Genetics and Molecular Research

Molecular cloning and expression analysis of *Fem1b* from oriental river prawn *Macrobrachium nipponense*

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ABSTRACT. Feminization-1 homolog b (*Fem1b*) is one of the genes essential for male development and play central roles in sex determination of *Caenorhabditis elegans*. In this study, we cloned and characterized the full-length *Fem1b* cDNA from the freshwater prawn *Macrobrachium nipponense* (*MnFem1b*) in different tissues and at different developmental stages. Real-time quantitative reverse polymerase chain reaction (RT-qPCR) showed that the *MnFem1b* gene was expressed in all investigated tissues, with the highest expression level found in the testes. The results revealed that the *MnFem1b* gene might play roles in aspects of development of the male prawn phenotype. The RT-qPCR also revealed that *MnFem1b* mRNA expression was significantly increased at 10 days after metamorphosis. The expression

levels in all investigated tissues showed a certain degree of sexually dimorphism, the expression levels in males were significantly higher than those in females ($P < 0.05$). Notably, the highest expression of *MnFem1b* was found in the testes. The expression of *MnFem1b* in different tissues indicates that it plays multiple biological functions in *M. nipponense*.

Key words: *Macrobrachium nipponense*; Expression pattern; Feminization-1 homolog b (*Fem1b*); Crustacean; RT-qPCR; Post-larval

INTRODUCTION

The feminization-1 (*Fem1*) gene is well known for its function as a signal transducing regulator affecting sex determination in the nematode *Caenorhabditis elegans*. The *Fem1* gene of *C. elegans* encodes an ankyrin (ANK) repeat protein that negatively regulates the transcription factor TRA-1, and functions as an E3 ubiquitin ligase substrate recognition subunit (SRS) to target TRA-1 for ubiquitylation (Doniach and Hodgkin, 1984; Spence et al., 1990; Hodgkin, 2002; Jäger et al., 2004; Starostina et al., 2007). Except in nematodes, the *Fem1* gene family, which consists of at least three members designated *Fem1a*, *Fem1b*, and *Fem1c*, has also been identified in mammals. The other *Fem1* gene family members show a high degree of evolutionary conservation with that of *C. elegans*, indicating that they may play similar roles (Chan et al., 2000; Ventura-Holman and Maher, 2000; Krakow et al., 2001; Ventura-Holman et al., 2003). Recently, *Fem1* homologs have also been found in insects (Shi et al., 2013; Xiong et al., 2014) and crustaceans (Robinson et al., 2014; Song et al., 2015). This leads to a new line of questioning: whether the sex determination mechanism of arthropods is similar to that of nematodes. A single nucleotide polymorphism marker, occurring in a transcript of *Fem1*, has been found to be significantly associated with sex in *Penaeus monodon*, whereas the expression profile of three crab *Fem1* genes (designated *EsFem1a*, *EsFem1b*, and *EsFem1c*) indicated that they might function in crab early sex determination and late gonad development. Together, these results provide new insights into the crustacean sex-determining mechanism.

The oriental river prawn, *Macrobrachium nipponense* (Crustacea; Decapoda; Palaemonidae) is a commercial freshwater prawn. It is considered an important commercial species in aquaculture and a capture fishery resource in China (Fu et al., 2012), with a calculated capture of 251,000 tons in 2013 (Bureau of Fishery, 2014). Male oriental river prawns are believed to grow faster and achieve higher weights during harvest compared to females. Culturing of all-male populations may therefore be beneficial economically. Therefore, studies on sex-determination are of great importance to *M. nipponense* aquaculture. However, little information on sex-determination has been reported, and the sex-determination mechanism remains unclear in *M. nipponense*.

In the current study, we identify *Fem1b* homologs in the androgenic gland transcriptome (Jin et al., 2013) of *M. nipponense*. In addition, we report the full length of the cDNA sequence and amino acid characterization of the *Fem1b* gene of *M. nipponense* and analyze its structural characteristics. A phylogenetic tree was built, in order to establish *MnFem1b* orthology. Moreover, the expression levels of the *MnFem1b*gene in different adult tissues and

developmental stages of embryos and larvae were determined, using real-time quantitative polymerase chain reaction (RT-qPCR). These results will provide a better understanding and directions for future research on the mechanisms of sex determination in *M. nipponense*.

MATERIAL AND METHODS

Prawn and tissue preparation

Adult *M. nipponense* with an average body weight of 2.3-4.5 g were obtained from Tai Lake in Wuxi, China (120°13'44''E, 31°28'22''N). Different developmental stages and tissues of adult animals were used for total RNA extraction. Adult prawns were kept under laboratory breeding conditions at 23°C and acclimatized in a 500-L tank with aerated freshwater for 72 h before tissue collection. Embryos and larvae at different developmental stages were obtained from a breeding room. After prawn spawning, each embryonal developmental stage (cleavage stage, blastula stage, gastrula stage, five larval stages, and three post-larval stages) was collected and inspected using an Olympus SZX16 microscope. The samples were classified based on morphological characters, following the criteria of Chen et al. (2012). The ovary, testis, muscle, heart, androgenic gland, brain, eye stalk gill, and hepatopancreas were also collected from mature male and female prawns. The samples were washed with 1X 0.01 M phosphate-buffered saline, frozen in liquid nitrogen, and stored at -20°C until processed (Zhang et al., 2013; Jiang et al., 2015).

RNA isolation and reverse transcription

RNA was extracted from the different developmental stages and tissues fromat least three adult *M. nipponense*, using RNAiso plus kit (TaKaRa, Dalian, China) following the manufacturer protocol. The RNA was treated with RNase-free DNase I (Sangon, Shanghai, China), to remove any possible genomic DNA contamination. The quality and quantity of RNA were assessed by BioPhotometer (Eppendorf, Hamburg, Germany) at 260 and 280 nm, respectively. To check the integrity of each sample, 2 μL was analyzed on 1% agarose gel. The concentration of each RNA sample was adjusted to 1 mg/μL with nuclease-free water. First-strand cDNA synthesis was performed, using 1 μL total RNA, 4 μL 5X iScript reaction mix (Bio-Rad, USA), and 1 μL iScript reverse transcriptase to a final volume of 20 μL. Then, 15 μL water was added to reach a final volume of 20 μL. The reaction was incubated at 25°C for 5 min, at 42°C for 30 min, followed by 85°C for 5 min. The reverse transcribed cDNA was stored at -20°C until the RT-qPCR.

Rapid amplification of cDNA ends (RACE)

Based on the DNA sequence data obtained from the androgenic gland transcriptome search (Jin et al., 2013) (http://www.ffrc.cn/gene/list.asp), a pair of gene-specific primers were designed and synthesized to clone the full-length cDNA of *MnFem1b* by rapid amplification of cDNA ends (RACE), using testis RNA as a template. All primers used in RACE are presented in Table 1. Cloning the full-length *MnFem1b* cDNA sequence was performed using a 5' and 3' RACE cDNA Amplification Kit (TaKaRa) according to the manufacturer protocol.

The *MnFem1b* cDNA sequences were extended using 5'/3' RACE. The PCR products were subjected to electrophoresis on 1.5% agarose gels, to compare differences in product length. The DNA bands of the expected size were excised from the agarose gel and purified using a DNA gel extraction kit (Axygen, Union City, CA, USA). Amplified cDNA fragments were cloned in the pMD18-T vector (TaKaRa) and sequenced with M13 forward and reverse primers. Subsequently, at least five independent clones were sequenced from each cDNA and the sequences were verified and subjected to cluster analysis in NCBI (http:/www.ncbi.nlm.nih.gov/).

Table 1. Nucleotide sequences of primers used for Mn*Fem1b* cloning and expression analysis.

	Sequence (5'→3')	Code
Primers for 5'RACE PCR		
MnFem1b 5' GSP primer 1	TCCTGATTTCGCTTCAGTTCCA	GSP1
MnFem1b 5' GSP primer 2	ACATCTGTCGAACCTGGCATTA	GSP2
Primers for 3'RACE PCR		
MnFem1b 3' GSP primer 1	GGTAAGAGTGCAACCGAGAACTA	GSP3
MnFem1b 3' GSP primer 2	TCAAGTTCCGTATCACCTGGAAG	GSP4
Full RACE™ Kit primers		
3' RACE OUT	ATCCGACGAAGACAAACTCTACC	
3' RACE IN	CGCGGATCCTCCACTAGTGATTTCACTATAGG	
5' RACE OUT	CTCCACAATGGGCTTTTTCATCC	
5' RACE IN	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	
Primers for real-time PCR analysis		
MnFem1b 5' primer	CTCCACAATGGGCTTTTTCATCC	RT-F1
MnFem1b 3' primer	ATCCGACGAAGACAAACTCTACC	RT-R1
β-actin 5' primer	TATGCACTTCCTCATGCCATC	actinF
β-actin 3' primer	AGGAGGCGGCAGTGGTCAT	actinR

Nucleotide sequence and bioinformatic analyses

Based on the BLASTx algorithm (http:/www.ncbi.nlm.nih.gov/BLAST/), the *MnFem1b*sequence from the AG transcriptome of *M. nipponense* was found to be highly homologous to the Chinese mitten crab *Eriocheir sinensis Fem1b (EsFem1b*) gene family. The 5' and 3' sequences from the RACE were assembled with the fractional cDNA sequence homologous to each fragmental sequence using DNAMAN 5.0. Sequences were analyzed, based on the nucleotide and protein databases using the BLASTx and BLASTn programs (http:/www.ncbi.nlm.nih.gov/BLAST/). Protein prediction was performed using 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 using the motif scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan/). The functional domain analysis was performed using SMART GENOMES (http://smart.embl.de/smart/set_mode.cgi?GENOMIC=1). To predict the signal peptide, SignalP 4.1 Server was used (http://www.cbs.dtu.dk/services/SignalP/). Multiples of *Fem1* homologue b (Table 2) were formed using DNAMAN 5.0. Based on the amino acid sequences, a phylogenetic tree was constructed by means of a neighbor-joining method. The bootstrapping test was performed using 1000 replications with MEGA4 (Tamura et al., 2007).

Table 2. *Fem1b* information used for sequence alignment and phylogenic analysis.

Species	Molecular name/accession number	Order/family	Identity %
Homo sapiens	Fem-1a/AAH04988	Primates/Hominidae	29
	Fem-1b/AAH14558Fem-1c/AAH28369		51
			43
Mus musculus	Fem-1a/AAC82372	Rodentia/Muridae	30
	Fem-1b/AAC82373		51
	Fem-1c/NP_775599		35
Locusts migratoria manilensis	Fem-1a/BAL61211	Orthoptera/Acrididae	25
	Fem-1b/BAL61212		65
	Fem-1c/BAL61213		32
Litopenaeus vannamei	Fem-1/AHA90856	Decapoda/Penaeidae	32.66
Eriocheir sinensis	Fem-1a/KR108010	Decapoda/Varunidae	53
	Fem-1b/KR108011		81
	Fem-1c/KR108012		55
Zootermopsis nevadensis	Fem-1a/KDR09672.1	Isoptera/Termopsidae	25
	Fem-1b/ KDR11581		66
Drosophila melanogaster	Fem-1a/AAF57431	Diptera/Drosophilidae	40
	Fem-1b/AAM68397		43
Macrobrachium nipponense	Fem-1a/ KT258023	Decapoda/Palaemonidae	100

The table shows the identity percentage between different species *Fem1a*, *b*, and *c* and *MnFem1b*.

Real-time quantitative PCR analysis of *Fem1b*

The transcriptional levels of the *MnFem1b* gene from the embryonal to post-larval stages and various adult tissues were measured using the SYBR Green RT-qPCR analysis using a Bio-Rad iCycler iQ5 Real Time System (Bio-Rad). The *MnFem1b* and β-actin primers are listed in (Table 1) were used to amplify the *MnFem1b* transcript, and the PCR products were sequenced, to verify the specificity of the PCR primers. From each developmental stage and each tissue, three samples were collected. Each sample was analyzed in triplicate. Each PCR well contained 25 μL PCR mixture composed of 1μL cDNA (50 ng), 10 μL SsoFast™ EvaGreen® Supermix (Bio-Rad), 0.5 μL 10 μM gene-speciðc forward and reverse primers (Table 1), and 13 μL diethylpyrocarbonate-water (DEPC-water). The reaction mixture was initially incubated for 30 s at 95°C to activate the Hot Start Taq DNA polymerase, followed by 40 cycles of 10 s denaturation at 95°C and a 10 s extension at 60°C. The melting curve analysis was performed over a range of 65-95°C (in 0.5°C increments) for 10 s, to verify the generation of a single product. Amplification of β-actin (Zhang et al., 2013) as an internal reference was also carried out in the same sample (primer sequences are shown in Table 1). As a negative control, DEPC-water replaced the template. The relative copy number of *MnFem1b* mRNA was calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method (Livak and Schmittgen, 2001).

Statistical analysis

Data were statistically analyzed using SPSS v. 19 (SPSS, Chicago, IL, USA) by one-way analysis of variances (ANOVA) followed by LSD multiple comparisons. Significant differences among the group means were further compared using Duncan's multiple range tests. $P \leq 0.05$ was considered statistically significant. Results are reported as means ± SE (N = 3).

RESULTS

Sequence analysis of *MnFem1b*

The full-length cDNA sequence of *MnFem1b* was 2596 bp, and contained an open

reading frame of 2055 bp, encoding a 684-amino acid protein. The protein had an estimated molecular mass of 75.6638 kDa, an isoelectric point of 6.74 with an untranslated region of 183bp the 5' end, a 3'-untranslated region of 358 bp, and a complete poly A tail. The A+T and C+G contents in the sequence were 53.8 and 46.2%, respectively. This cDNA sequence was submitted to GenBank under accession No. KT258023. According to the blastx analysis, the sequence showed significant homology with the *E. sinensis EsFem1 a*, *b*, and *c* genes with an identity of 53, 81, and 55%, respectively. The conserved sequences and characteristic motifs of the chromatin organization modifier domain and N-terminal RGD, were identified in the deduced amino acid sequences of *MnFem1b*. The conserved motifs were located in the N- and C-terminal region, respectively (Figure 1A-B). The region with highest identity between *MnFem1b* and *EsFem1b* was the N-terminal third of the amino acid sequences, which were composed of six continuous ANK repeats (Figure 1A).

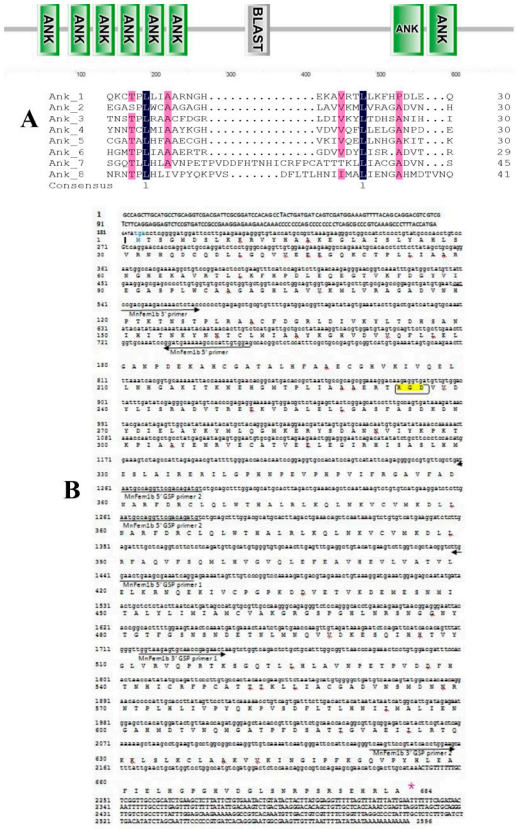

Figure 1. A. Domain analysis of *Fem1b* amino acid sequences in *Macrobrachium nipponense*. The SMART analysis of *MnFem1b* and the sequence alignment of eight ANK repeats. The boxes represent the ANK structural elements. Identical and similar residues are highlighted in black and pink, respectively. **B.** Nucleotide and deduced amino acid sequence of *MnFem1b*. The nucleotide sequence is displayed in the 5' to 3' direction and numbered at the left. The deduced amino acid sequence is shown in a single small letter amino acid code. The 3'- and 5'UTR are shown in capital letters. The codons are numbered at the left with the methionine (ATG) initiation codon (highlighted in blue text); an asterisk denotes the termination codon (TAA). The RACE and RT-qPCR primers are marked with arrows. RGD is highlighted in yellow.

The signal peptide analysis identified no signal peptides in the amino acid sequence of *MnFem1b*. The subcellular localization analysis revealed that the location of the predicted protein was in the cytoplasm. The amino acid sequence of *MnFem1b*, when analyzed by functional domain, had eight ANK repeats, which consisted of approximately33 amino acids (Figure 1A). Compared with *EsFem1b*, which has nine ANK repeats, *MnFem1b*has eight ANK repeats, six of which are located at the N-terminal in both *MnFem1b* and *EsFem1b*.

ExPASy were used to determine the protein functional sites in *MnFem1b*. The mature protein was composed of three N-glycosylation sites, one cAMP-and cGMP-dependent protein kinase sites, 13casein kinase II phosphorylation sites, eight N-myristoylation sites, 10 protein kinase C phosphorylation sites, one cell attachment sequence (RGD) and one tyrosine kinase phosphorylation site (Table 3).

Table 3. Functional site analysis of mature *MnFem1b* protein.

Function site	AA position
N-glycosylation site	157-160 (NNTC), 472-475 (NRSN), 478-481 (NYTG)
cAMP- and cGMP-dependent protein kinase phosphorylation site	630-633 (KKLS)
Casein kinase II phosphorylation site	3-6 (SGMD), 217-220 (TKNE), 233-236 (TRGD), 243-246 (SRAD), 248-251 (TREE),265-268(SDKD),313-316(TVEE),417-420(TVLE),439-442(TVKD),487-490(SNDE),533-536(TPVD),612-615(TPFD), 677-680 (SRSE)
N-myristoylation site	19-24 (GLAISL), 114-119 (GADVNH), 213-218 (GSKITK), 260-265 (GASFAS), 354-359 (GAVFAD), 476-481 (GGNYTG), 484-489 (GSNSND), 559-564 (GADVNS)
Protein Kinase C- phosphorylation site	7-9 (SLK), 29-31 (SVK), 81-83 (TVK), 153-155 (TNK), 265-267 (SDK), 439-441 (TVK), 551-553 (TTK), 628-630 (TQK), 633-635 (SLK), 673-675 (SNR)
Cell attachment sequence	234-236 (RGD)
Tyrosine kinase phosphorylation site	232-240 (RTRgDVVdY)

Homology and phylogenetic analysis of *MnFem1b*

The *MnFem1b*amino acid sequence was compared with the sequences of previously described *Fem1b* proteins (Figure 2). The *MnFem1b* protein shared high identity with other species, including *Zootermopsis nevadensis* (KDR19644.1) 66% and *Locusta migratoria manilensis* (BAL61212.1) 65%. However, *MnFem1b* showed the highest homologywith another crustacean, *E. sinensis* (KR108011) *EsFem1b*, with an identity of 81% (Table 2). The neighbor-joining phylogenetic tree of *MnFem1b* was constructed based on *Fem1a*, *b*, and *c* homologs from other species, using the MEGA 4.0 software (Figure 3) tree included Fem gene from crustaceans and other species. The species included in the phylogenetic tree are summarized in Table 2.

Tissue distribution of *MnFem1b* mRNA

The expression patterns of *MnFem1b* in the testis, ovary, heart, hepatopancreas, brain, muscle, eyestalk, gill, and androgenic gland tissues were examined. The RT-qPCR analysis of *MnFem1b* mRNA indicated that it was expressed in all examined tissues in the adult prawns, with the highest expression in the testis, followed by the hepatopancreas. The lowest levels were detected in the muscle tissue (Figure 4). In addition, we observed sexually dimorphic expression of *MnFem1b*; the mRNA expression was higher in male compared to in female tissues ($P < 0.05$; Figure 4).

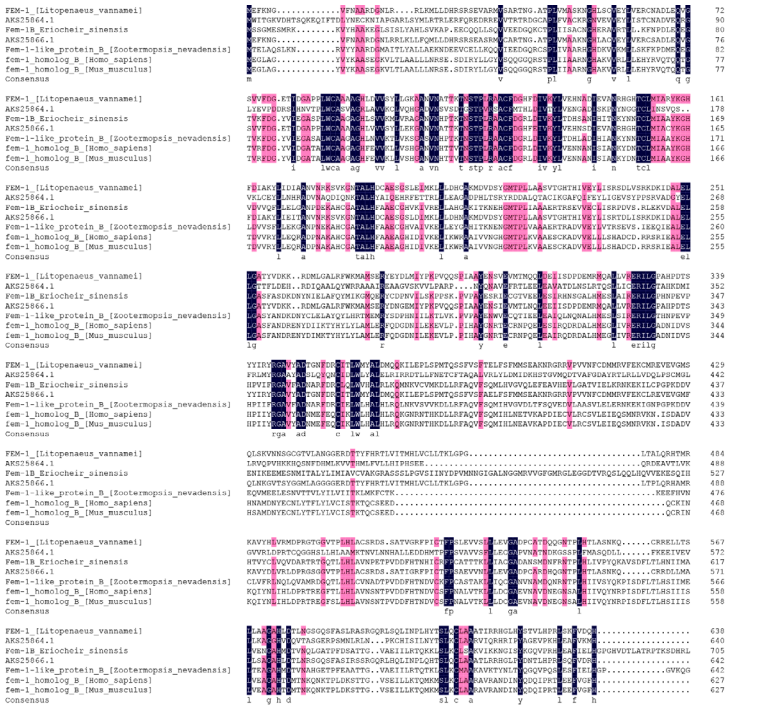

Figure 2. Multiple alignments of *Macrobrachium nipponense Fem1b* sequence with that of other species, using DNAMAN. The deduced amino acid sequences are summarized in Table 2. Identical amino acid residues are highlighted in black and similar amino acids are highlighted in pink. Dotted inserts are added to maximize sequence identity.

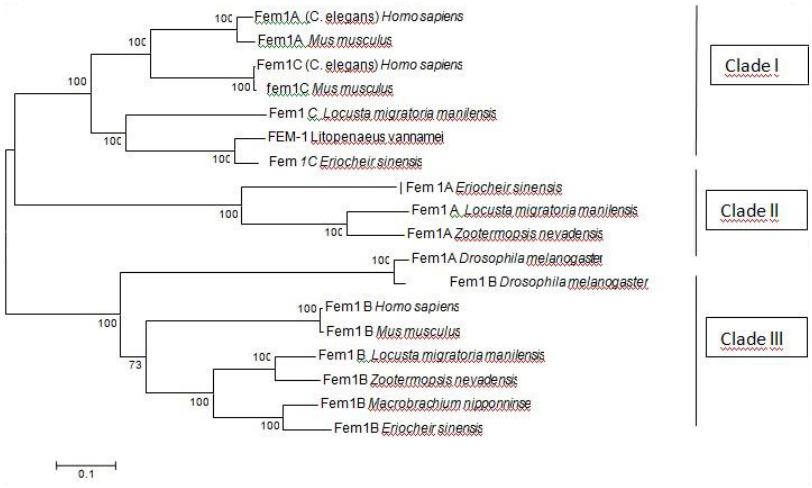

Figure 3. Neighbor-joining phylogenetic tree of *MnFem1b*and *Fem1b* homologs. The species used in the phylogenetic tree are summarized in Table 2. Bootstrap analysis of 1000 replicates was carried out to determine the confidence of tree node positions. The numbers indicated at the tree nodes represent the bootstrap values. Numbers in the branches represented the bootstrap values (%) from100 replicates.

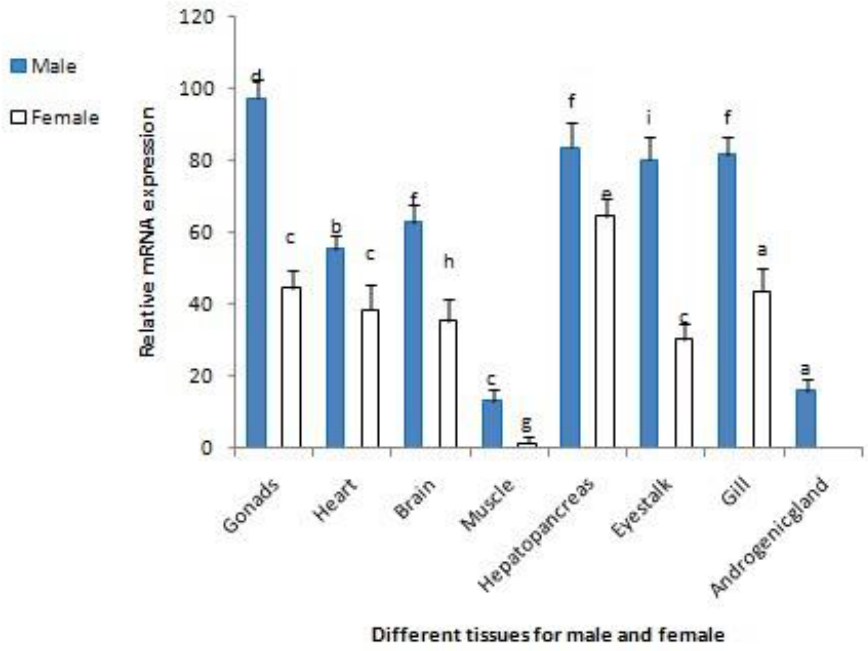

Figure 4. Distribution of *Fem1b* mRNA in different tissues of male and female prawns. Data are reported as the mean fold change (means ± standard error of the mean, N = 3). Statistical significance was calculated by one-way analysis of variance followed by multiple comparison tests. Bars with different letters were significant at $P < 0.05$. Gonads: testis and ovary.

Expression analysis of *MnFem1b* mRNA during embryo, larvae, and post-larval stages

MnFem1b mRNA expression levels were examined using RT-qPCR on *M. nipponense* embryos at different developmental stages, including both larval and post-larval stages. The results revealed that the *MnFem1b* gene was expressed at all developmental stages. The expression level increased gradually from the cleavage stage to the gastrula stage, it abruptly decreased during the pre-nauplius stage, and then increased again during the pre-zoea stage. This was followed by a gradual decrease until the expression reached its lowest level at day 10 of the larval stage. After metamorphosis, the expression of *MnFem1b* mRNA abruptly increased with increasing number of days after metamorphosis peaking at day 10 of the post-larval stage (Figure 5). Subsequently, expression both increases and decreases throughout the larval phases.

DISCUSSION

ANK repeats are composed of several modules of approximately33 amino acids. It is one of the most common protein-protein interaction motifs, functioning directly or indirectly in signal transduction, transcriptional regulation, and developmental regulation (Lambert et al., 1990; Bork, 1993; Batchelor et al., 1998; Baumgartner et al., 1998; Li et al., 2009). They

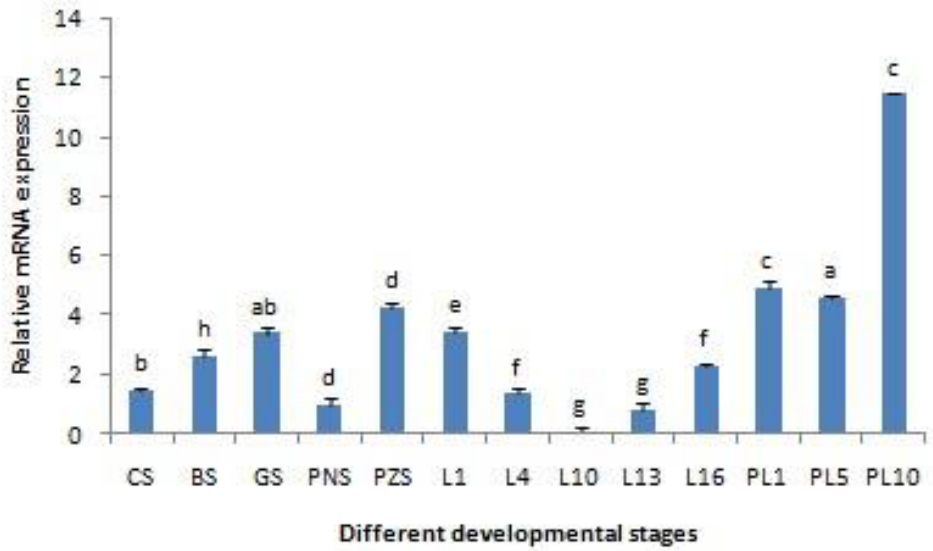

Figure 5. Temporal expression of *MnFem1b* during different developmental stages. Data are reported as means ± standard error of the three repeated samples during the embryo, larva, and post-larva stages. Bars with different letters were significant at $P < 0.05$. CS = cleavage stage; BS = blastula stage; GS = gastrula stage; PNS = pre-nauplius stage; PZS = pre-zoea stage; L1 = first day larva after hatching; LX = larval day X after hatching; P1 = first day post-larva after metamorphosis; PLX = post-larval day X after metamorphosis.

are highly conserved among diverse organisms, and the number of repeats ranges from seven to nine in *Fem1*. In *C. elegans*, seven ANK repeats were identified (Spence et al., 1990), whereas nine repeats were found in the freshwater pearl mussel *Hyriopsis schlegelii* (Xiong et al., 2014). For different members of *Fem1* (*a*, *b*, and *c*), the number of ANK repeats vary. Nine, eight, and nine ANK repeats have been detected in mouse and human *Fem1a*, *Fem1b*, and *Fem1c*, respectively (Ventura-Holman et al., 1998, 2003), and eight repeats were found in locust *Fem1* (*a*, *b*, and *c*) (Shi et al., 2013). In the mitten crab, the *Fem1a*, *Fem1b*, and *Fem1c* amino acid sequences have eight, nine, and eight repeats, respectively (Song et al., 2015). In our paper, *MnFem1b*contained eight ANK repeats, six of which were located in the N-terminal. The difference in *Fem1b* between prawns and other species might imply an evolutionary conservation and functional variation involved in nematode sex determination.

A neighbor-joining phylogenetic tree was constructed, based on the amino acid sequences of *Fem1* from various species. It indicated that individual members of the *Fem1* family were strongly conserved in the evolution across lineages. All *Fem1b* were clustered into one clade. *MnFem1b* clustered with the crabs *Fem1b* which indicated the highest closer relationship than fem1b from other species. The phylogenetic analysis revealed that the strong and stable conservation of the *Fem1* family was not simply based on a general ANK repeat consensus, but also on the sequence of the whole amino acid sequence.

The tissue distribution profile illustrated that the *MnFem1b* gene was expressed across several tissues, which indicates that it might have various functions in different tissues. The tissue expression profile of *MnFem1b* was similar to that found in human and Chinese mitten crab, displaying a high expression level in testis (Chan et al., 1999; Song et al., 2015). In

contrast, it showed some differences with the expression level patterns found in mouse, in which the highest expression levels were found in the heart and skeletal muscle (Ventura-Holman et al., 1998). *MnFem1b*also showed a certain degree of sexually dimorphic expression with the expression level in males being significantly higher than in females ($P < 0.05$). Notably, *MnFem1b* showed the highest level of expression in the *M. nipponense* testis, which was also found in *EsFem1b* in crabs, indicating their potential role in aspects of the male phenotype (Song et al., 2015).

In the current study, *MnFem1b* mRNA was expressed differently during different developmental stages of *M. nipponense*. The expression level gradually increased with embryonic development from the cleavage to the gastrula stage followed by a significant decrease in the pre-nauplius stage. It then reached a peak expression level in the pre-zoea stage. The blastula and gastrula stages are two key stages of cell differentiation and organ formation (Zhang et al., 2010). The embryonic development expression results indicate that the functions of *MnFem1b* were lasting and complex and might be involved in the sex determination. Our results are different from the results found in crabs and *C. elegans*, in which the authors inferred that the *Fem1* of both species were maternal genes that might help protect the identity and integrity of the germ line (Johnson and Spence, 2011; Song et al., 2015). Therefore, we speculate that *MnFem1b* may have other important roles during the embryonic development and organogenesis of *M. nipponense*. After hatching, the *M. nipponense* larvae need to undergo changes in morphology and appendage characteristics. During this time, the expression of *MnFem1b* remained at a low level, increasing markedly just after the metamorphosis. This suggests that *MnFem1b*playsa certain role in the metamorphosis and morphological differentiation (Unni et al. 2003; Burghardt et al. 2005). In our previous study (Zhang et al., 2015), we found that primordial germ cells in *M. nipponense* appeared at 10-15 days post-larvae with external sexual forms occurring at 5-10 days post-larvae. The patterns of *MnFem1b*expression in post-larvae tended to coincide with the occurrence of primordial germ cells (PGCs) and external sexual forms of *M. nipponense*, suggesting that *MnFem1b*is involved in sex differentiation in *M. nipponense*.

In conclusion, we cloned the full length novel gene *MnFem1b* and investigated its transcriptional patterns in different tissues and development stages in *M. nipponense*. Important roles of *MnFem1b* may be found in the embryonic development and in post-larval external sexual formation of *M. nipponense*. From this study, we can infer that *MnFem1b*might have multiple biological roles. Further detailed investigations should be carried out to elucidate the specific functions of *MnFem1b*.

Conflicts of interest

The authors declare no conflict of interest.

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