Final Report

Identification and expression analysis of sex-related *Transformer-2* gene in giant river prawn, *Macrobrachium rosenbergii*

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Abstract

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Abstract:

The *Transformer-2* (*Tra-2*) gene is a member of the *Sxl-Tra*/*Tra-2-Dsx* pathway, which regulates the sex determination pathway in *Drosophila melanogaster*. In the present study, a homologue of *Tra-2* was identified in the giant river prawn (*Macrobrachium rosenbergii*). Two variants were isolated and characterized namely, *MrTra-2a* and *MrTra-2b*. Deletion of a 61-bp region from *MrTra-2b* caused a translational frameshift resulting in a shorter *Mr*Tra-2b protein. A conserved RNA recognition motif (RRM) was present in both isoforms of *Mr*Tra-2 suggesting their functions in RNA-mediated processes similar to Tra-2 in other species. The expression of *MrTra-2a* and *MrTra-2b* in adult tissues was ubiquitous. Neither sex- nor tissue-specific splicing of *MrTra-2* were detected in *M. rosenbergii*. Both *MrTra-2* variants were expressed in the male and female gonad without a sexually dimorphic pattern. The expression of *MrTra-2a* and *MrTra-2b* was detected at the first day after hatching and exhibited a dynamic expression profile during development with the highest expression from *MrTra-2b* in L3 and PL10 for the larval (L) and post-larval (PL) stages, respectively. In PL30, *MrTra-2b* and *MrSxl1*—the gene at the top of the pathway—showed a significantly higher expression level over the *insulin-like androgenic gland factor* (*IAG*) gene of *M. rosenbergii*, whereas the opposite was evident in the juvenile stage and the androgenic gland. It is hypothesized that *MrTra-2*, together with *MrSxl*, plays a role in either initiating or regulating the expression of *MrIAG*, which is required for the sexual development in *M. rosenbergii*.

Keywords : *Transformer-2*; *Tra-2*; sex determination; *Macrobrachium rosenbergii*; giant river prawn

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1. Abstract

The *Transformer-2* (*Tra-2*) gene is a member of the *Sxl-Tra*/*Tra-2-Dsx* pathway, which regulates the sex determination pathway in *Drosophila melanogaster*. In the present study, a homologue of *Tra-2* was identified in the giant river prawn (*Macrobrachium rosenbergii*). Two variants were isolated and characterized namely, *MrTra-2a* and *MrTra-2b*. Deletion of a 61-bp region from *MrTra-2b* caused a translational frameshift resulting in a shorter *Mr*Tra-2b protein. A conserved RNA recognition motif (RRM) was present in both isoforms of *Mr*Tra-2 suggesting their functions in RNA-mediated processes similar to Tra-2 in other species. The expression of *MrTra-2a* and *MrTra-2b* in adult tissues was ubiquitous. Neither sex- nor tissue-specific splicing of *MrTra-2* were detected in *M. rosenbergii*. Both *MrTra-2* variants were expressed in the male and female gonad without a sexually dimorphic pattern. The expression of *MrTra-2a* and *MrTra-2b* was detected at the first day after hatching and exhibited a dynamic expression profile during development with the highest expression from *MrTra-2b* in L3 and PL10 for the larval (L) and post-larval (PL) stages, respectively. In PL30, *MrTra-2b* and *MrSxl1*—the gene at the top of the pathway—showed a significantly higher expression level over the *insulin-like androgenic gland factor* (*IAG*) gene of *M. rosenbergii*, whereas the opposite was evident in the juvenile stage and the androgenic gland. It is hypothesized that *MrTra-2*, together with *MrSxl*, plays a role in either initiating or regulating the expression of *MrIAG*, which is required for the sexual development in *M. rosenbergii*.

2. Executive summary

Sex determination is a fundamental mechanism to direct any living organism to follow the male or female pathway. Regulation of the sex-determining cascade has been well documented in the model organism *Drosophila melanogaster* (reviewed in Cline, 1993). The primary target of the sex-determining signal is the *Sex-lethal* (*Sxl*), which is primarily expressed in both sexes but functionally active only in females where it controls mRNA processing of the downstream *Transformer* (*Tra*) gene in the hierarchy (Inoue et al., 1990; Bell et al., 1991). The Transformer protein (Tra), together with Tra-2, subsequently direct alternative splicing of the *Double sex* (*Dsx*) gene, where as a result, sex-specific isoforms of

the Dsx protein are produced and serve as transcriptional regulators to control other processes of sexual differentiation and behavior in *D. melanogaster* (Inoue et al., 1992).

The giant river prawn (*Macrobrachium rosenbergii*), a commercially cultured crustacean, exhibits a sexually dimorphic growth pattern where males grow much faster and consequently reach a larger size at harvest than females (Sagi et al., 1986). Monosex culture of all-male *M. rosenbergii* has the best yield and the highest profit compared to all-female or mixed cultures (Nair et al., 2006). Therefore, there is a financial incentive in researching sex manipulation to produce all-male *M. rosenbergii*. How sex is established in *M. rosenbergii* is poorly understood and, as a consequence, limits its production. Cytological studies have not yet successfully differentiated sex chromosomes from autosomes (Justo et al., 1991), although the ZZ/ZW sex-determining system has been proposed (Malecha et al., 1992). Androgenic glands (AGs) have been recognized as a male-specific endocrine organ responsible for the production of the androgenic gland hormone (AGH) to induce the development of male characteristics in *M. rosenbergii* (Nagamine et al., 1980 a,b). Silencing of the *insulin-like androgenic gland factor* gene (*IAG*) is able to induce sex reversal in *M. rosenbergii* (Ventura et al., 2009, 2012). However, it is still not known whether the gene is responsible for sex determination or merely for subsequent processes in male development. Identification of sex-related genes is indispensable to give a better insight underlying the sex-determining mechanism of *M. rosenbergii*.

Several genes homologous to those in the *Sxl-Tra*/*Tra-2-Dsx* pathway have been discovered in many crustacean species, such as *Sxl* in the oriental river prawn, *M. nipponense* (Zhang et al., 2013a) and the Chinese mitten crab, *Eriocheir sinensis* (Shen et al., 2014), *Tra* and *Dsx* in the water flea, *Daphnia magna* (Kato et al., 2010, 2011), and *Tra-2* in *M. nipponense* (Zhang et al., 2013b), the black tiger shrimp, *Penaeus monodon* (Leelatanawit et al., 2009), the Chinese shrimp, *Fenneropenaeus chinensis* (Li et al., 2012) and the Chinese mitten crab, *E. sinensis* (Luo, 2015). These genes in crustaceans show high sequence similarity with their homologs in insects and most of them have have more than one variants generated from the alternative splicing mechanism. Recently three isoforms of *MrSxl* have been identified in *M. rosenbergii* (McMillan et al., 2018). Analysis of the expression profiles showed that *MrSxl* was present before the onset of *MrIAG* and that the relationship between these two genes may be necessary to the subsequent process of sexual differentiation. The findings of a number of genes in the conserved *Sxl-Tra/Tra-2-Dsx* pathway in crustaceans, including *MrSxl* in *M. rosenbergii*, have generated interest in whether or not such a pathway exists in *M. rosenbergii*. Therefore, this research was aimed to identify full-length cDNA

sequences of *Tra-2* gene in *M. rosenbergii* (*MrTra-2*) and to determine its spatiotemporal expression profile during larval (L) and post-larval (PL) stages, and in adult tissues. Comparing expression levels of *MrIAG*, *MrSxl* and *MrTra-2* were also conducted to show the relationship of these sex-related genes.

Here, two full-length cDNAs of *MrTra-2*, designated as *MrTra-2a* and *MrTra-2b*, were identified. Analysis of the cDNA sequences identified a presence of 61 bp in *MrTra-2b,* which led to a translational frameshift followed by an early termination of *Mr*Tra-2b protein synthesis resulting in a shorter *Mr*Tra-2b (268 residues) compared to *Mr*Tra-2a (271 residues). Analysis of the deduced amino acid sequences showed that a conserved RNA recognition motif (RRM), two arginine/serine-rich regions (RS1 and RS2), a linker and a poly glycine region were present in both isoforms and conserved among Tra-2 proteins from other crustaceans. A phylogenetic tree constructed showed distinct clades of the Tra-2 proteins of vertebrates, crustaceans and insects. Two *Macrobrachium* Tra-2, *Mr*Tra-2 of *M. rosenbergii* and *Mn*Tra-2 of *M. nipponense*, were placed on the same node of the tree.

Analysis of the temporal gene expression showed that *MrTra-2a* and *MrTra-2b* were ubiquitously expressed in all adult tissues examined. The expression level of *MrTra-2a* was highest in hepatopancreas and muscle while that of *MrTra-2b* was highest in hepatopancreas. Both variants did not show a dimorphic expression pattern and had a comparable expression level between the testis and the ovary. The spatial expression analysis during L and PL stages showed that the two *MrTra-2* variants were expressed as early as the first day after hatching and remained detectable in all subsequent stages. *MrTra-2b* had a higher expression level over *MrTra-2a* at most adult tissues and developmental stages. In larvae, the expression level of *MrTra-2b* reached its peak at L3 but remained low in other larval stages. Its expression was rapidly raised in PL1, when the metamorphosis of *M. rosenbergii* occurred, and significantly increased in PL10 before a decrease of its expression was observed in the following PLs. The expression of *MrTra-2a* stayed relatively low in all, except L3, stages.

The expression of *MrTra-2b* was quantitatively compared to that of *MrSxl1*, which is one of the three variants of *MrSxl*—the gene placed at the top of the *Sxl-Tra/Tra-2-Dsx* pathway. *MrSxl1* was significantly expressed more than *MrTra-2b* at L3, L7 and PL1. It is possible that the up-regulated expression of *MrTra-2b* in L3 and PL1 was probably in response to the function of *MrSxl* in the *Sxl-Tra/Tra-2-Dsx* pathway. The expression of *MrSxl1* and *MrTra-2b* was substantially higher than that of *MrIAG* in PL30 when sexual organs were not yet developed. The expression of *MrIAG* was later elevated in juveniles and became significantly higher than that of *MrSxl1* and *MrTra-2b*. In adult males, *MrIAG* was

expressed only in androgenic glands (AGs) and had over 10,000-fold higher than that of *MrSxl1* and *MrTra-2b*. Silencing of *MrSxl*, *MrTra-2* and *MrIAG* may reveal how these genes are functionally related and whether or not they are mutually regulated. A comprehensive investigation of how the *Sxl*/*Tra*/*Tra-2*/*Dsx* pathway functions, especially in cooperation with *MrIAG*, may uncover the mechanism underlying sexual development, if not sex determination, of *M. rosenbergii* and possibly bring an understanding how the sex is established in crustaceans.

In conclusion, this study characterized two alternatively spliced variants of *MrTra-2* and exhibited their spatiotemporal expression profiles, together with their relative expression with other sex-related genes. Analyses of the gene expression suggested that *MrTra-2* and the *Sxl*/*Tra*/*Tra-2*/*Dsx* pathway may play an important role either during or after metamorphosis when the male-specific *MrIAG* gene was initiated. Further investigations, such as gene silencing, are needed to disclose the biological functions of *MrTra-2* in *M. rosenbergii*. The benefit of this research is not only limited to the giant river prawn but also offers a great opportunity to other researchers in the research community to apply the knowledge to understand sex determination and development in other commercially important crustaceans such as the white shrimp, *Litopenaeus vannamei* and the black tiger shrimp, *P. monodon*. Finally, the outputs of this research would bring further studies in developing biotechnology to produce monosex population, which will increase the prawn production and provide more profit and income to farmers of the country.

3. Objectives

3.1 To identify a full-length cDNA of *M. rosenbergii Transformer-2* gene (*MrTra-2*) and its variants

3.2 To determine distribution of *MrTra-2* transcripts in adult tissues

3.3 To exhibit temporal expression of *MrTra-2* during larval and post-larval development

3.4 To compare expression levels of three sex-related genes including *MrSxl*, *MrIAG* and *MrTra-2* in post-larvae and in the androgenic gland

4. Research methodology

This research was conducted in the laboratory of aquaculture genetics, Department of Aquaculture, Faculty of Fisheries, Kasetsart University. The experiments were divided into four parts covering the objectives of this study.

4.1 Identification of *MrTra-2* and its transcript variants

Rapid amplification of cDNA ends (RACE) was used to amplify cDNAs of *MrTra-2* in *M. rosenbergii*. *Tra-2*-specific primers were designed based on analysis of full-length cDNA sequences of known *Tra-2* genes in crustaceans including *MnTra-2* (accession number JX221052) from the oriental river prawn, *M. nipponense*, *FcTra-2a* (AFU60543), *FcTra-2b* (AFU60544) and *FcTra-2a* (AFU60545) from the Chinese white shrimp, *F. chinensis*. The deduced amino acid sequences of these crustacean Tra-2 proteins were aligned using online bioinformatics analysis tools (www.ebi.ac.uk) to identify the most conserved region among them. Nucleotide sequences encoding the conserved amino acid region previously identified were obtained. An alignment of the nucleotide sequences was then conducted and conserved regions with high percentages of identical nucleotides were used in primer design using Primer3 (primer3.ut.ee) and OligoCalc (www.biotools.nubic.northwestern.edu/). Primers were ordered from First BASE Laboratories Sdn Bhd, Malaysia. All primers used in this research are listed in Table 1.

Adult orange-blue-clawed males weighing approximately 80 g each were obtained from a local farm in Nakhon Pathom province, Thailand. They were acclimated to laboratory conditions in 250-liter fiberglass tanks and were provided with an artificial feed diet twice a day for 7 days at which time tissue sampling was undertaken strictly in accordance with the Animal for Scientific Purposes Act A.D. 2015 (Permit number U1-01820-2558). Testis samples from three males were collected and pooled. They were immediately frozen in liquid nitrogen and stored at -80 °C until used. Total RNA of the testis samples was then extracted using TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's instructions. RNA quality and quantity were assessed using denaturing agarose gel electrophoresis and a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Polyadenylated RNA was isolated from the total RNA extracted from the testis of *M. rosenbergii* using Illustra™ QuickPrep Micro mRNA Purification Kit (GE Healthcare, Piscataway, NJ). Synthesis of the 5′- and 3′-rapid amplification of cDNA ends (RACE)-ready cDNAs was performed using SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. The Universal Primer A Mix (Clontech, Mountain View, CA) and *MrTra-2*-specific primers (Table 1) were used in 5′- and 3′-RACE PCR reactions (30 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min). Amplicons were gel-purified using NucleoTrap Gel Extraction Kit (GE Healthcare, Piscataway, NJ) and cloned into pGEM-T® easy vector (Promega, Madison, WI).

Recombinant plasmids were transformed into *Escherichia coli* DH5α competent cells. Positive clones from blue-white colony screening were isolated and sequenced (Macrogen Inc., Korea).

Table 1. Nucleotide sequences of primers for cloning and expression analysis

Primer	Sequence (5′ → 3′)	Experiment
F:MnTra2-1	CATGGAAGCAGAGAGGACCC	RT-PCR of *MrTra-2*
R:MnTra2-1	TTAGCTTCTGTGGCATCATCC	RT-PCR of *MrTra-2*
F:MnTra2-2	GAGAGACAGCTTCACCACCTC	RT-PCR of *MrTra-2*
R:MnTra2-2	CCTAATCCTGCGGCCATC	RT-PCR of *MrTra-2*
5RACE-Tra2	GGATGCCAAAACTGGACGGTCACGTGGC	5′-RACE of *MrTra-2*
3RACE-Tra2	GCAGAGAGGACCCTAGCCCCAGTACGTG	3′-RACE of *MrTra-2*
F:Tra2a_RT	TACTCGCCACGTCGCTACAG	RT-PCR and qRT-PCR of *MrTra-2a*
F:Tra2b_RT	GCTCTAGATGTTCGATCCGG	RT-PCR and qRT-PCR of *MrTra-2b*
R:Tra2ab_RT	GAATGGCAACTGCATCTACAATG	RT-PCR and qRT-PCR of *MrTra-2a* and *MrTra-2b*
F:Sxl1_RT	ATGCAGGACTAAACAACACGAG	qRT-PCR of *MrSxl1*
R:Sxl1_RT	TCTGACGGGTCACATTGTTATAG	qRT-PCR of *MrSxl1*
F:IAG_RT	GACAGCGTAAGGAGAAGCCC	qRT-PCR of *MrIAG*
R:IAG_RT	TATAGGACAGGGACGGGATG	qRT-PCR of *MrIAG*
F:β-actin_RT	TTCACCATCGGCATTGAGAGGTTC	RT-PCR and qRT-PCR of *β-actin*
R:β-actin_RT	CACGTCGCACTTCATGATGGAGTT	RT-PCR and qRT-PCR of *β-actin*

Full-length cDNAs were assemble and aligned with the *Tra-2* of crustaceans to confirm that *MrTra-2* of *M. rosenbergii* were successfully identified. The nucleotide sequences of *MrTra-2* were submitted to the GenBank database held by NCBI (www.ncbi.nlm.nih.gov/genbank/). The full-length cDNA sequences were translated using ExPASy Translation tool (web.expasy.org/translate) to obtain deduced amino acids of *Mr*Tra-2 proteins. Nucleotide and deduced amino acid sequences were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). Amino acid sequences of Tra-2 proteins from other species were obtained from the UniProtKB database (Table 2). Multiple sequence alignments were performed using the MUSCLE algorithm (Edgar, 2004). A phylogenetic tree was constructed using the maximum likelihood method implemented in the MEGA software version 7.0 (Kumar et al., 2016). The reliability of the tree topology was assessed using 1,000 bootstrap replicates.

Table 2. Information of Tra-2 proteins used in sequence alignment and phylogeny

Protein Name	UniProtKB accession number	Species	Common name
Tra2B_Rat	P62997	*Rattus norvegicus*	Rat
Tra2B_Bovine	Q3ZBT6	*Bos taurus*	Bovine
Tra2B_Human	P62995	*Homo sapiens*	Human
Tra2B_Mouse	P62996	*Mus musculus*	Mouse
Tra2B_Chick	Q9DDU8	*Gallus gallus*	Chicken
Tra2B_Turkey	G1MV83	*Meleagris gallopavo*	Turkey
Tra2B3_latipes	Q6I6X6	*Oryzias latipes*	Japanese rice fish
Tra2C_sinensis	APJ36538	*Eriocheir sinensis*	Chinese mitten crab
Tra2A_monodon	ACD13597	*Penaeus monodon*	Giant tiger shrimp
Tra2C_chinensis	K9MBG8	*Fenneropenaeus chinensis*	Chinese shrimp
Tra2_nipponense	X2C2W4	*Macrobrachium nipponense*	Oriental river prawn
Tra2_beetle	W0HDL6	*Tribolium castaneum*	Red flour beetle
Tra2_honeybee	I1Z8F1	*Apis mellifera*	Honeybee
Tra2D_silkworm	Q58ZF8	*Bombyx mori*	Silkworm
Tra2D_butterfly	A0A212ER44	*Danaus plexippus*	Monarch butterfly
Tra2_darkwing	E7AXY2	*Bradysia coprophila*	Dark-winged fungus gnat
Tra2_gnat	E7AXY1	*Sciara ocellaris*	Fungus gnat
Tra2_melanogaster	P19018	*Drosophila melanogaster*	Fruit fly
Tra2_domestica	Q5I705	*Musca domestica*	House fly
Tra2_cuprina	C5HJY6	*Lucilia cuprina*	Sheep blowfly
Tra2_grandis	D6CHG5	*Anastrepha grandis*	Cucurbit fruit fly
Tra2_capitata	B3TZZ6	*Ceratitis capitata*	Mediterranean fruit fly
Tra2_dorsalis	A0A034WNW1	*Bactrocera dorsalis*	Oriental fruit fly

4.2 Analysis of spatial distribution of *MrTra-2* transcripts in adult tissues

Adult females (approximately 40 g each) and adult orange-blue-clawed males (approximately 80 g each) were acclimated as mentioned above. Nine tissue types were dissected—the eyestalk, gills, heart, hemocyte, hepatopancreas, muscle, testis and *vas deferens* from males and only the ovary from females. Three replicates of tissue samples, with each consisting of a pool from three females or three males, were collected. Hemolymph samples from each male were collected from the ventral sinus using a sterile

syringe containing an equal volume of anticoagulant solution (10% trisodium citrate dihydrate in RMPI 1640 medium with L-glutamine; Gibco BRL) followed by centrifugation at 850×*g* and 4 °C for 10 min to isolate hemocytes. In order to obtain hypertrophied androgenic glands (hAGs), several orange-blue-clawed adult males weighing approximately 80 g each were acclimated. Eyestalks were unilaterally ablated and maintained for 14 days to produce hAGs with an increase activity of cell proliferation and protein synthesis compared to normal AGs (Kim et al., 2002; Phoungpetchara et al., 2011). Three replicates of the hAGs with approximately 15-20 glands each were collected at 14 days after eyestalk ablation. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until used. Total RNA was extracted and RNA quality and quantity were assessed as mentioned above.

Total RNA was treated with *DNase* I (Thermo Fisher Scientific, Carlsbad, CA) and purified using RNA Clean & Concentrator-5 column (Zymo Research, Irvine, CA). First-strand cDNAs were synthesized from 1 µg total RNA using iScript™ reverse transcription supermix for RT-qPCR (Bio-Rad, Hercules, CA). Quantitative RT-PCR reactions were measured using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) according to the manufacturer's protocols. Primer combinations were designed to quantitatively analyze the expression of each *MrTra-2* variant (Table 1). The expression of the *β-actin* gene of *M. rosenbergii* was used as an endogenous control. The reactions were performed in triplicate and the quantitative RT-PCR program was conducted at the following condition: a cycle of 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 15 s. Relative expression ratios were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Statistical analysis was conducted using the SPSS software version 13.0 with Student's t-test or one-way ANOVA followed by *post hoc* Duncan's multiple range test (DMRT).

4.3 Analysis of temporal distribution of *MrTra-2* transcripts at different developmental stages

Samples were collected at different developmental stages from offspring of gravid prawns. Larval stages (L) were classified according to external characteristics (Tayamen and Brown, 1999). Intact larvae at stages L1, L3, L7 and L11 were collected and approximately 30-100 individuals in the same stage were pooled in each replicate. Post-larvae (PL) emerged when the larvae underwent metamorphosis. Cephalothorax samples at PL1, PL10, PL20 and PL30 (where 1, 10, 20 and 30 represent days after metamorphosis) were dissected and a pool sample of 5-10 individuals in the same stage was used in each replicate. Male juveniles were

collected at ~PL100 and sexed according to the presence of the appendix masculina, a male secondary sexual characteristic, at the second pleopod. Cephalothorax samples, specifically at the fifth walking legs where AGs are located, were dissected from the male juveniles. Three replicates—a pool of three cephalothorax samples each—were prepared. Total RNA was extracted and RNA quality and quantity were assessed as mentioned above. The samples were subjected to quantitative RT-PCR as mentioned in the previous section.

4.4 Comparison of transcription levels of sex-related genes in *M. rosenbergii*

Two sex-related genes, *MrIAG* and *MrSxl*, have been identified in *M. rosenbergii*. *MrIAG* encodes androgenic gland hormone (AGH), which is involved in the development of male characteristics. Expression of *MrIAG* was detected prior to the presence of male gonopores and appendix masculina and maintained throughout the development (Ventura et al., 2009). In adults, *MrIAG* was strictly expressed only in the androgenic gland of male prawns. *MrSxl* was expressed as early as the first day after hatching. Its expression is detected in all developmental stages and in many adult tissues including the androgenic gland, testis and ovary (McMillan et al., 2018). Expression levels of *MrTra-2* and *MrSxl* covering developmental stages and in adult tissues were compared. Quantitative RT-PCR in 10 adult tissues and in L and PL samples were performed as previously described. Statistical analysis was conducted using the SPSS software version 13.0 with unpaired Student's t-test. A comparison of expression levels of *MrTra-2*, *MrSxl* and *MrIAG* in PL30, juveniles and hAGs was conducted using qRT-PCR and statistically analyzed with one-way ANOVA followed by *post hoc* Duncan's multiple range test (DMRT). All primers used in the expression analysis are listed in Table 1.

5. Results

5.1 Identification of *MrTra-2* specific primers

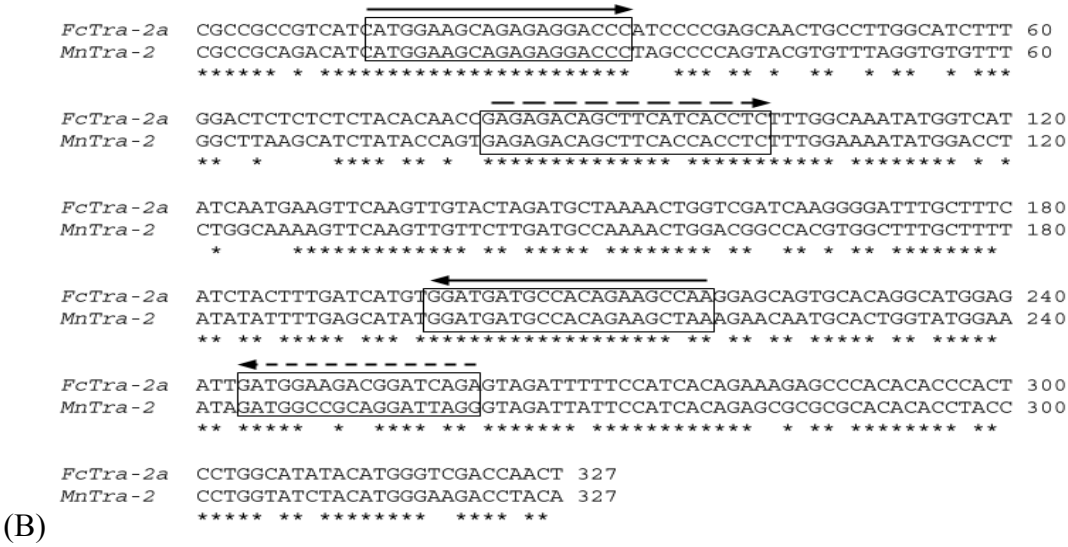
Rapid amplification of complementary DNA (cDNA) ends (RACE) is a powerful technique to isolate *M. rosenbergii Tra-2* cDNAs when partial sequences of *Tra-2* were available. Due to a lack of sequence information of *Tra-2* in *M. rosenbergii*, sequence analysis of known *Tra-2* genes from other crustacean species evolutionarily related to *M. rosenbergii* was performed. *Tra-2*-conserved regions were identified and used in designing *Tra-2*-specific primers.

First, amino acid sequences of four crustacean Tra-2 proteins, including *MnTra-2* from the oriental river prawn, *M. nipponense*, and *FcTra-2a*, *FcTra-2b* and *FcTra-2c* from

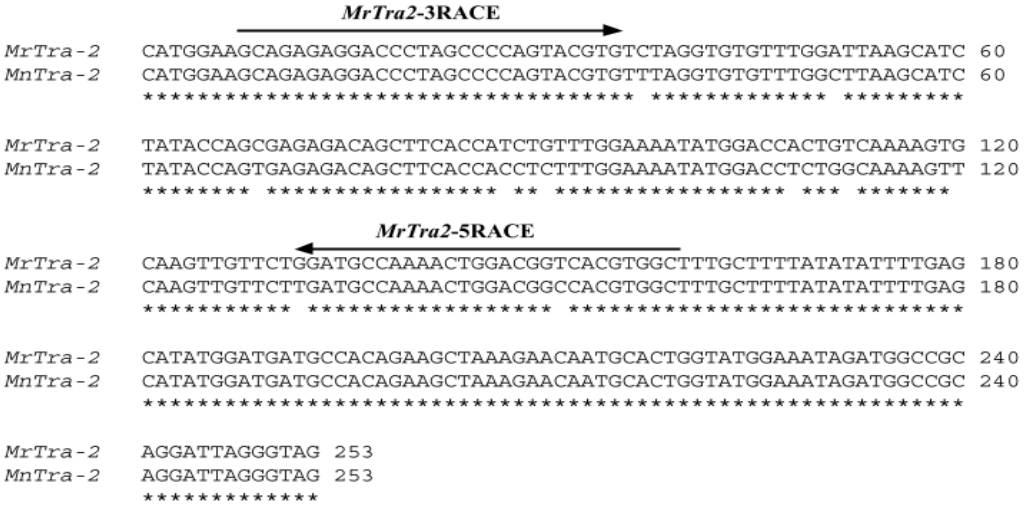
the Chinese shrimp, *F. chinensis*, were aligned. A conserved region with 89% identity of amino acid residues was identified (Fig. 1A). Nucleotide sequences encoding the conserved region were obtained and aligned. The three variants of *FcTra-2* had 100% identity of the selected nucleotide sequences; hence, *FcTra-2a* was chosen as a representative of *FcTra-2* in the subsequent analysis. The selected nucleotide sequences of *MnTra-2* and *FcTra-2a* were aligned and two primer pairs were designed (Fig. 1B). The primers were used in RT-PCR to exhibit an existence of *Tra-2* in *M. rosenbergii* (*MrTra-2*). PCR products were cloned and sequenced to obtain partial nucleotide sequences of *MrTra-2*. A pairwise alignment of a 253-bp amplicon of *M. rosenbergii* had 96.44% identity to the same region of *MnTra-2* of *M. nipponense* indicating that the partial nucleotide sequence of *MrTra-2* was successfully identified. *MrTra-2*-specific primers were designed based on the 253-bp sequence and used in 5′- and 3′-RACE experiments to isolate full-length cDNAs of *MrTra-2* (Fig. 1C).

(A)

Figure 1. Sequence alignment to obtain *MrTra-2* specific primers. (A) Amino acid sequence alignment of three isoforms of *Fc*Tra-2 proteins (*Fc*Tra-2a, *Fc*Tra-2b and *Fc*Tra-2c) from the Chinese shrimp, *Fenneropeneaus chinensis,* and *Mn*Tra-2 from the oriental river prawn, *Macrobrachium nipponense*. An asterisk (*), a colon (:) and a period (.) indicate identical, strongly and weakly similar amino acid properties, respectively. The conserved region was highlighted.



(B)



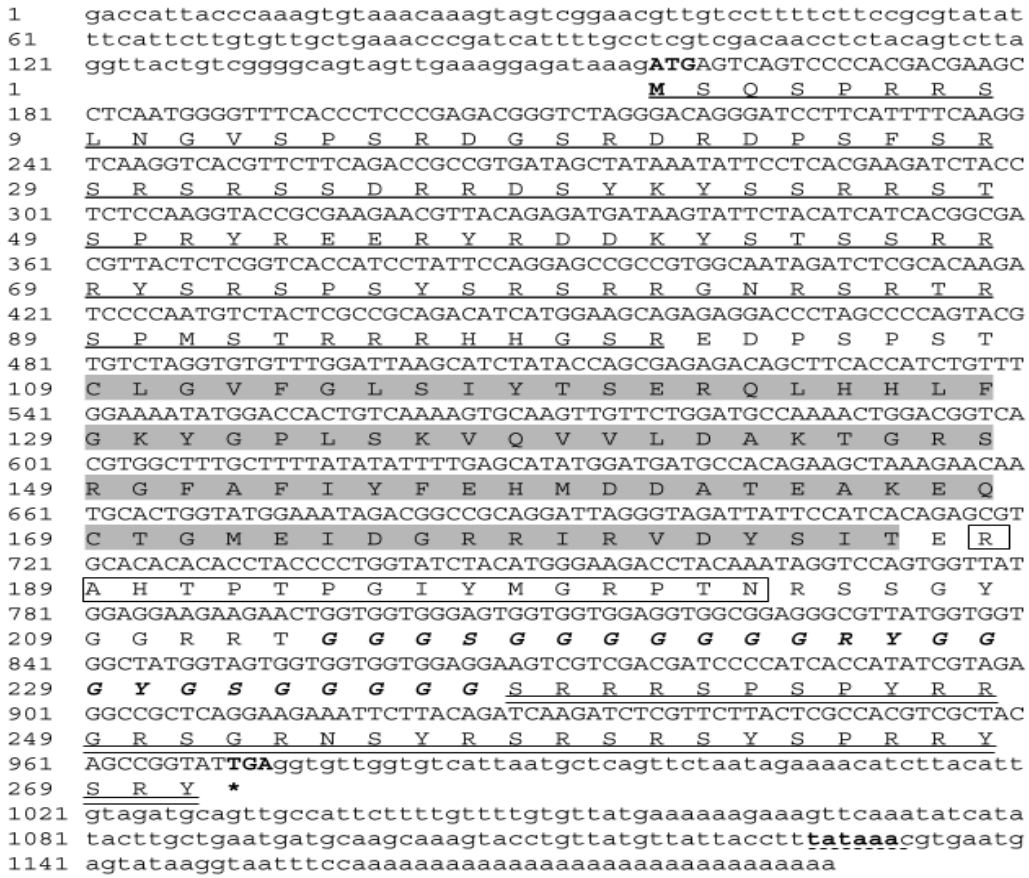
(C)

Figure 1. (continued) Sequence alignment to obtain *MrTra-2* specific primers. (B) Alignment of nucleotides encoding the amino acid residues highlighted in (A). An asterisk (*) indicates identical nucleotides. Conserved regions where primers were designed are boxed. Two primer pairs are shown with thick (→) and dashed (--) arrows. Right and left arrows indicate forward and reverse primers, respectively. (C) Alignment of partial nucleotide sequences between *MnTra-2* from *M. nipponense* and *MrTra-2* from *M. rosenbergii*. An asterisk (*) indicates identical nucleotides. Nucleotides where RACE primers were designed are indicated with arrows.

5.2 Full-length cDNA sequences and variants of *MrTra-2*

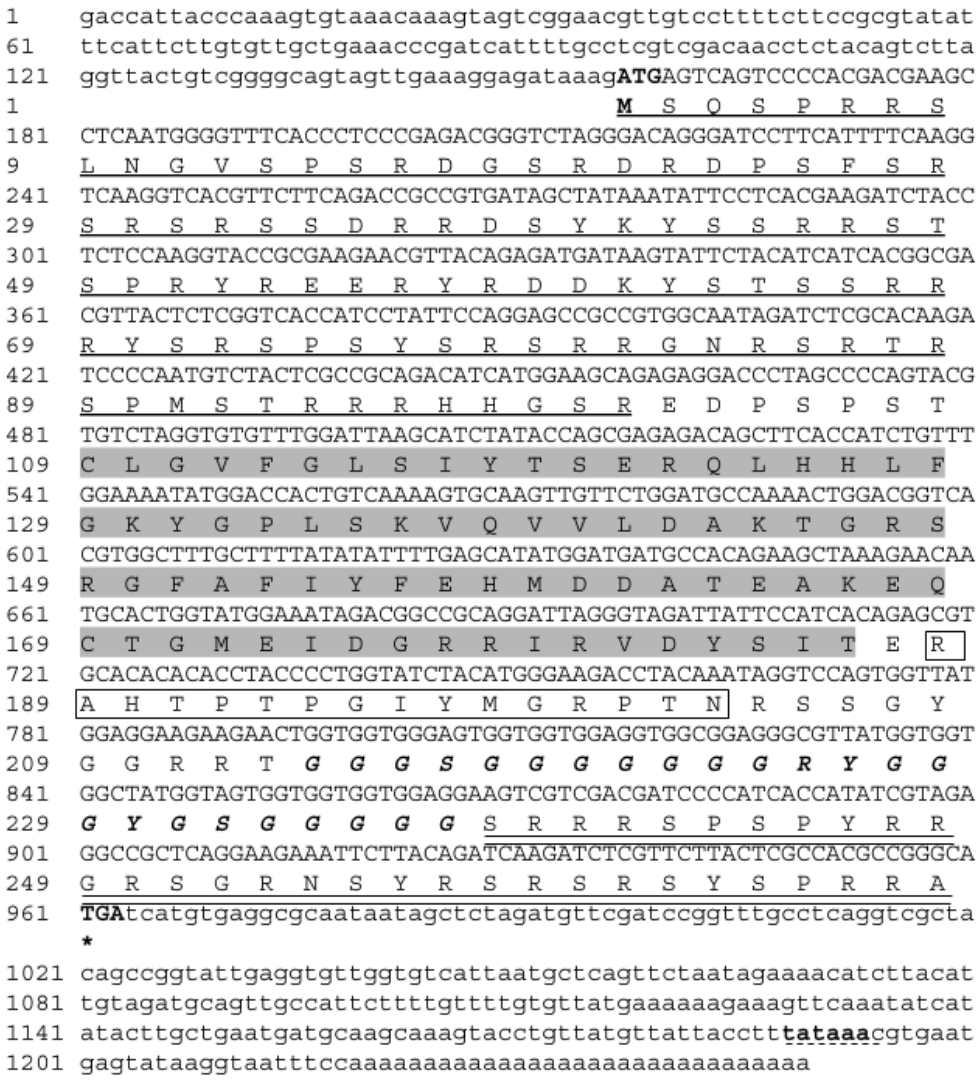
Two full-length cDNAs of *MrTra-2* designated as *MrTra-2a* and *MrTra-2b* were successfully identified (Fig. 2A-B). The nucleotide sequences were submitted to the GenBank database with accession numbers MH137935 and MH137936 for *MrTra-2a* and

MrTra-2b, respectively. The full-length cDNA of *MrTra-2a* was 1,188 bp, which consisted of a 156-bp 5′-untranslated region (UTR), a predicted 816-bp open reading frame (ORF) followed by a 216-bp 3′UTR. The full-length cDNA of *MrTra-2b* was 1,249 bp and shared the 156-bp 5′UTR and the start codon as in *MrTra-2a*. *MrTra-2b* had a predicted 807-bp ORF and a 286-bp 3′UTR. A polyadenylation signal (TAATAAA) was present at 25 bp upstream from the poly(A) tail in both variants of *MrTra-2* cDNAs. The deduced amino acid sequences of *Mr*Tra-2a and *Mr*Tra-2b proteins were 271 and 268 residues, respectively. Analysis of the amino acid sequences identified a conserved RNA recognition motif (RRM), two arginine/serine-rich regions (RS1 and RS2), a linker and a poly glycine region in both isoforms of *Mr*Tra-2 proteins.



(A)

Figure 2. Nucleotide (above) and deduced amino acid (below) sequences of the full-length cDNAs of (A) *MrTra-2a* and (B) *MrTra-2b*. Start codon is in bold and stop codon is in bold marked with an asterisk (*). RNA recognition motif (RRM) is shaded. Two arginine/serine-rich (RS) regions are underlined by a single line (RS1) and a double line (RS2). A linker is boxed and a poly glycine region is in bold and italicized. Polyadenylation signal is in bold marked with a dashed line.



(B)

Figure 2. (continued) Nucleotide (above) and deduced amino acid (below) sequences of the full-length cDNAs of (A) *MrTra-2a* and (B) *MrTra-2b*.

An alignment of the two cDNA variants identified a 61-bp region, which was present only in *MrTra-2b*, but not *MrTra-2a* (Fig. 3A). The presence of the 61-bp region led to a translational frameshift followed by an early termination of protein synthesis of *Mr*Tra-2b. It resulted in a shorter *Mr*Tra-2b protein (268 residues) compared to *Mr*Tra-2a (271 residues). Alignment of the deduced amino acid sequences revealed that the difference between the two isoforms was at the C-terminal end while the N-terminus and the central region were identical (Fig. 3B).

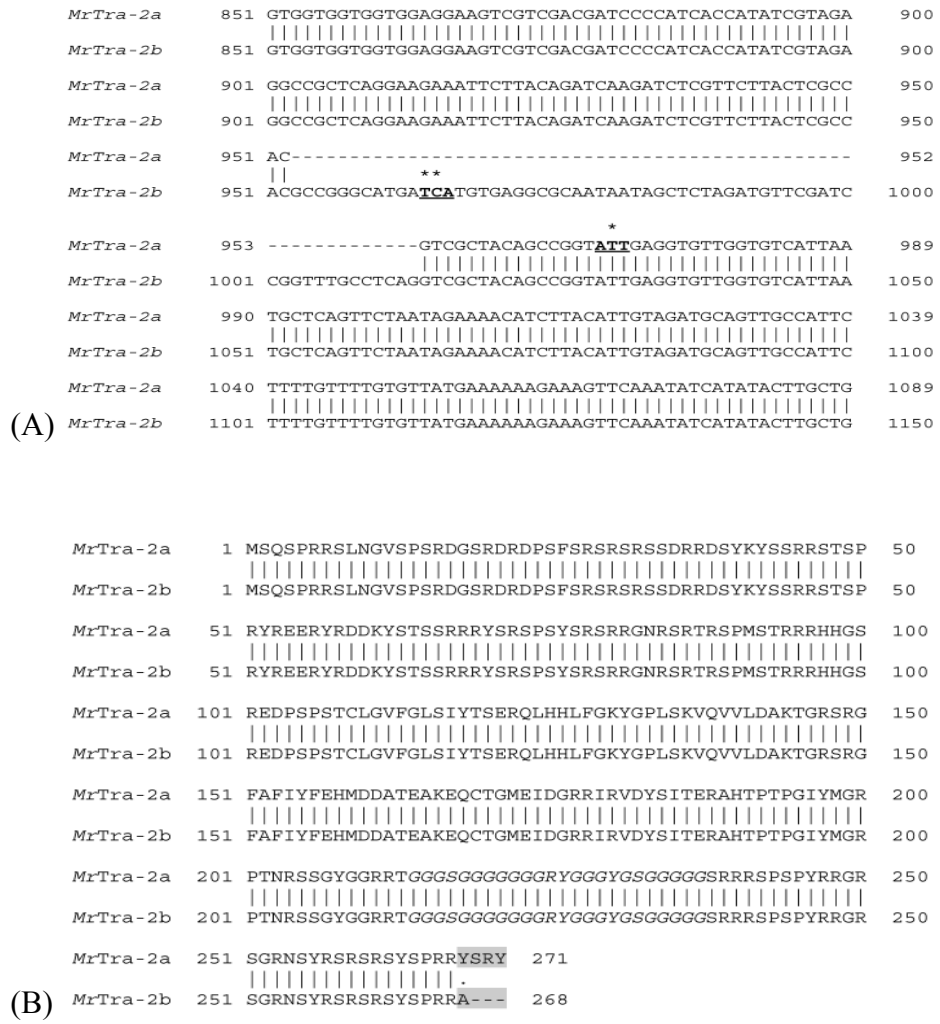
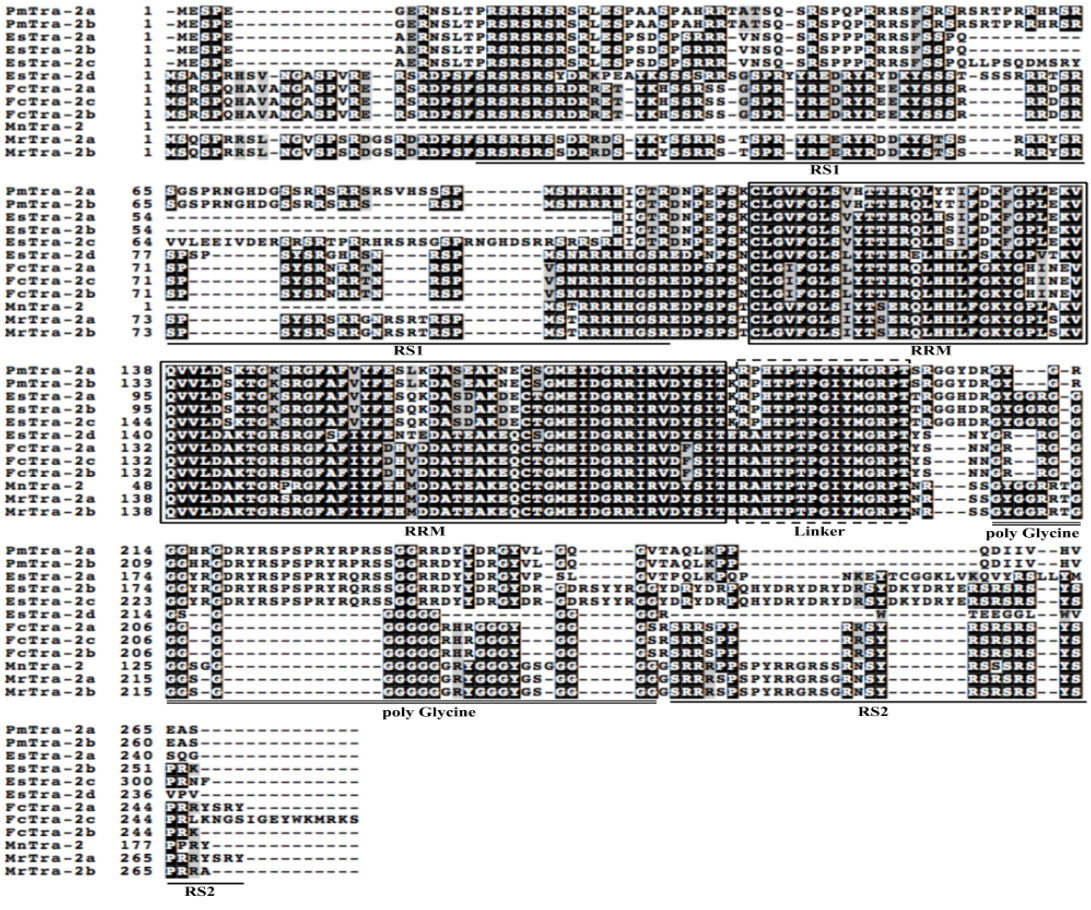


Figure 3. Pairwise alignment of (A) nucleotides and (B) amino acid residues between *MrTra-2a* and *MrTra-2b* of *M. rosenbergii*. Only the indicated nucleotide positions (851 - 1089 of *MrTra-2a* and 851 - 1150 of *MrTra-2b*) are shown in (A). Stop codons are indicated with bold and underline. The stop codons of *MrTra-2a* and *MrTra-2b* are shown with * and **, respectively. (B) Alignment of deduced amino acid sequences of *Mr*Tra-2a and *Mr*Tra-2b protein isoforms. A horizontal line (|) indicates identical residues. A period (.) indicates similar amino acid properties. A dash (-) indicates a deletion. Non-identical amino acid residues are shaded.

5.3 Multiple sequence alignment and phylogenetic tree construction

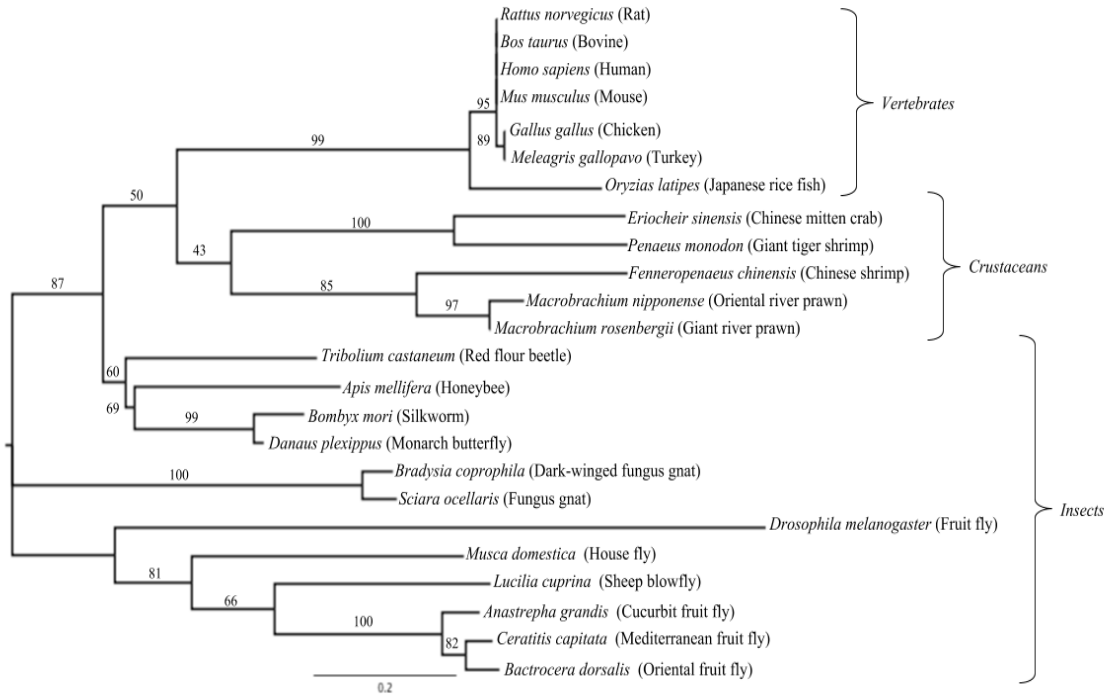
The deduced amino acid sequences of the two *Mr*Tra-2 isoforms were used in a multiple sequence alignment with the other Tra-2 proteins of crustaceans including two isoforms from the giant tiger shrimp (*P. monodon*), four isoforms from the Chinese mitten

crab (*E. sinensis*), three isoforms from the Chinese shrimp (*F. chinensis*) and one isoform from the oriental river prawn (*M. nipponense*). The alignment showed that an RRM, two RS (RS1 and RS2), a linker and a poly glycine region were present in all of the Tra-2 proteins. The RRM and the linker were conserved across the crustacean Tra-2 more than the other regions (Fig. 4A). A phylogenetic tree was constructed from the deduced amino acids of Tra-2 proteins from 24 species (7 vertebrates, 5 crustaceans and 12 insects). Only the longest isoform of Tra-2 in each species was used in the tree construction. The phylogenetic tree clearly showed three distinct clades of the vertebrate, crustaceans and insect Tra-2 proteins, and placed Tra-2 of *M. rosenbergii* and *M. nipponense* on the same node supporting the evolutionary relationship of these two species from the *Macrobrachium* genus (Fig. 4B) .



(A)

Figure 4. Multiple sequence alignment and phylogenetic tree of Tra-2 homologues. (A) Multiple sequence alignment of *Mr*Tra-2 with Tra-2 homologues from crustaceans. Identical and similar amino acid residues are highlighted in black and gray, respectively. An RNA recognition motif (RRM), two arginine-lysine rich regions (RS1 and RS2), a linker and a poly glycine regions are indicated.



(B)

Figure 4. (continued) Multiple sequence alignment and phylogenetic tree of Tra-2 homologues. (B) Phylogenetic tree of *Mr*Tra-2 and Tra-2 homologues from other species. The tree was constructed using the maximum likelihood method with 1,000 bootstrap replicates. The numbers of the tree branches indicate the bootstrap values. Sequence names, accession numbers and organisms are summarized in Table 2.

5.4 Spatial distribution of MrTra-2 in adult tissues

The expression of *MrTra-2* cDNA variants in 10 adult tissues including hypertrophied androgenic glands (hAG), eyestalk, gills, hemocyte, heart, hepatopancreas, muscle, ovary, testis and *vas deferens* was examined. Standard RT-PCR showed that both *MrTra-2a* and *MrTra-2b* were ubiquitously expressed in all adult tissues examined (Fig. 5).

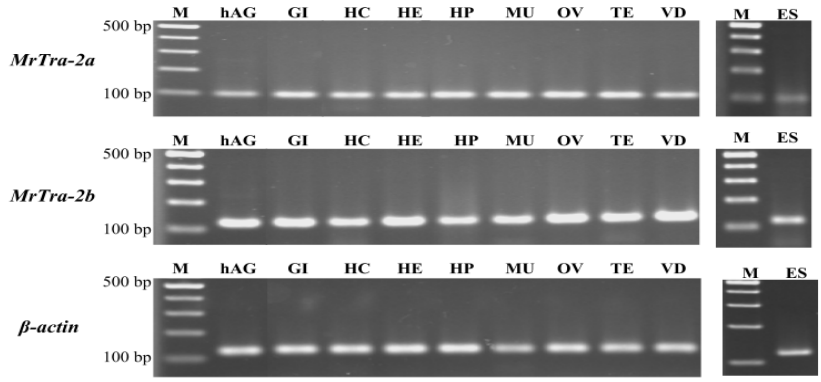


Figure 5. RT-PCR of *MrTra-2a*, *MrTra-2b* and *β-actin* in adult tissues of *M. rosenbergii* on 2% agarose gel electrophoresis. Lane M: 100 bp DNA ladder, hAG: hypertrophied androgenic gland, GI: gills, HC: hemocyte, HE: heart, HP: hepatopancreas, MU: muscle, OV, ovary, TE: testis, VD: *vas deferens*, ES: eyestalk

Quantitative real-time RT-PCR (qRT-PCR) was performed to measure expression levels of *MrTra-2a* and *MrTra-2b* transcripts in adult tissues. The expression levels of each variant were normalized with those of the internal control (*β-actin*) followed by analysis of the relative expression. The results showed that *MrTra-2a* had the highest expression in hepatopancreas and muscle with approximately 11-fold greater than that in hemocyte, where its expression was lowest (Fig. 6A). *MrTra-2b* had the highest and lowest expression in hepatopancreas and hAG, respectively, with a 31-fold difference (Fig. 6B). Both *MrTra-2* variants did not have a sexually dimorphic expression pattern and showed a comparable expression level between testis and ovary. Statistical analysis of the expression level between both variants in each tissue showed that *MrTra-2b* was significantly expressed more than *MrTra-2a* in most adult tissues including gills, hemocyte, heart and hepatopancreas at $P < 0.05$, and ovary and testis at $P < 0.01$ (Fig. 6C). The highest relative expression level of *MrTra-2b* compared to *MrTra-2a* was in testis (5.7 fold). The expression between *MrTra-2a* and *MrTra-2b* were statistically equivalent in hAG, eyestalk, muscle and *vas deferens* ($P > 0.05$).

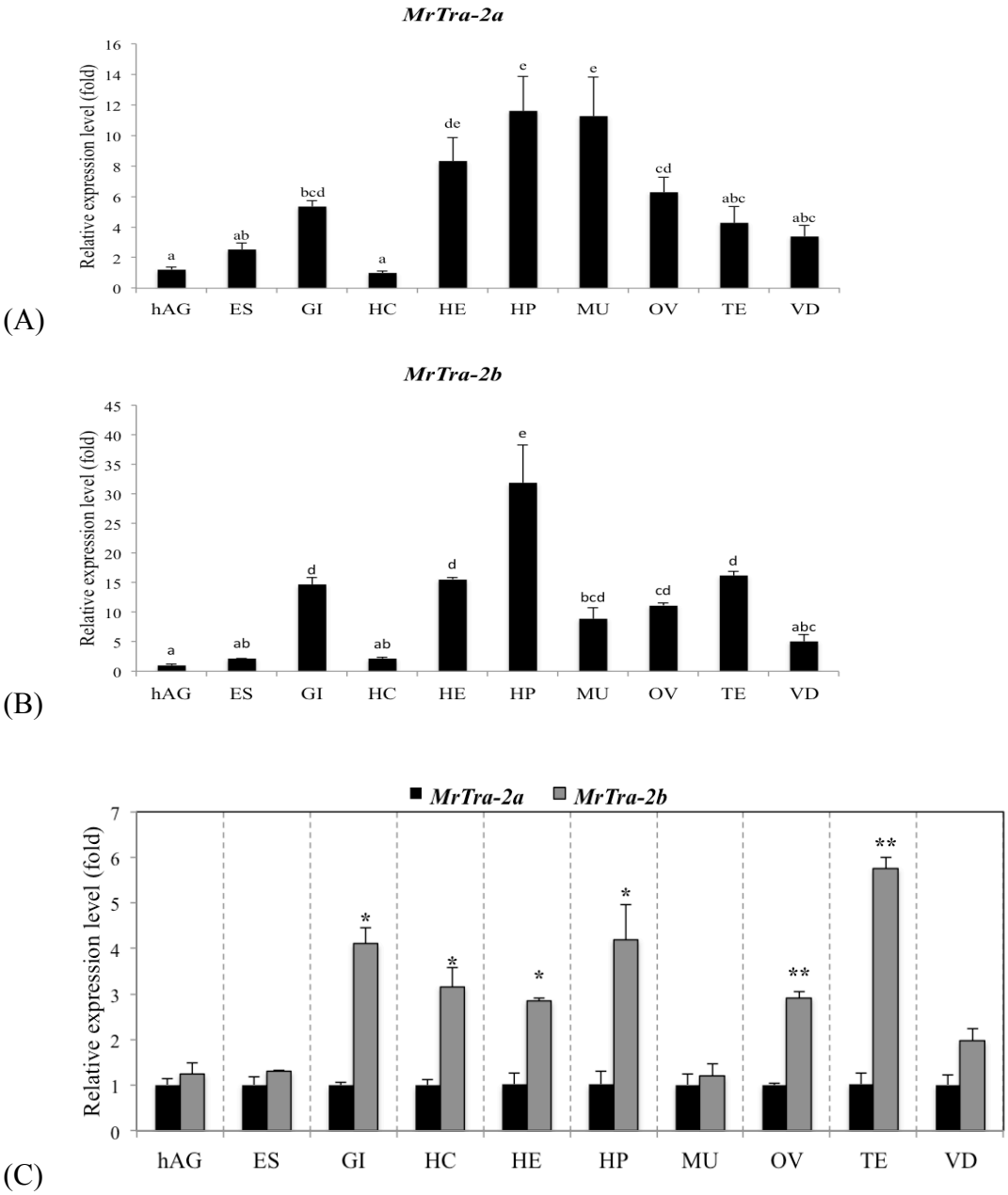


Figure 6. Spatial distribution of *MrTra-2* variants in adult tissues of *M. rosenbergii*. Relative expression levels of (A) *MrTra-2a*, (B) *MrTra-2b* in the adult tissues and (C) *MrTra-2a* and *MrTra-2b* in each adult tissue. Data are presented as means and standard deviations (means ± SD; n = 3). Statistical differences were determined using one-way ANOVA followed by *post hoc* Duncan's multiple range test (DMRT) at $P < 0.05$ for (A) and (B), and unpaired Student's *t*-test for (C). * and ** indicate a statistical significance at $P < 0.05$ and $P < 0.01$, respectively. hAG: hypertrophied androgenic gland, ES: eyestalk, GI: gills, HC: hemocyte, HE: heart, HP: hepatopancreas, MU: muscle, OV: ovary, TE: testis, VD: *vas deferens*.

5.5 Temporal distribution of MrTra-2 during developmental stages

Expression of *MrTra-2a* and *MrTra-2b* throughout larval (L) and post-larval (PL) stages were examined. Standard RT-PCR showed that both *MrTra-2a* and *MrTra-2b* were expressed as early as the first day after hatching (L1) and detectable at all of the following developmental stages examined (Fig. 7).

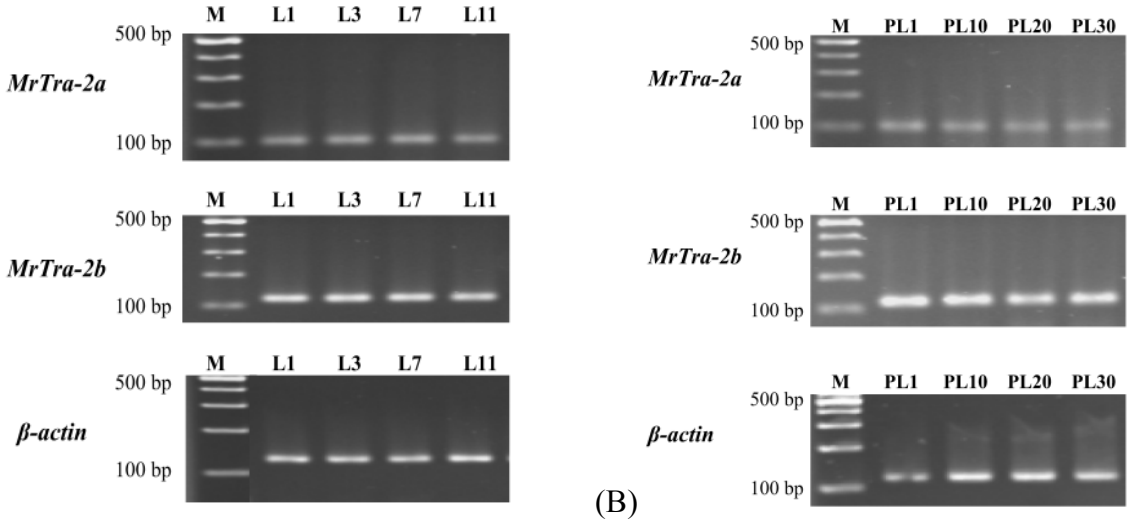


Figure 7. RT-PCR of *MrTra-2a*, *MrTra-2b* and *β-actin* at different developmental stages of *M. rosenbergii* on 2% agarose gel electrophoresis. Larval stages (L) at L1, L3, L7 and L11 are shown as indicated. Post-larval stages (PL) at 1, 10, 20 and 30 days after metamorphosis are presented as PL1, PL10, PL20 and PL30, respectively. Lane M is 100 bp DNA ladder.

Quantitative expression levels of *MrTra-2a* and *MrTra-2b* were measured and normalized with the internal control (*β-actin*). The lowest expression was observed from *MrTra-2* at L11 and used as a basal level for analysis of the relative expression (fold). In larvae (L), the expression of both variants was relatively low at all L stages, except L3 where their expression was raised and became significantly higher than the others (Fig. 8). Statistical analysis showed that the expression level of *MrTra-2a* and *MrTra-2b* at L3 was approximately 10-fold and 16-fold, respectively, higher than the lowest (*MrTra-2a* at L11). Both *MrTra-2* variants showed a significant up-regulation when larvae at L11 underwent metamorphosis and became post-larvae (PL1). During the PL development, the expression of *MrTra-2a* had no significant difference at all stages while *MrTra-2b* was expressed

dynamically (Fig. 8). The expression of *MrTra-2b* was highest at PL10 (approximately 13-fold higher than the lowest) followed by a slight decrease in the subsequent stages (PL20 and PL30).

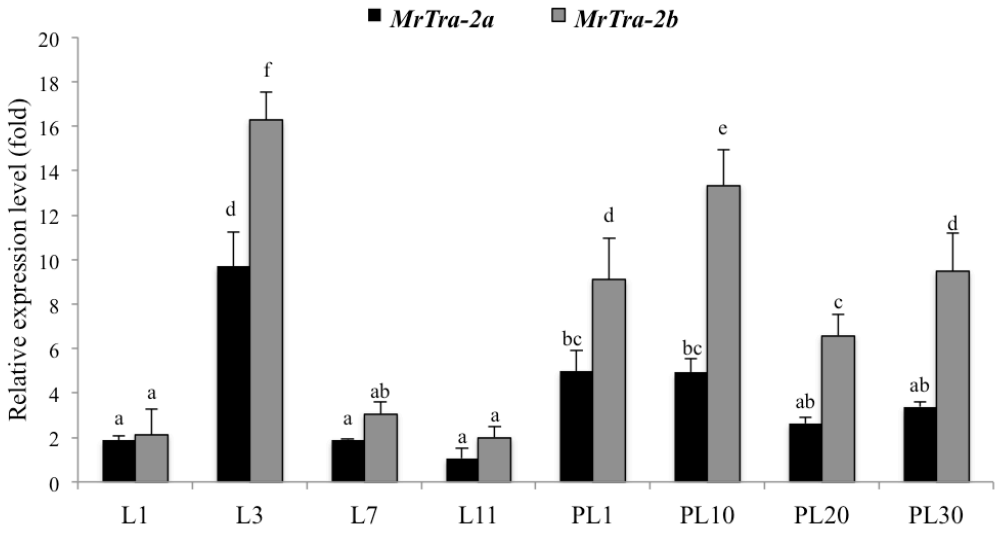
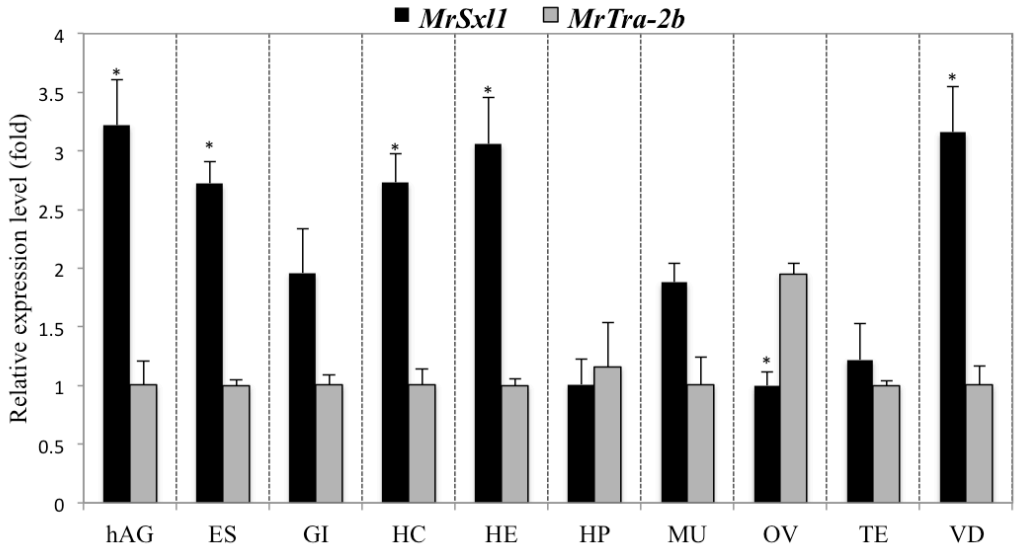


Figure 8. Temporal distribution of *MrTra-2a* and *MrTra-2b* at different developmental stages of *M. rosenbergii*. Larval stages (L) at L1, L3, L7 and L11 are shown as indicated. Post-larval stages (PL) at 1, 10, 20 and 30 days after metamorphosis are presented as PL1, PL10, PL20 and PL30, respectively. Data are presented as means and standard deviations (means ± SD; n = 3). Statistical differences were determined using one-way ANOVA followed by *post hoc* Duncan's multiple range test (DMRT) at $P < 0.05$.

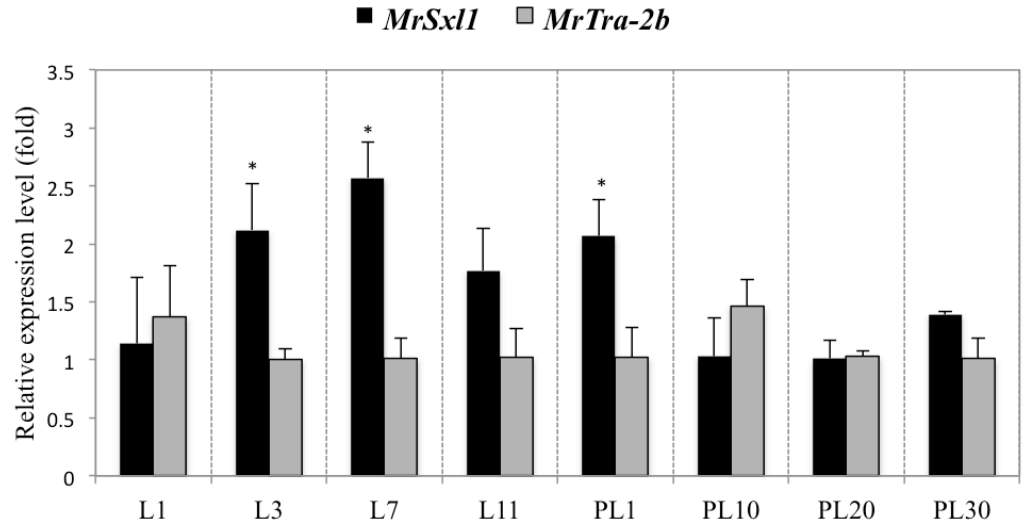
5.6 Expression profiles of MrTra-2 and other sex-related genes

Recently, a homologue of *Sex-lethal* (*Sxl*), the first gene of the *Sxl-Tra/Tra-2-Dsx* sex-determining hierarchy in *Drosophila*, was identified in *M. rosenbergii* (McMillan et al., 2018). Three variants of *MrSxl* were characterized and the gene expression analysis showed that *MrSxl1* had the highest expression level in all adult tissues and developmental stages examined. Here, *MrSxl1* and *MrTra-2b*, the variants with the highest expression level of *MrSxl* and *MrTra-2*, respectively, in most adult tissues and developmental stages of *M. rosenbergii*, were chosen for comparison of their expression profiles. In adult tissues, statistical analyses showed that *MrSxl1* was expressed in a significantly higher level than *MrTra-2b* in hAG, eyestalk, hemocyte, heart and *vas deferens*, but lower in ovary ($P < 0.05$; Fig. 9A). In larvae, *MrSxl1* was expressed significantly higher than *MrTra-2b* at L3 and L7

($P < 0.05$) but had no difference at L1 and L11 (Fig. 9B). In post-larvae, the expression of *MrSxl1* was significantly higher over *MrTra-2b* only at PL1 ($P < 0.05$).



(A)



(B)

Figure 9. Relative expression levels of *MrSxl1* and *MrTra-2b* in adult tissues (A) and at developmental stages (B) of *M. rosenbergii*. Data are presented as means and standard deviations (means ± SD; n = 3). Statistical differences were determined using unpaired Student's *t*-test. An asterisk (*) indicates a significant difference ($P < 0.05$). hAG: hypertrophied androgenic gland, ES: eyestalk, GI: gills, HC: hemocyte, HE: heart, HP: hepatopancreas, MU: muscle, OV: ovary, TE: testis, VD: *vas deferens*. Larval stages (L) at L1, L3, L7 and L11 are shown as indicated. Post-larval stages (PL) at 1, 10, 20 and 30 days after metamorphosis are presented as PL1, PL10, PL20 and PL30, respectively.

Another sex-related gene used in the expression analysis is *MrIAG*, which is responsible to the development of male characteristics in *M. rosenbergii* (Ventura et al., 2009). The expression of *MrIAG* was male-specific and first detected in PL20 prior to an appearance of external male organs (appendix masculina and male gonopore). In adult males, *MrIAG* was strictly expressed only in AGs, where the androgenic gland hormone—the male-specific hormone—was produced. Here, the expression of *MrTra-2b*, *MrSxl1* and *MrIAG* in PL30, male juveniles and hAG was quantitatively compared. Statistical analysis showed that the relative expression levels of *MrSxl1* and *MrTra-2b* were significantly higher (approximately 180-fold and 170-fold, respectively) than that of *MrIAG* at PL30 (Fig. 10A). The expression of *MrIAG* was elevated in juveniles and became significantly higher (approximately 45-fold) than that of *MrSxl1* and *MrTra-2b* (Fig. 10B). A similar observation to the juvenile was in hAGs where *MrIAG* was dramatically expressed over 10,000-fold higher than *MrSxl1* and *MrTra-2b* (Fig. 10C).

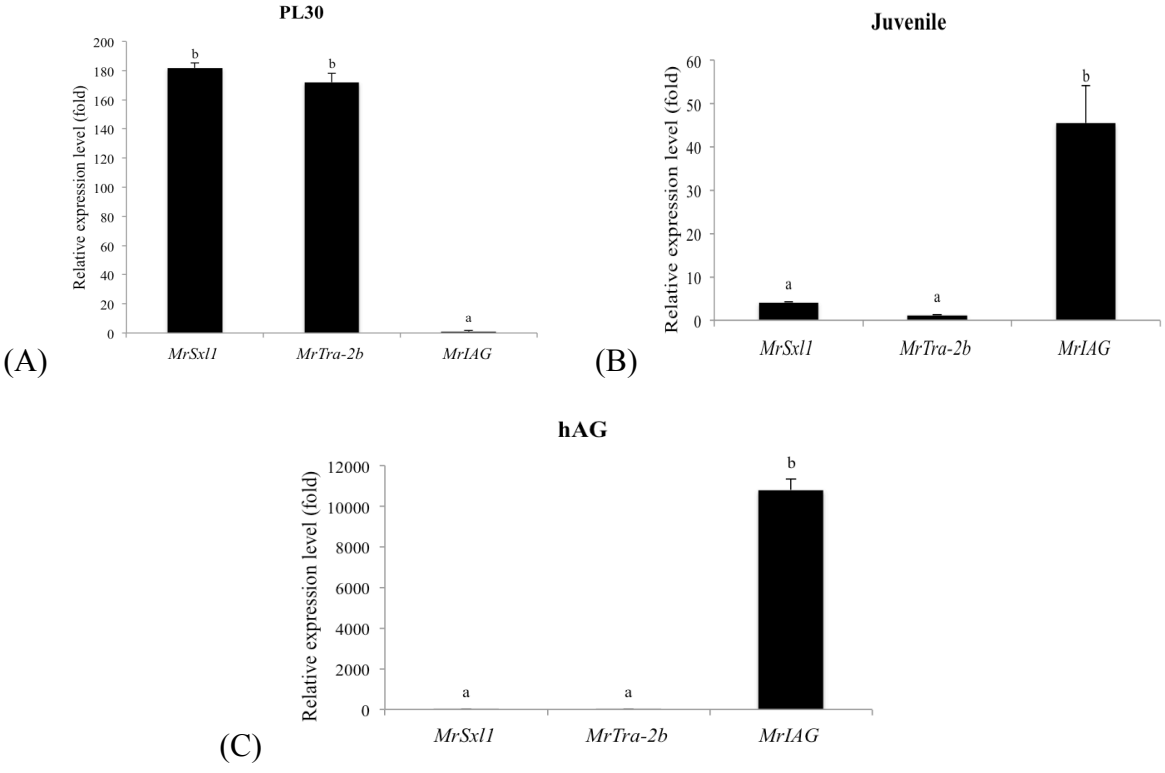


Figure 10. Relative expression levels of *MrSxl1*, *MrTra-2b* and *MrIAG* in (A) post-larvae at 30 days after hatching (PL30), (B) male juveniles and (C) hypertrophied androgenic glands (hAG) of *M. rosenbergii*. Data are presented as means and standard deviations (means ± SD; n = 3). Statistical differences were determined using one-way ANOVA followed by *post hoc* Duncan's multiple range test (DMRT) at $P < 0.05$.

6. Discussion

In this study, a *Tra-2* homologue from *M. rosenbergii* (*MrTra-2*) was identified and two variants (*MrTra-2a* and *MrTra-2b*) of the full-length cDNAs of *MrTra-2* were characterized. They were possibly generated from the same primary mRNA, which was processed through an alternative splicing mechanism to produce different transcript variants. Alternatively spliced variants of *Tra-2* have been previously reported in other crustaceans including *FcTra-2* of the Chinese shrimp, *F. chinensis* (Li et al., 2012), *EsTra-2* of the Chinese mitten crab, *E. sinensis* (Luo, 2015) and *PmTra-2* of the giant tiger shrimp, *P. monodon* (Leelatanawit et al., 2009), except *MnTra-2* of the oriental river prawn, *M. nipponense* whose only one isoform was identified (Zhang et al., 2013b).

The spatial expression analysis showed that both *MrTra-2* variants were ubiquitously expressed in all of the examined adult tissues. Neither tissue- nor sex-specific variants of *MrTra-2* were detected in *M. rosenbergii*, similar to the previous studies of *Tra-2* in other crustaceans (Li et al., 2012; Zhang et al., 2013b). Both *MrTra-2a* and *MrTra-2b* had a comparable expression level in the gonads of both sexes of *M. rosenbergii* without a sexually dimorphic pattern, which is unlike *FcTra-2* and *MnTra-2* whose expression was significantly higher in the testis than the ovary (Li et al., 2012; Zhang et al., 2013b). The absence of sex-specific variants in crustaceans suggested that *Tra-2* might be involved in the gonad development of crustaceans but not sexually regulated.

MrTra-2 was dynamically expressed throughout the larval and post-larval stages, implying its roles during the early development of *M. rosenbergii*. The significant increase of *MrTra-2* at L3 and PL1 was correlated with a rapid up-regulation of *MrSxl*, the gene located at the top of the *Sxl*/*Tra*/*Tra-2*/*Dsx* pathway (McMillan et al., 2018). It is hypothesized that the pathway was initiated as early as when a prawn hatched (L1), then elevated at L3 when *MrSxl* was highly expressed and subsequently activated the downstream targets, including *MrTra-2*, in the pathway. A similar observation has been reported in *MnSxl* and *MnTra-2* of *M. nipponense* (Zhang et al., 2013a,b), the species with the close evolutionary relationship to *M. rosenbergii*, emphasizing the importance of *Sxl* and *Tra-2* during the development of these species. The up-regulation *MrTra-2* after metamorphosis is in accordance with a sharp increase of not ony *MrSxl* (McMillan et al., 2018) but also *Mr-CYP15A1*, a gene encoding the cytochrome P450 that catalyzes epoxidation of methyl farnesoate (MF) to juvenile hormone III (JHIII) and enables metamorphosis (Ventura et al., 2013). In branchiopod crustaceans, for example *Daphnia magna*, MF is the sex determinant and directs the development of male pathway in response to environmental signals (Olmstead and Leblanc, 2002). How the

Sxl/*Tra*/*Tra-2*/*Dsx* pathway and MF are functionally related, especially regarding the sex-determining mechanism, in *M. rosenbergii* is yet to be determined.

The up-regulation of *MrTra-2* at PL1 and PL10 was prior to the first expression of *MrIAG* at PL20 (Ventura et al., 2009). Although a temporal silencing of *MrIAG* is sufficient to sexually reverse the male to be female, how the expression of *MrIAG* is regulated remains unknown. The increased expression of *MrTra-2* in PL was possibly to response the activation of *MrSxl* from top of the pathway followed by an initiation of *MrIAG* in later PL stages. The expression level of *MrIAG* increased and became higher than *MrSxl* and *MrTra-2* in juveniles and adult hAGs. Silencing of *MrSxl*, *MrTra-2* and/or simultaneously with that of *MrIAG* may reveal how these genes are functionally related and whether or not they are mutually regulated. A comprehensive investigation of how the *Sxl*/*Tra*/*Tra-2*/*Dsx* pathway functions, especially in cooperation with *MrIAG*, may uncover the mechanism underlying sexual development, if not sex determination, in *M. rosenbergii* and possibly bring an understanding of how the sex is established in crustaceans.

In conclusion, this study characterized two alternatively spliced variants of *MrTra-2* and exhibited their spatiotemporal expression profiles, together with their relative expression with other sex-related genes.. It was hypothesized that *MrTra-2* was possibly involved in sex development rather than sex determination. Analyses of the gene expression suggested that *MrTra-2* and the *Sxl*/*Tra*/*Tra-2*/*Dsx* pathway played important roles either during or after metamorphosis when the male-specific *MrIAG* gene was initiated. Further investigations, such as gene silencing, are needed to disclose the biological functions of *MrTra-2* in *M. rosenbergii*.

7. Output

7.1 Complete cDNA sequences of two variants of *MrTra-2* (*MrTra-2a* and *MrTra-2b*)

7.2 Deduced amino acid residues of two isoforms of *Mr*Tra-2 protein

7.3 Multiple sequence alignments and a phylogenetic tree of *Mr*Tra-2 and Tra-2 from other species

7.4 Spatial expression profiles of *MrTra-2a* and *MrTra-2b* in adult tissues

7.5 Temporal expression profile of *MrTra-2a* and *MrTra-2b* during larval and post-larval developmental stages

7.6 Comparison of *MrTra-2b* and *MrSxl1* in adult tissues, larvae and post-larvae

7.7 Comparison of *MrTra-2b*, *MrSxl1* and *MrIAG* expression levels in post-larvae, juveniles and androgenic glands

7.8 A manuscript in preparation entitled 'Characterization and Expression Analysis of *Transformer-2* Gene in Giant River Prawn, *Macrobrachium rosenbergii*' which will be submitted to *Gene* (Impact factor 2.32)

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