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Characteristics of Cyclin B and its potential role in regulating oogenesis in the red claw crayfish (*Cherax quadricarinatus*)

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ABSTRACT. Cyclin B is a regulatory subunit of maturation-promoting factor (MPF), which has a key role in the induction of meiotic maturation of oocytes. MPF has been studied in a wide variety of animal species; however, its expression in crustaceans is poorly characterized. In this study, the complete cDNA sequence of Cyclin B was cloned from the red claw crayfish, *Cherax quadricarinatus*, and its spatiotemporal expression profiles were analyzed. Cyclin B cDNA (1779 bp) encoded a 401 amino acid protein with a calculated molecular weight of 45.1 kDa. Quantitative real-time PCR demonstrated that Cyclin B mRNA was expressed mainly in the ovarian tissue and that the expression decreased as the ovaries developed. Immunofluorescence analysis revealed that the Cyclin B protein relocated from the cytoplasm to the nucleus during oogenesis. These findings suggest that Cyclin B plays an important role in gametogenesis and gonad development in *C. quadricarinatus*.

Key words: Cyclin B; cDNA cloning; Cellular localization; Spatiotemporal expression profile; Oogenesis; *Cherax quadricarinatus*

INTRODUCTION

The red claw crayfish, *Cherax quadricarinatus* (von Martens, 1868), is a crustacean species commercially farmed in several countries worldwide because of its potential size, tolerance to a broad range of temperatures and water quality conditions, and large market potential. Over the past decades, considerable progress has been made in the study of its reproductive biology (Sagi et al., 1996; Abdu et al., 2000, 2002; Rodríguez-González et al., 2006; Li et al., 2007, 2010, 2011; Vazquez et al., 2008; Wang et al., 2013a), but the molecular mechanisms of oocyte maturation are rarely studied (Wang et al., 2013b). Hence, a better understanding of these molecular mechanisms is important for the artificial control of breeding and could promote large-scale industrial culture in China.

Cyclin B is a prevalent regulatory protein in cells. Progression through the cell cycle is regulated by a complex of cyclin-dependent kinase 2 (Cdc2) and cyclin B (also called maturation or M-phase promoting factor, MPF), which allows cells to proceed through the G2-M checkpoint (Minshull et al., 1990; Lees and Harlow, 1993; Li et al., 2004). Although the role of MPF in oocyte maturation has been studied in a wide variety of animal species, little information is available for crustaceans. Thus far, the molecular characteristics of crustacean Cdc2 kinase have only been reported for *Eriocheir sinensis* (Qiu and Liu, 2009), *Macrobrachium nipponense* (Wu, 2009), and *C. quadricarinatus* (Wang et al., 2013b). Cyclin B transcripts have been identified and characterized in *Marsupenaeus japonicus* (Qiu and Yamano, 2005), *E. sinensis* (Fang and Qiu, 2009), *Penaeus monodon* (Qiu et al., 2007; Visudtiphole et al., 2009), *M. nipponense* (Wu, 2009), and *Macrobrachium rosenbergii* (Fang, 2008).

After characterizing the, spatiotemporal expression profiles and cellular localization of *C. quadricarinatus* Cdc2 kinase (Wang et al., 2013b), we cloned, for the first time, the complete cDNA sequence of Cyclin B from *C. quadricarinatus*, and we evaluated its temporal and spatial expression profiles during ovarian and embryonic development. In order to understand the role of Cyclin B in oocyte maturation in crustaceans, we then examined the localization of the Cyclin B protein during oogenesis by immunofluorescence.

MATERIAL AND METHODS

Sample preparation

C. quadricarinatus crayfish were supplied by the CaoJing Aquaculture Demonstration Garden Hatchery (Shanghai, China) and purchased from a local shrimp farm. A total of 100 female and 10 male crayfish (initial weight = 20.64 ± 1.53 g) were reared indoors in 10 and 1 x 300-L plastic containers separately under controlled conditions [27 ± 1°C; pH = 6.5-7.5, dissolved oxygen (DO) > 6 mg/L and 10:14 dark:light photoperiod] for 90 days. Two tiles and PVC tubes (5-cm diameter, 20-cm length) were distributed as shelters along the bottom of the containers. The crayfish were fed commercial pellets (Hongma Feed Company, Shanghai, China) containing 35% crude protein and 8% lipid, which is regarded as an optimum diet for gonadal development (Rodríguez-González et al., 2009a,b). After 15 days of feeding adaptation, 10 females (intermolt) were randomly collected at regular intervals (15 days) and weighed. Wounded or incomplete specimens were discarded. The crayfish were then placed in an ice bath for 1-2 min until they were lightly anesthetized, and were sacrificed to obtain various tissues, including ovaries (OV), testes (TT), antennal gland (AG), muscle (MU), he-

patopancreas (HP), hemocytes (HC), intestine (IT), and brain (B). The tissue samples were snap-frozen in liquid nitrogen and conserved at -80°C for subsequent nucleic acid analysis.

In addition, ovaries were dissected and weighed to calculate the gonadosomatic index (GSI) values (wet weight of the gonads/total body weight x 100) and fixed in 0.01 M phosphate-buffered saline (PBS) containing 4% paraformaldehyde at 4°C for 12 h. After washing with PBS three times, the ovaries were dehydrated in 10, 20, and 30% saccharose-PBS solutions, respectively, at 4°C, and then embedded in Tissue-Tek Optimal Cutting Temperature compound (Sakure, USA). Standard frozen sections 8-µm-thick were produced using a microtome (Leica, Germany). The sections were used for immunofluorescence (IF) analyses and conventional histological observation to determine developmental stages.

Ovarian development was classified into four stages according to ovarian color changes and GSI, as previously described (Wang et al., 2013b). The ovarian stage of each crayfish was further confirmed by conventional histology (hematoxylin/eosin staining). Two male crayfish were placed into a container in which the female crayfish had a mature ovary. After a spermatophore was observed on the female reproductive hole, the male was removed. The fertilized eggs and embryos were classified into five stages as previously described (Wang et al., 2013b). Specimens were placed in liquid nitrogen and stored at -80°C for subsequent total RNA extraction and quantitative real-time PCR (qRT-PCR) analysis. Three biological samples (N = 3) were analyzed.

Nucleic acid preparation and first-strand cDNA synthesis

Total RNA was extracted from various tissues using Tissue RNA Rapid Extraction Kit (Yuanpinghao Biotech Co., Ltd., Tianjin, China) according to the manufacturer protocol. The quantity and quality of RNA was examined by UV-spectrophotometry (OD_{260}/OD_{280}) and agarose gel electrophoresis, respectively. Total RNA (500 ng) from various tissues was reverse transcribed into first-strand cDNA using a PrimeScript RT Master Mix Perfect Real Time Kit (TaKaRa, Japan) according to the manufacturer instructions.

Degenerate RT-PCR (reverse transcription polymerase chain reaction)

The degenerate primers Cyclin B-F and Cyclin B-R (Table 1) were designed based on a highly conserved domain from known Cyclin B gene sequences, and were used to amplify the target fragment of the Cyclin B gene. First-strand cDNA was subjected to PCR amplification using Premix Ex Taq™ Hot Start Version (TaKaRa, Japan), and degenerate primers (20 µM) were used at final concentrations in a 25-µL reaction for 94°C for 2 min, followed by 35 cycles of 98°C for 10 s, 40°C for 30 s, 72°C for 1 min, and a final elongation step at 72°C for 6 min. PCR products were separated on 1.5% agarose gel and purified with an E.Z.N.A® Gel Extraction Kit (Omiga BioTek, USA). The purified PCR products were cloned into a pMD19-T vector (TaKaRa) overnight. The recombinant plasmid was transformed into competent *Escherichia coli* cells and positive clones were sequenced at Shanghai Biosune Bio Co. Ltd., China. The retrieved sequences were verified and compared with those of other known Cyclin B sequences using the BLASTX program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast).

Table 1. Primer sequences.

Primers	Sequences	Code
Degenerate primers to target Cyclin B		
Cyclin B Forward	5'-ATWGCWAGYAAATAYGAAGARA-3'	DP-F
Cyclin B Reverse	5'-TCCATIARRTAYTTKGCWARIGTA-3'	DP-R
Primers for 5'-RACE and 3'-RACE PCR		
Cyclin B 5'-RACE primer	5'-GGCTAGGGTGTGTTGGGCTGCATCA-3'	5'-R
Cyclin B 3'-RACE primer	5'-CACCAAAAATGAAATTCGCAAAATGGA-3'	3'-R
Primers for qRT-PCR		
Cyclin B Forward	5'-GAAATTCGCAAAATGGAAGTAAA-3'	Q-F
Cyclin B Reverse	5'-ATTTGGCTAGGGTGTGTTGG-3'	Q-R
18S rRNA Forward	5'-CATGCCCGTTCTTAGTTGGT-3'	18S-F
18S rRNA Reverse	5'-GTGCGGCCCAGAAATATAAA-3'	18S-R

Rapid amplification of cDNA ends (RACE)

Full-length Cyclin B cDNA was obtained by 5'- and 3'-RACE using the SMARTer™RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer protocol. The 5'- and 3'-RACE primers (Table 1) were designed based on the cDNA sequence obtained for Cyclin B. The 3'-RACE PCR program was performed for 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min. The 5'-RACE program was performed by touchdown PCR for five cycles of 94°C for 30 s, 72°C for 3 min, followed by five cycles of 94°C for 30 s, 70°C for 30 s, 72°C for 3 min, and a final step at 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min. The amplified cDNA fragments were cloned and sequenced as described for degenerate RT-PCR above.

Multiple sequence alignment and phylogenetic analysis

Deduced protein sequences of Cyclin B from various species were retrieved from GenBank and phylogenetically compared with those of *C. quadricarinatus*. Multiple alignments were carried out using ClustalX. A bootstrapped neighbor-joining phylogenetic tree was constructed using the MEGA software version4.0 (http://www.megasoftware.net/). The reliability of the branching was tested using bootstrap re-sampling with 1000 pseudo replicates.

qRT-PCR analysis

mRNA expression was analyzed by qRT-PCR during the tissue-dependent and different stages of ovarian and embryonic development, according to the following method. Gene-specific primers (Table 1) were designed based on the cloned Cyclin B cDNA sequence to produce an amplicon of 133 bp. qRT-PCR was performed in a C1000™ Thermal Cycler (Bio-Rad CFX 96™ Real-Time System) according to the manufacturer protocol. The final volume of each qRT-PCR reaction was 25 μL, which contained 12.5 μL 2X SYBR Premix ExTaq (TaKaRa, Japan), 1.0 μL diluted cDNA template (100 ng RNA), 9.5 μL PCR-grade water, and 1.0 μL of each 10 μM primer. PCR conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 59°C for 30 s. Each sample was run in triplicate and the results were normalized to the selected control gene 18S rRNA of *C. quadricarinatus* (AF235966). The primers of 18S rRNA (Table 1) were designed to produce an amplicon of 176 bp.

Cyclin B expression levels were calculated by the $2^{-\Delta\Delta Ct}$ comparative CT method (Li-

vak and Schmittgen, 2001). Mean and standard deviations were calculated from triplicate experiments, and presented as *n*-fold differences in expression relative to 18S rRNA. Data were analyzed using the CFX Manager™ software (version 1.0). Data are reported as mean values ± standard error (SE). The homogeneity of variance was confirmed and comparison between means was performed with a one-way ANOVA. Duncan's procedure was used for multiple comparisons between groups. Differences were regarded as significant when $P < 0.05$. All statistical analyses were performed by STATISTICA 8.0 (Statsoft, USA).

Immunofluorescence

Immunofluorescence (IF) was carried out using *Fast* Tissue ImmunoFluorescence® Staining Kit (The Protein Biotechnologies Company, Portland, USA) according to the manufacturer protocol. Briefly, after washing three times, frozen sections were incubated overnight at 4°C with Cyclin B rabbit monoclonal antibody (Epitomics, USA) (1:100), and were then rinsed three times. A preliminary test confirmed that the Cyclin B rabbit monoclonal antibody was specific for Cyclin B in *C. quadricarinatus*. Subsequently, the tissue sections were incubated with a secondary antibody (goat anti-rabbit IgG) conjugated with horseradish peroxidase (Bio-Rad) at 1:10000 for 10 min, and samples were then rinsed three times. Bright light was avoided when working with the secondary antibody to minimize photo bleaching of the fluorescent dye. Finally, samples were mounted with fluorescent mounting medium, incubated until dry, and visualized under a immunofluorescence microscope (Olympus BX51, Japan). A negative control was set up, which did not include the primary antibody. Immunoreactive signals were visualized using diaminobenzidine (Sigma) as the substrate. Sections were counterstained with hematoxylin-eosin.

RESULTS

Cloning and characterization of *C. quadricarinatus* Cyclin B cDNA

Using degenerate PCR amplification, a cDNA fragment of 184 bp was obtained and, following a search for homologous sequences deposited in GenBank, was confirmed to be a partial sequence of the Cyclin B gene. The full-length cDNA of Cyclin B was successfully identified by RACE-PCR. Cyclin *B* was 1779 bp in length and contained an open reading frame (ORF) of 1203 bp, corresponding to 401 amino acid residues and 5' and 3' UTRs (untranslated region) of 72 and 504 bp, respectively (accession No. JX880246.1, Figure 1). The predicted molecular mass and pI value of the deduced Cyclin B protein were 45.1 and 8.84 kDa, respectively.

Two cyclin domains were identified as the deduced amino acid sequences of *C. quadricarinatus* Cyclin B. Furthermore, some sites or domains characteristic for Cyclin B were found to be highly conserved in *C. quadricarinatus* Cyclin B (Figure 1). These conserved domains included two cytoplasmic polyadenylation elements CPEs (U/AUUUUAU/A), one K-box (TGTGAT), one signature motif (position 170-204aa), the cyclin destruction box (RXALGXIXN) (position 34-42aa), which was necessary for Cyclin B to inhibit cell division from the middle to the late period, and a specific AMP-dependent PKA site (FLRRXSK) (position 275-281aa) (Minshull et al., 1989).

CTAATAAACCCCGCCAGCCAGCCAGCCAGCCCCTCCATCACTGCTACTACTGTTACTGCTGCTGCTGTAG 70
ACATGTCTGTGAGAAGTAACATGCAGCCGTCAGTGATTGGGTTTGATGAGAATGGGCCAAGGAAAGTAGA 140
M S V R S N M Q P S V I G F D E N G P R K V E 23
GGCAAAGGTTCTACAAGGTCCTATTTTGCACAGACCTGCACTGGGAGAAGTTGGTAATCGCAGTGTATCA 210
A K V L Q G P I L H R P A L G E V G N R S V S 46
CTCCGTGGGCTTAAGGCTCCAGTGAAACCTGTGGATGTTGCTAGGAAAAGACCAGTATTGACTAGACCCA 280
L R G L K A P V K P V D V A R K R P V L T R P T 70
CAAAACCCAAGTCTAATTCCATTTCATTATGCAGAGTGTCTGGTAAAGAAAATGTGGGCCTGCAAGAGTG 350
K P K S N S I S L C R V S G K E N V G L Q E C 93
TGAAAAAGTGGAGGAAGAAATGGATGTGGAGGAAGTTGCTAAGGTGGAAGAGTTATCAGTTGCTTTTTCT 420
E K V E E E M D V E E V A K V E E L S V A F S 116
ACTCAGTGTCTCAATGTTGAAGATATAGATGCTGAAAATGGTGGAAACCCTCAGCTAGTGTCGGAGTATG 490
T Q C L N V E D I D A E N G G N P Q L V S E Y V 140
TGAACGACATTTACAAATACTTAAGAGAATTAGAGGAGGGCAGCAAGGTACAACCTCGTTATTTGGACGG 560
N D I Y K Y L R E L E E G S K V Q P R Y L D G 163
ACAGGTAGTCACAGGAAAAATGCGAGCTATTCTCATTGACTGGTTGGTTCAGGTGCACCTACGCTTCATG 630
Q V V T G K M R A I L I D W L V Q V H L R F M 186
CTTCTCCAGGAAACTTTGTACCTTACTGTGGCTATCATTGATAGGTATCTCCAGCGTGAAAGAACTGTCT 700
L L Q E T L Y L T V A I I D R Y L Q R E R T V S 210
CTCGCAGTAAACTGCAGTTAGTTGGCGTGACAGCCATGTTTATAGCGAGTAAATATGAAGAAATGTATTC 770
R S K L Q L V G V T A M F I A S K Y E E M Y S 233
TCCAGACATTGCAGATTTTGCATACATCACAGATAAGGCATACACCAAAAATGAAATTCGCAAAATGGAA 840
P D I A D F A Y I T D K A Y T K N E I R K M E 256
GTAAATATTCTCAAAACACTAGATTTCAATGTATCCTATCCACTTCCATTGCATTTTCTCCGAAGAAACA 910
V N I L K T L D F N V S Y P L P L H F L R R N S 280
GTAAAGCTGGATCAGTTGATGCAGCCCAACACACCCTGGCTAAGTACCTCATGGAGCTTTGTCTACCGGA 980
K A G S V D A A Q H T L A K Y L M E L C L P E 303
ATATTGCATGTGTCATTATAAAGCCTCTATGATTGCCGCTGCTGCCTTGTGCTTTTCACTAAAGGTGCTG 1050
Y C M C H Y K A S M I A A A A L C F S L K V L 326
GATGGCAGTCCATGGAATGACACGCTTGTGTACTATTCTTGCAACACTGAAGAACAGCTCATGCCAGTTA 1120
D G S P W N D T L V Y Y S C N T E E Q L M P V I 350
TTTGCAAGATGGCTGCTATAGTTGTTAAGAGTATATCGTCAAAACAACAGGCTGTGAAACAGAAGTACAA 1190
C K M A A I V V K S I S S K Q Q A V K Q K Y K 373
GACCAATAAGCAGATGAAAATCAGTGATATTCCCCAGCTTCATTCTCAAACTGTGAAAAAGCTAGCAGAG 1260
T N K Q M K I S D I P Q L H S Q T V K K L A E 396
AAGGGTGCTCTCACTTGAGGTAACACCTGCTTAGATCATAGTCTTGTACAAATTGAATTATTTTACTGGT 1330
K G A L T 401
AATGTAAATACTAGGTTTAGAAGCCAGTCATTTTAAATTGATCTTGTGATATTTTAGTTTATATGTACAT 1400
GAACATATTTTTTTTTACCCTTGGATGAATGCATTTATACTGTAATGAGGCTTGTAAATGCTTGAGCACA 1470
GCTCTTACCACAGCTTGTAGAGCATGTTTCCTCTGTATATTCACTGTAAGACAGGAGGCATCTATTGCCA 1540
ATACATGTATTTTATTTTATATTGCTGTAGTCTTGCAGAATTTAATTTTTTTCATTTTCAAAGTATCTAA 1610
TAATTAACAAATTTCAAAAACTGACACTGCAGTTATTTGGCTATCAATGTATTGCCAAGTATTACATATT 1680
CCTAAAGTTGAAACAAATTTGGGTGTCAGCTGTAAGGTCTGCAAGTTA*ATTAAA*AATGGCACTCCTTTTT 1750
AAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1779

Figure 1. Nucleotide and deduced amino acid sequences of *Cherax quadricarinatus* Cyclin B. The eukaryotic potential polyadenylation signal ATTAAA is boldfaced and italicized. Two cytoplasmic polyadenylation elements, CPEs (U/AUUUUAU/A) and a K-box (TGTGAT) are marked by squares and boldfaced, respectively. The cyclin destruction box (RXALGXIXN), signature motif and PKA site (FLRRXSK) are s double-underlined, underlined, and broken underlined, respectively.

Multiple sequence alignment and phylogenetic analysis

Homology between *C. quadricarinatus* Cyclin B and deduced amino acid sequences of Cyclin B from other species was explored via multiple sequence alignment using ClustalX. The *C. quadricarinatus* Cyclin B shared 69% similarity with *Metapenaeus ensis* (GenBank accession No. ADI86225.1) and *Metapenaeus affinis* (ADI86226.1), 68% with *Marsupenaeus japonicus* (AAV37462.1) and *Penaeus monodon* (ACH72068.1), 65 and 64% with *Macrobrachium rosenbergii* (ADP95148.1) and *Marsupenaeus japonicus* (ADB44902.1), respectively, and 63 and 60% with *Scylla paramamosain* (ACN54752.1) and *E. sinensis* (ACC77698.1), respectively. Homology with *Dreissena polymorpha* (AAC35952.1) and *Haliotis diversicolor supertexta* (ADP06655.1) was lower at 48 and 47%, respectively, and that with *Xenopus laevis* (NP_001081268.1), *Danio rerio* (AAH45937.1), *Gallus gallus* (NP_001004369.1), and *Mus musculus* (NP_031656.2) Cyclin B2 was 47, 46, 45, and 43%, respectively.

Phylogenetic analysis of Cyclin B from representative crustaceans, mollusks, mammals, and fishes (Figure 2) produced an NJ-phylogenetic tree that contained three distinct branches. Here, the *C. quadricarinatus* Cyclin B clustered with those of crustaceans and then mollusks, while sequences from mammals and fishes formed a separate cluster.

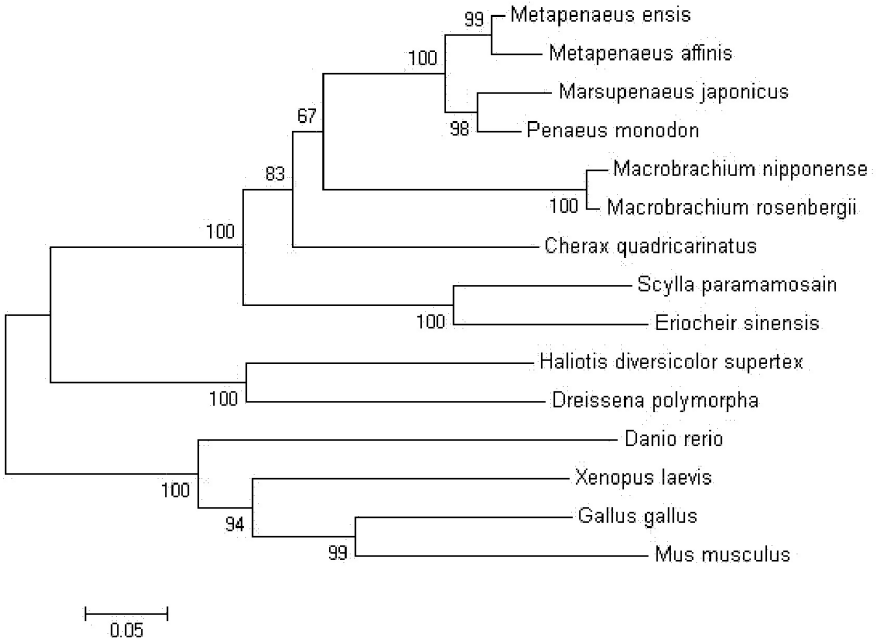

Figure 2. Neighbor-joining phylogenetic tree of the Cyclin B protein using the deduced amino acids sequences of *Cherax quadricarinatus* and other species obtained from GenBank database under the following accession No.: *Metapenaeus ensis* (ADI86225.1); *Metapenaeus affinis* (ADI86226.1); *Marsupenaeus japonicus* (AAV37462.1); *Penaeus monodon* (ACH72068.1); *Macrobrachium rosenbergii* (ADP95148.1); *Marsupenaeus japonicus* (ADB44902.1); Scylla paramamosain (ACN54752.1); Eriocheir sinensis (ACC77698.1); Dreissena polymorpha (AAC35952.1); *Haliotis diversicolor supertexta* (ADP06655.1); *Xenopus laevis* Cyclin B2 (NP_001081268.1); *Danio rerio* Cyclin B2 (AAH45937.1); *Gallus gallus* Cyclin B2 (NP_001004369.1); *Mus musculus* Cyclin B2 (NP_031656.2). The numbers in the phylogram nodes indicate percent bootstrap support for the phylogeny. The bar at the bottom indicates 2% amino acid divergence in sequences.

Pattern of Cyclin B expression

As shown in Figure 3, the relative expression of Cyclin B mRNA in ovarian tissue was significantly higher than that in other tissues ($P < 0.05$). Cyclin B was more abundantly expressed in ovaries than in embryos ($P < 0.01$) (Figures 4 and 5). As the development of ovaries progressed, the expression of Cyclin B mRNA decreased ($P < 0.05$) (Figure 4). Furthermore, the level of Cyclin B mRNA also decreased as embryonic development progressed ($P < 0.05$). The highest expression of Cyclin B mRNA was found in stages I, with lower levels in stages II and III, and the lowest levels in stages IV and V (Figure 5).

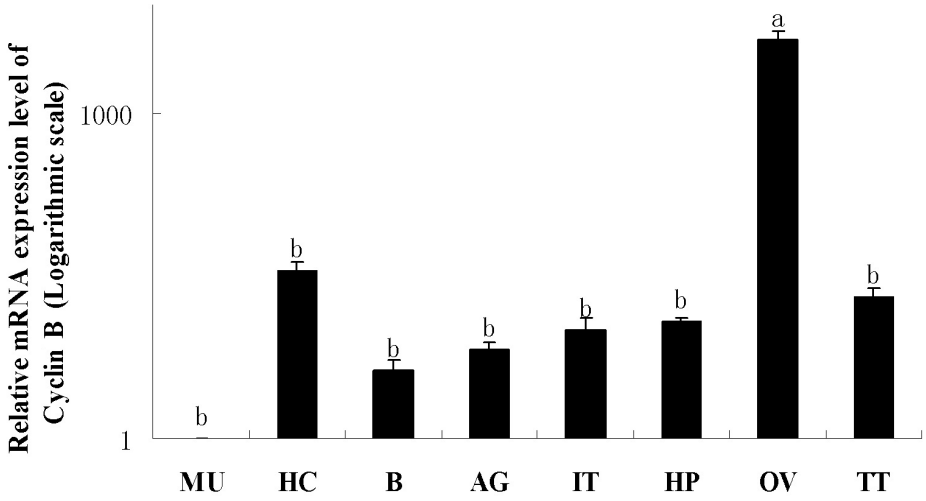

Figure 3. Tissue-dependent Cyclin B mRNA expression in *Cherax quadricarinatus*. ovaries (OV), testes (TT), antennal gland (AG), muscle (MU), hepatopancreas (HP), hemocytes (HC), intestine (IT), and brain (B).

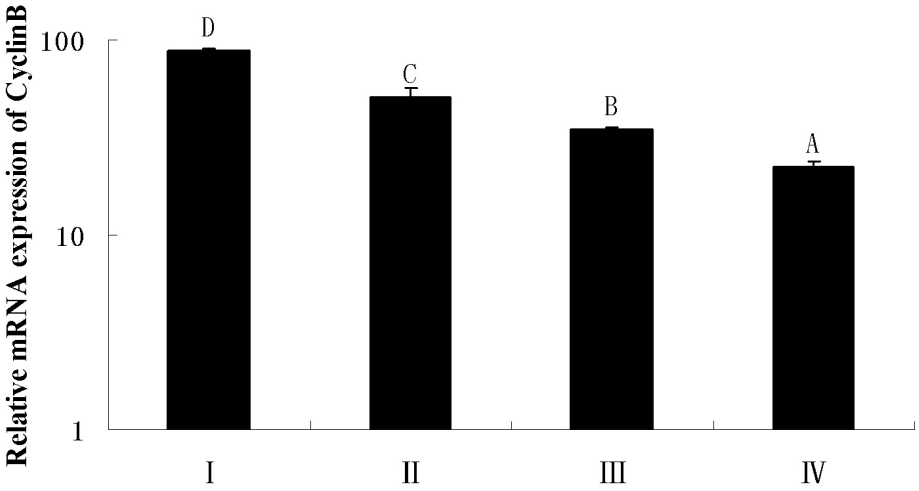

Figure 4. Relative expression of Cyclin B transcripts at different stages of ovarian development.

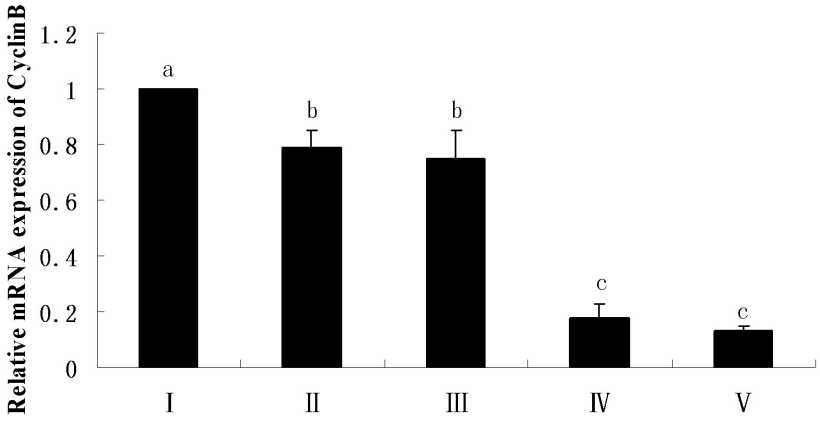

Figure 5. Relative expression of Cyclin B transcripts at different stages of embryonic development.

Localization of Cyclin B in ovaries

Localization of the Cyclin B protein was studied by IF. Specificity of the Cyclin B rabbit monoclonal antibody for *C. quadricarinatus* Cyclin B was confirmed in a preliminary test. Immunoreactive Cyclin B is shown in green. As the development of oocytes progressed, the signals became weaker in the ooplasm and stronger in the nucleus (Figure 6A and B). No positive signal was found in the negative control, which was not treated with the primary antibody.

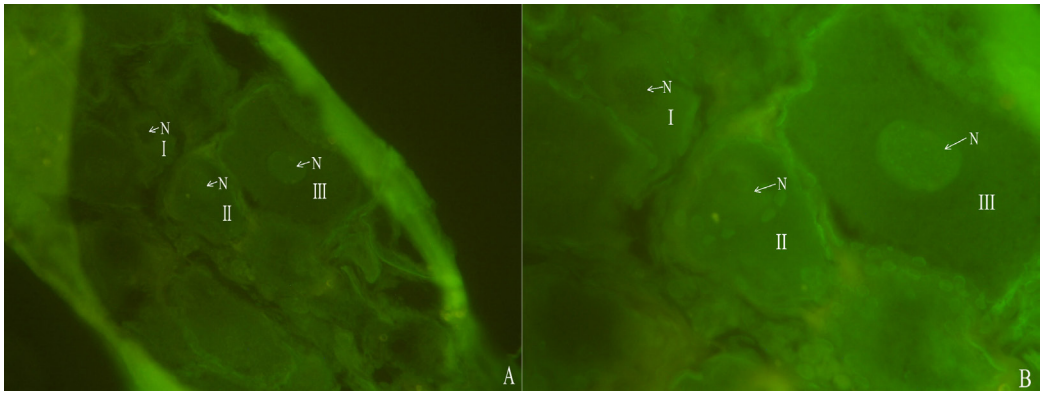

Figure 6. Immunofluorescence localization of Cyclin B protein in *Cherax quadricarinatus* ovary. Positive immunoreactive signals are shown in green. I, stage I oocyte; II, stage II oocyte; III stage III oocyte. N, nuclear. Magnification 200X for A; 400X for B.

DISCUSSION

In this study, the full-length cDNA of the *C. quadricarinatus* Cyclin B gene was characterized for the first time. Characteristic Cyclin B domains were found to be highly conserved (Figure 1). However, a polyadenylation signal sequence (AATAAA), which had previously been found in *E. sinensis* (Fang and Qiu, 2009), *P. monodon* (Qiu et al., 2007; Visudtiphole et al., 2009), *M. japonicus* (Qiu and Yamano, 2005), and *M. nipponense* (Wu, 2009) was ab-

sent within 30 nucleotides of the poly(A)-tail in *C. quadricarinatus*. The sequence ATTAAA, which was found within 30 nucleotides of the poly(A)-tail in this study, might be a potential polyadenylation signal sequence (MacDonald and Redondo, 2002). The sequence AATATA, which is similar to the polyadenylation signal sequence (AATAAA), was also only found within 30 nucleotides of the poly(A)-tail in the *M. rosenbergii* Cyclin B gene (Fang, 2008).

To date, at least two cyclin B subtypes (Cyclin B1 and Cyclin B2) have been discovered in vertebrates. Only a single B-type cyclin with a 3'UTR of 504 bp was identified in the present study. This is similar to that observed in *M. nipponense* (Wu, 2009), *M. rosenbergii* (Fang, 2008), and *E. sinensis* (Fang and Qiu, 2009), which also have long 3'UTRs consisting of 989, 1014, and 2400 bp, respectively. To our knowledge, the existence of Cyclin B2 has not been reported in invertebrates. In contrast, three forms of Cyclin B generated by 3'UTRs of three different lengths were reported in *M. japonicus* (Qiu and Yamano, 2005) and *P. monodon* (Visudtiphole et al., 2009). These studies indicated that the presence of various Cyclin B forms as a result of 3'UTR polymorphism might involve post-transcriptional regulation of gene expression and cellular and subcellular localization of the transcripts (Dalby and Glover, 1992; Qiu and Yamano, 2005; Visudtiphole et al., 2009).

qRT-PCR analysis showed that Cyclin B transcripts were ubiquitously distributed in various tissues of *M. nipponense*, and that high-levels were found in ovaries (Wu, 2009). In *E. sinensis*, Cyclin B transcripts were highly expressed in the testes and ovaries, and were expressed at lower levels in heart tissue, but were not detected in muscle, gill, and hepatopancreas tissue when examined by RT-PCR (Qiu and Liu, 2009). In *M. rosenbergii*, high levels of Cyclin B transcripts were also detected in the testes and ovaries and at lower levels were observed in the heart, hepatopancreas, gills, and muscle tissue (Fang, 2008). Moreover, *P. monodon* examined by RT-PCR revealed that cyclin B transcripts were most abundantly expressed in ovaries, followed by the testes, pleopods, and stomach, and low levels of expression were observed in the eyestalk, heart, and intestinal tissue (Visudtiphole et al., 2009). In the present study, Cyclin B transcripts were also found ubiquitously in all tissues, but the level of expression in ovarian tissue was significantly higher than that in other tissues when examined by qRT-PCR (Figure 3). The differences observed in the tissue distribution pattern might be due to qRT-PCR having higher sensitivity than RT-PCR, and differences between species (Wu, 2009). Differences in testicular expression in different species are probably due to the samples being obtained at different developmental stages (Wang et al., 2013b). However, the level of Cyclin B mRNA was consistently high in the ovaries, indicating that Cyclin B plays an important role in crustacean ovarian development as a component of MPF.

The detailed spatio-temporal expression profiles of *C. quadricarinatus* Cyclin B were further investigated during oogenesis and embryonic development. Cyclin B mRNA was more abundantly expressed in ovaries than in embryos ($P < 0.01$). However, the expression patterns of Cyclin B transcripts during oogenesis differ between various crustacean species. The levels of Cyclin B expression during the reproductive cycle of *M. japonicus* as analyzed by qRT-PCR were not significantly different (Qiu and Yamano, 2005). In *P. monodon*, the level of Cyclin B expression in stage IV ovaries was significantly greater than that in stage I (primary vitellogenesis), but not in stages II (vitellogenesis), or III (cortical rod) (Visudtiphole et al., 2009). The best explanation for this is that the total RNA was isolated from ovarian tissue contains various developmental stages of oocytes, since *M. japonicus* and *P. monodon* spawn repeatedly during a single breeding season, and, therefore, even though mature, ovaries also contain immature oocytes as noted by Qiu and Yamano (2005). However, semi-quantitative RT-PCR

analysis revealed that the amount of Cyclin B mRNA in *M. rosenbergii* was significantly higher during the previtellogenic and late vitellogenic stages, and was low during the early and middle vitellogenic stages (Fang, 2008). In *M. nipponense*, qRT-PCR revealed that the Cyclin B transcript is present in relatively high quantities at the perinucleolus stage, decreases at the nucleolus fusion stage, increases slightly at the oil globule stage, peaks during the yolk granule stage, and then drops remarkably at the paracmasis stage (Wu, 2009). In *E. sinensis,* the level of the cyclin B transcript is highest in stage I, drops remarkably at stage II, and reaches the lowest level at stage III, after which it begins to increase from stage IV until high expression is observed at the germinal vesicle breakdown (GVBD) stage (Fang and Qiu, 2008). The difference in Cyclin B expression might be associated with oogonial proliferation (mitosis) and oocyte meiotic maturation (Fang, 2008; Wu, 2009). In the present study, the color of the ovaries at each development stage was the same, and the pattern of Cyclin B mRNA expression in the ovaries decreased during oogenesis. At stages I and II, the shrimp oogonia undergo active mitosis and their number increases, and the level of Cyclin B mRNA significantly increased. At stages III and IV, the growing oocytes accumulate yolk and there are low levels of proliferation and the cell cycle is arrested at the first meiotic prophase, and during this phase, the level of Cyclin B mRNA falls to the lowest level. Following yolk accumulation, the fully-grown oocytes resume meiosis and GVBD occurs at the final maturation stage. The level of Cyclin B mRNA may increase again, which we could not detect because the ovary stage IV preceded the final whole maturation stage.

In the present study, immunofluorescence analysis revealed that the Cyclin B protein relocated from the cytoplasm to the nucleus during ovarian development (Figure 6A and B). Similar results have been reported for Cdc2 in *E. sinensis* (Qiu and Liu, 2009) and *C. quadricarinatus* (Wang et al., 2013b). In *M. japonicus*, *in situ* hybridization showed that the short transcript was expressed in the ova as early as the oogonia stage and that most accumulation occurred at the perinucleolus stage (Qiu and Yamano, 2005). Previous studies in mice and starfish demonstrated that intracellular relocation of Cyclin B-associated Cdc2 kinase is an essential step for acquiring meiotic resumption competence (Ookata et al., 1992) and induction of GVBD (Mitra and Schultz, 1996). The relocated Cyclin B protein in *C. quadricarinatus* is most likely an activated form associated with Cdc2 kinase, and the activated MPF mediates the dramatic changes that are required in nuclear and cytoskeletal architecture for accurate chromosome segregation as reported by Qiu and Liu (2009) and Wang et al. (2013b) in *E. sinensis* and *C. quadricarinatus*.

In conclusion, we successfully cloned and sequenced the full-length cDNA of Cyclin B, characterized the mRNA distribution in various tissues, and investigated the pattern of expression in different stages of gonadal and embryonic development in *Cherax quadricarinatus*. Cyclin B mRNA was mainly expressed in the ovaries and its levels decreased during ovarian development. Immunofluorescence analysis revealed that the Cyclin B protein relocated from the cytoplasm to the nucleus over the course of ovarian development.

Conflicts of interest

The authors declare no conflict of interest.

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