Vol.64: e21210292, 2021
https://doi.org/10.1590/1678-4324-2021210292
ISSN 1678-4324 Online Edition

Article - Biological and Applied Sciences

Cloning and Expression of NADPH-cytochrome P450 Reductase Gene in Chinese Mitten Crab, *Eriocheir sinensis*

Xianggang Gao[1]
https://orcid.org/0000-0002-6757-5701

Chongbo He[1*]
https://orcid.org/0000-0001-9767-4367

Ying Xia[2]
https://orcid.org/0000-0003-3512-0742

Xiangbo Bao[1]
https://orcid.org/0000-0001-9030-5980

Yulong Li[1]
https://orcid.org/0000-0002-5412-4880

Weidong Liu[1]
https://orcid.org/0000-0003-0931-1262

[1]Key Lab of Marine Fishery Molecular Biology of Liaoning Province, Liaoning Ocean and Fisheries Science Research Institute, Dalian Liaoning, China; [2]Dalian Eco-Environmental Monitoring Center of Liaoning Province, Dalian Liaoning, China.

Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Alexandre Rasi Aoki

Received: 2020.10.30; Accepted: 2021.05.06.

*Correspondence: chongbohe@163.com; Tel.: +86-0411-84697003 (C.H.).

HIGHLIGHTS

- *EsCPR* was isolated from *E. sinensis* using RT-PCR and RACE methods.
- *EsCPR* mRNA was markedly expressed in the hepatopancreas and stomach of *E. sinensis*.
- The results showed a higher CPR expression level in the premolt than other stages.

Abstract: NADPH-cytochromeP450 reductase (CPR) is one of the most important components of the cytochrome P450 enzyme system. In this study, a gene encoding CPR (named *EsCPR*) was isolated from *Eriocheir sinensis* using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) methods. Analysis of the nucleotide sequence revealed a cDNA full-length of 3717 bp with an open reading frame of 2046 bp, a 5′-untranslated region of 42 bp, and a long 3′-untryganslated region of 1628bp, which encodes a protein of 681 amino acids with a predicted molecular weight of 30.7 kDa and an estimated pI of 4.82. The mature peptide shares amino acid of *E. sinensis* identity 82 % - 89 % to the CPR from *Penaeus vannamei* and *Chionoecetes opilio.* Tissues and developmental stage-dependent expression of *EsCPR* mRNA was investigated by real-time quantitative PCR. *EsCPR* mRNA was markedly expressed in the hepatopancreas and stomach. These results would provide valuable information for further study on the interactions between CPR and cytochrome P450 enzyme systems.

Keywords: *Eriocheir sinensis*; Molting; NADPH-cytochrome P450 reductase gene; Cloning; Gene expression.

INTRODUCTION

The steroid hormone 20-hydroxyecdysone, which is produced from cholesterol via a series of oxidation steps, is the physiologically active molting hormone that controls crustacean development. The final step of its biosynthesis, has been reported to occur in the microsomal or mitochondrial fractions of the eye stalk of the insects at a certain developmental stage [1-3].

Cytochrome P450 is involved in the metabolism of a wide range of foreign compounds such as insecticides and plant secondary metabolites, as well as participating in the regulation of endogenous substrates [4,5]. The catalytic reaction of P450 enzymes require the electron donor, NADPH-cytochrome P450 reductase (CPR) [6]. CPR has several conserved functional domains, including the sites for binding flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH), which are involved in the transfer of electrons from NADPH to the central heme-group of P450s through a series of redox-coupled reactions [5]. CPR also shuttles electrons to other oxygenase enzymes, including cytochrome b5 [7,8] and heme oxygenase [9] found in most eukaryotes. As a component of the microsomal P450 electron transport system, CPR plays an essential role in the transfer of reducing equivalents from NADPH to various P450 molecules [10]. The P450 reductase in insects was first purified from the housefly Musca domestica [11], and was shown to participate in P450-dependent drug metabolism [12]. Antisera raised against the enzyme were used for isolating a cDNA from the abdominal tissue of phenobarbital-treated flies [13,14]. However, very limited information concerning the role of P450 reductase in the biosynthesis of ecdysteroid is available [1].

Chinese mitten crab (*Eriocheir sinensis*), one of the most important aquaculture species, has been widely farmed in ponds, reservoirs, and lakes in China [15]. Although *E. sinensis* is considered as an annoying invasive species in Europe and North America, it is considered as a native delicacy in China [16]. Being a catadromous crustacean, *E. sinensis* needs to undergo 18 molting states, including larval developmental molting, juvenile growth molting, and adult reproductive molting to finally become an adult crab[17]. Molting is a cyclic process that occurs throughout the life history of *E. sinensis* and is essential for metamorphosis, growth, and reproduction. Developmental molting is closely correlated with metamorphosis, and growth-related molting determines the growth and size of a crab. Abnormal molting might lead to development and growth deficiency or even death. The terminal or reproductive molting is closely associated with the onset of reproductive maturity, and advancement of reproductive molting might lead to precocity, which has adverse effects on the growth of *E. sinensis* [18]. CPR is a key enzyme that directly participates in the periodic molting process. Study on the *E. sinensis* CPR (*EsCPR*) gene is important to understand brachyuran metabolism as well as the effects of different arthropods. In this study, we isolated a CPR from *E. sinensis*, and examined its physiological relevance to the expression of ecdysone 20-hydroxylation during the molting of *E. sinensis*.

MATERIAL AND METHODS

Healthy juvenile Chinese mitten crabs (body weight 70.2 ± 9.6 g) with good vitality were collected from the Liaohe River in northeastern China, and acclimatized in freshwater inside a breeding room. The crabs were cultured in individual aquaculture tanks in a re-circulating-closed artificial system with an aeration system at room temperature. The crabs were mainly fed on alternate days with a diet containing aquaculture feed. Intermolt crabs were used for the experiments. Ten types of tissues, including the Y organs, eye stalk, horacic ganglion, cerebral ganglion, heart, stomach, hepatopancreas, muscle and gills were separately collected, and immediately frozen in liquid nitrogen and stored at -80℃ until use.

According to the morphological changes in setogenesis during the molting cycle, the crabs were first divided into five groups: early-postmolt (A), late-postmolt (B), intermolt (C), premolt (D), and ecdysis (E). The setae of the second maxilla were carefully sampled from the premolt group crabs and microscopically observed to identify precisely the premolt substages according to the method described by Tian[19]. The premolt group was then classified into 2 subgroups: premolt ($D^{1–2}$) and premolt ($D^{3–4}$). The crabs at different periods of molting cycle were thus divided into six groups, including the two subgroups of the premolt stage, with each group having at least three crabs. The hepatopancreas tissues from the crabs of different groups were dissected and immediately frozen in liquid nitrogen. Total RNA was extracted from these tissues by using the RNAprep pure Tissue Kit (Tiangen, China) according to the manufacturer's instructions. The quality of RNA was assessed by formaldehyde agarose gel electrophoresis and was quantitated spectrophotometrically.

The published CPR sequences from *Penaeus vannamei* were used as query sequences in a BLAST search of *E. sinensis* hepatopancreas transcriptome shotgun assembly database. In order to obtain CPR, multiple alignment and homology cloning were performed for the conserved regions of crustacean CPR. Gene-specific 5′ and 3′ primers were designed according to these partial cDNA sequences. All primers are listed in Table 1. The first-strand cDNA (5′ cDNA and 3′ cDNA) was reverse transcribed using a 3′-Full RACE Core Set with PrimeScript™ RTase (Takara, China) and 5′-Full RACE Kit with TAP (Takara, China), and polymerase chain reaction (PCR) amplifications for 3′ and 5′ rapid amplification of cDNA ends (RACE) were performed following the manufacturer's instructions. The 3-step PCR program for 3′ RACE was as follows: holding at 94 ˚C for 3 min, followed by 20 cycles at 94 ˚C for 30 s, 60 ˚C for 30 s and 72 ˚C for 1 min, and a final extension step at 72 ˚C for 10 min. The PCR program for 5′ RACE was as follows: 3 min at 94 ˚C, followed by 30 cycles at 94 ˚C for 30 s, 60 ˚C for 30 s, and 72 ˚C for 1 min. The full length of each *EsCPR* was obtained by alignment and assembly of the sequencing result for 5′ and 3′ RACE products. The PCR products were separated in 1% agarose gel via electrophoresis, and the gel was then stained with ethidium bromide.

Table 1. Primer sequences used in this study.

Primer name	Sequence (5′-3′)	Application
EsCPR-1	GATGCTGGAGACCATGTGGCT	RACE degenerate primers
EsCPR-2	TCTCCACAGACATACAAGTGGCC	RACE degenerate primers
EsCPR-3	TCAGGTGCACAACACAGCCGCCTAG	3′ RACE
EsCPR-4	CCATAACGGGTCGCTTCCTTGGCC	5′ RACE
EsCPR-5	GGTTGCCGTAACAAGGACAAGGAC	RT-qPCR analysis
EsCPR-6	CCAGTGTTGGCTGTTGCTGGC	RT-qPCR analysis
Actin-R	CTCCTGCTTGCTGATCCACATC	House-keeper gene
Actin-S	GCATCCACGAGACCACTTACA	House-keeper gene

The open reading frame (ORF) of CPR cDNA was identified using the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptide was predicted utilizing the Sigal P3.0 server (http://www.cbs. dtu.dk/services/SignalP), and protein domain features were predicted using SMART (http://smart.embl-heidelberg.de/). Multiple alignment of *EsCPR* and CPRs sequences in *E. sinensis* and other species was performed using BioEdit with manual checks. A phylogenetic tree was constructed by MEGA 5.0 with neighbor-joining method against bootstrap of 1,000 times on the basis of the catalytic domain.

RESULTS

The electrophoresis result verified the integrity of total RNA extracted from various tissues of *E. sinensis* (Figure 1). A cDNA fragment of approximately 680 bp was amplified by RT-PCR using degenerate primers. The fragment exhibited high sequence identity with other known CPR sequences in the GenBank database. The full-length *E. sinensis* CPR cDNA was obtained by 5′- and 3′-RACE. This sequence was named as *EsCPR* and had been submitted to GenBank (accession number KT159167). *EsCPR* contained a 2046-bp open reading frame encoding a protein of 681 amino acids. The predicted isoelectric point and molecular mass of the protein were 4.82 and 30.7 kDa, respectively. The protein contained the hallmark of arthropod CPR, including the FMN-, FAD- and NADPH-binding domains (Figure. 2). A hydrophobic transmembrane region consisting of 22 amino acid residues was predicted at the N-terminus of protein, and no signal peptide cleavage site was found in the secondary structure of the protein, indicating that the enzyme is a cytoplasmic protein.

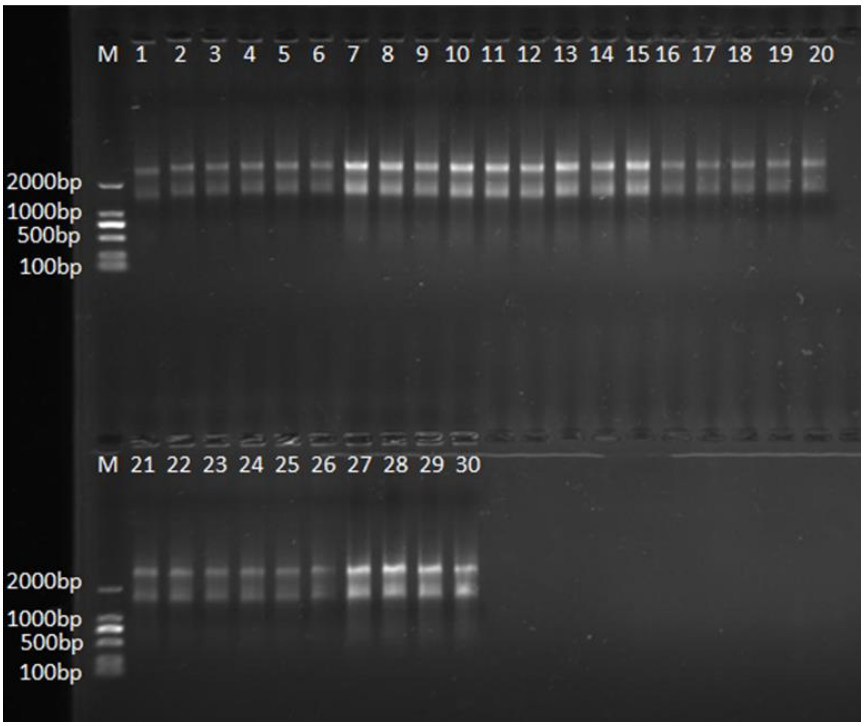


Figure 1. The electrophoretic electrophoresis results of total RNA various tissues in *E. sinensis*.1-3: heart; 4-6: Y organs; 7-9: eye stalk; 10-12: hepatopancreas; 13-15: muscle; 16-18: horacic ganglion; 19-21: cerebral ganglion; 22-24: intestines; 25-27:gills; 28-30:stomach.

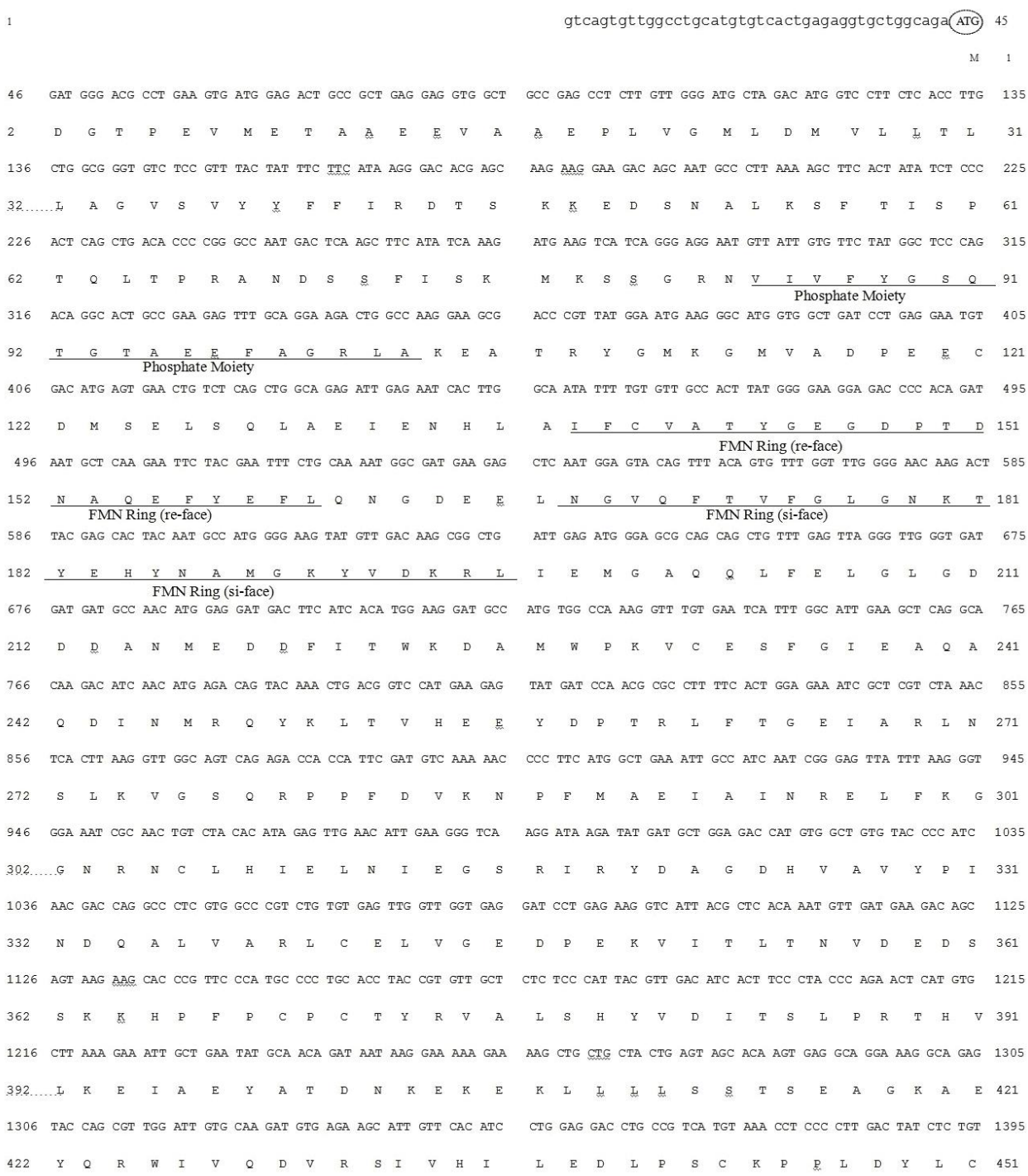
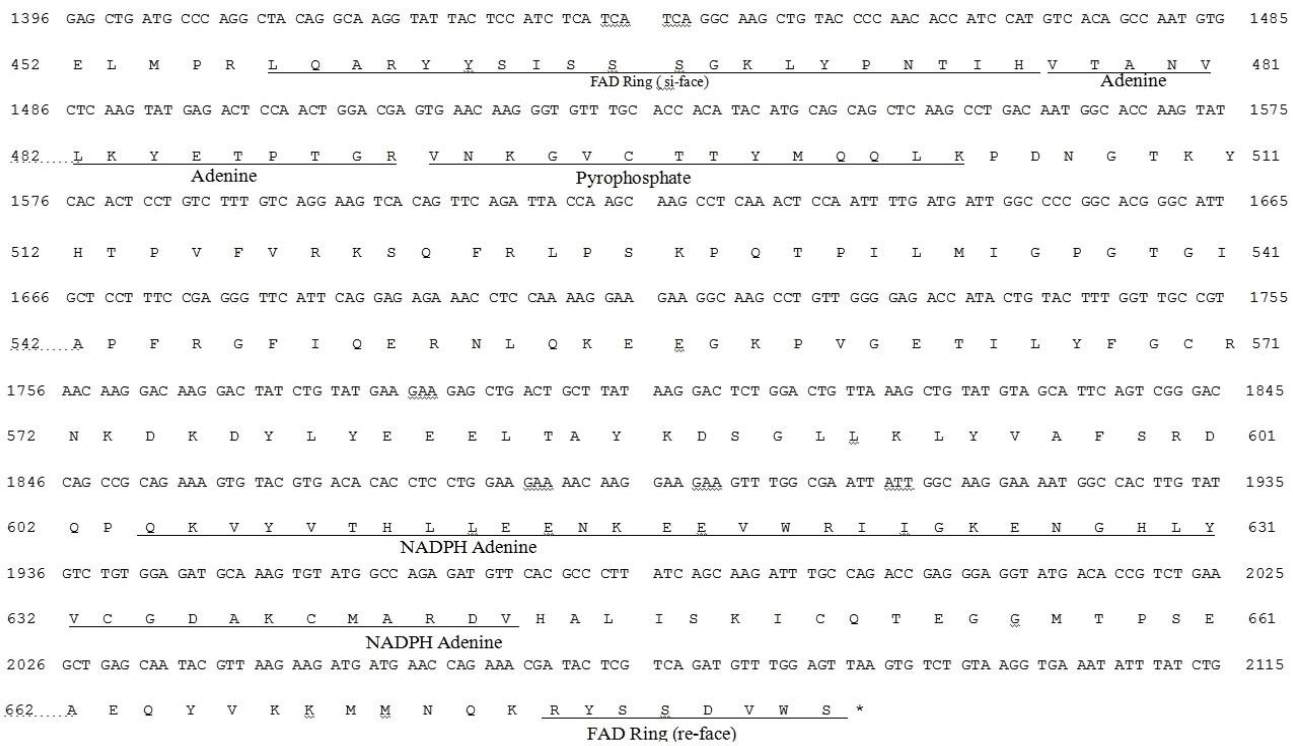


Figure 2. The nucleotide sequence and deduced amino acid sequence of *EsCPR* gene including 3′ and 5′UTR in the *E. sinensis*. The poly A sequences are underlined. The asterisk indicates the stop codon. The sequences of AATAAA as a canonical polyadenylation signal site are double underlined. The functional regions are identified and labeled.



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aattatggataagatatttaggtatgtcatgatggccagcaacagccaacactggttatgtgtgtgaggatggtggtgcttcctcacagtatgcacgcacacactcagac

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caccatgaaccacaaaaattaggtttctatggatggcaaattttcaaaaaaaaaaaaaaaaaaaaa3717

Figure 2 (continued). The nucleotide sequence and deduced amino acid sequence of *EsCPR* gene including 3′ and 5′UTR in the *E. sinensis*. The poly A sequences are underlined. The asterisk indicates the stop codon. The sequences of AATAAA as a canonical polyadenylation signal site are double underlined. The functional regions are identified and labeled.

In order to gain some insight into the relationship among the CPRs from the taxonomically diverse arthropod species, the amino acid sequences of *EsCPR* and 14 other CPRs taken from NCBI were subjected to phylogenetic analysis (Figure. 3). A neighbor-joining tree generated from the analysis showed that despite most of the proteins sharing a high level of sequence identity, they were well segregated and clustered into distinct branches. According to the constructed phylogenetic tree, species from the same arthropod phylum were grouped together with strong bootstrap supports.The mature peptide shares amino acid of *E. sinensis* identity 82 % - 89 % to the CPR from *P. vannamei* and *Chionoecetes opilio.* It can be seen that *E. sinensis*, *C. opilio* and *P. vannamei* were clustered into a single group, suggesting a much closer evolutionary relationship than with other species.

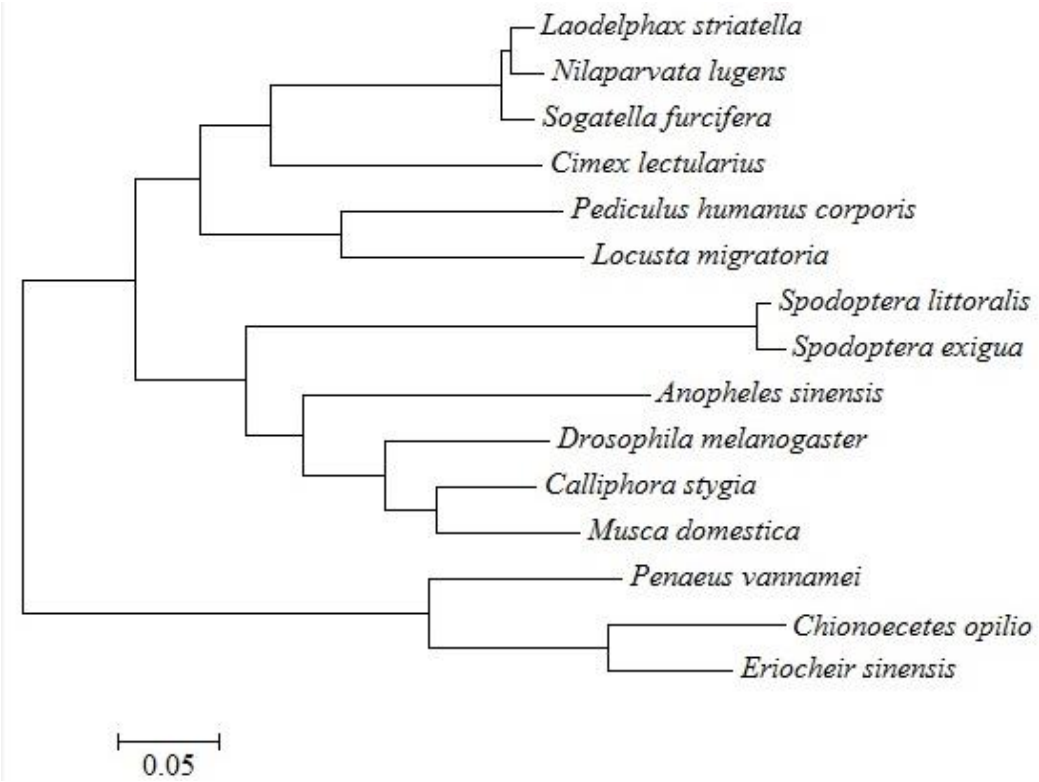

Figure 3. N-J phylogenetic tree based on CPR amino acid sequences. GenBank accession numbers are as follows: *Pediculus humanus corporis* (XM_002423935); *Eriocheir sinensis* (KT159167); *Laodelphax striatella* (KJ668698); *Sogatella furcifera* (KJ017970); *Nilaparvata lugens* (KF591574); *Calliphora stygia* (KJ702307); *Cimex lectularius* (JQ178363); *Drosophila melanogaster* (NM_057810); *Anopheles funestus* (EF152578); *Musca domestica* (NM_001286889); *Spodoptera littoralis* (JX310073); *Spodoptera exigua* (HQ852049); *Locusta migratoria* (KF984040.1); *Penaeus vannamei*(XM_027357984.1); *Chionoecetes opilio*(JACEEZ010001542.1).

Analysis of the transcript level of *EsCPR* in ten different tissues of the intermolt adult crab by the end-point RT-PCR showed that *EsCPR* was predominately expressed in the hepatopancreas and stomach, with lower levels of expression in the other tissues. Examination of the tissue-specific expression of *EsCPR* in the premoult, postmoult and intermoult stages showed that *EsCPR* was predominantly expressed during the premoult stage, with lower level of expression in the postmoult and intermoult stages (Figure. 4). *EsCPR* showed almost equally high expression in the stomach of premolt crabs as in the hepaopancreas of premolt and intermoult. *EsCPR* was almost exclusively expressed in the eye stalk of postmoult, with only minor expression in the hepatopancreas. The data indicated that the *EsCPR* was predominantly expressed in the hepatopancreas, thus a detailed analysis of the expression of *EsCPR* in the hepatopancreas at different moult stages was performed to determine if the level of the transcript would fluctuate during development.

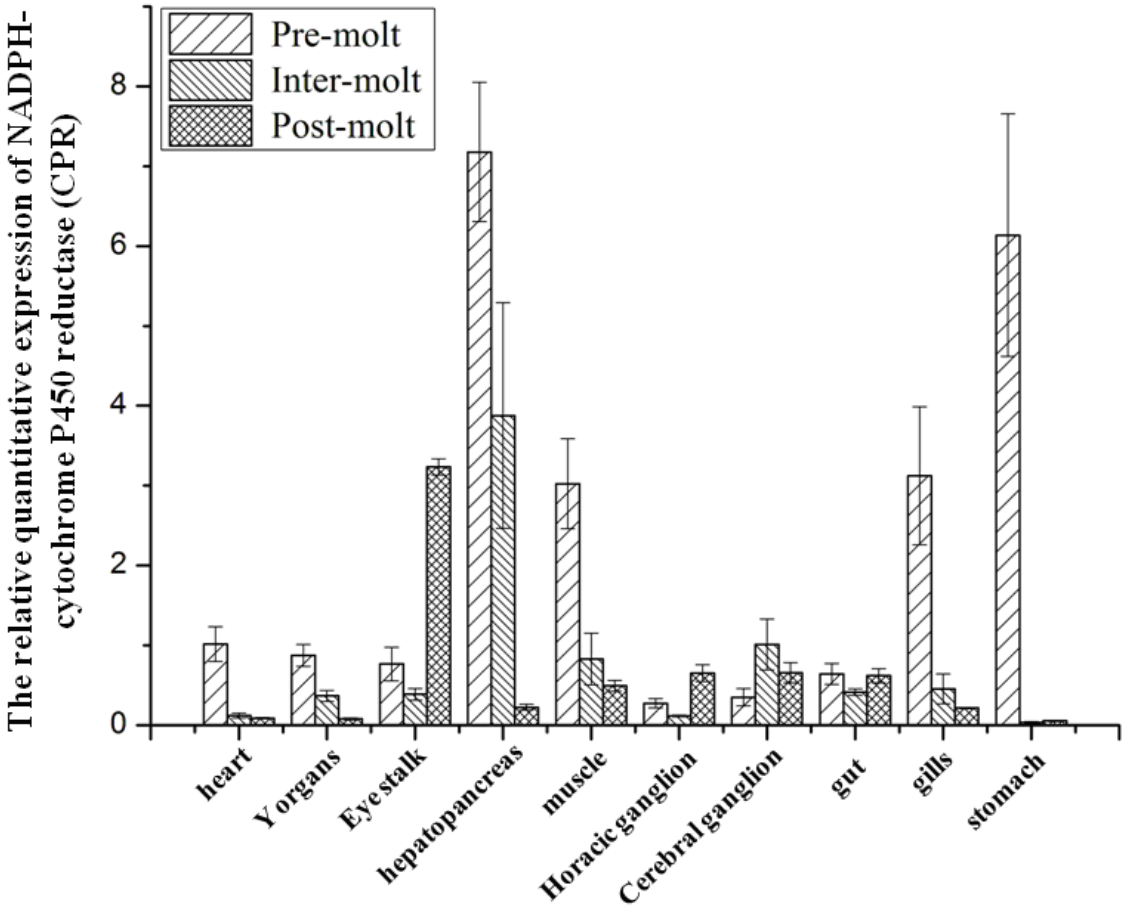

Figure 4. The relative expression of *EsCPR* in ten tissues from crabs of different molting stages.

DISCUSSION

In both vertebrates and invertebrates, xenobiotic metabolism is concentrated in hepatic-like tissues. Many mammalian CPRs that contribute to these activities are highly expressed in the liver. The identification of the CPR cDNA in *E. sinensis* not only extended the insect CPR family, but would also facilitate future functional study to investigate the interaction of the enzyme with other components of the cytochrome P450 enzyme systems.

The expression of CPR at different developmental stages in crustaceans has been rarely studied. The expression of CPR in the hepatopancreas varied across different developmental stages. The molt analysis showed a higher CPR expression level in the premolt stage than in the intermolt and postmolt stages. The expression analysis revealed that *EsCPR* participated not only in physiological growth but also in crustacen molting process.

The results demonstrated that the moulting stage of *E. sinensis* is dependent on the expression of *EsCPR* gene in the hepatopancreas and genes with similar expression patterns. As moulting is initiated by a surge of ecdysteroids, the levels of these hormones change dramatically during development. The level of *EsCPR* expression was higher in the premoult, with lower level found in the post- and intermoult stages. We have previously demonstrated that the hepatopancreas is the major site of the initial CPR-mediated reaction leading to the molting in *E. sinensis*. Published data showed that the gene is involved not only in hydrolyzing endogenous compounds in the early stage of embryogenesis[1], but also in synthesizing cuticular components throughout adult emergence[20]. The activity of P450 is dependent on CPR and a large number of individual P450s then catalyzed a many biology process in each organisms. Taken together, the finding of the present study constituted an initial effort to establish a foundation for utilizing *EsCPR* as a novel target to manage molting process in *E. sinensis*, also, it will shed more light on the understanding of the molecular basis of ecdysone and developmental regulation in crustaceans. Future studies on the inducible expression of the *EsCPR* gene by ecdysone and the potential role of *EsCPR* in the mechanism of molting are urgently needed.

Funding: This research was funded by the National Natural Science Foundation of China (#31302178), and the Scientific Research Program of Ocean and Fisheries Administration of Liaoning Province (201834).
Conflicts of Interest: The authors declare no conflict of interest.

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