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Molecular Cloning and Expression Analysis of Extra Sex Combs Gene during Development in *Macrobrachium nipponense*

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Abstract

The extra sex combs (Esc) gene first identified in Drosophia encodes a transcriptional repressor of homeotic genes belonging to the polycomb group (PcG) gene and is primarily expressed in the early embryos. In this study, we have isolated the full-length cDNA of an Esc gene from the testis of oriental river prawn (*Macmbrachium nipponense*) according to the established expressed sequence tags (ESTs) information using Rapid Amplification of the cDNA Ends (RACE) technique, designated as MnEsc. The full-length cDNA of MnEsc is 1,461bp in size and has an open reading frame (ORF) of 1,068bp, encoding a 355-amino acid protein. Real-time quantitative PCR analyses revealed that the expression level of MnEsc varied significantly in the developing embryo, postembryonic larval and adult tissues. Real-time quantitative PCR showed the MnEsc gene was expressed in testis, liver, ovary, brain, abdominal ganglion, heart, intestine and muscle with the highest level of expression in ovary and brain. In vivo silencing of the gene, the dsRNA of MnEsc caused a significantly decrease in target gene expression level in brain and ovary tissues, but no exterior appearance change of experimental shrimp was observed.

Keywords: *Macrobrachium nipponense*, extra sex combs, real-time quantitative PCR, RNAi.

Introduction

The polycomb group (PcG) genes are chromatin regulators of the homeotic and other developmental genes found in many different organisms, including fruit fly (Struhl and Akam, 1985; Lewis *et al*.,1978; Li *et al*., 2012), mammals (Morey *et a*l., 2010), and even plants (Kohler *et al*., 2010). These genes have received a lot of attention due to their important roles in the control of tissue and organ development during embryogenesis, tumorigenesis, chromosome X-inactivation, genomic imprinting, and so on (Oktaba *et al*., 2008; Bracken *et al*., 2009; Zhang *et al*., 2012). At least 11 PcG genes have been described and there may be up to 40, including Additional sex combs (JÜrgens, 1985), Enhancer ofzeste (Jones and Gelbart, 1990), extra sex combs (Struhl, 1981), pleiohomeotic (Girton and Jeon, 1994), polycomb (Lewis, 1978), polycomblike (Duncan, 1982), polyhomeotic (Dura *et al*., 1985), posterior sex combs (JÜrgens, 1985), sex combs extra (Breen and Duncan, 1986), sex combs on midleg (JÜrgens, 1985), super sex combs (Ingham, 1984) and enhancer of polycomb (Sato *et al*., 1983). The extra sex combs (Esc) gene, originally identified in *Drosophila melanogaster*, was found to be expressed primarily during the early embryogenesis (Gutjahr *et al*., 1995; Sathe *et al*., 1995; Simon *et al*., 1995) and play important roles in determining segment identity (Struhl *et al*., 1981) and in hedgehog signaling pathway (Norihisa *et al*., 2004). Its product is essential for histone H3 K27 methylation and the establishment of PcG silencing of homeotic genes of the Bithorax and Antennapedia complexes (Katsuhito *et al*., 2008). The Drosophila embryos lacking maternal Esc displayed complete derepression of all homeotic genes and conversion of all segments to the identity of the 14th parasegment (Struhl *et al*., 1981). In mouse, considerable evidence also suggested mutation of Esc homolog embryonic ectoderm development (EED) resulted in embryonic lethality (Faust *et al*., 1998). In Medaka, hypomorphic knock-down of oleed (Oryzias latipes embryonic ectoderm development) using morpholino antisense oligonucleotides resulted in the fusion of eyes (Norihisa *et al*., 2004). Taken together, these observations support Esc as one of the best biomarkers for development of embryogenesis and larvae, which was experimentally demonstrated in the houseflies, butterflies and grasshoppers (Simon *et al*., 1995; Joyce *et al*., 1997). Previous study confirmed

that the crustaceans have dramatically close evolution relationship with insects (Budd *et al*., 2009), we hypothesize that MnEsc is involved in the embryonic development and phenotypic differentiation in crustaceans.

The oriental river prawn, *Macrobrachium nipponense* (Crustacea; Decapoda; Palaemonidae) is a commercial freshwater prawn. It is considered as an important fishery resource in China with an annual production of 205,010 tons (Bureau of Fishery, 2009). The identification of a germ cell specific marker in mitten crab will aid in the study of the molecular event involved in the determination of the development of these cells (Wang *et al.*, 2012). In recent years, our laboratory has performed some molecular research on the *M. nipponense* development system. In this study, we cloned a full length Esc cDNA from *M. nipponense* and analyzed its expression pattern throughout developmental stages, which could help to understand the regulatory mechanism of early embryonic development, postembryonic development and phenotypic differentiation in the oriental river prawn. In addition, we also investigated the ability of dsRNA to inhibit MnEsc mRNA expression in *M. nipponense* in vivo.

Materials and Methods

Embryo, Larvae and Tissue Collections

Several female and male healthy adult shrimps with wet weight of 1.26~4.25g were obtained from Tai Lake in Wuxi, China (120°13'44"E, 31°28'22"N). All of these samples were transferred to lab breeding conditions and maintained in a 500-liter tank with aerated freshwater for 72 h before tissue collection. The different developmental stages of eggs and larval were obtained from our breeding room. After prawn spawning, each developmental stage of embryos (cleavage stage; blastula stage; gastrula stage; nauplius stage; protozoea stage; zoea stage) was collected according to morphological methods following the criteria by Chen *et al*. (2012). Larvae were collected every 3 days between 1 day post-hatching larvae (L1) and L13. Post-larvae were collected every 4 days from 1 to 20 day after the metamorphosis (P1~P20), and every 10 days from 21 to 30 day. A variety of tissues, including ovary, testis, muscle, heart, abdominal ganglion, brain, liver and intestine were also collected. The ovary was collected from mature female shrimps, but testis, muscle, heart, abdominal ganglion, brain and intestine were collected from mature male shrimps. The samples were washed with 1 × PBS (phosphate-buffered saline, 0.01 M), frozen directly in liquid nitrogen and stored at -80 °C until processed.

RNA Isolation and Reverse Transcription (RT)

Total RNA was extracted from embryos and larvae at different stages of development and was also from other tissues in mature shrimps with RNAiso Plus Reagent (TaKaRa Bio Inc., Japan) in accordance with the protocol of the manufacturer. The isolated RNA was treated with RNase-free DNase I (Sangon, shanghai, China) to eliminate possible genomic DNA contamination. The concentration of each total RNA sample was then measured by BioPhotometer (Eppendorf, Hamburg, Germany), and 2μL was analyzed on a 1% agarose gel to check the integrity. The first-strand cDNA synthesis for real-time quantitative PCR was performed for each RNA using 1μg of total RNA, 4μL 5× iScript reaction mix (BIO-RAD, CA, USA) and 1μL iScript reverse transcriptase in a final volume of 20 μL. The reaction was incubated at 25℃ for 5min, then 42℃ for 30min followed by 85℃ for 5min. The cDNA of real-time quantitative PCR was kept at -20℃.

5′- and 3′-RACE Amplification

Total RNA was extracted from testis of mature shrimps. Concentrations were measured by BioPhotometer (Eppendorf, Hamburg, Germany) and 1μL was analysed on a 1% agarose gel to check the integrity. The full length of the MnEsc gene was obtained by using the 5′ RACE cDNA and 3′ RACE cDNA amplification kit (TaKaRa Bio Inc., Japan). 3′ and 5′-RACE cDNAs were then used for the 3′/5′-RACE PCR with 3′ gene-specific primer (3GSP1, 3GSP2) and 5′ gene-specific primer (5GSP1, 5GSP2) that were designed on the basis of EST sequence of MnEsc (GenBank accession no. JK526490) obtained from the *M. nipponense* testis cDNA library (Qiao *et al*., 2012). All primers used in this study were shown in Table 1. The two primers of Esc-F and Esc-R validated the MnEsc fragments from the EST cDNA library. The 3′-RACE and 5′-RACE were performed with 3′-full RACE Core Set Ver.2.0 Kit and 5′-full RACE Kit (TaKaRa Bio Inc., Japan) according to the manufacturer's instructions.

The PCR fragments were subjected to electrophoresis on a 1.5% agarose gel to compare the length difference. Amplified cDNA fragments were cloned into the pMD18-T vector (TaKaRa Bio Inc., Japan) following the instructions provided by the manufacturer. The recombination was then transformed into the *ESCherichia coli* DH5α (Qiagen, Germany) competent cells which were identified by blue/white screening and confirmed by PCR. At least five positive clones were sequenced in both directions using an automatic DNA sequencer (Applied Biosystems ABI-3730, USA) and these resulting sequences were verified and subjected to cluster analysis in NCBI.

Bioinformatics Analyses

Sequences were analyzed based on the nucleotide and protein databases using the BLASTX

Table 1. Primers used in this study

Name	Sequence (5′→3′)	Primer description
Esc-F	TCACTTGCTACCATGCAGTGCG	Esc fragment
Esc-R	TCCCAACAGCTAGCACTTTTGC	
Esc-3GSP1	CCTTGTGGCTACCTTTGG	FWD first primer for 3′ RACE
Esc-3GSP2	GTTGGGAACCAGTTAGGC	FWD second primer for 3′ RACE
Esc-5GSP1	AGGTAGCCACAAGGATATCTGTCCTG	RVS first primer for 5′ RACE
Esc-5GSP2	TGCTGCTAAGATTGGCCTTCCTG	RVS first primer for 5′ RACE
3′RACE OUT	TACCGTCGTTCCACTAGTGATTT	RVS first primer for 3′ RACE
3′RACE IN	CGCGGATCCTCCACTAGTGATTTCACTATAGG	RVS second primer for 3′ RACE
5′RACE OUT	CATGGCTACATGCTGACAGCCTA	FWD first primer for 5′ RACE
5′RACE IN	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	FWD second primer for 5′ RACE
Esc-RTF	TGGCTACCTTTGGTGGAGTAGAG	FWD primer for Esc expression
Esc-RTR	TGCAGGTGCCCTCAATGTC	RVS primer for Esc expression
β-actinF	TATGCACTTCCTCATGCCATC	FWD primer for *β*-actin expression
β-actinR	AGGAGGCGGCAGTGGTCAT	RVS primer for *β*-actin expression
Esc-dsF	GAAGACCATGGACAGCCAAT	For MnEsc dsRNA synthesis
Esc-dsR	GAATGATCCATGCCACATGA	
GFP-F	GTCAGTGGAGAGGGTGAAGG	For G FP dsRNA synthesis
GFP-R	AAAGGGCAGATTGTGTGGAC	

and BLASTN program (Altschul *et al*., 1990). The protein prediction was performed using the ORF Finder tool (http://www.ncbi.nlm.nih.gov /gorf/gorf.html). The motif was performed with the motif scan program (http://hits.isb-sib.ch/cgi-bin/motif scan/). Esc deduced amino acid sequences from *M. nipponense* and representative invertebrates were compared by multiple sequence alignment using ClustalX. A neighbor-joining (NJ) phylogenetic tree was constructed using MEGA4.0 (Tamura *et al*., 2007).

Real-Time Quantitative PCR Expression Analysis

The MnEsc mRNA expression at different stages from embryo to post-larva, and various adult tissues was measured by a SsoFast^{TM} EvaGreen$^{®}$ (BIO-RAD, CA, USA) real-time quantitative PCR analysis in Bio-Rad iCycler iQ5 Real-Time System (BIO-RAD, CA, USA). Gene-specific primers (Table 1) were used to amplify the Esc transcript, and the PCR products were sequenced to verify the specificity of the PCR primers. Amplifications were performed on a 96-well plate with a 25μL reaction volume containing 1μL cDNA (50ng), 10μL SsoFast^{TM} EvaGreen$^{®}$ Supermix (BIO-RAD, CA, USA), 0.5μL 10μM of gene specific forward and reverse primers (Table 1), and 13 μL of DEPC-water. The PCR temperature profile was 95°C for 30 s followed by 40 cycles of 94°C for 15 s, 58°C for 20 s and 72°C for 20 s , with a 0.5°C/5s incremental increase from 60°C to 95°C. Each sample was run in triplicate along with the internal control gene. To ensure that only one PCR product was amplified and detected, the dissociation curve analysis of amplification products was performed at the end of each PCR reaction. Amplification of *β*-actin as an internal reference was also carried out in the same sample (Table 1). The relative copy number of MnEsc mRNA was calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method (Livak *et al*., 2001).

ds RNA Preparation

PCR fragments containing *M. nipponense* Esc or green fluorescent protein (GFP) open-reading frame were amplified using gene-specific primers containing T7 promoter site at the 5′ ends of the gene-specific primers. The MnEsc dsRNA and GFP dsRNA synthesis primers are shown in Table 1. The PCR products were purified with a gel extraction kit (Sangon, shanghai, China). dsRNA was synthesized in vitro using Transcript Aid^{TM} T7 High Yield Transcription kit (Fermentas, Inc., USA) according to the manufacturer's instructions. The dsRNA was purified by ethanol precipitation and dissolved in RNase-free water. Purity and integrity of the dsRNA were examined by standard agarose gel electrophoresis. Concentration of the dsRNA was measured at 260 nm by using a BioPhotometer (Eppendorf, Hamburg, Germany), and then kept at -20℃ until used.

For the short-term in vivo dsRNA injection experiment, 30 health mature female *M. nipponenses* (each weighing 1.6-2.3g) were selected to inject into heart. The female shrimps were assigned to three treatment groups: MnEsc-dsRNA injected (n=10), GFP-dsRNA injected (n=10) and vehicle injected (n=10). Each shrimp was injected with 4μg MnEsc-dsRNA or 4μg GFP-dsRNA, or a similar volume vehicle. The MnEsc mRNA expression of the brain and ovaries were investigated to detect the interference efficiency by real-time quantitative PCR after injection for 4 days and 1 week.

Statistical Analysis

All data were presented as mean ± SE (standard error of the mean). Statistical analysis was performed

using SPSS13.0. Statistical significance was determined using one-way ANOVA and post hoc Duncan multiple range tests. Significance was set at $P<0.05$.

Results

Cloning and Characterization of the MnEsc Gene

The nucleotide sequence reported in this study has been deposited in the GenBank DNA database under Accession No. JX221051. The full-length cDNA sequence of MnEsc was comprised of 1,461bp with a 1,068bp open reading frame (ORF) encoding 356 amino acid residues which display an estimated molecular mass of 40.201kDa and the isoelectric point of 7.09. The 5' and 3' untranslated region (UTR) contain 105 bp and 288 bp, respectively. The clone included the canonical polyadenylation signal (AATAAA), typical of eukaryotic mRNA, and poly (A) tail. Conserved sequence and characteristic motifs of Five WD40 repeats were identified in the deduced amino acid sequences of MnEsc (Figure 1). In addition, the N-terminal region contains a potential nuclear localization signal (NLS) (residues 42-54, Figure 1).

Secondary structure of MnEsc was composed of alpha helix (1.1%), extended strand (51.0%) and Loop (47.9%). Matured protein composed of 6 casein kinase II phosphorylation sites, 6 protein kinase C phosphorylation sites, an amidation site, 5 N-myristoylation sites, and 3 N-glycosylation sites (Figure 1). It has multiple potential functional sites for phosphorylation, Methylation and glycosylation.

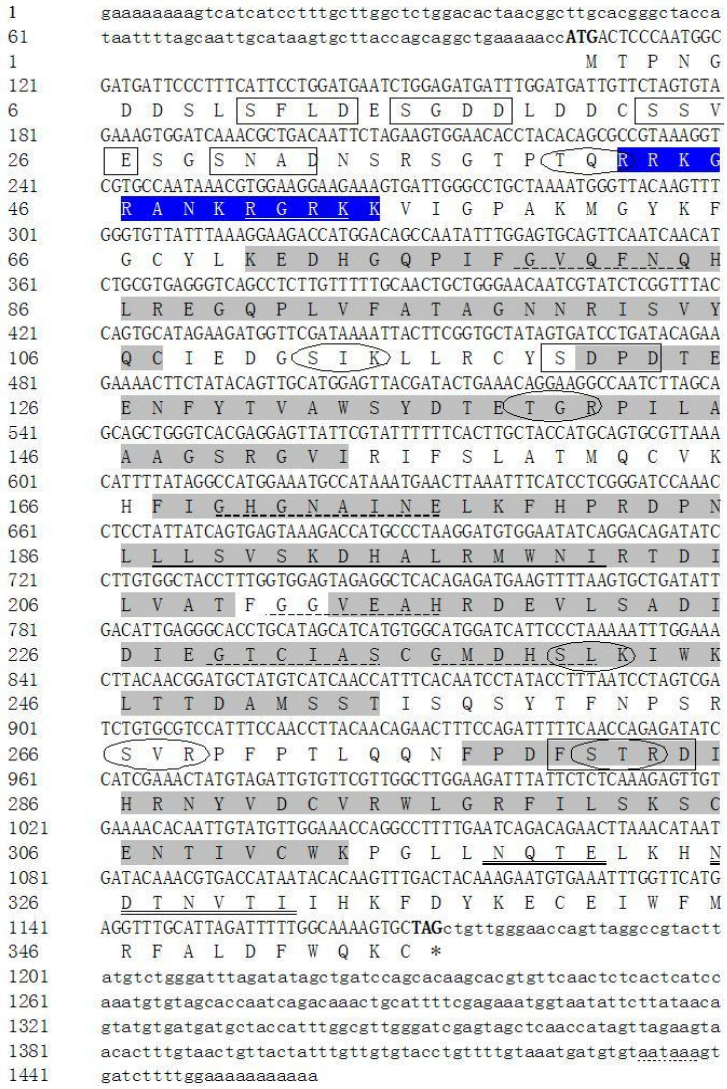

Figure 1. Nucleotide and deduced amino acid sequence of full-length Esc cDNA in *M. nipponense*. 3' UTR and 5' UTR are listed with lowercase letters. ORF is showed by capital letters. WD repeats profile are marked with shadow, WD repeats signature was marked with a single underline, and nuclear localization signal (NLS) was marked with blue. Putative protein kinase C phosphorylation sites, Casein kinase II phosphorylation sites, Amidation site, N-myristoylation sites and N-glycosylation sites are annotated respectively by a circle, a box, a inward hyperbola, an dashed underline and an double underline. aataaa was marked with an dot line.

Homology and Phylogenetic Analysis of MnEsc

Bioinformatic analyses with BLASTx searches of the public DNA sequences showed that the coding sequence (CDS) of *M. nipponense* displayed a high degree of identity with many Escs of arthropods. Sequence comparisons of the Esc deduced amino acids showed similarity of 67%, 65%, 64% and 63% to that of the *Schistocerca Americana* (AAC05332.1), *Danaus plexippus* (EHJ72379.1), *Junonia coenia* (AAC05331.1) and *Bombyx mori* (NP_001188366.1), respectively (Figure 2). Furthermore, based on the results of the alignment of Esc sequences of the different organisms, the phylogenetic trees were constructed based on the neighbor-joining method using the complete Esc proteins deposited in NCBI by ClustalW1.81 and MEGA4.0. The NJ tree showed that MnEsc is closely related to *Lepeophtheirus*

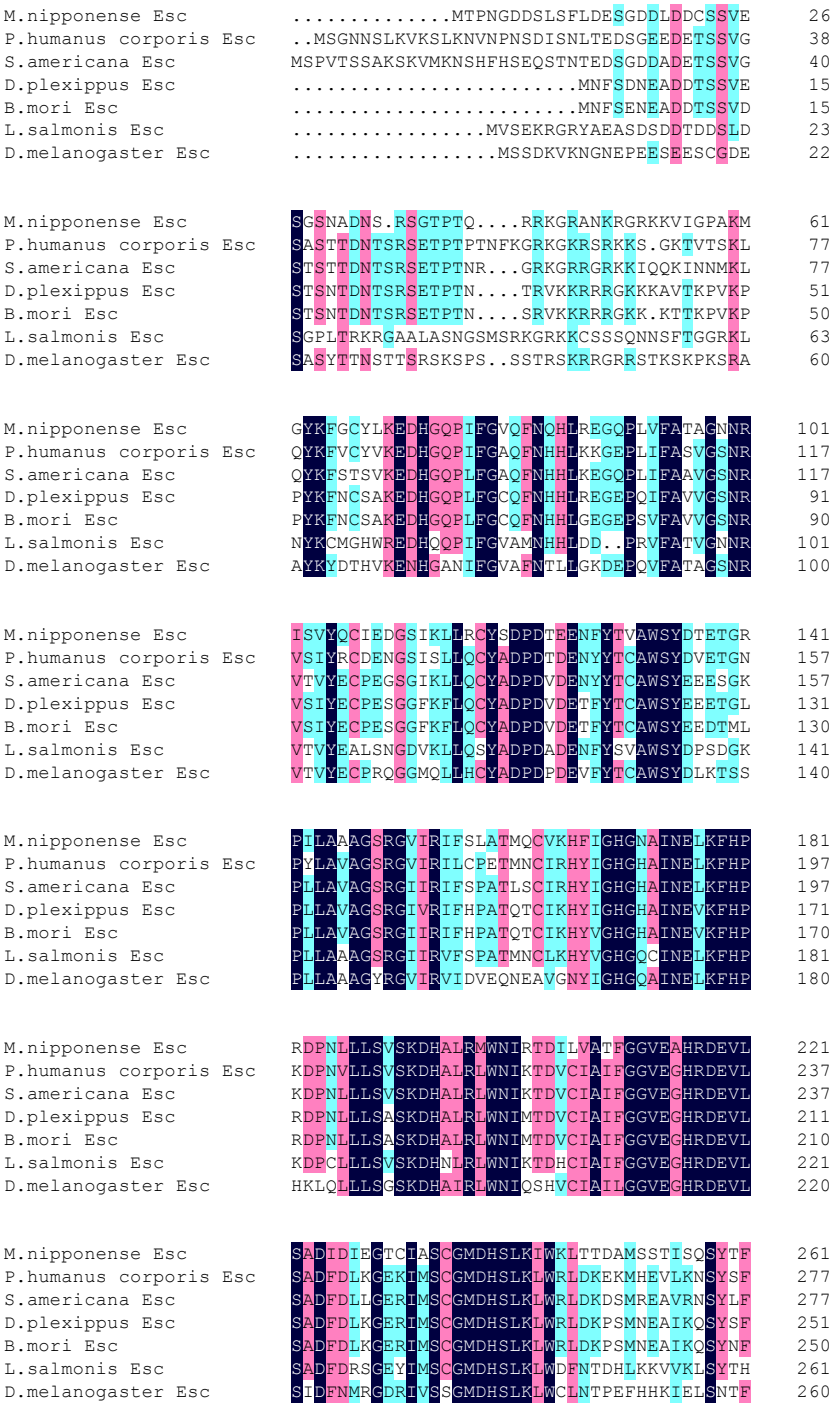

Figure 2. Alignment of *M. nipponense* Esc amino acid sequences with other species, Species names are abbreviated at the left and represent *Pediculus humanus corporis* (XP_002427573.1), *Schistocerca Americana* (AAC05332.1), *Bombyx mori* (NP_001188366.1), *Drosophila melanogaster* (NP_477431.1), *Lepeophtheirus salmonis* (ACO12729.1) and *Danaus plexippus* (EHJ72379.1).

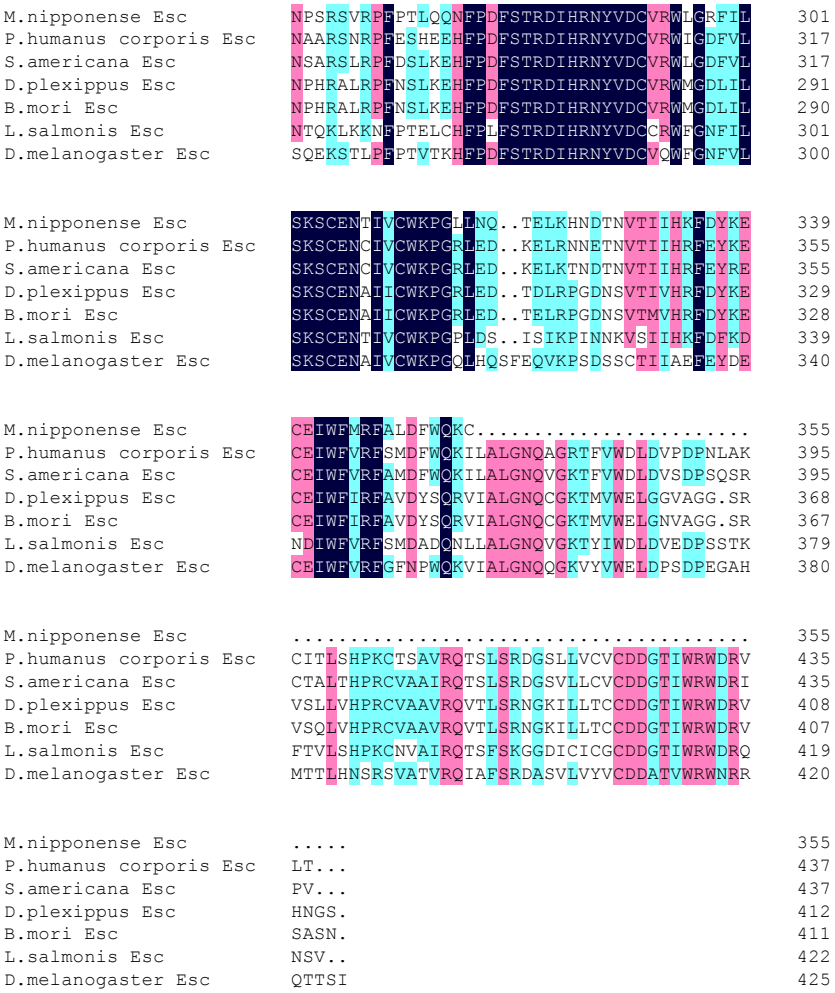

Figure 2. (Continued).

salmonis and *Culex quinquefasciatus*, separated from its homologues of flies (Figure 3).

Temporal Expression Pattern and Tissues Distribution of MnEsc

The relative expression levels of MnEsc normalized to *β*-actin during the developmental stages of embryo and postembryonic were determined by real-time quantitative PCR. During the embryo stages, the expression level of the MnEsc was stronger in the blastula stage (BS) than in the cleavage stage (CS) ($P<0.05$), and then it abruptly decreased to the lowest level at the gastrul stage (GS). Subsequently, the expression of the MnEsc stayed a low level from the nauplius stage (NS) to zoea stage (ZS) (Figure 4). During the postembryonic, the expression of MnEsc mRNA abruptly increased form 1 day post-hatching larvae (L1) to L4 and peaked at the L4, but it then gradually decreased from the L7 to L13 and reached the lowest level, as well as the one day before the metamorphosis. After metamorphosis, the larvae transition into post-larvae that resemble miniature adults. During the post-larva, it stayed a lowest level (Figure 5).

Real-time quantitative PCR analysis results showed that the relative expressions of MnEsc mRNA was widely distributed in all investigated tissues, including the testis, ovary, brain, abdominal ganglion, heart, intestine, eyestalk and muscle with the highest expression in brain and ovary (Figure 6).

dsRNA Interference Results

To establish a RNA interference technique in *M. nipponense* for usage in research of gene function, double strand RNA was systhesized using in vitro transcription. The MnEsc gene was examined by real-time quantitative PCR technique for determining the validity of RNA interference. The MnEsc gene expression was analyzed at different time points, i.e 4 days, 1 week after injection of dsRNA for examining the effect of RNA interference. The results were shown in Figure 7. The gene expression between GFP-dsRNA-injected and vehicle-injected control groups did not differ significantly from one another. After injection for 4 days and 1 week, the decrease of MnEsc expression could be observed in different tissues (ovary and brain) (Figure 7).

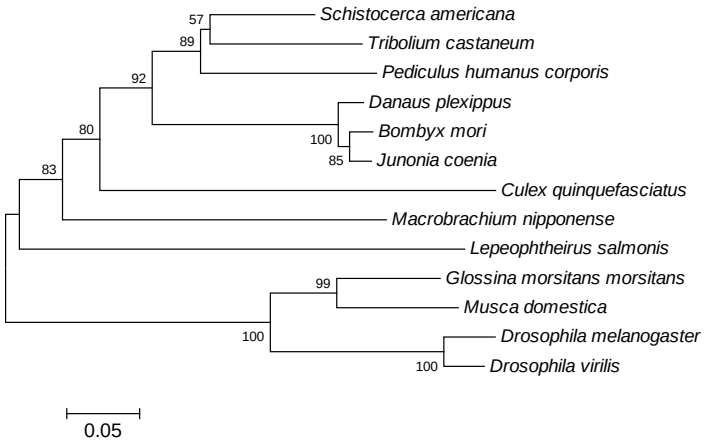

Figure 3. A phylogenetic tree was constructed based on the comparisons of amino acid sequences. Species names are listed on the right of the tree. Their accession number in GenBank as followed: *Pediculus humanus corpori*s (XP_002427573.1), *Schistocerca Americana* (AAC05332.1), *Danaus plexippus* (EHJ72379.1), *Glossina morsitans morsitans* (ADD18468.1), *Bombyx mori* (NP_001188366.1, *Drosophila melanogaster* (NP_477431.1), *Drosophila virilis* (XP_002052298.1), *Junonia coenia* (AAC05331.1), Culex quinquefasciatusand (XP_001842089.1), *Tribolium castaneum* (XP_973780.1), *Musca domestica* (AAC05333.1), *Lepeophtheirus salmonis* (ACO12729.1).

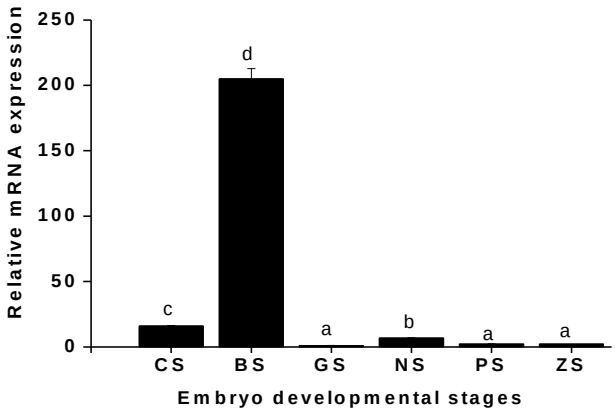

Figure 4. The expression profile of MnEsc at the different development stages of embryos were revealed by real-time quantitative PCR. The amount of MnEsc mRNA was normalized to the β-actin transcript level. Data are shown as means ± SE of three repeated samples during the embryos, larvae and post-larvae. CS-cleavage stage; BS-blastula stage; GS-gastrula stage; NS-nauplius stage; PS-protozoea stage; ZS-zoea stage.

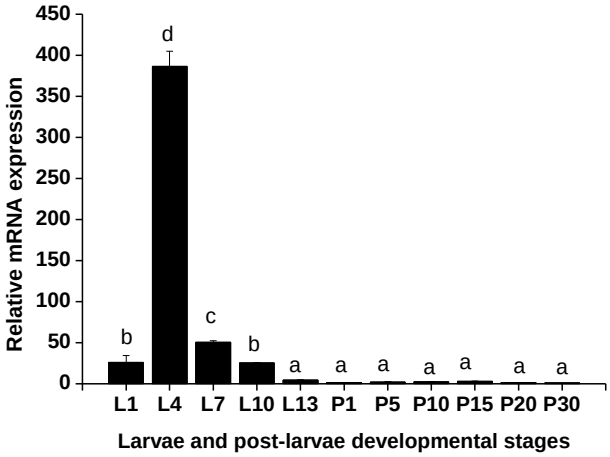

Figure 5. The temporal expression of MnEsc at the different development stages of larvae before the metamorphosis and post-larvae after the metamorphosis were revealed by real-time quantitative PCR. The amount of MnEsc mRNA was normalized to the β-actin transcript level. Data are shown as means ± SE of three repeated samples during the embryos, larvae and post-larvae. L1- the first day larvae after hatching, P1- the first day post-larvae after metamorphosis, and so on.

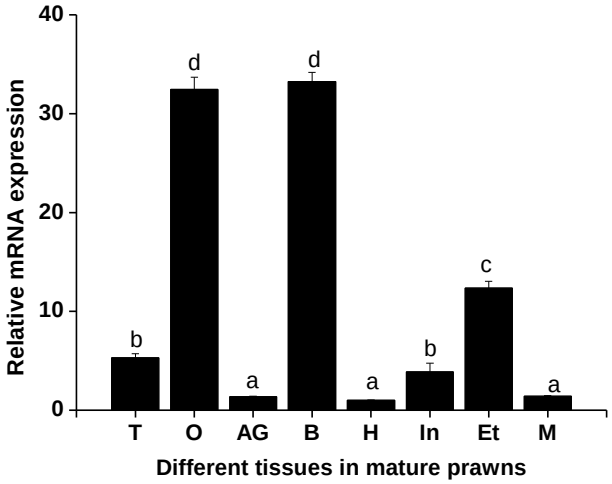

Figure 6. The expression profile of MnEsc in different tissues was revealed by real-time quantitative PCR. The amount of Esc mRNA was normalized to the *β*-actin transcript level. Data are shown as means ± SE of three replicates in various tissues. T-testis; O-ovary; B-brain; AG-abdominal ganglion; H-heart; In-intestine; Et-Eyestalk; M-muscle.

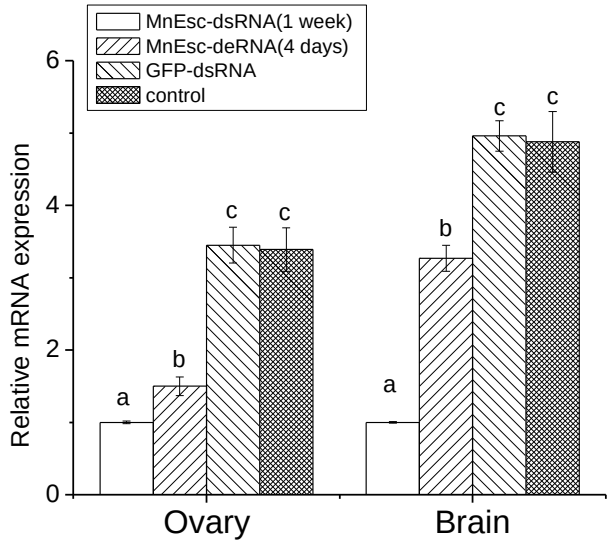

Figure 7. Relative MnEsc expression in the ovary and brain of control and RNAi-treated mature females.

Discussion

In the present study, we have successfully cloned the full-length cDNA of Esc gene based on the EST sequence from a *M. nipponense* testis cDNA library. The cDNA sequence encoded a protein of 355 amino acids having a molecular weight of 40.201 kDa. From the result of alignment, this deduced amino acid sequence shares high homology with those previously reported arthropoda Esc proteins, which confirmed the high conservation of this protein. The insect extra sex combs protein contains multiple WD repeats (Gutjahr *et al*., 1995; Sathe *et al*., 1995; Simon *et al*., 1995), which was essential for its function as a repressor of homeotic genes (Sathe *et al*., 1995). Conserved sequence of five WD40 repeated motifs were identified in the deduced amino acid sequences of Esc (van der Voorn and Ploegh, 1992; Neer *et al*., 1994). The widespread distribution of the WD40 repeats throughout MnEsc protein implies that its function is mainly by contacting other proteins (Gutjahr *et al*., 1995; Komachi *et al*., 1994; Whiteway *et al*., 1994).

Previous studies indicated that Esc product was required only in the very early stages of embryonic development (Gutjahr *et al*., 1995; Simon *et al*., 1995; Sathe *et al*., 1995). In the present study, expression of Esc mRNA increased at the early embryo, and then drop to a low level during larval growth folloeing increasing again during pupal stages, which is consistent with a transient role in establishing stable long term PcG-mediated repression (Sathe *et al*., 1995; Katsuhito *et al*., 2008). Our study was the first to report the involvement of Esc in the development of embryo and postembryonic and in other tissues of *M. nipponense*. Real-time quantitative PCR analysis

indicated that MnEsc is mainly expressed in the blastula stage of embryonic and the fourth larvae, which was similar with previous studies in the early developmental embryos of *Drosophila* (Gutjahr *et al*., 1995) and *medaka* (Norihisa *et al*., 2004). These data indicated that the MnEsc might be related to the embryogenesis and organogenesis of the *M. nipponense*. The Esc gene regulated the body segment and controlled the terminal pattern of leg sex comb differentiation of male *Drosophila* (Chiyoko *et al*., 1965; Papaceit *et al*., 1991; Struh *et al*., 1982). During the postembryonic stage of *M. nipponense*, we found that the expression of MnEsc displayed a transient increasing in 4 days post-hatching larvae, and then its expression in the post-larvae did not change significantly after metamorphosis. In a follow up study (unpublished), the eyestalk and branchiostegal spine of *M. nipponense* are developed from 4 days post-hatching larvae to 11 days post-hatching larvae according to morphological observation. Therefore, we speculated that the expression pattern of MnEsc during postembryonic development may concern to pre-metamorphic larval changes of morphology and the organogenesis of larvae.

The genetic evidence indicates that the maternal Esc contribution deposited in the oocyte is critical for early embryonic development (Struhl, 1981). At very late stages of *Drosophila* embryo, Esc is again expressed zygotically in specific regions of the brain of *Drosophila* (Gutjahr *et al*., 1995). The results of present study showed that MnEsc mRNA expressed in all detected tissues. The highest level is observed in brain and ovary of the oriental river prawn. These data indicate that the MnEsc mRNA may be also maternally deposited in the oocyte, its biological function may be controlled by central nervous system.

RNAi experiment showed that the decrease of MnEsc mRNA expression could be observed in different tissues after injection of MnEsc-dsRNA for four days and 1 week, indicating that RNA interference could be induced systemically and the interference effect could be transported among different tissues. But no exterior appearance change of experimental shrimps was observed. This may due to two reasons: firstly, the time is not long enough from interference to the observation, the *M. nipponense* have not been molted to change the phenotype. Secondly, experimental shrimps are mature, their phenotype is not easy to change after interference.

In conclusion, this study is the first report of an Esc gene in the oriental river prawn, *M. nipponense*. MnEsc may play important roles in the embryogenesis, oogenesis and morphological differentiation of the early larva, and may be used as a molecular marker for future studies of *M. nipponense* embryonic development. Moreover, a stable RNA interference technology could provide an effective molecular biological approach for the identification of gene function and antiviral research in *M. nipponense*.

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