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Comparative linkage map development and identification of an autosomal locus for insensitive acetylcholinesterase-mediated insecticide resistance in *Culex tritaeniorhynchus*

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Abstract

A comparative linkage map for *Culex tritaeniorhynchus* was constructed based on restriction fragment length polymorphism markers using cDNAs from *Aedes aegypti*. Linear orders of marker loci in *Cx. tritaeniorhynchus* were identical to *Culex pipiens* wherein chromosomes 2 and 3 reflect whole-arm rearrangements compared to *A. aegypti.* However, the sex determination locus in *Cx. tritaeniorhynchus* maps to chromosome 3, in contrast to *Cx. pipiens* and *Ae. aegypti* where it is located on chromosome 1. Our results indicate that insensitive acetylcholinesterase (AChE)-mediated organophosphate resistance is controlled by a single major gene (AChE^R) on chromosome 2, while the AChE structural gene (*Ace*) is located on chromosome 1. No evidence for a second *Ace* gene was observed, even under very low stringency hybridization conditions.

Keywords: *Culex tritaeniorhynchus*, linkage map, RFLP, acetylcholinesterase, insecticide resistance.

Introduction

The mosquito *Culex tritaeniorhynchus* is distributed throughout most of Asia and Africa, and is the main vector of Japanese encephalitis (JE) virus. JE is the most common cause of epidemic encephalitis in the world, with an estimated world-wide incidence of 45 000 cases each year, primarily in children. Approximately 25% of cases are fatal and 50% develop permanent neuropsychiatric sequelae (Vaughn & Hoke, 1992).

The larval habitat is mainly rice fields and shallow marshes, and frequently *Cx. tritaeniorhynchus* is the dominant mosquito species in these habitats. Adult females prefer to blood-feed on animals such as swine and cattle, but also frequently blood-feed on birds (Sirivanakarn, 1976; Tanaka *et al*., 1979; Harbach, 1988). The natural JE transmission cycle involves aquatic birds. However, frequently they transmit JE virus to swine, the major amplifier vertebrate host of the virus, and then occasionally to humans.

Despite the availability of an effective vaccine, mosquito control represents an important strategy for prevention of JE transmission and epidemic outbreaks in most developing countries (Vaughn & Hoke, 1992). This strategy is threatened by the recent evolution of high levels of resistance to organophosphate (OP) and carbamate insecticides among *Cx. tritaeniorhynchus* populations in Japan (Kamimura & Maruyama, 1983; Yasutomi & Takahashi, 1987) and other countries (Karunaratne *et al*., 1998; Thomas *et al*., 2000).

In addition to JE virus, *Cx. tritaeniorhynchus* is considered to be an excellent potential vector of West Nile virus in Pakistan (Hayes *et al*., 1980) and in India (Ilkal *et al*., 1997). Sindbis virus also was isolated from a pooled sample of these mosquitoes collected from Saudi Arabia (Wills *et al*., 1985).

Genetic studies of *Cx. tritaeniorhynchus* have involved a limited number of mutant marker and isozyme marker loci, and a simple linkage map has been constructed (Baker *et al*., 1977). Because few markers common to *Culex pipiens* or *Aedes aegypti* are included in this linkage map, detailed comparisons of genome organization in *Cx. tritaeniorhynchus* to other mosquito species are lacking (Matthews & Munstermann, 1994). We recently constructed comparative restriction fragment length polymorphism (RFLP) linkage maps for several mosquito species using a common set of cDNA clones developed from *Ae. aegypti* as marker loci

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(Severson *et al.*, 1993; Severson *et al.*, 1995; Ferdig *et al.*, 1999; Mori *et al.*, 1999). These investigations largely supported previous suggestions (Matthews & Munstermann, 1994) that mosquito chromosome evolution largely reflects whole-arm translocations. The most significant re-arrangements were observed between *Ae. aegypti* and *Cx. pipiens* (Mori *et al.*, 1999). In this study, a comparative linkage map for *Cx. tritaeniorhynchus* was constructed with a subset of *Ae. aegypti* RFLP markers. In addition, we investigated the genetic relationship between insensitive acetylcholinesterase-mediated insecticide resistance ($AChE^R$) and an acetylcholinesterase structural gene (*Ace*).

Results and discussion

Thirty-four of forty-six *Ae. aegypti* cDNA clones tested as probes to genomic Southern blots representing the *Cx. tritaeniorhynchus* Kochi, Pakistan and Toyama strains reflected informative strain specific polymorphisms (Fig. 1). These results are consistent with observations for other culicine species (Severson *et al.*, 1994). Twenty-six of the forty-six clones were previously shown to be informative among selected *Cx. pipiens* strains (Mori *et al.*, 1999), and twenty-four of these twenty-six clones were also informative among the *Cx. tritaeniorhynchus* strains examined in the present study. Fourteen of these clones that provided broad coverage of the *Cx. pipiens* genome (Mori *et al.*, 1999) were selected to construct the comparative map for *Cx. tritaeniorhynchus*. Because all hybridizations were performed under high stringency conditions, clones selected for linkage analysis clearly represent highly conserved homologous gene sequences among *Aedes* and *Culex* spp.

Segregation ratios for the backcross (BC_1) population generally fit the expected 1 : 1 Mendelian ratios, although a slight excess of heterozygotes was observed for 3 loci on chromosome 1 (Table 1). For each of these loci, an excess of heterozygotes was obtained. With most chromosome 3 loci, we observed significant sex-specific biases in segregation ratios because of their linkage with the male-determining factor (M^3) at the sex determination locus.

Segregation ratios observed with the F_1 intercross populations were more variable, with a general trend toward a significant excess of heterozygotes at some loci (data not shown). We have observed similar results with every mosquito species examined to date (Severson *et al.*, 1993, 1995; Ferdig *et al.*, 1998; Mori *et al.*, 1999). This phenomena likely represents lethal gene effects on survival of particular homozygote classes (Matthews & Munstermann, 1994; Matthews & Craig, 1989).

Previous genetic studies with morphological mutant and isozyme markers in *Cx. tritaeniorhynchus* have consistently shown that recombination does not occur in females, but does occur in males (Baker & Rabbani, 1970; Sakai *et al.*, 1972; Baker & Sakai, 1973a, 1973b, 1974). Because these studies were only able to examine 2 or 3 loci in individual crosses, we elected to re-confirm this phenomena

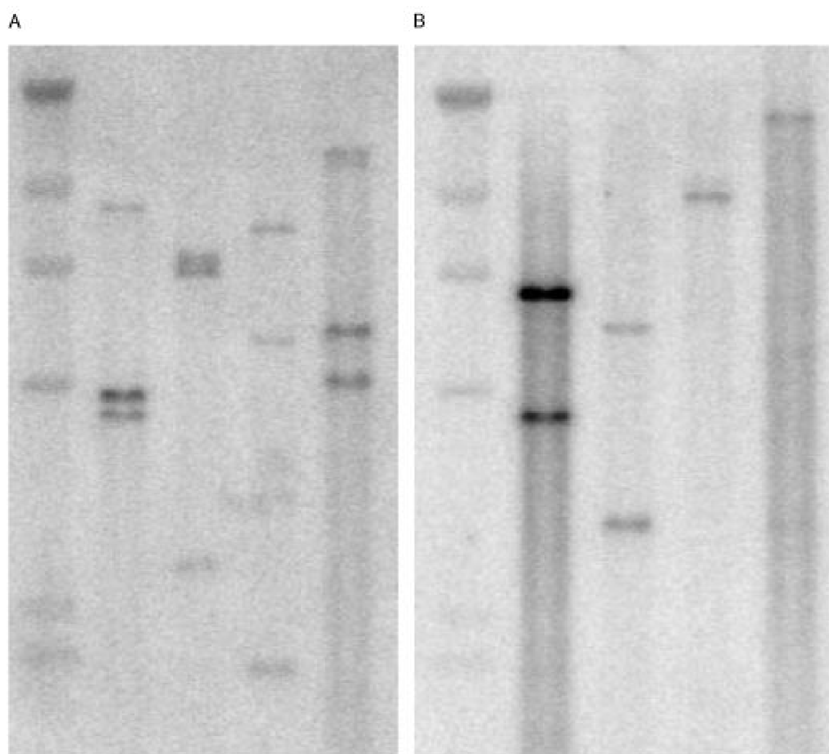

Figure 1. Autoradiographs of *Culex tritaeniorhynchus* genomic Southern blots used to screen for RFLPs with *Aedes aegypti* cDNA clones among pooled DNA samples representing mosquito strains. DNA was digested with *Eco*RI. (A) Hybridization with clone LF264; (B) hybridization with clone LF335. For each clone: lane 1 = *Hin*dIII-digested lambda phage DNA markers; lane 2 = *Ae. aegypti* Liverpool strain; lane 3–5 = *Cx. tritaeniorhynchus*, lane 3 = Kochi strain, lane 4 = Toyama strain, lane 5 = Pakistan strain.

Table 1. Segregation of markers in the (Toyama ♂ × Kochi ♀) ♂ × (Toyama ♀) BC_1 progeny

	H : P†	χ^2
Chromosome 1		
LF188	97 : 68	5.10*
Female	51 : 35	2.98
Male	46 : 33	2.14
Ace	100 : 66	6.96**
Female	52 : 34	3.77
Male	48 : 32	3.20
TY7	97 : 69	4.72*
Female	53 : 33	4.65*
Male	44 : 36	0.80
LF159	93 : 73	2.41
Female	50 : 36	2.28
Male	43 : 37	0.45
Chromosome 2		
LF128	94 : 72	2.92
Female	50 : 36	2.28
Male	44 : 36	0.80
LF106	91 : 75	1.54
Female	51 : 35	2.98
Male	40 : 40	0.00
$AChE^R$	92 : 74	1.95
Female	48 : 38	1.16
Male	44 : 36	0.80
LF115	95 : 71	3.47
Female	53 : 33	4.65*
Male	42 : 38	0.20
LF264	86 : 79	0.30
Female	49 : 37	1.67
Male	37 : 42	0.32
Chromosome 3		
DDC	84 : 82	0.02
Female	84 : 2	78.19**
Male	0 : 80	80.00**
LF335	84 : 82	0.02
Female	84 : 2	78.19**
Male	0 : 80	80.00**
LF168	94 : 68	4.17*
Female	68 : 16	32.19**
Male	26 : 52	8.67**
LF386	97 : 69	4.72*
Female	67 : 19	26.79**
Male	30 : 50	5.00*
LF218	89 : 77	0.87
Female	50 : 36	2.28
Male	39 : 41	0.05

†H = heterozygote; P = Toyama strain parental type.
**P* < 0.05; ***P* < 0.01; loci tested for expected 1 : 1 ratio.

using RFLP markers to perform whole genome analyses in both F_1 intercross and BC_1 populations. Our results indicate that recombination frequencies observed among F_1 intercross progeny are about one-half that observed with BC_1 progeny across all markers and chromosomes (Fig. 2). This result is expected if no recombination occurred in the F_1 females that produced the intercross populations, but recombination did occur in the F_1 males in both the intercross and BC_1 populations. The lack of recombination in male *Drosophila melanogaster* is well-known, and no recombination is observed in female *Bombyx mori* (Rasmussen, 1977). To our knowledge, *Cx. tritaeniorhynchus* represents the only mosquito species in which female sex-specific restrictions on recombination have been observed. The mechanism remains unknown.

Linkage group and linear order of all RFLP markers examined seem to be completely conserved between *Cx. tritaeniorhynchus* and *Cx. pipiens* (Mori *et al.*, 1999). However, note that our designated chromosome 3 for *Cx. tritaeniorhynchus* is homologous to chromosome 2 in *Cx. pipiens.* We used chromosome designations that are consistent with the published literature for both species.

Sex determination in most culicines is determined by a single autosomal locus on chromosome 1, with maleness representing the heterogametic sex (Gilchrist & Haldane, 1947). Some mapping efforts with *Cx. tritaeniorhynchus* are consistent with other culicines, because the sex determination locus in certain populations does map to chromosome 1 with the male-determining allele (M^1) being dominant (Baker *et al.*, 1971; Selinger, 1972). However, Baker & Sakai (1976) reported that the sex determination locus in a strain from Pakistan mapped to chromosome 3 and designated a new male-determining allele (M^3) for this locus. An earlier report (Baker *et al.*, 1977) identified chromosome 3 linked sex determination in four strains from Japan, while strains from Pakistan, Bangladesh and Taiwan exhibited chromosome 1 linked sex determination. Takahashi (1985) determined that strains from Japan, Taiwan and Thailand all exhibited chromosome 3 linked sex determination. Of interest, the sex determination locus in our segregating populations of *Cx. tritaeniorhynchus* maps to chromosome 3 (Fig. 2). These results indicate that the M^3 locus is present in our strain from Pakistan and two strains from Japan. The presence of the M^3 locus in a Pakistan strain in this study and a previous report (Baker *et al.*, 1977) is possibly due to inadvertent gene introgression from Japanese strains in the laboratory or perhaps both the M^1 and M^3 alleles are segregating within geographical regions.

The genetic mechanisms for insecticide resistance can be classified as metabolically based, either through gene amplification or gene up-regulation, or as due to structural changes in insecticide target molecules (Hemingway & Ranson, 2000). The exact mechanism can vary among mosquito populations. For example, the primary mechanism of resistance to carbamates among *Cx. tritaeniorhynchus* in Sri Lanka was a consequence of elevated esterase activity (Karunaratne *et al.*, 1998). Conversely, Kamimura & Maruyama (1983) reported very high levels of resistance to OP and carbamate insecticides among *Cx. tritaeniorhynchus* collected in Toyama, Japan in 1982, which was later shown to be due to insensitivity of AChE (Takahashi & Yasutomi, 1987).

We investigated AChE activity among BC_1 progeny of highly OP susceptible (Kochi) and resistant (Toyama)

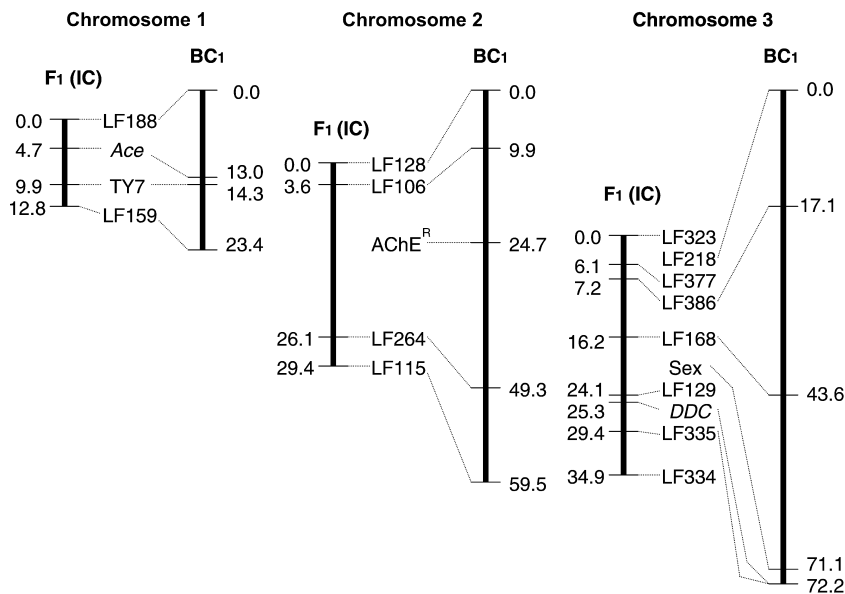

Figure 2. Linkage maps of *Culex tritaeniorhynchus* based on recombination frequencies observed among F_1 intercross and BC_1 progeny. F_1 (IC): (Kochi × Pakistan) F_1 intercross. BC_1: (Toyama ♂ × Kochi ♀) ♂ × (Toyama ♀) backcross. Map distances are listed in Kosambi centiMorgans.

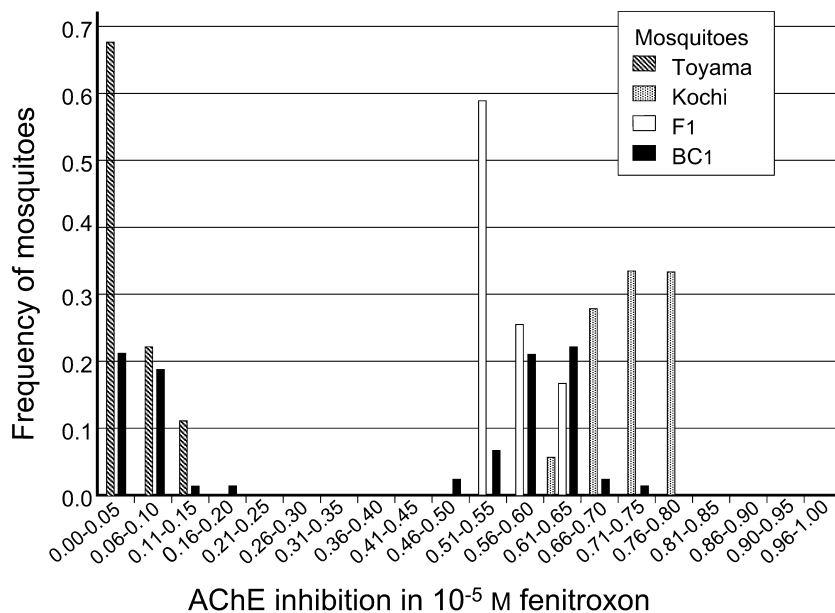

Figure 3. Distribution of AChE inhibition with fenitroxon in resistant (Toyama) and susceptible (Kochi) strains of *Culex tritaeniorhynchus*, and their F_1 and BC_1 progeny.

Cx. tritaeniorhynchus strains under conditions (10 μM fenitroxon) that clearly distinguish the two strains (Fig. 3). With the BC_1 progeny, a bimodal distribution was observed showing either (1) near complete AChE inhibition or (2) high AChE activity. This is consistent with resistance being due to the effects of a single major effect gene.

We also used RFLP marker analysis to map both the positions of OP resistance due to insensitive AChE ($AChE^R$) and the AChE structural gene (*Ace*). The *Cx. tritaeniorhynchus Ace* locus position was based on heterologous probing with a full-length *Ae. aegypti Ace* cDNA (Anthony *et al.*, 1995). To determine if multiple gene effects for OP resistance were evident, a whole genome scan for quantitative trait loci (QTL) was initially performed for the BC_1 population. However, only a single QTL was identified on chromosome 2. Thereafter, we directly mapped $AChE^R$ as a discreet locus within the same QTL interval (Fig. 2). The *Cx. tritaeniorhynchus Ace* locus maps to chromosome 1, and no evidence for a second *Ace* locus was observed (Fig. 2). That is, no non-chromosome 1 linked restriction fragments were observed with Southern blots probed with the *Ace* cDNA, even under very low stringency hybridization and wash conditions. These results are completely consistent with previous findings by Takahashi & Yasutomi (1987) that the $AChE^R$ locus maps to chromosome 2. In addition, Mamiya *et al.* (1997, 1998) observed that AChE from resistant and susceptible strains showed

variation in several biochemical parameters, suggesting that structural differences exist between the two forms.

The AChE^R locus in *Cx. tritaeniorhynchus* and all other mosquito species either represents a second yet to be identified *Ace* gene or an undefined gene product that interacts directly with AChE to promote conformational changes that result in resistance to OPs and carbamates. The literature reflects conflicting support for both scenarios.

The primary support for the putative existence of two unlinked *Ace* genes is provided by *Cx. pipiens* where two electrophoretically distinct isoforms of AChE have been identified, only one of which seems to be involved in AChE-mediated insecticide resistance (Bourguet *et al.*, 1996). However, only a single AChE enzyme is evident in all other mosquitoes examined to date, including *Ae. aegypti*, *Anopheles gambiae*, *A. stephensi*, *Culiseta longeareolata* and *Culex hortensis* (Bourguet *et al.*, 1997).

Support for the existence of a novel gene product that influences AChE activity through conformational changes comes largely from the inability to identify a second *Ace* gene in any mosquito species. Even with *Cx. pipiens*, only a single *Ace* gene has been identified that is located on chromosome 1, while the AChE^R locus maps to chromosome 2 (Malcolm *et al.*, 1998). While insensitive AChE-mediated insecticide resistance has not been reported for *Ae. aegypti*, only a single chromosome 1 linked *Ace* locus has been identified (Severson *et al.*, 1997). Direct evidence for two independent *Ace* loci exists only for *An. stephensi* (Malcolm & Hall, 1990), wherein an *Ace* cDNA clone reportedly hybridized to sites on chromosomes 2 and 3 with polytene chromosome preparations. However, hybridizations of the same probe sequence to Southern blots of total genomic DNA suggested only a single locus even under low stringency hybridization conditions. Furthermore, only a single cDNA for the *Ace* gene in *An. stephensi* has been cloned (Hall & Malcolm, 1991).

Resolution of the exact molecular basis for AChE-mediated resistance in mosquitoes will likely only be achieved through the isolation and characterization of the AChE^R gene. It is noteworthy, however, that among a wide range of non-mosquito insects that have been studied, their *Ace* gene sequences not only show considerable identities, but also the same underlying resistance-associated amino acid substitutions (reviewed by ffrench-Constant *et al.*, 1998). Given the importance of AChE, it would seem most parsimonious for mosquitoes to select for a gene product with a modifying effect on its activity rather than a highly divergent, yet functional, homologue.

Experimental procedures

Mosquitoes

Three *Cx. tritaeniorhynchus* strains maintained at the Research Center of Communicable Diseases (RCCD) in Japan were used to prepare segregating populations. The Pakistan strain was initiated from mosquitoes collected at Karachi, Pakistan by Dr Kamimura, Toyama Medical and Pharmacological University. The Kochi strain was originally provided by Dr Matsuzaki, Kochi Women's College in Kochi prefecture, Japan. The Toyama strain, which carries very high levels of resistance to OP and carbamate insecticides (Takahashi & Yasutomi, 1987; Mamiya *et al.*, 1997), was provided to RCCD Japan by Dr Kamimura.

Three segregating populations were prepared and investigated. Two populations were F_1 intercrosses between Pakistan and Kochi strains. In cross 1, an F_1 generation was produced using mass matings between twenty Kochi females and twenty Pakistan males, and F_1 intercross progeny ($n = 213$) were produced from sibling matings. In cross 2, the F_1 generation ($n = 95$) was produced using Pakistan females and Kochi males. The third population was a BC_1 ($n = 166$) initiated from Kochi strain females and Toyama strain males. F_1 males were backcrossed to Toyama females, because the lack of AChE inhibition is a recessive trait.

Mosquito larvae were reared on a suspension of dried beef liver powder. Individual egg rafts were reared in separate rearing pans and pupae transferred to cages for adult eclosion and sibling matings. P and F_1 generation pupae were separated by sex before adult emergence. Adults were kept in $20 \times 20 \times 30$ cm cages and provided cotton soaked with 2% sugar solution *ad libitum*. Adult females were blood-fed on rats 1 week after emergence.

Mosquitoes were reared and maintained in an environmental chamber at 25 °C, 80% RH and 16 h light and 8 h dark cycle with a 30 min crepuscular period at the beginning and end of each light cycle. F_1 intercross adults were frozen at −80 °C on the day of emergence. BC_1 progeny were preserved in 100% ethanol following measurements of AChE activity.

Molecular techniques

DNA extractions from individual and pools of mosquitoes representing both males and females of each of the parental strains, digestion with *Eco*RI, Southern blotting and hybridizations were performed as described elsewhere (Severson, 1997). Low stringency hybridizations were performed at 50 °C, and membranes were washed for 15 min each at room temperature and at 50 °C in 2x SSC/0.1% SDS. With ethanol preserved mosquitoes, DNA was extracted as above after allowing the carcasses to air dry on filter paper at room temperature overnight. All of the RFLP markers used in this study are *Ae. aegypti* cDNA clones (Severson *et al.*, 1993; D. W. Severson, unpublished data). Markers were selected from clones previously mapped in *Cx. pipiens* with the exception of LF159 and LF417 (Mori *et al.*, 1999).

Linkage analysis

Linkage analysis was performed using the MAPMAKER/QTL computer program (Lander *et al.*, 1987). The default minimum threshold LOD score of 3.0 was used to identify linkage between loci. Linkage data from the two independent F_1 intercrosses were integrated using the JoinMap computer program, with the default threshold values for identifying linkage and linear orders between loci (Stam, 1993).

Measurement of acetylcholinesterase inhibition

AChE inhibition was measured in the BC_1 progeny of Toyama and Kochi strains. Mosquito heads were individually homogenized on

ice in 600 µl of 50 mM Tris-HCl (pH 8.05) containing 0.1% Triton X-100 with a polytron. The homogenate was centrifuged at 900 ***g*** for 10 min at 4 °C, and then the supernatant was tested for AChE activity. The DTNB method of Ellman *et al.* (1961) was adopted with minor modification to detect the inhibition of AChE activity by microtiter plate assay. AChE inhibition was assayed in a 240 µl reaction containing 40 µl of the supernate in each well, at final concentration of 40 mM Tris-HCl (pH 8.05), 1.8 mM acetylthiocholine (ATCh), 1 mM 5,5′-dithiobis 2-nitrobenzoic acid, 1.8 mM $NaHCO_3$, and either: (1) 10 µM of fenitroxon or (2) an equal volume of H_2O. Residual AChE activity was measured at 40 min following preparation of reaction mixtures. Procedures for reaction conditions, measurements of optical density and calculations of enzyme activity followed Mamiya *et al.* (1997). Fenitroxon was purchased from Wako Pure Chemical Industries, Ltd, Tokyo, Japan.

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