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RESEARCH PAPER

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Putative Pacemakers in Crayfish *Procambarus clarkii* Show Circadian Oscillations in Levels of mRNA for Clock Genes under Different Light Rhythm

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Abstract

In the circadian rhythm mechanism, *cycle* (*Cyc*)*, clock* (*Clk*)*, cryptochrome* (*Cry*) are three core clock genes that constitute complex mechanism controlling organisms to follow a 24-hour oscillation cycle. In the present study, the major clock genes, i.e. *PcCyc*, *PcClk*, *PcCry1* were cloned from crayfish *Procambarus clarkii*. The putative functions as well as physical and chemical properties of the resolved proteins were analyzed by various bioinformatic methods. Real-time quantitative PCR technique was used to explore the mRNA expression of the *PcCyc*, *PcClk*, *PcCry1* in brain, eyestalk, and hepatopancreas tissues of *P. clarkii.* In order to examine circadian rhythm of selected clock genes in the species, the experiment was carried out under three different light conditions, namely: (a) 12 h of light and dark periods per day (12LD), (b) 24 h of constant darkness (24DD) and (c) 72 h of constant darkness (72DD). The obtained results showed essential correlation in the mRNA expression levels of *PcCyc* and *PcClk* genes under similar light conditions. It indirectly suggests that the PcCYC and PcCLK proteins might be involved in the transcriptomic regulatory mechanism of all other genes within the core-clock network. The mRNA expression levels of the *PcCry1* gene were highly variable, differing significantly in relation to the various illumination conditions and sampled tissues. The obtained results seem to follow the pattern evidencing the sensitivity of *PcCry1* gene to the light signals. It firmly supports the gene's role in the conversion mechanism of light signals into optical signals, which is engaged in the generation and control of circadian rhythmicity.

Introduction

The circadian clock is widespread mechanism observed from bacteria and fungi to plants and animals, being responsible for generation and control self-sustained oscillations of physiology, biochemistry and behavior in the course of the 24 h periodicity of day and night cycles (Young & Kay, 2001). It has been confirmed that circadian clock genes regulate organism's homeostasis, including: inflammation responses, blood sugar balance, synthesis and catabolism (Sancar *et al.*, 2015), etc. The biological foundation of circadian clock rhythm relies on the positive feedback loop of protein factors, such as CLK/BMAL1 and the negative feedback loop that depends on protein factors, such as CRY. It was evidenced that alike clock genes as: *Period* (*Per*), *Timeless* (*Tim*) and *pigment dispersing factor* (*pdf*) also regulate the mechanism. Generally, the circadian rhythm mechanism consists the central clock and the peripheral clocks of organization, which together maintain biological cycles (Lee *et al.*, 1998; Rudic *et al.*, 2004; Fuhr *et al.*, 2015). So far, the extensive studies on clock genes in *Drosophila* have provided many important information on its biological regulating

effects (Plautz *et al.*, 1997). Accordingly, in the *Drosophila* system the *clock* (*Clk*) and *cycle* (*Cyc*) genes produce proteins CLK and CYC that form CLK-CYC heterodimers, which enter the nucleus as a transcription factor, and bind to the E-box region (CACGTG) in the promoter site of downstream *Per* and *Tim* genes. The transcription of *Per* and *Tim* genes is activated, and the product proteins PER and TIM monomers accumulate in the cytoplasm and form PER-TIM heterodimer, then enter the nucleus and bind to CLK-CYC. As a result, CLK-CYC cannot bind to the E-box region, thereby suppressing the transcription of *Per* and *Tim* genes and forming a complete Per/Tim feedback loop (Beaver & Giebultowicz, 2004).

In vertebrates the *Arntl* gene, sometimes called *Bmal1* (brain and muscle ARNT-like protein 1) is known as homologous of *Cyc* gene, being considered as one of the most important core transcription factors regulating the circadian clock. The gene encodes BMAL1 protein, which is classified as bHLH/PAS transcription factor (Ikeda & Nomura, 1997; Zhang *et al.*, 2013). In turns, the *Clk* gene is the first cloned circadian clock gene in mammals, which has laid the foundation to consecutive studies (King *et al.*, 1997). Both CLK and Bmal1 proteins are members of the basic helix-loop-helix (bHLH)/PER-ARNT-SIM (PAS) super family of transcription factors. Moreover, *Clk* and *Bmal1* genes show high similarity, encoding both a DNA-binding and protein dimerization domains, which are strongly conservative. Another important core clock gene *Cry* was firstly found in plants, but further studies evidenced that it has homologues in various animals (Sancar, 2003). The encoded by *Cry* gene, the blue/UV-A absorbing photo protein, called as cryptochrome (CRY), which beside its transcription repression function is known as an photoreceptor that directly modulates light input into the circadian clock (Stanewsky, 2002; Hoang *et al.*, 2008; Song *et al.*, 2007; Heyers *et al.*, 2007; Ozturk *et al.*, 2011). Subsequent studies found two types of cryptochrome genes (*Cry1* and *Cry2*) in crustaceans, which suggests biological clock in crustaceans is more similar to that in butterflies than in fruit flies (Tilden *et al.*, 2011).

Up to date, many clock genes have been identified or their expressional patterns and functions have been detected in crustaceans. For instance, Zhang *et al.* (2013) cloned the clock genes of *Eurydice pulchra*, and found that the tidal rhythm is driven by a special circadian pacemaker. Chesmore *et al.* (2016) discovered that the clock genes in *Homarus americanus* are highly conserved. In turns, O'Grady *et al.* (2016) reported the sequence of major genes in the circadian clock of the *Talitrus saltator* and identified that the clock-related genes showed temporal changes in expression. It was found that in *Daphnia pulex* genes of many functional groupings, e.g. related to immunity, oxidative detoxification and sensory process exhibit 24 h rhythms in their expression patterns under diel conditions (Rund *et al.*, 2016).

Multiple endogenous and exogenous factors, such as: environmental temperature, light condition and physiological status, usually regulate the fluctuation of circadian clock gene transcript levels (Boothroyd *et al.*, 2007; Gegear *et al.*, 2008; Yan *et al.*, 2013). Photoperiodism is the length of light or dark period per day, which has been identified to be related to varieties of associated physiological states including diapause, seasonal morphs and growth rate in many insects (Saunders, 2010). It is widely accepted that a circadian clock is involved in the photoperiodic response (Goto, 2013). Fanjul-Moles and colleagues (2004) assumed that circadian rhythm of *P. clarkii* is regulated by blue light through the hypothetical additional retinal photoreceptors. Intriguingly, biochemical analyses showed that the circadian physiological functions of PER, TIM and CLK proteins and their composition in the cytoplasm and nucleus of the retina and optic lobes varies in time according to diurnal oscillations (Escamilla-Chimal *et al.*, 2010). In addition, sensitivity of rhodopsin in the retina of *P. clarkii* to light can cause changes in rhythm, which in turn leads to an increase or decrease in glucagon (CHH).

Based on the previous researches, we know that the core clock proteins (CLK, CYC, TIM, PER) interact in the brain, regulating the diel rhythm of *P. clarkii*. The light signals are received through the tissue of eyestalk in *P. clarkii*, which regulates the transcription of CRY protein, and then participates in regulating the clock network. In the crayfish *P. clarkii*, circadian pacemaker localization has been proved to be in the protocerebrum of the cerebroid ganglion (Escamilla-Chimal *et al.*, 2010; Nelson-Mora *et al.*, 2013). The sinus gland was found to be located in the eyestalk and formed by the axonal assembly of a specific neurons group that constitutes the X-organ (García & Aréchiga, 1998). The most relevant source of the core biological clock control is attributed to the X-organ-sinus gland and brain tissue of circadian pacemaker. Therefore, the eyestalk and brain are both central clock tissues, which receive and process external signals, playing a central role in the regulation of biological clock genes. It is known that the central circadian clock synchronizes the peripheral clocks in the tissues of hepatopancreas and cardiac neuromuscular system (Christie *et al.*, 2018).

The red-swamp crayfish *Procambarus clarkii* is an important freshwater economic species in aquatic products market of China (the total annual production in 2019 is 1.638,700 million tons, market value is ~54.92 billion dollars). In the process of aquaculture, clarifying the regularities of behavior, physiology, feeding and reproduction of economic species and their molecular background can provide scientific elaboration of sustainable aquaculture models, for instance, for better food intake, decrease of aggressive behaviour, cannibalism, etc. Although clock genes have been studied thoroughly in model animals, the regulatory mechanism in aquatic crustaceans is still unclear. Since the discovery of clock genes in crustaceans, most of

carried out research have focused mainly on the mechanisms maintaining rhythmic oscillations and their effects on the rhythm of life activities (Fanjul-Moles & Prieto-Sagredo, 2003; Grabek & Chabot, 2012; Manfrin *et al.*, 2015). Here we cloned full-length or partial cDNA of *Cyc*, *Clk*, and *Cry1* (denoted as *PcCyc*, *PcClk*, and *PcCry1*) and investigated the expression patterns of the circadian clock genes in different tissues (brain, eyestalk, and hepatopancreas) from *P. clarkii* as well as under three different lighting conditions (12LD, 24DD, 72DD).

Materials and Methods

Ethics Statement

Animal welfare and experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Normal University [SOXR (Jiangsu) 2015–028]. The authors confirmed that animals did not suffer unnecessarily at any stage of experiments in this study.

Animals and Experimental Design

All the adult males of *Procambarus clarkii* used in this study were field-collected from Xuzhou City, Jiangsu, China. The healthy crayfish were acclimatized for two weeks in the laboratory aquaria with adequate aeration at 25°C in a 12 h of light and dark regimes of daily photoperiod. All specimens were fed with commercially crayfish food with daily dosage 3% of specimens biomass. The aquaria were equipped with polyvinyl tubes simulating burrows, which allowed the crayfish to hide from the light. After acclimatization, the crayfish were divided into three groups. The first group was directly used for examination after two weeks of acclimation. The second and third group (N=63 per each group) was exposed to continuous darkness (DD) for 24 h and 72 h, respectively. At the end of each treatment, nine specimens from each group were randomly selected at seven different times (Day1, 7:00 am, 11:00 am, 15:00 pm, 19:00 pm, 23:00 pm. Day2, 3:00 am, 7:00 am). All samples from each group at different time points and from different tissues (brain, eyestalk, and hepatopancreas) of *P. clarkii* were preserved in RNA*later* RNA Stabilization Reagent (QIAGEN, Germany) after dissection. The tissue samples were cut into tiny particles (less than 0.2 mm thick) and placed in separate tubes, then kept overnight at 4°C and finally stored at -20°C before total RNA isolation.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the all collected tissues of *P. clarkii* using TransZol^{TM} Up Plus RNA Kit (TransGen, Beijing, China) according to the product manual. RNA purity was verified by measuring the absorbance at 260 and 280 nm by a NanoDrop ND2000C spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA integrity was checked by electrophoresis on 1.0% agarose gel. The cDNA for each sample was synthesized from the same amount of total RNA (0.5 μg) by PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) following the manufacturer's protocol. Finally, the obtained cDNAs were stored at -80˚C until further processing.

Cloning of Full-length cDNAs of *PcCyc*, *PcClk* and *PcCry1*

The full-length *PcCyc* and *PcCry1* cDNA, partical *PcClk* cDNA were derived by using the rapid amplification of cDNA ends (RACE) method. The partial cDNA sequences of *Cyc*, *Clk*, and *Cry1* was used to design degenerate primers, based on the nucleotide sequence data available for *P. clarkii* in the NCBI GenBank (Accession code: SPR044128) (Shen *et al.*, 2014).

The PCR reactions were conducted using first-strand cDNA from the eyestalk tissue as template. The PCR program included denaturation stage at 95°C for 5 min, following 29 cycles of annealing at 95°C for 30 s and elongation at 62°C for 30 s, then the final extension stage was carried out at 72°C for 1 min. The PCR reactions were completed an additional extension stage at 72°C for 10 min. The product was evaluated by 1.0% agarose gel electrophoresis to exclude the presence of nonspecific amplification and primer dimers.

Sequence and Phylogenetic Analysis

Bioinformatics analyses of the obtained *PcCyc*, *PcClk*, and *PcCry1* gene sequences were performed using the BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/) on National Center for Biotechnology Information (NCBI) to verify the full-length integrity of cDNA sequences. The online ORF Finder (http://ncbi.nlm.nih.gov/gorf/gorf.html) was used for finding open reading frames of the analyzed clock genes. The deduced proteins domains were analyzed using SMART online analysis programs (http://smart.embl-heidelberg.de/) and Motif Scan online server (https://myhits.isb-sib.ch/cgi-bin/motif_scan) (Hulo *et al.*, 2008). The SignalP 4.1 online server (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen & Krogh, 1998; Petersen *et al.*, 2011) was used to locate the signal peptide of the CYC, CLK and CRY1 proteins. The PSIPRED online server (http://bioinf.cs.ucl.ac.uk/psipred_new/) was used to resolve secondary structure of each protein. The three-dimensional (3D) structure of PcCYC, PcCLK, and PcCRY1 were constructed using SWISS-MODEL (https://swissmodel.expasy.org/).

Quantitative Real-time PCR

One microgram of previously isolated RNA was used to synthetize the first-strand cDNA with the

Primescript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). Next, one microliter (0.5 µg/µL) of 10-fold diluted of amplified cDNA template was used for quantitative real-time PCR. The RT-qPCR reactions were performed in a total of 20 µL reaction mixture using SYBR® *Premix Ex Taq*™ II kit (TaKaRa, Japan). The RT-qPCR conditions were as follows: a pre-denaturation stage at 95°C for 10 min, followed by 40 cycles of quantification stage: 95°C for 15 s and 60°C for 1 min as well as melting curves analysis stage: 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The relative expression level of target gene was analyzed by compare Ct ($2^{-\Delta\Delta Ct}$) method (Livak & Schmittgen, 2001). The reactions and samples were carried out in triplicate and the expression levels were normalized to that of *β-actin* reference gene. All the primers used for RT-qPCR were shown in Table 1. RT-qPCR data (see data in Appendix_data_file_S1) were analyzed using SPSS software 13.0 (SPSS Inc., Chicago, IL, USA) with one-way analysis of variance (ANOVA) and Duncan's multiple comparative analysis. For each value category the mean ± standard deviation (SD) values were reported. The weightiness of observed differences in expression levels were determined by applied statistical tests, assuming the significance criterion at α=0.05.

Results

Cloning and Sequence Analysis

One fragments (312 bp) was amplified by 5'-RACE technique from the 5' end of each cDNA and several other fragments (1,185 bp and 1,129 bp) were amplified by 3'-RACE technique. The full-length cDNA of the studied genes (nucleotide and amino acid sequences shown in Appendix_data_file_S1) was verified by BLAST from the National Center for Biotechnology Information (NCBI). The sequence data indicated that the partial *PcCyc* cDNA contained a 143 bp 5'-UTR and 2,010 bp open reading frame (ORF), encoding 669 amino acids. The ORF contained a typical bHLH/PAS transcription factor structure. Additionally, the translation starting codon was determined as ATG (located at nucleotide 144-146) and stop codon was identified as TGA. The GC and the AT contents in the *PcCyc* was 43.5% and 56.5%, respectively (Supplementary Figure S1). The Blastn search in NCBI showed a nucleotide homology similarity at the level of 75%-93%. The resulted *PcCyc* cDNA sequence was deposited in the NCBI GenBank under accession numbers MN908587 and MN939545. The *PcClk* cDNA was 1,940 bp in length and contained a 183 bp of the 3'-UTR. The open reading frame (ORF) contained 1,182 bp which encoding 393 amino acids. The ORF of the *PcClk* gene also contained a typical bHLH/PAS transcription factor structure. The identified the start codon was ATG, the stop codon was TAA (located at 1180-1182) and the obtained *PcClk* gene sequence has an AT content of 54.8% and GC content of 45.2%. The NCBI Blastn nucleotide homology similarity interval was 74%-98% (Supplementary Figure S2). The *PcClk* cDNA sequence was deposited in the GenBank under accession number MN908585. The full-length cDNA sequence of *PcCry1* was composed of 3,366 nucleotides, including a 5'-UTR of 116 nucleotides, an open read frame of 1,650 nucleotides, and a 3'-UTR of 240 nucleotides. The ORF encodes 393 amino acids, and the start codon was ATG (located at 117-119), while the stop codon was TGA (located at 1,767-1,769). The AT content of the *PcCry1* gene sequence was 55.7%, while the GC content was 44.3% (Supplementary Figure S3). The complete *PcCry1* cDNA sequence was deposited in the GenBank database (accession no. MN908586).

Phylogenetic Analysis

The phylogenetic tree was constructed based on CYC and CLK protein homologous sequences using maximum likelihood method. The ML tree revealed two major subgroups; one branch comprised insects (marked in yellow), while the second one included crustaceans. Interestingly, the tree showed that the *PcCyc* gene was clustered with *Cyc* genes of crustaceans, being very related to *Cyc* gene of *Pacifastacus leniusculus*, with an amino acid similarity of 93% (Figure 1). The present result supported wide-known phylogenetic relationship, clustering of the crustaceans CLKs into two separate monophyletic clades. The PcCLK was most related to CLK of *Pacifastacus leniusculus* (Figure 2). The PcCRY1 was clustered with two CRY1 proteins of other crustaceans. More specifically, it was most closely related to *Euphausia superba* CRY1, being placed in the joint group together with sequence of *Hyalella azteca* among all analyzed crustaceans' sequences (Figure 3).

Table 1. Primers used for real-time quantification PCR detection

Primer	Sequence (5'-3')	Purpose
qβ-actin-F	GCACCATCCACCATGAAGATTA	qRT-PCR
qβ-actin-R	CGTGAAAGGGAAGCCAAGATG	qRT-PCR
qCycle-F	CYGATGTTCCACAGGGACTTAC	qRT-PCR
qCycle-R	GAATCGGCCTCCTCTTTCAA	qRT-PCR
qClk-F	AAGATGGAGTGCTGGACTTTAG	qRT-PCR
qClk-R	GCATAAGGGAGCTCTGAAACAA	qRT-PCR
qCry-F	AGTCAGTGCAACGAGCTGCGAAAT	qRT-PCR
qCry-R	ACGTGGAGGTGATGGGTAGTGGTC	qRT-PCR

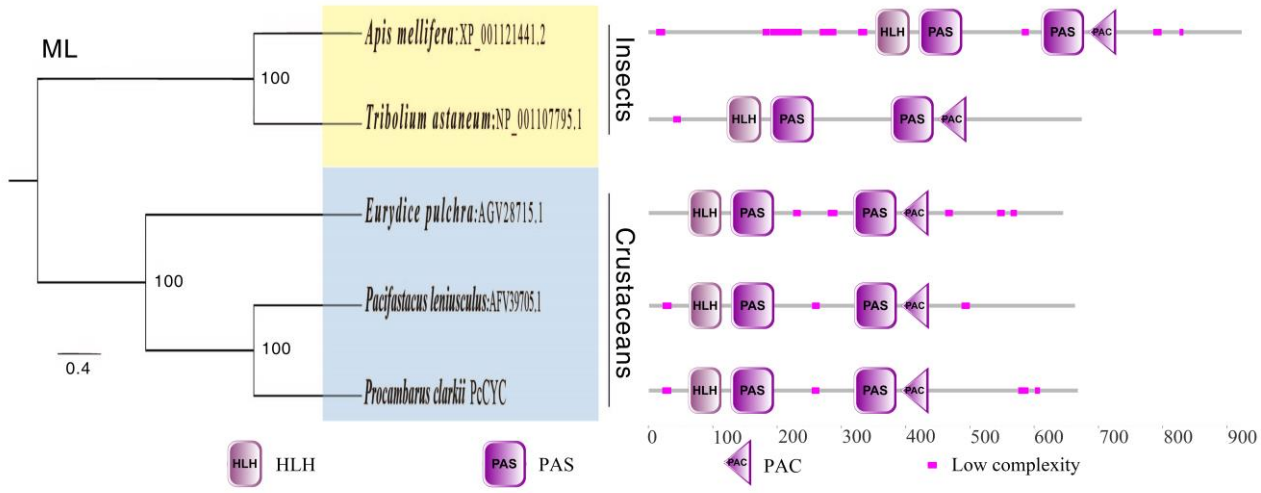

Figure 1. Phylogenetic analysis of CYC and its homologs. The values on the tree nodes are Neighbor-Joining bootstrap values and different branches are covered with specific colors. The domain structures of CYC are shown and their motifs are indicated by separate colors and shapes.

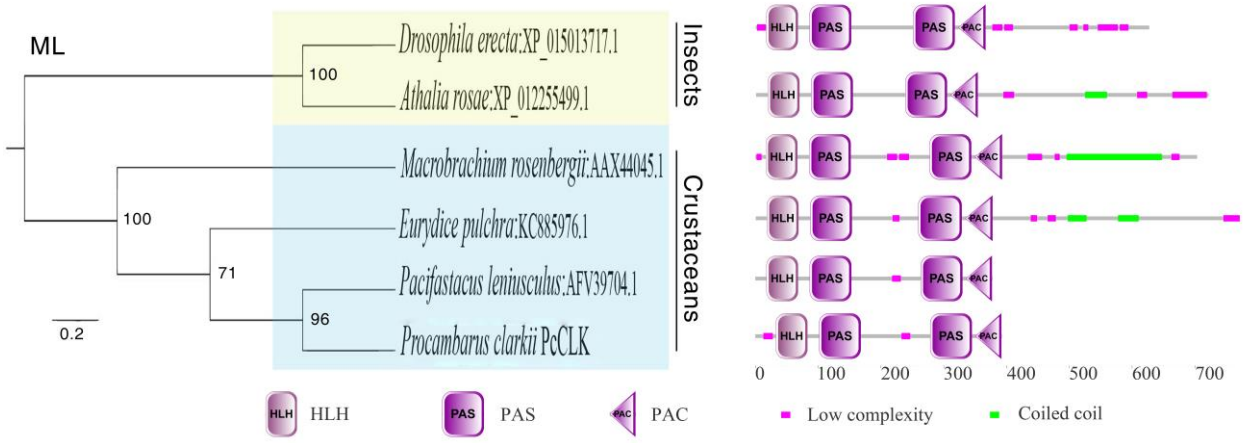

Figure 2. Phylogenetic analysis of CLK and its homologs. The values on the tree nodes are Neighbor-Joining bootstrap values and different branches are covered with specific colors. The domain structures of CLK are shown and their motifs are indicated by separate colors and shapes.

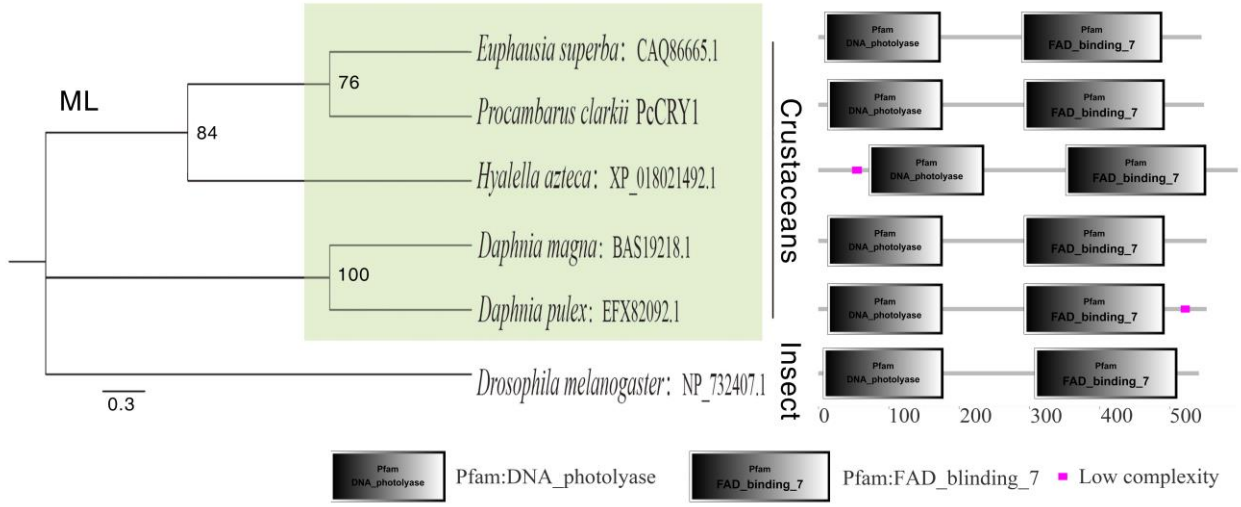

Figure 3. Phylogenetic analysis of CRY1 and its homologs. The values on the tree nodes are Neighbor-Joining bootstrap values and different branches are covered with specific colors. The domain structures of CRY1 are shown and their motifs are indicated by separate colors and shapes.

Structural Analyses

Protein motif domains scanning from SMART diagram database indicated that both PcCYC and PcCLK proteins contain a BHLH/PAS domain complex, which includes one helix-loop-helix (HLH) and two Per-Arnt-Sim (PAS) domains. In turns, two domains were identified in the PcCRY1 protein. One was PHR (photolyaserelated), the homologous sequence of N-terminal and photolyase, which was a region that binds to FMN (flavin mononucleotide). The second one was the C-terminus, which had no obvious homologous sequence with the known protein region and was a binding region with other proteins. Motif search results indicated that the PcCYC protein may contain 19 casein kinase II (CK2) phosphorylation sites, 14 protein kinase C (PKC) phosphorylation sites, 12 myristoylation sites, etc. The PcCLK protein may contain five casein kinase II (CK2) phosphorylation sites, and seven protein kinase C (PKC) phosphorylation sites, etc. Moreover, the PcCRY1 protein may include the following functional motifs: 7 casein kinase II (CK2) phosphorylation sites, 8 protein kinase C (PKC) phosphorylation sites, and 4 myristoylation sites, etc. The three-dimensional structure analysis of the PcCYC revealed that it contains 11 α helices, 12 β-sheets, and 24 irregular curls. The 3-D structure of the PcCLK includes 8 α helices, 13 β-sheets, and 22 irregular curls. The PcCRY1 consists of 18 α helices, 6 β-sheets, and 25 irregular curls (Supplementary Figure S4 A-C).

The mRNA Expression Patterns of the *PcCyc*, *PcClk* and *PcCry1* in Different Tissues

Under three lighting conditions, we investigated the mRNA expression patterns of the *PcCyc*, *PcClk*, and *PcCry1* in different tissues (brain, eyestalk, and hepatopancreas) of *P. clarkii* by RT-qPCR technique. It can be seen that no matter how the light changes, the *PcCyc* gene basically follows the 24-hour rhythm oscillation in eyestalk and hepatopancreas, and the rhythm oscillation in brain was broken with the change of the photoperiod. In this study, under the photoperiod condition of 12LD per day, the *PcCyc* gene in brain and eyestalk basically had a similar expression pattern, but under the continuous darkening condition for 24h (24DD), this regularity was disrupted. Under the 24DD condition, the peak of the *PcCyc* gene expression in the eyestalk was 4 hours earlier than that under 12LD condition. As for 72DD, the rhythm oscillation recovered, but it was slightly different from the rhythm of 12LD (Figure 4).

The results of RT-qPCR showed that the *PcClk* gene basically follows a 24-hour rhythm oscillation in the brain, eyestalk and hepatopancreas in any of three lighting conditions, while the 24DD rhythm oscillation in the brain deviates slightly. Under the 12LD condition, the expression levels of the *PcClk* gene in brain and eyestalk were similar, but the similarity was changed under 24DD continuous darkening. However, the expression of the *PcClk* mRNA was oscillated under the

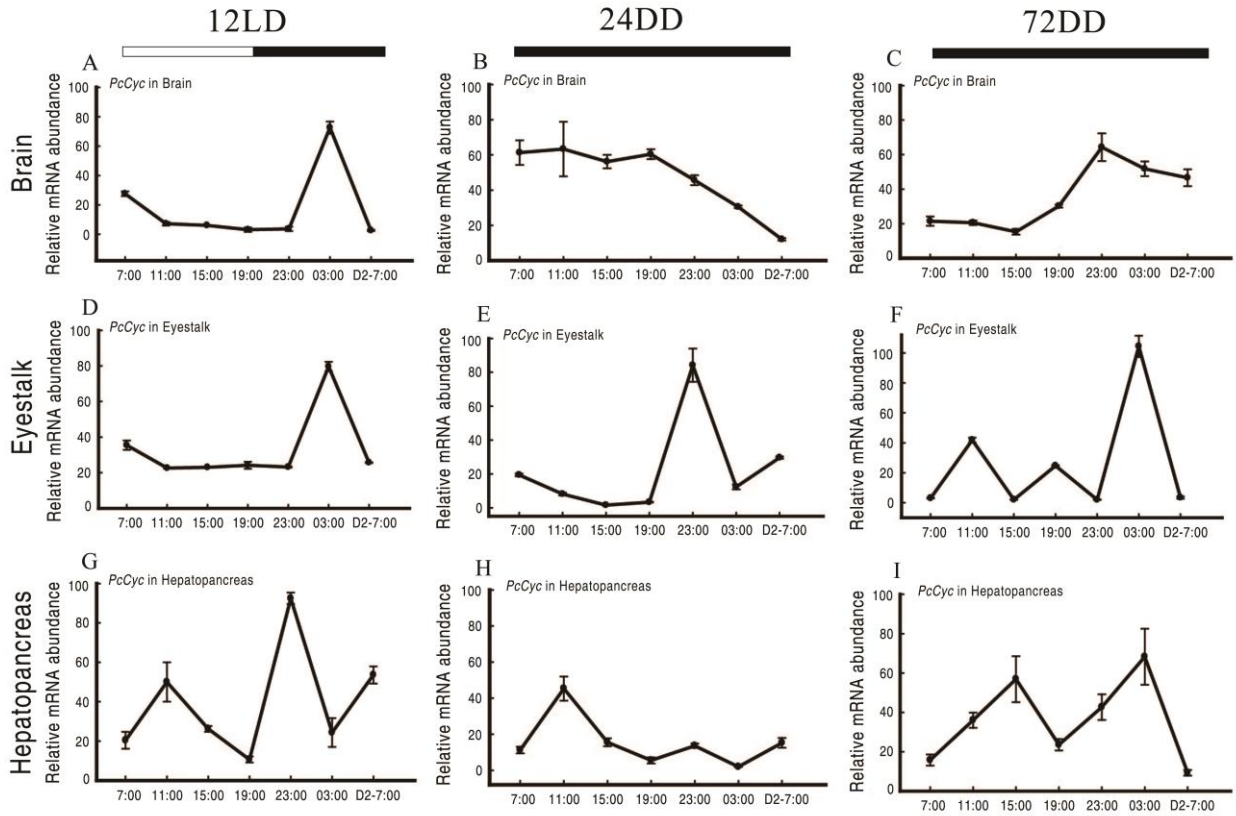

Figure 4. Temporal profiles of the *PcCyc* mRNA expression in the brain, eyestalk, and hepatopancreas of *Procambarus clarkii* in 12LD (A, D, G), 24DD (B, E, H), and 72DD (C, F, I). D2-7:00, the second day 7:00 am.

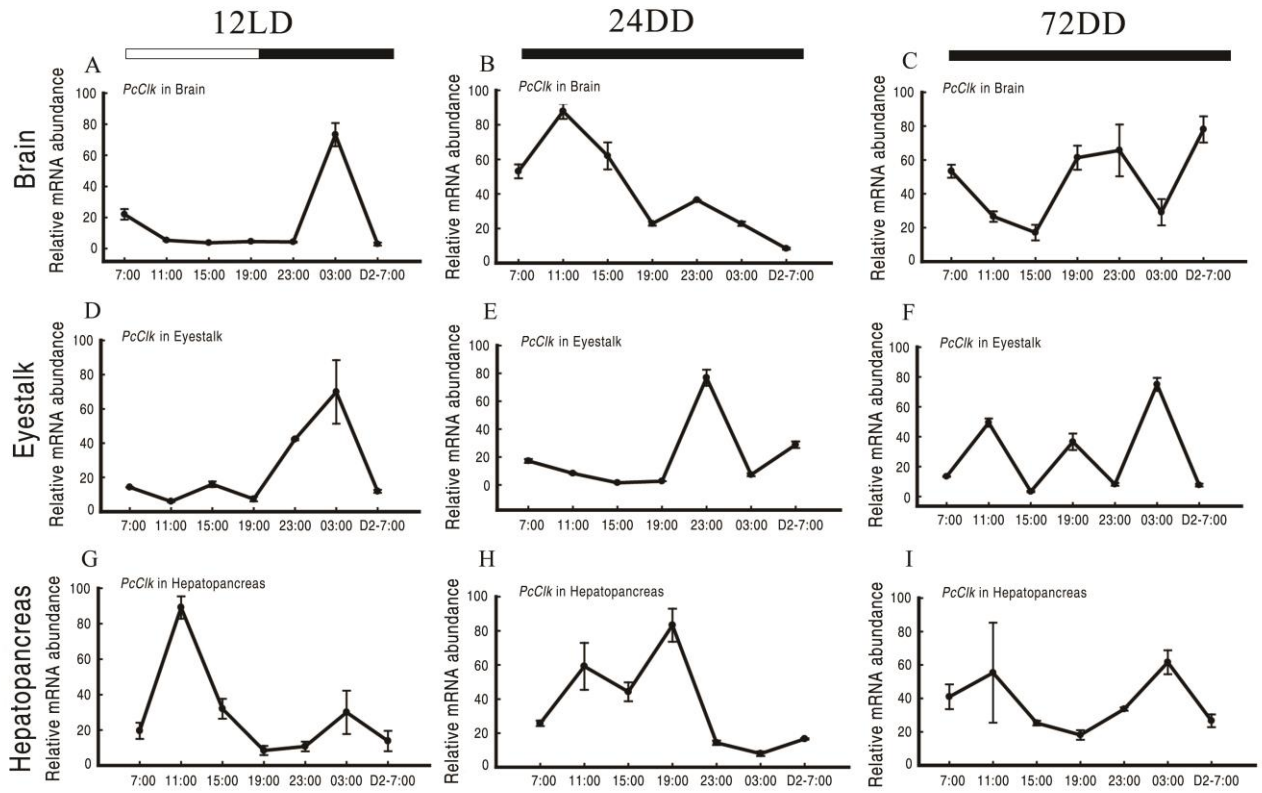

Figure 5. Temporal profiles of the *PcClk* mRNA expression in the brain, eyestalk, and hepatopancreas of *Procambarus clarkii* in 12LD (A, D, G), 24DD (B, E, H), and 72DD (C, F, I). D2-7:00, the second day 7:00 am.

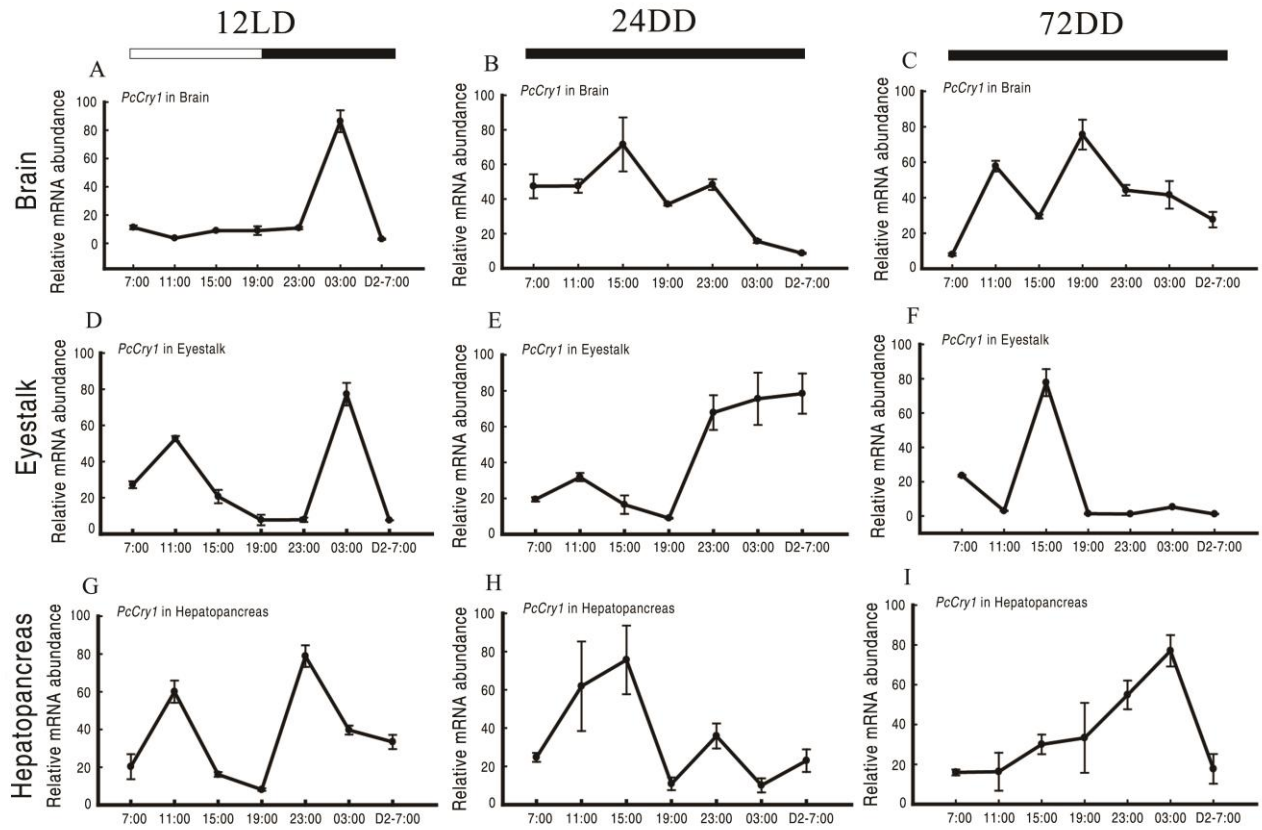

Figure 6. Temporal profiles of the *PcCry1* mRNA expression in the brain, eyestalk, and hepatopancreas of *Procambarus clarkii* in 12LD (A, D, G), 24DD (B, E, H), and 72DD (C, F, I). D2-7:00, the second day 7:00 am.

72DD condition. In the eyestalk of 12LD, the significant peak of the *PcClk* mRNA accumulation appeared at 3:00 am. In 24DD, the peak of expression was 4 h ahead of 12LD. Under the 72DD condition, there were three significant peaks of the *PcClk* gene expression. But they all basically follow the 24-hour rhythm oscillation. The expression of the *PcClk* mRNA in hepatopancreas showed a peak delay in 24DD compared with 12LD conditions. In 72DD, the law of rhythm oscillation of 12LD was restored (Figure 5). The mRNA expression levels of *PcCry1* gene among sampled tissues basically followed the 24-hour oscillating rhythm under the illumination conditions of 12LD and 72DD (Figure 6). Under the 12LD photoperiod the expression peak of the *PcCry1* gene in brain was detected at 3:00 am (Figure 6A). However, there was confusion in 24DD, and then it gradually showed stability in 72DD. In the eyestalk (Figure 6D), it can be seen that there is a significant peak of the *PcCry1* gene expression at 3:00 am and 11:00 am under 12LD. In 24DD, the rhythm was completely disturbed then the expression level of the *PcCry1* gene tended to be stable, and there was a peak at 11:00 am. As for hepatopancreas, the first peak appeared at 11:00 am and the second one appeared at 23:00 pm in 12LD. When the light conditions changed to 24DD, the expression of the *PcCry1* gene was chaotic and then took stable expression pattern under 72DD condition, where the only one peak appeared at 3:00 am. This indicates that the expression of the *PcCry1* gene ran counter to the 24-hour oscillation rhythm in brain and eyestalk under 24DD illumination conditions.

Discussion

In the present study, we cloned full-length cDNA sequence of *cycle* and *cryptochrome* genes, and partial cDNA sequence of *clock* gene from the red-swamp crayfish *Procambarus clarkii*. Our analysis revealed the mRNA expression patterns of the examined genes in different tissues of *P. clarkii* and under various diel illumination conditions. The mRNA expression levels of the *PcCyc*, *PcClk*, and *PcCry1* were rhythmic and analogous in LD cycle in the brain and eyestalk of *P. clarkii*. One significant peak in the *PcCyc* transcription level during the day in *P. clarkii* was the same as that pattern of expression from the commonly observed unimodal in the whole bodies or selected organs of insects, e.g., mosquitos (Gentile *et al.*, 2006) and aphids (Cortes *et al.*, 2010).

We recorded diel rhythms of the *PcCyc* mRNA levels in different photoperiods, where under the 24h period of constant darkness (24DD) expression of the *PcCyc* rhythmic oscillation were preserved, except from the brain tissue and the expression of the *PcCyc* rhythmic oscillation resumed running in 72DD. Under the condition of 72DD, the oscillation rhythm recovers, but it was different from the rhythm law of 12LD. We inferred that *P. clarkii* has an endogenous clock with a daily rhythm in the *PcCyc* mRNA levels. Similar expression rhythm pattern of *Cyc* has been reported for *Acyrthosiphon pisum* (Cortes *et al.*, 2010), *Thermobia domestica* (Kamae *et al.*, 2010), and *Aedes aegypti* (Chahad-Ehlers *et al.*, 2013). Under 12LD condition, the expression of the *PcClk* had a 24-hour amplitude rhythm in all three tissues. Despite scare state of knowledge on *Clk* genes in crustaceans, it can be spotted that *Clk* mRNA abundance was not only found in the nervous system but also some surrounding tissues of *Macrobrachium rosenbergii* (Yang *et al.*, 2006). Nevertheless, no rhythmic expression of either *Clk* or *Cyc* was found by characterizing transcriptional activity of clock genes in different organs of *Eurdice pulchra* (Zhang *et al.*, 2013). The levels of *Clk* and *Cyc* mRNA oscillate over the day in whole *Daphnia pulex* bodies (Bernatowicz *et al.*, 2016). However, the rhythm was disrupted in 24DD. It has been suggested that the rhythm of the crayfish circadian outputs is coordinated by a neuro-endocrine feedback loop established between sinus gland and protocerebrum (Hernández &Fuentes-Pardo, 2001; Nelson-Mora *et al.*, 2013).

As the relative mRNA abundance result showed, the *PcCyc* and *PcClk* were approximately phase congruent. Similar expression patterns of *Cyc* and *Clk* was observed in the heads of the mosquitos *Aedes aegypti*, *Anopheles gambiae*, and *Culex quinquefasciatus* (Gentile *et al.*, 2009; Ptitsyn *et al.*, 2011).

Escamilla-Chimal *et al.* (2010) used antibodies to detect the expression of CLK protein in different tissues under different illumination conditions. The results revealed that CLK protein showed peak protein expression at 12:00 am in eyestalk tissue under 12LD light conditions. In 24DD light conditions, protein expression peaks at 16:00 pm in brain tissue. As for the feedback system, the CYC and the CLK proteins form a heterodimer to function together, so it was speculated that these two genes might have similar mRNA expression levels (Escamilla-Chimal *et al.*, 2010). Accumulation of the clock gene proteins was delayed by 6-8 hours than mRNA production (Price *et al.*, 1998; Kloss *et al.*, 2001). Together, our results on the mRNA expression levels of the *PcCyc* and *PcClk* genes of *P. clarkii* in eyestalk were consistent with it. However, compared to in eyestalk, there was a four-hour delay in the rhythmic expression of the two genes in brain, the reason for that needs further research. It was noteworthy that the protein accumulation of the *PcCyc* and *PcClk* genes in brain was basically consistent with the rule of protein accumulation delay of 8 h under 24DD and 72DD conditions. This results support the hypothesis on the formation of heterodimers between CYC protein and CLK protein in the central rhythmic oscillatory tissue; however, further studies are still needed.

In the brain and eyestalk of *P. clarkii*, the oscillations of mRNA expression of the *PcCyc* and the *PcClk* genes in the 24DD condition were disrupted compared to the condition of 12LD, while the rhythm

oscillation recovered at 72DD, but it was different with the previous 12LD rhythm pattern. The hepatopancreas tissue in *P. clarkii* belongs to the peripheral oscillator cycle. Delayed phenomenon occurred at the peak period under 24DD conditions, and then the rhythmic oscillation law of 12LD was restored under 72DD illumination conditions. The hepatopancreas mainly regulates the fluctuation of the hormone level in organism, and participates in the regulation of the physiological and biochemical response of the body (Duka & Ahearn, 2014). As the changes in the environment, the clock genes make corresponding regulatory changes to response. So as time goes on, slowly restore the original rhythmic oscillations, and organism adapts to changes in the environment. Furthermore, we found high fluctuations in brain tissue, demonstrating that the clock genes *PcCyc* and *PcClk* have significant endogenous free-running rhythms and are subjected to brain dominance. Similarly, the mammalian's "central clock" of the circadian rhythm control is located in the hypothalamic supraoptic nucleus (SCN) (Coelho *et al.*, 2015). The 24-hour rhythm oscillation cycle of clock genes in mRNA expression levels was also found in *E. pulchra* (Zhang *et al.*, 2013), but not observed in *M. rosenbergii* (Yang *et al.*, 2006).

Contrastingly to eyestalk tissue, Fanjul-Moles *et al.* (2004) detected different CRY protein circulation rhythm in *P. clarkii* brain tissue under different light conditions by application of *Drosophila* polyclonal antibody detection method. In the present study the *PcCry1* gene had a peak expression at 3:00 am, but Fanjul-Moles and colleagues (2004) measured that CRY protein had a peak expression at 19:00 pm, which was not consistent with the clock gene expression to protein accumulation of 6-8 hours delay (Kloss *et al.*, 1998; Kloss *et al.*, 2001). Here we suspected that the CRY protein might be involved in some complex regulatory networks that we didn't find. In the eyestalk, there was a significant peak of expression of the *PcCry1* gene at 3:00 am and 11:00 am, and the results from Fanjul-Moles *et al.* (2004) showed CRY protein at 3:00 am and 19:00 pm has two peak periods, and the time taken from the peak of *Cry* gene mRNA expression to the peak of protein accumulation may be different due to the different networks involved in the regulation of clock genes under light compensation and darkness conditions. In the peripheral oscillating tissue hepatopancreas, there was a peak of expression at 11:00 am and 23:00 pm. The peak at 11:00 am may be related to the illumination at 7:00 am. In turns, the second expression activity peak at 23:00 pm may be associated to the fact that crayfish were generally nocturnally active (Fanju-Moles *et al.*, 2004). Moreover, different types of *cryptochrome* gene show different oscillation models in various organisms. In *A. pisum* and *A. gambiae*, *cry1* was transcribed constantly throughout the day, but in their heads, only *cry2* was rhythmically expressed, with peaks in mRNA accumulation occurring at early and late night (Cortes *et al.*, 2010; Rund, 2011). In *D. pulex*, *crya* and *cryb*, which homologues are *cry1* and *cry2*, show remarkable daily oscillations at the mRNA level (Bernatowicz *et al.*, 2016). Daily differences in mRNA expression described for *cry1* in some arthropods, e.g., the presence of peak mRNA accumulation of its homologue (*crya*) between evening and midnight in *D. pulex*, differ from that in both brain and peripheral organs of *D. melanogaster*, whose peak appear in the morning/midday (Emery *et al.*, 1998; Zheng *et al.*, 2008). Our study only analyzed mRNA expression levels as a basic inquiry and made inferences. In order to better verify that genes can be combined with protein analysis.

Functional domain analysis of the PcCYC protein revealed a typical bHLH/PAS transcription factor domain, including 1 HLH domain and 2 PAS domains, which is ubiquitous in CYC proteins of *Drosophila* (Ripperger & Schibler, 2006), mice (Rudic *et al.*, 2004), and humans (Shirai *et al.*, 2006). Meanwhile, the same domain appeared in the PcCLK protein, which is widespread in CLK proteins of *Drosophila* (Bae *et al.*, 1998), mouse (Steeves *et al.*, 1999), and *Arabidopsis* (Whitmore *et al.*, 1998). It was speculated that the domain's E-box region was the binding site of the PcCLK when interacting with other proteins. In the clock-regulated network, it can form a heterodimer with the PcCYC protein to activate transcription of the downstream genes *Per*, *Tim*, etc, which acts as positive feedback (Escamilla-Chimal *et al.*, 2010). At the same time, the *PcClk* gene can interact with *vrille* (*vri*) and *Par Domain Protein 1* (*Pdp1*) genes to form a second feedback regulation system (Cyran *et al.*, 2003). The result was similar with the previous studies on other crustaceans, such as: *M. rosenbergi* (Yang *et al.*, 2006) and *E. pulchra* (Zhang *et al.*, 2013). Therefore, these results confirmed that CLK protein was involved in the clock regulation system and cooperates with BMAL1 protein to activate transcription.

The amino acid sequence of the *PcCry1* was blast by homologous sequence alignment, and no other type of *Cry* gene (e.g. called *Cry2* in other species) was obtained in this study. This result supports that the data obtained in the current work have no possibility to detect two proteins products of two different *Cry* genes in the crayfish (Escamilla-Chimal & Fanjul-Moles, 2008). Furthermore, the protein may have two domains that were involved in two different functions, just like other species (Green, 2004). To this date, the existence status of both types *Cry* gene in *P. clarkii* have not been determined. Previous studies revealed that *Drosophila* has only one CRY protein (dCRY), which functions mainly as a blue light receptor (Emery *et al.*, 1998). Mice have two CRY proteins (mCRY1 and mCRY2), which are not directly used as photoreceptors but as effective transcriptional repressors (Reppert & Weaver, 2002). In turns, two CRY proteins (dpCRY1 and dpCRY2) are present in monarch butterfly *Danaus plexippus*. The dpCRY1 is a degenerating photoreceptor of the function similar to dCRY. The function of dpCRY2 is comparable to mCRY1, being a powerful transcription factor (Zhu *et*

al., 2005). The results in our study showed that the *PcCry1* might be controlled by a light-induced suppression mechanism that is known to regulate *cry1* in other insets. The function of the PcCRY1 protein in *P. clarkii* seems to be more similar to the dCRY protein of *Drosophila*. The PcCRY1 protein contained a typical PHR domain that was similar to DNA photolyase but did not possess DNA repair activity (Hitomi *et al.*, 2000; Ng & Pakrasi, 2001). PHR binds to FAD and tetrahydrofolate, being a region that senses light signals (Brudler *et al.*, 2003). Nevertheless, further studies are needed for exploring the regulatory functions and mechanism of the studies clock genes in the red swamp crayfish *P. clarkii*.

Conclusion

In summary, we cloned and characterized full-length or partial cDNA sequence of the major clock genes *PcCyc*, *PcClk*, *PcCry1* from crayfish *Procambarus clarkii*. This study focused on the mRNA expression levels of the circadian clock genes, i.e. *PcCyc*, *PcClk*, *PcCry1* in brain, eyestalk, and hepatopancreas tissues of *P. clarkii* under different light conditions (12LD, 24DD, and 72DD). Our results revealed original data on the mRNA expression of these three core clock genes in different tissues of *P. clarkii* under various illumination conditions. The mRNA expression levels of the *PcCyc*, *PcClk*, and *PcCry1* were rhythmic and analogous under LD cycle in the brain and eyestalk of *P. clarkii*. The examined clock genes had significant endogenous free-running rhythms and were dominated by the brain. This results support the statement on the formation of heterodimers between PcCYC and PcCLK proteins in the central rhythmic oscillatory tissue.

Electronic Supplementary Material

This article contains supplementary figures and appendix data (Appendix data file S1), which are available to authorized users.

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