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RESEARCH REPORT

The first identification of a C-type lectin gene (*CqCTL*) in *Cherax quadricarinatus*: sequence features and expression profiles

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Abstract

As pattern recognition receptors (PRRs), C-type lectins (CTLs) have important roles in the recognition and clearance of pathogens by the innate immune system. In the present study, the first *Cherax quadricarinatus* CTL gene (designated *CqCTL*) was cloned and characterized. The complete cDNA sequence of *CqCTL* contained an open reading frame (ORF) of 543 bp, which encoded a protein of 180 amino acids. A carbohydrate recognition domain (CRD) containing four conserved cysteines (Cys^{48}, Cys^{59}, Cys^{76}, Cys^{177}) and the EPD (Glu^{80}-Pro^{81}-Asn^{82}) and QPD (Gln^{146}-Pro^{147}-Asn^{148}) motifs were identified in the deduced amino acid sequence of CqCTL. The deduced tertiary structure of CqCTL revealed two α helices，five β sheets and two disulfide bonds. CqCTL exhibited high similarity with previously identified CTLs from other species. The mRNA transcripts of CqCTL were ubiquitously detectable in all the tested tissues, with the highest expression level in hepatopancreas. These results provide useful information on the potential role of CqCTL in the innate immune system of *C. quadricarinatus*, and lay the foundation for further studies on the CTLs of crustacean.

Key Words: *Cherax quadricarinatus*; C-type lectin; innate immunity

Introduction

In the long evolutionary process, under the double selection pressure of pathogens and the environment, organisms gradually evolved two sets of complex immune mechanisms to protect the body from infection, namely innate immune system and acquired immune system. However, invertebrates lack adaptive immune which produce specific antibodies, and only have nonspecific innate immune. The innate immune system is composed of cellular immunity and humoral immunity, which has the ability to bind to the surface conservative molecules of pathogenic bacteria (Iwanaga and Lee, 2005, Zelensky and Gready, 2005). These evolutionarily conserved pathogen-associated molecular patterns (PAMPs) are essential for the survival of microorganisms, but they are not present in the host animals, so they become the fatal weakness of microorganisms (Gottar *et al.*,2002; Janeway and Medzhitov, 2002). Invertebrates distinguish "self" and potentially harmful "non-self" mainly by pattern recognition receptors (PRRs), which recognize the invading pathogen PAMPs and transmit signals to innate immune cells (Hoffmann *et al.*, 1999; Janeway, 1989). This recognition of PRRs to PAMPs enables the host to respond quickly and extensively to infection, which is a typical characteristic of the innate immune system (Yu *et al.*, 2002).

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Table 1 Nucleotide sequences of primers used in this study

Primers	Sequence (5'-3')	Brief information
CqCTL-race-F1	CAAGGCTGAGGTGGTGAGAGG	Gene specific primer for RACE
CqCTL-race-F2	TCCGTTCTGGTGTATGGCTC	Gene specific primer for RACE
CqCTL-race-R1	TTGAGCCATACACCAGAACG	Gene specific primer for RACE
CqCTL-race-R2	GCTGACAGATGAACGGTAGGAG	Gene specific primer for RACE
UPML	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	Universal primers for RACE
UPMS	CTAATACGACTCACTATAGGGC	Universal primers for RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	Universal primers for RACE
3'CDS	AAGCAGTGGTATCAACGCAGAGTAC(T)30V N	Oligo (dT) for cDNA synthesizing
5-AP-DG	AAGCAGTGGTATCAACGCAGAGTACGCGGGGGGGGGG	Anchor primer for 5' RACE
M13F	TGTAAAACGACGGCCAGT	Vector primer for sequencing
M13R	CAGGAAACAGCTATGACC	Vector primer for sequencing
CqCTL-qRT-F	ATGGTGAAGGCATGTGTGACG	Gene specific primer for real-time PCR
CqCTL-qRT-R	GCAGACCAAGGTCTCTTGCTCA	Gene specific primer for real-time PCR
18S rRNA-qRT-F	AATGGTTGGACGAGAAGGAA	Internal control for real-time PCR
18S rRNA-qRT-R	CCAACTAAACACCCTGCTGATA	Internal control for real-time PCR

Lectin is a membrane or soluble PRR, and it plays a prominent role in autoimmune recognition and the clearance of invasive microbes (Yu and Kanost, 2004). Among the lectins, C-type lectins (CTLs) are the most widely studied proteins, which are a group of Ca^{2+}-dependent protein superfamily involved in agglutination and widely distributed in almost all organisms (Dodd and Drickamer, 2001; Lakhtin *et al.*, 2011). The main feature of the CTLs superfamily is that it contains at least one carbohydrate recognition domain (CRD), which has specific structures to identify specific sugars (Dodd and Drickamer, 2001). Each CRD contains four conserved cysteines and is involved in forming two pairs of disulfide bonds to stabilize the CRD conformation (Zelensky and Gready, 2005). CTLs perform a variety of immune functions, such as microbial agglutination, antibacterial or antiviral responses, cell adhesion, opsonization, phenoloxidase activation, nodule formation, phagocytosis, and maintenance of gastrointestinal immune system homeostasis (Ofek *et al.*, 2000; Wang and Wang, 2013; Li *et al.*, 2019). In recent years, CTLs in an increasing number of crustaceans have been reported to participate in innate immunity against pathogens. For example, *LvCTL3* in pacific white shrimp *Penaeus vannamei* reduced the mortality of shrimp infected by *Vibrio parahaemolyticus* and white spot syndrome virus (WSSV) (Li *et al.*, 2014). PtCLec1 in the swimming crab *Portunus trituberculatus* revealed antimicrobial activity, and promoted the clearance of *Vibrio alginolyticus in vivo* and hemocyte phagocytosis *in vitro* (Su *et al.*, 2020). Both *FcLectin* and *Fc-hsL* in Chinese shrimp *Penaeus chinensis* and *PmAV* in black tiger shrimp *Penaeus monodon* showed antiviral activity (Luo *et al.*, 2003; Liu *et al.*, 2007; Sun *et al.*, 2008).

The red claw crayfish, *Cherax quadricarinatus*, is a species native to northern Queensland, Australia and Southeast Papua New Guinea (Karplus *et al.*, 2003). It is a kind of freshwater breed, which has been raised for human consumption since 1985, and has gradually become an important economic species (Garcia-Ulloa *et al.*, 2003). The worldwide shrimp/crayfish cultivation industry is seriously affected by viral pathogens, particularly WSSV, and the cultivation industry has led to significant economic losses (Liu *et al.*, 2013). However, the study of its molecular immunology is insufficient. Only a few genes especially PRRs have been identified, such as serum lectin (*CqL*) (Sanchez-Salgado *et al.*, 2019), Toll receptor (*CqToll*) (Li *et al.*, 2017), and hypervariable immunoglobulin domain-containing receptor (*CqDscam*) (Li *et al.*, 2015). Therefore, it has become the focus of attention to understand the immune ability and defense mechanism of *C. quadricarinatus*.

In our present study, the first *C. quadricarinatus* CTL gene (designated *CqCTL*) was reported. The full-length cDNA of *CqCTL* was obtained via a rapid amplification of cDNA ends (RACE) technique. The expression characteristics of *CqCTL* were analyzed in different tissues. The purpose of this study was to preliminarily investigate the sequence characteristics and tissue distribution, and to provide useful information for its further study.

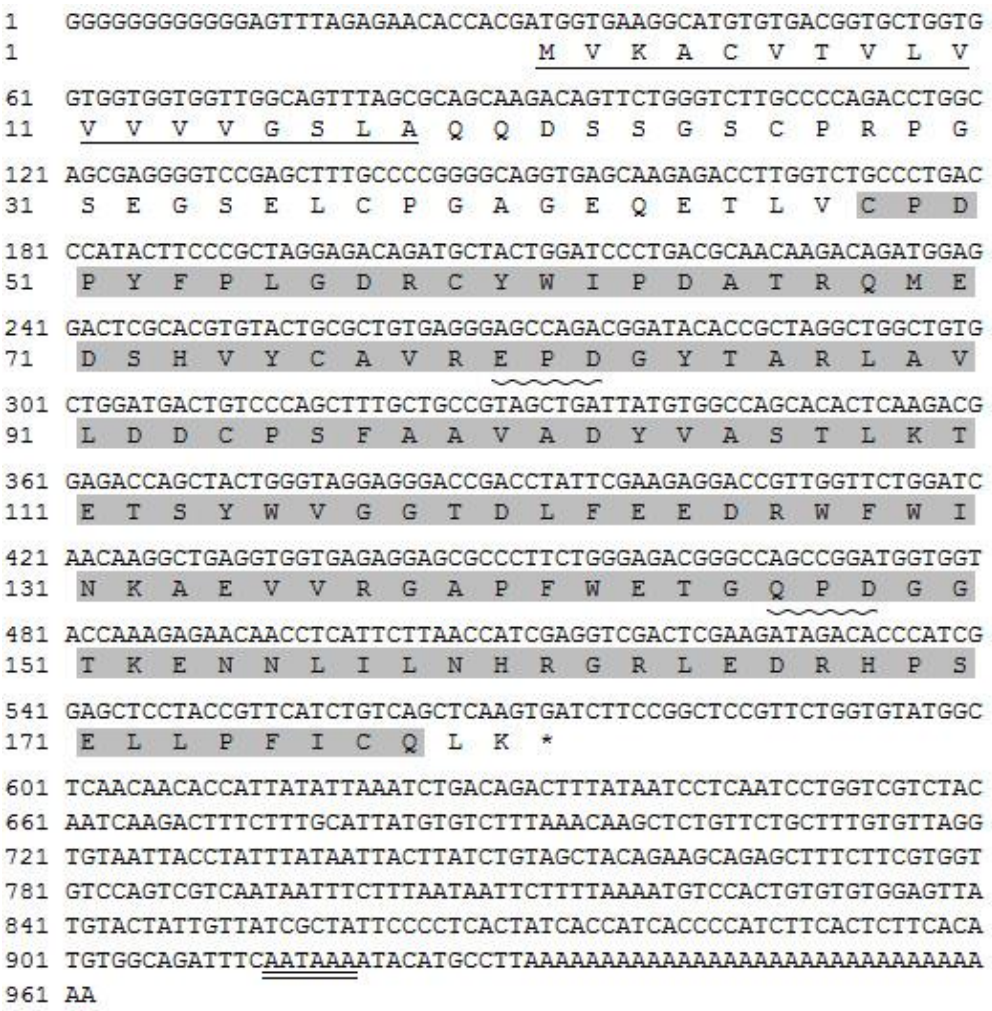

Fig. 1 Nucleotide and deduced amino acid sequence of CqCTL. The predicted signal peptide was in underline. The polyadenylation consensus signal was indicated in double underscore. The wavy line was the sugar binding site. The predicted carbohydrate recognition domain (CRD) was in shade. The asterisk indicated the stop codon. The amino acid sequence of CqCTL has been submitted to GenBank with the accession number MN944107

Materials and methods

Crayfish and samples collection

The healthy adult red claw crayfish used in this study were obtained from a crayfish farm in Boxing, Shandong Province, China. To determine the tissue distribution of *CqCTL*, tissues of the stomach, gill, testis, nerve, intestine, eyestalk, epithelium, muscle and hepatopancreas were sampled from five adult red claw crayfish, and immediately frozen in liquid nitrogen and then stored at -80 °C until total RNA extraction. Hemocytes were collected from the ventral sinus of five adult red claw crayfish using a sterile syringe which preloaded with an equal volume of anticoagulant buffer (NaCl 450 mmol L^{-1}, KCl 10 mmol L^{-1}, EDTA·2Na 10 mmol L^{-1}, and HEPES 10 mmol L^{-1}, pH 7.45), centrifugation at 800 g for 10 min at 4 °C, the supernatant was removed, and immediately frozen in liquid nitrogen and then deposited at -80 °C until use.

Total RNA extraction and cDNA synthesis

MiniBEST Universal RNA Extraction kit (9767, TaKaRa, China) was used to extract total RNA from ten tissues of *Cherax quadricarinatus* in accordance with the manufacturer's instructions. Degradation and contamination of RNA were determined by electrophoresis on a 1% agarose gels. The quality and quantity of RNA were checked using a NanoDrop Lite (Thermo Scientific, USA). Synthesis of the first-strand cDNA for the quantitative real-time PCR (qPCR) and the 3'-end RACE was carried out by transScrip® One-Step gDNA Removal and cDNA Synthesis SuperMix (AT311, TransGen Biotech, China) using anchored oligo(dT) primer (3'CDS) according to the manufacturer's instructions. To amplify the 5'-end of the cDNA sequence, a poly (C) tail was added to the first-strand cDNA using the terminal deoxynucleotide transferase (TdT). The obtained cDNA was stored at -20 °C for cloning and expression quantity analysis.

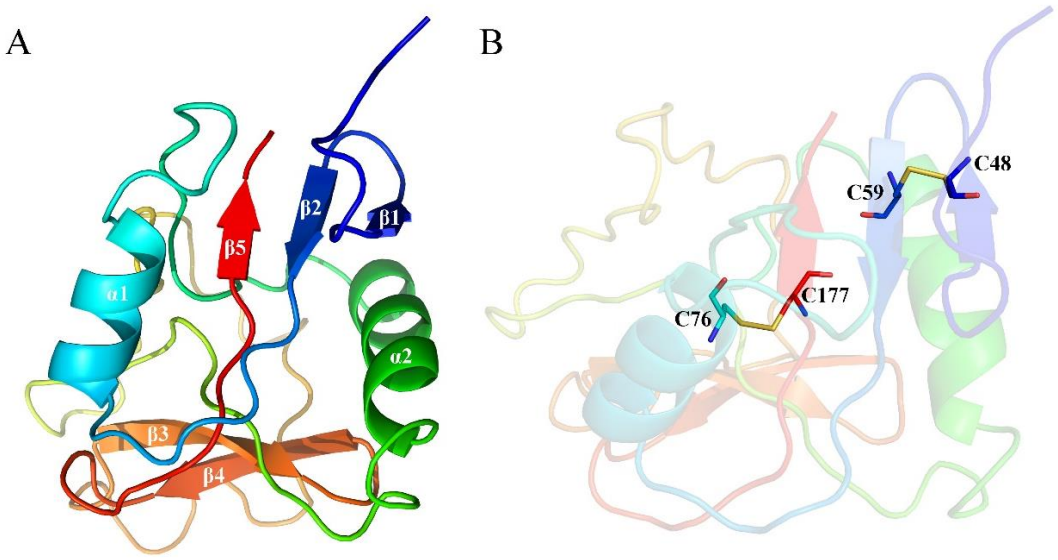

Fig. 2 (A) The three-dimensional (3D) structure of CqCTL modeled by SWISS-MODEL. (B) The disulfide bonds formed by conserved cysteine

Cloning and sequencing of CqCTL

The partial cDNA sequence of *CqCTL* was derived from the transcript that was obtained by the transcriptome of *C. quadricarinatus.* Based on the 901 bp mRNA linear transcript, primers for *CqCTL* (CqCTL-race-F1/2 and CqCTL-race-R1/2) were designed to clone the full-length cDNA of *CqCTL* by the RACE technique. The Universal Primer A Mix (UPML: UPMS= 1:5) combined with CqCTL-race-F1 was used to amplify 3'- end, and the Anchor oligo(dG) primer 5-AP-DG combined with CqCTL-race-R1 was used to amplify 5'- end. Subsequently, PCR products were used as the template for the nested PCR, which was performed with Nested Universal Primer A (NUP) and CqCTL-race-F2 or CqCTL-race-R2. The PCR was performed in 50 µL reaction volume containing 19 µL sterile distilled H_2O, 25 µL of 2 × *EasyTaq* DNA Polymerase (AP111, TransGen Biotech, China), 2 µL of each primer (10 µmol L^{-1}), and 2 µL of DNA template (~10 ng L^{-1}). The PCR amplification program included a denaturation for 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 57 °C, and 45 s at 72 °C, and finally 10 min at 72 °C. The PCR products were gel-purified and ligated into the pEASY®-T1 Simple Cloning Vector (CT111, TransGen Biotech, China), and then transformed into the competent cells of *Escherichia coli Trans*1-T1 (CD501, TransGen Biotech, China). M13 primers (M13F and M13R) were used for colony PCR to identify the potentially positive recombinants, and the positive recombinant clones were picked for sequencing. The primers are listed in Table 1.

Bioinformatics analysis of CqCTL

The nucleotide and amino acid sequence homologs of CqCTL were searched via the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast). Multiple sequence alignment of *CqCTL* with CTLs from other animals was generated using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The domain features of CqCTL were predicted by the Simple Modular Architecture Research Tool (SMART, http://smart.emblheidelberg.de/). The location of signal peptide was predicted by SignalP 3.0 program (www.cbs.dtu.dk/services/SignalP). The structure of CqCTL was predicted by the SWISS-MODEL Server and Repository (http://swissmodel.expasy.org/). The phylogenetic tree was constructed based on the amino acid sequences alignment by the neighbor-joining (NJ) method with MEGA 7.0 software, bootstrap trials were replicated 1000 times. The potential disulfide bonds and their position were predicted by the ScanProsite program (http://www.expasy.ch/tools/scanprosite/).

Quantification analysis of CqCTL *mRNA expression*

The mRNA expression quantities of *CqCTL* in different tissues were checked by the qPCR technique. All qPCR reactions were performed with *TransStart*® Top Green qPCR superMix (AQ131, TransGen Biotech, China) using about 100 ng cDNA template and 0.2 µM of each primer (Table 1), in an ABI 7300 Real-Time PCR system (Thermo Fisher, USA). The amplification program included a denaturation for 10 s at 94 °C, followed by 40 cycles of 5 s at 94 °C, and 31 s at 60 °C. The mRNA transcriptional level of *CqCTL* was normalized to that of the 18S rRNA gene for each sample. Three biological replicates were set for each tissue, and each experiment was performed in three independent replicates.

Statistical analysis

The results of qPCR analyses were based on the melting curve analysis of the PCR products and

the comparative C_T ($2^{-\Delta\Delta Ct}$) method (stomach as the reference tissue) was used to analyze the relative expression level of *CqCTL* mRNA (Livak and Schmittgen, 2001). Final results were expressed as means ± standard deviation (SD). The statistical analysis was performed by one-way analysis of variance (one-way ANOVA) followed by Duncan's multiple range tests by using SPSS Statistic 19.0 software to detect the significant intergroup differences. The *p* values less than 0.05 were considered as statistically significant.

Result

Cloning and characterization of CqCTL *cDNA*

The full-length cDNA sequence of *CqCTL* was obtained by RACE and submitted to GenBank under the accession No. MN944107. The gene contained a 543 bp open reading frame (ORF), a 30 bp 5'-untranslated region (UTR) and 389 bp 3'-UTR, including a polyadenylation consensus signal site (AATAAA) and a poly A tail, a total length of 962 bp (Fig. 1). The ORF encoded 180 amino acids with a calculated molecular weight of 19.89 kDa and a theoretical pI of 4.57. A signal peptide containing 18 amino acid residues and a single CRD domain were predicted by the SMART program. There were conserved EPD (Glu^{80}-Pro^{81}-Asn^{82}) and QPD (Gln^{146}-Pro^{147}-Asn^{148}) motifs in the CRD domain, which determine the specificity of ligand binding (Fig. 1).

The protein homologous modeling was performed to generate the three-dimensional (3D) structure of the CqCTL based on its amino acid sequence. The template protein was human CTL domain family 4 member C (PDB code: 4ZES) (Jegouzo *et al.*, 2015). The tertiary structure of CqCTL revealed two α helices and five β sheets (Fig. 2A). Two β sheets at the N-terminal (β1 and β2) and one at C- terminal (β5), which are close together to form an antiparallel β sheet. The β3 and β4 sheets form the second antiparallel β sheet slice. The disulfide bond formed by Cys^{48} and Cys^{59} connect β1 and β2. Another disulfide bond formed by Cys^{76} and Cys^{177} connect α1 and β5 (Fig. 2B).

Multiple alignments and phylogeny relationship of CqCTL

The deduced amino acid sequence of CqCTL exhibited high similarity with previously identified CTLs, 34.56 % with *Penaeus merguiensis* PmCTL (AGS42196) and 32.75 % with *Scylla paramamosain* SpCTL (AGL46986). The alignment of the protein sequence of *CqCTL* was performed to determine its identity, compared with those of previously identified CTLs (Fig. 3). A comparison of CTLs revealed that they had four conserved cysteines (Cys^{48}, Cys^{59}, Cys^{76}, Cys^{177}) involved in the formation of disulfide bonds, The N- terminal of CqCTL also contains two additional cysteines (Cys^{26}, Cys^{37}), indicating that CqCTL is a standard long CTL.

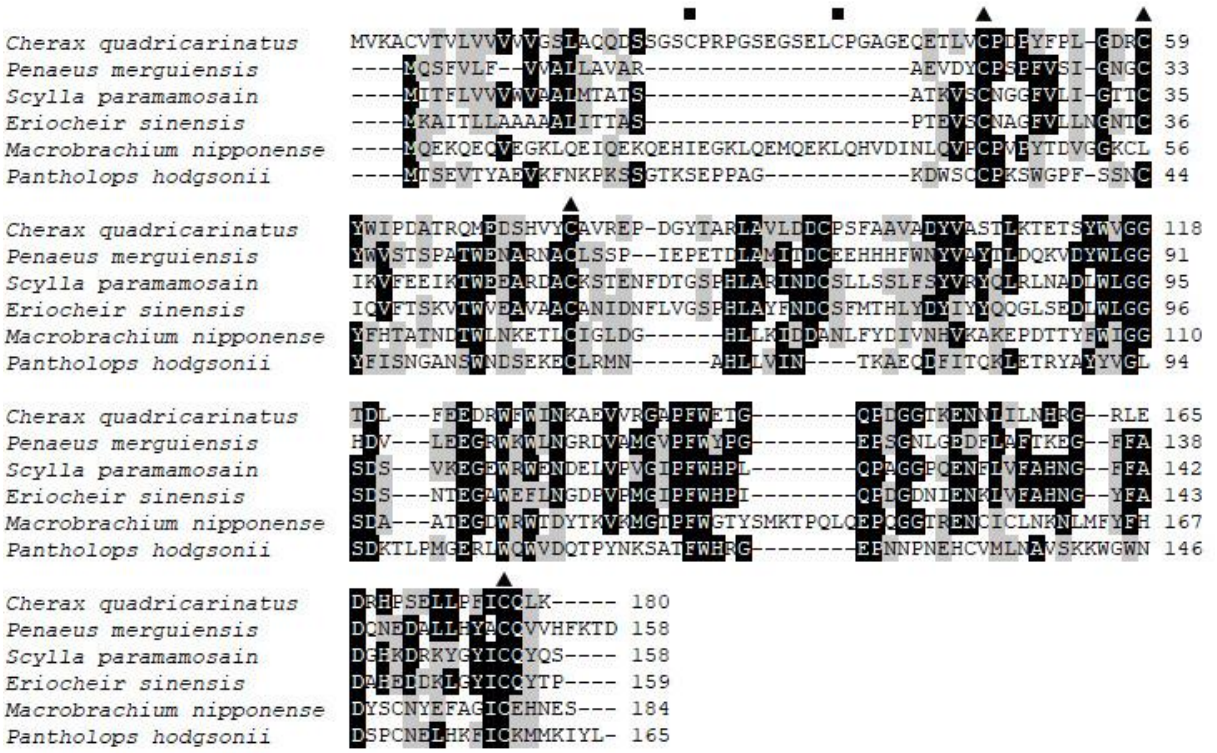

Fig. 3 Multiple alignments of CqCTL with previous known C-type lectins (CTLs). The triangle indicated four conserved cysteines, and the square indicated two additional cysteines. The same amino acid residues were shaded in black and the similar amino acids were shaded in grey. Gaps were indicated by dashes to improve the alignment. The sequences and their accession numbers are as follows: *Penaeus merguiensis* (AGS42196), *Scylla paramamosain* (AHH02662), *Eriocheir sinensis* (AFF59978), *Macrobrachium rosenbergii* (AFN20600), and *Pantholops hodgsonii* (XP_005959959)

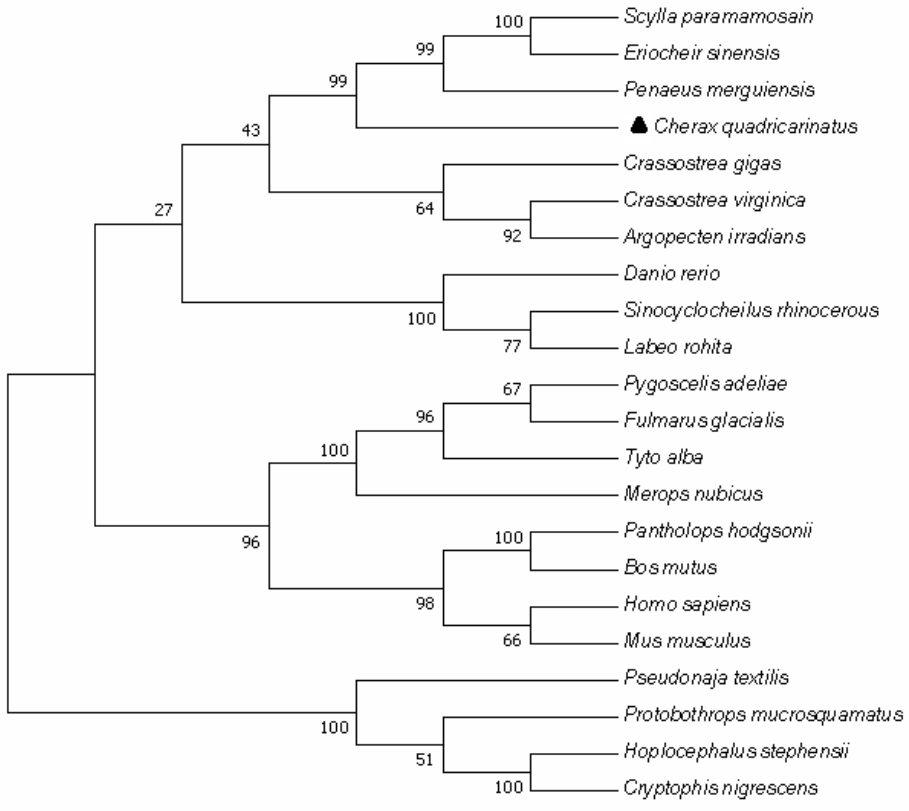

Fig. 4 Neighbor-Joining (NJ) phylogenic tree of CqCTL constructed based on the protein sequences of CTLs from different organisms. To derive confidence value for the phylogeny analysis, bootstrap trials were 1000 replicates. The black triangle indicated the CTL protein of *C. quadricarinatus*. The numbers at the forks indicated the bootstrap value. Species and their protein accession numbers are as follows: *Scylla paramamosain* (AHH02662), *Eriocheir sinensis* (AFF59978), *Penaeus merguiensis* (AGS42196), *Crassostrea gigas* (XP_011415449), *Crassostrea virginica* (XP_022293075), *Argopecten irradians* (ACS72239), *Danio rerio* (XP_021327322), *Sinocyclocheilus rhinocerous* (XP_016423812), *Labeo rohita* (RXN24634), *Pygoscelis adeliae* (XP_009320153), *Fulmarus glacialis* (XP_009583679), *Tyto alba* (XP_009974840), *Merops nubicus* (XP_008945916), *Pantholops hodgsonii* (XP_005959959), *Bos mutus* (XP_014337887), *Homo sapiens* (NP_001007034), *Mus musculus* (NP_001004159), *Pseudonaja textilis* (XP_026579441), *Protobothrops mucrosquamatus* (XP_015681994), *Hoplocephalus stephensii* (ABP94113), and *Cryptophis nigrescens* (ACC67946)

An NJ phylogenetic tree based on amino acid sequences of CqCTL and other animal CTLs was constructed to evaluate their molecular evolutionary relationships. CqCTL was clustered most closely with CTLs of *S. paramamisain*, *Eriocheir sinensis*, and *P. merguiensis*, and then with mollusks, including *Crassostrea gigas*, *Crassostrea virginica*, and *Argopecten irradians*. The vertebrate CTLs formed other clusters, including the fish CTLs (*Danio rerio*, *Sinocyclocheilus rhinocerous*, and *Labeo rohita*), bird CTLs (*Pygoscelis adeliae*, *Fulmarus glacialis*, *Tyto alba*, and *Merops nubicus*), mammal CTLs (*Pantholops hodgsonii*, *Bos mutus*, *Homo sapiens*, and *Mus musculus*) and reptile CTLs (*Pseudonaja textilis*, *Protobothtops mucrosquamatus*, *Hoplocephalus stephensii*, and *Cryptophis nigrescens*). (Fig. 4).

The tissue distribution of CqCTL *mRNA*

CqCTL mRNA transcripts were detected by qPCR in ten different tissues using 16S rRNA as an internal control. *CqCTL* mRNA transcripts were detectable in all the tested tissues; the highest mRNA transcripts level was in hepatopancreas, which was 22412.88-fold ($p < 0.05$) of that in the stomach, then muscle, epithelium, hemocytes, eyestalk, intestine, and nerve, which were 160.00-fold, 50.82-fold, 23.98-fold, 19.19-fold, 12.81-fold, and 12.75-fold ($p < 0.05$) of that in the stomach, respectively (Fig. 5) The mRNA expression levels were low in the stomach, gill, and testis.

Discussion

CTL, as an important PRR, is ubiquitous in animals and plants, and participates in innate immunity by non-self recognition, binding and clearing the invading pathogenic microorganisms (Dodd and Drickamer, 2001; Vasta *et al.*, 2004). In recent years, the research on CTL of crustacean has been gradually carried out. However, CTL has not

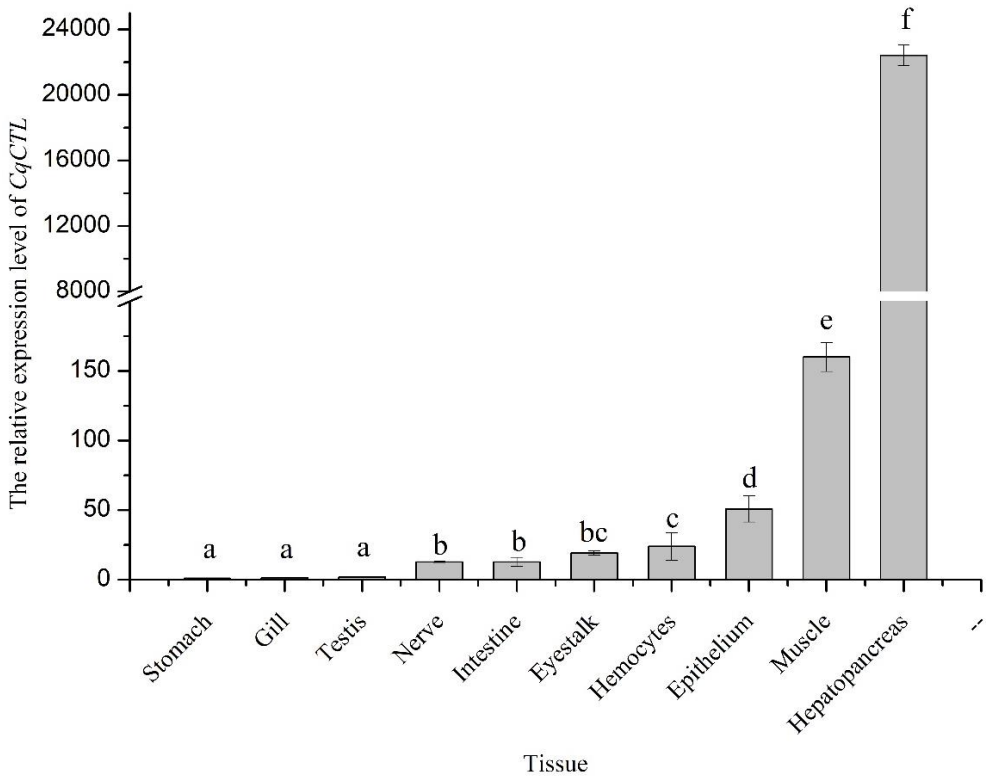

Fig. 5 Relative expression of *CqCTL* in different tissues. The 18S rRNA gene was used as an internal control to calibrate the cDNA template for each sample. Each vertical bar represents the mean ± SD (n = 3), and bars with different characters were significantly different ($p < 0.05$), while bars with same characters were not significantly different

been reported in *C. quadricarinatus*. In the present study, a novel C-type lectin (*CqCTL*) was identified in the red claw crayfish *C. quadricarinatus*, which was 962 bp and encoded a protein of 180 amino acids. We analyzed the sequence characteristics and tissue distribution, laying a foundation for further study on its functional characteristics.

Bioinformatics analysis revealed that *CqCTL* contained a CRD domain, which has conserved EPD and QPD motifs. It is known that EPN or QPD motifs are important to the binding of mannose or galactose and their derivatives, respectively (Zelensky and Gready, 2003; Zelensky and Gready, 2005). In many lower invertebrates, EPN may also be replaced by EPD or EPS (Wang and Wang, 2013). CqCTL contains EPD and QPD motifs, indicating that it may be able to combine with mannose, galactose, and their derivatives. Besides, the WND motif is important to carbohydrate-binding and is highly conserved in vertebrate lectins (Iwanaga and Lee, 2005), whereas invertebrates show a lack of WND motif or a mutation to a similar motif during evolution, for example, the LvLectin-1 and LvLectin-2 of the *P. vannamei* lack the WND motif but still respond to *Listonella anguillarum* and WSSV (Wei *et al.*, 2012). Like lvlectin-1 and lvlectin-2, CqCTL does not have WND or its similar motifs. The CRD domain contains two conserved disulfide bonds (Cys^{48}-Cys^{59} and Cys^{76}-Cys^{177}), which maintain the structural stability of CqCTL and form a fundamental and stable two-double-loop structure (Zelensky and Gready, 2005). The N-terminal of the CRD domain contains two additional cysteine residues, indicating that the CqCTL CRD is the “long” type, like the CTLs of other aquatic organisms (Yu *et al.*, 2013; Li *et al.*, 2019). The amino acid sequence of CqCTL had more than 30 % similarities with other identified CTLs. Phylogenetic analysis of the different CTLs sequences showed that CqCTL clustered with homologs from the *S. paramamisain*, *E. sinensis*, and *P. merguiensis*. The results indicate that CqCTL is a new member of the CTL family.

To investigate the potential functions of *CqCTL* in red claw crayfish, the tissue expression of the *CqCTL* gene was analyzed. The *CqCTL* mRNA transcripts could be detectable in all the sampled tissues; expression was highest in hepatopancreas. It is well known that the hepatopancreas is the main organ of immune defense in crustaceans (Lee *et al.*, 2000; Jiravanichpaisal *et al.*, 2006). Hepatopancreas is an important tissue producing immune response factors, including humoral immune and cellular immune response (Lee *et al.*, 2000; Wang *et al.*, 2013). In previous studies, the expression level of CTLs was highest in hepatopancreas, while the expression level was relatively low in other tissues, such as *P. monodon* (Ma *et al.*, 2008), *P. merguiensis* (Rattanaporn and Utarabhand, 2011)

and *P. chinensis* (Xu *et al.*, 2010). The high mRNA expression levels of *CqCTL* in hepatopancreas indicated that it might be involved in the innate immunity of red claw crayfish.

In conclusion, the full-length cDNAs of *CqCTL* were cloned and characterized from the red claw crayfish, the structure of CqCTL was modeled, and its tissue distribution was detected. CqCTL was found to be widely distributed in tissues, with the highest expression in hepatopancreas, and may participate in the immune response. The results obtained from this study would provide useful information on the potential role of CqCTL in the innate immune system of *C. quadricarinatus*,and lay the foundation for further studies on the CTLs of crustacean.

Acknowledgements

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