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Sequence analysis of the acetylcholinesterase 1 translated from messenger ribonucleic acid of the nervous system of crayfish (*Cherax quadricarinatus*)

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Abstract

Crayfish are the animal models used as biomarkers of exposure to pesticides. Some compounds such as organophosphates work by blocking Acetylcholinesterase (AChE), an enzyme that hydrolyzes the neurotransmitter acetylcholine on cholinergic synapses. In insects recurrent exposure to pesticides produces resistance, and the molecular analysis has identified mutations as the cause of this resistance. Till date, there are a few sequences reported for crustaceans. Even more, there are no reports of sequences in any species of crayfish that allow make comparative studies. In this study, we obtained the specific sequence of messenger Ribonucleic acid (mRNA) for the AChE1 protein from the crayfish, *Cherax quadricarinatus*. The mRNA was isolated from abdominal nerve cord of *C. quadricarinatus*. Degenerate oligonucleotides were used and the 1242 nucleotides sequence was directly obtained from PCR products. We have deposited this sequence with the accession number [GenBank KT007499]. Analysis of the 414 amino acid residues of the putative AChE protein showed a high homology to the Esterase-lipase super family. We identified 60% to 64% similarity of AChE1 in insects and crustaceans with well-conserved AChE1 main domains. We concluded that the partial sequence analysis to this putative mRNA and the comparative studies with other species clearly shows that the sequence corresponds to the translated product Acetylcholinesterase-1.

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Introduction

The enzyme Acetylcholinesterase (AChE: EC 3.1.1.7), belongs to the family of serine hydrolase, plays a crucial role in the hydrolysis of neurotransmitter acetylcholine into choline and acetic acid within the synaptic clefts of cholinergic synapse. Other roles of AChE include neurite outgrowth, synapse formation, learning/memory and tau phosphorylation (for review, see Karczmar, 2010). Moreover, AChE active site is the main target for pesticides, organophosphates and carbamates, and has been studied widely in insects. In invertebrates, the first *Ace* gene was cloned from *Drosophila melanogaster* (Hall and Spierer, 1986), and was reported to be the only gene encoding AChE in its genome. It was considered for several years as the unique *Ace* gene in all insects. However, cloning and phylogenetic studies confirmed the existence of two *Ace* genes in insects: the *Ace-1 a*nd the *Ace-2* encoding for AChE1 and AChE2 proteins, respectively (Weill *et al*., 2002; Huchard *et al*., 2006; Wu *et al*., 2010; Kim and Lee, 2013).

The *Ace-1* gene was reported for the first time in *Anopheles gambiae* (Weill *et al*., 2002). Apparently, the genome of *Drosophila* spp. contains only *Ace-2* gene due to the loss of *Ace-1* gene in the evolutionary process during their differentiation into *Drosophilidae* and *Culicidae* from a common ancestor. For this reason, the *Ace-2* gene encoding the protein with synaptic function is only present in true flies (Weill *et al*., 2002; Huchard *et al*., 2006; Kim *et al*., 2013; Cha and Lee, 2015). Other studies suggest a putative functional transition from *Ace-1* to *Ace-2*, as reported for the local Hymenopteran insects. In species, such as *Culens pipiens* and *A. gambiae*, that possess both *Ace* genes, it was shown that AChE2 protein is minimally involved in enzymatic activity and probably exhibiting non-synaptic functions (Huchard *et al*., 2006; Zhao *et al*., 2013). Recent phylogenetic studies have hypothesized that both the *Ace-1* and *Ace-2* genes arose by duplication, occurring before the split of ecdysozoa, so that both genes would exist in most arthropods (Weil *et al*., 2002; Huchard *et al*., 2006; Pezzementi and Chatonnet, 2010; Cha and Lee 2015), and also in the Subphylum *Crustacea*.

The decapod crustaceans, specifically the crayfish, has been widely used as an animal model in physiological studies, but very few studies are found at the molecular level. Currently, there are only three sequences in the GenBank identified as AChE1 in Crustaceans. Two corresponded to *Tigriopus japonicus* and *Lepeophtheirus salmonis* (Kaur *et al*., 2015), and the other to *Daphnia magna* with 97% homology with the hypothetical protein DAPPUDRAFT_39257 of *Daphnia pulex*. Numerous reports consider crayfish as a biomarker of toxicity when exposed to organophosphorus compounds, pesticides and metals that inhibit AChE activity (Repetto *et al*., 1988; Escartin and Porter, 1996; Devi and Fingerman, 1995; Vioque-Fernández *et al*., 2007). However, until today, the *Ace* gene has not been reported in any species of crayfish. The specific gene sequence and characterize the protein encoded, is essential to accurately study the biochemical processes that cause toxicity or resistance to environmental pollutants that can harm these species. This topic is important because depending on the region, the crayfish are a major aquaculture resource or predator in some ecosystems. In order to enrich the studies in Crustaceans, we obtained the partial sequence of putative Ace gene from mRNA of the crayfish nervous system, and carried out the comparative sequence analysis with other species.

Material and methods

Animals

We used twenty adult crayfish *Cherax quadricarinatus*, which were commercially acquired in México. The animals were placed in appropriate laboratory conditions under a 12:12 light-dark cycle with free access to food for two weeks. We have been handling the crayfish in this research in accordance with the general principles on the use of animals of the Society for Neuroscience (2015).

Total RNA extraction, cDNA synthesis and PCR conditions

We used all twenty abdominal nerve cord of *C. quadricarinatus* to extract total RNA with Trizol® (Invitrogen Co., USA) according to the instructions of the manufacturer. In order to detect the expression of *ace* mRNA, RT-PCR (reverse-transcription PCR) was performed on total RNA. We designed degenerate oligonucleotides considering the conserved regions of invertebrate sequences obtained from the databases available in GenBank. The oligonucleotide synthesis was developed by Invitrogen and Biotechnology Institute, UNAM. Resulting cDNAs were amplified for 35 cycles with the following thermocycling conditions (denaturation - 94° C for 30 s, annealing - 58°C for 30 s, extension - 72°C for 50 s) using GeneAMP PCR System 9700 Gold (Applied Biosystems, USA).

Electrophoresis and sequencing procedures

The endpoint PCR products were analyzed by 1.5% agarose gel electrophoresis (Invitrogen) in TAE buffer (40 mM Tris Base, 1 mM EDTA and 20 mM acetic acid) pH 7.5, pre-stained with ethidium bromide and the image was digitized and analyzed with the Molecular Imaging program (Kodak, Nusloch, Germany).

Sequencing was performed directly on PCR products on the CEQ 8000 (Beckman Coulter, USA) using the Quick Starter Kit (Beckman Coulter).

The PCR products were purified with QIAquick Gel Extraction Kit (Qiagen). The reads were obtained for duplicate and triplicate unitigs, and reported sequences were used as a guide to assembly the consensus sequence.

Sequence analysis

The analysis was performed by consulting ExPASy proteomics server (http://web.expasy.org/traslate/) for translation of nucleotide sequence to a protein sequence. The AchE sequences of different species were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html). BLAST research was performed (http://www.ncbi.nlm.nih.gov/blast) and the alignment was performed with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Phylogenetic analysis

With the same sequences obtained for the alignment, we performed the phylogenetic analysis that consists of 11 species of arthropods including crayfish and a vertebrate *T. californica*, whose sequence is considered to be the prototype of AChE. A phylogenetic tree is a Neighbor-joining without distance corrections and bootstrap 1000 was performed with MEGA4 (Tamura *et al.*, 2007).

Results

We designed degenerate oligonucleotides based on the alignment of several arthropods. We also selected the sequence fragments obtained from crayfish for specific oligonucleotides.

The pairs of oligonucleotides used and size of PCR products are shown schematically in the Fig. 1A. In the Fig. 1B, we showed the representative gels of electrophoresis of the amplicons by PCR that were subsequently sequenced.

Table 1. AChE reference sequences from Gen Bank.

Accession number	Protein	*Species*
AIY62313.1	acetylcholinesterase 1A	*Lepeophtheirus salmonis]*
AIU38228.1	acetylcholinesterase 1	*Tigriopus japonicus*
BAM36064.1	acetylcholinesterase	*Daphnia magna*
EFX89932.1	hypothetical protein DAPPUDRAFT_39257	*Daphnia pulex*
ABB89946.1	*ace-1* type acetylcholinesterase	*Blattella germanica*
BAF46107.1	acetylcholinesterase (partial)	*Pediculus humanus capitis*
ABY75631.1	acetylcholinesterase-1 precursor	*Locusta migratoria manilensis*
ADA60183.1	acetylcholinesterase 1	*Liposcelis paeta*
ADF43750.1	acetylcholinesterase (partial)	*Stegobium paniceum*
ABX44668.1	acetylcholinesterase (partial)	*Alphitobius diaperinus*
CAA27169.1	acetylcholinesterase	*Torpedo californica*
KT007499	acetylcholinesterase 1 (partial)	*Cherax quadricarinatus*

We have established a consensus sequence of 1242 nucleotides from the nervous system mRNA of a crayfish, *Cherax quadricarinatus,* that encode for 414 amino acids. The nucleotide sequence was assembled from raw sequences. In the Fig. 2, we showed the nucleotide and deduced amino acid sequences. The sequence obtained from the abdominal cord was used in BLASTP software to search and identify sequences with the highest match.

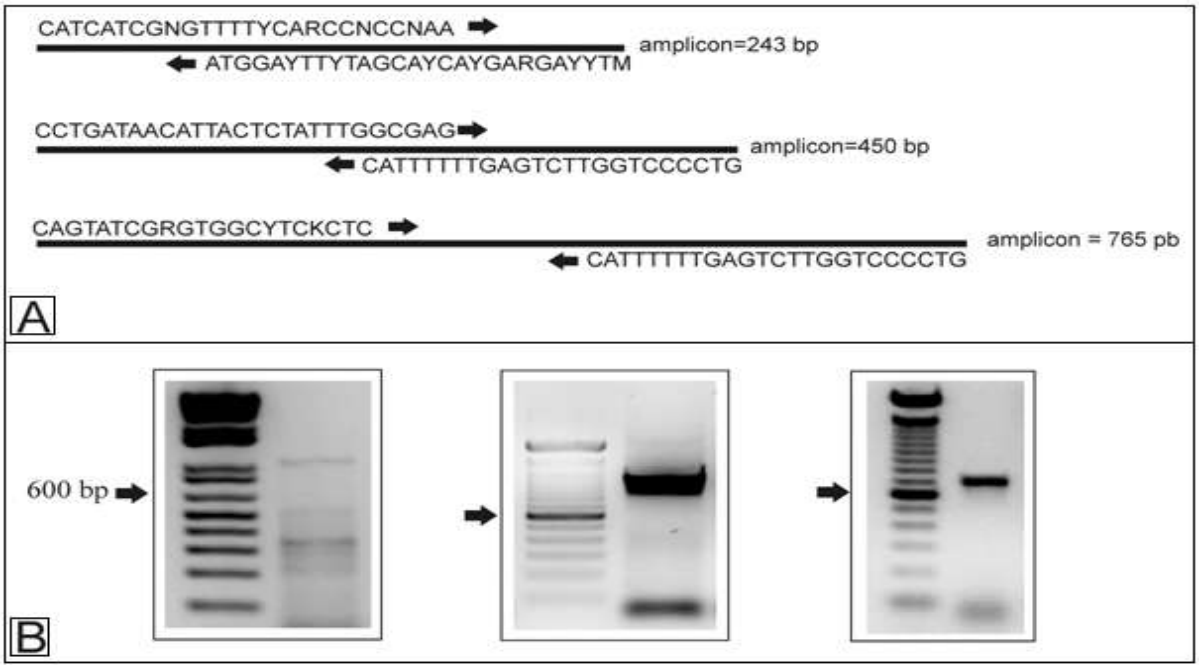

Fig. 1. A. Oligonucleotide pairs used in PCRs. Degenerate and specific oligonucleotides were designed from the invertebrate alignments. The line represents the DNA fragment (not to scale) and the expected size of the amplicon is indicated at the right end of each DNA fragment. The sequence of sense and antisense oligonucleotides were shown above and below of each line respectively. B. Representative electrophoresis PCR products. According to the three gels, the first line represents the 100 bp ladder DNA marker and in the second lane the amplicon obtained. As a reference, the gross arrows indicate the 600 bp fragment.

Fig. 3 shows the AChE amino acid sequence of several species aligned with the sequence obtained from crayfish. The alignment included insect and crustacean sequences. By convention, the amino acid position is related to the prototype AChE protein of *Torpedo californica (electric ray)* (Schumacher *et al*., 1986; Sussman *et al*., 1991).

From the amino acid sequence of the putative AChE1 of *C. quadricarinatus*, we have identified highly conserved residues that contribute to the conformation of five main domains of this protein. They are: catalytic triad, the oxyanion hole, the acyl binding pocket (ABP), the catalytic anionic site (CAS) and the peripheral anionic site (PAS). The analysis between the deduced amino acids of crayfish and sequences reported in other species allowed us to identify the canonical signature FGESAG (a. a. 197-202), a feature of the active site of AChE. Also, the conserved residues of the catalytic triad Ser200, Glu327 and His440 were seen.

The catalytic triad is located near the bottom of a deep and narrow gorge lined with the rings of 14 aromatic amino acid residues, which is described in *T. californica*. The crayfish sequence starts at position 172, so we identified only the last nine residues of ABP in the following positions: Trp233, Trp279, Phe288, Phe290, Trp330, Phe331, Tyr334, Trp432 and Tyr442. We identified 3 differences regarding them: first, at position 279 exist a gap; second, at position 330 there is a Trp (W330) and third, at

position 442, both insects and crustaceans retain Asp (D442). In the alignment (Fig. 3) It can be noticed that all insects and *L. salmonis* have a Tyr (Y) at position 330, only *D. pulex, D. magna* and *T. japonicus* retain a Phe (F) as in *Torpedo californica*. The amino acid residues at the peripheral position 279 and at catalytic position 330 also contribute to the anionic sites.

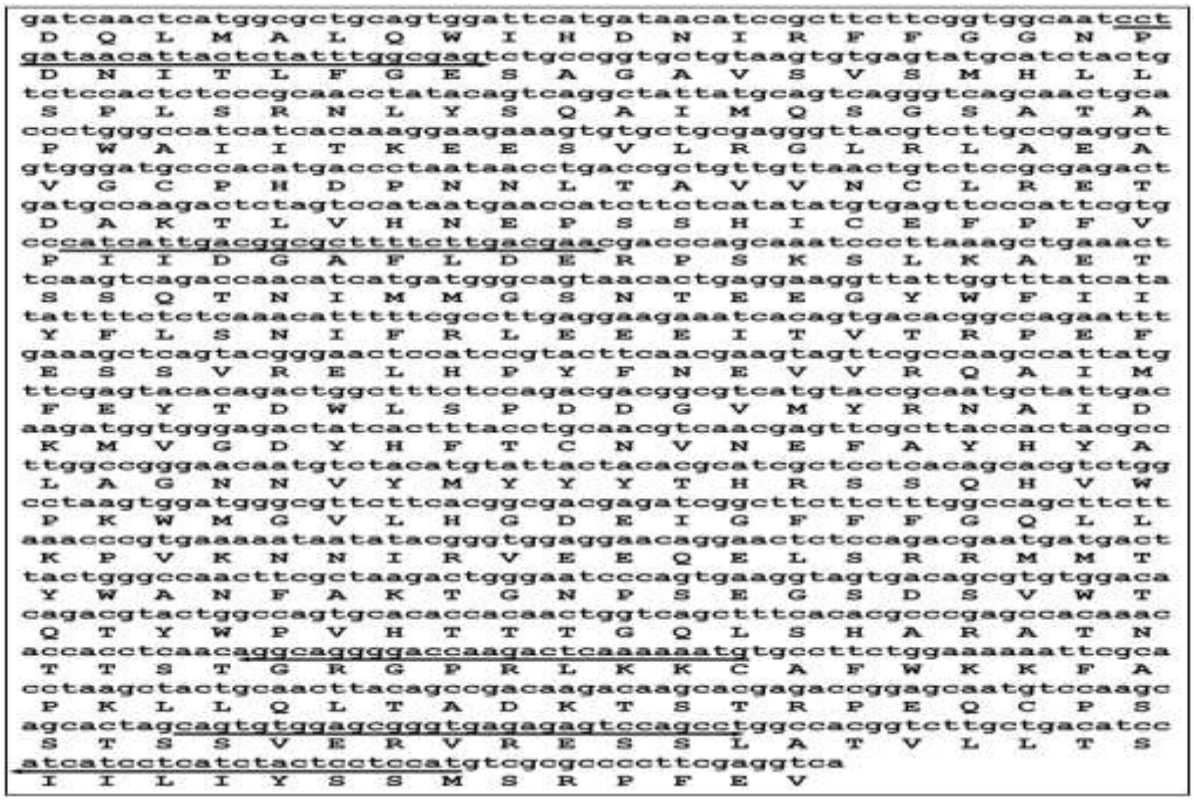

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the *Ace-1* from crayfish *Cherax quadricarinatus*. Arrows indicate the position of degenerate and specific primers used. The nucleotide sequence has the accession number [GenBank KT007499].

In addition, in *T. californica,* amino acids Gly118, Gly119 and Ala201 are part of oxyanionic hole, among which, Ala201 is conserved in all species included the crayfish. We also identified two amino acids, Glu199 and Glu443 among the three conserved (Asp72, Glu199 and Glu443) which influence the potential gradient along the whole length of the active-site gorge.

In the sequence of crayfish, we identified four of the six cysteines that form the inner chain disulfide bonds Cys254, Cys265, Cys402 and Cys521 (see Fig. 3).

Finally, we have identified five amino acids in the backdoor site, which is known to play an important role in the removal of the products of the catalytic reaction (acetate and choline). The three amino acids that contribute to this site are Trp84, Trp432 and Asp442; Trp432 is preserved in all these species, Asp442 changes to Tyr442 only in *T. californica*; also are important the two adjacent amino acids Tyr329 and Met439 in insects. Tyr329 is highly conserved; the exceptions were *S. paniceum* with Phe329 and two crustaceans: *T. japonicus* Phe 329 and *L salmonis* Asn329. Met439 in insects was substituted with Leu439 in all crustaceans. These amino acids are different in *T. californica* Ser329 and Ile 439; for easy location the amino acids at positions 329 and 439 are marked with an arrow in the Fig. 3.

The phylogenetic tree analysis was performed with 11 arthropods, including the crayfish, *C. quadricarinatus*, and in addition one vertebrate, *T californica*. We applied the same sequences that we

employed in the alignment with the highest homology with the consensus sequence of crayfish (see Table 1). In this analysis, we included two species of Crustaceans (*T. japonicus* and *L. salmonis*). Although, with the BLAST analysis, these sequences were not identified as similar to the sequence of *C. quadricarinatus*, we considered it is necessary to include because there are few Crustacean sequences reported, and also, both contains the characteristic domains of AChE1.

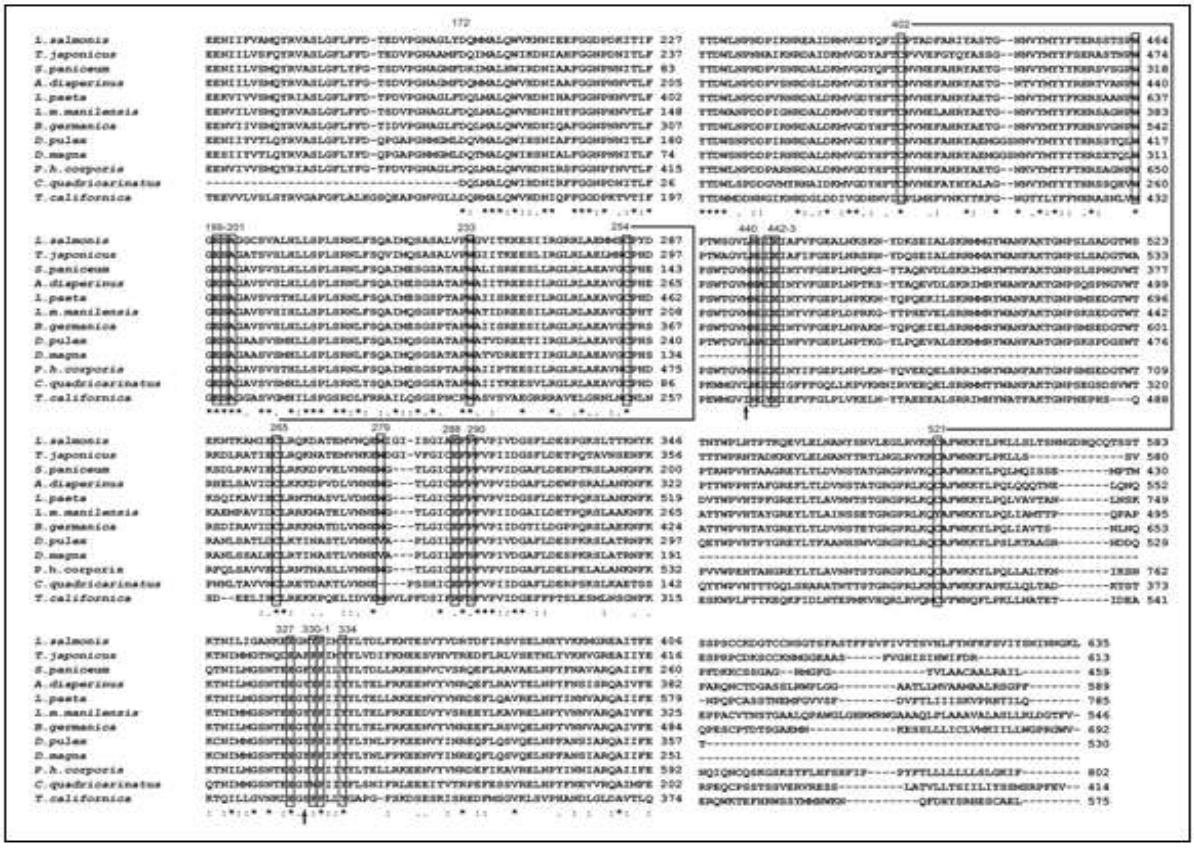

Fig. 3. Comparative alignments of deduced amino acid sequences from crayfish and several species. The initial part of the alignments were trimmed. The initial amino acid obtained from crayfish corresponded to the position 172 according to the *T. californica*. Conserved amino acids are delimited by boxes and the numbers on the top side correspond to the position of *T. californica*, by convention. Conserved disulfide bridges are indicated by connecting pairs of Cysteines with a black line..

Leaving out *T. californica* for the analysis, the phylogenetic tree has a common origin for AChE1 in all these arthropods (Fig. 4). The first branch-bifurcation corresponds to the Crustaceans of Maxilipoda class (*T. japonicus and L. salmonis*) which is a common line for Crustaceans and insects. The second branch-bifurcation corresponds to Branchiopoda class of Crustaceans (*D. pulex and D. magna*), a common line for insects and the Malacostraca class Crustacean which correspond to *C. quadricarinatus* that evolved independently. The other branch corresponds to a common origin, for the class of Insecta that include Lepidoptera, Coleoptera and Orthoptera, which were selected specifically for this study.

Discussion

The great interest in studying the synaptic catalytic activity of AChE is due to this enzyme is inhibited by organophosphates and carbamates which are widely used as pesticides to control the pests of a wide variety of insects. These studies have generated extensive information at the molecular level, e.g. to determine the amino acid change that confers resistance to these pesticides (Mutero *et al.*, 1994; Wu *et al.*, 2010), and have expanded the databases of

sequences reported in insects. A recent study compiled 181 genes and 11 *Aces*-like gene sequences, allowing the characterization of conserved domains and the evolutionary origin of the two *Ace* genes that exist from Choanoflagellata to Arthropod, with the exception for Cyclorrhapha which possess only the *Ace-2* gene (Weill *et al*., 2002; Huchard *et al*., 2006; Wu *et al*., 2010; Kim *et al*., 2013; Cha *and Lee*, 2015). In Crustaceans, the knowledge of genes that encode the AChE enzyme is minimal. The only genome of crustaceans that has been completely sequenced is in *Daphnia pulex*, a planktonic crustacean commonly called as the water flea. In this specie, the hypothetical protein DAPPUDRAFT_39257 presents the conserved domains, characteristic of AChE1 were included in the comparative alignment studies in this study. Although the latest phylogenetic studies agree with two paralogous genes coding for acetylcholinesterase in arthropods (Pezementi and Chatonet, 2010; Cha and Lee, 2015), currently, in the GenBank there are only 3 species reported for AChE´ crustaceans: *D. magna*, *T. japonicus* and *L. salmonis*. In addition, we identified two hypothetical proteins DAPPUDRAFT_104600 and DAPPUDRAFT_39257 of *D. pulex* that have conserved domains of the family of Esterases; we used for the analysis only the sequences that showed the higher homology with AChE1 of insects.

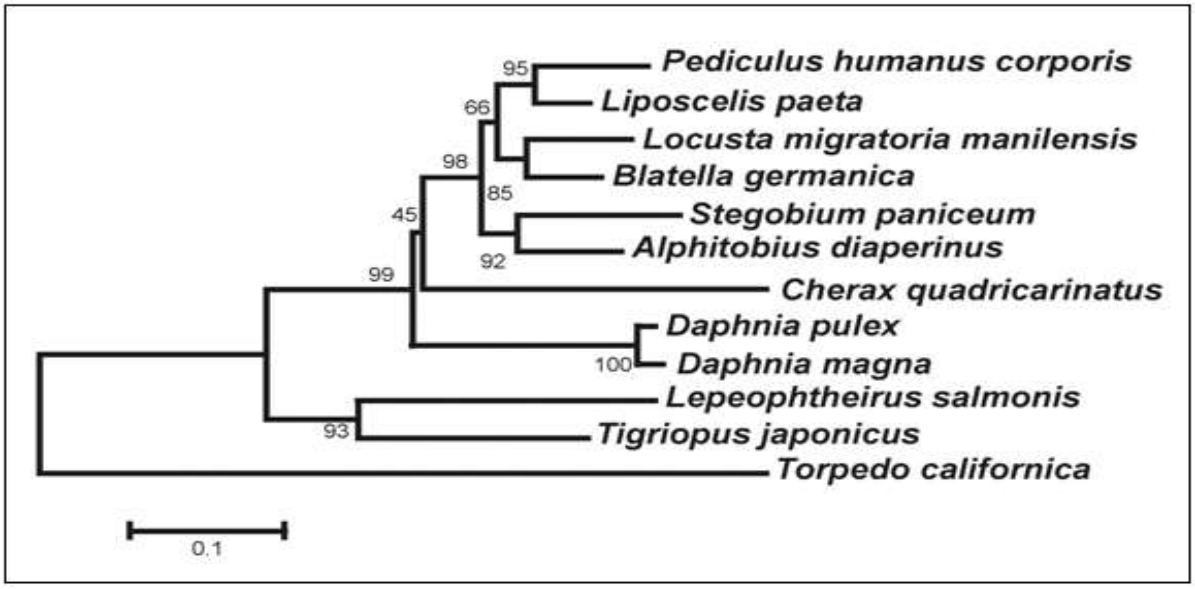

Fig. 4. Phylogenetic tree obtained with Mega4 using the Neighbor-Joining method. The evolutionary distances were computed using the Poisson correction method with 1000 bootstrap. Note that the crayfish *Cherax quadricarinatus* appear as an independent evolution of other Crustaceans from of the third branch-bifurcation. As expected, a total independent branch is for the vertebrate *Torpedo californica*.

The deduced amino acid sequence obtained from the ventral nerve cord of crayfish, *Cherax quadricarinatus,* has 60% to 64 % homology with different species of insects. This Rank of AChE1 protein similarity of crayfish is very close to that in *L salmonis*, as reported recently (Kaur *et al*., 2015). The best match was with AChE1 of *Liposcelis paeta*. The sequence of 414 amino acids obtained in the crayfish approximately represent the 64% of the complete sequences reported in other species, which include the crustaceans *Tigriopus japonicus* and *Lepeophtheirus salmonis*.

In crayfish, it has been well established that acetylcholine is a neurotransmitter in the nervous system, the neurons in the abdominal cord have a high level of cytoplasmic AChE and approximately 8% of neurons are immuno-positive to this enzyme (Habig *et al*., 1988; Braun and Mulloney, 1994). Recent studies indicate that in crayfish, the AChE has around 90 times higher enzymatic activity in the nervous system that in muscle tissue (Vioque-

Fernández *et. al.*, 2007). Although in the muscle of crayfish it has been measured both AChE and Butyrylcholinesterase (BChE) activities. AChE was also identified by immunocytochemistry in the transverse tubules of deep abdominal muscles, whose function is still unknown, because glutamate is the usual neuromuscular transmitter in crayfish (Escartin and Porter, 1996; van Harreveld and Mendelson, 1959; Takeuchi and Takeuchi, 1964).

We expect that the sequence reported in this study will allow for future studies to establish the role of acetylcholinesterase in various organs of crayfish and clarify if the differences in its activity are due to the differential expression of AChE1 or AChE2. We suggest this, because there is one evidence that both genes have evolved in different rate, expressing different proteins that retain their essential enzymatic characteristics (Cha and Lee, 2015). In addition, it has been confirmed that in the species that express both enzymes, AChE1 has the classical role at the synaptic level, but AChE2 function is unknown (Huchard *et al.*, 2006; Kim *et al.*, 2006; Zhao *et al.*, 2013). Besides that, it has been reported when there is a differential expression of both genes in *C. elegans* neuromuscular junction; *Ace-1* is expressed in presynaptic cells, unlike *Ace-2* which is expressed in postsynaptic cells (Combes *et al.*, 2003).

In this study, we compared the deduced sequence of crayfish with other species which allowed us to identify preserved amino acids and the characteristics of AChE1 as the catalytic triad, two of the three conserved disulfide bridges. In addition to the amino acids Trp432, and Asp442 for the "backdoor" which were preserved as in the insects (Cha and Lee, 2015). In the sequence of the crayfish, we identified nine amino acids of the fourteen conserved aromatic residues that lined the catalytic gorge as reported in *T. californica* (Sussman *et al.*, 1991). Although we identified two conserved amino acids that differ for crayfish in the positions 279 and 330. The amino acids Trp279 and PheF330 contribute to the catalytic and peripheral anionic site, respectively, it would be interesting to analyze the structural modifications in the protein, because it is known that PheF330 and Tyr121 are juxtaposed approximately half of the gorge, and causes constriction. In addition, TrpW279 is one aromatic residue at the entrance to the gorge and its absence surely modifies the interaction with their ligand (Silman and Sussman 2008; Massoulie *et al.*, 1993).

In a previous study, Weill *et al.*, (2002) identified specific amino acids that are conserved in both AChEs in insects. We compared these characteristics with the sequence obtained for crayfish. However, we did not identify any amino acid residues that were characteristic of AChE2 only of AChE1.

The phylogenetic analysis of this study with five AChE1 sequences of Crustaceans suggests divergence among Crustaceans with an independent evolution between species of classes Maxillopoda, Malacostraca and Branchiopoda (Fig. 4). We also confirm the preservation of this enzyme with a very important role in synaptic transmission activity and its evolution from a common ancestor. Although approximately the initial 172 amino acids of the proteins AChE1 reported in other species were not sequenced for crayfish, we considered that the homology of the sequence reported for the crayfish is plentiful conserved in the segment of 414 amino acids, which allow us to propose that the translation sequence undoubtedly corresponds to the AChE1 enzyme.

In Conclusion, the analysis of this partial sequence obtained from the nervous system mRNA of crayfish shows homology to the *Ace-1* gene described for insects and crustaceans. The identification of conserved domains of AChE protein and the catalytic triad leads us to propose that the putative gene sequence correspond to the acetylcholinesterase type 1 of crayfish, *Cherax quadricarinatus*.

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Competing interests

Participating authors declare that they have no competing interests.

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