Molecular cloning, expression pattern analysis, and in situ hybridization of a *Transformer-2* gene in the oriental freshwater prawn, *Macrobrachium nipponense* (de Haan, 1849)

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Abstract

In this study, we isolated a full-length cDNA sequence from *Macrobrachium nipponense* and investigated its gene function. We named the gene *Mntra-2a* because of high similarities and close evolutionary divergence with arthropod *tra-2*. The full-length cDNA of *Mntra-2a* was 1293 bp, consisting of a 212 bp 5' UTR, a 268 bp 3' UTR, and an ORF of 813 bp encoding 270 amino acids. It contained an RNA recognition motif and a linker region. Real-time PCR analysis showed that *Mntra-2a* was highly expressed in the gonads of both males and females. Further in situ hybridization analysis showed that *Mntra-2a* was mainly located in oocytes and spermatocytes. During embryogenesis, *Mntra-2a* expression was higher in the cleavage and nauplius stages. During the ovarian reproductive cycle, *Mntra-2a* expression reached a peak at Ovary V and decreased to the lowest level at Ovary IV. These results indicated that *Mntra-2a* probably played important roles in embryonic development and early gonad development in *M. nipponense*. Our results provide basic information for further functional studies of *tra-2* in *M. nipponense*.

Keywords *Macrobrachium nipponense* · Transformer-2 · Temporal and spatial expression · In situ hybridization

Introduction

The oriental river prawn, *Macrobrachium nipponense*, is an economically important freshwater prawn and is widely farmed in China with an annual production of almost 272,592 tons in 2016 (Bureau of Fishery 2016). The male individuals of the oriental river prawn grow faster than female individuals of the species. The average weight of commodity male individuals is 2–2.5 times that of females. An all-male culture will help to increase the yield and economic value of *M. nipponense* and could be achieved through sexual control technology. Therefore, it is important

to identify sex-determining genes and their regulatory mechanism.

Animals are under a variety of sex determination methods and this is currently the focus of research. Gender-determining methods in insects that are closely related to crustaceans are currently studied, especially with model species such as *Drosophila* (Burtis and Baker 1989; Martín et al. 2011). The sex determination mechanism of crustaceans is relatively complex and impacted by environmental factors. At present, studies in the sex determination mechanism of crustaceans are limited, and most species-related studies are still undefined. Insulin-like androgenic gland factor (IAG), a unique gene in crustaceans, plays an important role in sex differentiation, but its specific molecular mechanism is not yet clear (Ventura et al. 2008; Chung et al. 2011; Ma et al. 2013; Vega-Alpizar et al. 2017; Guo et al. 2018).

Insect sex determination involves a series of bottom-up regulatory genes (Wilkins 1995). Sex-lethal (*sxl*), which regulates female-specific splicing of the precursor mRNA of the *transformer* (*tra*) gene, is the major switch gene at the top of the cascade. (Chen et al. 2017). The functional *tra* protein interacts with the protein product of the *transformer-2* (*tra-2*)

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gene, which leads to female-specific splicing of the mRNA of *doublesex* (*dsx*) in the cascade. (Sievert et al. 1997; Chen et al. 2017). *Transformer-2* (*tra-2*) is an important cofactor for sex determinations in most insect species (Hediger et al. 2010; Sarno et al. 2010; Schetelig et al. 2012; Geuverink et al. 2017). Studies of *tra-2* knockdown in insects revealed participation of *tra-2* in female-specific splicing of *tra* mRNAs (Burghardt et al. 2005; Concha and Scott 2009; Sarno et al. 2010; Martín et al. 2011; Nissen et al. 2012; Geuverink et al. 2017). In some species of insect, *tra-2* has alternative isoforms which all encode for a basic RNA recognition motif (RRM) (Niu et al. 2005; Martín et al. 2011; Nissen et al. 2012; Shukla and Palli 2013). Only one *tra-2* isoform was detected in other insect species (Burghardt et al. 2005; Concha and Scott 2009; Salvemini et al. 2003; Sarno et al. 2010; Schetelig et al. 2012; Liu et al. 2015). In crustacean species, Leelatanawit et al. (2009) cloned a homologous gene similar to *Drosophila tra-2* in *Penaeus monodon*, but it has no gender differences in alternative splicing patterns and expression levels and may not participate in sex-related physiological processes. In *Eriocheir sinensis* and *Fenneropenaeus chinensis*, different isoforms of *tra-2* were cloned. The expression of *tra-2c* in the ovary was significantly higher than that in the testis, and the expression level in the ovary before gonad differentiation increased sharply (Li et al. 2012; Luo et al. 2017).

In decapod crustaceans, as in vertebrates, the vitellogenesis oocytes is a huge growing oocyte, the cytoplasmic is filled with eosinophilic cells. The early vitellogenesis oocyte contains yolk granules with a nucleus located in the center of oocyte. In the late vitellogenesis stage, the oocytes are mature with increased volume and the nucleus moves to the edge of the oocyte. Eventually, the germinal vesicle breaks down and the nuclear membrane disappears, and the follicular cell layer of the egg becomes extremely thin (Li et al. 2018). The testes were made up of many seminiferous tubules containing germ cells that were distinguished by nuclear size and cell shape at different stages of development. The collected tissue was present in the epithelial cells surrounding the testicular tubules too. Spermatogenesis begins with the mitotic proliferation of spermatogonia in the germinative zone, and the germination is located on one side of the liners of the tubule (Kim et al. 2014). Then, spermatogonia become spermatocytes that pass through meiosis. Spermatocytes differentiate into spermatozoa through spermatids in the seminiferous tubules (Zhang and Qiu 2010).

In the present study, we cloned the *tra-2* gene from multiple cDNA libraries of *M. nipponense* (Gu et al. 2017; Qiao et al. 2017). Our aim was to obtain a full cDNA clone encoding the *tra-2* gene to characterize the nucleotide and to examine the expression pattern of the *tra-2* gene in early embryonic developmental stages, ovary development, adult tissues, and histological positioning in the gonad of *M.*

nipponense. This study can provide insight into sex determination by the *tra-2* gene in *M. nipponense* and lay the foundation for functional studies of the *Mntra-2* gene in the future.

Materials and methods

Experimental animals and sampling

Adult healthy prawns (*M. nipponense*) were collected from Tai Lake in Wuxi, China (120°13'44"E, 31°28'22"N) in June 2017. Male prawns weighed 2.8 ± 0.5 g and females weighed 1.8 ± 0.5 g. All prawns were transferred to the aquarium and cultured in an inflated freshwater pool in an indoor facility and fed with parudina twice per day. The different developmental stages of embryos were obtained from our laboratory. After prawn spawning, the developmental stages of embryos were continuously observed and collected under microscope.

Nucleotide sequence and bioinformatics analysis

Total RNA was extracted from testis was subjected to 5' and 3' RACE cDNA syntheses using a 5'-full RACE Kit and 3'-full RACE Core Set Ver. 2.0 Kit (TaKaRa, Japan). Two-step PCR cloning with gene-specific primers (listed in Table 1) was carried out as described (Li et al. 2018). The ORF Finder program (<https://www.ncbi.nlm.nih.gov/orffinder/>), BLASTX, and BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST/>) were used to deduce amino acid sequences. The spatial structure was predicted by I-TASSER (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>). The phylogenetic tree was generated by Molecular Evolutionary Genetics Analysis (MEGA 5.1) by the neighbor-joining method and bootstrapping replications were 1000.

Tissue expression analysis by quantitative real-time PCR

After 1 week culture in the laboratory, eyestalk, brain, heart, hepatopancreas, gill, muscle, ovary and testis were dissected from mature prawns ($n = 5$). The developmental of embryos is divided into seven stages [from the unfertilized egg (UE) to the first-day larvae after hatching (L1)] based on the study of Bai et al. (2016). The samples were dissected separately, immediately frozen in liquid nitrogen and stored at -80°C until processed.

The procedures for RNA isolation and cDNA synthesis were as described (Li et al. 2018). The expression profiles of *Mntra-2* in different tissues were determined using qPCR assays (CWBIO, China) (Zhang et al. 2013; Li et al. 2018). The relative copy numbers of *Mntra-2* mRNA were calculated according to the $2^{-\Delta\Delta\text{CT}}$ comparative CT method (Livak and

Table 1 Primers of sequence used

Primer name	Sequence (5' → 3')	Description
5'-RACE outer	CATGGCTACATGCTGACAGCCTA	Primer for 5'-RACE
5'-RACE inner	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	Primer for 5'-RACE
Tra-2-5'R	GGACTGACTCATCTTTACCTC	Primer for 5'-RACE
3'-RACE outer	TACCGTCGTTCCACTAGTGATTT	Primer for 3'-RACE
3'-RACE inner	CGCGGATCCTCCACTAGTGATTTCACTATAGG	Primer for 3'-RACE
Tra-2-3'F	ACTCGCCACGCCGGGCATGA	Primer for 3'-RACE
Tra-2-qF	TTCATTTTCAAGGTCAAGGTCACG	Primer for RT-PCR
Tra-2-qR	GAATAAGATGGTGACCGGGAGTA	Primer for RT-PCR
β-actinF	TATGCACTTCCTCATGCCATC	Primer for RT-PCR
β-actinR	AGGAGGCGGCAGTGGTCAT	Primer for RT-PCR
Tra-2-probe	GTATTCTACATCATCACGACGACGTTACTCCCGGTCAC	Probe for ISH
Tra-2-control	GTATTCTACATCATCACGACGACGTTACTCCCGGTCAC	Control for ISH

Schmittgen 2001). Differences in expression levels were considered significant at $P < 0.05$.

Expression profiles of *tra-2* in the ovarian cycle

The ovarian stage was determined based on the color of the oocytes, according to the criteria as stated (Qiao et al. 2015): stage I, transparent; stage II, yellow; stage III, light green; stage IV, dark green; and stage V, gray. Ovarian samples were treated in the same way as the previous tissue. Then we used qPCR to detect the expression level.

In situ hybridization (ISH)

Tissue samples were embedded in paraffin as described (Li et al. 2018). ISH was performed on 4-μm-thick formalin-fixed paraffin-embedded ovary and testis sections using a Zytofast PLUS CISH implementation kit. The slides were examined under a light microscope. The antisense and sense probes of chromogenic ISH studies were designed by Primer5 software based on the cDNA sequence of *Mntra-2a*. Both antisense and sense probes were hybridized with the slide. The antisense probe (Tra-2-probe) was prepared for the experimental group, whereas a sense probe (Tra-2-control) was prepared for the control group (Table 1).

Data analysis

All data are expressed as mean ± standard deviation. Data-processing software and method are as described in Li et al. (2018). The significance level was set to $P < 0.05$.

Results

Molecular cloning and structural analysis of *tra-2* gene

The full-length cDNA of *Mntra-2a* was 1293 bp (GenBank accession number, MH540105) encoding 270 amino acids. The 5'-untranslated region (UTR) was 212 bp long, and the 3'-UTR was 268 bp long. The termination codon and polyadenylation signal locating on the 3'-RACE PCR product revealed the integrity of the sequences we cloned. We found a conserved RRM and linker region in the amino acid sequence of *Mntra-2a* (Fig. 1).

The deduced peptides of *Mntra-2a* contains 270 amino acids, the conserved RRM (Cys 109-Val 181), two arginine/serine-rich regions (RS1: Met 1-Arg 102; RS2: Gly 230-Ala 270) and a linker region (Asp 182-Arg 202) (Fig. 1). The predicted 3D structure of *Mntra-2a* is similar to *F. chinensis*, both had two α-helix; four main β-sheets and one poly-glycine region (Fig. 2) (Li et al. 2012).

Multiple alignments of *Mntra-2a* and *tra-2* homologs from several invertebrates were performed using MEGA5.1 and DNAMAN 6.0 software. The RRM domain and the linker region had a high similarity between different *tra-2* genes, while the two RS regions had a very low similarity in the sequence (Fig. 3). Phylogenetic analysis indicated that *Mntra-2a* and *tra-2* (Zhang et al. 2013) were clustered into a clade and classified into one clade of *tra-2* in other arthropods (Fig. 4).

Fig. 1 Nucleotide and deduced amino acid sequence of *Macrobrachium nipponense* transformer-2a gene (*Mntra-2a*) cDNA. 5' UTR and 3' UTR are listed with lowercase letters. ORF are showed by capital letters. The translation starts codon (ATG), termination codon (TGA), polyadenylation signal (AATAAA) are underlined. Serine/arginine-rich domains (dotted line) and an RNA recognition motif are marked with red

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1      caaacatggagtccttagcgccacgcgcacttccctcctaatacctggcccggtctcccacc
61     attacccaaagtgtaaacaaagtagtcgggacgttgctcctttcttccacttatatttca
121    atcctgtgttgctgaacccgatcattttgctcgtcgtaagccccagggttcttgcgtt
181    actgttggtgtagtagttgaaaggaggtaaagATGAGTCAGTCCCCCTGACGAAGCCTCA
1      M S Q S P R R S L
241    ATGGGGTTTCACCCCTCCCGAGACAGATCTAGGGACAGGGATCCTTCATTTCAAGGTCAA
10     N G V S P S R D R S R D R D P S F S R S
301    GGTCACGTTCTCAGACCGCCGTGATAGCTATAAATACTCTTCACGAAGATCTACCTCTC
30     R S R S S D R R D S Y K Y S S R R S T S
361    CAAGATACCGTGAAGAACGTTACAGAGATGATAAGTATTCTACATCATCACGACGACGTT
50     P R Y R E E R Y R D D K Y S T S S R R R
421    ACTCCCGGTCAACATCTTATTCAGGAGCCGCCGTGGCAATAGATCTCGACAAGATCCC
70     Y S R S P S Y S R S R R G N R S R T R S
481    CAATGTCTACTCGCCGAGACATCATGGAAGCAGAGAGGACCCTAGCCCCAGTACGTGTT
90     P M S T R R R H H G S R E D P S P S T C
541    TAGGTGTGTTTGGCTTAAGCATCTATACCAGTGAGAGACAGCTTCACCACCTCTTTGGAA
110    L G V F G L S I Y T S E R Q L H H L F G
601    AATATGGACCTCTGCGAAAAGTTCAAGTTGTTCTTGATGCCAAAAGTGGACGGTCACGTG
130    K Y G P L A K V Q V V L D A K T G R S R
661    GCTTTGCTTTTATATATTTTGTAGCATATGGATGATGCCACAGAAGCTAAAGAACAATGCA
150    G F A F I Y F E H M D D A T E A K E Q C
      RRM
721    CTGGTATGGAATAGATGGCCGAGGATTAGGGTAGATTATTCATCACAGAGCGCGCGC
170    T G M E I D G R R I R V D Y S I T E R A
781    ACACACCTACCCCTGGTATCTACATGGGAAGACCTACAAATAGGTCCAGTGGATATGGAG
190    H T P T P G I Y M G R P T N R S S G Y G
841    GAAGAAGAACTGGTGGTGGTAGTGGTGGTGGCGGTGGTGGTGGAGGCCGTTATGGTGGTG
210    G R R T G G G S G G G G G G G R Y G G
901    GCTATGGTAGTGGTGGTGGTGGAGGAAGTCGTCGACGATCCCCATCACCTTATCGTA
230    G Y G S G G G G G S R R R S P S P Y R
961    GAGGCCGCTCAGGAAGAAATTCTTACAGATCAAGATCTCGTTCTTACTCGCCACGCCGGG
250    R G R S G R N S Y R S R S R S Y S P R R
1021   CATGAtcatgtgaggcgcaataaaagctctagatgttcgatccggtttgcctcaggtcgc
270    A *
1081   tacagccggtattgaggtgttggtgtcattaatgctcagttctaataagaaaacatcttat
1141   attgtagatacagttgccattctttgttttatgaaaaaagaaagttcaaatgtcatgta
1201   cttgctgaatgatgcaagaaaagtaacctgttatgttacctttataatcatgaatgagtag
1261   gtataaggtaatgtgcaaaaaaaaaaaaaaaaaa

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Tissue-specific expression patterns of *Mntra-2a*

The expression levels were analyzed in adult prawn tissues by qPCR, and the results showed that *Mntra-2a* mRNA was distributed in all the tissues of the prawns (Fig. 5). The expression of *Mntra-2a* was highest in the testis ($P < 0.05$), followed by the ovary ($P < 0.05$). In the eyestalk, heart, gill, and muscle, *Mntra-2a* was expressed more highly in the male than in the female. The amount of expression in the brain and hepatopancreas, however, was the opposite.

Expression of the *Mntra-2a* gene during embryo stages

In different developmental stages, the *Mntra-2a* expression pattern was analyzed by qPCR (from cleavage stage to the first-day larvae after hatching) (Fig. 6). A relatively high level of *Mntra-2a* expression was observed in the cleavage and nauplius stages of embryos ($P < 0.05$).

Fig. 4 Phylogenetic analysis of *tra-2* family members. The diagram was generated by the neighbor-joining method using the MEGA 5.1 program. Bootstrapping replications were 1000. GenBank accession numbers are in brackets

Fig. 5 The expressions of *Mntra-2a* revealed by qPCR in different tissues. The amount of two genes mRNA was normalized to the β -actin transcript level. Data are shown as mean $\pm$ SD ($n=5$ prawns). *E* eyestalk, *Br* brain, *H* heart, *HE* hepatopancreas, *G* gill, *M* muscle, *O* ovary, *T* testis; statistical analyses were performed with one-way ANOVA analysis. Bars with different letters were considered significant at $P < 0.05$

Fig. 6 The expressions of *Mntra-2a* revealed by qPCR in different embryo stages. The amount of two genes mRNA was normalized to the β -actin transcript level. *UE* unfertilized egg, *CS* cleavage stage, *BS* blastula stage, *GS* gastrula stage, *NS* nauplius stage, *ZS* zoea stage, *L1* the first-day larvae after hatching. Data are shown as mean $\pm$ SD ($n=5$ prawns). Statistical analyses were performed with one-way ANOVA analysis. Bars with different letters were considered significant at $P < 0.05$

Fig. 7 Quantitative analysis of *Mntra-2a* transcripts using real-time PCR in different development stages of ovaries. *OI* undeveloped stage, *OII* developing stage, *OIII* nearly-ripe stage, *OIV* ripe stage, *OV* spent stage. Data are shown as mean $\pm$ SD ($n=5$ prawns). Statistical analyses were performed with one-way ANOVA analysis. Bars with different letters were considered significant at $P < 0.05$

Expression of the *Mntra-2a* gene in different developmental stages of the ovaries

Figure 7 shows that the patterns of *Mntra-2a* expression in the ovary obtained using qPCR. The results confirmed a regular expression throughout ovary maturation. The *Mntra-2a* transcript level was high at the spent stage (stage V) and reduced to a minimum at the ripe stage (stage IV). As ovary maturation proceeded, the expression of *Mntra-2a* gradually decreased.

Localization of the *Mntra-2a* gene in the gonad

The cellular localization of the *Mntra-2a* was examined in different development stages of ovaries by ISH. The *Mntra-2a* mRNA was visualized in all the oocyte including the yolk granule, nucleus, and cytoplasmic membrane (Fig. 8b). In mature testis, the *Mntra-2a* mRNA was visualized in spermatogonia during spermatogenesis (Fig. 8c).

Fig. 8 Location of *Mntra-2a* gene was detected in the gonads by in situ hybridization. **a** Photograph of *M. nipponense* ovary in ovarian cycle. *OC* oocyte, *N* nucleus, *Y* yolk granule, *FC* follicle cell. **b** Histological section of ovary at different ovary stages of *M. nipponense*. *OG* oogonium, *OC* oocyte, *N* nucleus, *CM* cytoplasmic membrane, *Y* yolk granule, *FC* follicle cell, *FM* follicle membrane. **c** Histological section of testis of *M. nipponense*. *CT* collecting tissue, *SG* spermatogonium, *SC* spermatocyte, *SC1* primary spermatocyte, *SC2* second spermatocyte, *ST* spermatid, *S* sperm. Scale bars: **a** $\times 100$, **b** $\times 400$; **c** $\times 400$

Discussion

A *tra-2* gene in *Macrobrachium nipponense* has been studied in previous research (Zhang et al. 2013). *Tra-2* is also found in other arthropod species such as *D. melanogaster* (Mattox et al. 1990), *Bombyx mori* (Niu et al. 2005), *Sciara ocellaris* (Martín et al. 2011), *E. sinensis* (Luo et al. 2017), and *F. chinensis* (Li et al. 2012). In this study, we identified another *tra-2* gene in *M. nipponense* that we named *Mntra-2a* and performed a series of analyses such as amino acid translation, motif identification and comparison, and phylogenetic analysis to characterize the gene. Further analysis of the predicted amino acid sequence alignments showed it contained an RRM, an RRM-linker region, a poly-glycine region, and two arginine/serine-rich domains (RS/SR) (Li et al. 2012; Zhang et al. 2013; Luo et al. 2017). The RRM domain of *tra-2* protein mainly used RNA junctions. The *tra* protein RRM is highly conserved among different species. RRM is not a characteristic domain of *tra-2* protein, however, most RNA-binding proteins have this domain. Downstream of the RRM, we also discovered an RVDY domain. RVDY is mainly involved in the dephosphorylation of the *tra-2* protein after binding to protein phosphatase 1. RVDF is followed by three consecutive conserved amino acids: serine/isoleucine/threonine (Dauwalder et al. 1996). The linker domain is a characteristic domain of the *tra-2* protein, and the linker region shows very high amino acid sequence conservation. Through the analysis of protein sequence deletions and mutations, the integrity of the linker region is known to be critical for the *tra-2* protein. Once the linker region protein sequence changes, the sex differentiation and sperm growth of the individual can be affected. This indicates that the linker domain maintains *tra-2*. The function of the protein is crucial and can influence the sex differentiation and function of germ cells (Martín et al. 2011). The RS domain is widely involved in protein binding and can directly combine exon splicing enhancement factors to regulate the process of downstream gene selective cleavage (Shen et al. 2004).

The amino acid sequence analysis showed the *Mntra-2a* has 72–97% identity to previously reported arthropods *tra-2* genes. The highest similarity was compared with the *tra-2* (97%) characterized by Zhang et al. (2013). We found a high

degree of similarity in the RRM and RRM-linker region of *M. nipponense* (98.94%) (Zhang et al. 2013); *F. chinensis tra-2* (89.36%) (Li et al. 2012); and *E. sinensis tra-2* (79.79%) (Luo et al. 2017). In comparing the RS1 domain of the reported insect and crustacean *tra-2*, diversity was mainly observed (Fig. 3). The number of RS/SR repeats in the RS1 domain of other reported species is 11–18 and the number in *Mntra-2a* was also 11. However, in others homologs, the number of repeats in RS1 was 1, 13, and 7 in RS1 of *M. nipponense* (Zhang et al. 2013), *P. monodon* (Leelatanawit et al. 2009), and *D. pulex* (Colbourne et al. 2011), respectively. Furthermore, the *Mntra-2a* protein included a poly-glycine region (Fig. 3), which was similar in *M. nipponense* (Zhang et al. 2013), *F. chinensis* (Li et al. 2012), and *E. sinensis* (Luo et al. 2017).

Among the reported species, we have found that *tra-2* pre-mRNA can generate multiple alternatively spliced mRNAs encoding multiple isoforms of *tra-2* protein by alternative splicing pathway (Mattox et al. 1990; Niu et al. 2005; Li et al. 2012; Luo et al. 2017). Zhang et al. (2013) proposed that *M. nipponense* has a single *tra-2* mRNA. Comparing *Mntra-2a* and the other *tra-2* from *M. nipponense* (Zhang et al. 2013), we found that *Mntra-2a* had 90 aa more than the other *tra-2* in N-terminus, and *Mntra-2a* has 12 aa less than the other *tra-2* in C-terminus. This was the same as the results reported for various crustaceans. We expect there are more subtypes waiting to be discovered. In the phylogenetic tree, *tra-2* from different subtypes of the same species shared the same branch, which indicated that the difference in the same species was small but the difference between different species was larger (Fig. 4). The result of the phylogenetic trees also confirmed this.

To characterize the expression pattern of *Mntra-2a* in different tissues from *M. nipponense*, the spatial distribution of *Mntra-2a* transcripts was analyzed with qPCR. We found that *Mntra-2a* was highly expressed in the testis and ovary which is consistent with reports on other crustaceans, such as *F. chinensis* (Li et al. 2012). In *E. sinensis*, different subtypes of *Estra-2* show different expression patterns. Only *Estra-2c* is expressed highly in gonads. *Estra-2c* was expressed more highly in the ovary than in testis (Luo et al. 2017). In our research, *Mntra-2* was expressed higher in the muscle than in other tissue (Zhang et al. 2013). There is a ubiquitous but differential distribution of *Mntra-2a* transcripts in gonad and among most of the tissues. The expression pattern of *Mntra-2a* suggested that it may act in a concentration-dependent manner, rather than being either present or absent. Further study was needed to examine the expression pattern of *Mntra-2a* in different embryo stages of *M. nipponense*. The *Mntra-2a* expression level increased rapidly at the cleavage and nauplius stages. In other words, *Mntra-2a* may be related to cell division of early fertilized eggs and differentiation of tissues in *M. nipponense*.

Although abundant *Mntra-2a* transcripts existed in ovaries, further studies showed that *Mntra-2a* might not play a role in ovary development as in other species such as *E. sinensis* (Luo et al. 2017), *D. melanogaster* (Mattox et al. 1990), and *Apis mellifera* (Gempe et al. 2009). *Mntra-2a* had the highest expression level in the final stage (stage V) but levels decreased along with ovary maturation and reached the lowest level in the ovary after oviposition (stage IV). Although the expression level of *Mntra-2a* in the ovary was higher, it seemed that its expression decreased with the ovary maturation processes in prawns. After completing the task in the early developmental stage in the ovary, the expression level of *Mntra-2a* began to decline.

Subsequently, we analyzed the histological location of the *Mntra-2a* in the gonad by ISH technology. The results revealed that *Mntra-2a* mRNA was visualized in all the oocyte include yolk granule, nucleus, and cytoplasmic membrane. We found that the *Mntra-2a* signal appeared only in oocyte cells but not in other cells. The signal was in the nucleus in stage I. With the development of the ovary, the signal began to distribute in various regions of the oocyte cell. The change in signal strength, however, was not the same as the real-time PCR result. We speculate that this may be related to the operation of ISH. In mature testis, the *Mntra-2a* mRNA signal was only visualized in spermatogonia during spermatogenesis, but no signals were observed in spermatocyte and sperm. This indicated that the *Mntra-2a* mainly promoted spermatogonia development, rather than other cells. This result further confirmed that *Mntra-2a* advanced early gonad development in *M. nipponense*. To the best of our knowledge, this study was the first to analyze the expression of *Mntra-2a* in the gonads by ISH.

In summary, we reported the cloning, sequence analysis, temporal and spatial expression, and histological location of *Mntra-2a* in *M. nipponense*. The conservation of key amino acids and motifs in the gene supported a common evolutionary origin and function. Histological localization was also studied by ISH. To understand thoroughly the function of *Mntra-2a*, further research on potential subtypes of *Mntra-2* in *M. nipponense* is required.

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Compliance with ethical standards

Conflict of interest We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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