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Differential expression of ubiquitin-conjugating enzyme E2r in the developing ovary and testis of penaeid shrimp *Marsupenaeus japonicus*

Bingling Shen · Ziping Zhang · Yilei Wang · Guodong Wang · Yun Chen · Peng Lin · Shuhong Wang · Zhihua Zou

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Abstract In order to identify genes involved in oogenesis and spermatogenesis in penaeid shrimp *Marsupenaeus japonicus*, a modified *a*nnealing *c*ontrol *p*rimer (ACP) system was adapted to identify genes differentially expressed in ovary and testis at different developmental stages. By using 20 pairs of ACP primers, 8 differentially expressed genes were obtained. One of these genes is ubiquitin-conjugating enzyme E2r (*UBE2r*). Bioinformatics analyses show that this gene encodes a protein of 241 amino acids with a predicted molecular mass of 27.4 kDa. Real time PCR analyses demonstrated that the expression level changed significantly in the developing testis and ovary. In the stage 2 of testis, it reached its highest expression level, the lowest expression level present in the stage 1 of ovary. The significantly different expression levels in developing testis and ovary suggest that *UBE2r* has an important role in oogenesis and spermatogenesis. This article is the first report of *UBE2r* in crustaceans and also is the first report showing that *UBE2r* is differentially expressed at different stages of the developing ovary and testis in an animal.

B. Shen · Y. Wang (✉) · G. Wang · Y. Chen · P. Lin · S. Wang · Z. Zou
The Key Laboratory of Science and Technology for Aquaculture and Food Safety, Fisheries College, Jimei University, Xiamen, Fujian 361021, China
e-mail: ylwang@jmu.edu.cn

Z. Zhang (✉)
Molecular Biosciences Research Group, Department of Chemistry & Biochemistry, Texas State University, San Marcos, TX 78666, USA
e-mail: Ziping.Zhang@txstate.edu

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Introduction

Penaeid shrimp *Marsupenaeus japonicus* is an important commercial species in aquaculture. Penaeid shrimp hatcheries have faced problems of declines in egg quality and low nauplii survival. Improvement in the production of penaeid shrimp is seen to be of economic significance. In order to develop novel methods for the control of gonad maturation in penaeid shrimp and therefore boost yields, it is critical to understand the molecular mechanisms involved in testis and ovary development. Recently, a few genes have brought insight into this area, for example: shrimp vitellogenin [1], ovarian cortical rod protein [2], thrombospondin [3], cathepsin C [4], ribosomal protein L24 [5], androgenic gland hormone [6] and insulin-like androgenic gland factor [7] have now been cloned from gonad tissues of penaeid shrimp and other crustaceans.

An *a*nnealing *c*ontrol *p*rimer (ACP) system was used to identify genes differentially expressed in ovary and testis at different developmental stages. The ACP system is based on principles of the unique tripartite structure of the primers: a 3′ end region with a target core nucleotide sequence that substantially complements the template nucleic acid for hybridization; a 5′ end region with a non-target universal nucleotide sequence; and a poly(dI) linker bridging the 3′ and 5′ end sequences. The ACP linker prevents annealing of the 5′ end non-target sequence to the template and facilitates primer hybridization at the 3′ end to the target sequence at specific temperatures, resulting in a dramatic improvement of annealing specificity [8]. In this study, we employed a modified ACP system developed in

our laboratory [9] to profile gene expression of the ovary and testis of *M. japonicus* at different developing stages. By using 20 pairs of ACP primers, 8 differentially expressed genes were obtained. One of these genes we identified is a component of ubiquitin proteasome pathway (UPP), the penaeid shrimp ubiquitin-conjugating enzyme E2r (*UBE2r*) gene.

UPP is a major means in eukaryotic cells for targeted protein proteolysis [10]. The system generally includes three classes of ubiquitin enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s or UBC) and ubiquitin protein ligases (E3s). In this pathway, ubiquitin is activated by E1 in an ATP-dependent process, then the activated ubiquitin is transferred to a reactive cysteine residue to E2 ubiquitin-conjugating enzyme, finally, the E2 is brought to the substrate by binding the ubiquitin-protein ligase E3 which can mark the protein for degradation by the 26S proteasome [11]. UPP is involved in numerous cellular processes, such as cell cycle progression [12], organelle biogenesis [13], and transcriptional regulation [14]. Recently some reports demonstrated that UPP contributes to several control mechanisms of gametogenesis. Sutovsky et al. showed that abnormal sperm are tagged by ubiquitin in the epididymis of mammals demonstrating a role of UPP in the control of the sperm quality [15]. Another function of UPP is selective destruction of sperm mitochondria, so that the mammalian mitochondrial DNA shows a nearly complete maternal inheritance [16]. In ascidians, the ubiquitin–proteasome system participates in fertilization, particularly in the degradation of the proteinaceous egg coat [17, 18].

UBE2r, also called UBC3 or CDC 34, is one of ubiquitin conjugating enzymes in the UPP system. It is phosphorylated by protein kinase CK2 [19, 20]. UBC3 has been isolated from several organisms, such as saccharomyces, xenopus and human. All of these proteins have a highly conserved catalytic domain that is common to all E2s and each one has a special carboxy-terminal extension which is different from other E2s. CDC34 is essential in regulation of the cell cycle at the G1–S transition in *Saccharomyces cerevisiae*, involving the degradation of p40sic1p, which is an inhibitor of the cell cycle [21, 22]. In eggs of *Xenopus laevis*, CDC34 is required in the initiation of DNA replication. It appears to regulate the initiation function of Cdk2-cyclin E [23]. In humans, E2r (CDC34) functions in the regulation of the cell cycle by way of chromosome segregation at the onset of anaphase [24]. Pati et al. [25] reported that UBE2r (CDC34) may have an impact on cAMP-inducible gene regulation during both meiotic and mitotic cell cycles, and CDC34 significantly increased in meiotic and postmeiotic haploid germ cells.

Research about the function of ubiquitin-conjugating enzymes in the developing ovary and testis of crustaceans is unavailable. Most of the reports on the ubiquitin-proteasome system in crustaceans are concerned with molting [26–28]. Currently, no *UBE2r* gene has been identified from crustaceans. Here we report the cloning of *UBE2r* gene and its' expression pattern at different developmental stages of testis and ovary in *M. japonicus.*

Material and methods

Animals

Penaeid shrimp, *M. japonicus* were captured in Xiamen Bay, P.R. China. After transport to the laboratory, animals were housed in aerated, 300 L maintenance tanks filled with sand-filtered seawater obtained from the capture site. According to the gonad somatic index (GSI = gonad weight/body weight), the female shrimp were classified into five stages: stage 1 (GSI = 0.43 ± 0.02), stage 2 (GSI = 0.89 ± 0.02), stage 3 (GSI = 2.45 ± 0.33), stage 4 (GSI = 6.30 ± 0.31) and stage 5 (GSI = 8.95 ± 1.75), the male shrimp were classified into three stages: stage 1 (GSI = 0.33 ± 0.04), stage 2 (GSI = 0.45 ± 0.12) and stage 3 (GSI = 0.57 ± 0.06). Five penaeid shrimp at each developmental stage were used for the experiment.

RNA isolation and cDNA synthesis for ACP system

Tissues from ovary (stage 1) and testis (stage 1) of *M. japonicus* were rapidly dissected and prepared according to previously described methods [5] and snap frozen in liquid nitrogen. Total RNA was isolated from these samples. Subsequently, total RNA was used for the synthesis of the first-strand cDNA by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42°C in a final reaction volume of 20 μl containing 3 μg purified total RNA, 4 μl of 5× reaction buffer (Promega, Madison, WI), 5 μl of 10 mM dNTPs, 1 μl of 10 μM cDNA synthesis primer dT-ACP, and 1 μl of M-MLV transcriptase (200 U/μl; Promega, Madison, WI). Followed by heating at 94°C for 2 min to inactivate the reaction, the first-strand cDNA samples were diluted by the addition of 80 μl ultra-purified water.

Polymerase chain reaction (PCR) was conducted by using 20 pairs of arbitrary ACPs to synthesize the second-strand cDNAs under annealing conditions. Because of the specific tripartite sequence regions of ACP primer, the 3′ end core portion of the dT-ACP is prevented from annealing to first strand cDNAs and only the 3′ end core portion of the arbitrary ACP anneals to the first-strand cDNAs. Arbitrary ACPs contain random sequence 10-mers as the 3′ end core sequences, and only those ACPs that are sufficiently complementary to a region of a first-strand

cDNAs will anneal [29]. For the PCR reaction, ovary and testis cDNAs were used as templates for amplifications using different sets of arbitrary ACPs. PCR analysis were performed in a final volume of 25 μl containing 1 μl of the cDNAs, 2.5 μl of 10× PCR buffer, 1.5 μl of 10 mM dNTP, 1 μl arbitrary ACP and dT-ACP, 0.5 μl Taq polymerase (200 U/μl). After incubation at 94°C 2 min, 50°C for 5 min and 72°C for 1 min, followed by 40 cycles of 94°C for 30 s, 65°C for 40 s and 72°C for 1 min, after which 72°C for 7 min. PCR product was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Differentially expressed bands were cloned into pMD-T18 vector (TAKALA). The cloned DNA was sequenced and analyzed by BLAST search (http://www.ncbi.nlm.nih.gov).

5′-RACE amplification of *UBE2r* gene

Total RNA of testis was used for the synthesis of first-strand cDNAs by reverse transcriptase for the 5′ rapid amplification of cDNA ends (RACE). Procedure was performed according to the manufacturer's instructions (Clontech). Briefly, 1 μg total RNA from the testis was reverse-transcribed to 5′-RACE-ready cDNA with Oligo(dT)17 and SMART oligonucleotide. PCR was then carried out using the Advantage 2 PCR kit (Clontech) with the primer UBE2r GSP1 and the universal primer (Table 1) mixture provided with the SMART RACE cDNA amplification kit (Clontech). The PCR product was 50-fold diluted in Tricine-EDTA buffer (10 mM Tricine-KOH (pH 8.5), 1 mM EDTA), and a nested PCR was performed using the primer UBE2r GSP2 and the nested universal primer (Table 1) supplied in the SMART RACE cDNA amplification kit. All PCR was carried out in a Perkin-Elmer 9700 thermal cycler (Applied Biosystems, Foster City, CA) according to the instructions of the SMART RACE cDNA amplification kit. PCR products were cloned into pGEM-T (Promega) and sequenced.

Nucleotide sequence and bioinformatics analyses

Nucleotide and predicted amino acid sequence data were compiled and aligned with sequences in EMBL, GenBank, DDBJ, SwissProt, PIR and PRF databases using the BLAST and/or FASTA algorithms (http://www.ncbi.nlm.nih.gov) to determine gene identity. Matches were considered to be significant only when the probability was smaller than 1×10^{-4} using BLASTN and BLASTX with default parameters. Amino acid sequences were predicted using BLASTX (http://www.ncbi.nlm.nih.gov) in combination with 6-frame translation by BCM Search Launcher (http://emboss.bioinformatics.nl/). Statistical analyses of protein sequences were carried out using an online program at http://ca.expasy.org/tools/pi_tool.html [30] and by CDD at: http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi [31]. UBE2r amino acid signature proposed by the Prosite database was systematically compared with the deduced sequence of *M. japonicus UBE2r* gene (http://www.expasy.ch/prosite/). Protein multiple-alignments were performed by BioEdit (http://www.mbio.ncsu.edu/BioEdit/). Phylogenetic relationships were deduced and dendrograms were drawn by using MEGA 4 program (http://www.megasoftware.net/) [32].

Real time PCR analysis of *UBE2r*

Evaluation of *UBE2r* gene expression levels in different developing testis and ovary of penaeid shrimp was achieved by real time quantitative PCR kinetics using the SYBR Green I chemistry. Primers for *UBE2r* and 18S rRNA (Table 1) were designed using primer express 3 software (Applied Biosystems) and tested to ensure amplification of single discrete bands with no primer–dimers. An aliquot of 2 μg of total RNA pre-treated with DNase I was used as template for cDNA synthesis in 20 μl reactions with random hexamers using the M-MLV Reverse transcriptase first-strand synthesis system for

Table 1 Primers used in the experiments

Primer name	Sequence
dT-ACP	AAGCAGTGGTATCAACGCAGAGTTTTTTTTTTTTTTTTTTTT
ACP19	AAGCAGTGGTATCAACGCAGAGTIIIIICTCTGCGAGC
Nested	AAGCAGTGGTATCAACGCAGAGT
universal primer	CTAATACGACTCACTATAGGGC
UBE2r-GSP1	GCCGTCACGCTCAGCCTCAC
UBE2r-GSP2	GGAATCCCTCCAGCGACGGTA
UBE2r real time F primer	CTGTCGGTGATTTCCTTGCTAA
UBE2r real time R primer	ATTCCTTGTCCTTGCCCTTG
18S rRNA real time F primer	CGTCGCTACTACCGATTGAATGGTC
18S rRNA real time R primer	TTCACCTACGGAAACCTTGTTACGACT

RT-PCR (Promega). For real time PCR, an amount of cDNA corresponding to 25 ng of input RNA was used in each reaction. Reactions were performed with the SYBR Green PCR Master Mix (TOYOBO), and analyzed in the ABI 7500 real time System. PCR products for *UBE2r* and 18S rRNA were ligated into pMD 18-T vector (TAKARA) and transformed in DH5α competent cells (Invitrogen). Minipreps of isolated plasmid DNA were then prepared (GENERAY) for sequencing to check the sequence of the real time PCR products. The comparative threshold cycle (CT) method (user Bulletin #2, the ABI PrismR 7500 Sequence Detector, PE Applied Biosystems) was used to calculate the relative concentrations. This method involves obtaining CT values for the *UBE2r*; normalizing to the housekeeping gene, 18S rRNA; and comparing the relative expression level among different developing stages of testis and ovary. Experiments were performed routinely with five females and five males of each stage with values presented as $2^{\Delta\Delta CT}$ for the expression levels of *UBE2r* normalized with 18S rRNA (ΔCT = CT of *UBE2r* minus CT of 18S rRNA, ΔΔCT = ΔCT of test sample minus ΔCT of calibrator sample). Data are expressed as mean and standard error of the mean (SEM) unless otherwise stated. *n* indicates the number of subjects tested. Statistical analysis of the normalized CT values (ΔCT) was performed with a one-way ANOVA and Student's t-test (the same gene in different tissues and different developing stages). Differences were considered significant at $P < 0.05$ (two-tailed test).

Results

Isolation of differentially expressed genes in ovary and testis of penaeid shrimp *M. japonicus*

By using the modified ACP system, eight products with different expression levels were identified and sequenced. Queries of publicly available databases using the BLAST algorithm showed that one of these products (from ACP 19) (Fig. 1) had high similarity to *UBE2r* gene. The length of the product was 516 bp (gray color highlighted from 961 to 1,477 bp in Fig. 2). Two gene specific primers, UBE2r-GSP1 (green color highlighted in Fig. 2) and UBE2r-GSP2 (yellow color highlighted in Fig. 2) were designed from the product for 5′-RACE. The full-length cDNA sequence of *UBE2r* was obtained by 5′-RACE method and confirmed by head-to-toe PCR amplification using *M. japonicus* testis cDNA as template. The nucleotide sequence obtained was 1,477 bp in length (GenBank Accession No. EU431335), including 651 bp of 5′ untranslated region (UTR), 726 bp of open reading frame, and 85 bp of 3′ UTR (excluding the poly(A)$^+$ tail) (Fig. 2). Analysis of the deduced protein

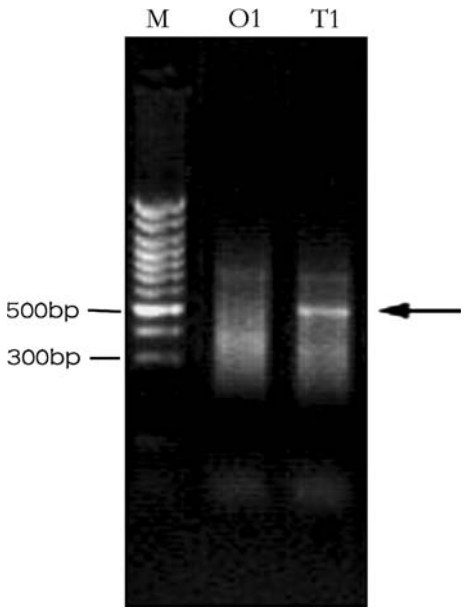


Fig. 1 ACP19 products of ACP system from ovary and testis were visualized by agarose gel electrophoresis and ethidium bromide staining. Candidate gene was indicated with arrowheads. The size of product is about 500 bp (M: marker, O1: stage 1 of ovarian development, T1: stage 1 of testicular development)

sequence of penaeid shrimp UBE2r showed that it comprises 241 amino acids (Fig. 2) with a predicted molecular weight of 27.3 kDa and isoelectric point of 3.97.

Structural characterization of *UBE2r* of *M. japonicus* and comparison with other known UBE2r proteins and protein motifs

Further bioinformatic analyses employing BLAST searches [33] of public DNA sequence databases showed that the coding sequence (CDS) of the *M. japonicus UBE2r* displayed a high degree of identity with a number of *UBE2r* of insects. For example, CDS of *M. japonicus UBE2r* shared 75% identity with the *UBE2r* of *Culex pipiens quinquefasciatus* (Accession No. XM_001843002), and 71% identity with the UBE2r of *Aedes aegypti* (Accession No. XM_001653422). Similarly, the predicted amino acid sequences of UBE2r shared 81% identity with *Aedes aegypti* (Accession No. XP_001653472) and *Culex pipiens quinquefasciatus* (Accession No. XP_001843054), shared 71% identity with *Mus musculus* (GenBank Accession No. CAC80335). Comparison of our deduced *M. japonicus* UBE2r sequence with the conserved domain databases revealed a significant alignment in the highly conserved region [31]. For example, when aligned with cd00195, the conserved domain length profile for UBE2r spanned 141 residues with a score of 167.728 bits. Furthermore, sequence alignment of UBE2r with sequences from the

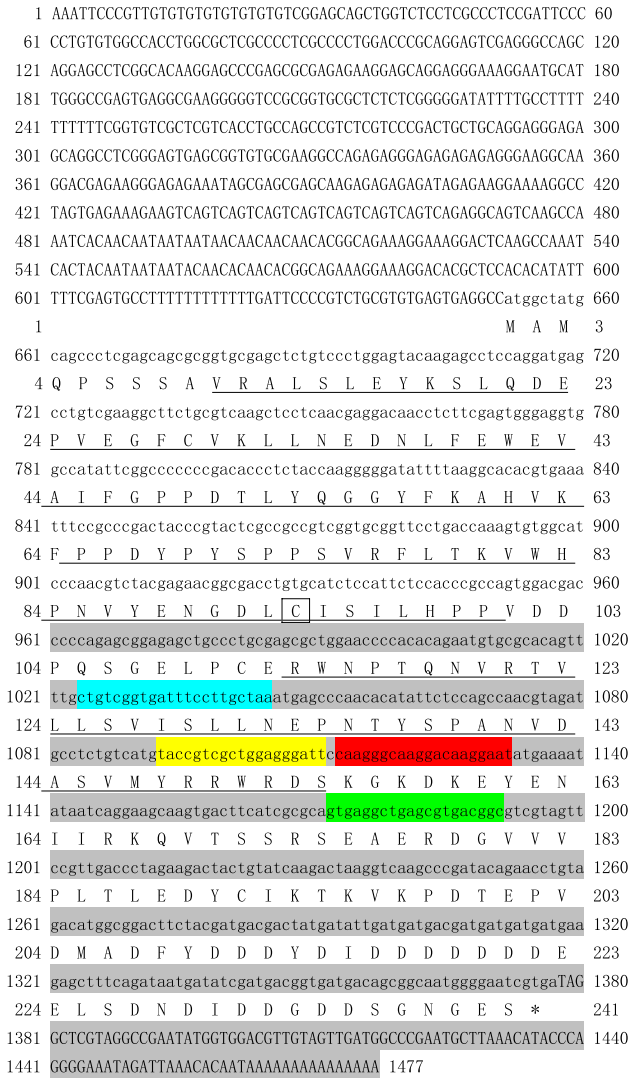


Fig. 2 Nucleotide and deduced amino acid sequences of the *UBE2r* gene. The nucleotide sequence is displayed in the 5′–3′ directions and numbered to the right and left. The derived amino acid sequence is shown in the single letter amino acid code. Codons are numbered at the left and right with the methionine initiation codon. An asterisk denotes the termination codon. The conserved domain (catalytic domain) of UBE2r is underlined. The box points to the active site cysteine residue of amino acid sequence of UBC domain. RT-PCR product is highlighted in grey color. 5′ RACE primers are highlighted in green color and yellow color, Real time PCR primers are highlighted in red color and blue color

Prosite Database identified several highly conserved amino acids (Fig. 3), corresponding to their importance in this region. Comparison of various UBE2r proteins using MEGA 4 generates trees of phylogenetic relationship of the topology (Fig. 4). As expected, UBE2r cloned in this project was found to root with its homologues of invertebrates such as *Nasonia Vitripennis*, *Aedes aegypti* and *Lycosa singoriensis*, separated from its homologues of vertebrates and plants such as *Mus musculus*, *Homo sapien* and *Arabidopsis thalianas*. Based on the highly conserved position of the sequence of the UBC domain (Fig. 3), the cysteine residue (Cys93) in this region is likely the active site, through which the thiol ester bond forms with ubiquitin (Fig. 3).

Gene expression of *M. japonicus UBE2r* in developing testis and ovary

M. japonicus UBE2r transcript (as detected by annealing control primer) was expressed at a higher level in the testis than in the ovary of *M. japonicus* (Fig. 1). This result was further validated by real time PCR with specific primers designed against *UBE2r*. In parallel, the cDNA of 18S rRNA which is recommended as the most stable reference target for real time PCR [34] was also amplified using respective primers as a control for cDNA template levels. Real time PCR results showed that the *UBE2r* expression level changed significantly in ovary and testis. In stage 2 of testis, it reached its highest expression level compared to others. The lowest expression level was in the stage 1 of ovary. Stage 4 and stage 5 of ovary were almost as low (Fig. 5).

Discussion

Many genes differentially expressed in the testis and ovary of *M. japonicus* have been identified in our on-going efforts to understand the role of genes involved in ovary and testis development in the penaeid shrimp. One of these genes, *UBE2r*, was isolated by a modified ACP system with the dT-ACP and ACP 19 primer pair (Table 1).

Although UBE2r/UBC3/CDC34 from different species differ in size, structure and function, all E2s have a conserved domain of roughly 16 kDa which is designated the UBC domain. This domain contains a centrally located cysteine residue for thiolester formation [35]. Our results showed that UBE2r of *M. japonicus* also has the cysteine residue (Cys93) in the UBC domain (Fig. 2). UBE2r/UBC3/CDC34s proteins contain a moderately conserved catalytic domain that is characteristic of all ubiquitin-conjugating enzymes and a C-terminal extension that is unique to UBE2r/UBC3/CDC34. UBE2r in *M. japonicus* has a special C-terminal that is different from other E2s. This characteristic C-terminal may be related to the different E2s having individual substrate recognition sites, and this individual site of UBE2r of *M. japonicus* responses to the distinct function of UBE2r in the developing testis and ovary.

UBE2r/UBC3/CDC34 plays important roles in the cell cycle. For example, a key negative regulator of mitosis, the protein kinase Wee 1, which is essential for cells to pass the S phase, was degraded in a CDC34-dependent fashion in *Xenopus* egg [36]. Oogenesis and spermatogenesis are driven by active mitosis and meiosis where protein

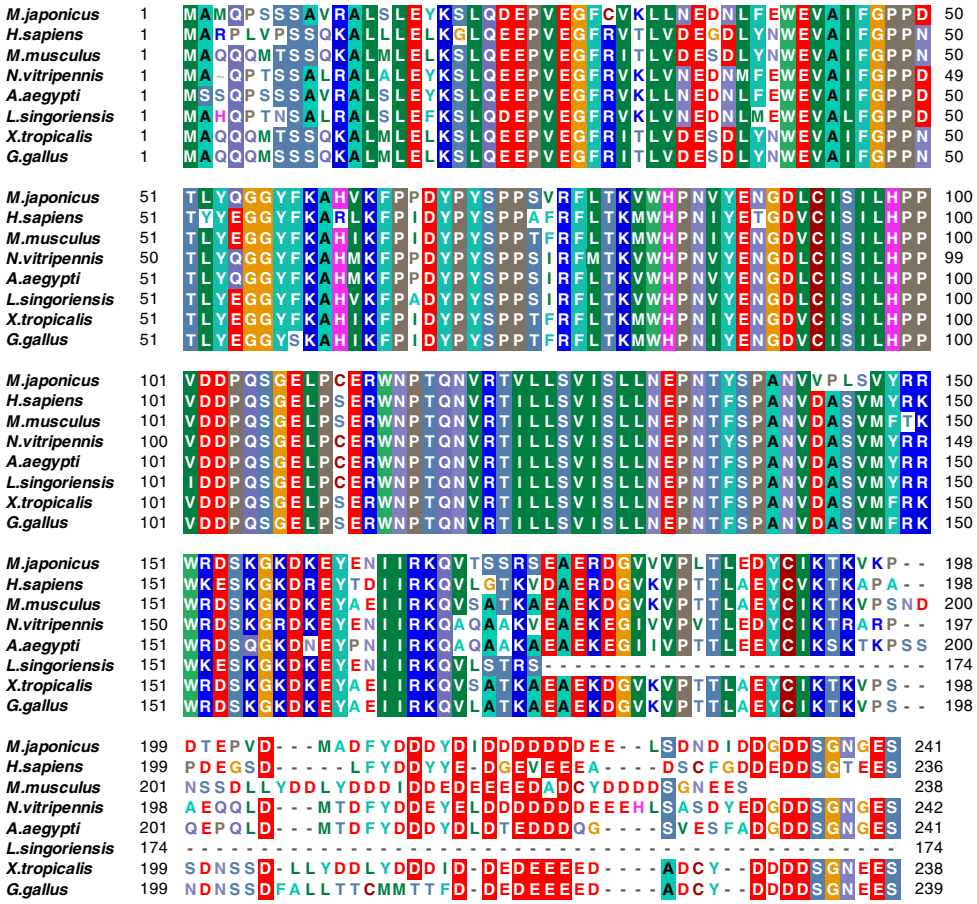


Fig. 3 Comparison of *M. japonicus* UBE2r amino acid sequence with those of other UBE2r homologues. Sequence alignment was performed with BioEdit (http://www.mbio.ncsu.edu/BioEdit/). Species names are abbreviated at the left and represent: *Marsupenaeus japonicus* Accession No: EU431335. *Homo sapiens*. Accession No: NP_004350. *Mus musculus*. Accession No: CAC80335. *Nasonia vitripennis*. Accession No: XM_001600129. *Aedes aegypti*. Accession No: XP_001653472. *Lycosa singoriensis*. Accession No: ABX75517. *Xenopus tropicalis*. Accession No: AAI24047. *Gallus gallus*. Accession No: NP_001026582

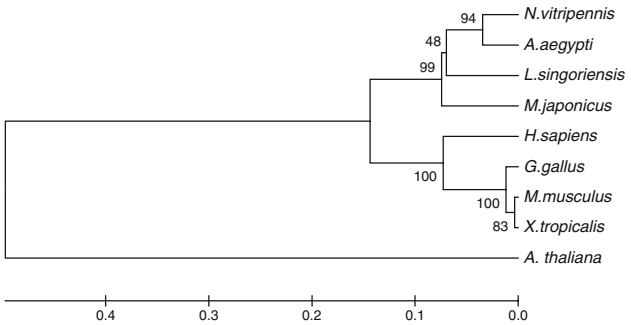
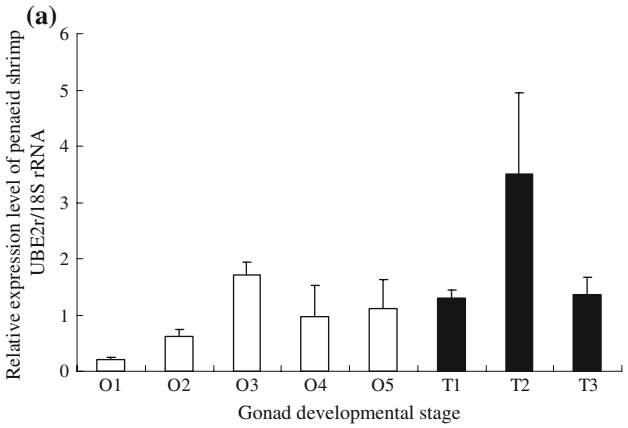


Fig. 4 Dendrogram of UBE2r/UBC3/CDC34 from different organisms based on amino acid sequence comparisons. Species names are abbreviated at the left and represent: *A. thaliana*, NP_568956. Others see Fig. 3

activation alternates with protein degradation [37, 38]. Our experiments showed that the expression level of *UBE2r* changed at different developmental stages of ovary and testis. In ovary, it reached the highest expression level at the stage 3 ovary and maintained a steady expression level at stage 4 and stage 5. In testis, the highest expression level of *UBE2r* appeared at stage 2. At stage 1 and stage 3 it was almost the same expression level. These data suggest that some specific proteins necessary for the cell cycle are synthesized before stage 3 of ovary. At stage 3, in order to continue the cell cycle, these proteins were degraded by the E2r-dependent ubiquitin pathway. At stage 4 and 5 of ovary, the steady expression of *UBE2r* may imply that the protein degradation also reached a steady level. At stage 2 of testis, the highest expression level of *UBE2r* may imply a mechanism to cope with the increased protein degradation that occurs in spermatogenesis. Furthermore, in the ovary of *Penaeus vannamei*, Jiang et al. [39] found that at the early developmental stage (stage 1), acidic and alkaline proteins as well as DNA and RNA are actively synthesized. At the late stages, however, the acidic and alkaline proteins decreased and neutral proteins increased. Based on these data we suggest that the lowest expression of *UBE2r* at stage 1 of ovary may related to the accumulation of acidic and alkaline proteins, and the high expression of *UBE2r* at the late stage may be associated with the degradation of some acidic and alkaline protein during the oogenesis. Articles published by our group have demonstrated active change or modification of proteins in testis of shrimp [40] may be related to the action of UBE2r.

UBE2r/UBC3/CDC34 and other ubiquitin conjugating enzymes have also been reported to play an important role in meiosis of spermatogenesis. For example, CDC34



(b)

	O1	O2	O3	O4	O5	T1	T2	T3
O1			**			*	**	*
O2			*				**	
O3	**	*					**	
O4							**	
O5							**	
T1	*	*					**	
T2	**	**	**	**	**	**		**
T3							**	

Fig. 5 (**a**) Bar graph showing expression of ubiquitin-conjugating enzyme E2r normalized to 18S rRNA in different developing stages of gonads in male and female penaeid shrimp. Data are expressed as means ± SEM of five separate individuals, each assayed in quadruplicate. (**b**) Asterisks indicate significant difference between different developing stages in ovary and testis ($P < 0.05$). (O: ovary, T: testis, number: stage of development). *Indicates significantly difference expression ($P < 0.05$), **Indicates most significantly difference expression ($P < 0.01$)

expression level is maximal in the postmeiotic phase of mammalian spermatogenensis [41]. Similarly, mutations in the UbcD1 gene of *Drosophila* disrupt male meiosis, leading to infertility [42]. Inactivation of the ubiquitin conjugating enzyme HR6B in mice causes male infertility [43]. These studies suggest that the ubiqutin conjugating enzymes, including UBE2r, may be necessary for the cell cycle and play a role in testicular developments especially in spermatogenensis. Even though further cytological study is required, our research suggests that UBE2r of *M. japonicus* may play a similar role, especially in spermatogenesis. More interestingly, the highest expression level of the *UBE2r* gene in testis may reflect the phenomenon that, in general, males have a higher rate of gamete production [44]. Therefore, meiosis in testis is much more active than in ovary.

Some data showed that the UPP system played a role in ovarian development. Ubiquitin C-terminal hydrolase L1 (UCH-L1) is a sperm-oocyte interactive binding or fusion protein on the plasma membrane. Its function is to block polyspermy in mouse oocytes [45]. Noma et al. reported that de-ubiquitylating enzyme Usp9x -involved in the gonadal development and oogenesis- is highly accumulated in the cytoplasm of Graaffian follicles of adult female *Drosophila* [46]. However, studies detailing the function of ubiquitin-conjugating enzyme during the ovary development and oogensis in animals are unavailable. Our results provide preliminary evidence to support that ubiquitin-conjugating enzymes, including UBE2r, play an important role in ovary development.

This study is the first to demonstrate that ubiquitin-conjugating enzymes UBE2r/UBC3/CDC34 are differentially expressed in developing ovary and testis and may play an important role in oogenesis and spermatogenesis in crustaceans. However, there is still much to understand in how a specific ubiquitin-conjugating enzymes E2r/UBC3/CDC34 gene might contribute to gonadal development. Although further studies are clearly required to detail the precise role of UBE2r/UBC3/CDC34 in oogenesis and spermatogenesis in the penaeid shrimp, the current study provides a novel insight into its importance in this process and represents an important starting point for the manipulation of oogenesis and spermatogenesis in this important marine aquaculture species.

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References

1. Quinitio ET, Hara A, Yamauchi K, Mizushima T, Fuji A (1989) Identification and characterization of vitellin in a hermophrodite shrimp, *Pandalus kessleri*. Comp Biochem Physiol B Biochem Mol Biol 94:445–451. doi:10.1016/0305-0491(89)90179-X
2. Khayat M, Babin PJ, Funkenstein B, Sammar M, Nagasawa H, Tietz A et al (2001) Molecular characterization and high expression during oocyte development of a shrimp ovarian cortical rod protein homologous to insect intestinal peritrophins. Biol Reprod 64:1090–1099. doi:10.1095/biolreprod64.4.1090
3. Yamano K, Qiu GF, Unuma T (2004) Molecular cloning and ovarian expression profiles of thrombospondin, a major component of cortical rods in mature oocytes of penaeid shrimp, *Marsupenaeus japonicus*. Biol Reprod 70:1670–1678. doi:10.1095/biolreprod.103.025379
4. Qiu GF, Yamano K, Unuma T (2005) Cathepsin C transcripts are differentially expressed in the final stages of oocyte maturation in kuruma prawn *Marsupenaeus japonicus*. Comp Biochem Physiol B 140:171–181. doi:10.1016/j.cbpc.2004.09.027
5. Zhang ZP, Wang YL, Jiang YH, Lin P, Jia XW, Zou ZH (2007) Ribosomal protein L24 is differentially expressed in ovary and testis of the marine shrimp *Marsupenaeus japonicus*. Comp Biochem Physiol B 147:466–474. doi:10.1016/j.cbpb.2007.02.013
6. Ohira T, Hasegawa Y, Tominaga S, Okuno A, Nagasawa H (2003) Molecular cloning and expression analysis of cDNAs encoding androgenic gland hormone precursors from two

porcellionidae species, *Porcellio scaber* and *P. dilatatus*. Zool Sci 20(1):75–81. doi:10.2108/zsj.20.75
7. Manor R, Weil S, Oren S, Glazer L, Aflalo ED, Ventura T et al (2007) Insulin and gender: an insulin-like gene expressed exclusively in the androgenic gland of the male crayfish. Gen Comp Endocrinol 150(2):326–336. doi:10.1016/j.ygcen.2006.09.006
8. Hwang IT, Kim YJ, Kim SH, Kwak CI, Gu YY, Chun JY (2003) Annealing control primer system for improving specificity of PCR amplification. Biotechniques 35:1180–1184
9. Xie FJ, Zhang ZP, Lin P, Wang YL (2007) Application of annealing control primer system to cloning of differentially expressed genes. Mark Sci 31(5):70–75. In Chinese
10. Wilkinson KD (2000) Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. Semin Cell Dev Biol 11:141–148. doi:10.1006/scdb.2000.0164
11. Haas AL, Warms JVB, Hershko A, Rose IA (1982) Ubiquitin-activating enzyme: mechanism and role in protein-ubiquitin conjugation. J Biol Chem 257:2543–2548
12. Goebl MG, Yochem J, Jentsch S, McGrath JP, Varshavsky A, Byers B (1988) The yeast cell cycle gene CDC34 encodes a ubiquitin-conjugating enzyme. Science 241:1331–1335. doi:10.1126/science.2842867
13. Spees JL, Chang SA, Mykles DL, Snyder MJ, Chang ES (2003) Molt cycle-dependent molecular chaperone and polyubiquitin gene expression in lobster. Cell Stress Chaperones 8(3):258–264. doi:10.1379/1466-1268(2003)008<0258:MCMCAP>2.0.CO;2
14. Hochstrasser M, Ellison MJ, Chau V, Varshavsky A (1991) The short-lived MATa2 transcriptional regulator is ubiquitinated in vivo. Proc Natl Acad Sci USA 88:4606–4610. doi:10.1073/pnas.88.11.4606
15. Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Thompson WE, Schatten G (2001) A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. J Cell Sci 114:1665–1675
16. Thompson WE, Ramalho-Santos J, Sutovsky P (2003) Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. Biol Reprod 69:254–260. doi:10.1095/biolreprod.102.010975
17. Sawada H, Sakai N, Abe Y, Tanaka E, Takahashi Y, Fujino J et al (2002) Extracellular ubiquitination and proteasome-mediated degradation of the ascidian sperm receptor. Proc Natl Acad Sci USA 99:1223–1228. doi:10.1073/pnas.032389499
18. Sakai N, Sawada MT, Sawada H (2004) Non-traditional roles of ubiquitin-proteasome system in fertilization and gametogenesis. Int J Biochem Cell Biol 36(5):776–784. doi:10.1016/S1357-2725(03)00263-2
19. Semplici F, Meggio F, Pinna LA, Oliviero S (2002) CK2-dependent phosphorylation of the E2 ubiquitin conjugating enzyme UBC3B induces its interaction with beta-TrCP and enhances beta-catenin degradation. Oncogene 21(25):3978–3987. doi:10.1038/sj.onc.1205574
20. Block K, Boyer TG, Yew PR (2001) Phosphorylation of the human ubiquitin-conjugating enzyme, CDC34, by casein kinase 2. J Biol Chem 276(44):41049–41058. doi:10.1074/jbc.M106453200
21. Goebl MG, Yochem J, Jentsch S, McGrath JP, Varshavsky A, Byers B (1988) The yeast cell cycle gene CDC34 encodes a ubiquitin-conjugating enzyme. Science 241:1331–1335. doi:10.1126/science.2842867
22. Schwob E, Böhm T, Mendenhall MD, Nasmyth K (1994) The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*. Cell 79(2):233–244. doi:10.1016/0092-8674(94)90193-7
23. Yew PR, Kirschner MW (1997) Proteolysis and DNA replication: the CDC34 requirement in the *Xenopus* egg cell cycle. Science 277(5332):1672–1676. doi:10.1126/science.277.5332.1672
24. Reymond F, Wirbelauer C, Krek W (2000) Association of human ubiquitin-conjugating enzyme CDC34 with the mitotic spindle in anaphase. J Cell Sci 113(Pt 10):1687–1694
25. Pati D, Meistrich ML, Plon SE (1999) Human Cdc34 and Rad6B ubiquitin-conjugating enzymes target repressors of cyclic AMP-induced transcription for proteolysis. Mol Cell Biol 19(7):5001–5013
26. Merlo E, Romano A (2007) Long-term memory consolidation depends on proteasome activity in the crab Chasmagnathus. Neuroscience 147(1):46–52. doi:10.1016/j.neuroscience.2007.04.022
27. Spees JL, Chang SA, Mykles DL, Snyder MJ, Chang ES (2003) Molt cycle-dependent molecular chaperone and polyubiquitin gene expression in lobster. Cell Stress Chaperones 8(3):258–264. doi:10.1379/1466-1268(2003)008<0258:MCMCAP>2.0.CO;2
28. Koenders A, Yu X, Chang ES, Mykles DL (2002) Ubiquitin and actin expression in claw muscles of land crab, *Gecarcinus lateralis*, and American lobster, *Homarus americanus*: differential expression of ubiquitin in two slow muscle fiber types during molt-induced atrophy. J Exp Zool 292(7):618–632. doi:10.1002/jez.10081
29. Xiang SC, Hyuk S, Nam HK (2005) Identification of metaphase II-specific gene transcripts in porcine oocytes and their expression in early stage embryos reproduction. Reprod Fertil Dev 17:625–631. doi:10.1071/RD05019
30. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD et al (2005) Protein identification and analysis tools on the ExPASy server. In: Walker JM (ed) The proteomics protocols handbook. Humana Press, Totowa, NJ, USA
31. Marchler-Bauer A, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S et al (2003) CDD: a curated Entrez database of conserved domain alignments. Nucleic Acids Res 31:383–387. doi:10.1093/nar/gkg087
32. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599. doi:10.1093/molbev/msm092
33. Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85:2444–2448. doi:10.1073/pnas.85.8.2444
34. Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR—a perspective. J Mol Endocrinol 34:597–601. doi:10.1677/jme.1.01755
35. Jentsch S (1992) The ubiquitin-conjugation system. Annu Rev Genet 26:179–207. doi:10.1146/annurev.ge.26.120192.001143
36. Michael WM, Newport J (1998) Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of Wee1. Science 282:1886–1889. doi:10.1126/science.282.5395.1886
37. Grondahl C, Lessl M, Faerge I, Hegele-Hartung C, Wassermann K, Ottesen JL (2000) Meiosis-activating sterol-mediated resumption of meiosis in mouse oocytes in vitro is influenced by protein synthesis inhibition and cholera toxin. Biol Reprod 62:775–780. doi:10.1095/biolreprod62.3.775
38. Wang YL, Zhang ZP, Li SJ (1996) Basic protein changes during spermatogenesis in *Metapenaeus ensis*. J Xiamen Univ Nat Sci 35:947–951. In Chinese
39. Jiang YH, Yan SF (2004) Cytochemical studies on oogenesis of *Penaeus vannamei*. J Jimei Univ Nat Sci 9(2):116–121. In Chinese
40. Zhang ZP, Wang YL (1993) Studies on anatomy, histology and histochemistry of the male reproductive system of *Penaeus penicillatus, Penaeus japonicus* and *Metapenaeus ensis*. J Xiamen Fish Coll 18(2):29–38. In Chinese

41. Clermont Y, Oko R, Hermo L (1993) Cell biology of mammalian spermatogenesis. In: Desjardins C, Ewing LL (eds) The cell and molecular biology of the testis. Oxford University Press, Oxford, UK, pp 332–376
42. Cenci G, Rawson RB, Belloni G, Castrillon DH, Tudor M, Petrucci R et al (1997) UbcD1, a *Drosophila* ubiquitin-conjugating enzyme required for proper telomere behavior. Genes Dev 11:863–875. doi:10.1101/gad.11.7.863
43. Roest HP, van Klaveren J, de Wit J, van Gurp CG, Koken MH, Vermey M et al (1996) Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification. Cell 86:799–810. doi:10.1016/S0092-8674(00)80154-3
44. Clutton-Brock TH (1991) The evolution of parental care. Princeton University Press, USA
45. Sekiguchi S, Kwon J, Yoshida E, Hamasaki H, Ichinose S, Hideshima M et al (2006) Localization of ubiquitin C-terminal hydrolase L1 in mouse ova and its function in the plasma membrane to block polyspermy. Am J Pathol 169(5):1722–1729. doi:10.2353/ajpath.2006.060301
46. Noma T, Kanai Y, Kanai-Azuma M, Ishii M, Fujisawa M, Kurohmaru M et al (2002) Stage- and sex-dependent expressions of Usp9x, an X-linked mouse ortholog of Drosophila Fat facets, during gonadal development and oogenesis in mice. Mech Dev 119(Suppl 1):S91–S95. doi:10.1016/S0925-4773(03)00098-4