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RESEARCH

Molecular Cloning and Characterization of a Novel P450 Gene Encoding CYP6BK18 From *Dastarcus helophoroides* (Coleoptera: Bothrideridae)

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ABSTRACT. A novel cDNA clone encoding a cytochrome P450 gene, named *CYP6BK18* (GenBank KC683905), was isolated by reverse transcription PCR from *Dastarcus helophoroides* (Fairmaire) (Coleoptera: Bothrideridae), a natural enemy of beetles. The full-length cDNA sequence is 1,659 bp, containing a 1,533 bp open reading frame predicting a 510-amino acid protein possessing a transmembrane domain with a calculated molecular weight of 59.4 kDa and a theoretical pI of 8.94. The deduced amino acid sequence of CYP6BK18 showed a 59% identity with CYP6BK17 (GenBank XP_970481.1) from *Tribolium castaneum*. Phylogenetic analysis indicated that CYP6BK18 was most closely related to CYP6BK17 and CYP6BK14 (GenBank EFA05731.1) from *T. castaneum.* Expression patterns of *CYP6BK18* in different tissues (head, oviduct, midgut, fat bodies, and Malpighian tubules), developmental stages (first- to sixth-instar larvae and adult) and 10 age groups of adult were analyzed by real-time quantitative PCR (RT-qPCR). The results showed that *CYP6BK18* was highly expressed in adulthood. Also, RT-qPCR analysis among different age groups of adult showed that *CYP6BK18* transcripts were abundant in the spawning period and peaked at the early stage of the adult development. Moreover, the tissue-specific expression levels of *CYP6BK18* were in the order of midgut, Malpighian tubes and fatty body from high to low. These results suggest that cytochrome P450 *CYP6BK18* may play a role in regulating the development and aging of *D. helophoroides*.

Key Words: *Dastarcus helophoroides*, cytochrome P450, CYP6 family, mRNA expression, aging

Dastarcus helophoroides (Fairmaire) (Coleoptera: Bothrideridae) is a natural predator of *Anoplophora glabripennis*, *Monochamus alternatus*, *Batocera horsfieldi*, *Massicus raddei,* and many other Cerambycidae insects (Wei et al. 2008). This parasitic beetle is mainly found in Japan and most provinces of China (Miura et al. 2003, Ren et al. 2012). *D. helophoroides* larvae are ectoparasitoids of late-instar larvae, pupae, and young adults of several long-horned beetle species, which makes it a potentially biological control agent for pest management (Wang and Hobbs 1995, Zhang and Yang 2006, Li et al. 2007).

Besides being the most valuable natural enemy of many long-horned beetles, *D. helophoroides* adult has a quite longer lifespan compared with other insects; it can survive eight or more years in laboratory (Wei et al. 2007). Thus, *D. helophoroides* could be used for studying the molecular and physiological basis of development and aging. To date, although the morphology and physiology of *D. helophoroides* has been widely reported, the molecular mechanism of development and aging is poorly understood (Lei et al. 2003). Cytochrome P450 superfamily of monooxygenases (P450s or CYPs) is a large family of heme-binding enzymes and is widely distributed in prokaryotes and eukaryotes from bacteria to plants and animals (Zhang et al. 2012). It may play a role in the growth and development of insects because of its involvement in regulation of endogenous substrates such as the biosynthesis and degradation of hormones, fatty acids and steroids, and in the catabolism and anabolism of xenobiotics such as drugs, insecticides, and plant toxins (Jeffrey 2008). However, few researches have investigated the P450 enzyme system in *D. helophoroides*. Therefore, a thorough understanding of the development mechanism of *D. helophoroides* at the P450s' molecular level is needed.

In insect, P450s can be detected in a wide range of tissues, while the highest monooxygenase activities are usually present in the midgut, fat bodies, and Malpighian tubules (Gilbert 2004). CYP6 gene family, initially isolated from *Musca domestica*, contained the largest proportion of the total number of insect P450 genes (Cohen et al. 1994, Feyereisen 2006). In the past, the majority of studies of CYP6 genes have focused primarily on the detoxification process of exogenous chemicals such as insecticides and drugs (Feyereisen 2012). However, as increasing numbers of CYP6 genes were identified from different insects' species, some CYP6 genes with special functions in the metabolism of endogenous compounds during development were revealed. For instance, *CYP6BQ13v2*, an allele of *CYP6BQ13*, isolated from the red flour beetle, *Tribolium castaneum*, was expressed in all life stages and associated with the synthesis of the insect molting hormone, further quantitative analysis revealed that *CYP6BQ13v2* was linked with the development of larva for its highly expression in preadult phase (Xu et al. 2009); *CYP6CM1*, an age-specific gene, isolated from the whitefly, *Bemisia tabaci*, was shown to play a potential role in the development process of whitefly based on its discriminating expression patterns in the adult phase (Jones et al. 2011). *CYP6A8*, identified from *Drosophila melanogaster*, was expressed at many developmental stages as well as in adult life and was found to catalyze the hydroxylation of lauric acid. It opened new views for insect CYP6 P450 enzymes, which may be involved in important physiological functions, such as regulating development and aging, through fatty acid metabolism (Helvig et al. 2004).

D. helophoroides is an insect which has a long lifespan. Instead of focusing on the role of P450 in neutralization of insecticides, we decided to choose CYP6 gene family as candidate genes in order to look at the role of these genes in the development and aging processes of *D. helophoroides*. In our previous study, 96 CYP6 fragments were isolated from *D. helophoroides* using five pairs of degenerate primers. Then the fragments were primitively classified into three subfamilies (CYP6BQ, CYP6BK, and CYP6BR; Wang et al. 2014). In this study, a novel full-length P450 gene, CYP6BK18 was successfully isolated, assembled, and identified based on our published CYP6 fragment (250 bp, GenBank JQ863088; Wang et al. 2014). Furthermore, we determined the expression profiles of CYP6BK18 in different developmental stages, 10 age groups of adult and various tissues using real-time

quantitative PCR (RT-qPCR). These results suggest that cytochrome P450 *CYP6BK18* potentially has an effect on aging process of *D. helophoroides.*

Materials and Methods

Insects. The *D. helophoroides* larvae and adult beetles were provided by the Laboratory of Forestry Pests Biological Control, College of Forestry, Northwest A&F University, Yangling, shaanxi province, China. First- instar larvae were inoculated into the pupa of the superworm *Zophobas morio* that acted as their food source in the preadulthood phase (first to sixth instar). Artificial forage for *D. helophoroides* adults contained silkworm pupa powder, sugar, yolk, and agar (Lei et al. 2005). All larvae and adults were reared and maintained in an air-conditioned room at 23 ± 1°C and 70–80% relative humidity, with a photoperiod of 16:8 (L:D) h. Every instar larvae, adults, and different tissues including head, oviduct, midgut, fat bodies, and Malpighian tubules were collected for extraction of total RNA utilized in RT-qPCR. Based on the surviving time from emergence date to the experiment date (December 2013), test adults were classified into 10 groups (0, 2, 6, 8, 10, 12, 16, 22, 24, and 26 months) to investigate the expression patterns of *CYP6BK18* in different age groups.

Isolation of Total RNA and Synthesis of First Strand cDNA. Total RNA used for cloning the full-length of P450 cDNA was extracted with UNIQ-10 column Trizol RNA extraction kit (Sangon, Shanghai, China) from adults *D. helophoroides*. Five adults were dissected to take their abdomen and were ground in a mortar and pestle in the presence of liquid nitrogen. Approximately 50 mg of powder was quickly transferred to a 1.5 ml centrifuge tube and homogenized with 500 µl Trizol reagent. The total RNA extraction and purification was carried out following the manufacturer's instructions of the reagent kit, including a DNase treatment (Takara, Beijing, China). In addition, ∼50 mg of adult powder from each age group and ∼50 mg larvae from each instar were whole-body extracted, respectively, which prepared for the total RNA used in preparation of cDNA template for RT-qPCR. In the tissue-specific experiment, various tissue (head, oviduct, midgut, fat bodies, and Malpighian tubules) samples were prepared by dissecting adults using dissection needle in physiological saline solution under a stereomicroscope (Motic SMZ-168, Shanghai, China). Tissues from 100 adults were homogenized with 1 ml Trizol reagent in the glass homogenizer.

Total RNA integrity was assessed by 1.2% formaldehyde agarose gel electrophoresis. RNA quantity and quality were assessed at the absorbance ratio of OD260/280 by using a UV-5500(PC) UV/VIS Spectrophotometer (Metash, Shanghai, China). Finally, the total RNA was dissolved in 20 µl diethylpyrocarbonate-treated water and stored at −80°C. The first-strand cDNA used for RT-qPCR was synthesized using 1 µg of DNase-treated total RNA by RevertAid First Strand cDNA Synthesis Kit (Fermentas, Shenzhen, China). The total volume of reverse transcription system was 25 µl. The procedures were used following the supplier's recommendations and the reaction mixture was stored at −20°C until further use.

The Full-Length cDNA Amplification of CYP6BK18. The amplification of 3′- and 5′- cDNA ends was conducted with SMARTer rapid amplification of cDNA ends (RACE) cDNA Amplification Kit (Clontech, Beijing, China). Approximately 1 and 2 µg total RNA were used to produce the cDNA templates for 3′ RACE and 5′ RACE, respectively. All the RACE PCR reactions were conducted in a S1000 PCR Thermal Cycler (Bio-Rad, Shanghai, China) using PrimeSTAR HS DNA polymerase (Takara).

For 3′ RACE, gene-specific primers (3′GSP1 and 3′GSP2, Table 1) designed on the basis of previously identified P450 cDNA fragment (GenBank JQ863088) (Wang et al. 2014) were used to amplify the 3′-ends of *CYP6BK18*. The 3′ RACE of *CYP6BK18* was performed directly using 3′ GSP1 (Sense) and the universal adaptor primer UPM (Antisense) (Table1). A nested PCR was conducted with 3′ GSP2 (Sense) and the nested universal adaptor primer NUP (Antisense; Table1). All the PCR reactions were conducted under the following conditions: an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products (360 bp) were checked by 1% agarose gel electrophoresis, then cloned into pUCm-T vectors and sequenced in both directions (Sangon, Shanghai, China).

Meanwhile, gene-specific primers (5′GSP1 and 5′GSP2, Table 1) designed based on the nucleotide sequences obtained from the 3′ RACE were used to amplify the 5′-ends of *CYP6BK18*. The 5′ RACE of *CYP6BK18* was carried out directly using the universal adaptor primer, UPM (Sense), with 5′ GSP1 (Antisense) (Table 1). A nested 5′ RACE was performed with the nested universal adaptor primer, NUP (Sense) and 5′ GSP2 (Antisense) (Table 1). All the 5′ RACE reactions were performed under the following conditions: an initial denaturation at 94°C for 3 min then cycled 30 times for 30 s at 94°C, 30 s at 60°C, and 2 min at 72°C followed by a final extension at 72°C for 10 min. The PCR products (1,592 bp) were checked then cloned and sequenced as described in 3′ RACE. All the gene-specific primers used in 3′ and 5′ RACE were designed utilizing Primer premier 5.0 (http://www.PremierBiosoft.com) and Oligo 6.0 (http://www.oligo.net).

After the 3′- and 5′- end sequences were obtained, contigs were assembled to produce putative full-length P450 sequences by using the DNAMAN 6.0 (http://dnaman.software.informer.com). To verify the full-length of putative P450 cDNA, a pair of primers (Full-1 and Full-2, Table1) was used to amplify the open reading frame (ORF) of *CYP6BK18* cDNA. To eliminate the potential error caused by Taq DNA polymerase, a thermal-stable high-fidelity PrimeSTAR HS DNA polymerase (Takara) was used to amplify the cDNA. All the PCR reactions were performed under the following conditions: an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min and a final extension at 72°C for 10 min. The PCR products (1,550 bp) were cloned and sequenced as described earlier.

Sequences Analysis and Phylogenetic Tree Construction. Obtained full-length cDNA sequences were aligned and edited by Clustal X 2.0 (Larkin et al. 2007) (http://clustalx.software.informer.com) and DNAMAN 6.0. The nucleotide and deduced amino acid sequences were compared with other similar P450 genes deposited in the GenBank database by using the BLAST algorithm at the NCBI website (http://blast.ncbi.nlm.nih.gov/). The assessment of transmembrane regions in the predicted CYP6BK18 protein was carried out with (http://genome.cbs.dtu.dk/services/TMHMM/). ProtScale analysis of protein hydrophobicity was performed with ExPASy protein analysis expert system (http://us.expasy.org/tools/protscale.html). The DictyOGlyc online software and NetPhos2.0 Server Analysis were used to search the protein post-translational modification of glycosylation sites (http://www.cbs.dtu.dk/services/DictyOGlyc/) and phosphorylation sites (http://www.cbs.dtu.dk/services/NetPhos/) while secondary structure was predicted by GOR method (GOR, is an information theory-based method for the prediction of secondary structures in proteins) (Garnier et al. 1996; http://npsa-pbil.ibcp.fr/cgi-bin/npsaautomat.pl?page=npsa gor4.html). The phylogenetic tree was constructed with MEGA 5.05 (http://www.megasoftware.net; Kumar et al. 2008) using the Neighbor-Joining (NJ) method with a 1,000 replicates bootstrap test.

RT-qPCR of CYP6BK18 mRNA. The total RNA for RT-qPCR was extracted from the different life stages, various adult groups and different tissues of *D. helophoroides* and treated with DNase (Takara) as described earlier. RT-qPCR was conducted on a Bio-rad IQ5 Thermol System using SYBR Green Mix (CWBIO, Beijing, China) with α-Tubulin and GAPDH as reference genes. The primers, F1and R1, were used to amplify *CYP6BK18*, whereas f2 and r2 were for α-Tubulin (GenBank AGJ51949.1), additionally, for GAPDH (GenBank AGJ51948.1), f3 and r3 were used. All the primers used in RT-qPCR were designed by Primer 3 (http://rodo.wi.mit.edu/; for primer information, see Table 2).

Each RT-qPCR reaction was carried out in a 25 µl reaction system containing 1 µl undiluted cDNA template, 10 µM of each primer, 9.5 µl

Table 1. Primers used for cloning

Experiments	Primer sequence 5′ to 3′
3′-RACE	3′GSP1: GAGGACACCATCTTGGAAACAGG (Sense) UPM: CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT (Antisense) 3′GSP2: GCGGTTTTCTGAGGAGAACTCAC (Sense) NUP: AAGCAGTGGTATCAACGCAGAGT (Antisense)
5′-RACE	UPM:CTAATACGACTCACTATAGGGCAAGCAGT GGTATCAACGCAGAGT (Sense) 5′GSP1: CTCCTTTAGCTGCCAAGATGAACGA (Antisense) NUP: AAGCAGTGGTATCAACGCAGAGT (Sense) 5′GSP2: CGAGAGGTAACTGGGTCTTCGTATTC (Antisense)
Full-length confirmation	Full-1: GTGTATTGTGTCTGACTGACCGAGAC (Sense) Full-2: TAAGCCAAATGTCTCCTTTAGCTGCC (Antisense)

Table 2. Primers Used for RT-qPCR

Genes	Primer names and sequences (5′ to 3′)	Product size (base pairs)
CYP6BK18	F1: GATCGAGTGCAAGTTTGTAATGTG (Sense) R1: TAATGTAGAAGTAGAGGAGGGTGG (Antisense)	147
α-Tubulin	f2: TCGGTGGTGGTACTGGGTCT (Sense) r2: ACGGCTGTTGAAACTTGAGGA (Antisense)	120
GAPDH	f3: TAACTTTGGCATCGTTGAGG (Sense) r3: AGCGGCGGGAATGATGTTTT (Antisense)	132

double distilled water and 12.5 µl SYBR Green Mix. All the reactions were performed as: 95°C for 3 min, 50 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Fluorescence was measured at the end of each cycle. After RT-qPCR, a melting curve analysis was performed by heating the PCR product from 65°C to 95°C to ensure the specificity of the amplified product. To compare the expression levels of *CYP6BK18* gene among the different life stages, various adult groups, and different tissues of *D. helophoroides*, the ΔCq method (Hellemans et al. 2007) was used to calculate the relative quantities of *CYP6BK18* mRNA. Values for mean expression and standard deviation were calculated from the results of three independent biological replicates. Statistical analysis of the RT-qPCR data was carried out using Bio-Rad 2.0 Standard Edition software and Excel 2003 (Microsoft, Dallas, TX).

Results

Nucleotide and Deduced Amino Acid Sequences of CYP6BK18. In our previous study, P450 cDNA fragments were isolated from *D. helophoroides* using a RT-PCR approach with degenerate primers (Wang et al. 2014). A fragment (GenBank JQ863088) with high homology to P450 superfamily was obtained. Using this fragment as a probe and by overlaying the cloned sequences a full-length of the P450 gene was obtained. The cDNA sequence was submitted to the P450 nomenclature committee and classified to the CYP6 family, which was qualified as *CYP6BK18*. The GenBank accession number of *CYP6BK18* was KC683905. Figure 1 shows the nucleotide and deduced amino acid sequence of *CYP6BK18.* The cDNA assembly yielded a sequence of 1,659 bp, containing a 1,533 bp ORF. The gene encoded a 510-amino acid protein, with a calculated molecular weight of 59.4 kDa and theoretical pI of 8.94. The start codon ATG is located at position 92–94, whereas the stop codon TAA is located at position 1622–1624. There is a 91-bp 5′-untranslated region (UTR) upstream of the start codon and a 35-bp 3′ UTR downstream of the stop codon. The nucleotides AATAAA at position 1615–1620 have the putative polyadenylation signal. The defining heme-binding sequence motif (FxxGxxxCxG) conserved among all P450 enzymes is found at residues 437–446 (Ranasinghe and Hobbs 1998, He et al. 2002).

TMHMM software analysis showed that the N-terminal of CYP6BK18 protein is a highly hydrophobic domain that contains a 30-residue-long transmembrane domain (residues 7–36; Fig. 2A). ProScale analysis showed that the maximum hydrophobicity of CYP6BK18 protein was 2.700 at residue 18, and the minimum value of −3.322 at residue 437 (Fig. 2B). DictyOGlyc projection showed the sugar-free glycosylation in CYP6BK18 (Fig. 2C). NetPhos 2.0 Server phosphorylation site analysis of CYP6BK18 portrayed that there were 10 serine (Ser) phosphorylation sites, 8 threonine (Thr) phosphorylation sites, and 8 tyrosine (Tyr) phosphorylation sites uniformly distributed throughout the polypeptide chain (Fig. 2D). The GOR IV method (Garnier et al. 1996) was used to predict the secondary protein structure of CYP6BK18. The protein structure mainly contains alpha helix (Hh), extended strand (Ee), and random coil (Cc) (Fig. 2E). The distribution of these elements is as follows: random coil, 47.25%; alpha helix, 34.31%; and extended strand, 18.43%.

Phylogenetic Relationship of CYP6BK18 With Other CYP Members. A BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of GenBank revealed that CYP6BK18 was closest in amino acid sequences to P450s in several members of CYP6 family. A search indicated that CYP6BK18 exhibited the greatest identity (59%) with *T. castaneum* CYP6BK17 (GenBank XP_970481.1) and a 55% amino acid identity with *T. castaneum* CYP6BK14 (GenBank FA05731.1), while CYP6BK18 shared weakest identity to CYP6N2 (42%; GenBank AFM08400.1) from *Anopheles funestus*. To analyze the sequence homology and phylogenetic relationships, 17 insect CYP6 genes were downloaded from GenBank and aligned with CYP6BK18 by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A NJ phylogenetic tree was constructed by MEGA 5.05 (Fig. 3). The phylogenetic tree showed that CYP6BK18 was most closely related to CYP6BK17 and CYP6BK14 from *T. castaneum*.

Expression Levels of CYP6BK18 in *D. helophoroides*. The relative expression patterns of *CYP6BK18* in *D. helophoroides* at different developmental stages are shown in Fig. 4A. Transcription levels for *CYP6BK18* mRNA were detectable at all tested developmental stages. The expression levels of *CYP6BK18* were highest in adulthood. In larval stages, *CYP6BK18* transcripts in the sixth instar were higher than that in the other stages in the preadulthood phases, while the mRNA levels in third instar were not significant. Figure 4B shows the relative expression profiles of *CYP6BK18* mRNA in 10 age groups of adult. It indicated that the *CYP6BK18* transcripts were observed at all tested groups. Generally, the whole trend of the *CYP6BK18* mRNA levels followed an arc. The expression levels of *CYPBK18* increased from the

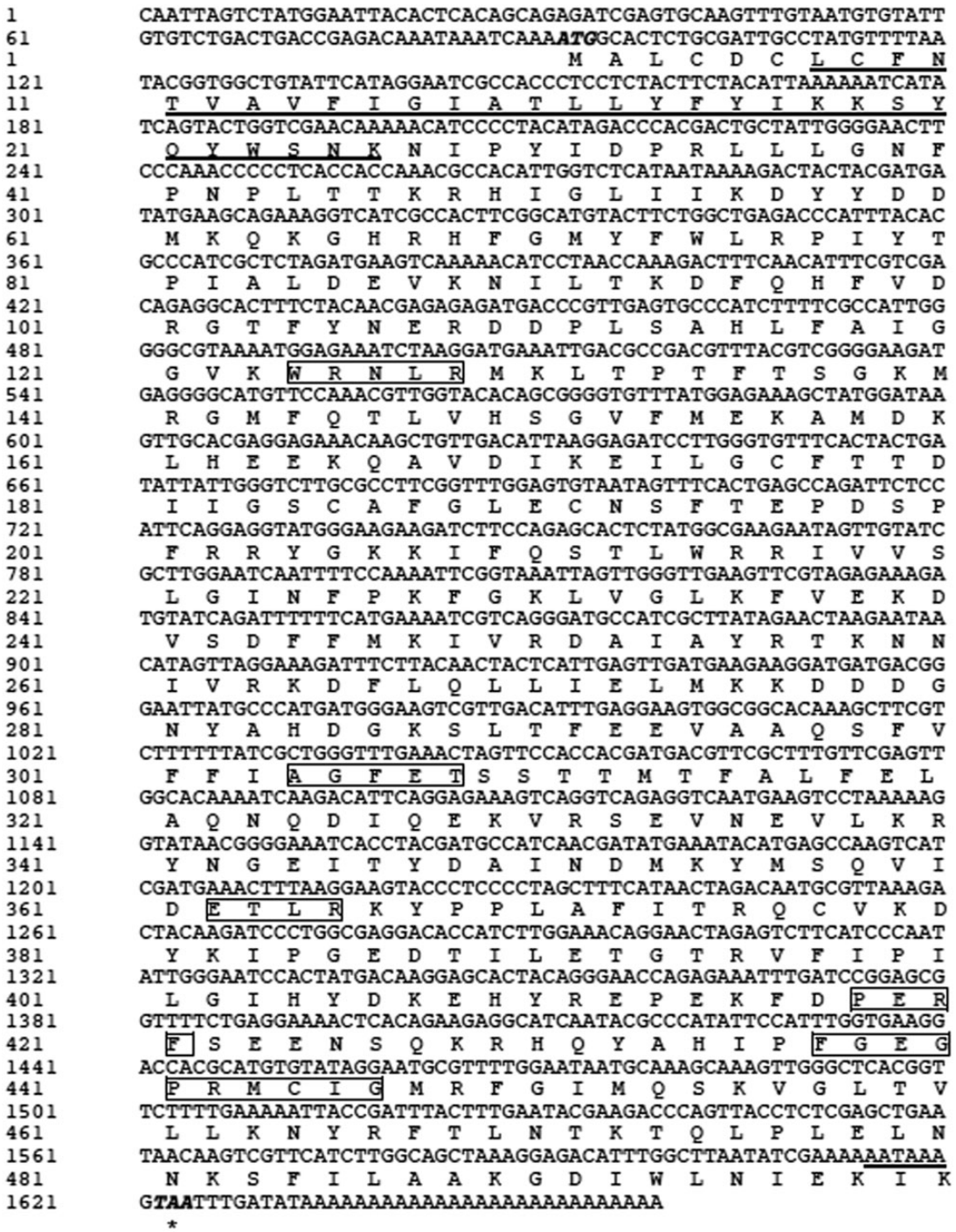

Fig. 1. Nucleotide and deduced amino acid sequence of *CYP6BK18*. The start codon ATG is indicated with bold italic and the stop codon TAA is indicated with bold italic by an asterisk. Polydenylation signal AATAAA is underlined. The heme-binding sequence motif FxxGxxxCxG and other conserved sequence motifs are marked by the boxed amino acids. The amino acids underlined illustrate the position of transmembrane region.

beginning and peaked around the eighth month. The transcription level then declined drastically from the 22nd to the 26th month.

The mRNA levels of *CYP6BK18* were significantly highest in the midgut of *D. helophoroides* (Fig. 5). Moreover, *CYP6BK18* transcripts in midgut were four- and sevenfolds higher than that in Malpighian tubules and fat body, respectively. However, the mRNA expression of *CYP6BK18* was barely detectable in the head and oviduct of *D. helophoroides*.

Discussion

With the wide spread of SMART RACE technique, an increasing numbers of the full-length P450 gene were cloned from diversity of insect species (Yeku and Frohman 2011). Further analysis found that the P450 genes have numerous roles in growth and development of insects such as detoxification of xenobiotics/insecticides, regulation of insect feeding, hormone resistance, and metabolism of endogenous compounds such as fatty acids (Claudianos et al. 2006, Gutierrez et al. 2007, Bass and Field 2011). *D. helophoroides* is an important natural enemy of the beetle, which has been used as a natural "pesticide" in areas of China (Wei et al. 2008). Studies on classification, natural distribution, biological characteristics, and artificial breeding technology (Ogura et al. 1999, Wei et al. 2009, Kijimoto et al. 2013) of *D. helophoroides* have been previously published. However, little is known

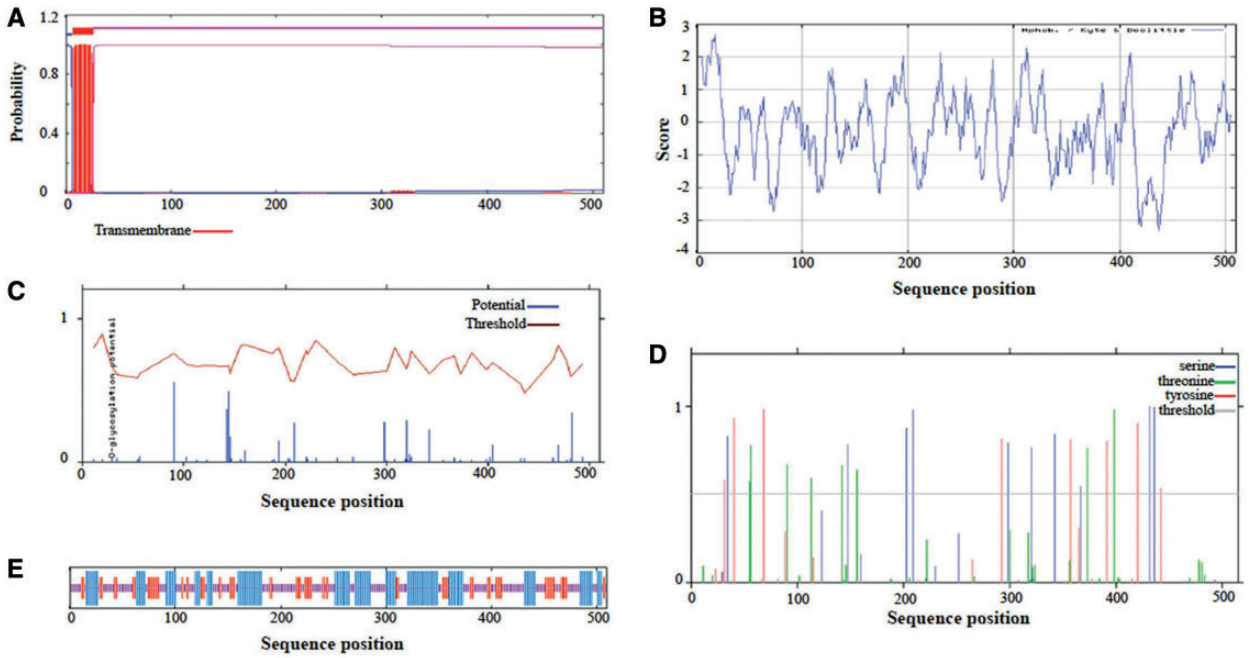

Fig. 2. Bioinformatic analysis of CYP6BK18 from *D. helophoroides*. (A) Transmembrane probabilities of the amino acid sequence CYP6BK18 with TMHMM analysis. (B) Hydrophobic character prediction of the amino acid sequence of CYP6BK18. (C) O-glycosylation site prediction of the amino acid sequence of CYP6BK18. (D) Phosphorylation site prediction of the amino acid sequence of CYP6BK18**. (E)** Secondary structure prediction of the amino acid sequence of CYP6BK18.

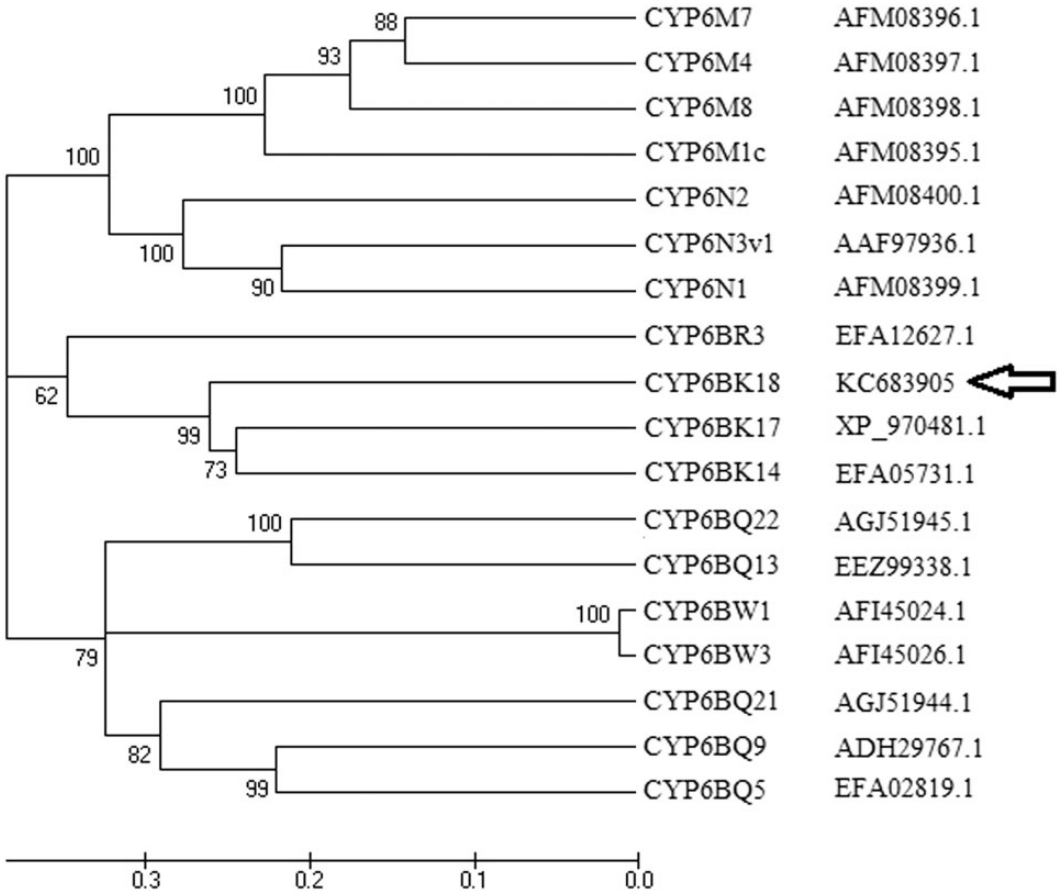

Fig. 3. Phylogenetic relationship among CYP6BK18 and other insect P450s. GenBank accession numbers are shown followed by the P450s' name. The phylogenetic tree was inferred using the NJ method. The consensus tree was inferred from 1,000 bootstrap replicates. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1,000 replicates), is shown next to the branches. Phylogenetic analyses were conducted in MEGA 5.05.

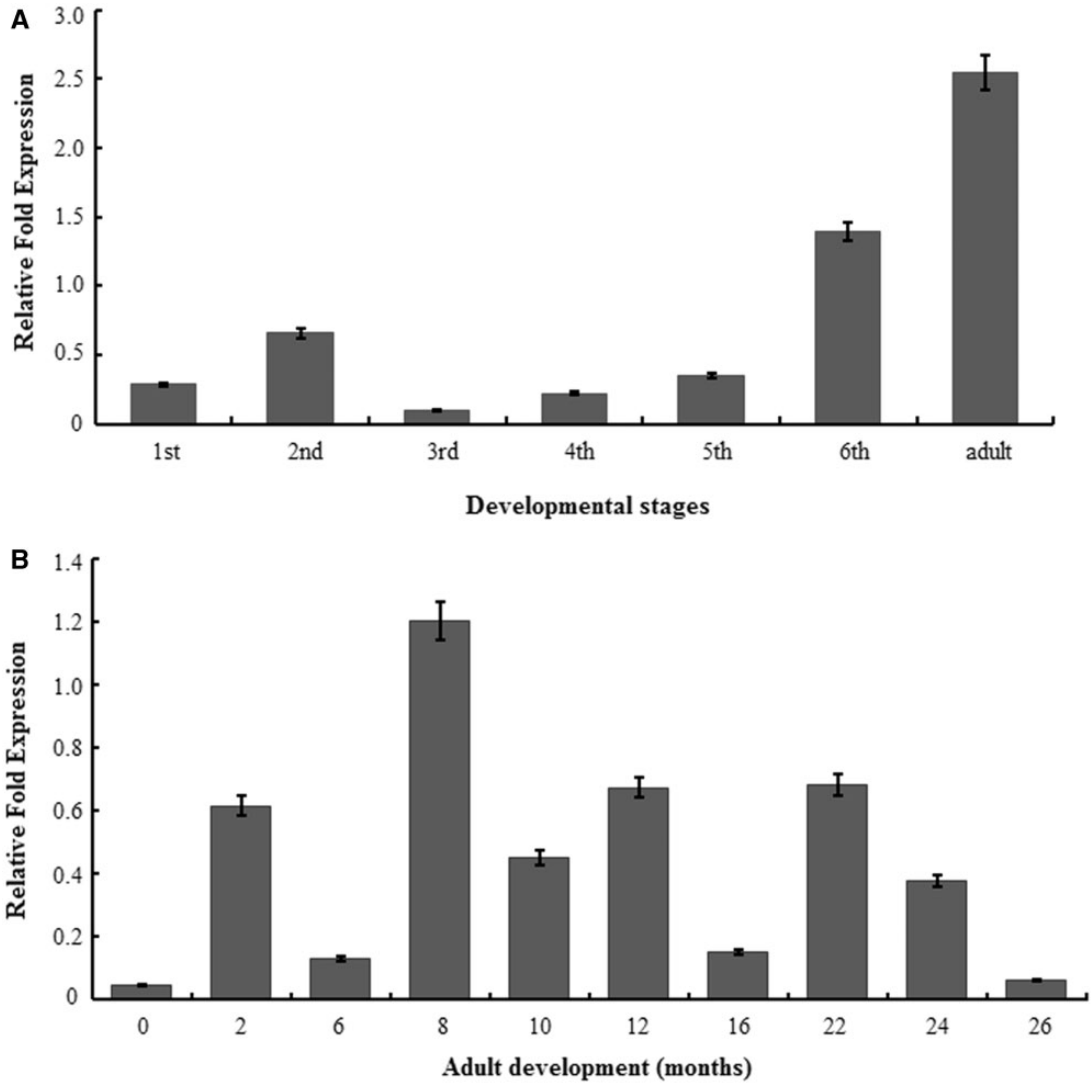

Fig. 4. Expression levels of *CYP6BK18* cDNA during the different developmental stages (first to sixth-instar larvae and adult) (**A**) and 10 different age groups of adult (0, 2, 6, 8, 10, 12, 16, 22, 24, and 26 months) (**B**) of *D. helophoroides*. The mRNA quantity is calculated using the ΔCq method; values are expressed as the mean relative quantity ± SEM.

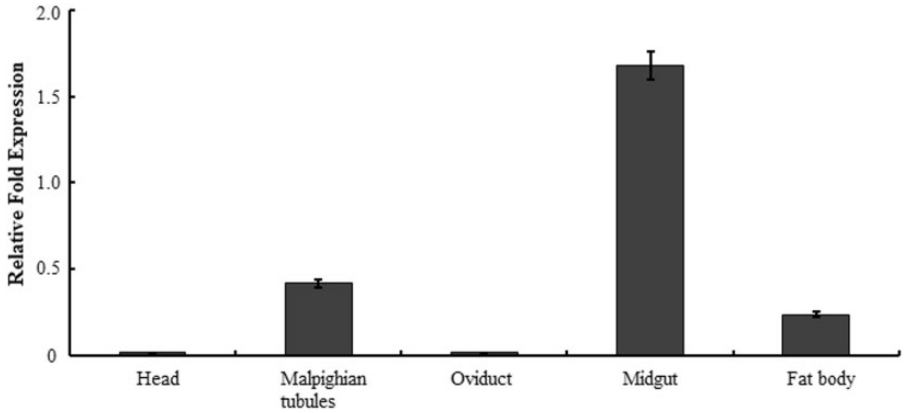

Fig. 5. The relative quantities of *CYP6BK18* mRNA in different tissues (head, oviduct, midgut, fat bodies, and Malpighian tubules). ΔCq method was used to calculate the mRNA quantity; data are expressed as the mean relative quantity ± SEM.

about the P450 system in *D. helophoroides*. Here, we isolated and characterized a novel cDNA clone encoding a cytochrome P450 gene, *CYP6BK18*. The cDNA sequence consists of 1,659 bp with an ORF of 1,533 bp encoding a 510 amino-acid protein with a molecular weight of 59.4 kDa, a theoretical pI of 8.94, and a polyadenylation signal at the 3′ coding region.

The deduced amino acid sequence of *CYP6BK18* features some highly conserved P450 motifs present in all other CYP6 family members, which are listed as follows (see in Fig. 1). First, the heme-binding sequence motif (FxxGxxxCxG), which is conserved among all CYP enzymes, is found in the deduced amino acid sequence (residues 437–446; Ranasinghe and Hobbs 1998, He et al. 2002). Second, the conserved motif WxxxR (residues 124–128) corresponds to a C-helix which has been identified in most eukaryotic P450s (Jiang et al. 2008). Third, the sequence motif (A/G) GxxT (residues 304–308), is an I-helix which is shared among several bacterial enzymes and is involved in substrate oxidation (Tripathi et al. 2013). Fourth, the K-helix ExxR sequence is found at residues 362–365. The K-helix acts as a salt bridge and is found in all reported P450 proteins (Graham-Lorence and Peterson 1996). Finally, The PERF sequences are found at residues 418–421 and are involved in heme binding (Kariakin et al. 2002). In addition, the bioinformatics analysis (see in Fig. 2) showed that CYP6BK18 has a hydrophobic domain that embraces a transmembrane domain located near the N-terminus; also, CYP6BK18 possesses phosphorylation sites but no glycosylation sites. The secondary structure of CYP6BK18 is mainly comprised of alpha helix (Hh), extended strand (Ee), and random coil (Cc). Phylogenetic analysis demonstrated that CYP6BK18 is most strongly associated with CYP6BK17 and CYP6BK14 from *T. castaneum* (see in Fig. 3).

The biology of *D. helophoroides* is unique. The *D. helophoroides* adult can survive eight or more years in the laboratory and spawn two to three times a year, but appears aging rapidly and the spawning rate decreased dramatically after the third year of incubating in adult (Su 2012). To date, the age-related P450 genes have long been reported in mammal and plants. It has been suggested that the age-related changes in CYP expression may be an important factor for deciding the efficacy and safety of endogenous compounds and environmental pollutants in human and rats (Yun et al. 2010). However, the studies on the age-related P450 genes in insects were limited. Recently, a great diversity of CYP6 genes have been identified and cloned from insects such as *Dr. melanogaster*, *M. domestica*, *Helicoverpa armigera*, cockroach, and silkworms (Scott and Wen 2001). Because insect CYP6 genes are widely involved in the metabolism of endogenous substrates and xenobiotics (Scott et al. 1998, Helvig et al. 2004, Xu et al. 2009), we hypothesized that CYP6 genes may play a role in aging.

CYP6 genes show life stage-specific or tissue-specific expression patterns depending on specific functions (Chung et al. 2009). For instance, *CYP6L1* was greatly expressed in the testicle and accessory gland of male *Blattella germanica* adult and might be related to the reproductive development (Wen and Scott 2001). *CYP6A2* transcripts were found in *Dr. melanogaster*, with highest levels in the fat body, midintestine, and Malpighian tube of larval and pupal phases (Brun et al. 1996, Saner et al. 1996). In *H. armigera*, the expression levels of *CYP6B2* mRNA were abundant in the midintestine, head, anterior intestine, and fat body at the larval stage (Wang and Hobbs 1995). The mRNA encoding *CYP6K1* in *B. germanica* was expressed in each life stage and increased with growth (Wen and Scott 2001). In this study, the expression patterns of *CYP6BK18* in larval (first to sixth instar and adult) and various stages of adult life of *D. helophoroides* were analyzed by RT-qPCR (see in Fig. 4). We found that *CYP6BK18* was expressed in both larval and adult stages; but the expression levels were higher in adulthood (see in Fig. 4A), suggesting that *CYP6BK18* expression may be associated with the *D. helophoroides* adult development. To further understand the role of P450 gene in *D. helophoroides* adult development, *CYP6BK18* mRNA expression was measured in different age groups of adult (see in Fig. 4B). We found that *CYP6BK18* transcripts could be detected in all tested sets and peaked around the eighth month that coincides with maturation of the adult. Normally, *D. helophoroides* begins to spawn after the first month of incubating in adult. Every year, the first spawning time lasts ∼2 months and then has 2–3 month dormant periods; soon after, entering the second spawning period and has 3 to 4 months dormant times (Lei et al. 2003). Interestingly, our results revealed that the *CYP6BK18* transcripts exhibited great amounts in almost all the spawning phases (the 2nd, 8th, 12th, 22nd, and 24th months), indicating that CYP6BK18 may potentially play an important role in the spawning process of *D. helophoroides* adult.

In addition, we determined *CYP6BK18* expression patterns in various tissues (head, oviduct, midgut, fat bodies, and Malpighian tubules) of *D. helophoroides* (see in Fig. 5). We found that *CYP6BK18* was mostly expressed in three tissues including midgut, Malpighian tubules, and fat body. The insect midgut is the largest portion of the digestive tract (Tzou et al. 2000), and the anterior portion of the midgut has been suggested to play some role in immunity (Senger et al. 2006). The insect Malpighian tubules have been shown to be the primary organs of excretion in insects and potential involved in immunity, also important for the metabolism and detoxification of xenobiotics (Dow and Davies 2006, Yang et al. 2007). The insect fat body is the main organ involved in energy metabolism and the vital storage site for glycogen, lipid, and protein (Costa-Leonardo et al. 2013), and also has other physiological functions such as innate immunity and detoxification (Liu et al. 2009). Here, the highest expression of *CYP6BK18* in the midgut indicated that it may be related to the metabolism of nutrients absorption as well as the digestion process in *D. helophoroides*.

In summary, a novel P450 gene, *CYP6BK18*, was isolated from *D. helophoroides*, and its expression profiles during different development stages, 10 age groups of adult and various tissues were detected. To fully illuminate the function of *CYP6BK18*, proteomic and biochemical studies are currently underway in our laboratory.

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