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Cloning and Expression of Ecdysone Receptor and Retinoid X Receptor from *Procambarus clarkii*: Induction by Eyestalk Ablation

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Abstract: Ecdysone receptor and retinoid X receptor are key regulators in molting. Here, full length ecdysone receptor (*PcEcR*) and retinoid X receptor (*PcRXR*) cDNAs from *Procambarus clarkii* were cloned. Full length cDNA of *PcEcR* has 2500 bp, encoding 576 amino acid proteins, and full length cDNA of *PcRXR* has 2593 bp, in which a 15 bp and a 204 bp insert/deletion splice variant regions in DNA binding domain and hinge domain were identified. The two splice variant regions in *PcRXR* result four isoforms: *PcRXR1*-4, encoding 525, 520, 457 and 452 amino acids respectively. *PcEcR* was highly expressed in the hepatopancreas and eyestalk and *PcRXR* was highly expressed in the eyestalk among eight examined tissues. Both *PcEcR* and *PcRXR* had induced expression after eyestalk ablation (ESA) in the three examined tissues. In muscle, *PcEcR* and *PcRXR* were upregulated after ESA, *PcEcR* reached the highest level on day 3 after ESA and increased 33.5-fold relative to day 0, and *PcRXR* reached highest the level on day 1 after ESA and increased 2.7-fold relative to day 0. In the hepatopancreas, *PcEcR* and *PcRXR* d*EcR*eased continuously after ESA, and the expression levels of *PcEcR* and *PcRXR* were only 0.7% and 1.7% on day 7 after ESA relative to day 0, respectively. In the ovaries, *PcEcR* was upregulated after ESA, reached the highest level on day 3 after ESA, increased 3.0-fold relative to day 0, and the expression level of *PcRXR* changed insignificantly after ESA ($p > 0.05$). The different responses of *PcEcR* and *PcRXR* after ESA indicates that different tissues play different roles (and coordinates their functions) in molting.

Keywords: ecdysone receptor; retinoid X receptor; *Procambarus clarkii*; molting; eyestalk ablation

1. Introduction

The red swamp crayfish *Procambarus clarkii* is a freshwater crayfish species, native to the Southeastern region of the United States, and has been introduced to countries in Asia, Africa, Europe and other regions of the world. The species is said to have invaded China at the beginning of the 20th century [1]. Since the 1990s, the crayfish has been farmed widely and it has become an important aquaculture crustacean in the southeastern part of China, especially in Jiangsu Province [2].

Like all arthropods, crayfish have a thin, but tough exoskeleton which is shed regularly during development, a process most commonly referred to as molting. Molting, as the most striking feature in anthropods, is indispensable for many biological processes, including growth, reproduction and metamorphosis. During the molting stage, the red swamp crayfish are prone to attacks from other crayfish which may result into death, given the fact that their exoskeletons are weak. For this reason therefore, the red swamp crayfish is not a good subject for high-density farming.

Ecdysteroid, as a lipophilic small molecule, performs its function in the nucleus. It binds to a nuclear receptor complex, which is constituted of two nuclear receptors: ecdysone receptor and retinoid X receptor (*RXR*), or ultraspiracle (*USP*), the homologue of *RXR* in insects [3,4]. After binding ecdysteroid, the *EcR-RXR* complex is activated. It regulates transcription of target genes, such as E75 and chitinase [5,6]. Moreover, the *EcR* can regulate the transcription of its own gene, as well as *EcR* and *RXR* molt-responsive genes [7,8].

Both *EcR* and *RXR* have all the conserved nuclear receptor structures, including the A/B, C, D, and E/F domains [9]. Among these conserved domains, the C domain is the most conserved. It is a DNA-binding domain, which binds ecdysone responsive elements in the promoters of molting-responsive genes. The moderately conserved domain is the E domain, which is a ligand-binding domain and more complex than the other domains. Besides its major role of ligand binding, it also mediates heterodimerization and regulates ligand-dependent transcriptional activation (AF-2) [10]. The less-conserved D domain is the hinge domain and links the DNA binding domain and ligand binding domain. The N-terminal A/B domain and the C-terminal F domain are always highly variable [10].

To date, *EcRs* and *RXRs* have been reported from many crustaceans, including the *EcR* and *RXR* from the fiddler crab *Uca pugilator* [11], the *EcR* from the land crab *Gecarcinus lateralis* [12], the *EcR* and *RXR* from the kuruma prawn *Marsupenaeus japonicus* [13], the *EcR* and *RXR* from the water flea *Daphina magna* [14], the *EcR* from the intertidal copepod *Tigriopus japonicas* [15], the *EcR* and *RXR* from the brown shrimp *Crangon crangon* [16], the *EcR* from the mysid shrimp *Americamysis bahia* [17], the *EcR* and *RXR* from the American lobster *Homarus americanus* [18], the *EcR* from the harpacticoid copepod *Amphiascus tenuiremis* [19], the *EcR* from the blue crab *Callinectes sapidus* [20], the *EcR* and *RXR* from the opossum shrimp *Neomysis integer* [21] and the *EcR* and *RXR* from the freshwater prawn *Macrobrachium nipponense* [22,23] and the *EcR* from the Chinese mitten crab *Eriocheir sinensis* [24].

Isoforms of *EcRs* and *RXRs* are always found in crustaceans. The variant regions among these isoforms occur frequently in the A/B domain, the hinge domain and the ligand binding domain. These variations affect transcriptional activation, dimerization, and presumably ligand binding. For example, four isoforms of *EcR* from the freshwater prawn *Macrobrachium nipponense*, which differ in the hinge and ligand binding domain, exhibit sex-specific dimorphic expression patterns [22].

To improve the basic knowledge about molting in *P. clarkii*, here we cloned the full length cDNA of *EcR* and *RXR* gene from the red swamp crayfish *P. clarkii*. We also described the expression of *PcEcR* and *PcRXR* in different tissues and in response to eyestalk ablation.

2. Results

2.1. Cloning of Full Length cDNAs of PcEcR and PcRXR

Using 5′ and 3′ RACE, we isolated full length cDNA sequences of *PcEcR* (Figure 1a) and *PcRXR* (Figure 1b). The 2500 bp full-length cDNA of *PcEcR* (KX673814) consisted of a 213 bp 5′ un-translated region (UTR), a 556 bp 3′ UTR with a poly(A) tail and a 1731 bp open reading frame (ORF), which encodes a deduced 576 amino acid proteins. Four variant forms of *PcRXR* were identified, designated *PcRXR1*, 2, 3, and 4 (KX673813, KX673815, KX673816, KX673817). It consists of two insert/deletion regions, one is 15 bp and another is 204 bp among these *PcRXR* variants. 15 bp insert/deletion region is located in DNA binding domain and is present in *PcRXR1* and *PcRXR3*, and the 204 bp insert/deletion region is located in hinge domain and is present in *PcRXR1* and *PcRXR2*. The longest cDNA of *PcRXRs*, *PcRXR1*, is 2593 bp, consisted of a 313 bp 5′ un-translated region (UTR), a 702 bp 3′ UTR with a poly(A) tail and a 1578 bp open reading frame (ORF), which encodes a deduced 525 amino acid proteins.

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1          TAGTGTTTTATCCATAAATAAAATACAAAAGTAACAAAACTAAGAGAAGATACGGAAATC
61         TGCAACAGTTTGCACAATAGAATTTACAAAGAGGCCGTACGTTTAACGGTGTCCGATTCC
121        GTCCCCTGTTATTCCCGGCACGAAGTGGTCTGGCATCGGCGACGACTTTGTCCTCTACCT
181        CCGACGACAGAGTGTGATGGTGTGCGCGTGACGATGGACTTCCCTCGCGACTCCAGCCAC
1                                                  M  D  F  P  R  D  S  S  H
241        CGGGGCCGCGGGCTGATGGGTCGGCGCTCACCCCCTACCCATCCTTCCCTGGTACACTCT
10          R  G  R  G  L  M  G  R  R  S  P  P  T  H  P  S  L  V  H  S
301        CTCGTCTCCCCTAAGCCCGACCCCTCGCCCTCCGCCTCCCCTTACGTCGTCGGCTCCCCT
30          L  V  S  P  K  P  D  P  S  P  S  A  S  P  Y  V  V  G  S  P
361        TTGATCGGAGAATCCCCCTCAGGGATCCCTCGTTCCCCGGTCTCGCCCCTAAGCATCATG
50          L  I  G  E  S  P  S  G  I  P  R  S  P  V  S  P  L  S  I  M
421        GTCAAGAACGAGCCCCCGGTGTCGCCCTCGGGGCCCTCAGACTATCCGGGGAACCCCAAG
70          V  K  N  E  P  P  V  S  P  S  G  P  S  D  Y  P  G  N  P  K
481        AAGCCGCGGTGCGACAGCGATTGGTCCCCCTCTCCAGGGGCCATGAGTGTGGACTCCCTC
90          K  P  R  C  D  S  D  W  S  P  S  P  G  A  M  S  V  D  S  L
541        TCACCGCCCCCGCAGAATCCCAACGGACTCTCTGGGGGCTCCATGGGGCATCCTTCGAAT
110         S  P  P  P  Q  N  P  N  G  L  S  G  G  S  M  G  H  P  S  N
601        GGCAGCGCGCTCTCCCCTATGTCCTCCTGCAGCTACGACCCCAGCTCACCCTACGTCCCC
130         G  S  A  L  S  P  M  S  S  C  S  Y  D  P  S  S  P  Y  V  P
661        AGATCAGGTCGAGATGACATGTCCCCCTCCTCCCTCACCAACTACGGCTCCGACTCCTAC
150         R  S  G  R  D  D  M  S  P  S  S  L  T  N  Y  G  S  D  S  Y
721        AGCGACCTCAAGAAGAAGAAAGGTCCCATACCCCGACAGGCAGAGGAGCTGTGCCTGGTG
170         S  D  L  K  K  K  K  G  P  I  P  R  Q  A  E  E  L  C  L  V
781        TGTGGGGACAGGGCCTCTGGATATCATTACAACGCGCTAACCTGTGAGGGATGCAAAGGT
190         C  G  D  R  A  S  G  Y  H  Y  N  A  L  T  C  E  G  C  K  G
841        TTCTTCCGAAGATCTATTACCAAGAATGCAGTATATCAGTGTAAATATGGCAACAATTGT
210         F  F  R  R  S  I  T  K  N  A  V  Y  Q  C  K  Y  G  N  N  C
901        GAAATGGACATGTATATGAGACGCAAGTGTCAAGAATGTCGCCTGAAGAAATGTCTCAGT
230         E  M  D  M  Y  M  R  R  K  C  Q  E  C  R  L  K  K  C  L  S
961        GTTGGCATGCGGCCAGAATGCGTGGTTCCCGAGTCTCAATGCCAGGTAAAACGTGAACAG
250         V  G  M  R  P  E  C  V  V  P  E  S  Q  C  Q  V  K  R  E  Q
1021       AAAAAAGCTAGGGATAAAGACAAAAAAGATTATCCCAGCCTTGGTTCCCCCATAGCTGAG
270         K  K  A  R  D  K  D  K  K  D  Y  P  S  L  G  S  P  I  A  E
1081       GAGAAGGCCATTCATTTTAGTCCAGTGAGTAATGATTGTAAACCCAAAGGATCACCAACT
290         E  K  A  I  H  F  S  P  V  S  N  D  C  K  P  K  G  S  P  T
1141       GCATCCGCTATGCAGTTCAAAAATCTTGTGGGAAGCAGTAACATCTCTCTAAGTCCTGTG
310         A  S  A  M  Q  F  K  N  L  V  G  S  S  N  I  S  L  S  P  V
1201       TCGGCAATTCCAAGATCAAATGTAAAGCCCTTGACTCGAGAGCAGGAAGAACTGATTCAC
330         S  A  I  P  R  S  N  V  K  P  L  T  R  E  Q  E  E  L  I  H
1261       ACGCTAGTCTATTATCAAGAAGAGTTTGAGCAGCCTTCAGAAGAAGAACTAAAGAAAATC
350         T  L  V  Y  Y  Q  E  E  F  E  Q  P  S  E  E  E  L  K  K  I
1321       AAATTTACCTTCGATGGTGAAGATACAAGTGACATGAGATTCAGGCACATAACCGAGATG
370         K  F  T  F  D  G  E  D  T  S  D  M  R  F  R  H  I  T  E  M
1381       ACGATCCTCACAGTTCAGCTCATTGTGGAATTCTCCAAGCAACTACCTGGTTTCGGCACT
390         T  I  L  T  V  Q  L  I  V  E  F  S  K  Q  L  P  G  F  G  T
1441       CTTCAACGAGAAGACCAGATTACTCTGCTCAAGGCATGCTCTTCTGAGGTGATGATGCTT
410         L  Q  R  E  D  Q  I  T  L  L  K  A  C  S  S  E  V  M  M  L
1501       AGAGCTGCACGGCGCTATGATTCCAAGACTGATTCAATTGTGTTTGGAAATAACTTTCCA
430         R  A  A  R  R  Y  D  S  K  T  D  S  I  V  F  G  N  N  F  P
1561       TATACACAACACTCCTATGAGTTAGCAGGCTTGGGAGAGTCGGCCGGTACACTTTTCCGC
450         Y  T  Q  H  S  Y  E  L  A  G  L  G  E  S  A  G  T  L  F  R
1621       TTCTGTCGTAATCTGTGTAAGATGAAGGTGGATAATGCAGAATATGCATTGTTAGCAGCT
470         F  C  R  N  L  C  K  M  K  V  D  N  A  E  Y  A  L  L  A  A
1681       ATTGCCATTTTCTCAGAGAGACCTAACCTAAAAGAACTCTCTAAAGTAGAAAAACTTCAA
490         I  A  I  F  S  E  R  P  N  L  K  E  L  S  K  V  E  K  L  Q
1741       GAAATATACCTCGAAGCATTGAAATCGTATGTGGAGAATCGACGAATGCCACGATCCGCA
510         E  I  Y  L  E  A  L  K  S  Y  V  E  N  R  R  M  P  R  S  A
1801       ATGGTGTTTGCAAAGTTGCTGAATATTCTCACAGAATTGCGAACCCTTGGAAACCTGAAC
530         M  V  F  A  K  L  L  N  I  L  T  E  L  R  T  L  G  N  L  N
1861       TCAGAGGTGTGCTTCTCTCTCACACTCAAGAACAAGCGACTTCCGCCTTTCTTGGCAGAG
550         S  E  V  C  F  S  L  T  L  K  N  K  R  L  P  P  F  L  A  E
1921       ATTTGGGATGTTACTGGATGTTAATGCTGAGCCACCGCCACTTCCCGGGTGCCATGTTAC
570         I  W  D  V  T  G  C  *
1981       TGAGGTCGACTTACGAATGTGAGAGGATGGTTGTGAGTCATCTGGGGGTGGTGGGTGGTC
2041       CCTACACAATGTACCTGCAGGCTGTGAGTGAGAGACTGATAATGCTGTGGGAGCAGCTTT
2101       ACACTGCCACCCAGGACCCACTTTCAGCCACCTGATGTGTCTATGTGCTAGGCCGCCGTT
2161       GTGACAGTGCTGTGCAGCCCGCCACCAGTGCAATGTAGAGTGTATAATGATGCGCCCAGT
2221       TTGTCCAAGGGGCAACAGAACATGCAATGTAGCCTTACTTATTATTATTCATTAGGATCC
2281       TTTCTTTAAGCTGTGAGGGGTGACACTGGCAATTGTTATATCATTGATCTCTCTCATGTG
2341       TGCGCATGCACACACACACACACACACAATCACTCACACACACACACACAACAAAATATA
2401       CAATGATATTCCAACCCATCCTCCAAAAAAATTATAATACTATATTGATTATGTATGGAA
2461       TGTACAAAATATGGACTGTTGTACCCCCAAAAAAAAAAAA
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(**a**)

Figure 1. *Cont.*

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1          GTGTGCCGTCTGGCTCAGTGTGCGCCGCTTGTGTCAGTGTTCGCTCCTGTGTACCACATG
61         TGTATATGTGGCGCGTCTCTACCACTGTAGAGCCCAAACGTTTCAGGTGTGCGTGAGGAG
121        AGTGAGTGTAGCAGGTGTAGACGAGTGCCGCACACCCACCAAGTGTAGAGACACAAGTGT
181        TAGTGTAAGTGTGGCCAAGTGTGGCTGGAGCAGCGGTGGCCAGGAAGAGTGTGGTGATGC
241        CCGCTGGTGTGGCCCTCTAGGCCAGCCACACCACCACCACCACACCTGCCACACTCCCGC
301        CACACCTGCCACCATGATTGTCATCAAGAAAGAGAAGCCGGTCATGTCCGTGTCGTCCAT
001                         M  I  V  I  K  K  E  K  P  V  M  S  V  S  S  I
361        CATACATGGCTCCCAACAGCGGGGGTGGACACCAGGTGAGCTGTATGATGACTGGGGCGG
017           I  H  G  S  Q  Q  R  G  W  T  P  G  E  L  Y  D  D  W  G  G
421        CCGCGCCGGCACACCAGGTCTGGAAATCGGCATGTCAGGGTCGGTGGAGCGGCAGTCACC
037           R  A  G  T  P  G  L  E  I  G  M  S  G  S  V  E  R  Q  S  P
481        CTTGAGCGTGGCGCCCGACACCCTACTCTCCCCCTCCTCCACCTCTACCTTCTCCACCAC
057           L  S  V  A  P  D  T  L  L  S  P  S  S  T  S  T  F  S  T  T
541        CAATGGCGGACCAGCCTCCCCAAGTATATCAACGCCTCCCTTCACCATTGGGTCGAGTGG
077           N  G  G  P  A  S  P  S  I  S  T  P  P  F  T  I  G  S  S  G
601        TAATATGCTCAGTTCCAGCAACAGCAGCAGCAACAGCCTGAGCACCTCACCCAACCAGTA
097           N  M  L  S  S  S  N  S  S  S  N  S  L  S  T  S  P  N  Q  Y
661        CCCTCCCAGTCACCCGTTGTCGGGCTCCAAGCACTTGTGTTCCATCTGTGGCGACCGCGC
117           P  P  S  H  P  L  S  G  S  K  H  L  C  S  I  C  G  D  R  A
721        CTCTGGCAAGCACTATGGCGTCTACAGCTGTGAGGGCTGCAAAGGATTTTTCAAACGAAC
137           S  G  K  H  Y  G  V  Y  S  C  E  G  C  K  G  F  F  K  R  T
781        AGTTCGCAAAGACCTAACTTATGCCTGTCGAGAGGACAGATCCTGCACCATTGACAAGAG
157           V  R  K  D  L  T  Y  A  C  R  E  D  R  S  C  T  I  D  K  R
841        ACAAAGGAACCGATGCCAGTATTGCCGATACCAGAAATGTTTATCCATGGGCATGAAGAG
177           Q  R  N  R  C  Q  Y  C  R  Y  Q  K  C  L  S  M  G  M  K  R
901        AGAAGCGGTCCAGGTAGGGGCAGTAGAGGAGGAGCGCCAACGTACAAAAGGAGACAAAGG
197           E  A  V  Q  V  G  A  V  E  E  E  R  Q  R  T  K  G  D  K  G
961        AGACGGCGACACAGAATCATCATGTGGAGGCATCTCGGATATGCCAATTGCAAGTATCCG
217           D  G  D  T  E  S  S  C  G  G  I  S  D  M  P  I  A  S  I  R
1021       TGAGGCAGAACTCAGCGTTGAACATACAAATGAGCATCCACTTGACCAAGGGAATCAAGA
237           E  A  E  L  S  V  E  H  T  N  E  H  P  L  D  Q  G  N  Q  D
1081       TGAACCCGCCCGATCTACGTCCCCATCGAGCCCAGCGATGACCACTCTCTCTCCTTCCTG
257           E  P  A  R  S  T  S  P  S  S  P  A  M  T  T  L  S  P  S  C
1141       TCTTCAGCCCATCTTGCCAGCAGATGTGACCATGGGCACTACCCTGGGGGGAGGGAGGGG
277           L  Q  P  I  L  P  A  D  V  T  M  G  T  T  L  G  G  G  R  G
1201       AGATGATGCAGTGAGGGGTGTTGGCCACAAGTTTCCAGCCCAAGCTTACAACACCAACTA
297           D  D  A  V  R  G  V  G  H  K  F  P  A  Q  A  Y  N  T  N  Y
1261       TCAGAGAGGCAACCAGGATGCTTTAACCAACATTTGCCAGGCTGCAGACAGACACTTAGT
317           Q  R  G  N  Q  D  A  L  T  N  I  C  Q  A  A  D  R  H  L  V
1321       CCAACTGGTAGAATGGGCCAAGCACATCCCACACTTCACAGACCTTCCTGTTGATGACCA
337           Q  L  V  E  W  A  K  H  I  P  H  F  T  D  L  P  V  D  D  Q
1381       AGTTGTCTTACTTAAGGCTGGTTGGAATGAACTACTTATTGCGTCCTTTTCACATCGAAG
357           V  V  L  L  K  A  G  W  N  E  L  L  I  A  S  F  S  H  R  S
1441       TATGGGAGTTGAAGACGGGATTGTACTAGCTACAGGACTCGTGGTACACAGAAGTAGTGC
377           M  G  V  E  D  G  I  V  L  A  T  G  L  V  V  H  R  S  S  A
1501       TCATCAGGAAGGTGTGGGGGCAATTTTTGACCGAGTGTTATCAGAGTTGGTTGCTAAAAT
397           H  Q  E  G  V  G  A  I  F  D  R  V  L  S  E  L  V  A  K  M
1561       GAAAGAGATGAAGATGGACAAGACAGAATTAGGCTGTCTGCGCTCCATAGTTCTCTATAA
417           K  E  M  K  M  D  K  T  E  L  G  C  L  R  S  I  V  L  Y  N
1621       CCCAGATGCTAAGGGACTCACATGCTGTAATGACGTGGAGATTCTACGCGAGAAGGTTTA
437           P  D  A  K  G  L  T  C  C  N  D  V  E  I  L  R  E  K  V  Y
1681       CGCAGCACTAGAAGAGTACACACGTACTAGCTACCCTGAAGAACCTGGCAGGTTTGCCAA
457           A  A  L  E  E  Y  T  R  T  S  Y  P  E  E  P  G  R  F  A  K
1741       GCTATTGCTACGACTACCAGCGCTCAGATCAATTGGCTTAAAATGCCTAGAGTATCTCTT
477           L  L  L  R  L  P  A  L  R  S  I  G  L  K  C  L  E  Y  L  F

1801       TTTGTTTAAGCTAATTGGAGATACACCTCTGGACAACTACTTGATGAAAATGCTTATGGA
497           L  F  K  L  I  G  D  T  P  L  D  N  Y  L  M  K  M  L  M  E
1861       AAACCCCAACAACACTTCCCCTTCAAGTTAAGATATGATAACACATGCAATATAAAAGCA
517           N  P  N  N  T  S  P  S  S  *
1921       GTCATGACTTAGTGTTTCTGTTAAACAGATTTTTAATCATATAGTGTACTATGAAATGAG
1981       CCTTACTGAACCACTGAGAATTTCTTATAAGGTCTTGATGTTGCTGTTCTTAGTATAAAG
2041       ACTAAATGTGTGTACATTATAATTGTTTTAATTATAGTGAGAGACAACTGACCTGTGAAG
2101       ACACTAGTAAAAACAAGGAAGTAGTTGGCATTGTCATGAACTGTGGCTAATTGGCAAGTG
2161       ATATATATATCTGCTAAGCATAGAAAAAAGCAAAGCAAAATAAACATGGTTGTGGTTGCA
2221       CGAGTGAAATTTGTTGCTATAAGACTGAACCAGTCGGGAGAGACTACCCAAGATAATGCG
2281       AGCTGCAATGGATACTAAATTGTTGCTGCCAAGAAAGTCCGGCCAGCTTTAAAACTCAAG
2341       GAAACTGATGAGGGATCCATTTCCCAATTAGTAATTATTACATTTTGCACATCCAAAGTA
2401       TTTATACACTTTAATTTGTTAAAAGTTTATATAAACAACCCATTTTATGTACTGATATTC
2461       AAAACTCATTGTTCCATGAGTATTATAATATGCTAATAACTTCTTTATTGTTTAGTGTTC
2521       AACTTTATTCCTAACACTTGTGCTTTCAGTGAAGAATTTACAATAAATGGAACGGCTTTG
2581       AAAAAAAAAAAAA
```

(**b**)

Figure 1. (**a**) Full-length cDNA of *PcEcR* and its encoded amino acid sequence; (**b**) Full-length cDNA of *PcRXR* and its encoded amino acid sequence. The two splice variant sequences: one with the red background is a 15 bp insertion/deletion alternatively spliced intron that only exists in *PcRXR1* and *PcRXR3*; the second with green background is a 204 bp insertion/deletion alternatively spliced intron that only exists in *PcRXR1* and *PcRXR2*.

2.2. Sequence Alignments and Phylogenic Trees of PcEcR and PcRXR

The alignment revealed that *PcEcR* and *PcRXR* has all the functional domains characteristic of nuclear receptors (A/B, C, D, E and F domains) (Figure 2). The C domain, which is the DNA-binding domain, is the most conserved in both *EcRs* and *RXRs*. DBD of *PcEcR* exhibits a high degree of identity (>86.3%) with the other *EcR* proteins, while DBD of *PcRXR* exhibits a high degree of identity (>82.6%) with the other *RXR* proteins. The E domain which is a ligand-binding domain, exhibit moderate conserved in both *EcRs* and *RXRs*. LBD of *PcEcR-1* shares a general degree of identity (>36.1%) with the compared *EcR* proteins, while LBD of *PcRXR-1* shares a general degree identity (>38.2%) with the compared *RXR* proteins. The most variable domains are the N-terminal A/B domain and the C-terminal F domain (Figure 2).

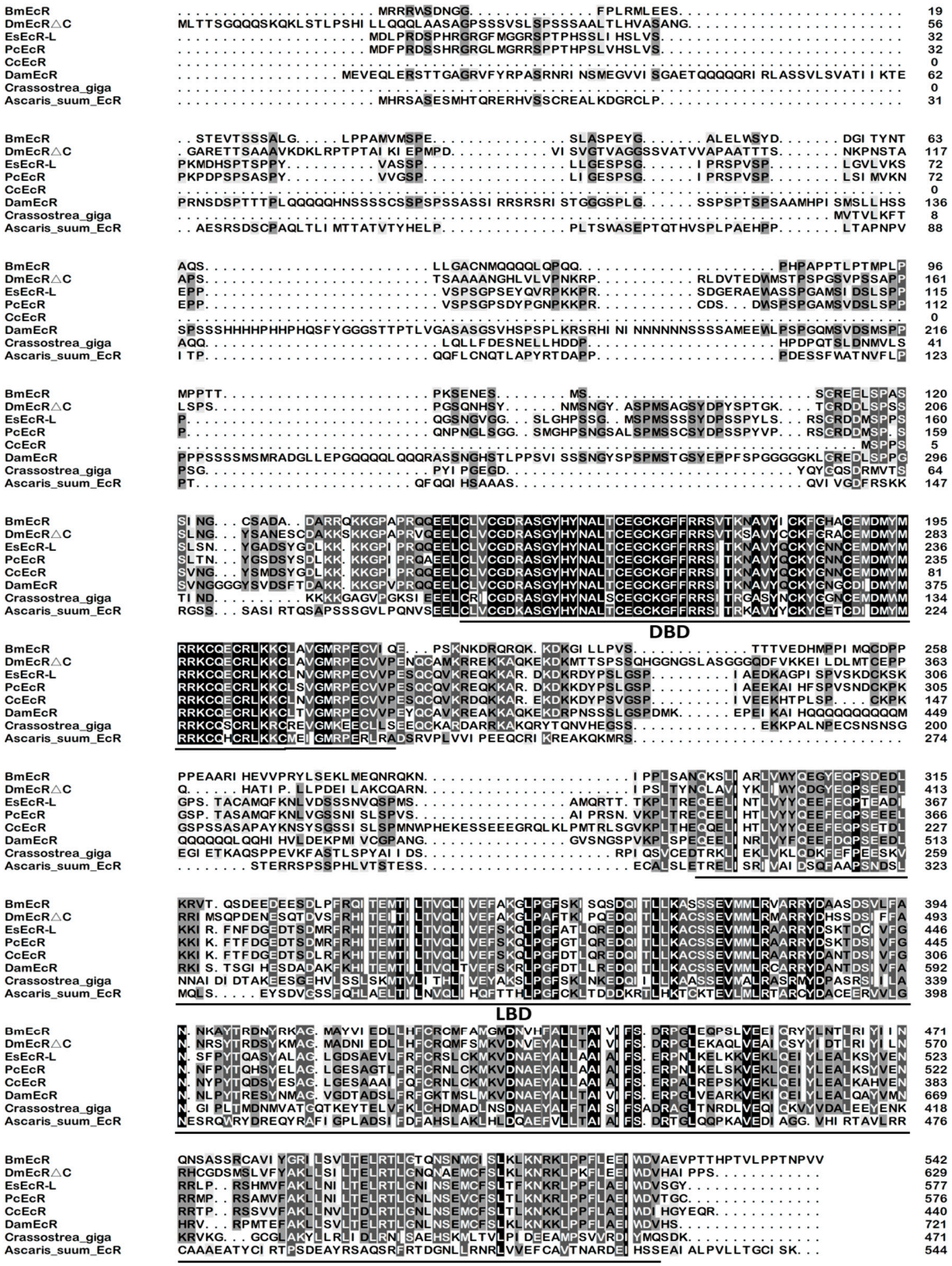
In the phylogenetic tree of *EcRs*, the crustacean group is clustered in one clade and the insect group in another (Figure 3a). In the phylogenetic tree of *RXRs*, *PcRXR1* and *PcRXR4* quickly clustered with all the other crustaceans, and the clade of the crustacean group was more close to the clade of vertebrate group and separated it from other arthropods' *RXRs* (Figure 3b).

2.3. Expression of PcEcR and PcRXR in Different Tissues

Both *PcEcR* and *PcRXR* were expressed in all eight tissues that were examined (Figure 4). It was observed that *PcEcR* was highly expressed in hepatopancreas and eyestalk, with the least expression in Testis. In the case of *PcRXR*, it was highly expressed in the eyestalk showing the lowest expression in muscle (Figure 4).

(**a**)

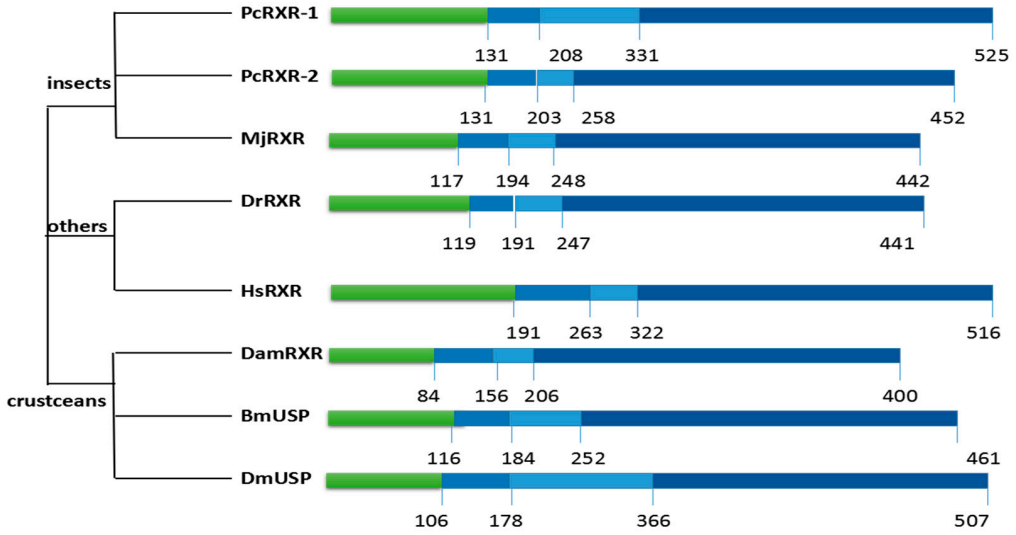
Figure 2. *Cont.*



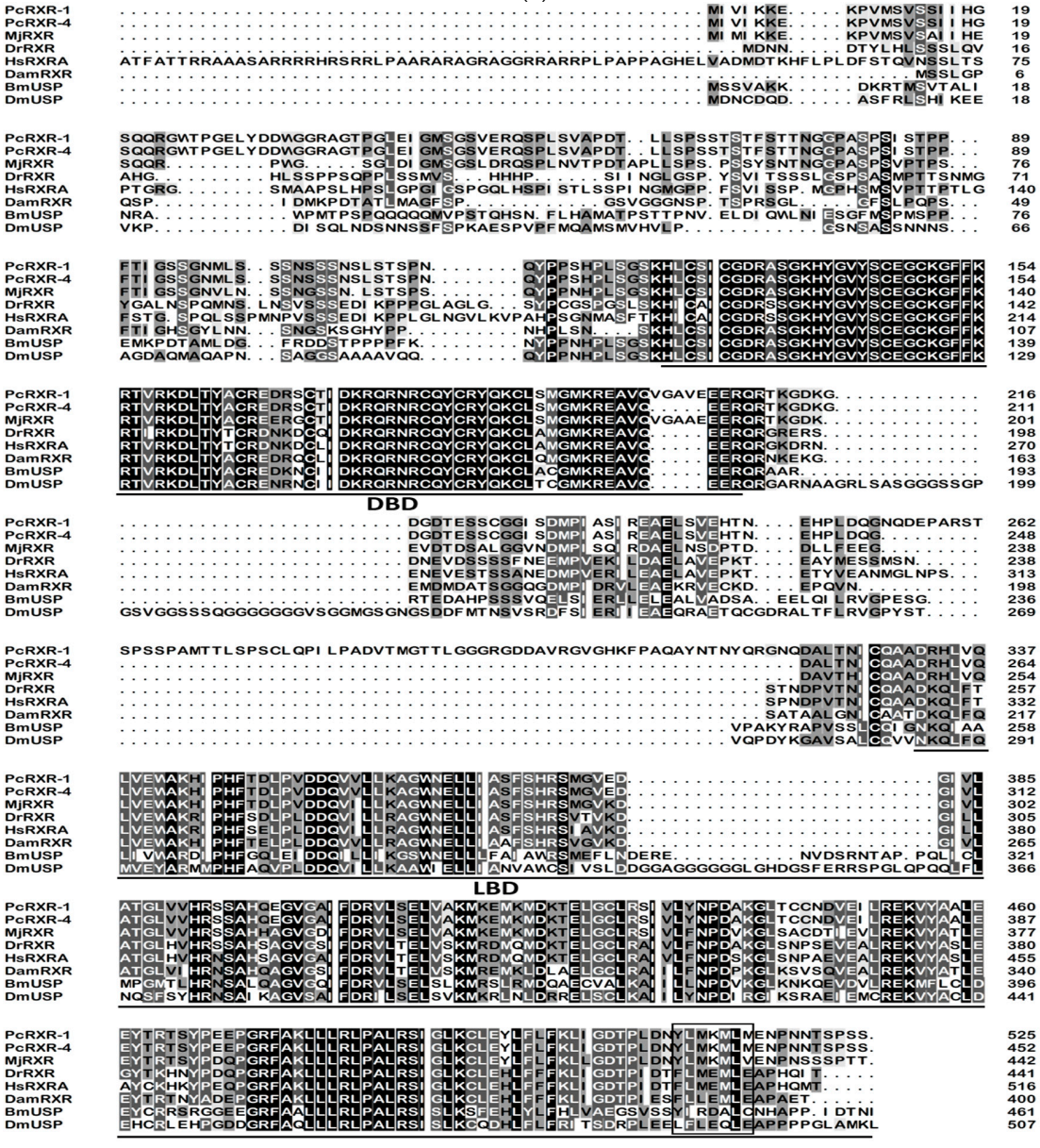
(**b**)

A

Figure 2. *Cont.*

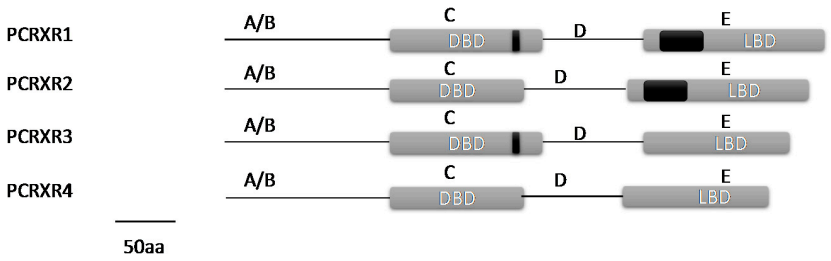


(**a**)



(**b**)

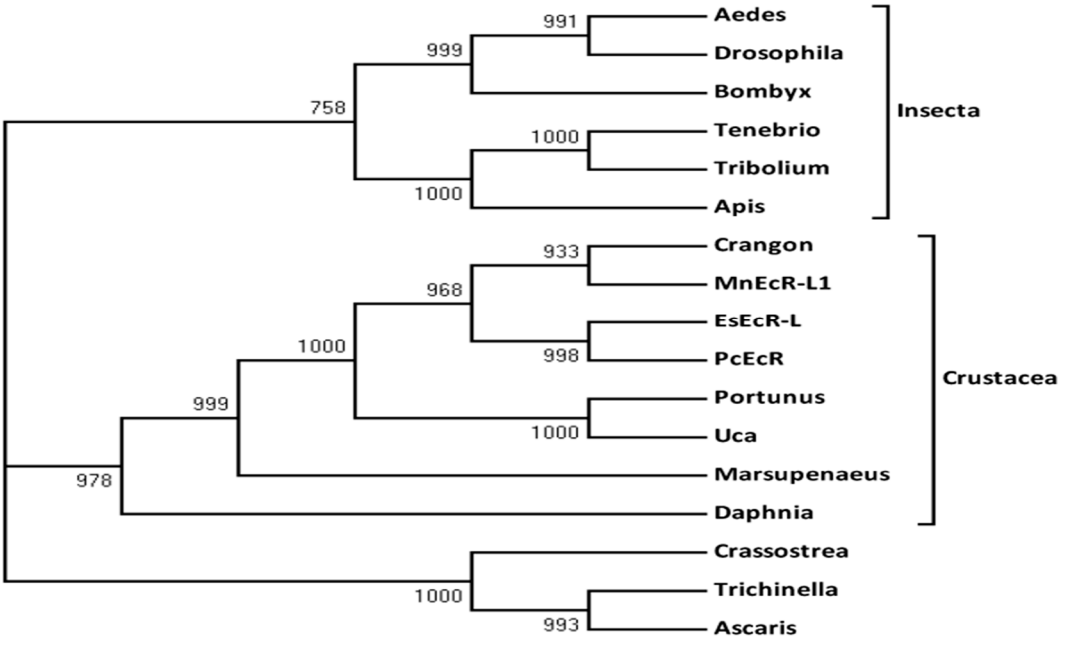
Figure 2. *Cont.*



(**c**)

B

Figure 2. (**A**) The former figure (**a**) is a simple schematic representation of both transcripts highlighting each domain region. The latter figure (**b**) is a comparison between deduced amino acid sequences of *PcEcR* with other seven *EcRs*. Amino acid residues that are identical or similar between all sequences are highlighted. The conserved DBD (DNA-binding domain) and LBD (ligand-binding domain) domains are underlined. Sequence names and accession numbers are supplied in the Methods section; (**B**) The first figure (**a**) is a simple schematic representation of both transcripts highlighting each domain region. The second figure (**b**) is a comparison between deduced amino acid sequences of *PcRXR1* and *PcRXR4* with other six *RXRs*. Amino acid residues that are identical or similar between all sequences are highlighted. The conserved DBD and LBD domains are underlined. Sequence names and accession numbers are supplied in the Methods section. The third figure (**c**) is a simple alignment of each domain region with *PcRXR* 1,2,3,4. PcRXR has all the functional domains characteristic of nuclear receptors (A/B, C, D, E domains). The gray represents the conserved DBD and LBD domains and the black represents these variants in domain.



A

Figure 3. *Cont.*

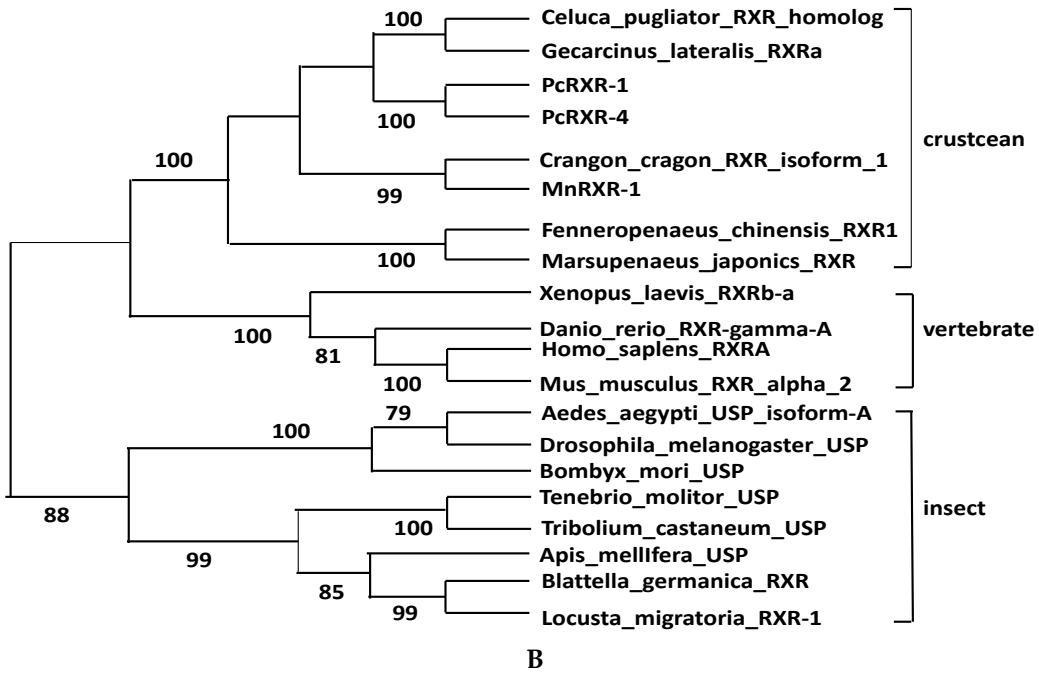


Figure 3. (**A**) Phylogenetic tree of *EcRs*. The tree was constructed using the neighbor-joining method. Numbers represent bootstrap values (%). Sequence names and accession numbers are supplied in the Methods section; (**B**) Phylogenetic tree of *RXRs*. The tree was constructed by use of the neighbor-joining method. Numbers represent bootstrap values (%). Sequence names and accession numbers are supplied in the Methods section.

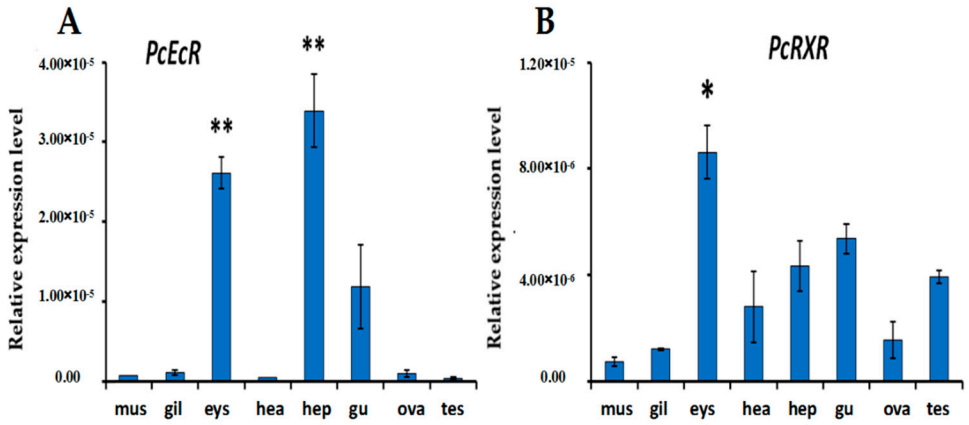


Figure 4. PCR analysis of relative expression levels of *PcEcR* (**A**) and *PcRXR* (**B**) in eight tissues of *P. clarkii*. mus: muscle; gil: gill; eys: eyestalk; hea: heart; hep: hepatopancreas; gu: gut; ova: ovary; tes: testis; Each data point represents the mean and standard deviation (n = 3 samples). The expression level in hepatopancreas was considerably higher than in other tissues (**: $p < 0.01$, *: $p < 0.05$; with Student's t-test).

2.4. The Induction Expression of PcEcR and PcRXR after Eyestalk Ablation

The expression of *PcEcR* and *PcRXR* were detected in three crayfish tissues at 0 days, 1 day, 3 days and 7 days after bilateral eyestalk ablation. As shown in Figure 5, the response of *PcEcR* and *PcRXR* to eyestalk ablation is different in different tissues. In muscle *PcEcR* and *PcRXR* were upregulated after ESA, *PcEcR* reached the highest level on day 3 after ESA and increased 33.5-fold relative to day 0, and *PcRXR* reached the highest level on day 1 after ESA and increased 2.7-fold

relative to day 0. In hepatopancreas, *PcEcR* and *PcRXR* were d*Ec*Reased continuously after ESA, the expression levels of *PcEcR* and *PcRXR* were only 0.7% and 1.7% in day 7 after ESA relative to day 0, respectively. In ovary, *PcEcR* were upregulated after ESA, reached the highest level on day 3 after ESA, and increased 3.0-fold relative to day 0, and the expression level of *PcRXR* changed insignificantly after ESA (p >0.05, Student's t-test).

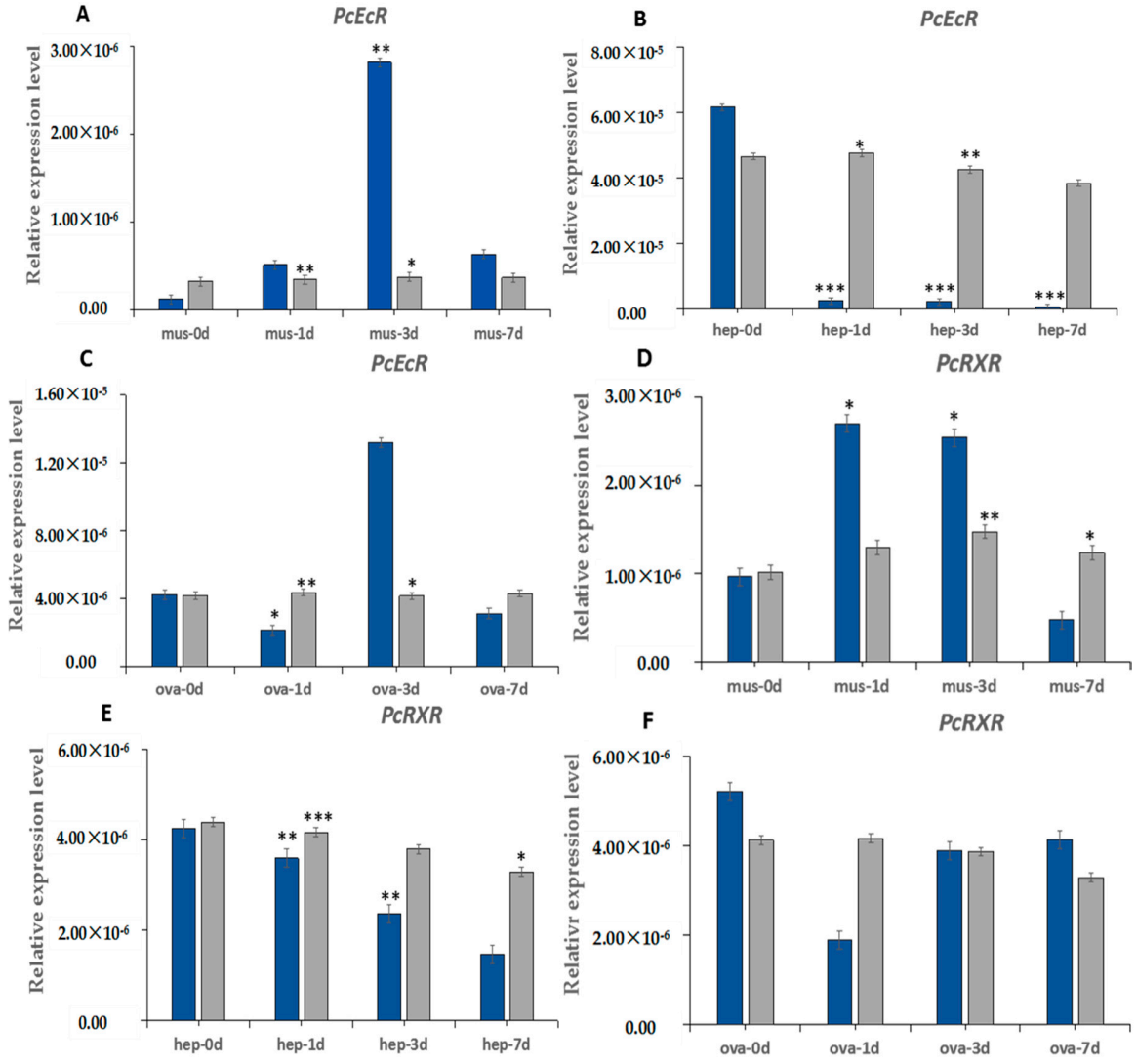


Figure 5. Expression level of *PcEcR* (**A**–**C**) and *PcRXR* (**D**–**F**) after eyestalk ablation in (**A**,**D**) muscle, in (**B**,**E**) hepatopancreas and in (**C**,**F**) ovary. *P. Clarkii:* mus: muscle; hep: hepatopancreas; ova: ovary; d: day. The blue represnts the expression by eyestalk ablation. The gray represents the expression without eyestalk ablation. Each data point represents the mean and standard deviation (n = 3 samples). Statistical analyses were performed with Student's t-test (***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$).

3. Discussion

3.1. Analysis of PcEcR and PcRXR

In the study, we have cloned two cDNAs encoding *PcEcR* and *PcRXR* from the red swamp crayfish. These play the role of an ecdysone receptor complex in *Procambarus clarkii*. They were found to be highly similar to known sequences of *EcR* and *RXR/USP* [21]. They also exhibit typical sequence domain structures of other *EcRs* and *RXRs* from insects and vertebrates [15]. The C domain, which is the DNA-binding domain, is the most conserved in both *EcRs* and *RXRs* [6]. DBD of *PcEcR* and *PcRXR*

exhibits a high degree of identity with the other proteins. The E domain which is a ligand-binding domain, exhibit moderate conservation in both *EcRs* and *RXRs*. LBD of *PcEcR* and *PcRXR* shares a general degree of identity with the compared proteins. This supports the notion that *PcEcR* and *PcRXR* can perform the functions similar to the other proteins.

In the A/B domain, variant sequences exist in insects caused by alternative splicing. Their expressions are regulated by different promoters, resulting in the different expression pattern of isoforms in a tissue-specific manner. Similarly to insects, the variant regions in crustaceans occur in the A/B domain [15], the hinge domain and the ligand domain [11,13,18]. Variant sequences in these regions presumably affect dimerization, transcription activation, and ligand binding. However, we have found a short 15 bp insertion/deletion region, which encoded 5 amino acids occurring in the DNA binding domain. These variants are produced by alternative splicing, and their expression is regulated by distinct promoters. The variable regions may bear some functional significance(s) in *RXR* binding or action, as steric hindrance or rigidity of Pro may alter flexibility or conformation within the molecule and importance of *RXR* signal transactivation properties or ligand affinities. The insertion is present in *PcRXR1* and *PcRXR*3 while absent in *PcRXR*2 and *PcRXR4*. It suggests the DNA binding activity between *PcRXR1*, 3 and *PcRXR*2, 4 may be different. A similar pattern is found in other crustacean *RXRs*, although the size or its position of an insertion in DBD and/or LBD varies [20]. Further studies are required to know whether these multiple variants of *PcRXR* have different properties in ligand binding, DNA binding and heterodimerization.

In the phylogenetic tree of *EcRs*, the crustacean group is clustered in one clade and the insect group in another. This suggests that *PcEcR* is different from that of insects. In the phylogenetic tree of *RXRs*, *PcRXR1* and *PcRXR4* quickly clustered with all the other crustaceans, and the clade of the crustacean group was closer to the clade of vertebrate group and separated it from other arthropods' *RXRs*. The vertebrate *RXR* binds retinoic acid preferentially and forms a homodimer. In contrast, the insect *RXR* has been identified as an orphan receptor, although a putative ligand, juvenile hormone, bound to *RXR* at higher concentrations than those causing physiological effects. The retinoic acid is the ligand of *PcRXR* in which LBD is highly conserved in that of crustacean *RXR* [13]. Thus, the crustacean *RXR* is closer to vertebrate *RXR* than to insect *RXR*.

3.2. Expression in Different Tissues and Synergistic Expression of PcEcR and PcRXR in Different Tissues

All internal tissues in crustaceans can be considered to be the target tissues of hemolymphatic ecdysteroids. All these tissues exhibit the co-presence of *EcR*/*RXR* expression, supporting the notion that they act as a heterodimer. However, the levels of their expression vary in different tissues with different levels of *PcEcR* and *PcRXR* expression. Both *PcEcR* and *PcRXR* were expressed in all eight tissues examined. It was observed that *PcEcR* was highly expressed in the hepatopancreas with the least expression in Testis. *PcEcR* was highly expressed in the hepatopancreas, which is consistent with *EcR* in *Macrobrachium nipponense* and *EcR* in *Eriocheir sinense*. The hepatopancreas is the major organ related to metabolism in animals; high expression levels of *PcEcR* in the hepatopancreas indicate that *EcR* is necessary for development in crayfish [6]. In the case of *PcRXR*, it was highly expressed in the eyestalk with the least expression in muscle. *PcRXR* was highly expressed constantly compared with *PcEcR* in testis and ovary indicating the possibility that *PcRXR* is other than ecdysteroids was required for development and maturation of reproductive tissues. For example, the expression of *CpRXR* and *MjRXR* gradually increased during ovarian maturation, which supports the importance of *RXR* in reproduction [13]. Reproduction in crustaceans is closely related to molting, and the underlying mechanism of reproductive processes is not yet well-understood.

3.3. Induction of PcEcR and PcRXR after ESA in Different Tissues

This is because the X-organ/sinus complex is located in the eyestalk [12,25–29]. Several important neuropeptides including MIH (molt-inhibiting hormone), gonad/vitellogenesis-inhibiting hormone, crustacean hyperglycemic hormone, and mandibular organ-inhibiting hormone are s*EcR*eted by the

X-organ/sinus complex in crustaceans [30,31]. These neuropeptide hormones regulate multiple physiological processes, such as metabolism, reproduction, and osmoregulation [32–34]. Also, it s*EcR*etes gonad-inhibiting hormone to regulate gonadal dysgenesis. Eyestalk ablation breaks the X organ-sinus gland complex functions or weakens it. Therefore, eyestalk ablation promotes molting and growth.

The response of *PcEcR* and *PcRXR* to eyestalk ablation is different in different tissues. In the hepatopancreas, *PcEcR* and *PcRXR* d*EcR*ease continuously after ESA. Both of them were upregulated in muscle and ovaries in general. The induction of gonad maturation and the molting results are affected by *EcR* and *RXR* after ESA. The process described for the *EcR* and *RXR* gene is indeed involved in molting. The effect of ecdysteroids is mediated by a receptor complex composed of ecdysone receptor (*EcR*) and retinoid X receptor (*RXR*) homolog in crustaceans. Overall, the *PcEcR*/*PcRXR* complex functions as a mediator of ecdysteroid signals. The hepatopancreas plays the role of a positive regulator in molting and reproduction. However, *PcEcR* and *PcRXR* were not upregulated continuously after ESA in muscle and ovary. The expression patterns of *EcR* and *RXR* did not coincide with the process of ecdysteroid titer and were different depending on different times. These imply that the expression of these genes was not controlled by ecdysteroid only. The expressions were also affected by the other factors. Also, a similar result was observed in Eriocheir sinensis [24]. The variable effect in different tissues after ESA indicates different tissues may have a notable difference in sensitivity to the concentration and a specific type of Ecds and may coordinate their inherent specific functions during molting and gonad maturation.

4. Material and Methods

4.1. Animal Collection, Preparation of Total RNA, and cDNA Synthesis

Crayfish *P. clarkii* that were about 10–20 grams weight were collected from a crayfish farm in Xuyi, Jiangsu Province, China. They were cultured in water tanks with adequate aeration at 20 °C in a natural photoperiod and fed with a commercial crayfish diet once a day. The methods of eyestalk ablation can be subdivided into two: unilateral resection and bilateral resection. The effects of bilateral resection are fast and significant but the mortality rate is high because the endocrine is not in control. Molting in the unilateral resection group is slower in comparison to that of the bilateral group [35]. The practice show that bilateral resection tend to have higher survival rates. In this here study, the experiments were conducted with respect to the bilateral resection due to its effects. In order to establish the expression levels, samples were collected from different tissues from 3 crayfish (1male and 2 female). For the eyestalk ablation experiment, crayfish (female) in the intermolt stage were chosen for the ablation of bilateral eyestalk using sterile surgical scissors. The same samples from different tissues were collected from crayfish at 0, 1, 3 and 7 days after eyestalk ablation. Tissue samples were frozen immediately in liquid nitrogen and then stored at −80 °C.

Total RNA from various tissues was isolated using the TRIzol® Reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. RNA integrity was evaluated by 1.5% agarose gel electrophoresis. The concentrations were measured and the purity of the RNA was determined by use of a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized according to the manufacturer's protocol using the SuperScript II RNase H reverse transcriptase first strand synthesis system (Invitrogen, Waltham, MA, USA).

4.2. Cloning and Sequencing of Full-Length PcEcR and PcRXR cDNA

Two partial cDNA sequences highly similar to published *EcRs* and *RXRs* (cDNA in Genbank) from our deep sequencing data were identified, respectively. Based on these two partial cDNA sequences, gene-specific 3′ and 5′ primers were designed for RACE PCR (rapid amplification of cDNA ends) (Table 1). 3′ and 5′ RACE cDNA were prepared from total RNA of *P. clarkii* (hepatopancreas), using a 3′-Full RACE Core Set Ver.2.0 Kit and 5′-Full RACE kit (Takara, Dalian, China) according to the

manufacturer's instructions, respectively. After performing two rounds of PCR to obtain 3′ and 5′ end fragments of *PcEcR* and *PcRXR*, the final PCR products were cloned into the pEASY-T1 vector (Transgen, Beijing, China). The recombinant plasmids were used to transform *E. coli* (*Escherichia coli*) TOP 10 competent cells, isolated, and sequenced.

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

Primer	Sequence (5′ to 3′)	Primer Description
PcEcR-3a-Outer	CTCACAGAATTGCGAACCCTT	3′ RACE Primer for first round
PcEcR-3a-Inner	CACCCAGGACCCACTTTCAG	3′ RACE Primer for second round
PcEcR-5a-Outer	GAGATGTTACTGCTTCCCAC	5′ RACE Primer for first round
PcEcR-5a-Inner	ATCCTTTGGGTTTACAATCA	5′ RACE Primer for second round
PcRXR-3a-Outer	CAATACTGGCATCGGTTCCT	3′ RACE Primer for first round
PcRXR-3a-Inner	CCGCCATTGGTGGTGGAGAA	3′ RACE Primer for second round
PcRXR-5a-Outer	AAAACAAGGAAGTAGTTGGC	5′ RACE Primer for first round
PcRXR-5a-Inner	TAAAACTCAAGGAAACTGATG	5′ RACE Primer for second round
Rt-*PcEcR*-F	CCTGTGAGGGATGCAAAGGT	FWD primer for *EsEcR* expression
Rt-*PcEcR*-R	GCATTGAGACTCGGGAACCA	RVS primer for *EsEcR* expression
Rt-*PcRXR*-F	CCTTCACCATTGGGTCGAGT	FWD primer for *EsEcR* expression
Rt-*PcRXR*-R	AGCTGTAGACGCCATAGTGC	RVS primer for *EsEcR* expression
Pc18S-F	ATCACGTCTCTGACCGCAAG	FWD primer for 18S expression
Pc18S-R	GACACTTGAAAGATGCGGCG	RVS primer for 18S expression

4.3. Sequence Alignments and Phylogenetic Analysis

The deduced amino acid sequences of *Procambrus clarkii EcR* were aligned with the seven known *EcRs* of other species, derived from the NCBI GenBank database: *AsEcR* from *Ascaris suum EcR* (ADY42041.1), *BmEcR* from *Bombyx mori* (BAA07890.1), *CcEcR* from *Crangon crangon* (Accession Number ACO44665.1), *CgEcR* from *Crassostrea gigas EcR* (EKC19773.1), *DamEcR* from *Daphnia magna* (BAF49029.1), *DmEcR* from *Drosophila melanogaster* (AAF57278.3), and *EsEcR-L* from *Eriocheir sinensis* (KF469222). A neighbor-joining tree was constructed from multiple sequence alignments with 16 other *EcR* protein sequences derived from the GenBank database using the molecular evolutionary genetics analysis (MEGA) software, version 3.1 (www.mega.co.nz). Bootstrap analysis of 1000 replicates was carried out to determine the confidence of tree branch positions. The names and the accession numbers of the *EcR* proteins used are as follows: *Crangon crangon EcR* (Accession Number ACO44665.1), *Daphnia magna EcR* (BAF49029.1), *Eriocheir sinensis EcR-L* (KF469222), *Macrobrachium nipponense EcR* (KC631613), *Marsupenaeus japonicus EcR* (Accession Number: BAF75375.1), *Portunus trituberculatus EcR* (AFH35032.1), *Uca pugilator EcR* (AAC33432.2), *Aedes aegypti EcR* (XP_001660279.1), *Apis mellifera EcR* isoform A (NP_001091685.2), *Bombyx mori EcR* (BAA07890.1), *Drosophila melanogaster EcR* (AAF57278.3), *Tenebrio molitor EcR* (CAA72296.1), *Tribolium castaneum EcR* isoform A (NP_001107650.1), *Ascaris suum EcR* (ADY42041.1), *Crassostrea gigas EcR* (EKC19773.1), and *Trichinella spiralis EcR* (XP_003376657.1).

The deduced amino acid sequences of *Procambrus clarkii RXR* were aligned with the six known *RXRs* of other species, derived from the NCBI GenBank database: *MjRXR* from *Marsupenaeus japonicus* (Accession Number: BAF75376), *DamRXR* from *Daphnia magna* (ABF74729), *DmUSP* from *Drosophila melanogaster* (NP_476781), *BmUSP* from *Bombyx mori* (NP_001037470), Dr*RXR* from *Danio rerio RXR*-γ-A (NP_571292) and Hs*RXR*A from *Homo sapiens* (AAH63827). *PcRXR1*, *PcRXR4* and 18 *RXR* proteins from other species were involved in neighbor-joining tree construction, their names and the accession numbers are as follows: *Celuca pugilator RXR* homolog (AAC32789), *Crangon crangon RXR* isoform 1 (Accession Number: ACO44668), *Fenneropenaeus chinensis RXR* (1130559), *Gecarcinus lateralis RXR*a (AAZ20368), *Marsupenaeus japonicus RXR* (BAF75376), *Aedes aegypti USP* isoform-A (AAG24886), *Apis mellifera USP* isoform-A (AAF73057), *Blattella germanica RXR* (CAH69897), *Bombyx mori USP* (NP_001037470), *Danio rerio RXR*-γ-A (NP_571292), *Drosophila melanogaster USP* (NP_476781), *Homo sapiens RXR*A

(AAH63827), *Macrobrachium nipponense RXR*-L (KC460323), *Marsupenaeus japonicus RXR* (Accession Number: BAF75376), *Mus musculus RXR* α 2 (AAB36777), *Tenebrio molitor USP* protein (CAB75361), *Tribolium castaneum USP* (CAL25729), and *Xenopus laevis RXR*b-a (AAI08461).

4.4. Quantitation of PcEcR and PcRXR Transcripts by Real-Time PCR

The quantitative real-time PCR assay was performed using the ABI 7500 system (Applied Biosystems, New York, NY, USA) to detect the expression levels of *PcEcR* and *PcRXR*. The expression of the 18S RNA gene of *Procambrus clarkii* (accession number: EU920952.1) was selected as the reference gene to be an internal and experiment control, using the primer pair *Pc18S*-F and *Pc18S*-R. The primers Rt-*PcEcR*-F and Rt-*PcEcR*-R were designed to detect the expression of *PcEcR*, and primers Rt-*PcRXR*-F and Rt-*PcRXR*-R were designed to detect the expression of *PcRXR*. The real-time PCR program was run at a temperature 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 34 s. PCR reactions were performed in triplicate for each sample, and the expression levels were normalized to that of the 18S RNA gene. All the primers used for quantitative real-time PCR are listed in Table 1.

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Author Contributions: Tian-Hao Dai performed the experimental work and statistical analysis, produced the tables and figures, and wrote the paper; Ali Sserwadda, Kun Song, Ya-Nan Zang read and made improvements to the manuscript; Huai-Shun Shen gave us the guide.

Conflicts of Interest: The authors declare no conflict of interest.

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