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**Journal of Chemical and Pharmaceutical Research, 2014, 6(7):425-431**

**Research Article**

**ISSN : 0975-7384**
**CODEN(USA) : JCPRC5**

# Molecular cloning and expression analysis of profilin in red swamp crayfish Procambarus clarkia

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**ABSTRACT**

*In this study, a novel cDNA encoding the profilin protein from red swamp crayfish Procambarus clarkii, designated as Procambarus clarkii profilin (pc-profilin), was cloned and identified. Its full-length cDNA contains an open reading frame (ORF) of 378 bp encoding a predicted protein of 125 amino acid residues. The pc-profilin protein exhibits a characteristic sequence structure of cd00148 PROF domain consisting actin interaction sites. Sequence alignment analysis showed that pc-profilin exhibits the highest amino acid sequence similarity (47%) to a profilin from Portunus trituberculatus. Real time RT-qPCR showed that pc-profilin mRNA is abundantly expressed in hemocytes and ovary. Moreover, it was found that with the continuous development of the ovary, expression level of pc-profilin was gradually increased from stage II to V, and subsequently decreased in stage VI. Our study suggests a possible important role for pc-profilin in the ovarian development and maturity in P. clarkii.*

**Key words:** Procambarus clarkii; profilin; mRNA; expression analysis; ovary development

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## INTRODUCTION

Profilin is a small ubiquitous protein originally described as actin-binding protein and is critical for cytoskeletal dynamics [1]. Profilins are potent regulators of actin filament dynamics, by promoting the exchange of ADP to ATP on actin and by the affinity of profilin-actin complexes for actin filament ends [2]. They act by sequestering actin monomers and inhibiting actin polymerization.

Apart from actin binding, profilins also have a role in cellular processes such as membrane trafficking, small-GTPase signaling and nuclear activities, in addition to neurological diseases and tumor formation [3]. Genetic studies have shown the importance of profilins for cell proliferation and differentiation. Profilin gene disruption leads to grossly impaired growth, motility and cytokinesis, and embryonic lethality in multicellular organisms, such as insects and mice [4,2]. Profilin-mutant hemocytes in *Drosophila* exhibited an increased phagocytic activity, showing that profilin is a critical regulator of phagocytosis in *Drosophila* [5]. Human breast cancer cell lines contain less profilin than normal breast epithelial cells, and raising the intracellular profilin level by transfection abolishes tumor growth in nude mice, suggesting that profilin plays a role as a tumor suppressor [6,7].

A series of studies indicated that profilin, like other actin-binding proteins, might be involved in controlling T-lymphocyte activation and effector functions, through participating in the reorganization of cytoskeleton dynamics at the immune synapse [8]. There is also some indirect evidence of a role of profilin in invertebrate immune response. Transcriptomic analysis of gills from the Pacific white shrimp *Litopenaeus vannamei* infected with white spot syndrome virus (WSSV) revealed an increase in the profilin mRNAs level at 6 h after infection [9]. In plant cells, the actin and actin-binding partners (including profilin) performed reorganization of the cytoskeleton in response to external and internal cues, and thus regulates several vital cellular processes, including reactions to the

pathogen attack and wounding [10]. Whereas, studies of profilin involved in development and maturity in crustacean were really very few.

Previously, we have observed that profilin expression level was been up-regulated in stage IV ovaries compared with stage III by using 2D-gel electrophoresis analysis in *P. clarkii* [11]. As the initial step in the determination of the function of profilin in ovary development, in this study we have obtained the full-length cDNA of pc-profilin using the rapid amplification of cDNA ends (RACE) technique, and investigated the distribution pattern of its mRNA in various tissues. Furthermore, the expression level of pc-profilin in *P. clarkii* ovary during different development stages was also analyzed.

## EXPERIMENTAL SECTION

### 2.1. Sample preparation

Domesticated female crayfish *P. clarkii* broodstock (length 10.5-12.5 cm, weight 28.2-35.5 g) were collected from the Jiangsu Baolong Breed Aquatics Company in Dafeng city, Jiangsu province, China. Brooders were maintained in 40-L aquaria in filtered aerated water at room temperature (25°C). A commercially available diet (Minghui Feed Co., Ltd., ZJ) for crayfish broodstock was fed to the animals twice daily.

### 2.2. Isolation of total RNA

After dissection, tissues (ovary, hemocyte, gill, lymphoid organ, intestine, hepatopancreas and heart) were frozen immediately in liquid nitrogen and stored at -80°C. Total RNA from various tissues was isolated by utilizing the Trizol reagent (Invitrogen) according to manufacturer's instruction. Briefly, 0.5 g of tissue was finely ground in liquid $N_2$ and lysed by adding 1.0 mL of Trizol reagent. The lysates were allowed to incubate for 5 min. Then 1.0 mL chloroform was added followed by vigorous vortexing for 15 s. Samples were then incubated for 5 min at room temperature and centrifuged for 15 min at 10,000 g. Following removal of the aqueous phase and the addition of 1.5 mL of isopropanol, samples were placed at -80°C overnight and then centrifuged for 15 min at 12,000 rpm. The RNA pellets were washed, sedimented and air-dried before being dissolved in diethyl pyrocarbonate (DEPC)-treated water. The total RNA was quantified by the RNA 6000 Nano assay in the Agilent 2100 Bioanalyzer (Applied Biosystems). The contaminating genomic DNA was removed by DNase I at 0.15 U $mg^{-1}$ total RNA treatments at 37°C for 30 min.

### 2.3. Molecular cloning of the full-length cDNA of pc-profilin

First-strand cDNAs were synthesized using Reverse Transcriptase XL (Takara, Bio, Otsu, Japan) according to manufacturer's instruction. A pair of degenerate primers P1: 5'-CAATGANNAAGGNCGCNATCTA-3' and P2: 5'-ATCCNACTNGCTCTNAGANNTG-3', whose design was based on the conserved region using the available sequences of crayfish profilin cDNA in the database, was used in the PCR. PCR conditions (32 cycles) were as follows: denature for 30 s at 94°C, annealing for 45 s at 54°C, and extension for 1 min at 72°C. PCR product was cloned into pMD18-T vector (Takara, Japan) and commercially sequenced by the ABI Prism automated sequencing method.

Subsequently, RACE technique was used to obtain the full-length cDNA of pc-profilin including the 3'- and 5'-untranslated regions (UTRs). Briefly, two specific primers P3: 5'-GTAGATGGTGAGCCGTCC-3' and P4: 5'-CCGTTAGTGAAGGCCTTGCAG-3' were synthesized based on the cDNA sequence obtained by the internal amplification above. For each 5' (primer P3) and 3'-RACE (primer P4), the cDNA was synthesized according to the manufacturer's protocol (SMART$^{TM}$ RACE cDNA Amplification Kit, Clontech, Japan). PCR conditions (32 cycles) were as follows: denature for 30 s at 94°C, annealing for 30 s at 68°C, and extension for 2 min at 72°C. PCR products of 5'- and 3'- RACE were both cloned and sequenced as described above. Three independent clones were sequenced from both ends.

Finally, the amplification of a single fragment of a full-length pc-profilin cDNA was carried out by PCR using the primer pair P5: 5'-GATCCATCGACGCTCTTCCG
ATC-3' and P6: 5'-GTACCCCTCTCTATGCCAGTCC-3'.

### 2.4. Sequence analysis and phylogenetic tree construction

Sequence homology was analyzed using the BLAST programs (http://www.ncbi.nlm.nih.gov/blast) in the GenBank database. The potential cleavage site of the signal peptide was predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignments of nucleotide and deduced amino acids were performed using Clustal W2 program (http://www.ebi.ac.uk/Tools/clustalw2/). Tertiary protein structure was modeled using the SWISS-MODEL automated protein modeling server
(http://www.expasy.ch/swissmod/SWISS-MODEL.html).

Phylogenetic analysis was conducted using MEGA 4.1 [12]. A phylogenetic tree was constructed using the neighbour-joining method (NJ) based on the Poisson-corrected distances. Node robustness was evaluated by the bootstrap method ($N$ = 1000 replications). Profilins of *Homo sapiens* (AAA36486, AAA03022, and AAI32955) were used as outgroups.

### 2.5. Real-time RT-qPCR assay

Real-time RT-qPCR was employed to quantitate changes in mRNA levels for the pc-profilin gene in a range of tissues. DNA-free total RNA from ovary, hemocyte, gill, lymphoid organ, intestine, hepatopancreas and heart was reverse transcribed with random hexamers to obtain cDNA using the Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The specific primers, P7: 5'-AGAGAACCTGGAGAACACCAG-3' and P8: 5'-TTCACTATGTCTTGAATTTG-3' were designed according to the full-length pc-profilin cDNA sequence. A 150 bp fragment of 18S rRNA was amplified using the primer pair P9: 5'-TGGTGCATGGCCGTTCTTA-3' and P10: 5'-AATTGCTGGAGATCCGTCGAC-3' according to the *P. clarkii* 18S rRNA gene (GenBank accession no. AF436001) as an internal control. DEPC-water for the replacement of RNA template was used as negative control. The SYBR Green RT-qPCR assay was conducted in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Data analysis of RT-qPCR was performed using the SDS software V2.0 (Applied Biosystems) according to the $2^{-\Delta\Delta Ct}$ algorithm [13]. The finally obtained data were subjected to one-way ANOVA followed by Duncan's test ($P$<0.05).

Subsequently, RT-qPCR was employed again for the transcript levels of pc-*profilin* gene in each stage in ovary development in *P. clarkii*. Briefly, DNA-free total RNA from ovary in stage II-VI was reverse transcribed using the Taqman Reverse Transcription kit as above.

## RESULTS AND DISCUSSION

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P. trituberculatus    MSWNSYIEN-LISSSHVQKAAIYGLDGSKWAASEGFEVSKEEFDAIKAGFNDTKNFSMSG 59
P. clarkii            MSWNTYVEN-LENTSGVTKAAIYGLDGSPWAASQGWTATPKQIQDIVKAFADPSSLRASG 59
L. vannamei           MSWQNYVDQQLMGSGFVSKAVIAGHDGTLWAKSDNIEPSREELVKLANSFTDQKGLAMTG 60
F. chinensis          MSWDQYVSKQLVESGNVKMGAICGLDGSVWAASPDLKITRDEVKTIANNFG-TDNFNTSG 59
P. monodon            MSWDQYVSKQLVESGNVKMGAICGLDGSIWAASPDLKITQEEVKTIANNFG-TDNFHTSG 59
                      ***: *:.: *  :. *  ..* * **: ** * .   : .:.  :   *  ..:  :*

P. trituberculatus    MRVGQTKFFFLSGSDDILRGKKETTGVHVAKTEQAIIIGYYDQASTSNLCATQVDCMADH 119
P. clarkii            IWLGEDKYFFLSSDSEVMRGKKGQNGVHICKTKSAIIIGYYEDPIQPGQCAKEVENVAEY 119
L. vannamei           VHMGGEKYFYLSGTDKVIRCKKGKAGMHCMKTLQAVLIAMFEDPIQPPQVASIVESLGEY 120
F. chinensis          VMLSGERFVFLRAEEGNLRAKKGKKFLHITKTNTALIMGISEEPIQPGCCTCTVEALGDY 119
P. monodon            VMLSGERYVFLRAEEGNMRAKKGKKFLHITKTNTAFIMGMCEEPVQPSCCSCTVEALGDY 119
                      : :.  ::.:* . .  :* **    :*  **  *.::.  ::.  .   :  *: :.::

P. trituberculatus    LKKSGY 125
P. clarkii            LKGQNY 125
L. vannamei           LISMTY 126
F. chinensis          LKGLNY 125
P. monodon            LKGLNY 125
                      *    *
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**Fig. 1 Multiple amino acid sequence alignment of pc-profilin (KJ150295) with that of other arthropod profilins: *Portunus trituberculatus* profilin (GU253912); *Litopenaeus vannamei* profilin (DQ907943); *Fenneropenaeus chinensis* profilin (FJ480175); *Penaeus monodon* profilin (EU106623). The symbol (*) indicates that the aligned residues are identical. Substitutions said to be conservative or semi-conservative by ClustalW are marked by (:) and (.), respectively.**

### 3.1. Cloning and sequence analysis of pc-profilin

First of all, part of pc-profilin cDNA sequence was obtained through the homologous cloning strategy. From this product, both 5'- and 3'-RACE reactions were performed. Finally, the products from these procedures provided the full-length cDNA for *P. clarkii* profilin. The complete sequence of pc-profilin cDNA contains 1177 nucleotides with a 189 bp of 5'-untranslated region (UTR), a 378 bp open reading frame (ORF), and a 610 bp 3'-UTR. The ORF encoded a predicted polypeptide of 125 amino acid residues (GenBank accession no. KJ150295). The calculated

molecular mass of the mature peptide is 13.76 kDa with a predicted isoelectric point (pI) of 5.34.

Searching for sequence similarities of known proteins by Blastx revealed that the amino acid sequence of the newly isolated gene is similar to *Portunus trituberculatus* profilin (47% identities; ADB27938), *Scylla paramamosain* (46% identities; ACY66446) and *Pseudodiaptomus annandalei* (46% identities; AGT28477) (Fig. 1). It also shows 46% identities to the profilin of chain A from *Acanthamoeba Castellanii* (1PRQ_A).

Pair-wise alignments between the complete sequence of shrimp profilin and that of crayfish profilin revealed that the predicted amino acid residues representing a cd00148 PROF domain consisting actin interaction sites, indicating that this protein likely belongs to the family of PROF superfamily. High sequence identity and similarity of the newly isolated molecular with other crustacean profilin molecular, suggests its possible role as a profilin in the cd00148 PROF family. Thus, it was named pc-profilin. Nothing of any potential cleavage site of the signal peptide was been predicted in pc-profilin according to the SignalP 3.0 server, implied its other possibility in protein assembly.

Its three-dimensional (3-D) structures have been determined for profilins as diverse as bovine [14] and *Arabidopsis* [15]. Although there is low conservation in the amino acid sequences of these proteins, the tertiary structures are all very similar. These structures consist of an antiparallel β sheet flanked by α helices on either side. Two long α helices on one side consist of the amino- and carboxyl termini (Fig. 2).

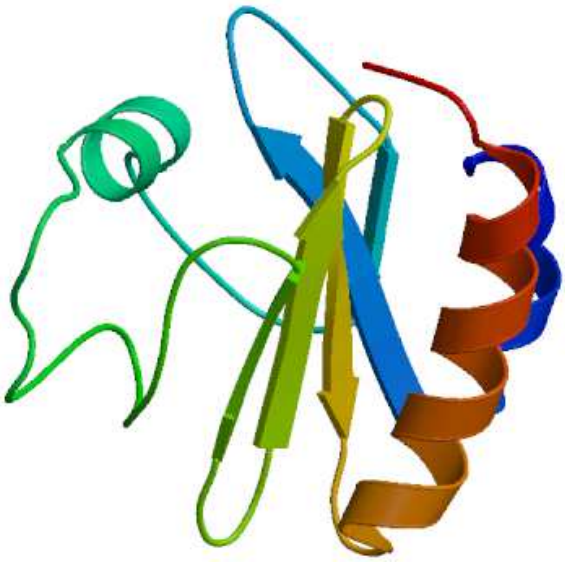

**Fig. 2 Hypothetical the three-dimensional (3-D) structure of pc-profilin. Tertiary protein structure was modeled using the** SWISS-MODEL automated protein modeling server.

### 3.2. Evolution of pc-profilin protein

Phylogenetic analysis was performed to characterize the evolutionary relationships of the pcprofilin and other invertebrate and invertebrate profilin protein subtypes using the Clustal W2 and MEGA 4 programs. As shown in Fig. 3, four clusters in the phylogenetic tree were identified which included group 1 (*H. sapiens* pro 1, pro 2, pro 3), group 2: Chelicerata (*B. tropicalis* pro, *S. medanensis* pro), group 3: Crustacea (*P. monodon* pro, *F. chinensis* pro, *P. clarkii* pro, *P. trituberculatus* pro, *C. rogercresseyi* pro, *L. salmonis* pro, *L. vannamei* pro), and group 4: Insecta (*D. melanogaster* pro, *A. pisum* pro, *A. gambiae* pro, *B. mori* pro). *P. clarkii* profilin was clustered into group 3: Crustacea. As *L. vannamei* profilin appears to be clustered into group 4 and is more closely related to the Insecta rather than Crustacea, we therefore suggest that *L. vannamei* profilin belongs to insecta subfamily.

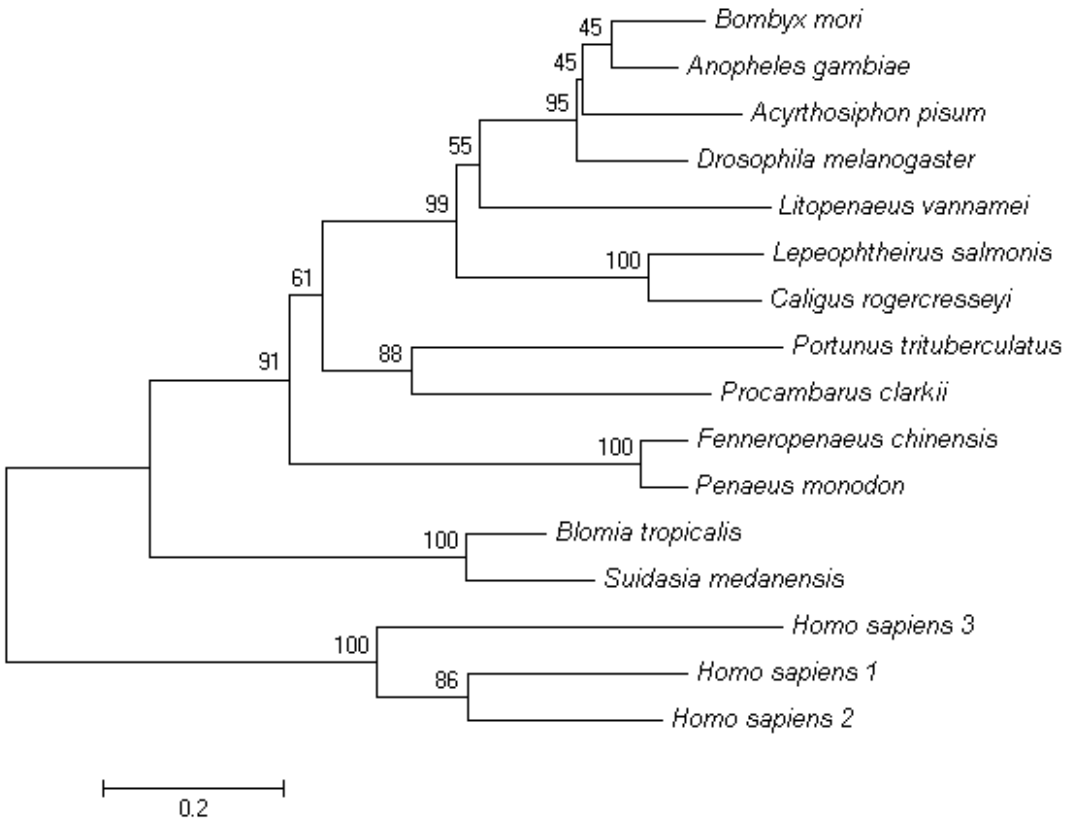


**Fig. 3 Phylogenetic analysis of profilin. Multiple alignments were constructed by ClustalW2 program. Treeview was used in tree construction. GenBank accession numbers are: *Fenneropenaeus chinensis*: FJ480175, *Litopenaeus vannamei*: DQ907943, *Penaeus monodon*: EU106623, *Portunus trituberculatus*: GU253912, *Bombyx mori*: NM_001043643, *Drosophila melanogaster*: NP_477016, *Anopheles gambiae*: XP_553744, *Acyrthosiphon pisum*: NP_001156129, *Lepeophtheirus salmonis*: ACO12777, *Caligus rogercresseyi*: ACO10829, *Blomia tropicalis*: AAQ24553, *Suidasia medanensis*: AAX34044, *Homo sapiens*: AAA36486, AAA03022, AAI32955.**

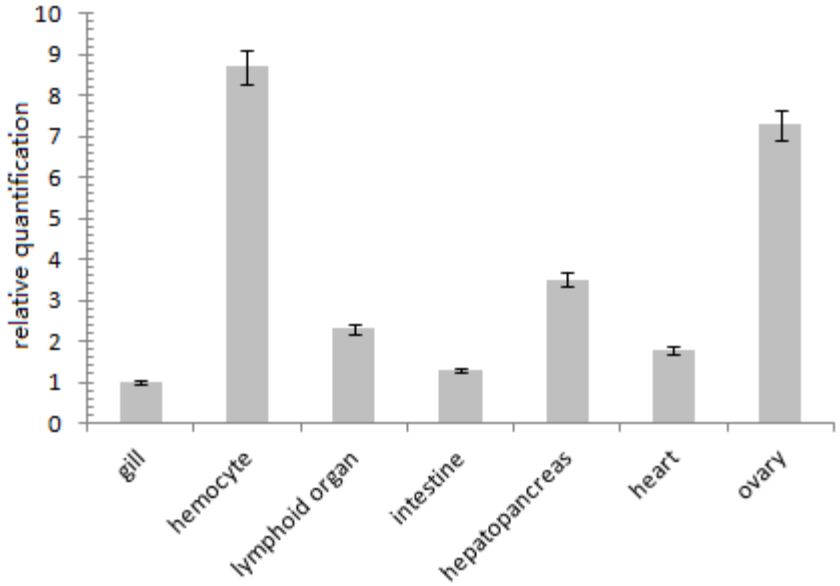


**Fig. 4 Real-time RT-qPCR assay for the expression analysis of pc-profilin mRNA in gill (control), hemocyte, lymphoid organ, intestine, hepatopancreas and heart in adults. Values are expressed as mean±S.D. from 3 different samples. Quantitative real-time PCR employed the relative quantification (RQ) ΔΔCt method. The threshold cycle (Ct) represents the PCR cycle at which an increase in SYBR Green fluorescence can first be detected above a baseline signal. The Ct value of each sample was normalized to the Ct value of the endogenous control 18s RNA gene (ΔCt) and then calibrated against ΔCt of control tissue (in this case gill). Fold change in expression was calculated as $RQ=2^{-\Delta\Delta Ct}$.**

### 3.3. Expression patterns of pc-profilin transcript in different tissues of *P. clarkii*

Real-time RT-qPCR data revealed a dynamic regulation of transcription of pc-profilin relative to 18S RNA in *P.*

*clarkii*. Tissue differential expression showed pc-profilin was broadly distributed in all tested tissues in adults (ovary, hemocyte, gill, lymphoid organ, intestine, hepatopancreas and heart), but the levels of expression were the highest in hemocyte (Fig. 4). Using gill expression value as a calibrator in determining the expression fold changes of pc-profilin in other tissues, the highest pc-profilin mRNA expression was found in hemocyte (8.7-fold higher), followed by ovary (7.3-fold higher), hepatopancreas (3.5-fold), and lymphoid organ (2.3-fold). Lowest levels were detected in heart (1.8-fold) and intestine (1.3-fold). A similar expression analysis results has been observed for profilin protein in *Portunus trituberculatus*, where the mRNA was found to be expressed in hemocyte and hepatopancreas but not in the muscle and eyes [16]. These results suggest that profilin was mostly distributed in hemocyte, ovary, and hepatopancreas in crustacean.

Fig. 5 shows the real-time RT-qPCR results for pc-profilin transcripts expression level in ovaries maintaining to different development stage. For these analyses, expression level was the lowest in stage II ovary, therefore this stage was used as the calibrator. It revealed that with the continuous development of the ovary, expression level of pc-profilin mRNA was gradually increased from stage II to V, and subsequently decreased in stage VI. Compared to expression level of stage II, pc-profilin mRNA expression in stage III was 3.5-fold greater in ovary. Even more pronounced changes were observed in stage IV (8.8-fold) and in stage V (11.2-fold). Expression in stage VI was about 6.7-fold higher than calibrator. Similar results was observed in a previous study from our group, in which we observed that the pc-profilin protein was dramatically up-regulated ($P$<0.05) in the ovarian proteome of stage IV compared to stage III by using a 2D-gel electrophoresis analysis [11], indicating that pc-profilin in *P. clarkii* ovary might play an important biological role during the ovarian development.

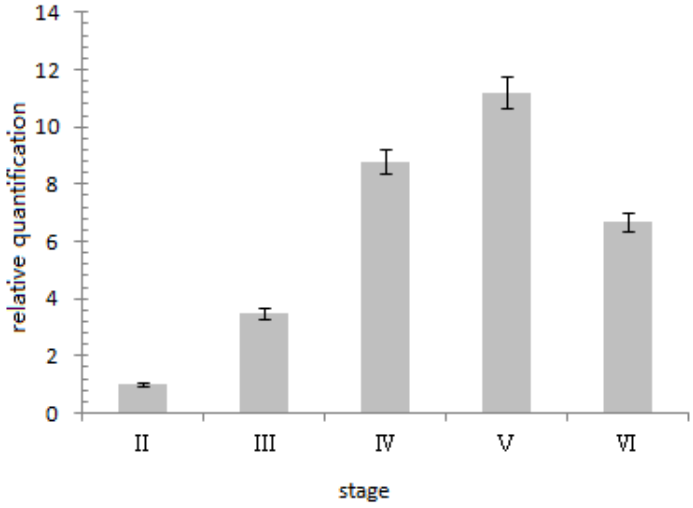


**Fig. 5 Real-time RT-qPCR assay for the expression analysis of pc-profilin mRNA in ovaries belonging to different development stages.**

In summary, we have accomplished the first step toward the determination of the function of profilin in ovary development in *P. clarkii*. The cDNA sequence for pc-profilin was determined, and the predicted protein sequence was found to be most similar to that from *Portunus trituberculatus* (47%). Pc-profilin mRNA is abundantly expressed in hemocytes and ovary, and its transcript expression level was greatest in stage V in ovary development. Our study suggests a possible role for pc-profilin in the ovary development and maturity in the crayfish *P. clarkii.* Thus, more evidence is needed to determine the importance of the *profilin* gene in gonad development in crustaceans.

**Acknowledgements**
This study was supported by the Special Fund for Agro-scientific Research in the Public Interest (201003070), Central Public-interest Scientific Institution Basal Research Fund (2013JBFM09), and the Natural Science Foundation of Jiangsu Province, China (Grant no. BK2012090).

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