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Comparison of Two Acetylcholinesterase Gene cDNAs of the Lesser Mealworm, *Alphitobius diaperinus*, in Insecticide Susceptible and Resistant Strains

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Two cDNAs encoding different acetylcholinesterase (AChE) genes (*AdAce1* and *AdAce2*) were sequenced and analyzed from the lesser mealworm, *Alphitobius diaperinus*. Both *AdAce1* and *AdAce2* were highly similar (95 and 93% amino acid identity, respectively) with the *Ace* genes of *Tribolium castaneum*. Both *AdAce1* and *AdAce2* have the conserved residues characteristic of AChE (catalytic triad, intra-disulfide bonds, and so on). Partial cDNA sequences of the *Alphitobius Ace* genes were compared between two tetrachlorvinphos resistant (Kennebec and Waycross) and one susceptible strain of beetles. Several single nucleotide polymorphisms (SNPs) were detected, but only one non-synonymous mutation was found (A271S in *AdAce2*). No SNPs were exclusively found in the resistant strains, the A271S mutation does not correspond to any mutations previously reported to alter sensitivity of AChE to organophosphates or carbamates, and the A271S was found only as a heterozygote in one individual from one of the resistant *A. diaperinus* strains. This suggests that tetrachlorvinphos resistance in the Kennebec and Waycross strains of *A. diaperinus* is not due to mutations in either AChE gene. The sequences of *AdAce1* and *AdAce2* provide new information about the evolution of these important genes in insects. Arch Insect Biochem Physiol. 67:130–138, 2008. © 2007 Wiley-Liss, Inc.

KEYWORDS: acetylcholinesterase; *Tribolium castaneum*; lesser mealworm; genotyping; *AdAce1; AdAce2*; Insecta

INTRODUCTION

The lesser mealworm, *Alphitobius diaperinus*, is a manure-breeding beetle that is the primary structural pest of the poultry industry in the United States (Axtell, 1999; Hinton and Moon, 2003). The lesser mealworm is also a reservoir of *Salmonella typhimurium*, *Escherichia coli*, tapeworms, avian leucosis virus, turkey coronavirus, turkey enterovirus (Avincini and Ueta, 1990; Axtell and Arends, 1990; Despins et al., 1994; Goodwin and Waltman, 1996; McAllister et al., 1996; Watson et al., 2000), and may serve as a source of *Campylobacter* contamination of poultry (Bates et al., 2004). High beetle populations consume significant amounts of bird feed (Savage, 1992). Under dry conditions in the broiler house, beetles bite the skin of birds resting at night. To prevent these bites, birds will rest for short periods and then move (Despins et al., 1987; Vaughan and Turner, 1984). This can affect the weight gain of chicks.

Organophosphate and carbamate insecticides have served as effective tools for control of the lesser mealworm, and tetrachlorvinphos continues to be used for this purpose in the United States. However, a recent study indicated that there were some populations of lesser mealworm in which a substantial portion of the population was highly resistant to tetrachlorvinphos (Hamm et al., 2006). The mechanism responsible for this resistance has not been determined.

Organophosphate and carbamate insecticides exert their toxic effects via inhibition of acetylcholinesterase (AChE). Recent studies have discovered

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that some insect species have a single *Ace* gene (*Drosophila melanogaster* and *Musca domestica*), while many other species have two *Ace* genes (*Culex pipiens, Bombyx mori, Myzus persicae,* among others). Beetles (Coleoptera) are the most evolutionarily successful metazoans, contributing 25% of all known animal species, far more than any other taxonomic order. Despite the diversity and economic importance of Coleoptera, *Ace* genes have been reported from only two beetles: *Leptinotarsa decemlineata* (Say) (Zhu et al., 1996) and *Tribolium castaneum* (http://www.hgsc.bcm.tmc/edu/projects/tribolium/). Evidence has accumulated that indicates a limited number of mutations in *Ace* are as-

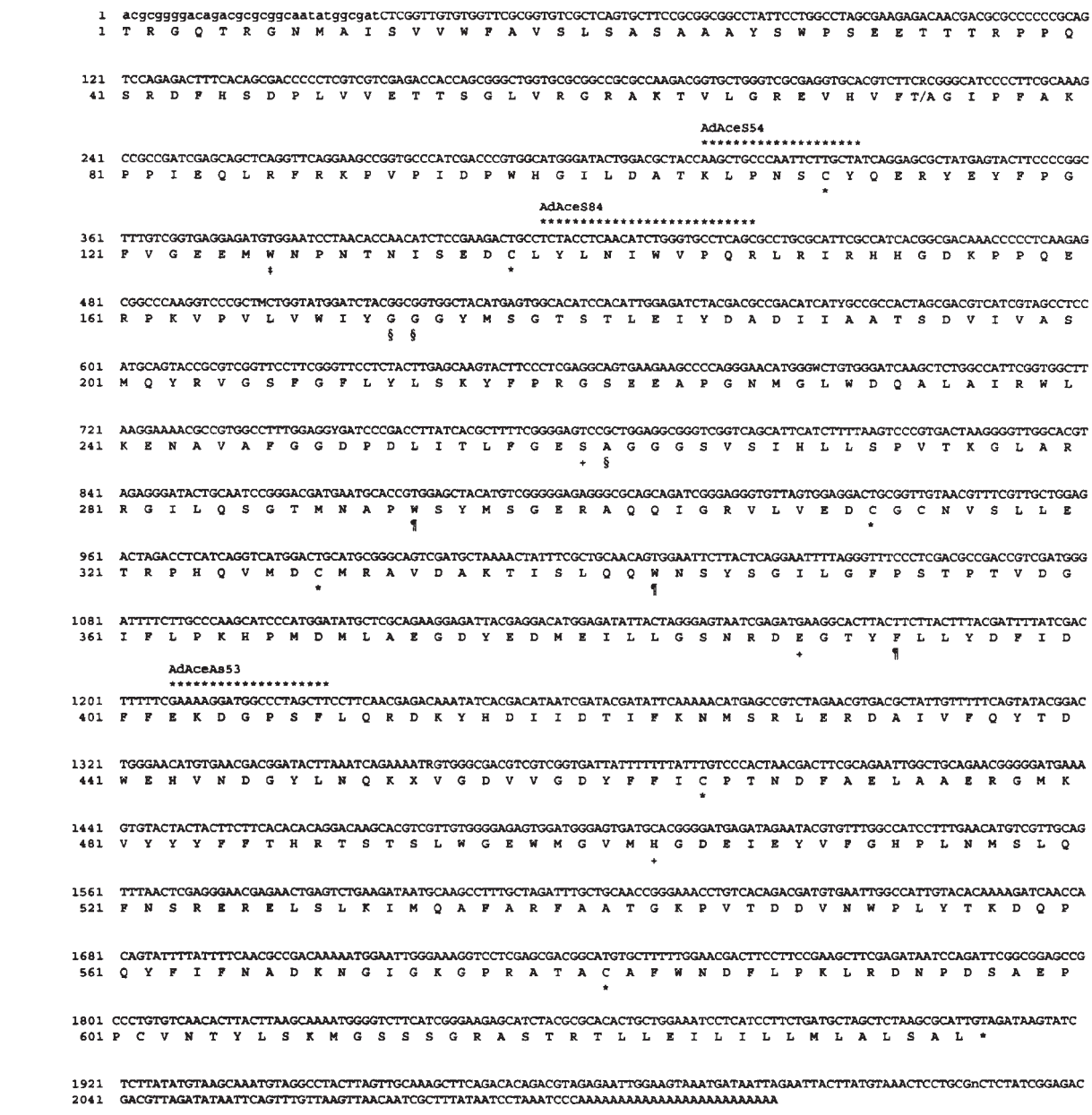

Fig. 1. The nucleotide and deduced amino acid sequences of *AdAce1* (*Drosophila Ace* orthologous) cDNA (Accession no. EU086056). The residues that make intra-disulfide bonds are marked with *, oxianion hole with §, catalytic triad with +, acyl binding site with ¶, and anionic subsite with ‡. The locations of the primers used for genotyping are indicated as **.

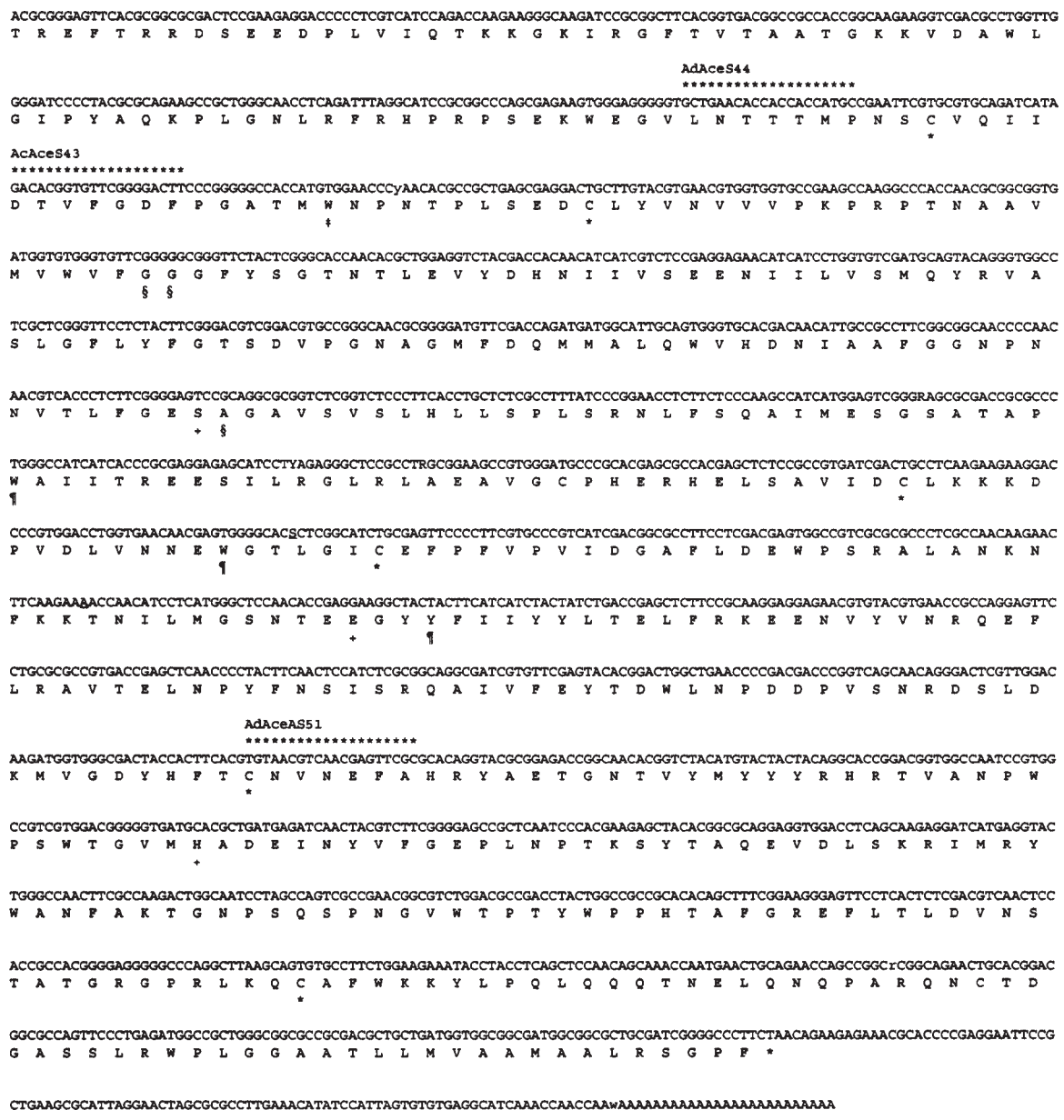

Fig. 2. The nucleotide and deduced amino acid sequences of *AdAce2* (the *Drosophila Ace* paralogous) cDNA (Accession no. EU086057). The residues that make intra-disulfide bonds are marked with *, oxianion hole with §, catalytic triad with +, acyl binding site with ¶, and anionic subsite with ‡. The locations of the primers used for genotyping are indicated as **.

Fig. 3. Phylogenetic tree of the Arthropoda acetylcholinesterases. One thousand bootstrap pseudo replicates were performed. Bootstrap values > 50% are indicated by a • (small filled circle). Taxonomy classification is based on NCBI database. The species and accession numbers are AA (*Schizaphis graminum*, AAK09373.1), AB (*Rhopalosiphum padi*, AAT76530.1), AC (*Aphis gossypii*, AAM94376.1), AD (*Sitobion avenae*, AAV68493.1), AE (*Myzus persicae*, AAN71600.1), BA (*Nephotettix cincticeps*, AAP87381.1), CA (*Blattella germanica*, ABB89946.1), DA (*Tribolium castaneum*, XP_973462.1), DB (AdAce2), EA (*Pediculus humanus corporis*, BAF46105.1), FA (*Helicoverpa assulta*, AAY42136.1), FB (*Helicoverpa armigera*, AAY59530.1), FC (*Chilo suppressalis*, ABO38111.1), FD (*Cydia pomonella*, ABB76666.1), FE (*Bombyx mandarina*, ABM66370.1), FF (*Bombyx mori*, BAF33338.1), FG (*Plutella xylostella*, AAY34743.1), GA (*Apis mellifera*, XP_393751.1), HA (*Culex*

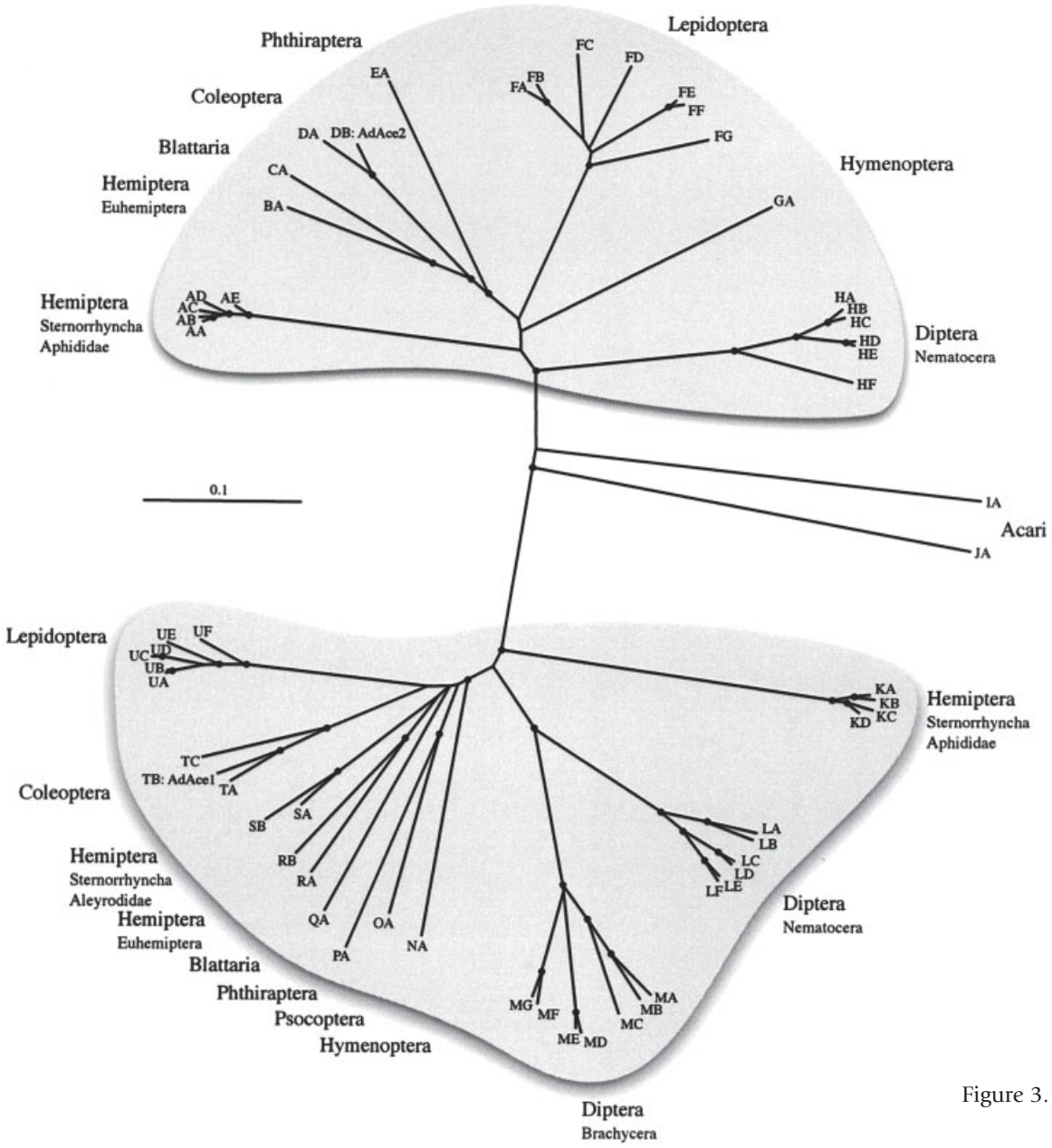

Figure 3.

pipiens pallens, AAV28503.1), HB (*Culex pipiens*, CAD33707.2), HC (*Culex tritaeniorhynchus*, BAD06210.1), HD (*Aedes aegypti*, ABN09910.1), HE (*Aedes albopictus*, BAE71346.1), HF (*Anopheles gambiae*, XP_321792.2), IA (*Tetranychus urticae*, AAO73450.1), JA (*Boophilus decoloratus*, CAA06980.1), KA (*M. persicae*, AAL99585.1), KB (*S. avenae*, AAU11286.1), KC (*A. gossypii*, AAM94375.1), KD (*R. padi*, AAU11285.1), LA (1808210A), LB (*Anopheles gambiae*, XP_310628.3), LC (*Aedes aegypti*, AAB35001.1), LD (*Ae. albopictus*, BAE71347.1), LE (*Cx. tritaeniorhynchus*, BAD06209.1), LF (*Cx. pipiens*, CAJ43752.1), MA (*Musca domestica*, AAK69132.1), MB (*M. domestica*, AAS45645.1), MC (*Lucilia cuprina*, AAC02779.1), MD (*Bactrocera oleae*, AAM69920.1), ME (*Bactrocera dorsalis*, CAD57142.1), MF (*Drosophila melanogaster*, CAA29326.1), MG (*D. pseudoobscura*, XP_1358489.1), NA (*Apis mellifera*, BAE06051.1), OA (*Liposcelis bostrychophila*, ABO31937.1), PA (*P. humanus corporis*, BAF46104.1), QA (*B. germanica*, ABB89947.1), RA (*N. cincticeps*, AAF65202.1), RB (*Nilaparvata lugens*, CAH65679.2), SA (*Bemisia tabaci*, CAE11222.1), SB (*Trialeurodes vaporariorum*, CAE11223.1), TA (*T. castaneum*, XP_970774.1), TB (AdAce1), TC (*Leptinotarsa decemlineata*, AAB00466.1), UA (*H. armigera*, AAM90333.1), UB (*H. assulta*, AAV65638.1), UC (*Bombyx mandarina*, ABM46999.1), UD (*Bombyx mori*, BAF33337.1), UE (*Cydia pomonella*, ABB76665.1), and UF (*Plutella xylostella*, AAK39639.1).

sociated with resistance (i.e., mutations that code for an organophosphate and/or carbamate insensitive AChE) in insects (Fournier, 2005; Kono and Tomita, 2006; Oh et al., 2006). In this study we examined if there was one or two *Ace* genes in *A. diaperinus*, and if mutations in *Ace* could be correlated with tetrachlorvinphos resistance.

MATERIALS AND METHODS

Strains of *Alphitobius diaperinus*

Three strains of lesser mealworm were used. The Denmark-S (susceptible) strain was obtained from Saturnia (Bjerringbrovej 48 2610 Rødovre, Denmark). Two strains (from Kennebec, Co., ME, and Waycross, GA) that contain high proportions of tetrachlorvinphos resistant individuals (Hamm et al., 2006) were also used. *A. diaperinus* colonies were maintained at 28°C with 60–70% RH, and provided a diet of cracked corn:wheat bran (95:5) *ad libitum*. Adult beetles from the Kennebec and Waycross strains were exposed to tetrachlorvinphos using a residual contact bioassay method as described previously (Hamm et al., 2006). Beetles that survived exposure to a concentration of tetrachlorvinphos that was 350-fold greater than the susceptible strain LC_{99} for 48 hr (i.e. resistant individuals) were used in genotyping.

Sequencing of the *Ace* Genes

Five Denmark-S adult beetles (84 mg) were used to isolate mRNA using a QuickPrep *Micro* mRNA purification kit (GE Healthcare, Waukesha, WI,), and cDNA was synthesized with 500 ng of mRNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). A fragment that encoded the *Ace* cDNA, orthologous *Drosophila Ace* (Fournier et al., 1989), was amplified by S11ACE (TAYGARTAYTTYCCiGGiTT), S1iACE (GARATGTGGAAYCCiAAYAC), and AS17ACE (CCiCCiCCRTAiAYCCA). A fragment that encoded the *Ace*-paralogous gene was amplified using DL3 (GCiACiATGTGGA AYCCiAA) and DR98 (GGYTTiCCiGTYTTiGCRAA) with the following thermal cycler program 95°C for 3 min, 35 cycles (95°C for 30 sec, 40°C for 30 sec, 72°C for 1.5 min) and a final extension at 72°C for 7 min. We obtained a fragment of 151 bp for the *Drosophila Ace* orthologous gene and a fragment of 825 bp for the paralogous gene. Gene specific primers for 5′- and 3′-RACE were designed based on these sequences. RACE was performed using the BD SMART RACE cDNA amplification kit (BD Biosciences, Mountain View, CA).

To compare the *Ace* alleles in the susceptible and tetrachlorvinphos-resistant beetles, the *Ace* cDNAs were sequenced from individual beetles. The mRNA was prepared using a PolyATract System 1000 (Promega, Madison, WI), and was concentrated with a Microcon YM-100 (Millipore, Billerica, MA). One fourth of the mRNA from a beetle was used in the RT-PCR (50°C for 30 sec, 94°C for 2 min, 40 cycles (95°C for 30 sec, 50°C 30 sec, and 72°C for 1 min) using the SuperScript III One-Step RT-PCR System and Platinum Taq (Invitrogen). Gene-specific primers, S54AdAce (AAGCTGCCCAATTCTTGCTA), S84AdAce (TCTACCTCAACATCTGGGTGCCTCAGC), and AS53AdAce (AAGCTAGGGCCATCCTTTTC) were used for the amplification of the *Drosophila Ace*- orthologous gene. Primers S44AdAce (GCTGAACACCACCACCATGC), S43AdAce (GACACGGTGTTCGGGGACTT), and AS51AdAce (GCGAACTCGTTGACGTTACA) were used to amplify the *Drosophila Ace* paralogous gene. DNA sequencing was performed with S84AdAce and AS53AdAce for the *Drosophila Ace* orthologous gene and with S43AdAce and AS51AdAce for the paralogous gene at the Cornell Biotechnology Resource Center.

RESULTS AND DISCUSSION

Ace Genes

We obtained the nearly complete ORFs for *AdAce1* and *AdAce2* (Figs. 1 and 2). Both genes show a high similarity (95 and 93% amino acid identity, respectively) with the predicted *Ace* genes of *T. castaneum* (XP_970774.1, XP_973462.1) (Figs. 3–5). Kyte-Doolittle hydropathy plots indicated the C-terminal of both AdAce1 and AdAce2 were hydrophobic (data not shown), and thus potentially exchanged for glycolipids.

The cDNA sequence of *AdAce1* (the *Drosophila Ace* orthologous gene, EU086056) was 2,123 bp;

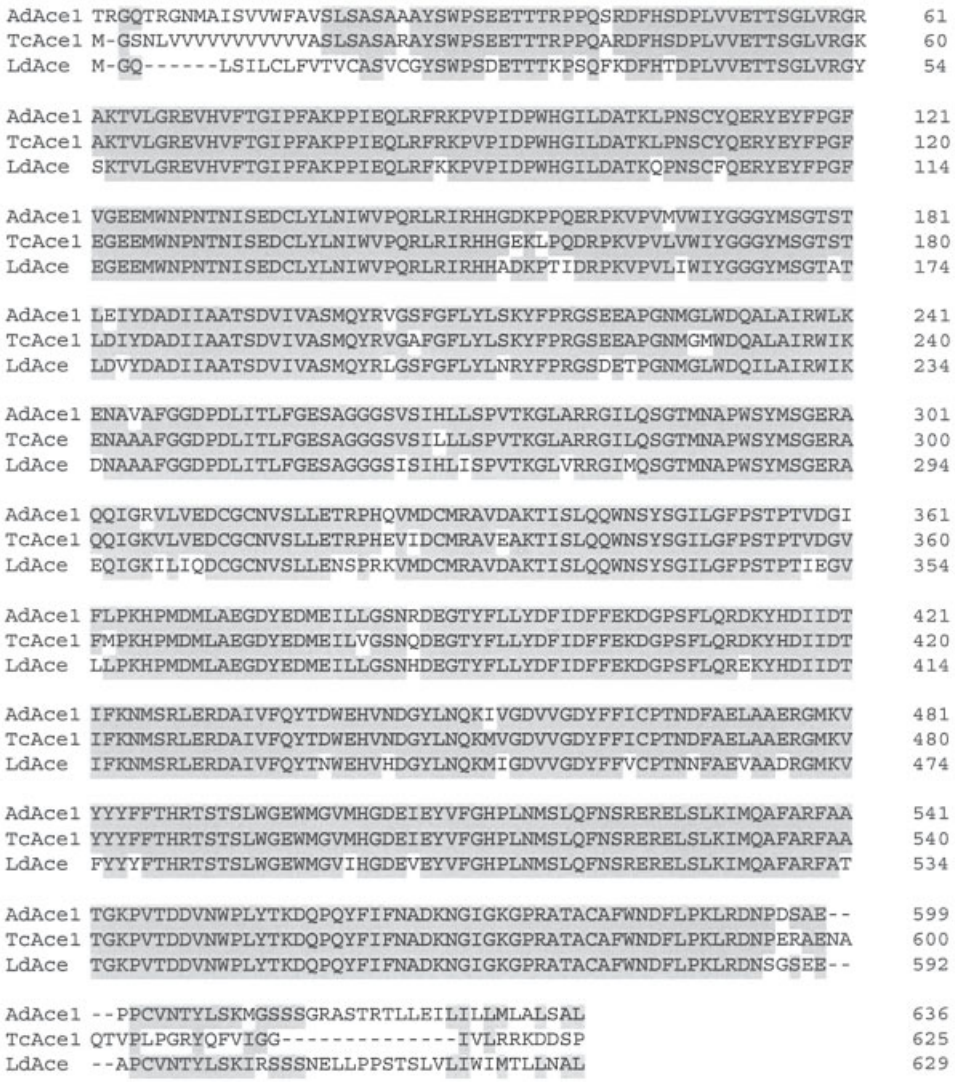

Fig. 4. Alignment of the deduced amino acid sequences from the *Drosophila Ace* orthologous genes in Coleoptera. Ad, Tc, and Ld represent *A. diaperinus, T. castaneum*, and *L. decemlineata*, respectively.

encoding 636 amino acid resides of an immature AChE (Fig. 1). The deduced amino acid sequence had the characteristic features of AChE, including the residues for the intra-molecular disulphide bonds (C110(67)- C137(94), C312(254)- C329(265), C465(402)- C582(521)), catalytic triad (S260(200), E389(327), H503(400)), protein dimerisation (C602), anionic subsite (W127(86)), oxianion hole (G172(118), G173(119), A261(201), and acyl binding site (W293(233), F352(290), F393(329)) (number in parentheses indicates the corresponding amino acid in *Torpedo* AChE). We could not unambiguously identify the translation start site because no stop codon was found in frame in the 5′ upstream region. If this transcript is similar in size to the *Ace* gene in the Colorado potato beetle, *L. decemlineata* (Zhu and Clark, 1995), it will be more than 13 kb in size. However, *AdAce1* has an initiation codon that is identical to the one tentatively identified in *L. decemlineata*. Given that *AdAce1* does not have any of the mutations associated with organophosphate and/or carbamate resistance in *Drosophila* (Mutero et al., 1994), *Lucilia cuprina* (Chen et al., 2001), or *M. domestica* AChEs (Kim et al., 2003; Kozaki et al., 2001; Walsh et al., 2001), we conclude that *AdAce1* encodes an organophosphate-sensitive AChE (characterized by M126, V205, G287, F352, and G390). This is consistent with the Denmark-S strain being insecticide susceptible.

We sequenced 1,895 bp encoding 591 amino acids of *AdAce2* (the *D. melanogaster Ace* paralogous AChE, EU086057) (Fig. 2). As also found for *AdAce1*,

Fig. 5. Alignments of the deduced amino acid sequences from the *Drosophila Ace* paralogous genes in Coleoptera. Ad and Tc represent *A. diaperinus* and *T. castaneum*, respectively.

the residues for the intramolecular disulphide bonds (C76(67)- C103(94), C275(254)- C295(265), C410(402)- C532(521)), catalytic triad (S208(200), E334(327), H448(440)), protein dimerisation (C558) anionic subsite (W93(86)), oxianion hole (G126(118), G127(119), A209(201)), and acyl binding site (W241(233), F298(290), F338(331)) were found in *AdAce2*. We were unable to complete the 5′-RACE for *AdAce2*, although we tried multiple variations of the protocol given by the manufacturer, including increased or decreased cation concentration, increasing the viscosity of the reaction mix by BSA or by using an alternative cation ($Mg2^{+}$ to Mn^{2+}). The alignment of this gene with the *Drosophila Ace* paralogous AChEs showed that, as expected for an insecticide-susceptible strain, beetles from the Denmark-S strain had an organophosphate and carbamate sensitive type.

Genotyping

A partial cDNA, covering the amino acid residues found to be responsible for insecticide resistance in other species, was sequenced from individual adults for both *Alphitobius Ace* genes to ascertain if resistance was due to a change in one or both genes. If resistance was due to a mutation in *AdAce1* or *AdAce2*, all resistant individuals should have a unique allele (i.e., different from the susceptible strain).

The *Drosophila Ace* orthologous gene, *AdAce1*, was sequenced from two susceptible Denmark-S, four Waycross (tetrachlorvinphos-resistant), and two Kennebec (tetrachlorvinphos-resistant) adults. The deduced amino acid sequences from all individuals were the same. There were six synonymous polymorphisms detected (data not shown).

The *Ace* paralogous gene, *AdAce2*, was sequenced from three susceptible (Denmark-S), five Waycross (tetrachlorvinphos-resistant), and five Kennebec (tetrachlorvinphos-resistant) adults. The sequences from all individuals were highly similar. One of the Denmark-S and one of the Waycross beetles had a A271(261)S mutation (detected as a heterozygote in both individuals). There were an additional 10 synonymous polymorphisms identified (data not shown). Given that neither *AdAce1* nor

AdAce2 were different between resistant and susceptible beetles, we conclude that the mechanism of tetrachlorvinphos resistance in these strains of *A. diaperinus* is not due to mutations in the *Ace* genes (i.e., is not an altered acetylcholinesterase).

Alignments of the deduced amino acid sequences from the *Drosophila Ace* orthologous and paralogous genes in Coleoptera are shown in Figures 4 and 5, respectively. As expected, the *Ace* orthologous sequences of the two Tenebrionidae, *A. diaperinus* and *T. castaneum*, were more similar to each other than to the *Leptinotarsa decemlineata* (Fig. 4). These three coleopteran sequences differed primarily at the N- and C-terminal regions, but showed expected conservation at most functionally important residues. Similarly, the *Ace* paralogous sequences from *A. diaperinus* and *T. castaneum* were highly similar, with the greatest number of differences found in the C-terminal region (Fig. 5).

The phylogenic tree of the Arthropod AChEs (Fig. 3) shows there are two major groups, with the Acari AChEs being intermediates. Each group is further divided into the subgroups, primarily by Order. *AdAce1* and *AdAce2* clustered with the other Coleoptera genes in both. This is consistent with the idea that beetles have two *Ace* genes. The mutations related to organophosphate resistance were first studied in *D. melanogaster* and *M. domestica*. However, studies of *Drosophila Ace* orthologous genes failed to identify mutations responsible for organophosphate resistance in other species. Subsequently, mutations in the *Drosophila Ace* paralogous genes were found to be associated with the resistance in some mosquitoes. The increasing number of insect genome sequences reveal that the ancestral condition, at least in Pterygota, is two copies of *Ace*. It also appears that mutations on the *Drosophila* and *Musca Ace* paralogous genes are more important than the mutations on the *Drosophila* orthologous genes, in terms of conferring organophosphate resistance, at least in many species.

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