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# Molecular cloning and expression analysis of two sex-lethal homolog genes during development in the oriental river prawn, *Macrobrachium nipponense*

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**ABSTRACT.** In this study, two *Sxl* gene homologs, designated as *Mnsxl1* and *Mnsxl2*, were cloned and characterized from the freshwater prawn *Macrobrachium nipponense* by rapid amplification of cDNA ends. The deduced amino acid sequences of *Mnsxl1* and *Mnsxl2* showed high sequence homology to the insect Sxl and contained conserved domains in two RNA-binding motifs. Real-time quantitative reverse transcription-polymerase chain reaction (RT-QPCR) showed that the *Mnsxl1* and *Mnsxl2* genes were expressed in all investigated tissues, with the highest level of expression in the intestine and liver. RT-QPCR also revealed that *Mnsxl1* and *Mnsxl2* mRNAs expressions were both significantly increased at 5 and 20 days post-larvae after metamorphosis. Thus, the results of the present study imply that *Mnsxl1* and *Mnsxl2* play

 

complex and important roles in the sex differentiation of *M. nipponense*.

**Key words:** *Macrobrachium nipponense*; Sex-lethal; Sex determination; Expression pattern; Crustacean

## INTRODUCTION

Sex-determining cascades in insects constitute an intensively studied system that evolves from bottom to top (Wilkins, 1995). As an RNA-binding protein, the gene *Sxl* controls sex determination, dosage compensation, and oogenesis in *Drosophila melanogaster* (Penalva and Sánchez, 2003; Serna et al., 2004). Its state of activity is set at around the blastoderm stage in somatic cells by the primary sex-determining signal, which is formed by the ratio of X chromosomes to sets of autosomes. If this ratio is 1.0 (XX:AA), *Sxl* is activated and a female develops. Alternatively, a ratio of 0.5 (X:AA) leaves *Sxl* inactive, and male development ensues (Cline and Meyer, 1996; Schütt and Nöthiger, 2000). So far, the fundamentals of the molecular regulation mechanism of the sex-determination pathway of *Drosophila* have been elucidated (Casper and Van Doren, 2009; Siwicki and Kravitz, 2009), but knowledge regarding sex determination in other insects remains at the chromosome or gene level.

Understanding tissue- and stage-specific gene regulation remains one of the central issues in developmental biology. *Sxl* was initially discovered as a master switch gene of sex determination in *Drosophila* (Cline, 1978). The *Sxl* gene has been implicated in germ cell proliferation, differentiation, and meiotic recombination during early oogenesis in *Drosophila* (Vied and Horabin, 2001; Vied et al., 2003). *Sxl* has also been isolated and characterized in other species such as *Chrysomya rufifacies* (Müller-Holtkamp, 1995), *Megaselia scalaris* (Sievert et al., 2000), *Ceratitis capitata* (Saccone et al., 1998), *Sciara ocellaris* (Ruiz et al., 2003) and *Bactrocera oleae* (Lagos et al., 2005). Although the *Sxl* sequence from these species shares a high degree of similarity with that from *Drosophila*, it is not sex-specifically spliced and does not appear to play the key discriminative role in controlling sex determination and dosage compensation that it plays in *Drosophila* (Traut et al., 2006). Considering the fact that crustaceans have a dramatically close evolution relationship with insects (Glenner et al., 2006; Budd and Telford, 2009), we hypothesized that the homolog *Mnsxl* from the oriental river prawn *Macrobrachium nipponense* is involved in the regulation of sex determination in crustaceans.

*M. nipponense* (Crustacea; Decapoda; Palaemonidae) is a commercial freshwater prawn. It is considered as an important fishery resource in China, with an annual production of 205,010 tons (Bureau of Fishery, Ministry of Agriculture, P.R.C., 2009). Male individuals of the oriental river prawn grow faster and reach higher weights at harvest than female individuals reach. Thus, culture of all-male populations would be dramatically economically beneficial. To understand the mechanism controlling sex maturation, a high-quality normalized cDNA library from testis tissue of this species has been established (Qiao et al., 2012). For this reason, molecular mechanisms regulating the expression of sex-specific and sex-determining genes in crustaceans have received great attention in recent years (Li et al., 2009; Kato et al., 2011; Ventura et al., 2012). In the present study, we aimed to obtain a full cDNA clone encoding the *Sxl* gene from the cDNA library of testis in *M. nipponense*, and to examine the expression pattern of the *Sxl* gene during the embryonic development, larvae, and post-larvae

 

stages of *M. nipponense*. This study may provide insight into the regulatory mechanism of sex determination in the oriental river prawn.

## MATERIAL AND METHODS

### Tissue preparation

Several healthy adult oriental river prawns with wet weight of 1.26 to 4.25 g were obtained from Tai Lake in Wuxi, China (31°28'22"N, 120°13'44''E). 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 eggs and larvae were obtained from our breeding room. After prawn spawning, each developmental stage of embryos was collected as per the morphological methods, following the criteria of Chen et al. (2012). Larvae were collected every 4 days between 1 day post-hatching (L1) and L13 (1 day before the metamorphosis). Post-larvae were collected every 5 days between 1 and 20 days after the metamorphosis (P1~P20), and every 10 days between P20 and P30. Several tissues, including ovary, testis, muscle, heart, abdominal ganglion, brain, liver, and intestine, were also collected. The samples were washed with 1X 0.01 M phosphate-buffered saline, frozen directly in liquid nitrogen, and stored at -80°C until processed.

### Total RNA extraction and reverse transcription

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, Shanghai, China) to eliminate possible genomic DNA contamination. The concentration of each total RNA sample was then measured using a BioPhotometer (Eppendorf), 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) according to manufacturer protocols. The cDNA was kept at -20°C for real-time quantitative reverse transcription-polymerase chain reaction (RT-QPCR).

### Rapid amplification of cDNA ends

Four gene-specific primer sets (Table 1) were designed on the basis of the expressed sequence tag of the sex-lethal homolog (GenBank accession No. JK526786) and sex-lethal-like protein (GenBank accession No. JK525705) obtained from the *M. nipponense* testis cDNA library. The full lengths of the *Mnsxl1* and *Mnsxl2* genes were obtained by using the SMART™ RACE cDNA Amplification Kit (Clontech, USA). For 5'-rapid amplification of cDNA ends (RACE), the primer sets consisted of 5'-CDS primer A (as the RT primer); four gene-specific primers of GSP1, GSP2, GSP3, and GSP4; and universal primer A mix (UPM) (Table 1). For 3'-RACE, the primer sets consisted of 3'-CDS primer A (as the RT primer); four gene-specific primers of GSP5, GSP6, GSP7, and GSP8; and UPM (Table 1). The same PCR conditions were followed as described in the manufacturer protocol of the Advantage™ 2 PAC Kit (Clontech).

**Table 1.** Primer sequences.

| Primer | Nucleotide sequence (5'→3') | Code |
|---|---|---|
| Primers for 5' RACE PCR | | |
| MnSxl1 5' GSP primer 1 | GCCAGGAGAAGACATAAAG | GSP1 |
| MnSxl1 5' GSP primer 2 | GGAGTCGGGTTTGTCAGGT | GSP2 |
| MnSxl2 5' GSP primer 1 | GCGTGGGAACTACAACAA | GSP3 |
| MnSxl2 5' GSP primer 2 | CAGCAGGGTGGTAAGATG | GSP4 |
| Primers for 3' RACE PCR | | |
| MnSxl1 3' GSP primer 1 | CCACCTGTGATGATACTTGACCTCC | GSP5 |
| MnSxl1 3' GSP primer 2 | TCTAACTGGTCTAGGGTGTAGGATCTGG | GSP6 |
| MnSxl2 3' GSP primer 1 | GTAACCACCACCAGTGCCAATGG | GSP7 |
| MnSxl2 3' GSP primer 2 | TCGGCTCAGTACCACCTGTGATG | GSP8 |
| Clontech™ Kit primesr | | |
| Universal primer A mix | CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGA GT | |
| Primers for real-time PCR analysis | | |
| MnSxl1 5' primer | AAGGTGGCACGGAACCGTTGG | RT-F1 |
| MnSxl1 3' primer | CCACCTGTGATGATACTTGACCTCC | RT-R1 |
| MnSxl2 5' primer | GCGTGGGAACTACAACAA | RT-F2 |
| MnSxl2 3' primer | TCGGCTCAGTACCACCTGTGATG | RT-R2 |
| β-actin 5' primer | TATGCACTTCCTCATGCCATC | β-actinF |
| β-actin 3' primer | AGGAGGCGGCAGTGGTCAT | β-actinR |

## Primer sequences

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) 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 bioinformatic 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/motifscan/). Sxl deduced amino acid sequences from *M. nipponense* and representative invertebrates were compared by multiple sequence alignment using ClustalX and the Box shade program (http://www.ch.embnet.org/software/BOX_form.html). A neighbor-joining phylogenetic tree was constructed using the MEGA version 4 software.

The *Mnsxl1* and *Mnsxl2* mRNA expressions at different stages, from embryo to post-larval, and in various adult tissues were measured by a SYBR Green real-time quantitative RT-PCR analysis in the CFX96TM Real-Time System (Bio-Rad, USA). Gene-specific primers (Table 1) were used to amplify the Sxl 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 *Mnsxl1* and *Mnsxl2* mRNA was calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method (Livak and Schmittgen, 2001).

### Statistical analysis

All data are reported as means ± SE (standard error; N = 3). Statistical analysis was performed using the SPSS 13.0 software. Statistical significance was determined using one-way ANOVA and *post hoc* Duncan multiple range tests. Significance was set at $P < 0.05$.

## RESULTS

### Cloning and identification of the *Mnsxl* cDNA

The full-length *Mnsxl1* cDNA was 1138 bp, and it included a 927-bp open reading frame (ORF) encoding a 308-amino acid protein with an estimated molecular mass of 33.404 kDa and isoelectric point of 8.71. The 5'- and 3'-untranslated regions (UTRs) were 104 bp and 107 bp, respectively (Figure 1). *Mnsxl2* cDNA, which was 1214 bp long, contained a 726-bp ORF and encoded a 241-amino acid protein with a predicted molecular mass of 26.706 kDa and an isoelectric point of 9.31. The 5'- and 3'-UTRs were 103 bp and 385 bp, respectively (Figure 2). The *Mnsxl1* and *Mnsxl2* cDNA sequences have been submitted to GenBank under the accession Nos. JX457334 and JX457335, respectively. The conserved sequence and characteristic motifs of two RNA recognition motifs domains were identified in the deduced amino acid sequences of *Mnsxl1* and *Mnsxl2*, and the conserved motifs were located in the N-terminal region and central region, respectively (Figure 3).

### Homology and phylogenetic analysis of *Mnsxl*

The amino acid sequences of *Mnsxl1* and *Mnsxl2* were compared with the sequences of previously reported Sxl proteins, showing that the *Mnsxl1* protein shared high identity to those of other species such as *Harpegnathos saltator* (EFN79874.1; 66%), *Daphnia pulex* (EFX75394.1; 62%), *Bombyx mori* (BAE86938.1; 59%), and *Rhynchosciara americana* (AAS45604.1; 56%). *Mnsxl2* shared 67% identity to the sex-lethal-like protein of *Acromyrmex echinatior* (EGI69813.1), 64 and 63% identity to the sex-lethal protein variant 2 (EFX75395.1) and variant 1 (EFX75394.1) of *D. pulex*, respectively, and 51% identity to the Sxl homolog of *Lepeophtheirus salmonis* (ACO12409.1). A neighbor-joining phylogenic tree was constructed based on reported arthropod Sxl amino acid sequences by using the MEGA 4.0 software. The tree constructed by the neighbor-joining method showed that *Mnsxl1* and *Mnsxl2* were not lined with crustaceans but with the Hymenoptera (Figure 4), suggesting a closer phylogenetic relationship with the latter.

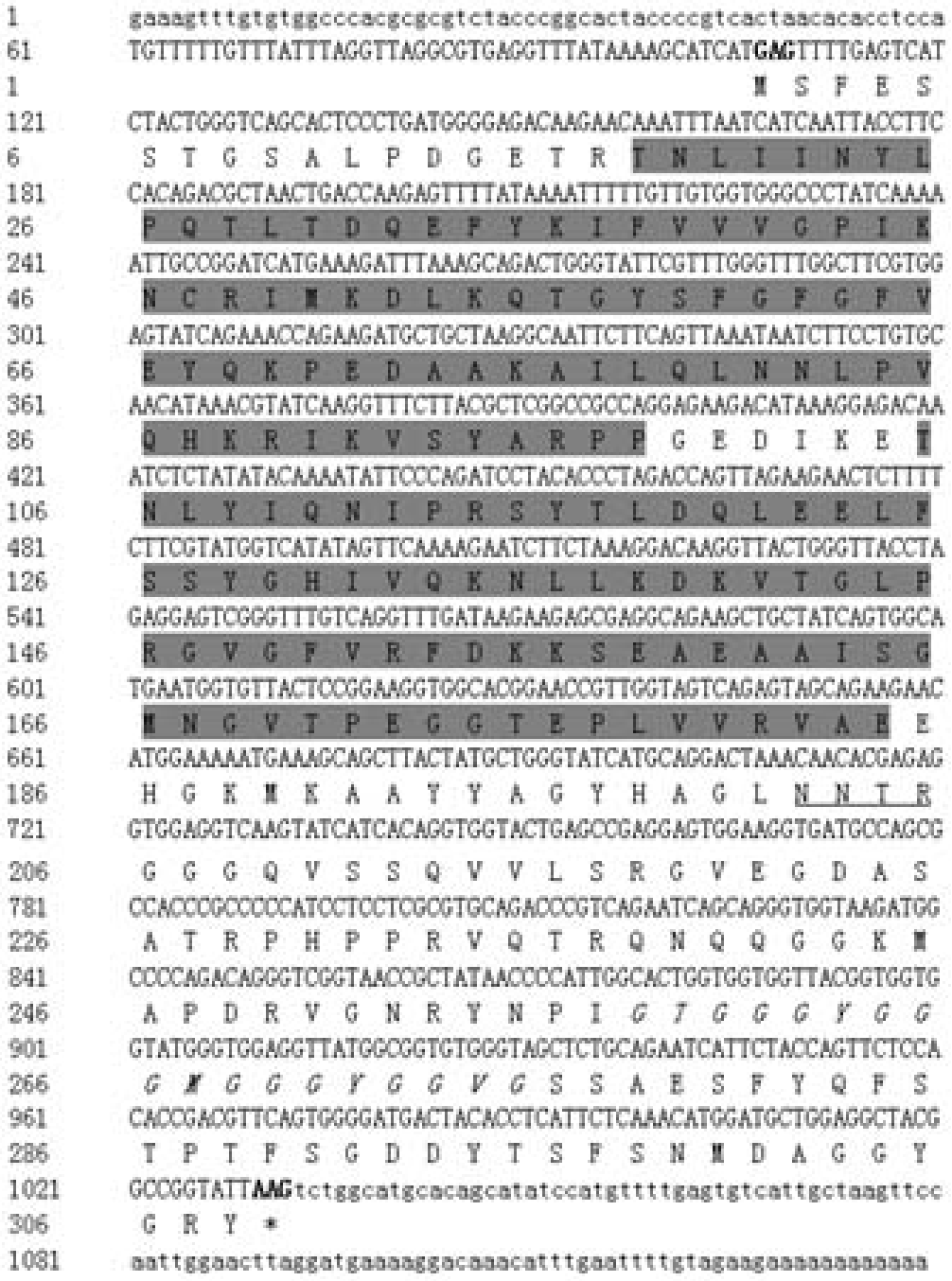


**Figure 1.** *Mnsxl1* nucleotide (above) and deduced amino acid (below) sequences.

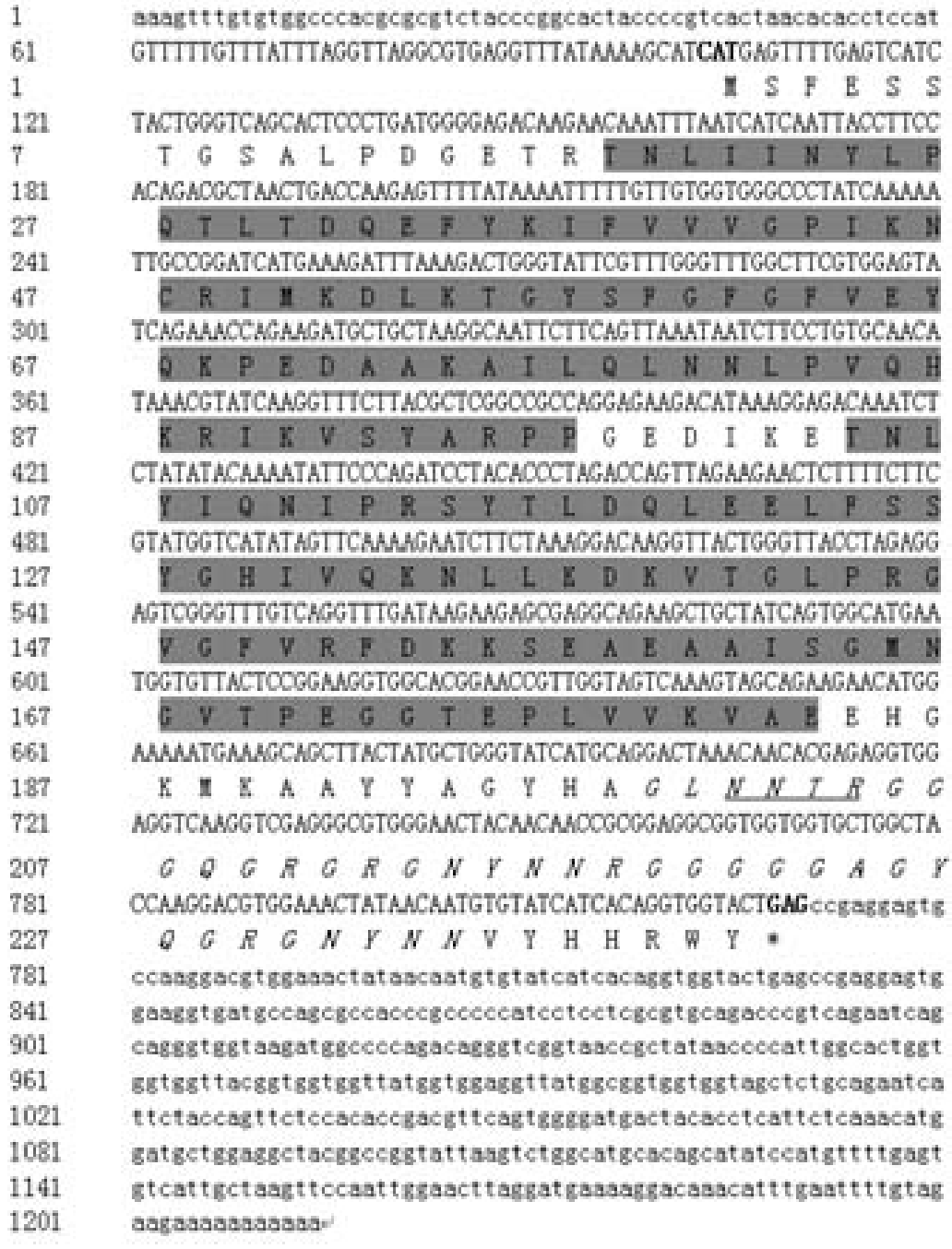

**Figure 2.** *Mnsxl2* nucleotide (above) and deduced amino acid (below) sequences.

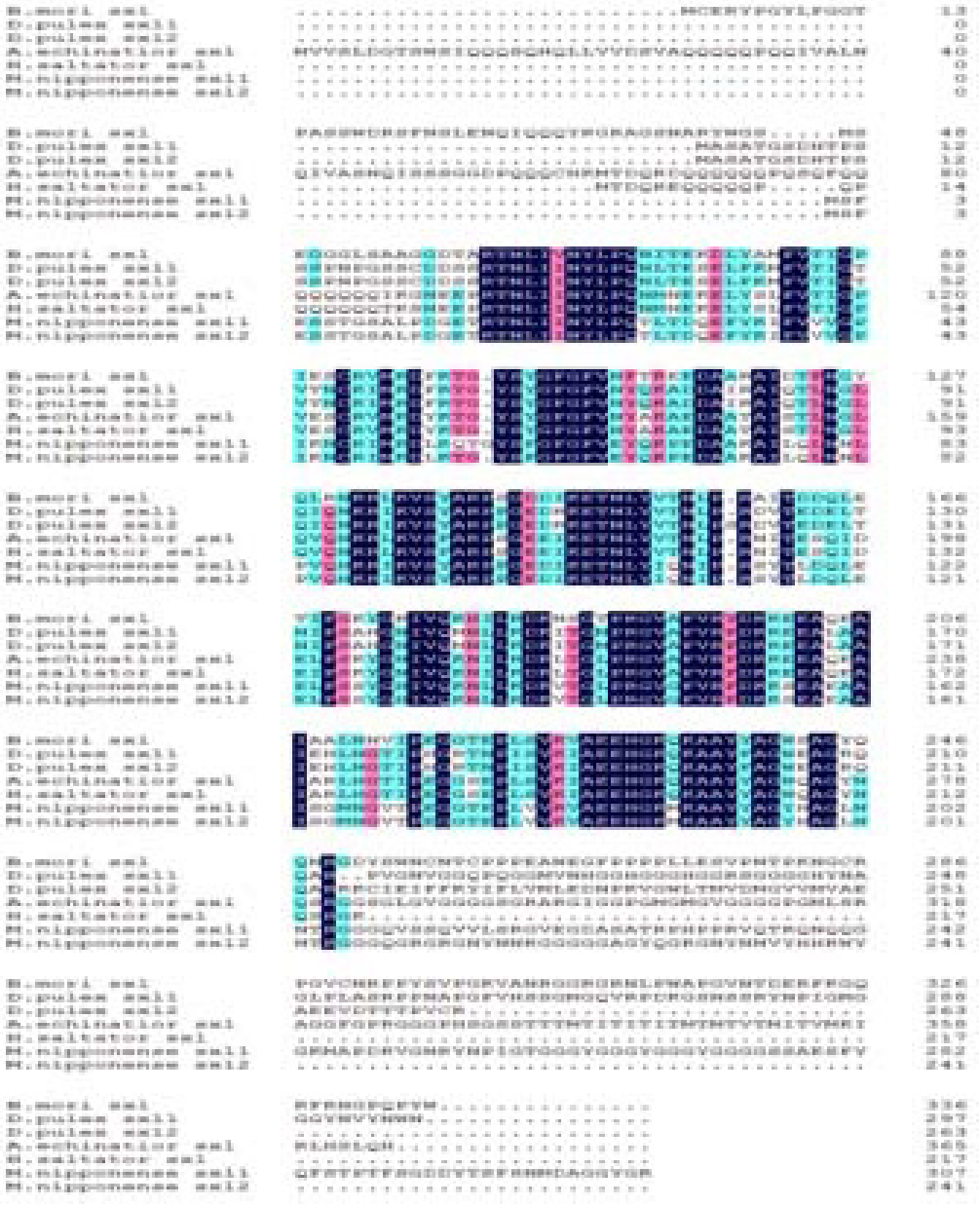

**Figure 3.** Alignment of *Macrobrachium nipponense* Sxl amino acid sequences with other species using DNAMAN.

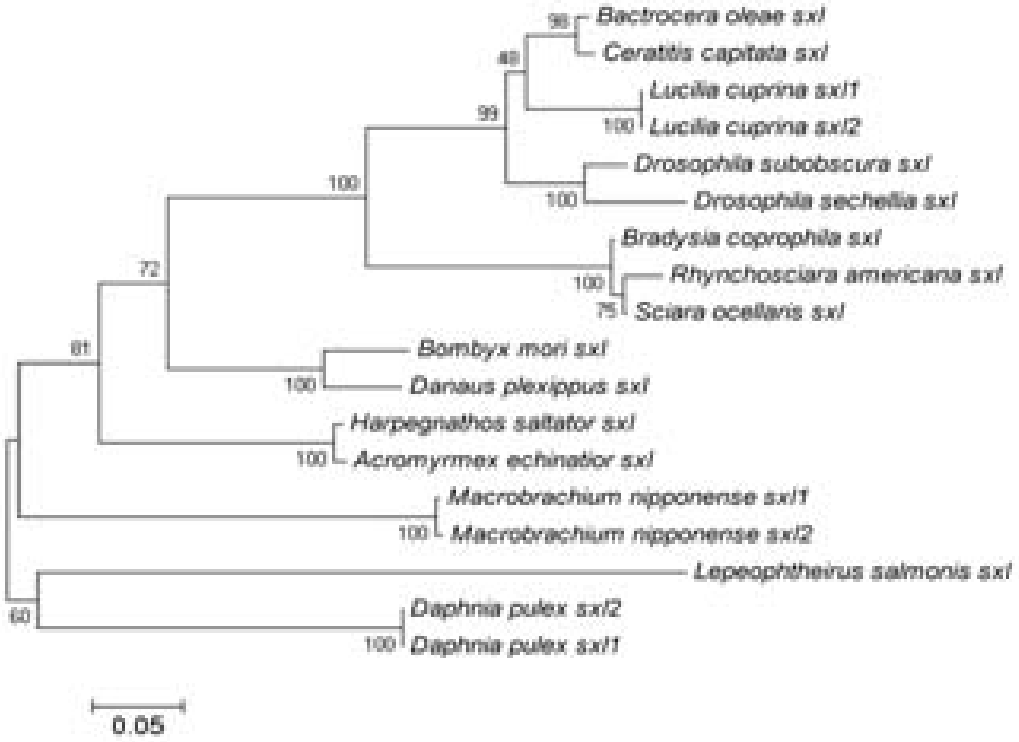


**Figure 4.** The neighbor-joining phylogenetic tree based on the sequences of Sxl from different species.

## Tissue distribution of *Mnsxl1* and *Mnsxl2* mRNA

Real-time quantitative RT-PCR analysis of *Mnsxl* mRNA indicated that the highest expression level was in the intestine (54.19-fold), followed by the liver (43.7-fold), muscles (9.06-fold), and testis (7.32-fold). The lowest levels were detected in the ovary tissue (1-fold). Similarly, the highest level of *Mnsxl2* was observed in the liver (52.18-fold) and then intestine (17.8-fold), and the lowest level was detected in the abdominal ganglion (1-fold) followed by that in muscles (1.23-fold) (Figure 5).

## Expression analysis of *Mnsxl1* and *Mnsxl2* mRNA during embryo, larvae, and post-larvae stages

*Mnsxl* mRNA expression levels were studied by performing real-time quantitative RT-PCR on embryos at different developmental stages, the larval and post-larval stages. The results revealed that both *Mnsxl* genes were expressed in all the developmental stages of the *M. nipponense* embryo. The expression level of *Mnsxl1* increased gradually from the cleavage stage (CS) to the gastrula stage (GS), and it was the highest at GS. Subsequently, it maintained a steady level from GS to the protozoea stage (PS), but declined abruptly at the zoea stage (ZS). However, the expression of *Mnsxl2* was slightly stronger in CS than in the blastula stage

(BS). After decreasing to a low level, the expression of *Mnsxl2* gradually increased with the development of the embryo and peaked at the nauplius stage (NS), where it maintained a steady level up to PS, and then again abruptly decreased at ZS (Figure 6).

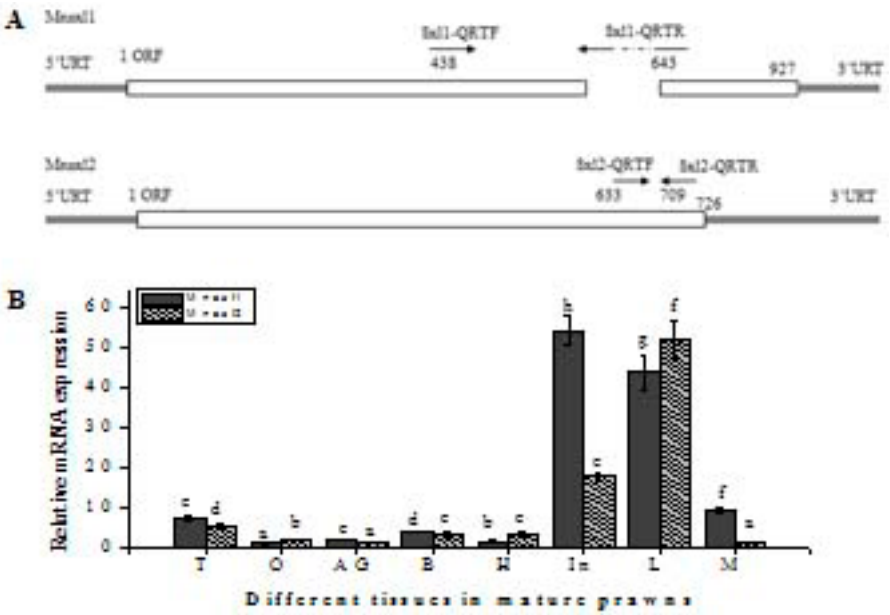


**Figure 5. A.** Diagrammatic representation of primer locations used in real-time quantitative RT-PCR. **B.** Relative expression levels of *Mnsxl1* and *Mnsxl2* transcripts quantified in various adult tissues.

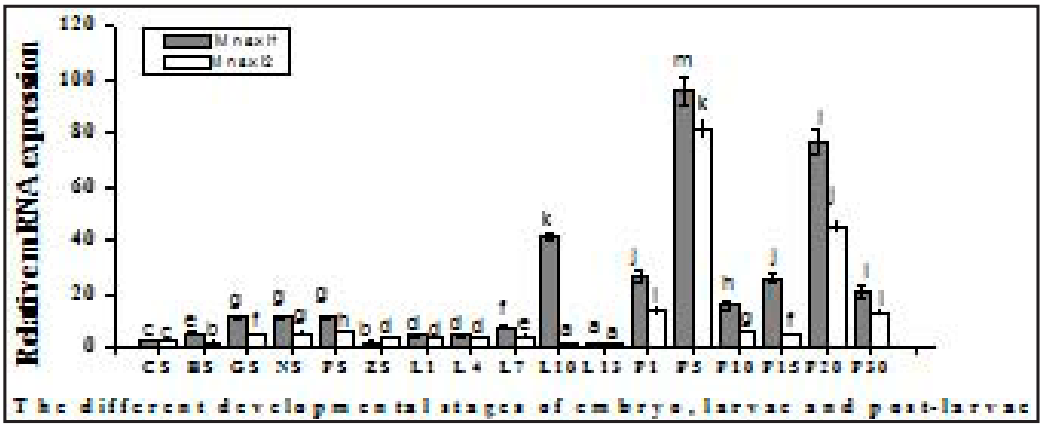


**Figure 6.** *Mnsxl1* and *Mnsxl2* mRNA expressions, normalized to β-actin, quantified in *Macrobrachium nipponense* during the embryos, larvae before the metamorphosis, and post-larvae after the metamorphosis.

During the larvae stage, the expression levels of *Mnsxl1* and *Mnsxl2* increased gradually with each day of larvae and reached the maximum at L10 (41.26-fold) or L7 (3.71-fold), respectively. The lowest expression levels of the *Mnsxls* were found at L13, as well as 1

day before metamorphosis (Figure 6). After metamorphosis, the larvae transitioned into post-larvae that resemble miniature adults. During the post-larval stage, the expression levels of *Mnsxl1* and *Mnsxl2* gradually increased from P1 to P5, reaching the highest level at P5 (96.18-fold and 81.66-fold. respectively), and then gradually decreased from P10 to P15. Subsequently, the *Mnsxl1* and *Mnsxl2* levels abruptly increased again at P20 (79.65-fold and 45.11-fold, respectively), but also abruptly declined at P30 (Figure 6).

## DISCUSSION

The sex-lethal gene (*Sxl*) is a master switch gene for sex determination. The sex-lethal protein belongs to the family of RNA-binding proteins, acting as a regulator of both alternative pre-mRNA splicing and translation (Sievert et al., 2000). In the present study, we cloned and identified two *Sxl* homologs in *M. nipponense* that we named *Mnsxl1* and *Mnsxl2*. *The* nucleotide sequence analysis showed that *Mnsxl1* and *Mnsxl2* shared 92.75% identity, but the *Mnsxl1* cDNA sequence lacked 80 bp compared with the sequence of *Mnsxl2*. Thus, we speculated that they are isoforms, which are rather common with Sxl and its homologs in insectssuch as *Lucilia cuprina* and *D. pulex* (Traut et al., 2006). The Sxl homologs of fly species contained three highly conserved domains, including two RNA-binding domains (RBD) and an Sxl-specific domain at the N-terminus (Sievert et al., 2000). The conserved sequence and characteristic motifs of RNA recognition motifs were identified in the deduced amino acid sequences of *Mnsxl1 and Mnsxl2,* which endow the Sxl protein with the capacity to bind to RNA. *T*he Sxl-specific domain in the *Sxl1* and *Sxl2* nucleotide sequences of *M. nipponense* was not observed, a reasonable explanation being that the Sxl-specific domain is not characteristic for Sxl in a wider range of insects; it was the most well-conserved part of this region in Diptera, but was not recognizable in non-dipterans (Traut et al., 2006). Compared with other invertebrate nucleotide sequences (Sievert et al., 2000; Lagos et al., 2005), the highest degree of similarity was observed in their two RBD domains (97.60~100%), suggesting that Mnsxl1 and Mnsxl2 were likely to perform similar functions for the capacity to bind to RNA. The C-terminal of the two isoforms was significantly different, suggesting that they have different spatial conformations and functions. Phylogenetic analysis revealed that *Mnsxl1* and *Mnsxl2* were closely related to Hymenoptera Sxl, but separated from their homologs of crustacean *L. salmonis* and *D. pulex*. The exact orthologous relation for Mnsxls needs further study when more Sxls are cloned from crustaceans.

Apart from *Drosophila*, Sxl has also been transcribed in adult gonads and somatic tissues of both sexes in *S. ocellaris* and *B. oleae* by northern blot analyses (Maine et al., 1985; Ruiz et al., 2003; Lagos et al., 2005), and *Sxl* expression was found to be extraordinarily high in the ovaries (Sievert et al., 2000; Ruiz et al., 2003; Cline et al., 2010). The current study was the first to report *Mnsxl* gene expression in the abdominal ganglion, which is part of the central nervous system in shrimp. In the examined tissues, the abdominal ganglion is the only organ reported to secrete hormones to induce ovary maturation in crustaceans (Kulkarni et al., 1991; Jin et al., 2003). Interestingly, the highest levels of *Mnsxl1 and Mnsxl2* mRNAs were detected in intestine and liver tissue, respectively, suggesting that Mnsxls are involved in other physiological functions of the shrimp.

The developmental process of oocytes in the prawn undergoes a series of cellular differentiations, in which differential genes are expressed temporally and spatially to en-

sure the proper development of oocytes (Qiu et al., 2005; Meeratana and Sobhon, 2007). In the present study, we found that the *Mnsxl1* and *Mnsxl2* mRNAs were expressed at every stage, including the cleavage stage during the embryonic development of M. nipponense, and had a similar expression pattern except at the blastula stage. It is believed that high levels of Sxl protein in fertilized eggs were derived from zygotic expression of the Sxl gene, and maternal Sxl mRNAs were not translated prior to fertilization (Salz et al., 1989; Bopp et al., 1991). The results of the present study showed that the expression of *Mnsxl1* was gradually increased with the development of the embryo, and reached a peak at the nauplius stage. The level of *Mnsxl2*, however, decreased at BS, and then gradually increased and peaked at PS, which was similar to the finding reported in the previous study (Bell et al., 1988; Keyes et al., 1992) on Drosophila that Sxl mRNA expression is zygotically activated at the blastoderm stage and decreased at late embryonic development. Because embryonic organogenesis of *M. nipponense* starts from the nauplius stage and is most active at the protozoea stage (Zhang et al., 2010), the expressions of *Mnsxl1* and *Mnsxl2* during embryogenesis and organogenesis in this study imply that *Mnsxl1* and *Mnsxl2* may play different roles in the embryonic development of *M. nipponense*.

During the larvae stage, *Mnsxl1* and *Mnsxl2* mRNA expressions gradually increased with the age of larvae and respectively peaked at L10 and L7 before the metamorphosis, but decreased to the lowest level at metamorphic climax in *M. nipponense*. We speculate that the expression patterns of *Mnsxl1* and *Mnsxl2* during larval development are necessary to stimulate pre-metamorphic larval changes of morphology and initiate metamorphosis, which is consistent with previous studies showing that the sex differentiation and sexually dimorphic development of the crustaceans begin after metamorphosis (Lee et al., 1994; Zhao, 2009). As is already known, changes of external sexual forms occur before those of the internal sexual forms (Zhu et al., 2011). In a follow-up study (unpublished), our laboratory found from histological sections that primordial germ cells (PGCs) of *M. nipponense* appeared in 10~15 days of post-larvae, and its external sexual forms occurred at 5~10 days post-larvae. The time of Mnsxl action in post-larvae tended to correlate with the occurrence time of PGCs and external sexual forms of *M. nipponense*, suggesting that the *Mnsxl* homologs were involved in sex differentiation and external sexual forms in *M. nipponense*.

In conclusion, our results together with literature evidence suggest that the *Mnsxl* homologs play important roles in the embryonic development and sex differentiation of *M. nipponense*. This study advances our understanding of the multiple biological functions of the *Mnsxl* genes, and lays a foundation for researching the regulation mechanism of the sex-determination pathway in *M. nipponense*.

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