CDNA CLONING AND EXPRESSION ANALYSIS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR ALPHA6 SUBUNIT IN THE ORIENTAL FRUIT FLY, *BACTROCERA DORSALIS* (DIPTERA: TEPHRITIDAE)

Wen-Jia Yang, Yi Tian, Lin Cong, Wei Dou and Jin-Jun Wang*
Key Laboratory of Entomology and Pest Control Engineering, College of Plant Protection, Southwest University, Chongqing 400716, People's Republic of China

E-mail: jjwang7008@yahoo.com; wangjinjun@swu.edu.cn

Abstract

Nicotinic acetylcholine receptors (nAChR) play major roles in the fast synapse cholinergic transmission in the insect central nervous system, and they are recognized as potential target sites for insecticides. In this study, the full-length cDNA sequence of the nAChR alpha6 subunit (*Bdor*α6) was isolated and characterized from the oriental fruit fly, *Bactrocera dorsalis*, using the Rapid Amplification of cDNA Ends (RACE) technique. The *Bdor*α6 cDNA was 2020 bp long, containing an open reading frame of 1467 bp, which encodes 488 amino acid residues with a predicated molecular mass of 55.6 kDa. Homology analysis indicated that *Bdor*α6 has typical features of a nAChR α subunit. Phylogenetic analysis with other insect nAChR subunits suggested that *Bdor*α6 was most closely related to *D*α6 of *Drosophila melanogaster*. The developmental changes and tissue-specificity of the relative expression levels of *Bdor*α6 were investigated using quantitative real-time PCR. The highest expression level was observed in the first instar larva and the lowest was in the pupa, and there was a descending expression level during the developmental period from the first instar larva to the pupa. The relative expression levels were 23-, 39-, 6-, 2- and 27-fold higher in the egg, first, second and third instar larvae and adult than in the pupa, respectively. Furthermore, *Bdor*α6 was expressed in the head and thorax at 109- and 15-fold higher levels than in the abdomen. The results provided some important baseline data for further molecular studies in *Bactrocera dorsalis*.

Key Words: alpha6 subunit, *Bdor*α6, nicotinic acetylcholine receptor, cloning, sequence analysis, quantitative real-time PCR.

Resumen

Los receptores nicotínicos de la acetilcolina (RnACh) juegan un papel importante en la rápida transmisión colinérgica de la sinapses del sistema nervioso central de insectos, y son reconocidos como sitios claves potenciales para dirigir los insecticidas. En este estudio, la secuencia completa de cADN de la subunidad nAChR alpha6 (*Bdor*α6) de la mosca oriental de la fruta, *Bactrocera dorsalis*, fue aislada y caracterizada utilizando la técnica de la rápida amplificación de cDNA extremos (RACE) . El cADN fue *Bdor*α6 2020 pb de longitud, que contiene un marco de lectura abierto de 1467 pb, que codifica 488 residuos de aminoácidos con una masa molecular de 55.6 basa kDa. El análisis de homología indicó que el *Bdor*α6 tiene caracteristicas típicas de una subunidad α de RnACh. El análisis filogenético de las subunidades de RnACh de otros insectos sugiere que el *Bdor*α*6* esta más estrechamente relacionado con el Dα6 de *Drosophila melanogaster*. Los cambios en el desarrollo y la especificidad de los tejidos de los niveles de expresión relativa del *Bdor*α6 fueron investigados utilizando RCP cuantitativo de tiempo real. El nivel de expresión más alto se observó en las larvas de primer estadio y el más bajo fue en las pupas, y hubo un nivel de expresión descendente durante el período de desarrollo de las larvas de primer estadio hasta las pupas. Los niveles relativos de expresión fueron 23 -, 39 -, 6 - 2 - y 27 veces más altos en los huevos, los primeros, segundos y terceros estadios de las larvas y los adultos que en las pupas, respectivamente. Además, el nivel del *Bdor*α6 en la cabeza y el tórax se expresó a 109 - y 15 veces mayor que en el abdomen. Los resultados provee una linea de base de información importante para futuros estudios moleculares sobre *Bactrocera dorsalis*.

Nicotinic acetylcholine receptors (nAChR) play major roles in the fast synapse cholinergic transmission in insect central nervous system (Gundelfinger & Schulz 2000). The nAChRs belong to the cys-loop superfamily of ligand-gated ion channels, which also includes γ-aminobutyric acid (GABA)-gated Cl- channels, glycine receptors, glutamate-gated Cl- channels, and 5-hy-

droxytryptamine type 3 (5-HT3) receptors (Lester et al. 2004). The nAChRs consist of 5 homologous subunits, typically 2 α subunits and 3 non-α subunits, but receptors consisting of only α subunits are also known (Couturier et al. 1990; Marshall et al. 1990). The α subunits are characterized by the presence of 2 adjacent cysteine residues in loop C, while the non-α subunits lack this cysteine doublet (Gotti & Clementi 2004; Sattelle et al. 2005). Each subunit has an N-terminal extracellular domain that includes a Cys-loop and acetylcholine (ACh) binding site, which is located at the interface of 2 subunits and is formed by 6 loops (loops A-F) with each of the adjacent subunits contributing 3 loops (Grutter & Changeux 2001). Each subunit also contains 4 transmembrane regions (TM1-4), of which TM2 donates most of the residues that line the ion channel, and the intracellular linker between TM3 and TM4, which is responsible for receptor desensitization and intracellular trafficking (Corriger et al. 2000; Karlin 2002).

Based on completed genome sequences, cloning of nAChR cDNAs has been performed for 2 dipteran species, *Drosophila melanogaster* (7 α and 3 β) and *Anopheles gambiae* (9 α and 1 β), possessing 10 nAChR subunit genes (Littleton & Ganetzky 2000; Jones et al. 2005), for the hymenopteran species, *Apis mellifera* (9 α and 2 β), possessing 11 nAChR subunit genes (Jones et al. 2006), and for the coleopteran species, *Tribolium castaneum* (11 α and 1 β), and Lepidopteran species, *Bombyx mori* (9 α and 3 β), both possessing 12 nAChR subunit genes (Jones & Sattelle 2007; Shao et al. 2007). In addition, many other insect nAChR subunit genes have also been identified such as *Manduca sexta*, *Myzus persicae*, *Schistocerca gregaria* (Tomizawa & Casida 2001), *Ctenocephalides felis* (Bass et al. 2006), *Sitobion avenae* (Qiu & Han 2007), *Liposcelis bostrychophila* (Tang et al. 2009), and *Apis cerana cerana* (Yu et al. 2011). The insect nAChRs have been recognized as potential target sites for insecticides such as neonicotinoid insecticides and spinosad (Tomizawa et al. 1999; Salgado & Sparks 2005). For instance, A Y151S mutation was identified in 2 nAChR subunits (*Nl*α1 and *Nl*α3) from neonicotinoid imidacloprid-resistant of *Nilaparvata lugens* and was strongly correlated with a substantial reduction in specific imidacloprid binding (Liu et al. 2005). In *D. melanogaster*, mutations in *D*α1 or *D*β2 nAChR subunits can confer resistance to neonicontinoids, and a knockout of the *D*α6 subunit of the nAChR confers high level of resistance to spinosad (Perry et al. 2007; Perry et al. 2008).

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), is one of the most destructive pests with a cosmopolitan distribution in tropical and subtropical regions. The oriental fruit fly is a polyphagous pest that damages more than 250 plant species including numerous fruits and vegetables (Clarke et al. 2005; Chen & Ye 2007). Measures taken to control the pest mostly involve using chemical insecticides. In recent years, this pest has developed high level resistance to organophosphates, pyrethroids, and avermectin (Hsu et al. 2004; Zhang et al. 2007). Resistance of *B. dorsalis* to spinosad has been developed in laboratory selection and cross-resistance experiments (Hsu & Feng 2006). Therefore, it is expected that the resistance to spinosad may also increase rapidly in orchards globally.

In order to develop insecticide resistance monitoring tools and understand the mechanism of insecticide resistance targeting nAChR in *B. dorsalis*, we report the cloning and characterization of the nicotinic acetylcholine receptor alpha6 cDNA and the expression patterns at different developmental stages and in different tagmata of adult flies using quantitative real-time PCR.

Materials and Methods

Insects

The oriental fruit fly, *Bactrocera dorsalis*, was originally collected from Fujian province, People's Republic of China. The adults were reared on an artificial diet consisting of water, yeast powder, honey, and Vitamin C (500:15:15:1) in glass cages. Every 2 days, a banana was put into the cage to collect eggs. After hatching, larvae were reared on banana in plastic basins with sand until pupation. The colonies were kept in a temperature controlled room at 27 ± 1 °C, 70 ± 5 %RH at 14:10 h L:D throughout development (Shen et al. 2010).

RNA Isolation and cDNA Preparation

Total RNA was isolated from the heads of adult flies (15 mg) using an RNA Isolation Kit (Watson Biotechnologies, Shanghai, China) following the manufacturer's instructions. The total RNA was treated with DNase (TaKaRa, Dalian, China) and dissolved in 40 µL DEPC-treated H_2O and stored at -80 °C until use. The first-strand cDNA was synthesized from 2 µg of DNase-treated RNA by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) with oligo (dT)18 primers according to the manufacturer's instructions. The reaction mixture was stored at -20 °C for future use.

Amplification of cDNA Fragments

Degenerate primers, Bd1, 5'-ATGAARTTYGGNWSNTGGACNTAYGA-3' and Bd2, 5'-GCNACCATRAACATDATRCARTTRAA-3', were designed based on the conserved motifs of nicotinic acetylcholine receptor family to amplify the cDNA fragments (Gao et al. 2007b). Degenerate

PCR was conducted using rTaq™ polymerase (TaKaRa, Dalian, China) in 25 µL, including 1 µL cDNA templates, 1 µL each primer (10 µM), 2 µL dNTP (2.5 mM), and 2.5 µL 10× PCR buffer (Mg^{2+} plus). Degenerate PCR amplifications were performed under the following conditions: initial denaturation at 95 °C for 3 min, followed by 34 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were then separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide (EB). A band of 428 bp cDNA was excised and purified using the Gel Extraction Mini Kit (Watson Biotechnologies, Shanghai).

The purified fragments were cloned into pGEM®-T Easy vector (Promega, Madison, Wisconsin). The ligation reactions were used for transformations with DH5α competent cells (Transgen, Beijing), the DNA inserts of the recombinant clones were identified by PCR with the primers used before, and further confirmed by sequencing in both directions with an ABI-PRISM 3730 sequencer (Invitrogen Life Technologies, Shanghai, China).

Rapid Amplification of cDNA Ends (RACE) and Cloning of the Open Reading Frame (ORF) of *Bdor*α6

The full-length cDNA of *Bdor*α6 was obtained by 5'/3' RACE techniques using a SMARTer™ RACE cDNA Amplification Kit (Clontech, California) according to the manufacturer's instructions. The 3'-RACE-ready cDNA was synthesized from 2 µg of total RNA at 42 °C for 1.5 h with SMARTScribe™ Reverse Transcriptase and the 3'-CDS primer, while the 5'-RACE-ready cDNA was synthesized with SMARTScribe™ Reverse Transcriptase, the 5'-CDS primer, and SMARTer IIA oligo.

Based on the 428 bp cDNA sequence, 4 gene specific primers were designed to amplify the full-length cDNA: 2 forward primers (in the first round reaction, GSP1: 5'-ACAAATGGCGAGTGGTATCTGC-3' and in the second round reaction, GSP2: 5'-CTTCTATCGCTCACGGTGTTTCTC-3′) for 5'-RACE PCR and 2 reverse primers (in the first round reaction, GSP3: 5'-GAGAAACACCGTGAGCGATAGAAG-3' and in the second round reaction, GSP4: 5'-CGCCGTCGGATTTGTATCGTGAA-3') for 3'-RACE PCR. Utilizing the gene specific primers, universal primer (UPM, provided by the Clontech kit) and nested universal primer (NUP, provided by the Clontech kit), the 3'-and 5'-RACE were conducted with PrimeSTAR™ HS DNA polymerase (TaKaRa, Dalian, China). Thermal cycling conditions were: pre-denaturation 3 min at 94 °C, 30 cycles of 94 °C for 30 sec, 60 °C -65 °C (according to the annealing temperature of the primer) for 30 sec and 72 °C for 1-1.5 min (according to the size of expected fragment) with a final extension of 10 min at 72 °C. The resulting PCR products of 5'-RACE and 3'-RACE were cloned into pGEM®-T Easy vector (Promega, Madison, Wisconsin) and sequenced as described above.

After the 5'-ends and 3'-RACE sequences were obtained, the contigs were assembled to produce putative full-length sequences by using DNAMAN 5.2.2 (Lynnon BioSoft, Quebec). To verify the full-length cDNA, the primers BdF, 5'-ACATGGACCCGTCGCTGCTAGTC-3' and BdR, 5'-CATTATCGTGCAATAAGCCTAAA-3', were designed based on the sequences obtained from 5'-untranslated region (UTR) and 3'-UTR of the gene and used to amplify the ORF of *Bdor*α6. The PCR products were purified, cloned, and sequenced as described above.

Sequence Analysis

A search for similar sequences was performed using BlastP in the non-redundant protein sequences (nr) database of the NCBI website (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was performed with DNAMAN 5.2.2 (Lynnon BioSoft, Quebec). The signal peptide was predicated by SignalP 3.0 (Bendtsen et al. 2004). Phosphorylation sites and *N*-linked glycosylation sites were identified by the PROSITE database (Falquet et al. 2002). Transmembrane domains were predicted by the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/). The phylogenetic tree construction by MEGA5.04 (Tamura et al. 2011) used the neighbor-joining method. Bootstrap values were calculated on 1000 replications.

Quantitative Real-Time PCR of *Bdor*α6

The expression levels of *Bdor*α6 mRNA relative to expression of α-tubulin at different developmental stages and in different tagmata of *B. dorsalis* were examined using quantitative real-time PCR. Total RNA was isolated from whole bodies at different developmental stages (including egg, first, second and third instar larvae, pupa, and adult) or from heads, thoraces, and abdomens of adult flies, using RNeasy Plus Mini Kit (with gDNA Elimator spin columns, Qiagen, Valencia, California). First strand cDNA was synthesized in a 10 µL reaction mixture using random hexamers by PrimeScript® RT reagent Kit (TaKaRa, Dalian, China). The quantitative real-time PCR was carried out by Mx3000P thermal cycler (Stratagene, La Jolla, California). The PCR amplifications were performed in 25 µL reactions systems containing 2 µL of template cDNA, 12.5 µL iQ™ SYBR® Green Supermix (BIO-RAD, Hercules, CA, USA) and 0.2 mM each of the primers 5'-ATTCGATGGCACGTATCACA-3' (forward) and 5'-GGGCACCAAGTTAGTCTGGA-3'

(reverse) for *Bdor*α6, and 5'-CGCATTCATG-GTTGATAACG-3' (forward) and 5'-GGGCAC-CAAGTTAGTCTGGA-3' (reverse) for α-tubulin. Amplification conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec. After reaction, a melting curve analysis from 60 °C to 95 °C was applied to all reactions to ensure consistency and specificity of the amplified product. The real-time PCR analysis was repeated 3 times in independent experiments. The data of different developmental stages and tagmata were normalized according to the expression level of α-tubulin gene (GenBank accession number: GU269902). When we tested the mRNA expression during the different life stages, the pupal stage served as the calibrator sample whose mRNA expression ratio would always equal 1. In addition, when testing mRNA expression in different tagmata, the abdomen served as the calibrator sample.

Statistics Analysis

The PCR efficiencies of *Bdor*α6 and α-tubulin were calculated by the Mxpro-Mx3000P version 4.01 (Stratagene, La Jolla, California). Data from the quantitative real-time PCR for both the developmental expression and different tagmata expression profiles were subjected to LSD test in analysis of variance (ANOVA) (SPSS 12.0 for Windows).

Results

Cloning and Characterization of *Bdor*α6

Degenerate primers Bd1 and Bd2 were used to amplify a 428 bp fragment from a cDNA preparation of *B. dorsalis*. Based on the sequence of the cDNA fragment, we obtained the full-length cDNA through the 5'/3' RACE technique. It was the closest similarity to other known insect α6-type nAChR subunits at amino acid levels; the gene was named as *Bdor*α6. The cDNA sequence and deduced amino acid sequence of the *Bdor*α6 is shown in Fig. 1. The complete cDNA of the *Bdor*α6 (GenBank accession number: JF974072) consists of 2020 nucleotides with an ORF of 1464 nucleotides encoding 488 amino acids. The cDNA includes a 5'-UTR located 206 bp upstream of the start codon (ATG) and 3'-UTR of 350 nucleotides that ended in a poly (A) tail. A possible consensus signal sequence for polyadenylation (AATAAA) is located 27 bp upstream of the poly (A) tail. The theoretical molecular mass of *Bdor*α6 based on the deduced amino acid sequence was calculated to be 55.6 kDa, with an isoelectric point of 5.41.

We performed a signal peptide scan using the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP) finding that the protein possesses a predicted 21-residue long signal peptide. The protein has 2 potential *N*-linked glycosylation sites (N-X-S/T) at positions 44 and 88 in the N-terminal extracellular domain. There are 15 potential phosphorylation sites for protein kinase C, casein kinase II, tyrosine kinase and cAMP- and cGMP- dependent protein kinase, 7 of which are located in the N-terminal extracellular domain, 1 located in the cytoplasmic TM1-TM2 linker, and 7 located in the cytoplasmic TM3-TM4 linker.

Phylogenetic Analysis

To understand the relationships among the *Bdor*α6 protein with other insect nAChRs, a phylogenetic tree was constructed using the neighbor-joining method (Fig. 2). Phylogenetic analysis indicated that the *Bdor*α6 was most closely related to *D*α6 and *Agam*α6 with these 3 subunits clustering together, and also showed the relationships with different subunits from other species. Sequence BLASTP revealed that the deduced amino acid sequence of *Bdor*α6 was highly conserved with alpha6 homolog in other insect species. Compared with proteins in alpha6 subunit from the NCBI database, *Bdor*α6 shared 94.53% identity with the *D*α6 of *D. melanogaster* (AAM13393), 87.78% identity with the *Agam*α6 of *A. gambiae* (AAU12509), 81.08% identity with the *Bm*α6 of *B. mori* (ABL67934), and 72.21% identity with *Amel*α6 of *A. mellifera* (AAY87894). Therefore, the cloned nAChR subunit gene should belong to α6-type.

Developmental and Tissue-Specific Expression Pattern of *Bdor*α6

Quantitative real-time PCR was employed to investigate the *Bdor*α6 gene expression levels during various developmental stages and different adult tagmatas. The result showed that the *Bdor*α6 gene was expressed in all stages, indicating that it has a role throughout the entire life cycle. The lowest mRNA level was found in pupa stage, and the relative expression levels of *Bdor*α6 was 23-, 39-, 6-, 2- and 27-fold higher in the egg, first, second and third instar larvae, and adult than in the pupa, respectively. Subsequently, the relative expression level of *Bdor*α6 in egg, second, third instar larvae, and pupa were significantly lower from that in the first instar larva ($P < 0.05$) (Fig. 3). There was a descending expression level of *Bdor*α6 during the developmental period from the first instar larva to the pupa.

The *Bdor*α6 mRNA was found in different tagmata of adult flies. The relative expression level of *Bdor*α6 was significantly different ($P < 0.05$) between different tagmata, and it was 109- and 15-fold higher in the head and thorax relative to the abdomen, respectively (Fig. 4).

```
1       acatgggcgacagacaacacgacctcgtcgttactgcaacttgtcttcgagcagctgtagcacttgcagctaattgtgctgaatcaacat
91      cgacacatcctctacaaatatacatacatatacttgtacatacttatacccacccgcccgcaacatcgacaacagttgaacgtgtgttta
181     ttgctcggtagcagaaatcagaggacATGGACCCGTCGCTGCTAGTCGTGTTGATCTTTCTTGTGATAATTAAAGAAAGCTGCCAAGGAC
1                                   M  D  P  S  L  L  V  V  L  I  F  L  V  I  I  K  E  S  C  Q  G

271     CACACGAAGAACGTTTACTTAACCACCTACTATCCACCTATAACACACTGGAGCGACCCGTATCGAACGAATCGGAACCGCTGGAAGTCA
22      P  H  E  E  R  L  L  N  H  L  L  S  T  Y  N  T  L  E  R  P  V  S  N  E  S  E  P  L  E  V

361     AATTCGGTTTAACGCTGCAACAAATTATTGATGTGGACGAGAAGAATCAACTTCTTATAACCAATCTTTGGCTTTCGTTGGAGTGGAATG
52      K  F  G  L  T  L  Q  Q  I  I  D  V  D  E  K  N  Q  L  L  I  T  N  L  W  L  S  L  E  W  N

451     ACTATAATTTACGATGGAATGAATCCGAGTATGGAGGCGTGAAAGACTTGCGGATAACGCCAAATAAGCTGTGGAAACCGGATGTGCTAA
82      D  Y  N  L  R  W  N  E  S  E  Y  G  G  V  K  D  L  R  I  T  P  N  K  L  W  K  P  D  V  L

541     TGTACAACAGCGCGGATGAGGGATTCGATGGCACGTATCACACCAACATTGTGGTCAAACATGGCGGCAGTTGTCTGTACGAGCCCCCTG
112     M  Y  N  S  A  D  E  G  F  D  G  T  Y  H  T  N  I  V  V  K  H  G  G  S  C  L  Y  E  P  P

631     GTATCTTCAAGAGCACATGCAAGATGGACATCACGTGGTTCCCATTTGATGACCAACACTGCGAAATGAAATTCGGTAGTTGGACTTACG
142     G  I  F  K  S  T  C  K  M  D  I  T  W  F  P  F  D  D  Q  H  C  E  M  K  F  G  S  W  T  Y

721     ATGGAAATCAGTTGGATTTGGTTTTGAGTTCCGAAGATGGAGGGGATCTTTCCGATTTCATAACAAATGGCGAGTGGTATCTGCTTGCAA
172     D  G  N  Q  L  D  L  V  L  S  S  E  D  G  G  D  L  S  D  F  I  T  N  G  E  W  Y  L  L  A

811     TGCCTGGCAAGAAGAATACCATCGTCTACGCGTGCTGCCCGGAACCCTACGTGGACGTCACATTCACGATACAAATCCGACGGCGTACAT
202     M  P  G  K  K  N  T  I  V  Y  A  C  C  P  E  P  Y  V  D  V  T  F  T  I  Q  I  R  R  R  T
                                          ####
901     TATATTATTTTTTCAATTTAATTGTGCCGTGTGTGCTGATATCATCGATGGCTTTATTAGGATTTACACTACCTCCCGATTCGGGCGAAA
232     L  Y  Y  F  F  N  L  I  V  P  C  V  L  I  S  S  M  A  L  L  G  F  T  L  P  P  D  S  G  E

991     AATTAACATTAGGAGTTACAATTCTTCTATCGCTCACGGTGTTTCTCAACCTTGTAGCTGAGACATTGCCCCAAGTATCTGATGCAATCC
262     K  L  T  L  G  V  T  I  L  L  S  L  T  V  F  L  N  L  V  A  E  T  L  P  Q  V  S  D  A  I

1081    CCTTGTTAGGCACCTACTTCAATTGCATCATGTTTATGGTGGCATCATCGGTGGTGCTGACGGTTGTTGTGCTCAACTATCATCATCGCA
292     P  L  L  G  T  Y  F  N  C  I  M  F  M  V  A  S  S  V  V  L  T  V  V  V  L  N  Y  H  H  R

1171    CGGCGGATATACACGAAATGCCGCCTTGGATTAAATCCGTTTTCCTGCAATGGCTGCCATGGATTTTACGTATGGGTCGTCCGGGCCGAA
322     T  A  D  I  H  E  M  P  P  W  I  K  S  V  F  L  Q  W  L  P  W  I  L  R  M  G  R  P  G  R

1261    AAATCACACGAAAAACAATTCTACTAAGCAATCGCATGAAAGAATTGGAATTAAAGGAACGTTCTTCCAAATCACTACTAGCCAATGTGC
352     K  I  T  R  K  T  I  L  L  S  N  R  M  K  E  L  E  L  K  E  R  S  S  K  S  L  L  A  N  V

1351    TGGATATCGATGATGATTTCCGGCATACGATCTCCGGTTCACAAACGGCGATCGGTTCATCAGCAAGCTTCGGTCGCCCCACAACGGTGG
382     L  D  I  D  D  D  F  R  H  T  I  S  G  S  Q  T  A  I  G  S  S  A  S  F  G  R  P  T  T  V

1441    AGGAGCATCATAACACAATAGGTTGCAATCATAAAGATTTACATTTGATTCTTAAAGAATTACAATTCATAACATCACGTATGCGTAAAT
412     E  E  H  H  N  T  I  G  C  N  H  K  D  L  H  L  I  L  K  E  L  Q  F  I  T  S  R  M  R  K

1531    CCGATGATGAGGCGGAATTAATAAGCGATTGGAAATTCGCGGCAATGGTTGTGGATAGATTTTGTTTAATTGTTTTTACGCTCTTTACAA
442     S  D  D  E  A  E  L  I  S  D  W  K  F  A  A  M  V  V  D  R  F  C  L  I  V  F  T  L  F  T

1621    TTATCGCAACAGTGACCGTTCTGCTGTCGGCTCCACACATTATCGTGCAATTAAgcctaaatgcatacgaggtttaagcataagcctagga
472     I  I  A  T  V  T  V  L  L  S  A  P  H  I  I  V  Q  *

1711    tagatatatggaaactgcatactttagatgaacaagatgaagaagaaatatttatttttccagaggaaaaatcacttacatacataatt
1801    acaaagaaaagcaaataatcgagaggagcaatgcaaattcaaagagatttagcacttacatagacgtagtttagtgaagatgatgagact
1891    actcaataattagaaaattatacgaaagacaattgaaaagttcaccaaaatcaatacaaaaaaattgttaattaattatttaagaaaaac
1981    tgaaaactagttaaaacaaaaaaaaaaaaaaaaaaaaaaa
```

Fig. 1. Nucleotide and deduced amino acid sequence of *Bdor*α6 cDNA from *Bactrocera dorsalis* (GenBank accession number: JF974072). The start codon is indicated with bold and the stop codon is indicated both with bold and an asterisk. The predicated signal peptide is double underlined. The Cys-loop domain is underlined with a dotted line. Potential *N*-linked glycosylation sites are boxed. The position of putative 4 trans-membrane domains (TM1-4) is shaded. The cysteine doublet (characteristics of nAChR α subunits) is marked with as `####`. The potential phosphorylation sites are underlined.

Discussion

In this study, we identified and characterized *Bdor*α6 cDNA from the oriental fruit fly, *B. dorsalis*. Alignment of the deduced amino acid sequence with the nAChR homologs revealed that *Bdor*α6 possesses typical nAChR subunit characteristics, such as a signal peptide, a long N-terminal extracellular domain, and 4 hydrophobic transmembrane domains (TM1-4). Other important amino acid residues conserved in nAChRs were also identified. The N-terminal domain includes a cysteine loop consisting of 2 cysteines separated by 13 residues, which is the best-conserved feature in all ligand-gated ion channels, and the ligand-binding site forming regions (loops A-F) (Grutter

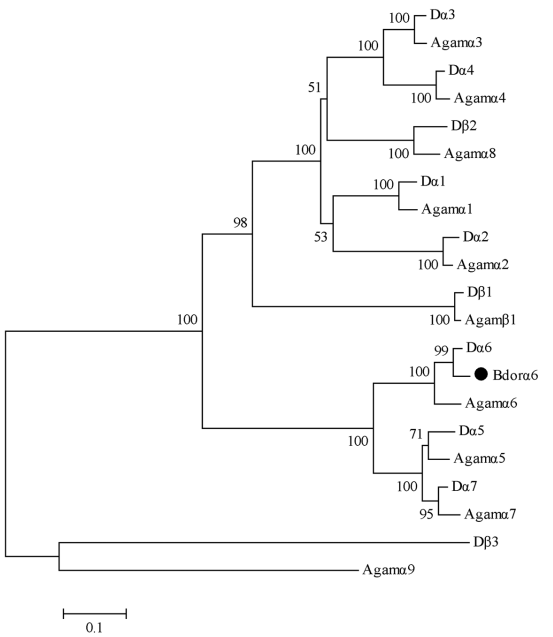

Fig. 2. Evolutionary relationships of deduced amino acid sequence of *Bdor*α6 with nAChR subunits of *Anopheles gambiae* and *Drosophila melanogaster* constructed using the neighbor-joining method. Bootstrap values with 1000 trials are indicated on branches. The scale bar represents the number of substitutions per site. Sequences (GenBank accession numbers) used: *Anopheles gambiae Agam*α1 (AAU12503), *Agam*α2 (AAU12504), *Agam*α3 (AAU12505), *Agam*α4 (AAU12506), *Agam*α5 (AAU12508), *Agam*α6 (AAU12509), *Agam*α7 (AAU12511), *Agam*α8 (AAU12512), *Agam*α9 (AAU12513), *Agam*β1 (AAU12514); *Drosophila melanogaster D*α1 (CAA30172), *D*α2 (CAA36517), *D*α3 (CAA75688), *D*α4 (CAB77445), *D*α5 (AAM13390), Dα6 (AAM13393), *D*α7 (CAD86936), *D*β1 (CAA27641), *D*β2 (CAA39211), *D*β3 (CAC48166).

& Changeux 2001; Karlin 2002). Additionally, because of the 2 adjacent cysteines in loop C, the proteins are defined as α-type subunit (Corringer et al. 2000). A BLASTP search against the NCBI protein database found that *Bdor*α6 sequence was highly similar to many other insect nAChR α subunits. With high similarity to the nAChR α6 subunits from *A. mellifera*, *A. gambiae*, and *D. melanogaster*, we deduce the *Bdor*α6 belongs to α6-type of nAChR.

During development process, the expression of nAChRs changes considerably (Gotti & Clementi 2004). In *D. melanogaster*, the expression of *D*α6 was most abundant in embryos and then decreased in both larvae and adults (Grauso et al. 2002). A similar developmental pattern in expression was found in rats and humans (Gotti & Clementi 2004). In adult *L. bostrychophila*, higher amounts of *Lb*α1 and *Lb*α8 transcripts were

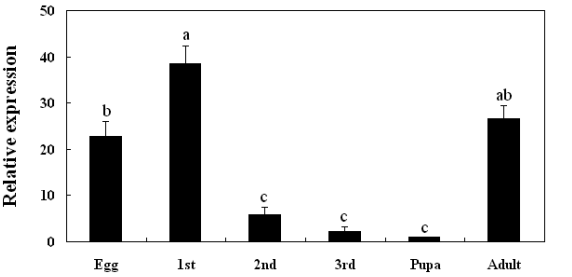

Fig. 3. The relative expression levels of the *Bdor*α6 in different developmental stages of *Bactrocera dorsalis* (bar graph represents Mean ± SE; different letters above each bar indicate significant differences, $P < 0.05$, LSD in ANOVA).

found compared to nymphal stages (Tang et al. 2009). Furthermore, the mRNA expression level of *Px*α8 was higher at adult stage than at either pupal or fourth instar larval stage in *P. xylostella* using semi-quantitative RT-PCR (Zhao et al. 2009). In the present study, *Bdor*α6 was found to be present at all life stages, and most abundant in the first instar larva followed by adult, egg, second and third larval stages and the lowest in pupa. Therefore, the relative high expression level of *Bdor*α6 in the first instar larva was unique and its significance will require further investigation.

The nAChRs are ligand-gated ion-channels expressed in many regions of the brain in insect. Several nAChR subunits transcripts from various species were detected in different tagmata. In the present study, *Bdor*α6 was found to be present in all tagmatas of adult flies the highest abundance in head followed by thorax and the lowest in abdomen. These results agreed well with the expression levels of nAChR genes *Md*α2, *Md*α5, and *Md*α6 found in *M. domestica*

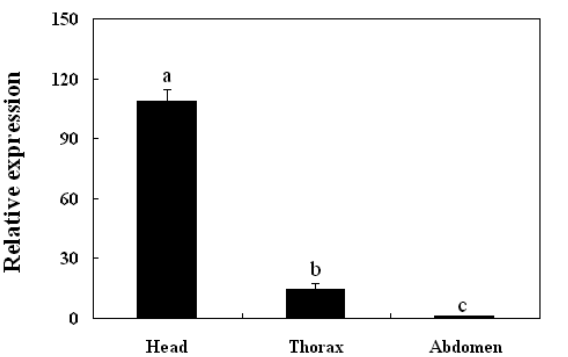

Fig. 4. The relative expression levels of the *Bdor*α6 in different tagmata of adult *Bactrocera dorsalis* (bar graph represents Mean ± SE; different letters above each bar indicate significant differences, $P < 0.05$, LSD in ANOVA).

(Gao et al. 2007a, b; Gao et al. 2007c). However, the highest levels of *Acc*β1and *Acc*β2 mRNA were found in the abdomen of Chinese honey bee (*A. cerana cerana*) (Yu et al. 2011). The relative expression level of *Md*β3 in the abdomen of *M. domestica* was also higher than in the thorax (Gao et al. 2007c). These results indicate that different nAChR subunits may exhibit distinct functions in different species. In *Drosophila*, *D*α2 transcripts and protein were exclusively in the CNS detected by *in situ* hybridization and immunohistochemistry, and suggested that the *D*α2 protein was a subunit of a synaptic nicotinic receptor (Jonas et al. 1994). In the honeybee, *Apis*α3 mRNA expression was restricted to the suboesophageal ganglia in larvae, while it is further expressed in the optic, dorsal and antennal lobes, and the calyces of mushroom bodies in adults (Thany et al. 2003). While the *B. dorsalis Bdor*α6 mRNA transcript was most abundant in the head, and this pattern is consistent with the idea that *Bdor*α6 is expressed in the central nervous system (CNS). The CNS of adult *B. dorsalis* is highly specialized and has a complex of brain and suboesophageal ganglia in head, a fusion of the thoracic and abdominal ganglia in thorax, and mainly distributes in the head. However, the localized distribution in the head at different developmental stages remains unknown. Therefore, further experiments need to be performed to have additional evidence to confirm this result.

In summary, we first cloned and characterized the cDNA of a nAChR subunit, *Bdor*α6, and analyzed its gene expression in different developmental stages and in different tagmata of adult *B. dorsalis*. Resistance to insecticides has become a major worldwide problem, and mutations of insect nAChR subunits may lead to high level of resistance (Liu et al. 2005; Perry et al. 2007). Our results may provide clues for understanding of physiological, pharmacological properties and mechanisms of insecticide resistance. In addition, determining the sequence of the nAChR cDNA can provide a first step to examine mutations that may give rise to insecticide resistance. Future work should focus on the understanding of the roles of nAChR in comprehending the insecticide-resistance mechanism of *B. dorsalis*.

Acknowledgments

This work was supported in part by the National Basic Research Program of China (2009CB125903), Natural Science Foundation of Chongqing (CSTC, 2009BA1042, JJA80020), the Program for Changjiang Scholars and Innovative Research Team in University (IRT0976), the Specialized Research Fund for the Doctoral Program of Higher Education (20100182120022), and the Earmarked Fund for Modern Agro-industry (Citrus) Technology Research System of China.

References Cited

Bass, C., Lansdell, S., Millar N., Schroeder I., Turberg A., Field L., and Williamson M. 2006. Molecular characterisation of nicotinic acetylcholine receptor subunits from the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae). Insect Biochem. Mol. Biol. 36: 86-96.

Bendtsen, J., Nielsen, H., Heijne, G., and Brunak, S. 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340: 783-795.

Chen, P., and Ye, H. 2007. Population dynamics of *Bactrocera dorsalis* (Diptera: Tephritidae) and analysis of factors influencing populations in Baoshanba, Yunnan, China. Entomol. Sci. 10: 141-147.

Clarke, A. R., Armstrong, K. F., Carmichael, A. E., Milne, J. R., Raghu, S., Roderick, G. K., and Yeates, D. K. 2005. Invasive phytophagous pests arising through a recent tropical evolutionary radiation: the *Bactrocera dorsalis* complex of fruit flies. Annu. Rev. Entomol. 50: 293-319.

Corringer, P. J., Le Novere, N. L., and Changeux, J. P. 2000. Nicotinic receptors at the amino acid level. Annu. Rev. Pharmacol. Toxicol. 40: 431-458.

Couturier, S., Bertrand, D., Matter, J. M., Hernandez, M. C., Bertrand, S., Millar, N., Valera, S., Barkas, T., and Ballivet, M. 1990. A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. Neuron 5: 847-856.

Falquet, L., Pagni, M., Bucher, P., Hulo, N., Sigrist, C. J. A., Hofmann, K., and Bairoch, A. 2002. The PROSITE database, its status in 2002. Nucleic Acids Res. 30: 235.

Gao, J. R., Deacutis, J. M., and Scott, J. G. 2007a. Characterization of the nicotinic acetylcholine receptor subunit gene *Md*α*2* from the house fly, *Musca domestica*. Arch. Insect Biochem. Physiol. 64: 30-42.

Gao, J. R., Deacutis, J. M., and Scott, J. G. 2007b. The nicotinic acetylcholine receptor subunit *Md*α*6* from *Musca domestica* is diversified via post-transcriptional modification. Insect Mol. Biol. 16: 325-334.

Gao, J. R., Deacutis, J. M., and Scott, J. G. 2007c. The nicotinic acetylcholine receptor subunits *Md*α*5* and *Md*α*3* on autosome 1 of *Musca domestica* are not involved in spinosad resistance. Insect Mol. Biol. 16: 691-701.

Gotti, C., and Clementi, F. 2004. Neuronal nicotinic receptors: from structure to pathology. Prog. Neurobiol. 74: 363-396.

Grauso, M., Reenan, R., Culetto, E., and Sattelle, D. 2002. Novel putative nicotinic acetylcholine receptor subunit genes, *D*α*5*, *D*α*6* and *D*α*7*, in *Drosophila melanogaster* identify a new and highly conserved target of adenosine deaminase acting on RNA-mediated A-to-I pre-mRNA editing. Genetics 160: 1519-1533.

Grutter, T., and Changeux, J. P. 2001. Nicotinic receptors in wonderland. Trends Biochem. Sci. 26: 459-463.

Gundelfinger, E., and Schulz, R. 2000. Insect nicotinic acetylcholine receptors: genes, structure, physiological and pharmacological properties. Handb. Exp. Pharmacol. 144: 496-521.

Hsu, J., and Feng, H. 2006. Development of resistance to spinosad in oriental fruit fly (Diptera: Tephritidae) in laboratory selection and cross-resistance. J. Econ. Entomol. 99: 931-936.

Hsu, J. C., Feng, H. T., and Wu, W. J. 2004. Resistance and synergistic effects of insecticides in *Bactrocera dorsalis* (Diptera: Tephritidae) in Taiwan. J. Econ. Entomol. 97: 1682-1688.

Jonas P. E., Phannavong B., Schuster R., Schröder C., and Gundelfinger E. D. 1994. Expression of the ligand-binding nicotinic acetylcholine receptor subunit *Dα2* in the *Drosophila* central nervous system. J. Neurobiol. 25: 1494-1508.

Jones, A. K., Grauso, M., and Sattelle, D. B. 2005. The nicotinic acetylcholine receptor gene family of the malaria mosquito, *Anopheles gambiae*. Genomics 85: 176-187.

Jones, A., Raymond-Delpech, V., Thany, S., Gauthier, M., and Sattelle D. 2006. The nicotinic acetylcholine receptor gene family of the honey bee *Apis mellifera*. Genome Res. 16: 1422-1430.

Jones, A., and Sattelle, D. 2007. The cys-loop ligand-gated ion channel gene superfamily of the red flour beetle, *Tribolium castaneum*. BMC Genomics 8: 327.

Karlin, A. 2002. Emerging structure of the nicotinic acetylcholine receptors. Nat. Rev. Neurosci. 3: 102-114.

Lester, H. A., Dibas, M. I., Dahan, D. S., Leite, J. F., and Dougherty, D. A. 2004. Cys-loop receptors: new twists and turns. Trends Neurosci. 27: 329-336.

Littleton, J. T., and Ganetzky, B. 2000. Ion channels and synaptic viewpoint organization: analysis of the *Drosophila* genome. Neuron 26: 35-43.

Liu, Z. W., Williamson, M. S., Lansdell, S. J., Denholm, I., Han, Z. J., and Millar N. S. 2005. A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in *Nilaparvata lugens* (brown planthopper). Proc. Natl. Acad. Sci. U. S. A. 102: 8420-8425.

Marshall, J., Buckingham, S., Shingai, R., Lunt, G., Goosey, M., Darlison, M., Sattelle, D., and Barnard, E. 1990. Sequence and functional expression of a single alpha subunit of an insect nicotinic acetylcholine receptor. The EMBO J. 9: 4391-4398.

Perry, T., McKenzie, J., and Batterham, P. 2007. A Dalpha6 knockout strain of *Drosophila melanogaster* confers a high level of resistance to spinosad. Insect Biochem. Mol. Biol. 37: 184-188.

Perry, T., Heckel, D., McKenzie J., and Batterham, P. 2008. Mutations in *Dα*1 or *D*β2 nicotinic acetylcholine receptor subunits can confer resistance to neonicotinoids in *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 38: 520-528.

Qiu, G., and Han, Z. 2007. Cloning and sequence analysis of nicotinic acetylcholine receptor subunits from the grain aphid, *Sitobion avenae* (Fabricius). Acta Entomol. Sin. 50: 762-768.

Salgado, V., and Sparks, T. 2005. The spinosyns: chemistry, biochemistry, mode of action, and resistance. Comprehensive Molec. Insect Sci. 6: 137-173.

Sattelle, D., Jones, A., Sattelle, B., Matsuda, K., Reenan, R., and Biggin, P. 2005. Edit, cut and paste in the nicotinic acetylcholine receptor gene family of *Drosophila melanogaster*. BioEssays 27: 366-376.

Shao, Y., Dong, K., and Zhang, C. 2007. The nicotinic acetylcholine receptor gene family of the silkworm, *Bombyx mori*. BMC Genomics 8: 324.

Shen, G. M., Jiang, H. B., Wang, X. N., and Wang, J. J. 2010. Evaluation of endogenous references for gene expression profiling in different tissues of the oriental fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae). BMC Mol. Biol. 11: 76.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731-2739.

Tang, P. A., Jiang, H. B., Xu, Y. Q., An, F. M., and Wang, J. J. 2009. Molecular characterization of two nicotinic acetylcholine receptor subunits from *Liposcelis bostrychophila* Badonnel (Psocoptera: Liposcelididae). Arch. Insect Biochem. Physiol. 72: 34-47.

Thany, S., Lenaers, G., Crozatier, M., Armengaud, C., and Gauthier, M. 2003. Identification and localization of the nicotinic acetylcholine receptor alpha3 mRNA in the brain of the honeybee, *Apis mellifera*. Insect Mol. Biol. 12: 255-262.

Tomizawa, M., and Casida, J. 2001. Structure and diversity of insect nicotinic acetylcholine receptors. Pest Manag. Sci. 57: 914-922.

Tomizawa, M., Latli, B., and Casida, J. E. 1999. Structure and function of insect nicotinic acetylcholine receptors studied with nicotinoid insecticide affinity probes, pp. 271-292 *In* I. Yamamoto and J. E. Casida [eds.], Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. Springer-Verlag. New York.

Yu, X., Wang, M., Kang, M., Liu, L., Guo, X., and Xu, B. 2011. Molecular cloning and characterization of two nicotinic acetylcholine receptor β subunit genes from *Apis cerana cerana*. Arch. Insect Biochem. Physiol. 77: 163-178.

Zhang, Y. P., Zeng, L., Lu, Y. Y., and Liang, G. W. 2007. Monitoring of insecticide resistance of *Bactrocera dorsalis* adults in South China. J. South China Agric. Univ. 28(3): 20-23.

Zhao, Y., Yang, Y. H., Wu, S. W., and Wu, Y. D. 2009. Cloning, sequence analysis and developmental expression of a cDNA encoding nicotinic acetylcholine receptor αsubunit from *Plutella xylostella* (L.)(Lepidoptera: Plutelidae). Acta Entomol. Sin. 52: 17-26.