PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2006) **273**, 2595–2604
doi:10.1098/rspb.2006.3621
Published online 18 July 2006

Acetylcholinesterase genes within the Diptera: takeover and loss in true flies

Elise Huchard[1], Michel Martinez[2], Haoues Alout[1], Emmanuel J. P. Douzery[1], Georges Lutfalla[3], Arnaud Berthomieu[1], Claire Berticat[1], Michel Raymond[1,*] and Mylène Weill[1]

[1]*Institut des Sciences de l'Evolution (UMR 5554 CNRS), C.C. 065, and* [3]*UMR 5124 CNRS, C.C.86, Université de Montpellier II, 34095 Montpellier cedex 5, France*

[2]*INRA, Unité d'Ecologie animale et Zoologie agricole, 2, place Pierre Viala, 34060 Montpellier Cedex 01, France*

It has recently been reported that the synaptic acetylcholinesterase (AChE) in mosquitoes is encoded by the *ace-1* gene, distinct and divergent from the *ace-2* gene, which performs this function in *Drosophila*. This is an unprecedented situation within the Diptera order because both *ace* genes derive from an old duplication and are present in most insects and arthropods. Nevertheless, *Drosophila* possesses only the *ace-2* gene. Thus, a secondary loss occurred during the evolution of Diptera, implying a vital function switch from one gene (*ace-1*) to the other (*ace-2*). We sampled 78 species, representing 50 families (27% of the Dipteran families) spread over all major subdivisions of the Diptera, and looked for *ace-1* and *ace-2* by systematic PCR screening to determine which taxonomic groups within the Diptera have this gene change. We show that this loss probably extends to all true flies (or Cyclorrhapha), a large monophyletic group of the Diptera. We also show that *ace-2* plays a non-detectable role in the synaptic AChE in a lower Diptera species, suggesting that it has non-synaptic functions. A relative molecular evolution rate test showed that the intensity of purifying selection on *ace-2* sequences is constant across the Diptera, irrespective of the presence or absence of *ace-1*, confirming the evolutionary importance of non-synaptic functions for this gene. We discuss the evolutionary scenarios for the takeover of *ace-2* and the loss of *ace-1*, taking into account our limited knowledge of non-synaptic functions of *ace* genes and some specific adaptations of true flies.

Keywords: acetylcholinesterase; evolution of gene function; Diptera; duplication; gene loss

1. INTRODUCTION

The recent discovery of the gene coding of the synaptic acetylcholinesterase (AChE) in mosquitoes has led to a paradox in their evolution. AChE stops neurotransmission in the sensorial synapses of insects by hydrolysing the neurotransmitter acetylcholine (Toutant 1989). The *Anopheles gambiae* genome contains two *ace* genes: *ace-1*, which encodes the main synaptic AChE (Weill *et al.* 2002) and *ace-2*, which has an unknown function. These two genes have only 53% similarity at the amino acid level, and the overall *ace* phylogeny suggests that they diverged before the diversification of the arthropods (Weill *et al.* 2002). Thus, both genes should be present in most arthropods and have already been formally identified in Hemiptera, Hymenoptera, Lepidoptera and Acari (Weill *et al.* 2002; Li & Han 2004; Russell *et al.* 2004; Lee *et al.* 2006). By contrast, the *Drosophila melanogaster* genome contains a single gene, *ace-2* (Weill *et al.* 2002), which encodes the synaptic AChE (Fournier *et al.* 1989). The absence of *ace-1* in the *D. melanogaster* genome can therefore be explained by a secondary loss. Consequently, within the Diptera, either *ace-1* (e.g. in mosquitoes) or *ace-2* (e.g. in flies) encodes the main AChE, implying that *ace-2* took over the function of *ace-1* during evolution.

The synaptic AChE is involved in a vital function, and it is assumed that this function has always been present during the evolution of insects. Apparently, AChE has been naturally selected for its particularly high enzymatic activity. It is one of the fastest known enzymes with up to 10^4 substrate molecules being hydrolysed per second by each enzyme molecule, and its enzymatic velocity seems to be limited only by the diffusion velocity of its substrate (Quinn 1987). This suggests that a slight reduction in its activity would somehow be translated to a significant fitness cost. In the mosquito *Culex pipiens*, a variant synaptic AChE (coded by *ace-1*) differing by one amino acid (glycine 119 changed to serine, or G119S) is found in insecticide-treated areas. This variant is insensitive to some insecticides and has a 60% reduced activity, which is associated with substantial fitness cost: about 11% per generation during the breeding season and 50–60% for survival during the overwintering season (Lenormand *et al.* 1998; Lenormand *et al.* 1999; Lenormand & Raymond 2000). The G119S mutation has also been detected in insecticide-resistant individuals in distant mosquito species (*An. gambiae* and *An. albimanus*, Weill *et al.* 2004), suggesting that the AChE function cannot be greatly modified (to increase insensitivity to some insecticides) without greatly affecting its optimal and vital activity.

* Author for correspondence (raymond@isem.univ-montp2.fr).

The electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2006.3621 or via http://www.journals.royalsoc.ac.uk.

Received 19 January 2006
Accepted 13 May 2006

Despite these physiological constraints, the gene encoding the synaptic AChE has apparently changed within the Diptera, with the ancestral gene (*ace-1*) being replaced by a divergent and distant gene (*ace-2*). This type of gene replacement, which pertains to a vital function, has never been observed previously (to our knowledge), and thus we have no conceptual framework for understanding how such a phenomenon is possible and what sort of selection has driven it. The *Ao* (aldehyde oxidase) gene in eukaryotes underwent a possibly related change; it derives neofunctionalization from a duplicate copy of *Xdh* (xanthine dehydrogenase), and *Xdh* underwent a second duplication in chordates. The new duplicated copy became a neofunctionalized *Ao* gene, with the first *Ao* gene subsequently disappearing from the vertebrate genome (Rodriguez-Trelles *et al.* 2003). However, the *Ao*/*Xdh* and *ace-1*/*ace-2* situations cannot be directly compared because the loss of *ace-1* is not associated with a new *ace-2* copy, which could have acquired (by neofunctionalization) the same functions as *ace-1*.

The first step in understanding this situation is to determine which taxonomic groups within the Diptera display this gene change. There are about 129 000 species described within the Diptera order, distributed among 185 families (McAlpine & Wood 1989; Grimaldi & Engel 2005; plus update from M.M.). The main synaptic AChE is encoded by *ace-1* in *Culex* and *Anopheles* mosquitoes (Culicidae family), and by *ace-2* in *D. melanogaster*. Both the housefly *Musca domestica* and the olive fruitfly *Bactrocera oleae* use *ace-2* for their cholinergic synapses, as shown by *ace-2* mutations providing insecticide resistance (Kozaki *et al.* 2001*a*; Walsh *et al.* 2001; Vontas *et al.* 2002). Currently, there is no information available on the presence or absence of *ace-1* in these two species. Species using *ace-2* for their cholinergic AChE belong to distinct families (Drosophilidae, Muscidae, and Tephritidae). This present study aims to determine the presence or absence of both *ace-1* and *ace-2* in the Diptera families. The information obtained from the study of these genes and their functions allowed us to propose some possible evolutionary scenarios.

2. MATERIAL AND METHODS

(a) *Insect samples*

Diptera species were either collected locally or obtained from various sources (particularly strains and identified preserved materials). Parasite species from the Braulidae, Nycteribiidae and Gasterophilidae families were freshly obtained from host species specialists, respectively (honeybee keeper, chiropterologist and veterinarian). References of samples used are reported in the electronic supplementary material. Most samples were identified by only one person in our team (M.M.).

(b) *PCR amplification*

DNA extraction was carried out using a DNeasy Tissue Kit (Qiagen) following the manufacturer's instructions. For *ace-1*, there are published sequences from only one Diptera family (Culicidae), *An. gambiae* strain KISUMU (AJ515150, exon3; AJ488492, exon 4–9) and strain YAO (AJ515149, exon 3; AJ515148, exon 4–9); *Aedes aegypti* (AAB35001); *C. pipiens* strain SLAB (AJ489456) and strain SR (AJ515147). Therefore, we also considered other insect *ace-1* sequences: *Aphis gossypii* strain S171B (AJ748114) and strain S1081K (AJ748115); *Schizaphis graminum* (Q9BMJ1). All these *ace-1* sequences were used to design four pairs of primers: Moustdir (5′ CCG-GGN-GCS-ACY-ATG-TGG-AA 3′) with Moustrev (5′ ACG-ATM-ACG-TTC-TCY-TCC-GA 3′); P6dir (5′ATM-GWG-TTY-GAG-TAC-ACS-GAY-TGG 3′) with P7rev (5′ GGC-AAA-RTT-KGW-CCA-GTA-TCK-CAT 3′); Ace1dir3 (5′ GAC-AAR-ATG-GTS-GGN-GAY-TAT-CA 3′) with Ace1rev4 (5′ CCR-TGC-ATM-ACR-CCN-GTC-CA 3′) or with P7rev. Polymerase chain reaction (PCR) comprised 35 cycles of 93 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. These four pairs of primers were systematically used for all samples.

We amplified *ace-2* using two pairs of degenerate primers: ace2dir4 (5′AAY-GCN-CCS-TGG-AGY-CAY-ATG-AC 3′) with ace2rev6 (5′ CCV-GAR-TAS-GAR-TTC-CAY-TGY-TG 3′), and ace2dir3 (5′ TGG-ATY-TAY-GGB-GGY-GGS-TTY-ATG 3′) with ace2rev3 (5′ GTC-ATR-TGR-CTC-C AS-GGN-GCR-TT 3′). These were designed by comparing published *ace-2* sequences from distant Diptera species: *D. melanogaster* (P07140), *M. domestica* (Q8MXC4), *Lucilia cuprina* (P91954), *B. oleae* (Q8MVZ4), *An. gambiae* (Q869C3) and *C. pipiens* (Q86GC8). The first pair of primers was systematically used, with the second being used if the first pair failed.

PCR products were directly sequenced with an ABI prism 310 sequencer using the Big Dye Terminator kit.

(c) *Diptera phylogeny*

The phylogenetic topology of the major taxonomic divisions in Diptera (infraorders or superfamilies illustrated in figure 1) and the identification of monophyletic groups were established according to published data (table 1; McAlpine & Wood 1989; Wiegmann 1993; Griffiths 1994; Cumming *et al.* 1995; Oosterbroek & Courtney 1995; Yeates & Wiegmann 1999; Yeates 2002; Grimaldi & Engel 2005).

(d) *Test of* ace-2 *molecular evolution rate*

We tested whether there was a faster molecular evolution of *ace-2* in groups also possessing *ace1* using the following relative rate test procedure. First, 28S rRNA sequences (fragment D7) were downloaded from data banks and used to construct a phylogeny of the Diptera families (electronic supplementary material). The phylogenetic tree was inferred by maximum likelihood (Felsenstein 1981) using a GTR model of nucleotide substitution with a gamma distribution of substitution rates among sites, as implemented in the Phyml program (Guindon & Gascuel 2003). A bootstrap analysis was then carried out using Seqboot (Felsenstein 1993), followed by a Phyml reconstruction. The majority-rule consensus tree was built using PAUP* (Swofford 1998). This topology was then used to connect 27 *ace-2* partial sequences included in our sample (indicated in bold, electronic supplementary material), plus 3 *ace-2* partial sequences from the literature (Drosophilidae: *D. melanogaster*, Fournier *et al.* 1989; Culicidae: *C. pipiens* and *An. gambiae*, Weill *et al.* 2002).

Three outgroups were branched according to the literature (Wheeler *et al.* 2001; Gaunt & Miles 2002). Likelihood ratio tests were then carried out on amino acids using the PAML program (Yang 1997), according to different evolutionary rate models for the various taxa: a single, global rate for all taxa; three different, local rates for Cyclorrhapha, non-Cyclorrhapha, and outgroups; and one rate for each of the 63 tree branches. Tests were also conducted on nucleotides to detect potential synonymous rate variations among taxa.

Table 1. Taxonomy of the species used for the construction of the Diptera phylogeny using 28S rRNA sequences (fragment D7), and the corresponding accession number. (The outgroup is a flea (order Siphonaptera).)

infraorder	family	species	accession
Culicomorpha	Culicidae	*Culex pipiens*	X93403
	Chironomidae	*Chironomus tentans*	X93412
	Simuliidae	*Simulium euryadminiculum*	X93377
Bibionomorpha	Bibionidae	*Dilophus febrilis*	X93375
Psychodomorpha	Psychodidae	*Psychoda cinerea*	X93404
	Scatopsidae	*Anapausis inermis*	X93374
Tipulomorpha	Tipulidae	*Tipula paludosa*	X93405
Stratiomyomorpha	Stratiomyidae	*Pachygaster leachii*	AF238524
Tabanomorpha	Tabanidae	*Tabanus sudeticus*	X93371
Asilomorpha	Bombyliidae	*Bombylius major*	AY456149
Aschiza	Lonchopteridae	*Lonchoptera lutea*	AF502991
	Syrphidae	*Rhingia nasica*	AF502998
Schizophora acalyptratae	Otitidae	*Ceroxys edwardsii*	AF503002
	Drosophilidae	*Drosophila melanogaster*	M21017
Schizophora calyptratae	Otitidae	*Ceroxys edwardsii*	AF503002
	Muscidae	*M. domestica*	AJ551427
	Calliphoridae	*Chrysomya albiceps*	AJ551433
	Calliphoridae	*L. cuprina*	AJ417709
	Tachinidae	*Tachina grossa*	AJ300130
	Gasterophilidae	*Gasterophilus intestinalis*	AJ551429
(outgroup)	Pulicidae	*Archaeopsylla erinace*	X93407

(e) *Quantification of* ace *genes expression during development*

Larvae of each instar and adults of the *C. pipiens* SLAB strain were used to extract RNA with Trizol (Life Technologies) and each sample was reverse transcribed. Real time quantitative PCR (Roche light cycler) was used to estimate the number of both *ace-1* and *ace-2* mRNA copies. Three PCRs were carried out for each developmental stage: one was specific for the *ace-1* gene (Moustdir and Moustdrev primers), the second was specific for the *ace-2* gene (ace2dir4 and ace2rev6 primers) and the last was specific for the G6PDH gene of which the mRNA expression level remains constant during the different developmental stages of *C. pipiens* (CpG6PDHdir GCGG CGGGACTTTGAG and CpG6PDHrev AATCCTGTT CCACCCCTTCA primers). Each cDNA template was analysed in triplicate. The ratio between the *ace* (*1* or *2*) and G6PDH arbitrary concentrations gave the pattern of expression for both *ace* genes during the development of *C. pipiens*.

(f) *Obtaining of* C. pipiens ace-2 *protein in S2* Drosophila *cells*

5′ and 3′ RACEs were carried out using the 'GENE RACER' kit from Invitrogen to give the complete *ace-2* cDNA of *C. pipiens*. The coding cDNA was then amplified using ace2dir ATGTCGTCGATTAGCATGGT and ace2rev GAATAATCTCAGCACGATTA primers and inserted into a pAc5.1/V5-His vector (Invitrogen). S2 cells (20×10^6) were transfected with the expression vector using Fugene6 (Roche) as a transfection reagent in OptiMEM medium according to the manufacturer's protocol. Cells were maintained in serum free Schneider's medium to avoid endogenous AChE activity due to foetal cow serum. Four days after transfection, the cells were collected and centrifuged at 1200 rpm for 3 min and then homogenized in 500 µl of phosphate buffer (0.25 M) containing 0.1% Triton X100. This was then centrifuged for 10 min at 10 000 rpm and the supernatant used as an AChE2 source.

(g) *Detection of* ace-1 *and* ace-2 *in the cholinergic activity of* C. pipiens

We used two methods to determine the contribution of *ace-2* in the cholinergic activity. For method 1, residual activity of AChE from both *ace-1* and *ace-2* were measured with respect to an increasing concentration of one inhibitor. For method 2, residual activity of AChE from both *ace-1* and *ace-2* were measured with respect to an increasing time in the presence of an inhibitor. For both methods, fresh samples (larvae, adults or adult heads) were homogenized in a phosphate buffer (0.25 M and pH 7) containing 1% Triton. The homogenates were then centrifuged (12 000*g* for 5 min) and the supernatants used for detecting enzyme activity. For method 1, extracts from whole larvae, adults and adult heads, and recombinant *ace-1* or *ace-2* proteins (from *C. pipiens*, produced in S2 cells) were incubated with 10^{-4} M of malaoxon (the oxon form of malathion, an organophosphate (OP) insecticide) for various times before adding a substrate solution of acetylthiocholine (10^{-3} M). For method 2, larvae from two different strains were used, one susceptible (SLAB strain; Georghiou *et al.* 1966) and one resistant (SR strain, Bourguet *et al.* 1996) to organophosphates (OP) and carbamate insecticides. *Ace-1* recombinant proteins from the SLAB or SR strains were obtained according to Weill *et al.* (2003). Six inhibitors were tested: propoxur and aldicarb (carbamate insecticides), malaoxon, paraoxon (the oxon form of parathion, OP), trichlorfon (OP) and eserine (an alkaloid from *Physostigma venenosum*). All inhibitors were purchased from Sigma, except propoxur, which was supplied by Bayer (Leverkusen, Germany). Eight dilutions from the initial concentration (1 M: 10^{-1}–10^{-8}) were used for each inhibitor. AChE residual activity was determined (Ellman *et al.* 1961) using acetylthiocholine (10^{-3} M) for each dilution, and was expressed as a percentage of initial activity (without inhibitor) against concentration.

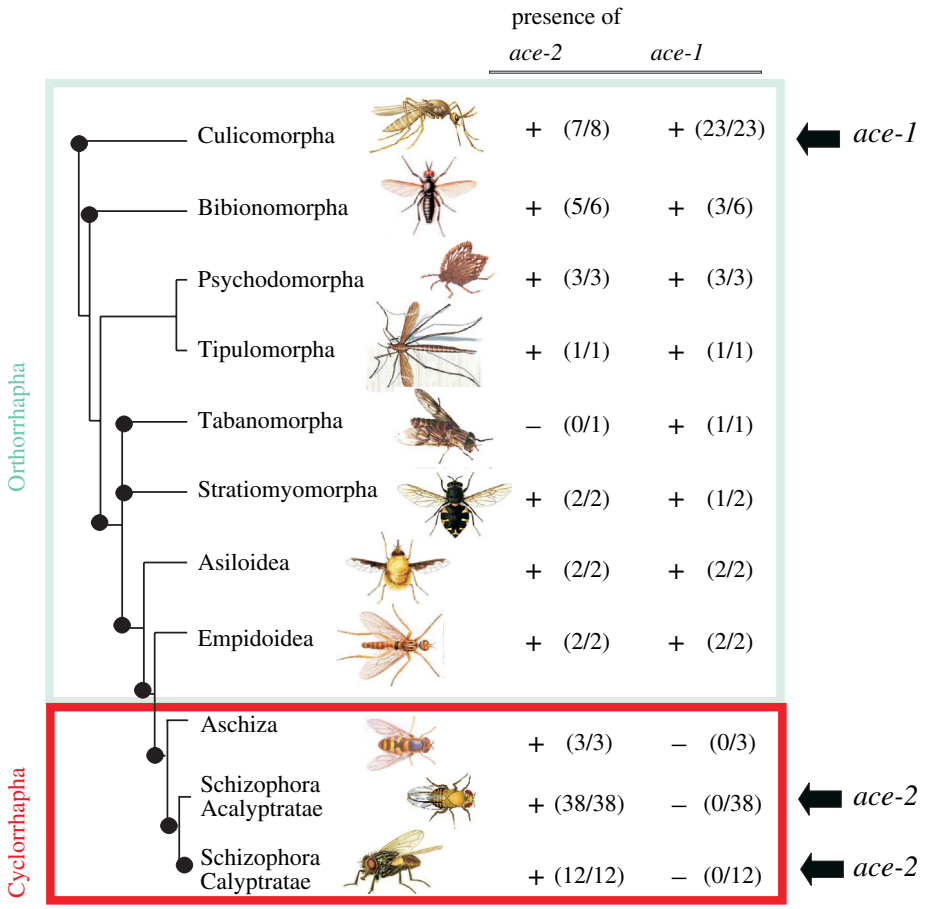

Figure 1. The distribution of *ace* genes in Diptera: presence (+) or absence (−) of *ace-1* and *ace-2* genes in the major divisions of the Diptera phylogeny. Cyclorrhapha or true flies are boxed in red and non-Cyclorrhapha in blue. *Ace* genes were amplified by PCR and sequenced. The gene is considered 'absent' when no amplification occurred after using four (for *ace-1*) or two (for *ace-2*) different pairs of primers. 'Presence' was always confirmed by sequencing. The number of 'positive' species over the total number of species sampled is given in parentheses. For Culicomorpha, data from 15 species investigated in a previous survey (Weill *et al.* 2002) are also included. Black circles on the phylogeny indicate monophyletic groups. When the information was available, black arrows indicate the *ace* gene is involved in the synthesis of the main synaptic AChE: *ace-1* for five species of Culicomorpha, whereas *ace-2* encodes the main synaptic AChE in two species of Schizophora Acalyptratae (*Drosophila melanogaster* and *Bactrocera oleae*) and in one species of Schizophora Calyptratae (*Musca domestica*). See text for explanations.

3. RESULTS

(a) *Distribution pattern of* ace-1 *and* ace-2 *in Diptera*

We sampled 78 species representing 50 families (27% of the dipteran families) and 10 infraorders (71% of the dipteran infraorders), and amplified the *ace-1* and *ace-2* genes using a wide range of degenerated primers to determine the distribution pattern of *ace-1* and *ace-2* among the different Diptera lineages. We found *ace-2* in 75 species, distributed in all major dipteran taxonomic divisions (figure 1). We found no amplified *ace-2* in three species: *Simulium ornatum* (Simuliidae, Culicomorpha), *Tabanus bromius* (Tabanidae, Tabanomorpha) and *Mycetophilidae* (Bibionomorpha).

By contrast, we found *ace-1* in 21 out of 25 species belonging to the non-Cyclorrhapha group (which includes mosquitoes), but in none of the 53 species of the suborder Cyclorrhapha (or 'true flies'), despite an intensive PCR investigation. As a control, we were able to amplify *ace-2* in all the species lacking *ace-1*. The Cyclorrhapha, in which we were unable to amplify *ace-1*, includes the Drosophilidae and in particular *D. melanogaster*, in which *ace-1* is known to be absent.

(b) *Evolution of* ace-2 *within the Diptera*

Likelihood ratio tests were carried out for 30 dipteran and 3 outgroup *ace-2* cDNA (138 nucleotide sites) and protein (45 amino acid sites) sequences, according to different evolutionary rate models for the various taxa. According to the global clock model, a single evolutionary rate is fixed for all taxa. According to the local clock model, three groups (outgroups, non-Cyclorrhapha and Cyclorrhapha) have their own evolutionary rate. According to the relaxed clock model, each branch of the tree has its own evolutionary rate (i.e. a standard maximum-likelihood analysis). The likelihood of each model is shown in table 2.

The log-likelihood values of the relaxed and global clock models were significantly different ($\chi^2_{31}=62.5\times2=125.0$, $p<10^{-5}$ for cDNA, and $\chi^2_{31}=39.2\times2=78.4$, $p<10^{-5}$ for proteins). This suggests that substitution rate contrasts accumulated, among the taxa compared, during *ace-2* evolution. However, the log-likelihood of the local clock model was not significantly different from the global clock model value ($\chi^2_2=1.6$, $p=0.45$ for cDNA, and $\chi^2_2=4.0$, $p=0.13$ for protein). This suggests that the *ace-2* evolutionary rate was not different between the

Table 2. Likelihood values of three different models of the molecular evolution of the *ace-2* fragment. (See text for explanations.)

	log likelihood	
model	cDNA (138 sites)	amino acids (45 sites)
global clock	−2736.6	−1180.7
local clock	−2735.8	−1178.7
relaxed clock	−2674.1	−1141.5

Cyclorrhapha (in which *ace-1* is absent) and the non-Cyclorrhapha (in which *ace-1* is present).

(c) *Quantification of* ace *gene expression during development*

In the mosquito *C. pipiens* (Culicidae family), *ace-2* is expressed, as we were able to quantify mRNA by quantitative PCR in all larval instars (figure 2). From the first instar, *ace-1* is also expressed, which is as expected for a gene encoding the main cholinergic AChE. As the levels of *ace-1* and *ace-2* were determined with different PCR primers, we were unable to directly compare their expression values. Nevertheless, the expression pattern of both genes followed the same trend, with the high level of expression in the newly hatched larvae, a moderately high level from the first instars, and the lowest level of expression in adults.

(d) *Detection of* ace-2 *in the cholinergic activity of* C. pipiens

We studied the AChE activity of whole individuals, heads or recombinant proteins from *ace-1* and *ace-2* in the presence of various AChE inhibitors, which allowed the potential discrimination of enzymatic characteristics, to determine the contribution of *ace-2* in the cholinergic AChE activity in a species possessing the two *ace* genes (*C. pipiens* mosquito, Culicidae family). The first was used in the same concentration of one inhibitor (malaoxon) with respect to increasing time (method 1). The activity curves obtained from whole larvae, adults and adult heads could be perfectly superimposed over the *ace-1* recombinant protein activity curve, and were very different from the *ace-2* recombinant protein activity curve (figure 3). This suggested either a very low or a localized enzymatic activity of the *ace-2* protein. We then measured the residual activity of AChE from both *ace-1* and *ace-2* at different concentrations of seven inhibitors (method 2). The activity curves obtained from whole larvae (either the susceptible or the resistant strain) could be superimposed over the corresponding recombinant *ace* protein activity curve. This suggested that the *ace-2* protein was minimally implicated in the main enzymatic activity of mosquitoes. We observed similar results for five inhibitors from two insecticide families (carbamates and OP) and for one plant alkaloid (figure 4).

4. DISCUSSION

(a) *How many losses of* ace-1 *within the Diptera?*

Diptera divides into paraphyletic lower Diptera (previously Nematocera), paraphyletic lower Brachycera (previously Orthorrhapha) and monophyletic Cyclorrhapha (or true flies). Our data suggest that *ace-1* was lost just before the emergence of Cyclorrhapha. This is supported

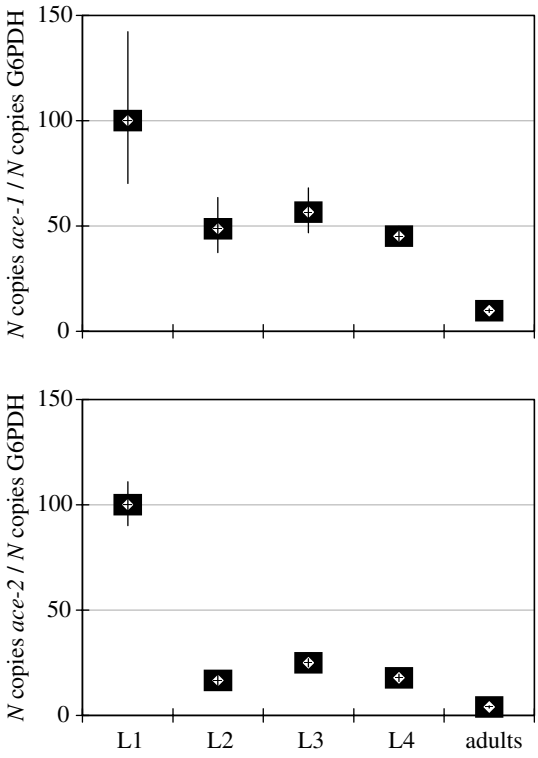

Figure 2. Quantitative reverse transcriptase (RT)-PCR carried out at different developmental stages of the mosquito *Culex pipiens*. The mRNA quantity is expressed as the ratio of the amount of mRNA of the studied gene to the amount of G6PDH mRNA used as a reference, the expression level of which remains constant during development. The value 100 was arbitrarily attributed to the highest ratio value observed. Only the overall profile should be considered when comparing *ace-1* and *ace-2*, see text for explanations.

by several points. First, the absence of *ace-1* in Cyclorrapha is based on negative PCR amplification, using 4 primer pairs from 53 species, sampled from the three main divisions (Aschiza, Schizophora Acalyptratae and Schizophora Calyptratae), representing 32 out of the 74 families (or 43%) of this taxonomic group. This is consistent with the confirmed absence from whole genome sequence of *ace-1* in members of the Drosophilidae family (Schizophora Acalyptratae), i.e. *D. melanogaster* (Weill *et al.* 2003) and *D. pseudoobscura* (P. Fort 2005, personal communication). It is also consistent with *M. domestica* (Schizophora Calyptratae) and *B. oleae* (Schizophora Acalyptratae) using *ace-2* for the cholinergic AChE. Second, whereas the clade of lower Diptera is paraphyletic, the Cyclorrapha is considered monophyletic, as supported by several unambiguous morphological synapomorphies (McAlpine & Wood 1989; Cumming *et al.* 1995; Yeates & Wiegmann 1999; Yeates 2002;

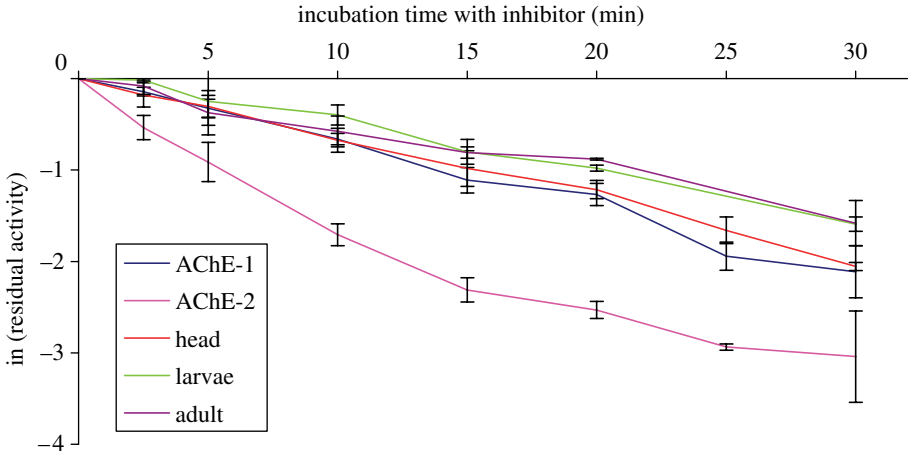

Figure 3. Study of AChE residual activity in *C. pipiens*, expressed as log (A/A_0), with A as the activity at time t and A_0 as the initial activity, against incubation time (minutes) with malaoxon. Results are shown for recombinant protein AChE1 or AChE2, and for whole larval, adults or head extracts.

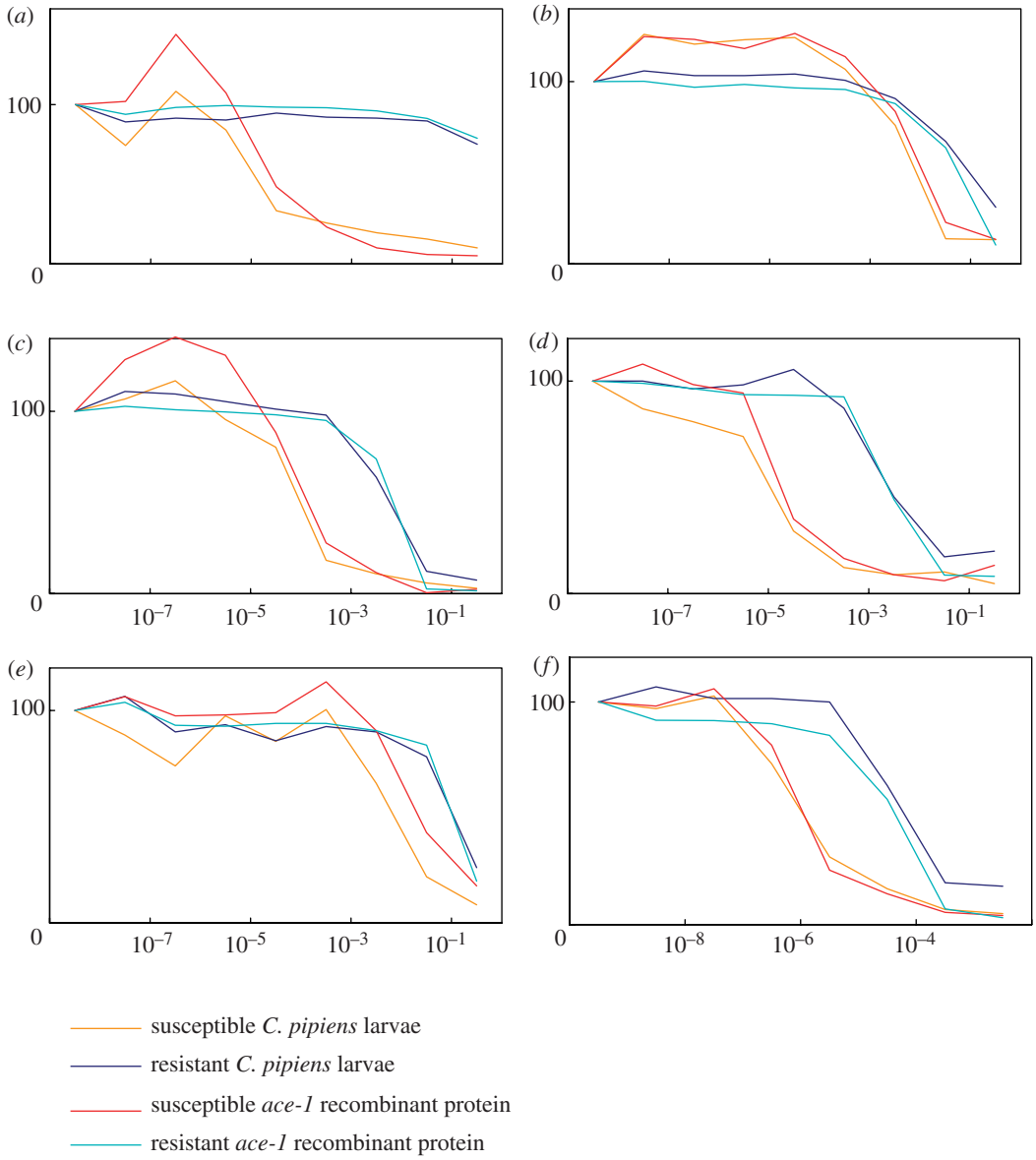

Figure 4. AChE residual activity of *C. pipiens* larvae and of recombinant *ace-1* proteins, (percentage of initial activity), for increasing concentrations of inhibitor (in mol l^{-1}). Both larvae and recombinant proteins are from a susceptible reference strain (SLAB) and an insecticide resistant strain (SR). Six different inhibitors were tested: (*a*) propoxur, (*b*) trichlorfon, (*c*) malaoxon, (*d*) paraoxon, (*e*) aldicarb and (*f*) eserine. See §2 for details. Yellow, susceptible larvae; dark blue, resistant larvae; red, susceptible *ace-1* recombinant protein; light blue, resistant *ace-1* recombinant protein.

Grimaldi & Engel 2005) and by molecular data (Wiegmann *et al.* 2003). Finally, *ace-1* is present in one lower Brachycera subdivision that is considered to be closest to the Cyclorrapha (i.e. Empidoidea, electronic supplementary material and figure 1), and is not detected in cyclorraphan families that are close to the lower Brachycera (i.e. Aschiza: Lonchopteridae, electronic supplementary material and figure 1).

For four lower Diptera species, corresponding to the Mycetophilidae, Sciaridae and Stratiomyidae families, *ace-1* was not amplified. This could be explained by a large gene divergence that prevents PCR amplification in certain groups. This is strengthened by the successful amplification of *ace-1* with another pair of primers in two other species belonging to Mycetophilidae and Stratiomyidae (electronic supplementary material), showing that *ace-1* has not been lost in these two families. Thus, the Sciaridea family is the only non-cyclorraphan family (among 18) in which *ace-1* was not found, although this family was only represented by one species in our sample. It is possible that outside of the Cyclorrapha, *ace-1* has been independently lost in some non-sampled subdivisions (e.g. Ptychopteromorpha, Blephariceromorpha, Axymyiomorpha, Xylophagomorpha, Nemestrinomorpha) or in lower taxonomic units such as the Sciaridae family, although this remains to be established.

In conclusion, *ace-1* has been lost in the Cyclorrapha sub order. In this group, *ace-2* is probably the only *ace* gene present, and encodes the cholinergic AChE (as already confirmed in three species). Thus, the replacement of the *ace-1* function by *ace-2* probably took place around the Jurassic/Cretaceous boundary, as palaeontological evidence suggests that the early cretaceous witnessed the nascent evolution of the Cyclorrapha, with the oldest cyclorraphan fossil being 140 Myr old (Wiegmann *et al.* 2003).

(b) *Understanding of the takeover of* ace-2 *in Cyclorrapha: proximate considerations*

Duplication and subsequent functional divergence of descendant genes has classically been recognized as being the source of new genes (Ohno 1970). Genome analysis has shown that many new functions are associated with gene duplication (He & Zhang 2005). However, the general rules governing functional divergence are unclear. The neofunctionalization hypothesis suggests that after duplication, one daughter gene retains the ancestral function while the other acquires new functions. By contrast, the subfunctionalization hypothesis suggests that the two copies share the ancestral function but differential, tissue-specific expressions. Recent studies have suggested that subfunctionalization is evolutionarily unstable. In other words, it is not generally the terminal fate of the duplicated genes and may evolve towards neofunctionalization, which is evolutionarily stable (He & Zhang 2005; Rastogi & Liberles 2005).

For example, the four *ace* genes in *Caenorhabdis elegans* (*ace-1*, *ace-2*, *ace-3* and *ace-4*) are the result of three independent duplications. These now code for AChE with different pharmacological and tissue distribution, except for *ace-4*, in which the new function is still unclear, being either non-catalytic and/or *cis*-regulating for *ace-3* (Combes *et al.* 2003). In insects, *ace-1* and *ace-2* (resulting from a duplication occurring before the emergence of the Arthropoda, Weill *et al.* 2002) have coexisted for a long time. This is probably because they acquired distinct functions (neofunctionalization). So far, the loss of *ace-1* occurring at the emergence of Cyclorrhapha is unique because it means that, at least in the cholinergic synapse, *ace-2* has retrieved initial functions of the ancestral gene. Thus, it is unclear how to classify this situation according to the various theories of duplication evolution.

The exact function of both *ace-1* and *ace-2* in non-Cyclorrhapha species must be known in order to propose a valid scenario for gene function replacement. In *Culicidae* (mosquitoes), which possess both *ace* genes, AChE activity from *ace-2* cannot be detected by enzymatic assays (figures 3 and 4). This suggests either a very low or a very localized enzymatic activity, suggesting that it plays a very small role in synaptic AChE. Thus, according to the literature and the present results, *ace-2* only encodes the main synaptic function only in true flies but not in other insects (for a review, see Weill *et al.* 2002). However, *ace-2* is expressed in all larval instars, as we were able to quantify the mRNA by quantitative PCR (figure 2). Also, the sequence conservation of *ace-2* across the insects suggests that this gene is being subjected to purifying selection, probably related to restricted synaptic functions and/or other functions. A relative molecular evolution rate test confirmed that the intensity of purifying selection on *ace-2* sequences is constant across the Diptera, irrespective of the presence or absence of *ace-1* (table 2). Non-synaptic functions have been described for cholinesterases, including developmental involvement in neurogenesis or synaptogenesis in *Drosophila* (Greenspan *et al.* 1980; Sternfeld *et al.* 1998). These non-synaptic functions are probably catalytic, possibly operating through the hydrolysis of the same substrate (acetylcholine or ACh) as the synaptic function (Cousin *et al.* 2005). This is probably essential for a possible neofunctionalization towards a synaptic function, as it is perhaps the case for *ace-2* in true flies. Possible non-synaptic functions of *ace-1* are yet to be investigated and if they exist, they may have changed or even disappeared in true flies.

Despite having very little knowledge about the various functions of both *ace-1* and *ace-2*, we know of one requirement for the takeover of *ace-2*. Both genes must have been co-expressed in cholinergic synapses before the loss of *ace-1*, and have, or have had, some degree of compensatory function for synaptic ACh hydrolysis. This is because synaptic activity is vitally important and therefore cannot have been interrupted, even temporarily, in the ancestors of true flies. *C. elegans* presents an example of functional compensation between two *ace* genes: AChE is supplied at the excitatory neuromuscular junction by both nerve cells, in which one *ace* gene is expressed, and by muscle cells, in which another *ace* gene is expressed (Culotti *et al.* 1981; Johnson *et al.* 1981; Combes *et al.* 2003). Here, this functional compensation appears to be stable because it is also found in *C. briggsae*, which diverged from *C. elegans* about 30 Myr (ago) (Grauso *et al.* 1998; Combes *et al.* 2000). Thus, although functional compensation or co-expression is a necessary condition, it is not sufficient for gene takeover and the subsequent loss of the other gene.

(c) *Understanding of the takeover of* ace-2 *in Cyclorrapha: ultimate considerations*

Cyclorrapha is the best defined and most diverse major lineage in Diptera, containing about 105 families and 72 000 species (Grimaldi & Engel 2005; plus update from M.M.). The tremendous success of Cyclorrhapha is linked to several key adaptations, such as fast flying in adults and desiccation resistance of pupae (McAlpine & Wood 1989). There are major differences between Cyclorrapha and other Diptera, particularly within the central nervous system, such as the circumversion (360° rotation) of the male terminalia and a regression of the larval head (McAlpine & Wood 1989; Melzer *et al.* 1995; Yeates *et al.* 2002; Grimaldi & Engel 2005). Thus, within the evolution of true flies, there are both innovations and regressions, which offer several possibilities for explaining *ace-2* takeover and the loss of *ace-1*.

A non-synaptic function of *ace-1* becoming useless during, for example, an organ regression could trigger the takeover of *ace-2*, because any non-synaptic function may have provided a strong evolutionary advantage for an *ace* gene (here, *ace-2* in the true flies). This was particularly true when both genes were co-expressed in cholinergic synapses, a possibly unstable situation in which random fluctuation and gene compensation could easily eliminate one of the genes. Ultimately, an *ace* gene with no non-synaptic function is doomed. There are several possible regression candidates for the loss of a non-synaptic function of *ace-1*. For example, the regression of the larval eye in true flies and, more generally, the regression of the larval cephalic sensory neurogenesis—the loss of muscle and muscle plaques on the side of the external tergites of the pupa, and of the accompanying nervous system etc. These possible candidates could be evaluated empirically by, for example, determining the level of expression of *ace-1* in the tissue of various orthorrhaphan species, particularly families close to the Cyclorrapha.

A phenotypic innovation involving *ace-2* could also be proposed. For example, an adaptive change concerning *ace-2* (e.g. an increase of AChE activity) could also favour *ace-2* in the *ace-1*/*ace-2* activity ratio in synapses. There have been several innovations in true flies that may have triggered a selected change in *ace-2*. For example, adults have a particularly complex organization of the photoreceptor synapses, characterized by a cholinergic presynaptic platform acting as a sort of signal amplification device (Meinertzhagen 1989; Edwards & Palka 1991; Yasuyama & Salvaterra 1999; Buschbeck 2000). This is interpreted as an adaptation to fast flying, which requires particularly efficient and fast receptor–neuron and neuron–neuron communication. In this situation, even a very slight increase in activity could be a significant selective advantage for *ace-2*, thus changing the *ace-1*/*ace-2* synaptic activity ratio. This could explain the *ace-2* takeover of the synaptic function although this requires that *ace-1* has no non-synaptic function for it to be subsequently lost.

In conclusion, the loss of *ace-1* has occurred in a well-defined taxonomic dipteran group, the Cyclorrhapha. Since they were initially duplicated, *ace-1* and *ace-2* have coexisted in arthropods and insects for a long time, probably because they acquired distinct functions. So far, the loss of *ace-1* at the emergence of Cyclorrhapha is unique because it means that *ace-2* must have retrieved, at least in the synapse, the initial functions of the ancestral gene. This event took place about 150 Myr (ago) and therefore cannot be directly investigated. This situation can be further clarified in several ways. In particular, we should identify all of the functions of both genes in non-Cyclorrhapha species to provide useful information that may strengthen or validate one of our possible scenarios involving nervous system innovation or regression in the true flies.

We thank N. Agusti, R. Allemand, T. Baldet, D. Bourguet, S. Bréchoire, C. Caceres, A. Callaghan, A. Casteignau, E. Cosson, J.-C. Delécolle, M. Denniff, L. Desprès, T. Disca, G. Duvallet, S. Gouinguene, P. Grébaut, P.-Y. Henry, V. Jamonneau, Y. Layec, B. Mathieu, A. Pomiamkowski, F. Renaud, D. Rey, V. Rufray, F. Schaffner, J. Scott, M. Valero and P. Vernon for Diptera samples, D. Bourguet and N. Pasteur for useful discussion and V. Durand for bibliographic help. This work was financed in part by ANR MoREvol. Contribution 2006.051 of the Institut des Sciences de l'Evolution de Montpellier (UMR CNRS 5554).

REFERENCES

Bourguet, D., Pasteur, N., Bisset, J. & Raymond, M. 1996 Determination of *ace-1* genotypes in single mosquitoes: toward an ecumenical biochemical test. *Pest. Biochem. Physiol.* **55**, 122–128. (doi:10.1006/pest.1996.0041)

Buschbeck, E. K. 2000 Neurobiological constraints and fly systematics: how different types of neural characters can contribute to a higher level dipteran phylogeny. *Evolution* **54**, 888–898.

Combes, D., Fedon, Y., Grauso, M., Toutant, J.-P. & Arpagaus, M. 2000 Four genes encode acetylcholinesterase in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*. cDNA sequence, genomic structures, mutations and *in vivo* expression. *J. Mol. Biol.* **300**, 727–742. (doi:10.1006/jmbi.2000.3917)

Combes, D., Fedon, Y., Toutant, J.-P. & Arpagaus, M. 2003 Multiple ace genes encoding acetylcholinesterase of *Coenorhabditis elegans* have distinct tissue expression. *Eur. J. Neurosci.* **18**, 497–512. (doi:10.1046/j.1460-9568.2003.02749.x)

Cousin, X., Strähle, U. & Chatonnet, A. 2005 Are there non-catalytic functions of acetylcholinesterases? Lessons from mutant animal models. *BioEssays* **27**, 189–200. (doi:10.1002/bies.20153)

Culotti, J. G., von Ehrenstein, G., Culotti, M. R. & Russell, R. L. 1981 A second class of acetylcholinesterase-deficient mutants of the nematode *Caenorhabditis elegans*. *Genetics* **97**, 281–305.

Cumming, J. M., Sinclair, B. J. & Wood, D. M. 1995 Homology and phylogenetic implications of male genitalia in Diptera–Eremoneura. *Entomologica Scandinavica* **26**, 120–151.

Edwards, J. S. & Palka, J. 1991 Insect neural evolution-a fugue or an opera? *The Neurosci.* **3**, 391–398.

Ellman, G. L., Courtney, K. D., Andres, V. & Feather-stone, R. M. 1961 A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95. (doi:10.1016/0006-2952(61)90145-9)

Felsenstein, J. 1981 Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.* **17**, 368–376. (doi:10.1007/BF01734359)

Felsenstein, J. 1993 PHYLIP (phylogeny inference package), v. 3.5c. Department of Genetics, University of Washington, Seattle WA.

Fournier, D., Karch, F., Bride, J. M., Hall, L. M. C., Bergé, J.-B. & Spierer, P. 1989 *Drosophila melanogaster* acetylcholinesterase gene, structure, evolution and mutations. *J. Mol. Evol.* **210**, 15–22.

Gaunt, M. W. & Miles, M. A. 2002 An insect molecular clock dates the origin of the insects and accords with paleontological and biogeographic landmarks. *Mol. Biol. Evol.* **19**, 748–761.

Georghiou, G. P., Metcalf, R. L. & Gidden, F. E. 1966 Carbamate-resistance in mosquitoes: selection of *Culex pipiens fatigans* Wied (=*Culex quinquefasciatus*) for resistance to Baygon. *Bull. World Health Organ.* **35**, 691–708.

Grauso, M., Culetto, E., Combes, D., Fedon, Y., Toutant, J.-P. & Arpagaus, M. 1998 Existence of four acetylcholinesterase genes in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*. *FEBS Lett.* **424**, 279–284. (doi:10.1016/S0014-5793(98)00191-4)

Greenspan, R. J., Finn, J. A. & Hall, J. C. 1980 Acetylcholinesterase mutants in *Drosophila* and their effects on the structure and function of the central nervous system. *J. Comp. Neurol.* **189**, 741–774. (doi:10.1002/cne.901890409)

Griffiths, G. C. D. 1994 Relationships among the major subgroups of Brachycera (Diptera): a critical review. *Can. Entomologist* **126**, 861–880.

Grimaldi, D. & Engel, M. S. 2005 *Evolution of the insects*. Cambridge, UK: Cambridge University Press.

Guindon, S. & Gascuel, O. 2003 A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52**, 696–704. (doi:10.1080/10635150390235520)

He, X. & Zhang, J. 2005 Rapid subfunctionalization accompanied by prolonged and substantial neofunctionalization in duplicate gene evolution. *Genetics* **169**, 1157–1164. (doi:10.1534/genetics.104.037051)

Johnson, C. D., Duckett, J. G., Culotti, J. G., Herman, R. K., Meneely, P. M. & Russell, R. L. 1981 An acetylcholinesterase-deficient mutant of the nematode *Caenorhabditis elegans*. *Genetics* **97**, 261–279.

Kozaki, T., Shono, T., Tomita, T. & Kono, Y. 2001*a* Fenitroxon insensitive acetylcholinesterases of the housefly, *Musca domestica*, associated with point mutations. *Insect Biochem. Mol. Biol.* **31**, 991–997. (doi:10.1016/S0965-1748(01)00047-9)

Lee, D.-W., Kim, S.-S., Shin, S. W., Kim, W. T. & Boo, K. S. 2006 Molecular characterization of two acetylcholinesterase genes from the oriental tobacco budworm, *Helicoverpa assulta* (Guenée). *Biochim. Biophys. Acta* **1760**, 125–133.

Lenormand, T. & Raymond, M. 2000 Clines with variable selection and variable migration: model and field studies. *Am. Nat.* **155**, 70–82. (doi:10.1086/303295)

Lenormand, T., Guillemaud, T., Bourguet, D. & Raymond, M. 1998 Evaluating gene flow using selected markers: a case study. *Genetics* **149**, 1383–1392.

Lenormand, T., Bourguet, D., Guillemaud, T. & Raymond, M. 1999 Tracking the evolution of insecticide resistance in the mosquito *Culex pipiens*. *Nature* **400**, 861–864. (doi:10.1038/23685)

Li, F. & Han, Z. 2004 Mutations in acetylcholinesterase associated with insecticide resistance in the cotton aphid, *Aphis gossypii* Glover. *Insect Biochem. Mol. Biol.* **34**, 397–405. (doi:10.1016/j.ibmb.2004.02.001)

McAlpine, J. F. & Wood, D. M. 1989 *Manual of nearctic Diptera*. Ottawa, Canada: Research Branch Agriculture.

Meinertzhagen, I. A. 1989 Fly photoreceptor synapses: their development, evolution, and plasticity. *J. Neurobiol.* **20**, 276–294. (doi:10.1002/neu.480200503)

Melzer, R. R., Panzinger, A., Reckel, F. & Smola, U. 1995 Central nervous system of brachyceran larvae (Insecta, Diptera). *Zoologischer Anzeiger* **234**, 113–123.

Ohno, S. 1970 *Evolution by gene duplication*. Heidelberg, Germany: Springer.

Oosterbroek, P. & Courtney, G. 1995 Phylogeny of the Nematocerous families of Diptera (Insecta). *Zool. J. Linn. Soc.* **115**, 267–311. (doi:10.1006/zjls.1995.0080)

Quinn, D. M. 1987 Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. *Chem. Rev.* **87**, 955–979. (doi:10.1021/cr00081a005)

Rastogi, S. & Liberles, D. A. 2005 Subfunctionalization of duplicated genes as a transition state to neofunctionalization. *BMC Evol. Biol.* **5**, 28–35. (doi:10.1186/1471-2148-5-28)

Rodriguez-Trelles, F., Tarrio, R. & Ayala, F. J. 2003 Convergent neofunctionalization by positive Darwinian selection after ancient recurrent duplications of the xanthine dehydrogenasegene. *Proc. Natl Acad. Sci. USA* **100**, 13 413–13 417. (doi:10.1073/pnas.1835646100)

Russell, R. J., Claudianos, C., Campbell, P. M., Horne, I., Sutherland, T. D. & Oakeshott, J. G. 2004 Two major classes of target site insensitivity mutations confer resistance to organophosphate and carbamate insecticides. *Pest. Biochem. Physiol.* **79**, 84–93. (doi:10.1016/j.pestbp.2004.03.002)

Sternfeld, M., Ming, G.-L., Song, H.-J., Sela, K., Timberg, R., Poo, M.-M. & Soreq, H. 1998 Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C-termini. *J. Neurosci.* **18**, 1240–1249.

Swofford, D.L. 1998 *PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4*. Sunderland, MA: Sinauer.

Toutant, J. P. 1989 Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms. *Prog. Neurobiol.* **32**, 423–446. (doi:10.1016/0301-0082(89)90031-2)

Vontas, J. G., Hejazi, M. J., Hawkes, N. J., Cosmidis, N., Loukas, M. & Hemingway, J. 2002 Resistance-associated point mutations of organophosphate acetylcholinesterase, in the olive fruit fly *Bactrocera oleae*. *Insect Mol. Biol.* **11**, 329–336. (doi:10.1046/j.1365-2583.2002.00343.x)

Walsh, S. B., Dolden, T. A., Moores, G. D., Kristensen, M., Lewis, T., Devonshire, A. L. & Williamson, M. S. 2001 Identification and characterization of mutations in housefly (*Musca domestica*) acetylcholinesterase involved in insecticide resistance. *Biochem. J.* **359**, 175–181. (doi:10.1042/0264-6021:3590175)

Weill, M., Fort, P., Berthomieu, A., Dubois, M.-P., Pasteur, N. & Raymond, M. 2002 A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the *ace* gene in *Drosophila*. *Proc. R. Soc. B* **269**, 2007–2016. (doi:10.1098/rspb.2002.2122)

Weill, M. *et al.* 2003 Insecticide resistance in mosquito vectors. *Nature* **423**, 136–137. (doi:10.1038/423136b)

Weill, M., Malcolm, M., Chandre, F., Mogensen, K., Berthomieu, A., Marquine, M. & Raymond, M. 2004 The unique mutation in *ace-1* giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Mol. Biol.* **13**, 1–7. (doi:10.1111/j.1365-2583.2004.00452.x)

Wheeler, W. C., Whiting, M., Wheeler, Q. D. & Carpenter, J. M. 2001 The phylogeny of the extant hexapod orders. *Cladistics* **17**, 113–169. (doi:10.1111/j.1096-0031.2001.tb00115.x)

Wiegmann, B. M. 1993 Evolutionary origin of the Cyclorrhapha (Diptera): tests of alternative morphological hypotheses. *Cladistics* **9**, 41–81. (doi:10.1111/j.1096-0031.1993.tb00208.x)

Wiegmann, B. M., Yeates, D. K., Thorne, J. L. & Kishino, H. 2003 Time flies, a new molecular time-scale for Brachyceran fly evolution without a clock. *Syst. Biol.* **52**, 745–756. (doi:10.1080/10635150390250965)

Yang, Z. 1997 PAML: a program package for phylogenetic analysis by maximum likelihood. *CABIOS* **13**, 555–556.

Yasuyama, K. & Salvaterra, P. M. 1999 Localization of choline acetyltransferase-expressing neurons in *Drosophila* nervous system. *Microsc. Res. Tech.* **45**, 65–79. (doi:10.1002/(SICI)1097-0029(19990415)45:2<65::AID-JEMT2>3.0.CO;2-0)

Yeates, D. K. 2002 Relationships of extant lower Brachycera (Diptera): a quantitative synthesis of morphological characters. *Zoologica Scripta* **1**, 105–121. (doi:10.1046/j.0300-3256.2001.00077.x)

Yeates, D. K. & Wiegmann, B. M. 1999 Congruence and controversy: toward a higher-level phylogeny of Diptera. *Annu. Rev. Entomol.* **44**, 397–428. (doi:10.1146/annurev.ento.44.1.397)

Yeates, D. K., David, J., Merritt, D. J. & Baker, C. H. 2002 The adult ventral nerve cord as a phylogenetic character in brachyceran Diptera. *Organ. Divers. Evol.* **2**, 89–96. (doi:10.1078/1439-6092-00037)