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Molecular Characterization, Genomic Organization, and Expression Analysis of Sperm Gelatinase Gene during Post-Embryonic Development in *Macrobrachium nipponense*

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Abstract

Crustacean sperm gelatinase first identified in *Macrobrachium rosenbergii* encodes a gelatinolytic activity protease belonging to a kind of sperm proteases and probably involve in the fertilization process, which could be inhibited by a male reproductive tract-specific peptidase inhibitor (MRPINK) specifically. In this study, we have isolated the full-length cDNA and its genomic DNA sequence of a sperm gelatinase homologue from the testis of oriental river prawn (*Macmbrachium nipponense*) according to the established expressed sequence tags (ESTs) information using Rapid Amplification of the cDNA Ends (RACE) technique, designated as *MnSG*. The full-length cDNA of *MnSG* is 575bp in size and has an open reading frame (ORF) of 384bp, encoding a 127-amino acid protein. Genomic DNA sequence contains 1 intron and 2 exons, and three microsatellite sequences, (ATAC)5(AC)13(AT)15, (TA)11, AT)4, (AT)11, (AT)11 and (TA)37, were identified in the intron. Real-time quantitative PCR analyses revealed that *MnSG* expresses specifically in the testis, while genomic DNA of *MnSG* exists in both male and female prawn. The expression level increase gradually during growth and development process of male juvenile prawn and sharply from PL30 to PL40.

Keywords: *Macrobrachium nipponense*, sperm gelatinase, sperm protease, fertilization, autumn propagation.

Introduction

Oriental river prawn *Macrobrachium nipponense*, is an important aquaculture species in China because of its high nutritional value to humans (Zhang *et al.*, 2010; Fu *et al.*, 2012), with an annual production of 230,248 t (Bureau of Fishery Ministry of Agriculture P.R.C., 2012). However, with the rapid development of the culture industry, sexual precocity and autumn propagation have become prevalent in cultured stocks, which lead to the oriental river prawn farming facing some problems with multigenerational together, high amount of feed and falling commodity rate. For this reason, effective controls of the autumn propagation become an urgent task for us. Therefore, to carry out the research of genes related with gonadal development and fertilization process in oriental river prawn, which not only provide information for molecular assisted breeding, but also has important application value to control phenomena such as autumn propagation in practice.

Mammalian spermatozoa contains a variety of proteins, and involvement of a large number of sperm proteases and their inhibitors in spermatogenesis and fertilization process, which play important roles in the regulation of sperm motility (hyperactivation) (Finkelstein *et al.*, 2013; Iwamoto and Gagnon, 1988; Lindemann and Kanous, 1989; Majumder *et al.*, 1990; Robert and Gagnon, 1995; Turner, 2005), capacitation, acrosome reaction (Breitbart, 2003; Breitbart and Etkovitz, 2010; Salicioni *et al.*, 2007; Visconti *et al.*, 1995) and sperm-egg fusion (Inoue *et al.*, 2013; Primakoff *et al.*, 1987; Stein *et al.*, 2004; Wolfsberg *et al.*, 1993; Yanagimachi, 1988) and many others. The main physiological functions of these sperm proteases and protease inhibitors involve in inhibiting and stabilizing enzyme system and chromosomes of sperm; immune suppression and defense response of the spermatozoa; supplying the metabolic activity during sperm metabolism; contributing to regulate the transportation of spermatozoa in the reproductive tract and sperm-egg fusion (Strzezek *et al.*, 2005).

Unlike sperm of mammals and other invertebrates, crustacean decapod spermatozoa are atypical because they are nonflagellated and nonmotile (Kleve *et al.*, 1980; Rios and Barros, 1997). The fertilization process in decapods differs markedly from those of motile sperm systems (Yang *et al.*, 2013). But in any case, the success of

fertilization in shrimp relies on two key steps: contact of the sperm anterior spike with the egg outer vestment and the subsequent acrosome reaction (AR) (Kruevaisayawan *et al.*, 2008). Therefore, the participation of sperm protease would be expected to play a key role in these processes. Up to now, sperm proteases have also been identified in *Sicyonia ingentis* (Chen *et al.*, 1994), *Rhynchocinetes typus* (Bustamante *et al.*, 2001; Rios and Barros, 1997), *Penaeus monodon* (Kruevaisayawan *et al.*, 2008) and *Portunus pelagicu*s (Sroyraya *et al.*, 2013). In addition, in the research of male reproductive system-related genes of *Macrobrachium rosenbergii*, a gene with gelatinolytic activity (sperm gelatinase) was identified, which could be inhibited by a male reproductive tract-specific peptidase inhibitor (MRPINK) specifically (Li*et al.*, 2008). The function of this gene is correlation with fertilization process intuitively from the localization of sperm gelatinase, which mainly located on the convex surface of the sperm base, and gelatinolytic activity decreased dramatically after *MSG* RNAi.

In order to study the role of sperm gelatinase in *M. nipponense*, a full length cDNA and genomic DNA sequence of sperm gelatinase gene (*MnSG*) was isolated and characterized from *M. nipponense*. Tissue expression profile of *MnSG* was determined using real-time quantitative PCR (RT-qPCR), and RT-qPCR also was used to determine the effects of larval development on the expression of *MnSG* gene and expression patterns during growth and development process of male juvenile prawn.

Materials and Methods

Prawns

Several healthy adult oriental river prawns with wet weight of 1.26 to 4.25 g were obtained from Tai Lake in Wuxi, China (120°13′44″ E, 31°28′22″ N). All of these samples were transferred to laboratory breeding conditions and maintained in a 500-L tank with aerated freshwater for 72 h before tissue collection. The different developmental stages of post-larvae were obtained from our breeding room. About four male and female postlarvaes were collected every 10 days between 1 and 60 days after the metamorphosis. The gender of postlarvaes was distinguished under integrated microscope. The space between two fifth paraeiopod of female is larger than male. Four adult male and female tissues, including ovary/testis, heart, abdominal ganglion, brain, hepatopancreas, and gut, were also collected. The samples were washed with 1X 0.01 M phosphate-buffered saline, and were frozen immediately in liquid nitrogen and stored at -80°C until total RNA extraction.

Total RNA and DNA Extraction

Total RNA was extracted using RNAiso Plus Reagent (TaKaRa, Japan) in accordance with the manufacturer protocol. The isolated RNA was treated with RNase-free DNase I (Sangon, China) to eliminate possible genomic DNA contamination. The concentration of each total RNA sample was then measured using a BioPhotometer (Eppendorf, Germany), and 2 µl was analyzed on a 1% agarose gel to check the integrity. The cDNA was synthesized from 5 µg total RNA using the PrimeScript™ RT-PCR Kit (TaKaRa, Japan) according to manufacturer protocols. The cDNA was kept at -20°C for real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Genomic DNA was extracted from caudalmuscle of male and female prawns using the DNAiso Reagent (TaKaRa, Japan), following recommended protocols.

cDNA and Genomic DNA Cloning of *MnSG*

Four gene-specific primer sets (Table 1) were designed on the basis of the expressed sequence tags of the sperm gelatinase-like protease (GenBank Accession No. EF647641) obtained from the *M. nipponense* testis cDNA library(Qiao *et al.*, 2012).The full length of the *MnSG* gene was obtained by using

Table 1. Universal and specific primers used in this study

Name	Sequence （5′→3′）	Description
MnSG-3GSP1	AGCACAGGAGTTCAAGGACA	FWD first primer for 3′ RACE
MnSG -3GSP2	GACGCTTCGGTATCCTACTT	FWDsecondprimer for 3′ RACE
MnSG -5GSP1	CTTATCCTCCTTGGGACATT	RVS first primer for 5′ RACE
MnSG -5GSP2	CCCAGGAAGTAGGATACCGA	RVS first primer for 5′ RACE
3′RACE OUT	TACCGTCGTTCCACTAGTGATTT	RVS first primer for 3′ RACE
3′RACE IN	CGCGGATCCTCCACTAGTGATTTCACTATAGG	RVS secondprimer for 3′ RACE
5′RACE OUT	CATGGCTACATGCTGACAGCCTA	FWD first primer for 5′ RACE
5′RACE IN	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	FWDsecondprimer for 5′ RACE
MnSG-GF	TGGAATCCTCTTGTTTGCCTTGGCAGTGAGTGTCG	FWD primer forMnSGgenomic DNA
MnSG-GR	TCACTTATCCTCCTTGGGACATTGTTCCTTGACG	RVS primer forMnSGgenomic DNA
MnSG -RTF	ACCCTAGCCCCAGTACGTGTT	FWD primer for MnSGexpression
MnSG -RTR	AGAGGTGGTGAAGCTGTCTCTCA	RVS primer for MnSG expression
β-actinF	TATGCACTTCCTCATGCCATC	FWD primer for β-actinexpression
β-actinR	AGGAGGCGGCAGTGGTCAT	RVS primer forβ-actinexpression

3′-full RACE Core Set Ver.2.0 Kit and 5′-full RACE Kit(TaKaRa, Japan). For 5'-rapid amplification of cDNA ends (RACE), the primer sets consisted of 5'-outer primer; 5'-inner primer; andtwo gene-specific primers of GSP1, GSP2 (Table 1). For 3'-RACE, the primer sets consisted of 3'-outer primer; 3'-inner primer;and two gene-specific primers of MnSGR1, MnSGR2 (Table 1). The same PCR conditions were followed as described in the manufacturer protocol.

Based on the obtained MnSG cDNA sequence, 1 pair of primer was designed to amplify the genomic DNA sequence (Table 1). PCR amplification was performed using the DNA template above with the primer pair of MnSG-GF and MnSG-GR (1 cycle of 94°C for 4 min; 35 cycles including denaturation at 94°C for 30s, annealing and extension at 68°C for 2 min; followed by 1 cycle of 72°C for 10 min). One specific band with the size around 1.3 kb was obtained. In order to avoid single primer amplification, the controls of only MnSG-GF and MnSG-GRPCR amplification were performed.

The PCR products were gel-purified and ligated into the pMD18-T vector (TaKaRa) following the instructions provided by the manufacturer. The recombination was then transformed into *Escherichia coli* DH5α (Qiagen, Germany) competent cells, which were identified by blue/white screening and confirmed by PCR. Three of the positive clones were sequenced in both directions using an automatic DNA sequencer (ABI Applied Biosystems Model 3730) and these resulting sequences were verified and subjected to cluster analysis by using the online database of the National Center for Biotechnology Information (NCBI).

Nucleotide Sequence and Bioinformatics Analyses

The searches for protein sequence similarities were conducted with the BLAST algorithm at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The protein prediction was performed using the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/). The ProtParam program (http://www.expasy.ch/tools/ protparam. html) was used to compute physical and chemical parameters of the amino acid sequence. The motif was searched with the motif scan program (http://hits.isb-sib.ch/cgi-bin/motif scan/).

Tissue, Post-embryonic Development (Larvae and Post-Larvaestages) Expression by Real-Time Quantitative PCR

Total RNA was isolated from the different stages of larva and post-larvae, as well as six target tissues (ovary/ testis, heart, abdominal ganglion, brain, hepatopancreas, andgut) according to the manufacturer's instructions. The isolated RNA wastreated with RNase-free DNase I (Sangon, China) to eliminate possiblegenomic DNA contamination. RNA was quantified by BioPhotometer (Eppendorf). Approximately 1 μg of RNA was reverse-transcribed byiscript™ cDNA Synthesis Kit perfect Real Time (Bio-Rad, USA) following the manufacturer's instruction.

Gene-specific primers (Table 1) were used to amplify the *MnSG* transcript, and the PCR products were sequenced to verify the specificity of the PCR primers. The β-actin primers (Table 1) were used to amplify the β-actin fragments that were used as an internal control. Amplifications were performed in a 96-well plate with a 20 μl reaction volume containing 10 μl 2 SYBR Green Premix *Ex Taq* (TaKaRa), 0.4 μl each 2 μM primer, 2 μl template, and 7.2 μl PCR-grade water. The thermal profile for SYBR Green real-time quantitative RT-PCR was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. DEPC-water was used as a negative control instead of the template. A relative standard curve was constructed using 10-fold serially diluted cDNA. Each sample was run in triplicate along with the internal control gene. To ensure that only one PCR product was amplified and detected, a dissociation curve analysis of amplification products was performed at the end of each PCR. The relative copy number of *MnSG* mRNA was calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method (Livak and Schmittgen, 2001).

Statistical Analysis

All data are presented as mean ± SEM (standard error of the mean; N = 3). Statistical differences were estimated by one-way ANOVA followed by Duncan's multiple range tests. A probability level of 0.05 was used to indicate significance. All statistics were performed using SPSS Statistics 17.0.

Results

Molecular Cloning and Sequence Analysis of cDNAs Encoding *MnSG*

The full-length *MnSG* cDNA was 575 bp, and it included a 384bp open reading frame (ORF) encoding a 127-amino acid protein with an estimated molecular mass of 13.995 kDa and isoelectric point of 4.82. The 5′- and 3′-untranslated regions (UTRs) were 33 bp and 158 bp, respectively (Figure 1). Sequence analysis using Signal P 3.0 Server showed that a 21 residues signal peptide would be cleaved from each *MnSG* precursor protein, yielding mature peptides of 106 residues. A polyadenylation signal, AATAAA, was present 128 bp downstream from the stop codon, TGA, and 12 bp upstream from a poly (A) tail. The *MnSG* cDNA sequences have been submitted to Gen Bank under the accession number AFM38794.

Gene Structure of *MnSG*

To clarify the organization of the genomic

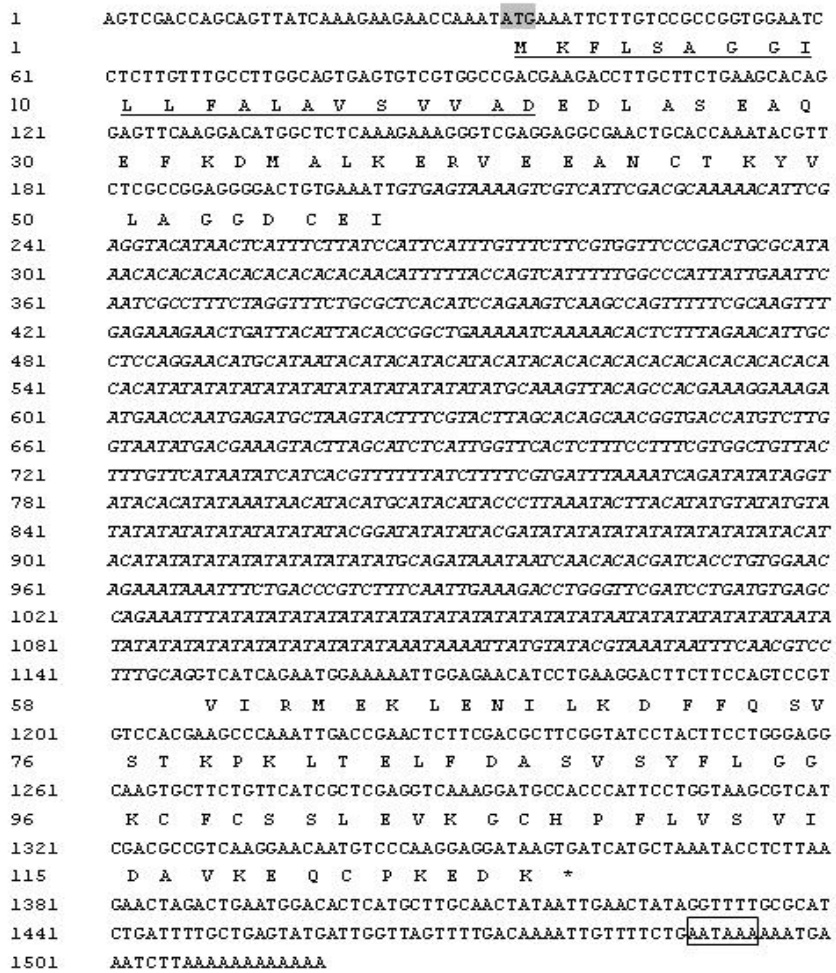

Figure 1. The DNA and deduced amino acid sequences of MnSG. DNA sequences were numbered from the first nucleotide. Deduced amino acid sequences were numbered relative to the first residue of the precursor peptide, and were shown in single-letter code below the relevant nucleotide sequence. The putative signal sequences were underlined by horizontal line. The intron sequences were in italic type. The nucleotide sequences encoding the start codon were shaded and the stop codon are underlined by asolid line, with an asterisk (*) below.

structure, we cloned and sequenced one full-length DNA sequence of *MnSG*. We aligned the full length of genomic DNA sequence with the cDNA sequence. The obtained genomic DNA sequence indicated that contain 1 intron and 2 exons (Figure 2), the intron is inserted between the 57th and 58th amino acid residues (Ile[57] and Val[58]).Splicing signals (AG, GT), as recognition sites, are found in the border of exons and introns. Three microsatellite sequences, (ATAC)5(AC)13(AT)15, (TA)11, (AT)4, (AT)11, (AT)11 and (TA)37, were identified in the intron.

Tissue Distribution of *MnSG* mRNA

Expression patternsand tissue distributionof MnSG was analyzed in six selected tissues of male and female prawn (testis or ovary, hepatopancreas, heart, brain, abdominal ganglion and gut) by using real-time quantitative RT-PCR. The results were listed in Figure 3.The result indicated that the highest expression level was in testis, the expression level were much fractionary in heart and gut of the male than testis, and no expression *level* was detected in the other selected tissues nearly. However, no *MnSG* transcripts were detected in all selected tissues of female prawn.

Expression Analysis of *MnSG* mRNA during Post-Embryonic Development (Larvae and Post-Larvaestages)

Stage-specific expression patterns of MnSG transcripts were determined in larval stage (first, fourth, seventh, tenth, thirteenth instar) and male post-larval stage (tenth, twentieth, thirtieth, fortieth, fiftieth, sixtieth day and sex mature individual) by using real-time quantitative RT-PCR (Figure 4). Average body length of the post-larval prawn of male and female under the different day-ages was listed in table 2, respectively. The results revealed that MnSG transcripts almost were not expressed during the larvae stage, which was before metamorphosis. After

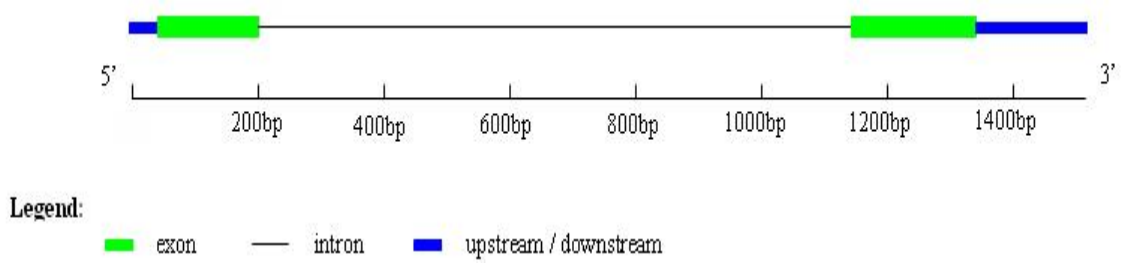

Figure 2. Schematic presentation of the MnSG from *M. nipponense*.

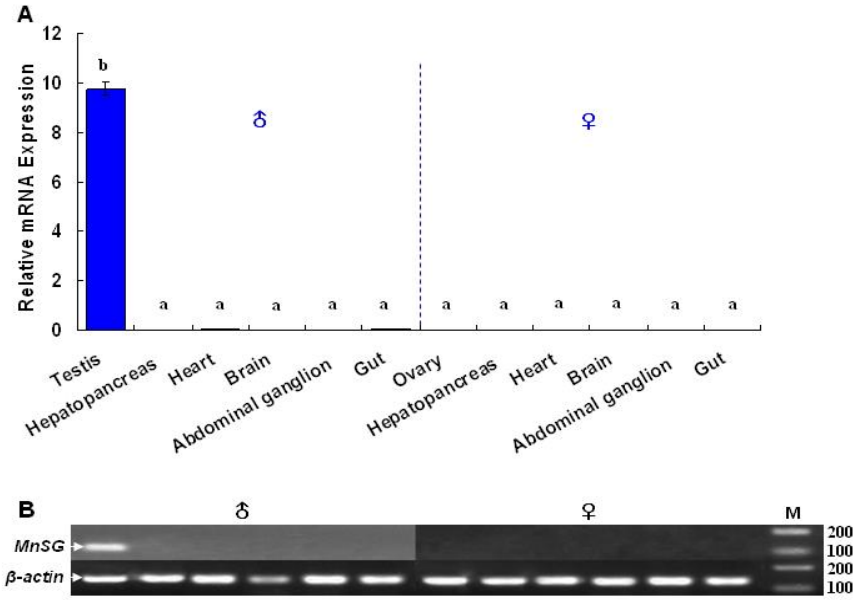

Figure 3. Expression characterization of MnSG in the various adult tissues was revealed by real-time quantitative PCR (A) and semi-quantitative PCR (B). The amount of MnSG mRNA was normalized to the β-actin transcript level. Data were shown as means±SD (standard deviation) of three separate individuals in the tissues. Bars with different letters were significantly different ($P < 0.05$).

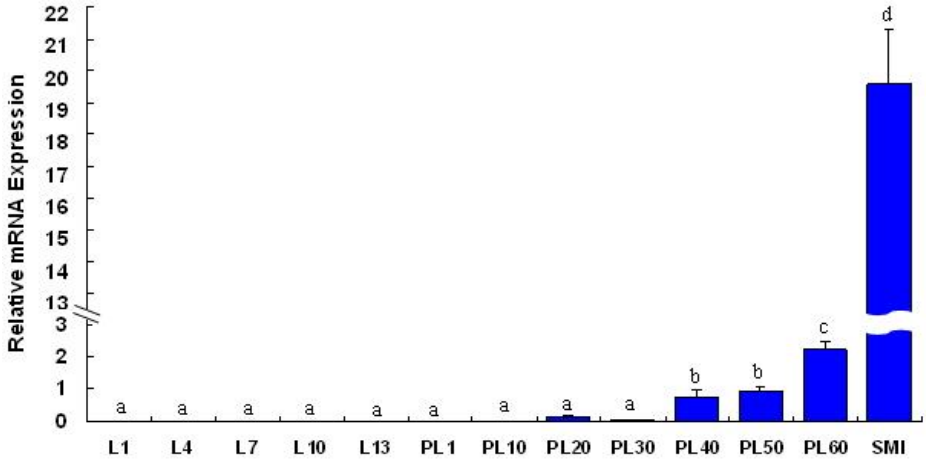

Figure 4. The temporal expression of MnSG in the different development larvae before the metamorphosis and post-larvae after the metamorphosis were revealed by real-time quantitative PCR. The amount of MnChtm RNA was normalized to the β-actin transcript level. Data were shown as means±SD of three repeated samples during the larvae and post-larvae. Bars with different letters were significantly different ($P<0.05$). L1- the first day mixed larval sample of female and male after hatching, PL1- the first day post-larva of male after metamorphosis, and so on. SMI represented the sexually mature individuals.

metamorphosis, the larvae instars transitioned into post-larvae that resemble miniature adults. During the post-larval stage, the expression levels of MnSG transcripts increased gradually and reached the highest expression until sex mature male prawn.

Disscussion

The participation of sperm protease would be expected to play a key role in sperm penetration of egg coats. Instead of sperm motility of mammals, fertilization process of most non-mammals relies on hydrolysis of the sperm protease. In the present study, we report the cloning of the first full-length cDNA and genomic DNA sequence of MnSG based on the EST sequence from an *M. nipponense* testis cDNA library. *The cDNA sequence* encoded a protein of 127 amino acids having a molecular weight of 13.995kDa. The deduced amino acid sequence only shares homology *with* sperm gelatinase-like protease of *M. rosenbergii*, and the identity reach up to 90%. Such a high identity illustrates the phylogenetic relationship of these two species is relative so much. Expression patterns and tissue distribution of *MnSG* was analyzed by using real-time quantitative RT-PCR, which demonstrate *MnSG* express specifically in the testis. However, ISH experiment of sperm gelatinase of *M. rosenbergii* (MSG) demonstrated the presence of the mRNA in the secretory epithelial cells ofvas deferens and terminal ampullae, and a further Northern blot analysis indicated *MSG* I smale reproductive tract specific (Li*et al.*, 2008).

A reasonable explanation about this result is the mRNA of *MnSG* is synthesized in testis and then transported to vas deferens and terminal ampullae. There are two reasonable grounds for why no expression level was detected in all selected tissues of female prawn: (1) *MnSG* exists in the discrepant chromosome of female and male; (2) *MnSG* express in the testis selectively by the regulation of genetic level and transcription level. On the basis of the first, we try to suppose that the female lack of this gene. The hypothesis contributes to find a discrepant marker in female and male, which can be applied for rapid detection of prawn gender in case the sex-related genes are knocked out. PCR amplification was performed to acquire the *MnSG* genomic sequence from ten female and male prawns. Although the gene only was amplified from a minority of female prawns of ten (Figure 5), in contrast to no one were not amplified from all selected ten male prawns, the hypothesis still was negated. Maybe, we just attribute this phenomenon to the methylated *MnSG* gene or its promoter region, as DNA methylation plays important role in the regulation of gene expression and those promoter-methylated genes often show tissue-specific expression patterns (Law and Jacobsen, 2010; Martienssen and Colot, 2001; Zhang *et al.*, 2006).

Expression level of *MnSG* showed an obvious upward trend during post-larvae stages of male juvenile prawn, and there was a remarkable increasement from PL30 to PL40. In our laboratory's follow-up study (unpublished), we found from histological sections that juvenile shrimp's external sexual forms occurred at PL5~ PL10, primordial germ cells (PGCs) appeared in PL10~ PL15 and the differentiation time of the testis during postlarval periods from 20th day-stage (PL20) to 40th (PL40). Gonadal differentiation time of *M. nipponensis* is earlier than kuruma prawn, *Marsupenaeus japonicas* (Nakamura, 1992), Pacific white shrimp, *Litopenaeus vannamei* (Zhao *et al.*, 2009)and giant freshwater prawn *Macrobrachium rosenbergii* (Zhu*et al.*, 2011), whereas posterior to green neon shrimp, *Neocaridina denticulata sinensis* (Yang *et al.*, 2010). Maybe, large increase in the expression level of *MnSG* from PL30 to PL40 reflects the rapid differentiation of testis during this period to a certain extent. In the other hand, the postlarval prawns were fed in aquarium (Table 2) is shorter than outside pool on the body length front. Surprisingly, such slow growth rate does not affect gonadal differentiation yet. Deeper reason

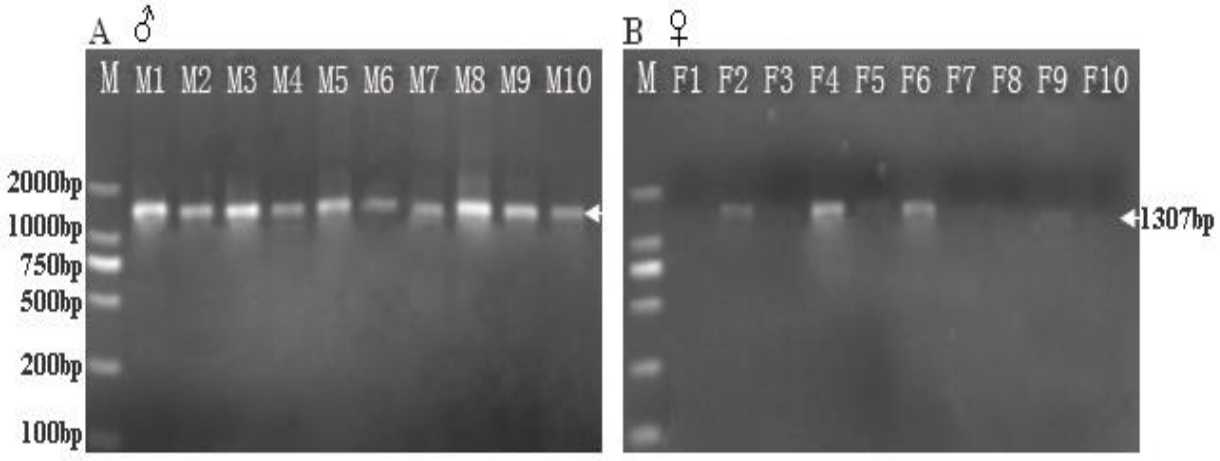

Figure 5. Electrophoretic image of amplified genomic DNA of MnSG in male (A) and female (B) prawn. M1 ~ M10, ten male prawns; F1 ~ F10, ten female prawns.

Table 2. Average body length of the post-larval prawn under the different day-ages

Day-ages (dph)	Body length of males (mm)	Body length of females (mm)
10	11.24±1.02 [a]	8.33±0.58 [a]
20	14.36±1.21 [a]	10.26±1.53 [a]
30	18.53±1.16 [a]	14.67±2.08 [a]
40	23.08±1.43 [a]	18.85±0.57 [a]
50	30.39±1.56[b]	23.40±1.26[b]
60	41.76±2.51[c]	32.33±1.15 [c]

is hard to expound, which needs further study in the future. Once the *MnSG* was verified relative with gonadal development and fertilization process, maybe sexual precocity and autumn propagation would be controlled effectively.

In conclusion, this study is the first report of the full-length cDNA and genomic DNA sequence of MnSG in the oriental river prawn, *M. nipponense*. The results show MnSG is expressed specifically in the testis, and the expression level increase gradually with the degree of development of testis. This study advances our understanding of the function of the *MnSG* gene in the fertilization process and gonadal development in *M. nipponense*. *MnSG* is an important candidateto control sexual precocity and autumn propagation in practice probably.

Acknowledgments

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