used for pest management in agriculture and public health. Target (AChE) insensitivity has been described in many species (see the review in Fournier & Mutéro 1994).

To identify the mutation(s) reducing target sensitivity and thus conferring insecticide resistance, genes encoding AChE (i.e. *ace* genes) have been cloned and sequenced. The first invertebrate *ace* gene was cloned in *Drosophila melanogaster*, by means of reverse genetics. The final identification of the gene was based on the homology with *Torpedo* AChE (Hall & Spierer 1986; Fournier *et al.* 1989). Evidence that this gene coded a functional AChE in cholinergic synapses came from the identification, in resistant strains, of point mutations providing insensitivity towards cholinergic insecticides (Fournier *et al.* 1993; Fournier & Mutéro 1994; Mutéro *et al.* 1994). Numerous studies in *D. melanogaster*, using the segmental aneuploidy technique and mutagenesis, indicated that only one gene encoded AChE (Hall & Kankel 1976; Greenspan *et al.* 1980; Fournier & Mutéro 1994). In this species, germline transformation of a minigene rescued lethal mutations, definitively demonstrating the presence of a unique gene coding for AChE in cholinergic synapses (Hoffmann *et al.* 1992). From this work in *Drosophila*, it was assumed that only one *ace* gene was present in insects.

In other arthropods, *ace* genes have been cloned by homology with the *ace* of *D. melanogaster*. In the housefly *Musca domestica* and the Colorado potato beetle *Leptinotarsa decemlineata*, the cloned *ace* genes seem to be involved in resistance, as indicated by the identification of one or several mutations in strains with an insensitive AChE (Zhu *et al.* 1996; Kozaki *et al.* 2001; Walsh *et al.* 2001). However, in several other arthropod species, the cloned *ace* gene codes for an AChE that is apparently not involved in resistance. Two lines of evidence support this conclusion: (i) absence of non-synonymous point mutations between susceptible and resistant strains (*Aphis gossypii*, *Nephotettix cincticeps*, *Boophilus microplus* (Baxter & Barker 1998; Hernandez *et al.* 1999; Menozzi 2000; Tomita *et al.* 2000)), (ii) independent segregation in crosses between the cloned *ace* gene and resistance (*Culex pipiens* (Malcolm *et al.* 1998) and *Cx. tritaeniorynchus* (Mori *et al.* 2001)). Involvement of *ace* genes in resistance in other arthropods

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is not known, either because insensitive AChE has not been described in some species (i.e. *Aedes aegypti*, *Anopheles gambiae*, *An. stephensi*), or because relevant evidence has not, to our knowledge, been published yet (e.g. *Lucilia cuprina*, *Schizaphis graminum*). So, for most insects studied, the gene encoding the OP target remains to be identified.

Two hypotheses may explain cases where the cloned *ace* gene did not show mutations associated with resistance: the 'modifier gene' hypothesis and the 'two *ace* genes' hypothesis. In the first case, the *ace* structural gene is indeed involved in the resistance, but resistance is the result of post-transcriptional or post-translational modifications controlled by a 'modifier' gene, leading to an AChE enzyme with distinct inhibition properties. Only the modifier gene is thus linked with resistance, explaining the genetic independence between resistance and the *ace* structural gene in crosses. Present data do not support this hypothesis. For example, alternative mRNA splicing of the *ace* gene in vertebrates gave rise to two polypeptides with identical catalytic properties (Massoulié *et al.* 1993).

In the second case, resistance is conferred by an *ace* gene that is different from the one already cloned. This hypothesis was first proposed when two types of AChE were found in the mosquito *Cx. pipiens*, with distinct catalytic properties (Bourguet *et al.* 1996). Although two *ace* genes have been identified in Arachnidae (Baxter & Barker 1998; Hernandez *et al.* 1999), intensive searches for a second *ace* gene in several insect species has remained unsuccessful (Severson *et al.* 1997; Menozzi 2000; Mori *et al.* 2001; Tomita *et al.* 2000; see however Gao *et al.* 2002). This indicates that if a second *ace* gene exists in insects, its divergence from the first one complicates the cloning by homology with the first gene by classical PCR and Southern blotting techniques.

Here, we have taken advantage of the available genomic sequences of *An. gambiae* to search for loci encoding for AChE proteins. We identified two loci, one being a new *ace* locus. This locus is present in several mosquito species and is tightly linked with insecticide resistance in *Cx. pipiens*. Comparison of available *ace* sequences indicates a complex evolution, including a modification of physiological function between the two genes within Diptera.

2. MATERIAL AND METHODS

(a) *Strains and crosses*

Five strains of *Cx. pipiens* were used: S-LAB, which is a standard insecticide-susceptible strain (Georghiou *et al.* 1966), SA1, SA4 and EDIT, which display only a sensitive AChE, and SR, which is homozygous for an insensitive AChE (Berticat *et al.* 2002).

(b) *Nomenclature of* ace *genes and numbering of amino acids*

For clarity, we propose a consistent nomenclature of *ace* genes across insects, using mosquitoes as the reference. Thus *ace-1* designates the locus coding for a cholinergic AChE (or AChE1), responsible for OP and carbamate resistance in *Cx. pipiens* (it was previously named *Ace.1*; Raymond *et al.* 2001) and *ace-2* refers to the second *ace* locus, not involved in insecticide resistance in *Cx. pipiens* (previously named *Ace.2*), its function being unknown in *Cx. pipiens*. The unique *ace* gene in *D. melanogaster*, being homologous to *ace-2* (see § 3), will be referred to as such. By convention, the numbering of amino acids corresponds to that of *Torpedo marmorata* AChE (Massoulié *et al.* 1992).

(c) *Inheritance of* ace-1

Noting the female parent first, F_1 crosses ($F_1 = S \times R$) and backcrosses ($F_1 \times$ S-LAB and S-LAB $\times F_1$) were obtained by mass-crossing adults. S refers to strains with a sensitive AChE and R designates the strains with an insensitive AChE. Some backcross larvae were treated with a dose ($4\ \mathrm{mg\ l^{-1}}$) of propoxur (a carbamate insecticide) killing 100% of susceptible larvae. Linkage of *ace-1* with propoxur resistance was studied in surviving larvae, by restriction fragment length polymorphism (RFLP) on a 320 bp PCR product of *ace-1* identifying *S* and *R* alleles. This experiment was performed three times independently, with S = SA1, S = SA4 and S = EDIT.

(d) *Database searches and gene assembly*

All searches were performed using sequences from the *An. gambiae* trace archive database through INFOBIOGEN (http://www.infobiogen.fr/) and NCBI Trace Archive Mega BLAST (http://www.ncbi.nlm.nih.gov/blast/) facilities. Genomic sequences encoding AChE were identified using TBLASTIN and BLASTN programs (Altschul *et al.* 1990). Downloaded genomic sequences were assembled using ABI Prism Auto-Assembler (v. 2.1, Perkin Elmer). Sequences were checked and corrected using ENSEMBL Trace Server facilities (http://trace.ensembl.org/). Two contigs of 5195 and 6975 bases (encoding AChE1 and AChE2, respectively) were assembled from 74 and 64 independent sequences (average redundancy 10.5 and 6.5). Identification of exons and proteic sequences was performed using a combination of FGENESH (http://www.sanger.uk) and BLASTX (http://www.ncbi.nlm.nih.gov). In the process of this manuscript being submitted, a full annotation of *An. gambiae* genome data appeared at the ENSEMBL website http://www.ensembl.org/Anopheles—gambiae/. We searched for cholinesterase signature (six motives, as defined by the InterPro Entry IPR000997) and identified seven potential proteins. Two of them were highly significant (i.e. showed matches for all six motives): ENSANGP00000016929, corresponding to AChE1 (gene located on chromosome 2R-7A), while ENSANGP00000020022 corresponded to AChE2 (gene located on chromosome X-1D). The other five showed lower similarity with cholinesterase signature (three motives: ENSANGP00000003191 (gene on chromosome 2R), two motives: ENSANGP00000017380, −5974, −5718 and −21598 (genes on chromosome 2L)). Subsequent BLAST searches indicated that –3191 is related to fatty acyl-CoA hydrolase, −17380 to esterase 6 and −5974, −5718 and –21598 to esterase B.

Ascidian genomic sequences for AChE were assembled from raw sequence data deposited at the NCBI Trace Archive (*Ciona savignyi*) and the Doe Joint Genome Institute (*Ciona intestinalis*, http://www.jgi.doe.gov/programs/ciona/ciona _mainpage.html). Searches in *Drosophila* databases were performed using Flybase facilities (http://www.fruitfly.org/).

(e) *Sequence comparisons*

Deduced *An. gambiae* AChE1 and AChE2 proteins as well as peptides deduced from *Cx. pipiens* and *Ae. aegypti* PCR fragments were aligned with previously known AChE proteins using the CLUSTALW program with a BLOSUM matrix and default settings (Thompson *et al.* 1994). A phylogenetic tree was constructed using the neighbour-joining algorithm of the Clustalw (v. DDBJ, http://hypernig.nig.ac.jp/homology/ex_clustalw-e.shtml). Bootstrap analysis (1000 counts and 111 seed values) was applied to estimate confidence levels for the tree topology. Construction of trees was done using TreeView (v. 1.6.6).

(f) *Accession numbers*

Accession numbers of the sequence retrieved for phylogenetic analysis are as follows. Craniata: *Homo sapiens*: NP_000046; *Bos taurus*: P23795; *Felis catus*: O62763; *Oryctolagus cuniculus*: Q29499; *Rattus norvegicus*: P36136; *Mus musculus*: P21836; *Gallus gallus*: CAC37792; *Danio rerio*: Q9DDE3; *Electrophorus electricus*: 6730113; *T. marmorata*: P07692; *T. californica*: P04058; *Bungarus fasciatus*: Q92035; *Myxine glutinosa* (Hagfish): Q92081. Cephalochordates: *Branchiostoma floridae*: O76998 and O76999; *Ba. lanceolatum*: Q95000 and Q95001. Urochordates: *Ciona intestinalis*: BN000069; *Ci. savignyi*: BN000070. Nematodes: *Caenorhabditis elegans* (1 to 4): P38433, O61371, O61459 and O61372; *C. briggsae* (1 to 4): Q27459, O61378, Q9NDG9 and Q9NDG8; *Dictyocaulus viviparus*: Q9GPL0. Insects: *An. gambiae* (1 and 2): BN000066 and BN00006; *Ae. aegypti* (1 and 2): AJ428049 and AAB3500; *An. stephensi*: P56161; *Cx. pipiens* AJ428047 (for *ace-1*) and Esther database (for *ace-2*); *D. melanogaster*: P07140; *Lu. cuprina*: P91954; *M. domestica*: AAK69132.1; *L. decemlineata*: Q27677; *Apis mellifera*: AAG43568; *N. cincticeps*: AF145235_1; *S. graminum*: Q9BMJ1. Arachnidae: *Rhipicephalus appendiculatus*: O62563; *B. microplus* (1 and 2): O45210 and O61864; *B. decoloratus*: O61987. Molluscs: *Loligo opalescens*: O97110.

(g) *Homologous cloning of* ace-1 *in other mosquitoes*

Mosquito DNA extraction was carried out following Rogers & Bendich (1988). Oligonucleotides PdirAGSG (5′ATMGWGT TYGAGTACACSGAYTGG3′) and PrevAGSG (5′GGCAAA RTTKGWCCAGTATCKCAT3′) amplified a 320 bp fragment (K fragment) on several mosquitoes' genomic DNA. PCR was run for 30 cycles (94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s). Sequences were performed directly on PCR products on an ABI prism 310 sequencer using the Big Dye Terminator kit. In order to detect the expression of *ace-1* mRNA, RT–PCR (reverse-transcription PCR) was performed on RNA extracted with Trizol (Life Technologie) according to the manufacturer's instructions.

Culex pipiens ace-1 genotype test: PCR K fragments were digested by *Eco*R1 and the digestion product was run on a 2% agarose gel. Restriction patterns showed two bands (106 bp and 214 bp) for homozygous *SS* mosquitoes and three bands (106 bp, 214 bp and 320 bp) for heterozygous *RS* mosquitoes.

(h) *Data deposition*

The nucleotide sequences of the genes encoding *An. gambiae* AChE1 and AChE2 proteins have been submitted to DDBJ/EMBL/GenBank with accession numbers BN000066 (*ace-1*) and BN000067 (*ace-2*). Partial *ace-1* nucleotide sequences of *Cx. pipiens* (S-Lab and SR strains) genomic DNA have been submitted with accession numbers AJ428047 and AJ428048, respectively. Partial *ace-1* nucleotide sequences were submitted for several mosquito species: *Ae. aegypti* (AJ428049), *Ae. albopictus* (AJ438598), *An. darlingi* (AJ438599), *An. sundaicus* (AJ438600), *An. minimus* (AJ438601), *An. moucheti* (AJ438602), *An. arabiensis* (AJ438603), *An. funestus* (AJ438604), *An. pseudopunctipennis* (AJ438605), *An. sacharovi* (AJ438606), *An. stephensi* (AJ438607), *An. albimanus* (AJ438608) and *An. nili* (AJ438609). *Ciona intestinalis* and *Ci. savignyi ace* genes have been submitted with accession numbers BN000069 and BN000070, respectively.

3. RESULTS

(a) *Two* ace *genes in* Anopheles gambiae

To identify genes encoding AChE in *An. gambiae*, we used the Tblastn program to search for homologues of human and *Drosophila* AChEs in the *Anopheles* raw genomic sequences deposited recently in public databases. Two distinct groups of fragments were identified that encoded peptides highly similar to *Drosophila* AChE. For each of them, we performed gene reconstruction by merging overlapping sequences. This produced two contigs (*ace-1* and *ace-2*) of 6975 and 5195 bases, respectively. Gene analysis with Fgenesh and Blastx showed that *ace-1* and *ace-2* are made of at least four and eight coding exons, encoding potential polypeptides of 534 and 569 amino acids, respectively. These polypeptides do not represent full-length proteins. Indeed, in the absence of cDNA sequences, we could not determine with a high level of confidence the 5′ and 3′ non-coding sequences, as well as the NH_2 and COOH termini of the proteins, which are not conserved among AChE proteins. Protein analysis confirmed that both proteins are highly homologous to *Drosophila* AChE (BLASTP: $p < e^{-180}$) and contain the canonical 'FGESAG' motif (around position S200, figure 1), characteristic of the active site of cholinesterases. In addition, the following characteristics of AChE are also found in both sequences: the choline binding site at W84, the three residues of the catalytic triad (S200, E327 and H440), the six cysteines potentially involved in three conserved disulphide bonds (67–94; 254–265; 402–521) and the aromatic residues lining the active site gorge (10 and 11 residues for AChE1 and AChE2, respectively). Interestingly, F290 is present and F288 is absent in both sequences, a property of all invertebrate AChE sequences, explaining a wider substrate specificity than vertebrate AChE (Vellom *et al.* 1993). Examination of the C-terminal ends of the deduced amino acid sequences showed, in all available dipteran AChEs, a hydrophobic peptide compatible with a signal for glycolipid addition, indicating that a portion of the C-terminus is cleaved post-translationally and replaced by a glycolipid anchor, as in *Drosophila* and several species of mosquitoes (Gnagey *et al.* 1987; Bourguet *et al.* 1996, 1997). It is also observed, in all cases, that a free cysteine is present in the C-terminus upstream of the putative cleavage site of the hydrophobic peptide (not shown in figure 1). This cysteine could be involved in an interchain disulphide bond linking the dimer of catalytic subunits (Bourguet *et al.* 1996).

Anopheles gambiae AChE1 and AChE2 (respectively encoded by *ace-1* and *ace-2*) are 53% similar and show, respectively, 76% and 55% amino acid similarity with AChE from the aphid *S. graminum* (gi|12958609), 53% and 98% with *An. stephensi* (gi|2494391), 54% and 95% with *Ae. aegypti* (gi|2133626) and 52% and 83% with *Drosophila* (gi|17136862). A major difference between AChE1 and AChE2 is a 31 amino acid insertion in the AChE2 sequence (boxed in figure 1). This region, which is usually referred to as 'the hydrophilic insertion' in *Drosophila* AChE, is

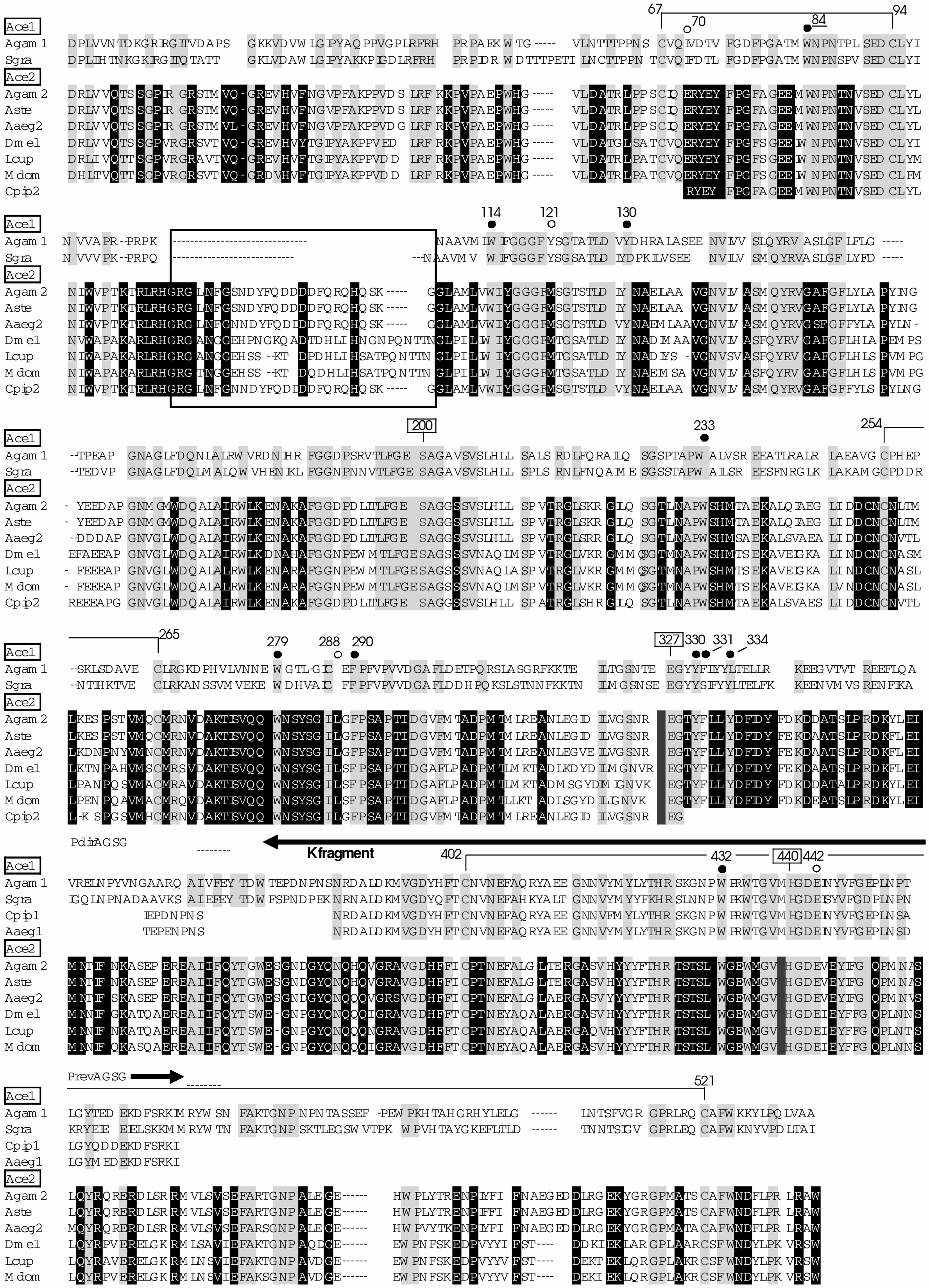

Figure 1. Alignment of AChE1 and AChE2 proteins of *Anopheles gambiae*, *Schizaphis graminum*, *An. stephensi*, *Aedes Aegypti*, *Drosophila melanogaster*, *Lucilia cuprina*, *Musca domestica* and *Culex pipiens*. By convention, numbering is that of *Torpedo*. The N- and C-terminal sequences are not represented because of their variability. Amino acids in grey are conserved for AChE1 and AChE2. Amino acids in black are specific to AChE2. The three residues composing the catalytic triad (S200, E327 and H440) are indicated by a boxed number. Circles represent the position of the 14 aromatic residues lining the active gorge in *Torpedo* AChE, 10 of which are present in all AChE1 or AChE2 (filled circles), the others being non-conserved (open circles). The choline binding site (W at position 84) is underlined. Three intrachain disulphide bridges are drawn between conserved Cys (arrows). The horizontal arrow in bold indicates the position of the amplified K fragment (amplified using PdirAGSG and PrevAGSG primers). The hypervariable region of AChE2, absent in AChE1, is boxed.

absent in vertebrate and nematode AChEs and could be a characteristic of the *ace-2* gene, at least in Diptera.

These data therefore demonstrate the presence of two *ace* genes in the *An. gambiae* genome, one coding for AChE1, closely related to *Schizaphis* AChE, and the other for AChE2, closely related to *Drosophila* AChE and other mosquito AChEs. The presence of additional *ace* genes is highly unlikely, as further searches in the *Anopheles* genomic database using less stringent parameters only detected alpha-esterases (EC 3.1.1) and carboxylesterases (EC 3.1.1.1) sequences (data not shown).

(b) *A single* ace *gene in* Drosophila melanogaster

To determine whether an *ace-1* homologue was present in *Drosophila*, we performed a similar *in silico* screening on this species genome. Tblastn searches readily detected the previously known *ace* gene, homologous to *An. gambiae ace-2*, but failed to detect any other sequence more closely related to *ace-1*. As above, searches with less stringent parameters only detected alpha- and carboxylesterases. This demonstrates that the *Drosophila* genome contains a single *ace* gene (named *ace-2*, following the nomenclature defined above).

(c) *At least two* ace *genes in other mosquitoes*

We next investigated whether a gene homologous to *An. gambiae ace-1* was present in other mosquito species. To do this, we followed a PCR strategy, based on the alignment of *An. gambiae* AChE1 and AChE2 with the protein sequences of other species. We designed degenerated oligonucleotides in an exonic region conserved between *An. gambiae* and *S. graminum* AChE1 sequences (K fragment, see figure 1), but divergent between *An. gambiae* AChE1 and AChE2. PCR amplification of genomic DNAs with PdirAGSG and PrevAGSG yielded a 320 bp K fragment in all species tested. DNA sequencing showed high identity at the nucleotide level between K fragments of *Anopheles*, *Culex* and *Aedes*. Most substitutions are silent ones, because the deduced protein sequences only differ from each other by five to six amino acids (figure 2*a*). The K fragment was also obtained by RT–PCR of *Cx. pipiens* mRNA, indicating that the *ace-1* gene is expressed as mRNA. This is consistent with the existence of two AChEs with distinct catalytic properties in *Cx. pipiens* (Bourguet *et al.* 1996).

(d) *Insecticide resistance and* ace-1 *in* Culex pipiens

To determine whether insecticide resistance is linked to *ace-1*, we first amplified and sequenced the K fragment from genomic DNA of a resistant *Cx. pipiens* (R strain). Sequence comparison of the K fragment between S and R strains showed variations only at the nucleotide level (three silent substitutions, figure 2*b*). One of these substitutions was found to affect an *Eco*R1 site and provided an easy diagnostic to differentiate *ace-1* loci from S and R strains by PCR–RFLP. Linkage between *ace-1* and propoxur resistance was performed in triplicates by treating backross larvae ((S × R) × S) at a dose lethal for susceptible individuals and analysing the survivors by PCR–RFLP. Propoxur exposure killed 50% of the larvae in all of the backcrosses, i.e. all expected susceptible individuals. All surviving larvae (100 for each backcross, 300 in total) displayed a heterozygous RFLP pattern, indicating that they all possessed the *ace-1* copy from the R strain (figure 2*c*). This demonstrates that *ace-1* and resistance are tightly linked (less than 1.0% at the 0.05 confidence level).

(e) *Phylogeny of* ace-1 *and* ace-2

To construct phylogenetic trees, we applied the neighbour-joining method to the conserved regions of *An. gambiae* AChE proteins and to those of 33 species, already deposited in GenBank. We also included partial sequences corresponding to the K fragment from *Cx. pipiens* and *Ae. aegypti*.

The unrooted distance tree (figure 3) illustrates the heterogeneity in the number of *ace* genes within and between phyla: in chordates, cephalochordates show at least two *ace* genes, whereas urochordates have only one *ace* gene, as deduced from the analysis of their complete genomes. In arthropods, Diptera show either one (i.e. *Drosophila*, belonging to the Brachycera suborder) or two (i.e. mosquitoes, belonging to the Nematocera suborder) *ace* genes. The overall topology of the tree shows that these two *ace* sequences have duplicated very early during evolution, probably before the separation between protostomes and deuterostomes. This is supported by the fact that AChE from different phyla (molluscs, nematodes and arthropods) are branched within sequences from the chordate phylum (craniata, cephalochordates and urochordates). Another clue is the presence of two distantly related AChE sequences within arthropods and nematodes. Thus, *ace-1* and *ace-2* found in insects probably derived from a very ancient duplication event. This indicates that the absence of *ace-1* in at least one Brachycera species results from a loss rather than from a recent duplication event in Nematocera.

4. DISCUSSION

(a) *How many* ace *genes in insects?*

Only two insect species, both Diptera, have had their genomes completely sequenced: *D. melanogaster* and *An. gambiae*. *In silico* gene detection in these two genomes disclosed that two genes (*ace-1* and *ace-2*) coding acetylcholinesterase proteins are present in *Anopheles*, whereas only one (*ace-2*) exists in *Drosophila*. The overall topology of the phylogenetic tree constructed from the available AChE sequences of 33 species (figure 3) indicates that the two *Anopheles* genes derived from a duplication that occurred very early in evolution, long before the differentiation of insects. Thus, the presence of these two *ace* genes is an ancestral character and insects will possess both genes, unless one was lost during the evolution of a particular group. Our data showed that such a loss occurred in the Diptera, at least within the Drosophilidae family. These results stress the fact that extrapolations derived from studies of *Drosophila* must be done with caution (the *ace* situation in *Drosophila* being representative neither of the Diptera order nor of the insect class).

(b) *Insecticide resistance and* ace *genes*

The toxicity of OP and carbamate insecticides is due to the inhibition of AChE activity in cholinergic synapses and resistance to these compounds is the result of a reduced inhibition of cholinergic AChE, a phenomenon that has developed following extensive and prolonged use of these

(*a*)

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         1                                                                                80
Ae alb   TEPENPNSNR DALDKMVGDY HFTCNVNEFA QRYAEEGNNV YMYLYTHRSK GNPWPRWTGV MHGDEINYVF GEPLNSDLGY
Ae aeg   TEPENPNSNR DALDKMVGDY HFTCNVNEFA QRYAEEGNNV YMYLYTHRSK GNPWPRWTGV MHGDEINYVF GEPLNSDLGY
An alb   TEPDNPNSNR DALDKMVGDY HFTCNVNEFA QRYAEEGNNV YMYLYTHRSK GNPWPRWTGV MHGDEINYVF GEPLNPTLGY
An gam   TEPDNPNSNR DALDKMVGDY HFTCNVNEFA QRYAEEGNNV YMYLYTHRSK GNPWPRWTGV MHGDEINYVF GEPLNPTLGY
An fun   TEPDNPNSNR DALDKMVGDY HFTCNVNEFA QRYAEEGNNV YMYLYTHRSK GNPWPRWTGV MHGDEINYVF GEPLNPSLGY
An nil   TEPDNPNSNR DALDKMVGDY HFTCNVNEFA QRYAEEGNNV YMYLYTHRSK GNPWPRWTGV MHGDEINYVF GEPLNPSLGY
An sac   TEPDNPNSNR DALDKMVGDY HFTCNVNEFA QRYAEEGNNV YMYLYTHRSK GNPWPRWTGV MHGDEINYVF GEPLNPSLGY
An pse   TEPDNPNSNR DALDKMVGDY HFTCNVNEFA QRYAEEGNNV YMYLYTHRSK GNPWPRWTGV MHGDEINYVF GEPLNPGLGY
Cx Pip   IEPDNPNSNR DALDKMVGDY HFTCNVNEFA QRYAEEGNNV FMYLYTHRSK GNPWPRWTGV MHGDEINYVF GEPLNSALGY

           81        91
Ae alb   MDDEKDFSRK I
Ae aeg   MEDEKDFSRK I
An alb   TDDEKGFSRK I
An gam   TEDEKDFSRK I
An fun   TEDEKDFSRK I
An nil   TEDEKDFSRK M
An sac   TDDEKDFSRK I
An pse   TEDEKDFSRK I
Cx Pip   QDDEKDFSRK I
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(*b*)

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                      •        20          •        40          •        60          •        80
Ace1-SLAB   ATTGAACCGGACAACCCGAACAGCAACCGTGACGCGCTGGACAAGATGGTCGGGATTATCACTTCACCTGCAACGTGAA
Ace1-SR     ATCGAACCGGACAACCCGAACAGCAACCGTGACGCGCTCGACAAGATGGTCGGGATTATCACTTCACCTGCAACGTGAA

             EcoR1    •       100          •       120          •       140          •       160
Ace1-SLAB   CGAATTCGCCCAGCGGTACGCCGAGGAGGGCAACAACGTGTTCATGTACCTGTACACGCACAGAAGCAAAGGAAATCCCT
Ace1-SR     CGAGTTCGCCCAGCGGTACGCCGAGGAGGGCAACAATGTGTTCATGTACCTGTACACGCACAGAAGCAAAGGAAATCCCT

                      •       180          •       200          •       220          •       240
Ace1-SLAB   GGCCGAGGTGGACCGGCGTGATGCACGGCGACGAGATCAACTACGTGTTTGGCGAACCGCTGAACTCGGCCCTCGGCTAC
Ace1-SR     GGCCGAGGTGGACTGGCGTGATGCACGGCGACGAGATCAACTACGTGTTTGGCGAACCGCTGAACTCGGCCCTCGGCTAC

                         •       260          •
Ace1-SLAB   CAGGACGACGAGAAGGACTTTAGCCGGAAAATT
Ace1-SR     CAGGACGACGAGAAGGACTTTAGCCGGAAAATT
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(*c*)

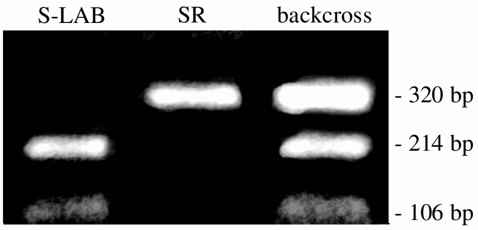

Figure 2. (*a*) Protein sequence comparison of the K fragment from several mosquitoes: Ae alb (*Aedes albopictus*), Aed aeg (*Aedes aegypti*), An alb (*Anopheles albimanus*), An gam (*An. gambiae*), An fun (*An. funestus*), An nil (*An. nili*), An sac (*An. sacharovi*), An pse (*An. pseudopunctipennis*), Cx pip (*Culex pipiens*). Variant amino acids are shaded. The *An. darlingi* protein sequence is identical to the *An. albimanus* sequence. *Anopheles sundaicus* is identical to *An. gambiae* and to *An. arabiensis*. *Anopheles moucheti* is identical to *An. funestus* and to *An. minimus*. *Anopheles stephensi* is identical to *An. sacharovi*. (*b*) Sequence comparison of the K fragments in the susceptible (S-LAB) and resistant (SR) strains. Variant nucleotides are shaded. The *Eco*R1 site used for the PCR-RFLP analysis is present only in the S-LAB strain. (*c*) Electrophoresis of the PCR-RFLP diagnostic test. The K fragment of the S-LAB strain digested with *Eco*R1 gives two bands of 214 bp and 106 bp. The SR profile gives one band of 320 bp and the surviving mosquitoes of the backcross give an heterozygote profile of three bands of 320 bp, 214 bp and 106 bp.

insecticides. It is established beyond doubt that resistance in *D. melanogaster* and *M. domestica* is caused by mutations at the *ace-2* locus (Mutéro *et al.* 1994; Walsh *et al.* 2001). It has been shown for some years that, in *Cx. pipiens*, resistance segregates independently from *ace-2* (Malcolm *et al.* 1998). By contrast, present data showing no recombinant between this character and *ace-1* provide strong evidence that the enzyme encoded by this locus is the cause of resistance. Thus, among Diptera, the same physiological function in cholinergic synapses is fulfilled by *ace-2* in at least two species of the Brachycera suborder (and more precisely of the Muscomorpha infraorder) and by *ace-1* in at least the Culicidae family, which belongs to the Nematocera suborder (McAlpine *et al.* 1981).

Table 1 lists insect species from which an *ace* gene was cloned, indicating its family (*ace-1* or *ace-2* as identified in the present study), as well as available data on the association of this gene and resistance. As in *Cx. pipiens*, resistance was shown to be independent from the *ace-2* locus in *Cx. tritaeniorhynchus*, the Coleoptera *L. decemlineata* and the Homoptera *Ap. gossypii* and *N. cincticeps*. Because the presence of *ace-1* is an ancestral character, this gene may exist in these species and explain resistance. We strongly suspect that this is the case for *Cx. tritaeniorhynchus* by analogy with

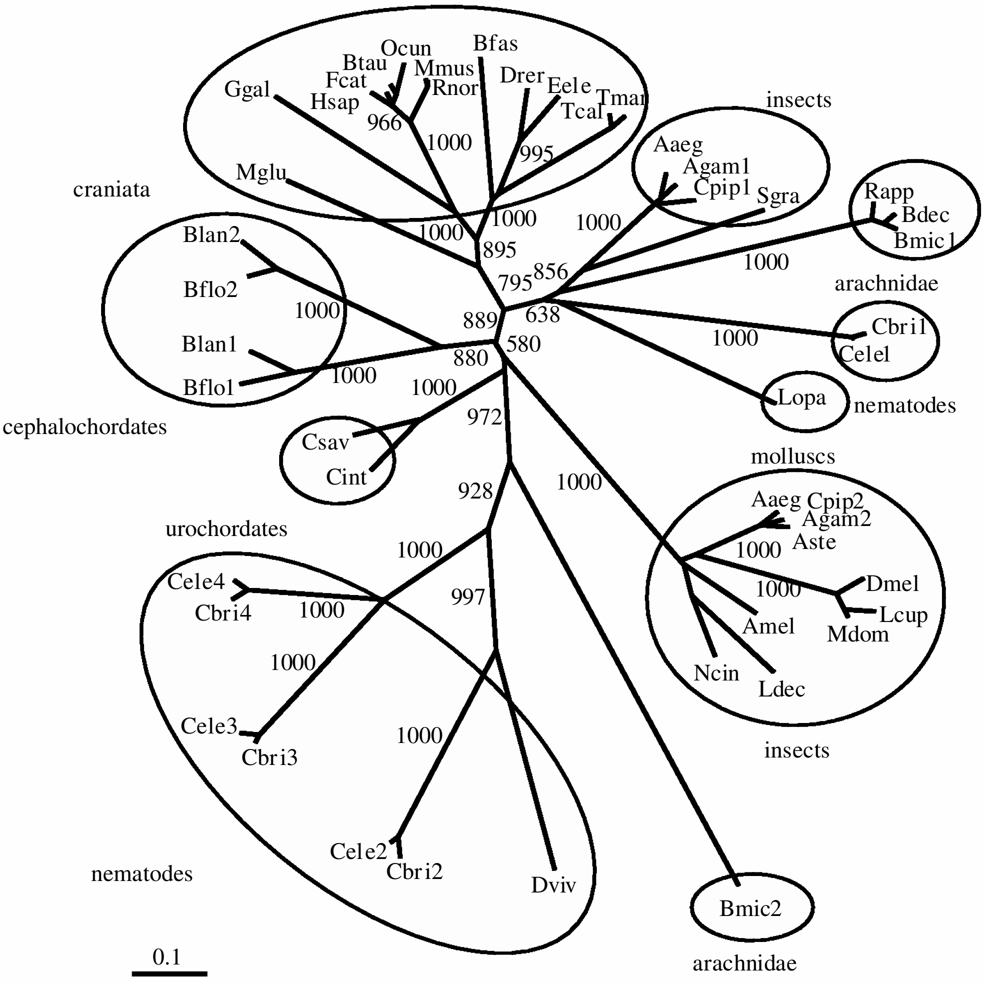

Figure 3. Phylogenetic tree of AChE proteins. Forty-seven protein sequences from 35 species were retrieved from the ESTHER database (http://www.ensam.inra.fr/cgi-bin/ace/index). Sequences were aligned and a bootstrapped unrooted tree was constructed as described in § 2. Only nodes supported by more than 50% bootstraps (i.e. scores above 500) are indicated. The scale bar represents 10% divergence. (Agam, *Anopheles gambiae*; Aaeg, *Aedes aegypti*; Aste, *Anopheles stephensi*; Cpip, *Culex pipiens*; Dmel, *Drosophila melanogaster*; Lcup, *Lucilia cuprina*; Mdom, *Musca domestica*; Ldec, *Leptinotarsa decemlineata*; Amel, *Apis mellifera*; Ncin, *Nephotettix cincticeps*; Sgra, *Schizaphis graminum*; Rapp, *Rhipicephalus appendiculatus*; Bmic, *Boophilus microplus*; Bdec, *Boophilus decoloratus*; Hsap, *Homo sapiens*; Btau, *Bos taurus*; Fcat, *Felis catus*; Ocun, *Oryctolagus cuniculus*; Rnor, *Rattus norvegicus*; Mmus, *Mus musculus*; Ggal, *Gallus gallus*; Drer, *Danio rerio*; Eele, *Electrophorus electricus*; Tmar, *Torpedo marmorata*; Tcal, *Torpedo californica*; Bfas, *Bungarus fasciatus*; Mglu, *Myxine glutinosa*; Bflo, *Branchiostoma floridae*; Blan, *Banchiostoma lanceolatum*; Cint, *Ciona intestinalis*; Csav, *Ciona savignyi*; Cele, *Caenorhabditis elegans*; Cbri, *Caenorhabditis briggsae*; Dviv, *Dictyocaulus viviparus*; Lopa, *Loligo opalescens*.)

Cx. pipiens, and probably for all Culicidae. However, here again caution should prevail in generalizing data obtained on Culicidae to other groups. An *ace-1* gene was formally identified in one Homoptera species (*S. graminum*), but no evidence has yet been published, to our knowledge, indicating that it caused resistance. In Arachnids (an arthropod class distinct from insects), resistance was not associated with any of the two *ace* genes cloned from *B. microplus*, although one appeared to belong to the *ace-1* family (see figure 3). Phylogeny of *ace* genes within the animal kingdom revealed that several duplications occurred at different steps of evolution and in different groups, one of the best-studied examples being the Nematode *C. elegans*, in which four *ace* genes have been identified (Combes *et al.* 2001). Such duplications offered potential for evolving differentiation of physiological functions, and until we have a better understanding of the overall trends in different groups we must remain open to situations that are different from those already described. Thus, we can only conclude that *ace-2* is the gene conferring resistance in species of Brachyceran Drosophilidae and Muscidae, and *ace-1* is the resistance gene of Nematocera Culicidae and possibly of other insect orders, although this latter conclusion is only tentative (figure 4). Due to the relatively high divergence between *ace-1* and *ace-2*, it is particularly important to know which one is the insecticide target, in view of designing new insecticides to improve pest control and overcome resistance problems.

(c) *Evolution of the physiological function of* ace *genes in Diptera*

We have established that the presence of a single *ace* gene in *Drosophila*, in contrast to two genes in Culicidae, is the result of the loss of *ace-1* at some stage of the evolution processes that differentiated Drosophilidae and Culicidae from their common ancestor. Resistance data provided evidence that synapse cholinergic function is ensured by *ace-1* in Culicidae and by *ace-2* in *Drosophila*. Thus, two distinct

Table 1. Distribution of *ace* genes in arthropods and their involvement in insecticide resistance. A question mark indicates an absence of information. Nomenclature of the *ace* genes is based on the mosquito *Culex pipiens*. See § 2b for explanation.

systematic				presence of *ace* genes			insecticide resistance	
class	order	suborder	species	*ace-1*	*ace-2*	references	*ace* gene involved	references or comments
Insect	Diptera	Brachycera	*Drosophila melanogaster*	absent	yes	Hall & Spierer (1986)	*ace-2*	Mutéro *et al.* (1994)
			Musca domestica	absent ?	yes	Williamson *et al.* (1992)	*ace-2*	Kozaki *et al.* (2001); Walsh *et al.* (2001)
			Lucilia cuprina	absent ?	yes	Chen *et al.* (2001)	?	not studied
		Nematocera	*Culex pipiens*	yes	yes	*ace-1*: this study; *ace-2*: Malcolm *et al.* (1998)	*ace-1*	this study; Malcolm *et al.* (1998)
			Culex tritaeniorynchus	likely	yes	Mori *et al.* (2001)	not *ace-2*	Mori *et al.* (2001)
			Aedes aegypti	yes	yes	*ace-1*: this study; *ace-2*: Anthony *et al.* (1995)	?	insensitive AChE not reported
			Anopheles gambiae	yes	yes	this study	?	insensitive AChE not reported
			Anopheles stephensi	yes	yes	*ace-1*: this study; *ace-2*: Malcolm & Hall (1990)	?	insensitive AChE not reported
	Coleoptera		*Leptinotarsa decemlineata*	?	yes	Zhu & Clark (1995)	perhaps not *ace-2*[a]	Zhu *et al.* (1996)
	Homoptera		*Aphis gosypii*	?	yes	Menozzi (2000)	not *ace-2*	Menozzi (2000)
			Schizaphis graminum	yes	?	Gao *et al.* (2002)	?	not studied yet
			Nephotettix cincticeps	?	yes	Tomita *et al.* (2000)	not *ace-2*	Tomita *et al.* (2000)
Arachnidae	Acarinae		*Boophilus microplus*	yes[b]	yes[b]	Baxter & Barker (1998); Hernandez *et al.* (1999)	not *ace-2*, not *ace-1*	Baxter & Barker (1998); Hernandez *et al.* (1999)

[a] Zhu *et al.* (1996) concluded the opposite. However, re-analysing their data (13 individuals analysed from a strain with 80% of resistant individuals) does not suggest a significant ($p > 0.05$) association between resistance and the presence of a particular mutation in *ace-2*.

[b] Two distinct *ace* genes (33% homology at the nucleotide level) have been found in this species, neither involved in insecticide resistance. Thus, at least three *ace* genes must be present in this species. Identification as *ace-1* is based on figure 1 and identification as *ace-2* is tentative.

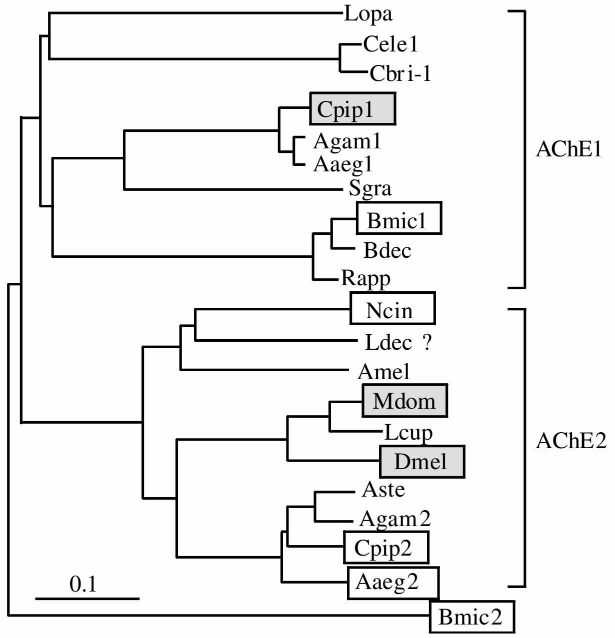

Figure 4. Cladogram of AChE1 and AChE2 proteins. Protein sequences from AChE1 and AChE2 classes were processed as in figure 1. The Bmic2 sequence was added as an external outgroup to root the cladogram. Shaded frames, proteins whose gene segregates with insecticide resistance; open frame, proteins whose genes do not segregate with insecticide resistance. The question mark for Ldec (*Leptinotarsa decemlineata*) is discussed in table 1. The scale bar represents 10% sequence divergence.

events have led to the present situation in *Drosophila*, the deletion of *ace-1* and the modification of *ace-2* function.

It is difficult to envision the acquisition of the main synaptic cholinergic function by *ace-2*, if this function was solely fulfilled by *ace-1* in the ancestral group. This is because the loss of *ace-1* would probably have been lethal: we know, for example, that a reduction of AChE activity in synapses observed with most insensitive AChE is associated with a severe fitness cost (Lenormand *et al.* 1999; Raymond *et al.* 2001). Thus, ancestral *ace-1* and *ace-2* genes must have been somehow overlapping for this particular function, allowing a compensatory effect, similar to those described in the nematode *C. elegans* (Culotti *et al.* 1981; Johnson *et al.* 1981; Grauso *et al.* 1998; Combes *et al.* 2001).

AChEs have other functions than neurotransmitter hydrolysis in cholinergic synapses (Massoulié *et al.* 1993) and, for example, striking cases of non-neuronal AChE activity have been described in parasitic nematodes (Lee 1996). Thus, *ace-1* deletion might also result in the loss of one or several of these functions. However, our knowledge on the non-cholinergic role of *ace* genes is too limited to speculate about their identity. In *Cx. pipiens*, the only evidence that *ace* genes have different functions is derived from their different relative activity in larvae and adults (Bourguet *et al.* 1996).

Thus, two non-exclusive hypotheses can explain the loss of *ace-1*: either a change in physiology occurred that abolished the requirement for *ace-1* specific functions, or a change in the ace-2 protein or its regulation led to a gain of function, compensating the loss of *ace-1* specific function. Although no definite evidence can discriminate between both hypotheses, it is intriguing that a major difference between *ace-1* and *ace-2* gene products is a 31-amino acid insertion in the AChE2 sequence (boxed in figure 1), which corresponds to a region of AChE2 that greatly diverges between Brachycera (as represented by the Drosophilidae, Muscidae and Calliphoridae) and Nematocera (represented by the Culicidae). The availability of additional *ace-1* and *ace-2* sequences from various insect orders, as well as the comparison of their biochemical and physiological properties, are needed to understand the specific features of AChE proteins and their implication in insecticide resistance.

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