

Identification of spliced mRNA isoforms of retinoid X receptor (RXR) in the Oriental freshwater prawn *Macrobrachium nipponense*

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ABSTRACT. Retinoid X receptors (RXR) are members of the nuclear receptor family that are conserved from invertebrates to vertebrates, and they play an essential role in regulating reproductive maturation, molting, and embryo development. In this study, five RXR isoforms, named RXRL2 (L, long form), RXRL3, RXRS1 (S, short form), RXRS2, and RXRS3, containing six domains from A to F, were cloned from the prawn *Macrobrachium nipponense* using 5'- and 3'- rapid amplification of cDNA ends. Differences among their structures were observed not only in the D and E domains but also in the A/B domain, which were previously found in insects but not in crustaceans. This is the first report to show that differences occur in the A/B domain of RXR in crustaceans. RXR expressions were also examined in various tissues including the ovary, testis, muscle, hepatopancreas, heart, gill, stomach, intestine, and cuticle. Expression pattern investigations indicated that the five isoforms were differentially expressed. RXRS3

was only detected in the ovary, and the other RXRs were abundant in the ovary and testis. These data suggested that RXR mediates a series of processes related to reproduction.

Key words: Retinoid X receptor; *Macrobrachium nipponense*; Cloning; Distribution; Isoforms

INTRODUCTION

Retinoid X receptors (RXRs) are the most highly conserved members of the nuclear receptors superfamily. RXR was first isolated from the cDNA library of human liver and kidney cells using human retinoic acid receptor (RAR) DNA in combination with regional cDNA (Mangelsdorf et al., 1990). RXRs have been reported in many species from invertebrates to vertebrates (Wiens et al., 2003). In vertebrates, RXR can bind with RAR to form a heterodimer. Upon activation by 9-cis retinoic acid (RA), the RXR/RAR heterodimer regulates the expression of genes involved in many distinct stages during early embryogenesis, organogenesis, and the transduction of the RA signal during prenatal development (Mark and Chambon, 2003; Mark et al., 2006). RXRs can also form homodimers or heterodimers with the liver X receptor (LXR), the thyroid hormone receptor (TR), the pregnane X receptor (PXR), and other nuclear receptors (Waxman, 1999). Hence, RXR mediates various regulatory activities that are important to the normal physiology of vertebrates. In invertebrates, especially crustaceans and insects, RXR can heterodimerize with the ecdysone receptor (EcR). The heterodimer EcR/RXR induces the expression of early ecdysone responsive genes, such as transcription factors E74, E75, and Broad Complex, which regulate further downstream cascade reactions by binding to hormone responsive elements (Li and White, 2003; Riddiford et al., 2003).

RXR shares the common six domains (A-F) with other nuclear receptors (Renaud and Moras, 2000; Aranda and Pascual, 2001; Bonneton et al., 2003). The A/B domain at the N-terminal contains a transcriptional activation function (AF-1) sequence in some members, and its length and amino acids vary among species (Aranda and Pascual, 2001; Cherbas et al., 2003). The C region is highly conserved and acts as a DNA binding domain (DBD), including two zinc-finger motifs (Devarakonda et al., 2003). The E region acts as a ligand-binding domain (LBD), harboring transactivation (AF-2) motifs and a hydrophobic pocket for ligands, and it is associated with heterodimerization (Wang et al., 2000; Hu et al., 2003). The D domain is a flexible hinge region linking the C and E domains. The F region is at the C-terminal, which varies in length and sequence and may be entirely absent in some species.

Due to splice variants in post-transcription, RXR has multiple isoforms in various organisms. In vertebrates, three isoforms RXR α , RXR β , and RXR γ , were identified (Tallafuss et al., 2006; He et al., 2009). The main differences among the three isoforms occur in the N-terminal sequences, and their tissue distributions are different. In *Sebastiscus marmoratus*, three isoforms were isolated with differences in the H1-H3, H7, and H12 helices. According to the expression of the three isoforms in embryo development stages, it was deduced that RXR α might be involved in the development of the neural crest, anterior hindbrain, tailbud, and in the formation of the fin and pharynx, whereas RXR β likely plays important roles in early development, and that RXR γ appears to play a role in the development and function of the nervous system and brain (He et al., 2009). In mature adult rodents, RXR α was found to

be abundant in the liver, kidney, spleen, and cuticle, while RXR β was expressed in almost all tissues, and predominantly in the central nervous system. In contrast, the expression of RXR γ was found to be limited to muscle and the peripheral nervous system (Chung and Cooney, 2003). In invertebrates, especially crustaceans and insects, the numbers of RXR isoforms also varies. For instance, in the land crab (*Gecarcinus lateralis*), nine splicing isoforms were produced by three splicing sites, with one site in the D domain and two in the E domain. More isoforms were produced in the gonad than in skeletal muscles, which indicate a greater complexity of the RXR isoforms in gonads (Kim et al., 2005). In the fiddler crab (*Uca pugilator*), four RXR isoforms were produced by two splicing sites, with one site in the D domain and the other in the E domain (Hopkins et al., 2008). In the cockroach (Bg, *Blattella germanica*), two RXR isoforms were isolated, named Bg-RXRL (long form) and Bg-RXRS (short form), with a 23-amino acid insertion/deletion in the H1-H3 helices of the LBD. During embryogenesis, Bg-RXRL was detected at the early stages, while Bg-RXRS was highly expressed in the middle and late stages. This expression pattern strongly suggested that RXR was related to processes in the embryogenesis and the Bg-RXRL might be of maternal origin (Maestro et al., 2005). Many insect RXR isoforms show differences in the A/B domain, except for *B. germanica* and migratory locust.

To date, RXR full sequences and their isoforms' expression patterns and functions have been isolated and detected in both land crab and fiddler crab independently, which both belong to the decapod crustacean group (Durica et al., 2002; Kim et al., 2005). The Oriental freshwater prawn, *Macrobrachium nipponense*, which also belongs to the Palaemonidae family of decapod crustaceans, is an economically and nutritionally important species. In order to gain a better understanding of the expression and function of RXR in crustaceans, in this study, we cloned and characterized the MnRXRs with respect to sequence comparison, 3D-model, phylogeny, and tissue expression. Together, these results have led to a hypothesis that crustacean RXRs present more isoforms than those of insects and vertebrates.

MATERIAL AND METHODS

Animals and sample collection

Adult prawns, *M. nipponense*, larger than 6 cm in length, were purchased from a market in Shanghai, China. They were sacrificed in order to collect tissue samples such as the ovary, testis, heart, muscle, hepatopancreas, intestine, and gill. All tissues were frozen immediately in liquid nitrogen and stored at -80°C until used.

Cloning of RXR cDNAs

Total RNA was extracted from the ovaries of prawns using Trizol reagent (Invitrogen, USA) as described in the manufacturer protocol, and treated with RNase-free DNaseI (Invitrogen). RNA was reverse transcribed to cDNA with oligo (dT) primers using RT M-MLV (RNase H-, TaKaRa, Japan). The cDNA was used as a template in subsequent polymerase chain reaction (PCR).

Degenerate primers, RXR-DF and RXR-DR (Table 1), were designed based on the highly conserved C domain of several homologous proteins in the GenBank database (<http://>

www.ncbi.nlm.nih.gov/genbank/), which were used to amplify a partial sequence encoding the *M. nipponense* homolog of RXR. PCR was performed in 25 μ L volumes including 1 μ L cDNA primer, 4 μ L PCR buffer, 2 μ L dNTP, 1 μ L upstream, 1 μ L downstream, 0.2 μ L DNA polymerase, and 15.8 μ L ddH₂O. Samples were run on a PCR machine, Mastercycle Pro S (Eppendorf, Germany), as follows: 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, then extension at 72°C for 45 s, then post-extension at 72°C for 5 min. Amplification products were resolved on 1.2% agarose gel, excised, recovered using a purification system, and then linked into a pGM-T vector, transformed into competent cells, screened white bacterial colonies, and then directly sequenced.

Rapid amplification of cDNA ends (RACE) was used to obtain full-length sequences. The cloning strategy is shown in Figure 1, and the primers are listed in Table 1. For the 3'-sequence, the 3'-Full RACE Core Set (TaKaRa) was used. The specific primers, 3RXRS1 and 3RXRS2 (Table 1), were designed according to the conserved sequence obtained above. Briefly, first-strand cDNA synthesis reactions were performed according to the 3'-Full RACE Core Set. Nested PCR was conducted with a gene-specific primer, 3RXRS1, and the 3'-RACE Outer Primer as the first-round PCR under the following conditions: 25 cycles (94°C for 30 s, 65°C for 30 s, and 72°C for 1 min) and then extension at 72°C for 10 min. Next, the second-round PCR was performed with the 3RXRS2 primer and the 3'-RACE Inner Primer under the same conditions. PCR products were cloned and sequenced as described above.

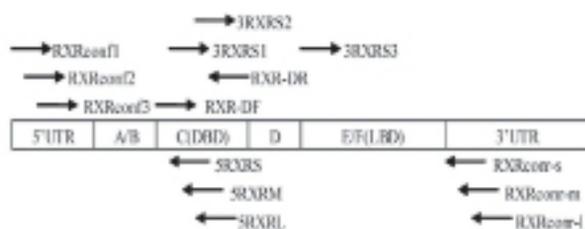


Figure 1. Location of the primers used for cloning of MnRXRs.

The SMARTTM RACE cDNA Amplification Kit (TaKaRa) was used to obtain the 5'-end sequences of each cDNA. The downstream specific primers were designed according to the sequences obtained by 3'-RACE, and were named 5RXRS, 5RXRM, and 5RXRL (Table 1). After the first-strand cDNA synthesis, first-round PCR was performed under the following conditions: 5 cycles (94°C for 30 s, 68°C for 30 s, and 72°C for 2 min), 20 cycles (94°C for 30 s, 65°C for 30 s, and 72°C for 3 min), and then extension at 72°C for 10 min. Second-round PCR was conducted using nested gene-specific primer and a nested universal primer. The PCR conditions were the same as those used for first-round PCR. PCR products were cloned and sequenced as described above.

The sequences obtained from 5'-RACE and 3'-RACE, along with the conserved sequence, was spliced using the Bioedit software. In order to check the integrity of the splicing, specific primers, RXRconf1, RXRconf2, RXRconf3, RXRconr-s, RXRconr-m, and RXRconr-l (Table 1) were designed according to the 5'-untranslated region (UTR) and 3'-UTR separately. PCR products were cloned and sequenced as described above.

Sequence comparison, phylogenetic analysis and 3D model

Multiple sequence alignment of the full-length amino acids of Mn-RXRs with known sequences (DQ067280, AF032983, BAF75376.1, NM_011305, P19793, ABF74729, AAF73057) was conducted using the ClustalX software. Phylogenetic analysis of the full-length RXRs was performed using the neighbor-joining method with 1000 bootstrap repetitions. The consensus phylogenetic tree was assembled using Mega 4.0. The 3D models of Mn-RXRs were created by the Phyre2 web server and labeled with Adobe Photoshop.

Expression of MnRXRs

Total RNA was extracted from prawn tissues, including the ovary, testis, heart, muscle, hepatopancreas, intestines, stomach, and gill, and treated with RNase-free DNaseI (Invitrogen). Subsequently, cDNAs were synthesized using the Superscript RNase H-reverse transcriptase first strand synthesis system, and quantified based on absorbance at 260 nm. PCR was performed with the gene-specific primers (RXRconr-s and RXRconf1, RXRconr-s and RXRconf2, RXRconr-s and RXRconf3, RXRconr-l and RXRconf2, and RXRconr-l and RXRconf3) under the following conditions: denaturation at 94°C for 3 min, 30 cycles (94°C for 30 s, 58°C for 30 s, and 72°C for 30 s), and post-extension at 72°C for 7 min. The amplification products were then resolved on 1.2% agarose gel. Meanwhile, β -actin was used as an internal standard with the same templates.

RESULTS

Cloning of *M. nipponense* RXR cDNA

A 176-bp product was obtained with PCR using degenerated RXR primers based on the partial cDNA sequences encoding the C domain of known RXRs. The deduced amino acid sequence was 91% identical to the DBD of *Celuca pugilator* and *Gecarcinus lateralis*, 84% identical to *Mus musculus* RXR α , and 87% identical with *Bombyx mori*, indicating that it could be a *M. nipponense* ortholog of RXRs (Figure 2).

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Up-RXR      IFFWYISCEGGKQFFRITVRNDLTYADREERSCTIDKRGMRQDYORYQKDLTIGANKRE
Gj-RXR      IFFWYISCEGGKQFFRITVRNDLTYADREERSCTIDKRGMRQDYORYQKDLTIGANKRE
Mj-RXR      IFFWYISCEGGKQFFRITVRNDLTYADREERSCTIDKRGMRQDYORYQKDLTIGANKRE
Daphnia-RXR IFFWYISCEGGKQFFRITVRNDLTYADREDRQDLIDKRGMRQDYORYQKDLTIGANKRE
Mn-RXR      IFFWYISCEGGKQFFRITVRNDLTYADREDFVCTIDKRGMRQDYORYQKDLTIGANKRE
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Figure 2. Comparison of deduced amino acid sequences of RXR DNA binding domains.

To obtain the entire cDNAs of RXR, 3'-RACE and 5'-RACE were performed on the basis of the partial sequence. In 3'-RACE, two bands approximately 600 bp and 900 bp in length were obtained. Although RACE-PCR conditions were optimized, three sequences were obtained with lengths of 561, 610, and 885 bp, respectively, after the PCR products were

cloned and sequenced. All three sequences were spliced with the conserved DBD sequence. Subsequently, the amino acid sequences, named Mn-RXRS (short), Mn-RXRM (medium) and Mn-RXRL (long) were confirmed as three isoforms of the RXR 3'-end based on BlastX. In order to obtain the 3'-terminal sequence of Mn-RXRL, the specific primer 3RXRS3 (Table 1) was designed. In 5'-RACE, three sequences, named Mn-RXR1, Mn-RXR2 and Mn-RXR3, were obtained by two bands after cloning and sequencing as for the 3'-RACE. They were also spliced with the conserved sequence, and blasted in the National Center of Biotechnology Information (NCBI), which confirmed that they were three isoforms of the RXR 5'-end. The 3'- and 5'-RACE results revealed nine sequences after splicing, named Mn-RXRS1, Mn-RXRS2, Mn-RXRS3, Mn-RXRM1, Mn-RXRM2, Mn-RXRM3, and Mn-RXRL1, Mn-RXRL2, and Mn-RXRL3. However, only five isoforms were ultimately obtained in this study. The longest cDNA (Mn-RXRL2) was 1689 bp, which consisted of a 354 bp 5'-UTR, an open reading frame (ORF) of 1011 bp encoding a 337 amino acid sequence, and a 333 bp 3'-UTR. The deduced amino acid sequence of Mn-RXRL2 contained all six domains. The conserved DBD domain included a P-box (FGCKG) and a D-box (CREDR), and the D domain contained the T-box (EAVQEERQ), which is related to the DNA binding domain (Figure 3). Its deduced amino acid was 80% identical to that of *Marsupenaeus japonicus*, 84% identical to Gl-RXRa, 83% identical to Gl-RXRb, 81% identical to Gl-RXRc, 76% identical to Gl-RXRd, 83% identical to Gl-RXRf, 83% identical to the *C. pugilator* RXR, and 68% identical to the *Apis mellifera* RXR. The information for the other four isoforms is described in Table 2.

Table 1. Primers used in this study.

Designation	Primer Sequence	Use
RXR-DF	5'-CACTAYGGSGTSTACAGCTG-3'	Initial amplification, FP
RXR-DR	5'-GCTTCYCKCTTCATSCCAT-3'	Initial amplification, RP
3RXRS1	5'-GGAGTATACAGCTGTGAGGGTTGCA-3'	3'-RACE first-round, FP
3RXRS2	5'-AGAAGTGTCTATCCATGGGCATG-3'	3'-RACE nested, FP
3RXRS3	5'-TGGGTGTTAAAGATGGCATTGTCT-3'	3'-RACE nested, FP
3' RACE Outer Primer	5'-TACCGTCGTTCCACTAGTGATTT-3'	3'-RACE first-round, RP
3' RACE Inner Primer	5'-CGCGGATCCTCCACTAGTGATTTCACTATAGG-3'	3'-RACE nested, RP
5RXRS	5'-CGTTCGCTGGCGCTCCTCCTGGAC-3'	5'-RACE, RP
5RXRM	5'-TGTCGTGTCAGCTGCCTGGCATGT-3'	5'-RACE, RP
5RXRL	5'-CCAAGCCCACCTCTATTCCCCCT-3'	5'-RACE, RP
RXRconf1	5'-TCGGTAAACGTCTTGGCCTAAG-3'	Splicing verification, FP
RXRconf2	5'-TCGTGTGACGTTGGCACTTTCG-3'	Splicing verification, FP
RXRconf3	5'-GCCATTGGTTCGTTTCATCCGTTG-3'	Splicing verification, FP
RXRconr-s	5'-ATGGGGCAATACCAGCTGTG-3'	Splicing verification, RP
RXRconr-m	5'-AGAACCCTCGTGCCGCATC-3'	Splicing verification, RP
RXRconr-l	5'-ACGATGGATCGTAAACATCC-3'	Splicing verification, RP
β-actinF	5'-CGACGGTCAGGTCATACCA-3'	Control of tissue expression, FP
β-actinR	5'-ACGTCGCACTTCATGATGGA-3'	Control of tissue expression, RP

FP = forward primer; RP = reverse primer.

Based on comparison of the five isoforms, four variant alternative splicing sites were obtained. A splicing site was located in the amino terminal that generated three variant 5'-terminal sequences. A second splicing site in the D domain produced three sequences that differed in the length of the T box, which is a highly conserved sequence adjacent to the DBD. A third splicing site in the H1-H3 helices of the LBD produced two forms. The last one was truncated in the D domain, and therefore lacked the E/F domain.

that all of the isoforms consisted of a small C domain linked to a larger LBD domain by a long hinge. Notably, in Mn-RXRL3, Mn-RXRS1 and Mn-RXRS2, the long hinge contained an α -helix. The LBD domains appeared to show some similarities and differences. On the one hand, compared with other species, the helices in the LBD of Mn-RXRs were shorter, and for this reason, the helices between H9-H12 were truncated. On the other hand, the number of helices in the LBD domains of Mn-RXRL2 and Mn-RXRL3 were obviously higher than those of the three others, which lack the H6-H8 helices.

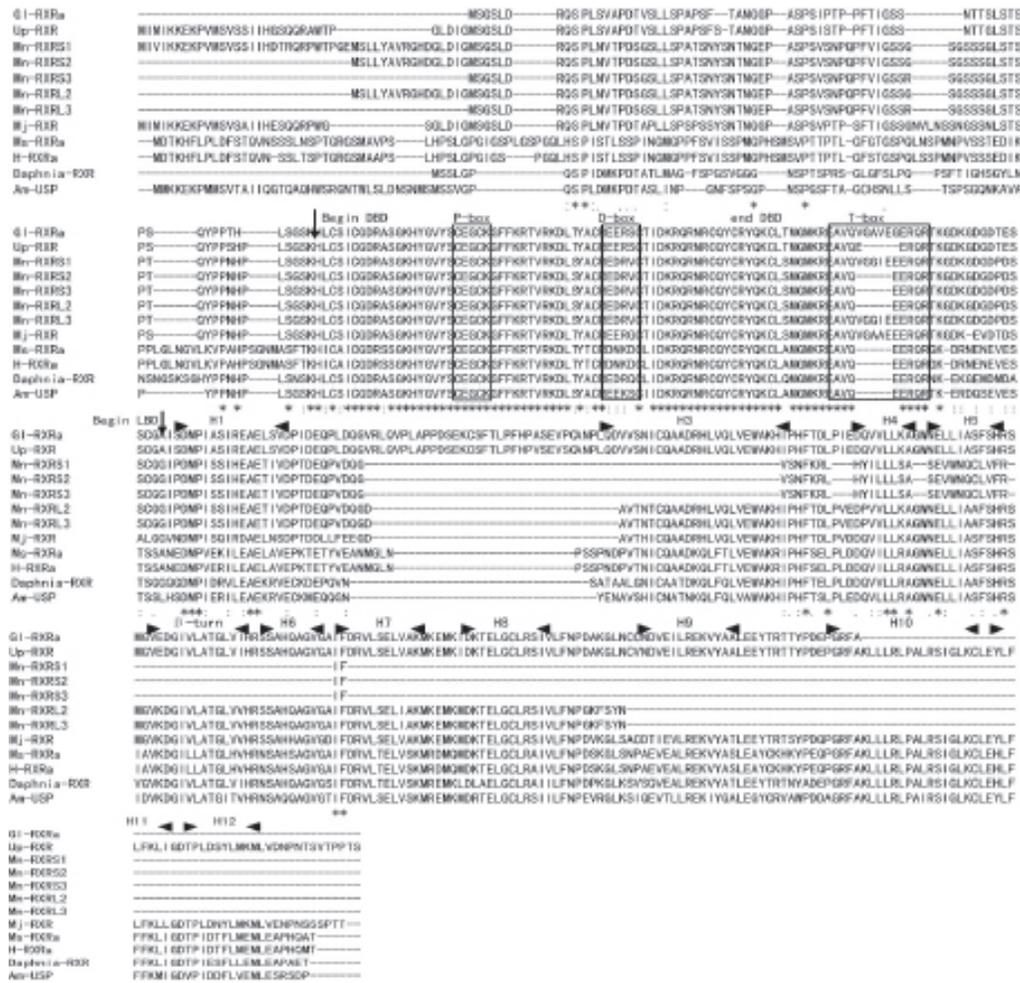


Figure 4. Multiple alignment of amino acid sequences of RXR cDNAs from crustaceans and vertebrates.

Since Mn-RXR sequences showed high homology with the other RXRs, neighboring analysis of the full-length RXR amino acid sequences was performed in order to gain perspective on their divergence pattern (Figure 6). RXRs of fiddler crab and land crab were in one branch, and RXRs of the prawns (*M. japonicus*, *M. nipponense*) were in the same branch,

Tissue expression of the prawn RXR

We performed reverse transcription-PCR (RT-PCR) to examine the tissue-specific expression profile of Mn-RXR. The five isoforms of Mn-RXR were tested in all of the tested tissues, and β -actin was used as an internal standard. The same template was used for the five isoforms and β -actin. The results are showed in Figure 7. Mn-RXRL2 was mainly expressed in the ovary, but was expressed at low levels in the testis, hepatopancreas, and heart. Mn-RXRL3 expression was the highest in the ovary and testis, but was weak in the muscle and hepatopancreas. Mn-RXRS1 was predominantly expressed in the ovary, and was mildly expressed in the gill and stomach. Mn-RXRS2 was expressed in the ovary and testis at very low levels. In contrast, Mn-RXRS3 was particularly abundant in the ovary.

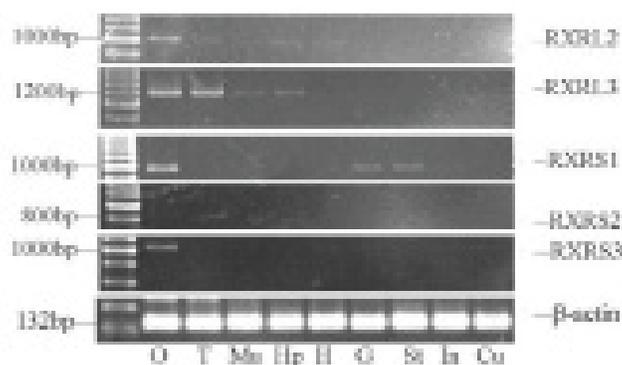


Figure 7. Expression of five isoforms of RXR in the crab tissues.

DISCUSSION

RXR is a highly conserved member of the steroid nuclear receptor family and plays important roles in several physiological processes of crustaceans, including reproduction and development. Most studies on RXR/USPs have focused on their structure, characterization, and function in vertebrates and invertebrates, whereas there are few reports on the molecular characterization of RXRs in decapod crustaceans. So far, entire cDNA sequences encoding crustacean RXRs have been obtained from fiddler crab *C. pugilator* (Durica and Hopkins, 1996; Chung et al., 1998), land crab *G. lateralis* (Kim et al., 2005), water flea *D. magna* (Wang et al., 2007), kuruma prawn *M. japonicus* (Asazuma et al., 2007), and Chinese shrimp *F. chinensis* (Priya et al., 2009). In this study, the full-length cDNA sequences of MnRXRs were isolated from *M. nipponense*.

Most studies have suggested that there are two or more isoforms of RXR, not only in the vertebrates, but also in invertebrates, including insects and crustaceans. Their common splicing sites were found to be mostly distributed in the T-box of the D domain and in the H1-H3 or H7/8 helices of the E domain (Hopkins et al., 2008), while several RXR isoforms were also shown to differ in their A/B domain sequences, especially in the insects (Horigane et al., 2008). In crustaceans, however, there was no evidence to verify that the isoforms differ

with respect to their A/B domains. In the present study, five sequences of RXR isoforms were found to differ not only in the D and E domains, but also in the A/B domain. The differences among the five isoforms were as follows. First, the A/B domain has three variant sequences, A/B (+16), A/B (+45), and A/B (-16). Second, there were two variant sequences in the T-box of the D domain, T (+12) and T (+7). Third, differences of the LBD were complicated and were distributed across the entire helix (Table 2, Figure 5). Therefore, the present study is the first to demonstrate differences in the A/B domain of crustacean RXRs.

In comparison to other RXRs, the lengths of the LBD of Mn-RXRs obtained in this study were shorter, because H9-H12 helices in the E domain were absent in the Mn-RXRs (Figure 5). Compared with the Up-RXR and Gl-RXR, the helices between H1-H3 of other RXRs were lacking the 33 amino acid (aa) insertion (Figure 3). In the fiddler crab *U. pugnator*, the results of GST-pull down experiments showed that UpEcR interacted only with UpRXR variants containing the 33 aa insertion, and not with those lacking the 33 aa insertion (Wu et al., 2004). The helices H9-H12 were also obviously truncated in all of the MnRXR isoforms. In *Drosophila* USP, H12 in the E domain is crucial for DNA binding, efficient ecysteroid binding by EcR, and transcriptional activation (Przibilla et al., 2004). In crustaceans and insects, RXR can heterodimerize with EcR. The heterodimer EcR-RXR/USP binds to sequence-specific DNA elements and induces the expression of a number of primary response genes, such as transcription factors E74, E75, and Broad Complex (Riddiford et al., 2003). Therefore, it was deduced that the function of these isoforms without the H9-12 region was affected. Moreover, the 3D structures were shown to be significantly different, especially in the LBD domain, which might affect their ligand binding functions.

In addition, other isoforms exist in *M. nipponense*, which were not obtained in this study. RXRs display a wider variety of isoforms in crustaceans than in insects and vertebrates. There were only two or three RXR/USP isoforms identified in an insect species (Kapitskaya et al., 1996; Jindra et al., 1997), and only three RXR isoforms have been shown to occur in vertebrates, including mice and zebrafish. However, there were potentially four (Up-RXR) or nine (Gl-RXR) alternatively spliced sequences generated at three splicing sites. In the prawn *M. nipponense*, there were five or more isoforms that were generated at four splicing sites. Consequently, multiple isoforms of RXR may represent a characteristic specific to crustacean.

Monitoring the expression of MnRXRs demonstrated that crustacean RXR isoforms showed different expression patterns from that of vertebrates and insects. In *S. marmoratus*, three RXR isoforms were expressed in all tissues examined, including the brain, eye, gill, heart, ovary, and testis. RXRa and RXRg were expressed at highest levels in the gill, which was consistent with results of Chinese shrimp *Fenneropenaeus chinensis*, which might suggest that RXR plays an important role in the structure and/or function of gills. RXRb was particularly abundant in gonads, which was similar to other work on mammals (He et al., 2009). In insects, such as in the penultimate and last larval instars of *B. germanica*, Bg-RXRS mRNA was the predominant form in the fat body and in the prothoracic gland. In the adult female, Bg-RXRS mRNA predominated in the fat body, whereas Bg-RXRL mRNA predominated in the ovary (Hopkins et al., 2008). In the prawn, the five RXR isoforms were all expressed in the ovary, and some of them were expressed in the testis, reinforcing the idea that RXR is involved in several physiological processes, including reproduction. RXRS3 was only detected in the ovary, suggesting that it is possibly very important for ovary development.

In summary, our study obtained five isoforms of RXR from *M. nipponense* by splicing

following RACE. Sequence comparison and 3D structures suggested that differences occurred in the A/B domain, which has not previously been shown in crustaceans, and that differences in the LBD might influence ligand-binding functions. Compared with vertebrate and other invertebrate species, our results suggest that multiple RXR isoforms might be a characteristic of crustaceans. The expression of RXRs was dominant in the ovary indicating that RXR likely plays an important role in ovary development.

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