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Expressed Sequence Tag Analysis for Identification and Characterization of Sex-Related Genes in the Giant Tiger Shrimp *Penaeus monodon*

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Sex-related genes expressed in vitellogenic ovaries of the giant tiger shrimp, *Penaeus monodon*, were identified by an EST approach. A total of 1051 clones were unidirectionally sequenced from the 5 terminus. Nucleotide sequences of 743 EST (70.7%) significantly matched known genes previously deposited in the GenBank (E-value $<10^{-4}$) whereas 308 ESTs (29.3%) were regarded as newly unidentified transcripts (E-value $>10^{-4}$). A total of 559 transcripts (87 contigs and 472 singletons) were obtained. *Thrombospondin* (*TSP*) and *peritrophin* (79 and 87 clones accounting for 7.5 and 8.3% of clones sequenced, respectively) predominated among characterized transcripts. Several full length transcripts (e.g. *cyclophilin*, *profillin* and *thioredoxin peroxidase*) were also isolated. A gene homologue encoding chromobox protein (*PMCBX*, ORF of 567 nucleotides encoding a protein of 188 amino acids) which is recognized as a new member of the *HP1* family was identified. Expression patterns of 14 of 25 sex-related gene homologues in ovaries and testes of *P. monodon* broodstock were examined by RT-PCR. *Female sterile* and *ovarian lipoprotein receptor* homologues were only expressed in ovaries whereas the remaining transcripts except *disulfide isomerase related P5 precursor* and *adenine nucleotide translocator 2* were higher expressed in ovaries than testes of *P. monodon* broodstock. A homologue of *ubiquitin specific proteinase 9, X chromosome* (*Usp9X*) revealed a preferential expression level in ovaries than testes of broodstock-sized *P. monodon* ($N = 13$ and 11, $P < 0.05$) but was only expressed in ovaries of 4-month-old shrimp ($N = 5$ for each sex).

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Introduction

The giant tiger shrimp, *Penaeus monodon*, is one of economically important cultured species (Rosenberry, 2003). Annual production of farmed *P. monodon* in Thailand alone had greater than 200,000 metric tons since 1993 (Asian Shrimp Culture Council, 1996). Farming of *P. monodon* in Thailand relies almost entirely on wild-caught broodstock for supply of juveniles because reproductive maturation of cultured *P. monodon* female is extremely low. As a result, breeding of pond-reared *P. monodon* is extremely difficult and rarely produced enough quality of larvae required by the industry. The high demand on wild female broodstock leads to overexploitation of the natural populations of *P. monodon* in Thai waters (Klinbunga *et al*., 1999). The price of a gravid wild female of *P. monodon* from the Andaman Sea (west of peninsular Thailand) is approximately $200 USD which is 3-5 times greater than for that from the Gulf of Thailand (east). This situation resulted from farmer demand since farmers believe that progeny of Andaman Sea *P. monodon* exhibit greater growth and possibly survival rates than do progeny from broodstock shrimp caught elsewhere in Thailand (Klinbunga *et al*., 1999 and 2001).

Besides problems from diseases, the lack of high quality wild and/or domesticated broodstock of *P. monodon* has possibly caused an occurrence of a large portion of stunted shrimp at the harvest time (less than 10 g rather than approximately 25 g body weight at 4 month cultivation period). The farmed production of *P. monodon* has significantly

decreased since the last few years. As a result, domesticated Pacific white shrimp, *Litopenaeus vannamei*, has recently been introduced to Thailand as a new cultured species (Khamnamtong *et al*., 2005) and initially contributed approximately 20000 MT (7.4%) of the cultured production in 2002 and dramatically increased to 220000 MT (73.3%) in 2004, respectively (Limsuwan, 2004).

The domestication and selective breeding programs of penaeid shrimp would provide a more reliable supply of seed stock and the improvement of their production efficiency (Makinouchi and Hirata, 1995; Clifford and Preston, 2006; Coman *et al*., 2006). The use of selectively bred stocks having improved culture performance, disease resistance and/or other commercially desired traits rather than the reliance on wild-caught stocks is a major determinant of sustainability of the shrimp industry (Clifford and Preston, 2006). Despite the potential benefits, the domestication of *P. monodon* has been remarkably slow in Thailand (Withyachumnarnkul *et al*., 1998) and is still at the initial stage.

In *P. monodon*, females exhibit approximately 10-20% greater growth rate than do males (Browdy, 1998). The diploid chromosome numbers of penaeid shrimp are $2N = 88$-92 where *P. monodon* possesses $2N = 88$ (Benzie, 1998). Neither sex chromosomes nor environmental sex determination has been reported in penaeid shrimp.

Eyestalk ablation is used commercially to induce ovarian maturation of penaeid shrimp but the technique leads to an eventual loss in egg quality and death of the spawner (Benzie, 1998). Therefore, predictable maturation and spawning of captive penaeid shrimp without the use of eyestalk ablation is a long-term goal for the industry (Quackenbush, 2001).

Apart from the control of reproductive maturation, manipulation of sex ratio is an important tool for crustacean aquaculture (Lyons and Li, 2002). An understanding of sex determination and differentiation is, therefore, necessary for designing appropriate breeding programs in penaeid species. Li *et al.* (2003) constructed genetic linkage maps of the kuruma shrimp, *Marsupenaeus japonicus*, based on AFLP analysis and revealed that sex of female progeny ($N = 54$) was tightly mapped to the linkage group 28 of the female map (LOD = 5.0) which led to the argument of female heterogamy (ZW) in this species. Moreover, triploidy affects the sex ratio in *Fenneropenaeus chinensis* (Li *et al*., 2003) and *M. japonicus* (Preston *et al*., 2004) where the female-to-male ratio was almost 4 : 1 in the former but all triploids were female in the latter. These further support complex heterogametic sex in penaeid shrimp (Preston *et al*., 2004).

The development of oocytes consists of a series of complex cellular events, in which different genes express to ensure the proper development of oocytes and to store transcripts and proteins as maternal factors for early embryogenesis (Qiu and Yamano, 2005). Different biotechnological approaches, for example; injection of vertebrate steroid hormones, neuro-transmitters and ecdysteroids (Benzie 1998; Okumura, 2004) and the use of specially formulated feed (Harrison, 1990) have been applied to induce the ovarian maturation of female shrimp but results are inconsistent owing to limited knowledge on genetic and hormonal control of penaeid species (Meusy and Payen, 1988; Okumura, 2004). Accordingly, an initial step toward understanding molecular mechanisms of ovarian (and oocyte) maturation and sex differentiation cascades in *P. monodon* is the identification and characterization of sex-related genes expressed in ovaries of this economically important species.

Recently, an EST approach (single-pass sequencing of randomly selected clones from cDNA libraries) has been successfully applied and recognized as an effective method for discovery of immune related genes in *L. vannamei* and *L. setiferus* (Gross *et al*., 2001), *M. japonicus* (Rojtinnakorn *et al*., 2002), *F. chinenesis* (Shen *et al*., 2004) and *P. monodon* (Supungul *et al*., 2004). In the present study, an ovarian cDNA library was constructed and 1051 randomly selected clones were sequenced to identify sex-related transcripts in *P. monodon*. The expression profiles of interesting ESTs in ovaries and testes of *P. monodon* were further examined using reverse transcription-polymerase chain reaction (RT-PCR). Transcripts only expressed in ovaries but not testes (and those expressed higher in ovaries than testes) of *P. monodon* are reported.

Materials and Methods

Experimental animals and RNA isolation. Female broodstock of *P. monodon* used for construction of a cDNA library was collected from Satun (west of peninsular Thailand). In addition, juvenile (4-month-old) and broodstock-sized shrimp used for RT-PCR analysis were purchased from a commercial farm in Chachoengsao (eastern Thailand) and wild-caught from Chonburi (Gulf of Thailand, east), respectively. Total RNA was extracted from ovaries or testes of each shrimp using TRI-REAGENT (Molecular Research Center). Messenger (m) RNA was further purified using a QuickPrep Micro mRNA Purification Kit (GE Healthcare) and kept under the absolute ethanol at −70°C prior to reverse transcription.

Construction of a normal cDNA library and EST analysis. Five microgram of mRNA from vitellogenic ovaries of wild *P. monodon* was reversed transcribed and second-stranded synthesized using a ZAP-cDNA Synthesis and Cloning Kit (BD Biosciences Clontech). Size-selected cDNAs (>500 bp) were cloned into dephosphorylated *Eco*RI/*Xho* I-digested Uni-ZAP® XR and transfected into *E. coli* XL1-Blue MRF'. The lambda library was converted into the pBluescript library by *in vivo* excision. Recombinant clones were selected by a *lacZ* system following standard protocols (Sambrook and Russell, 2001). Recombinant clones were randomly selected from those carrying insert sizes greater than 500 bp (Srisuparbh *et al*., 2003). Plasmid DNA was extracted and unidirectionally sequenced on a MegaBase 1000 automated DNA sequencer (GE Healthcare).

EST clustering and assembly. Nucleotide sequences of ESTs were compared with those previously deposited in the GenBank using

BLAST*N* and BLAST*X* (Altschul *et al*., 1990, available at http://www.ncbi.nlm.nih.gov). Significant matched nucleotides/proteins were considered when the E-value was $<10^{-4}$. Clustering and assembling of sequences were performed using TIGR Gene Indices Clustering Tools (TGICL) (Pertea *et al*., 2003) with CAP3 (Huang and Madan, 1999).

Phylogenetic analysis of a chromobox (CBX) gene homologue. Protein sequences of different isoforms of *CBX* from various species; *Apis mellifera* (*AMZGC*, XM_393875), *Homo sapiens* (*HSHP1γ*, U26312; *HSCBX3*, NM_007276; *HSHP1*, AF13660; *HSCBX5-HP1α*, CR457418; *HSCBX5*, NM_012117 and *HSCBX1*, NM_006807), *Mus musculus* (*MMCBX3*, NM_007624 and *MMCBX5*, NM_007626), *Gallus gallus* (*GGCBX3*, NM_204643 and *GGCBX1*, NM_204332), *Xenopus laevis* (*XLHP1γ*, AY168926; *XLCBX3*, BC046570 and *XLHP1α*, AF009820), *Cricetulus griseus* (*CGHP1α*, AY548740 and *CGHP1β*, AY548739), *Danio rerio* (*DRCBX1*, NM_199746), were retrieved from the GenBank and compared with that of *P. monodon*. Multiple alignments were carried out using ClustalW (Thompson *et al*., 1994). Sequences were bootstrapped 1000 times using Seqboot. The divergence between pairs of protein sequences was estimated using Prodist. A bootstrapped neighbor-joining tree (Saitou and Nei, 1987) was constructed to illustrate phylogenetic relationships among sequences using Neighbor and Consense. All phylogenetic programs described were routine in PHYLIP (Felsenstein, 1993).

RT-PCR of sex-related gene homologues. One microgram of total RNA extracted from ovaries or testes of juvenile and broodstock *P. monodon* was reverse-transcribed and 100 ng of the first strand cDNA was used as the template for amplification of sex-related gene homologues in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer and 1 U of Dynazyme^{TM} II DNA polymerase. *Elongation factor-1α* (*EF-1α*) or *β-actin* was included as a positive control. RT-PCR was initially performed by predenaturation at 94°C for 3 min followed by 25 (semi-quantitative RT-PCR for transcripts that show differential expression between ovaries and testes) or 30 (end-point RT-PCR for transcripts that were only expressed in ovaries but not testes) cycles of a 94°C denaturation for 30 s, a 53°C annealing for 60 s and a 72°C extension for 30 s. The final extension was carried out at 72°C for 7 min. Amplicons were electrophoretically analyzed through 1.5% agarose gels and visualized with a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001). The intensity of interesting genes and that of *EF-1α* or *β-actin* was quantified from the scanned photograph of the gel using the Quantity One software (BioRad). Relative expression levels of investigated transcripts (intensity of target/intensity of *EF-1α* or *β-actin*) in ovaries and testes of *P. monodon* were statistically tested using one way analysis of variance (ANOVA).

Results and Discussion

An ovarian cDNA library was established for isolation of genes involving ovarian (and oocyte) development in *P. monodon*. The primary titer of a cDNA library was approximately 4×10^6 pfu/ml. From 1051 recombinant clones sequenced, a total of 743 ESTs (70.7%) corresponded to known sequences in the GenBank (E-value $< 10^{-4}$) whereas the remaining sequences were regarded as novel (unknown) transcripts (29.3%, E-value $> 10^{-4}$). The percentage of unknown transcripts found in the present study was lower than that previously reported in hemocyte cDNA libraries of normal (44.7%) and WSSV-infected (47.0%) *M. japonicus* (Rojtinnakorn *et al*., 2002) but greater than the percentage of unknown transcript (E-value $> 10^{-2}$) in hemocyte and hepatopancreas cDNA libraries of *L. vannamei* (15.7%, Gross *et al*., 2001) and *L. setiferus* (29.5%, Gross *et al*., 2001).

The relationship between the number of clones sequenced and the accumulative numbers of unique transcripts indicated that the discovery rate of new transcripts still does not reach a plateau of saturation and is greater than 10% after 1051 recombinant clones were sequenced (Fig. 1). Therefore, additional unique transcripts can still be identified by sequencing a larger number of recombinant clones. Five

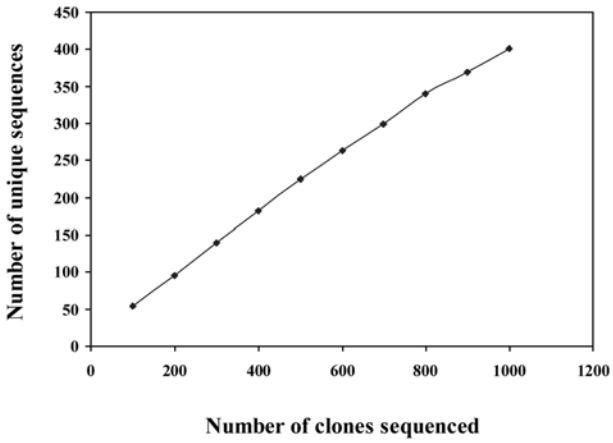

Fig. 1. Number of unique sequences plotted as a function of the accumulative number of clones sequenced from the ovarian cDNA library of *P. monodon*.

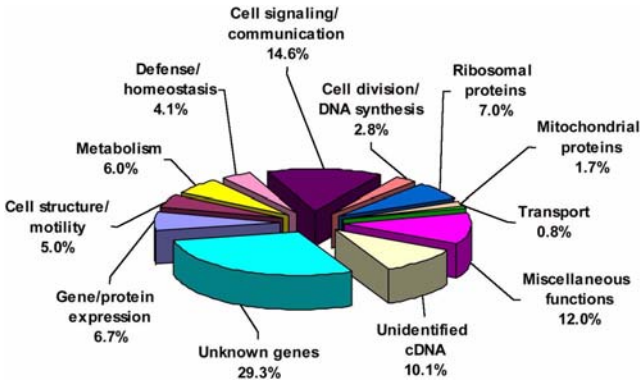

Fig. 2. Classification of genes identified in the ovarian cDNA libraries of *P. monodon* (E-value $< 10^{-4}$) owing to functional categories of their homologues.

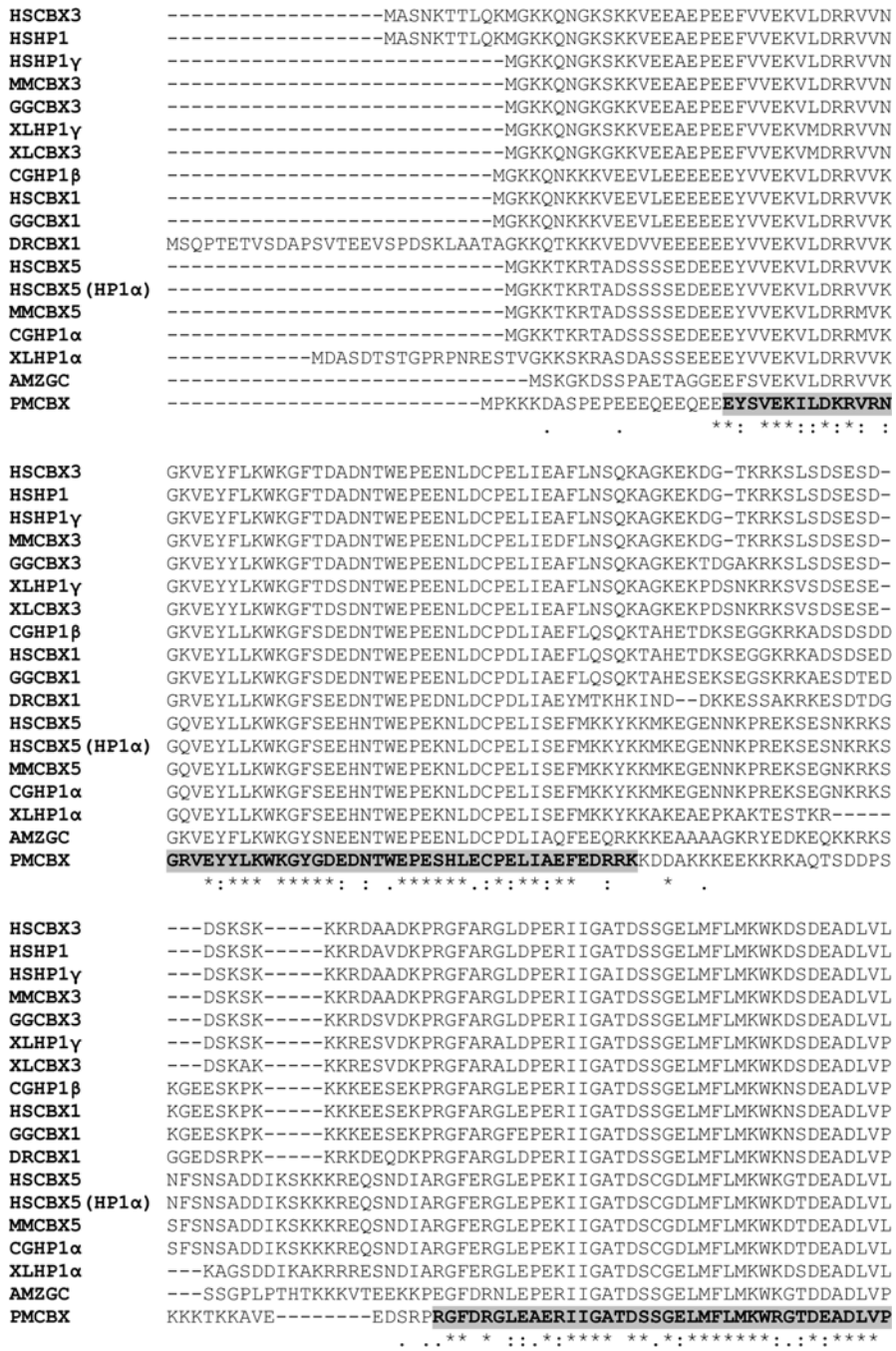

Fig. 3. Multiple alignments of *CBX* from various species and a *CBX* homologue of *P. monodon* (*PMCBX*). CHROMO (positions 21-73, E-value = 1.6e-29) and ChSh (positions 109-171, E-value = 2.0e-20) domains in *PMCBX* are boldfaced and highlighted.

hundred and fifty-nine transcripts (87 contigs and 472 singletons) were obtained after clustering analysis. The relatively high rate of gene discovery and a large number of transcripts obtained indicated that the established library is reasonably diverse.

Among known transcripts in the ovarian cDNA library of *P. monodon*, ESTs categorized as members of signaling and communication were predominant (14.6%) followed by those classified as members of miscellaneous function (12.0%), unidentified function (10.1%), gene expression and protein synthesis (6.7%), metabolism (6.0%) and internal/external structure (5.0%) groups. The remaining ESTs allocated to other functional categories were accounted for less than 5.0% of the characterized ESTs in this library (Fig. 2).

In a recent study by Leelatanawit *et al.* (2004), a total of 218 clones from subtraction suppressive hybridization (SSH) cDNA libraries between ovaries and testes of *P. monodon* broodstock were unidirectionally sequenced. Most of the

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HSCBX3          AKEANMKCPQIVIAFYEERLTWHSCPEDEAQ----------
HSHP1           AKEANMKCPQIVIAFYEERLTWHSCPEDEAQ----------
HSHP1γ          AKEANMKCPQIVIAFYEERLTWHSCPEDEAQ----------
MMCBX3          AKEANMKCPQIVIAFYEERLTWHSCPEDEAQ----------
GGCBX3          AKEANVKCPQIVIAFYEERLTWHSCPEDEAQ----------
XLHP1γ          AKEANVKCPQVVIAFYEERLTWHSCPEDEAQ----------
XLCBX3          AKEANVKCPQVVIAFYEERLTWHSCPEDEAQ----------
CGHP1β          AKEANVKCPQVVISFYEERLTWHSYPSEDDDKKDDKN-----
HSCBX1          AKEANVKCPQVVISFYEERLTWHSYPSEDDDKKDDKN-----
GGCBX1          AKEANIKCPQVVISFYEERLTWHSYPSEDDDKKEDKN-----
DRCBX1          AKEANVKCPQVVISFYEERLTWHSYPSEEEEKKDDKN-----
HSCBX5          AKEANVKCPQIVIAFYEERLTWHAYPEDAENKEKETAKS---
HSCBX5(HP1α)    AKEANVKCPQIVIAFYEERLTWHAYPEDAENKEKETAKS---
MMCBX5          AKEANVKCPQIVIAFYEERLTWHAYPEDAENKEKESAKS---
CGHP1α          AKEANVKCPQIVIAFYEERLTWHAYPEDAENKEKESTKS---
XLHP1α          AKEANLKCPQIVIAFYEERLTWHAYPEESESKEKEAVKS---
AMZGC           ARIANEKCPQIVIKFYEERLTWHSPAHDEESSVKADAE----
PMCBX           ARQANVRCPQVVIKFYEERLTWHSSTNDEEEGNHEADWILFF
                *: ** :***:** *********: . :  .
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Fig. 3. Continued.

expressed genes in ovaries encoded *thrombospondin* (*TSP*, 45 clones accounting for 28.7% of total investigated ESTs), *peritrophin* (17 clones, 10.8%), and unknown transcripts (78 clones, 49.7%). Conversely, almost all of the ESTs in *P. monodon* testes were unknown transcripts (59 clones, 96.7%).

In this study, homologues of *TSP* (79 clones, 7.5% of sequenced clones) and *peritrophin* (87 clones, 8.3%) were abundantly expressed in vitellogenic ovaries of *P. monodon* but surprisingly lower than those in previously reported SSH libraries (Leelatanawit *et al*., 2004). Recently, complete sequences of three closely related *TSP* homologues encoding the major cortical rod proteins of 1114, 1032, and 991 amino acids (GenBank accession numbers AB121209, AB121210, and AB121211) were isolated and characterized in *M. japonicus* (Yamano *et al*., 2004). *MjTSP* protein levels dramatically increased after eyestalk ablation (Okumura *et al*., 2006). In *P. monodon*, expression of *TSP* were specifically found in ovaries ($N = 20$) but not in testes ($N = 22$) of broodstock-sized *P. monodon* (Leelatanawit *et al*., 2004). In the present study, preferential expression of *TSP* in ovaries than testes of 4-month-old *P. monodon* was found ($p < 0.05$).

Peritrophin, a major component of cortical rods and is the precursor of the jelly layer of the shrimp eggs, is highly expressed during oocyte development of marine shrimp (Khayat *et al*., 2001). Synthesis of *peritrophin* in ovaries of *P. semisulcatus* is inhibited by crustacean hyperglycemic hormone (CHH) purified from the sinus gland extract of *M. japonicus* (Avarre *et al*., 2001). *Peritrophin* was not differentially expressed between ovaries and testes of *P. monodon* broodstock (Leelatanawit *et al*., 2004) and between different stages of ovarian development of *M. japonicus* after eyestalk ablation (Okumura *et al*., 2006). The recombinant *peritrophin*-like protein of *F. merguiensis* has the activity of binding Gram-negative bacteria and strong binding activity to chitin suggesting that it may also play an important role in the immune defense mechanisms (Du *et al*., 2006).

Several full length transcripts of functionally important genes including *chromobox protein* (*CBX*; ORF of 567 bp, encoding a polypeptide of 188 aa; Fig. 3), *cyclophilin 1* (519 bp, 172 aa), *cytochrome c oxidase subunit Va* (462 bp, 153 aa), *eIF-5A* (474 bp, 157 aa), *histone 1* (411 bp, 136 aa), *histone H2A* variant (399 bp, 132 aa), *profillin* (381 bp, 126 aa), *signal peptidase complex*; *sid2895p* (540 bp, 179 aa), *stress-associated endoplasmic reticulum protein 1* (201 bp, 66

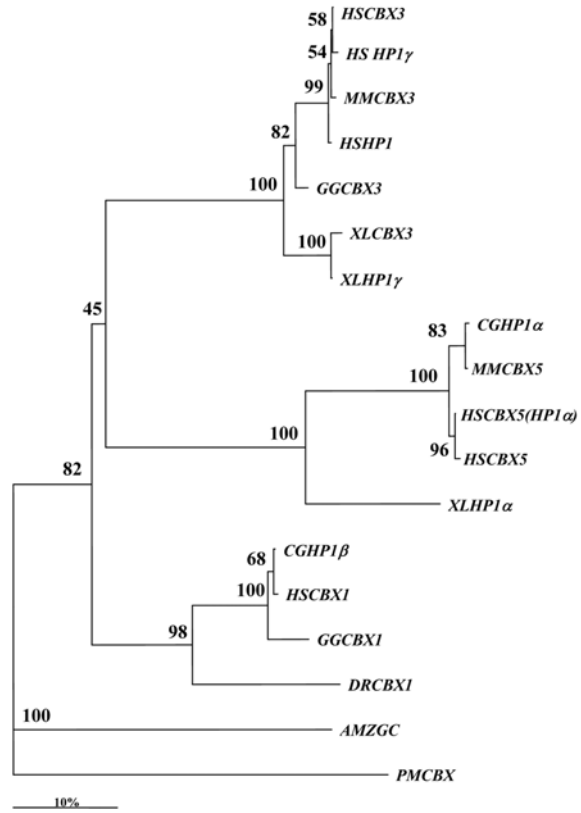

Fig. 4. A bootstrapped neighbor-joining tree illustrating relationships between a homologue of *CBX* from *P. monodon* (*PMCBX*), and that of various taxa. Values at the node represent the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original aligned protein sequences. A scale bar indicates 10% of protein sequence divergence.

Table 1. A summary of homologues of sex-linked or sex differentiation-related transcripts found in the ovarian cDNA library of *P. monodon*

Transcripts*	Matched species	Size (bp)	E-value	Linked**
CG9946-PA	*Drosophila melanogaster*	738	3e-65	X
Brain mitochondrial carrier protein 1	*Homo sapiens*	671	3e-43	X
Thioredoxin peroxidase	*Homo sapiens*	923	7e-68	X
NADPH-cytochrome P450 reductase	*Anopheles gambiae*	698	8e-26	X
Phosphatidylinositol 4 kinase	*Caenorhabditis elegans*	725	3e-13	X
ENSANGP00000019081	*Anopheles gambiae*	452	6e-22	X
Rab-protein 10 CG17060-PA	*Drosophila melanogaster*	892	1e-88	X
Ubiquitin specific protease 9	*Mus musculus*	891	5e-58	X
ENSANGP00000011950	*Anopheles gambiae*	866	2e-87	X
Female sterile (1) M3	*Drosophila melanogaster*	884	2e-07	X
Protein disulfide isomerase-related P5 precursor	*Caenorhabditis elegans*	651	3e-61	X
ENSANGP00000022750	*Anopheles gambiae*	666	2e-89	X
Ribosomal protein L1, isoform D	*Drosophila melanogaster*	611	7e-62	X
NADP-dependent leukotriene B4 12-hydroxy-dehydrogenase (15-oxoprostaglandin 13-reductase)	*Tribolium castaneum*	923	6e-37	Not known
Nuclear autoantigenic sperm protein	*Homo sapiens*	639	5e-25	1
X-linked eukaryotic translation initiation factor isoform 3	*Tribolium castaneum*	797	4e-93	X
CG1681-PA	*Drosophila melanogaster*	452	2e-09	X
Adenine nucleotide translocator 2	*Homo sapiens*	554	1e-93	X
Chromobox protein	*Gallus gallus*	745	2e-46	W
Small androgen receptor-interacting protein isoform 1	*Canis familiaris*	578	3e-30	1
Vitellogenin	*Fenneropenaeus merguiensis*	801	6e-97	Not known
Ovarian lipoprotein receptor	*Penaeus semisulcatus*	923	4e-94	Not known
Y-box protein Ct-p0	*Chironomus tentans*	846	3e-10	Not known
Polehole	*Drosophila melanogaster*	725	4e-09	1
Zonadhesin precursor	*Homo sapiens*	653	1e-10	7

*GenBank accession number EE332433-EE332457. **Localization in chromosomes of matched species

aa) and *thioredoxin peroxidase* (591 bp, 196 aa), homologues were also discovered (GenBank accession numbers EE332458-EE332467).

The *chromobox protein* (*CBX*) is composing of *CBX5* (or *heterochromatic 1α*; *HP1α*), *CBX1* (*HP1β*) and *CBX3* (*HP1γ*) which is an evolutionarily conserved family of proteins involved in the packaging of chromosomal domains into representative heterochromatic states (Jones *et al*., 2001) and W-linked in chicken (Yamaguchi *et al*., 1998). In the present study, a homologue of *CBX* (ORF = 567 bp, 188 aa) was identified in *P. monodon*. The chromatin organization modifier (CHROMO, positions 21-73; Fig. 3) domain and the chromo shadow domain (ChSh, positions 109-171; Fig. 3) were found in this putative non-secretory protein. Phylogenetic analysis suggested that *CBX5* (*HP1α*), *CBX1* (*HP1β*) and *CBX3* (*HP1γ*) in vertebrates were born from the gene duplication process (Fig. 4) and *PMCBX* is regarded as a new member of invertebrate *CBX*-related proteins.

Recent advances in the genetic mapping of *M. japonicus* (Li *et al*., 2003) and sex ratio alteration in *F. chinensis* (Li *et al*., 2003) and *M. japonicus* (Preston *et al*., 2004) implied possible female heterogamy (ZW) in penaeid shrimp. Accordingly, identification and characterization of SNP in sex-related (W-linked for *CBX* and X-linked for others; Table 1) gene homologues of the originally matched species of *P. monodon* ESTs may provide the possibility to further develop genomic sex determination markers for which no DNA markers are available in any penaeid species at present.

In addition, homologues of 25 different sex-related genes were also found from EST analysis (Table 1) and 10 of which were homologous to genes of *Drosophila* and *Anopheles*. Homologues of *female sterile* and *ovarian lipoprotein receptor* were specifically expressed in ovaries but not testes of *P. monodon* broodstock ($N = 5$ for each sex, Table 2 and Fig. 5). Both *female sterile* and its related protein, polehole are required for the activation of the Tor receptor which are important for eggshell integrity and embryonic development (Perrimon *et al*., 1986).

The fundamental controls of growth in penaeid shrimp are largely unstudied. Several genes encoding vertebrate-like growth factors and cell cycle regulating proteins (*cyclin*, *cyclin dependent kinase*, *cell division cycle 2* and *epithelial*

Table 2. Primer sequence and expression patterns of sex-related transcripts and the positive control (*elongation factor-1α* and *β-actin*) of *P. monodon*

Transcripts	Primer sequence	Expected size	Expression pattern*
Thioredoxin peroxidase	F: 5'-CGAAGTGGTTGCTTGCTCTA-3'	233	HO
	R: 5'-CTGGCAGGTCATTGATTGTT-3'		
Phosphatidylinositol 4 kinase	F: 5'-CAACGCCATCAACTCCATCAC-3'	335	HO
	R: 5'-CTTCCAGCACCACACAGTTTTAT-3'		
Rab-protein 10 CG17060-PA	F: 5'-CTATTACAGAGGGGCAATGGGC-3'	177	HO
	R: 5'-TTTTCTTTGGCAATGACACGCT-3'		
Ubiquitin specific protease 9	F: 5'-GGAAATGGACCTGGGCGG-3'	247	HO
	R: 5'-TCTTCTGGAACTGCTACCTCTGC-3'		
Female sterile	F: 5'-GCAATAACGGTGAACAAGGGA-3'	296	O
	R: 5'-GCAACCACATTAGTAGCCATA-3'		
Protein disulfide isomerase-related P5 precursor	F: 5'-GCCGTTGCCAATAAGGACGA-3'	180	OT
	R: 5'-TCACCCGCCTTGAGATTGGT-3'		
agCP13148	F: 5'-CATACCTCGCATCATCAGTG-3'	212	HO
	R: 5'-CCTCAGGAGACGATACAAAGC-3'		
Nuclear autoantigenic sperm protein	F: 5'-AGGAAATGGAAACTGATGTCGC-3'	301	O
	R: 5'-TTCTTAGCCATCTCTGGGTTGT-3'		
Adenine nucleotide translocator 2	F: 5'-GTCCGCATCCCAAAGGAACGAG-3'	239	OT
	R: 5'-CGAGCGAAGTCAAGGGGGTAGA-3'		
Chromobox protein	F: 5'-TGGGAACCTGAATCTCATCTTG-3'	303	HO
	R: 5'-ACGAACATTTGCCTGCCTTG-3'		
Small androgen receptor interacting protein	F: 5'-GGCTTAGTGACTGAACGCCTCTA-3'	155	HO
	R: 5'-GCTGCTCTACTACGCACAACAC-3'		
Ovarian lipoprotein receptor	F: 5'-CGGGATGAGTGCGAGAAGTGC-3'	354	O
	R: 5'-CAGGGGCTCCGAGTCAAAGA-3'		
Y-box protein Ct-p0	F: 5'-CGGAGACACAAGCCAAGCCT-3'	435	HO
	R: 5'-GGTGGAACCCAACCAGCAAC-3'		
Zonadhesin precursor	F: 5'-CCTGGGCGTAGCTAATCTTAAC-3'	177	HO
	R: 5'-TCGGTAGGGCCATATCCTCTCC-3'		
elongation factor-1α	F: 5'-ATGGTTGTCAACTTTGCCCC-3'	500	Control
	R: 5'-TTGACCTCCTTGATCACACC-3'		
β-actin	F: 5'-GGTATCCTCACCCTCAAGTA-3'	327	Control
	R: 5'-AAGAGCGAAACCTTCATAGA-3'		

*HO = higher expression in ovaries than testes ($P < 0.05$), OT = expression levels in ovaries and testes were not different ($P > 0.05$), O = specific expression in ovaries of broodstock-sized *P. monodon*.

growth factor 1) were identified. Additionally, *nuclear autoantigenic sperm protein* (*NASP*) which was first described in rabbit and designated a homologue to the *Xenopus* oocyte histone binding protein N1/N2 (Welch and O'Rand 1990) were also discovered. *NASP* is found in all dividing cells as either a somatic/embryonic (*sNASP*) or a testis/embryonic (*tNASP*) isoforms (Richardson *et al*., 2000). Overexpression of *tNASP* affects progression through the cell cycle. In mice, *HSP90* acts as a *tNASP*-binding partner (Alekseev *et al*., 2005). This transcript showed significant higher expression in ovaries than testes of broodstock-sized *P. monodon* ($N = 6$ for each sex, $P < 0.05$; Fig. 5).

A homologue of *protein disulfide isomerase* (*PDI*, also called *thyroid hormone binding protein*, *THBP*; Lee *et al*., 1996; Prim and Gilbert, 2001) and *adenine nucleotide translocator 2* did not show different expression levels between ovaries and testes of *P. monodon* broodstock ($P > 0.05$). However, several other transcripts (e.g. *CBX*, *phosphatidylinositol 4 kinase*, *thioredoxin peroxidase* and *USP9X*; $P < 0.05$; Table 2) illustrated the preferential expression in ovaries of *P. monodon*.

CBX was preferentially expressed in ovaries than testes of both juvenile ($N = 10$) and broodstock-sized *P. monodon* ($N = 12$, Fig. 5). Significant different expression levels of *ubiquitin specific protease* 9, *X chromosome* homologue (*Usp9X*, 247 bp, Fig. 6) were observed in broodstock-sized *P. monodon* ($N = 13$ and 11 of females and males, $P < 0.05$). Nevertheless, *Usp9X* was only expressed in ovaries but not testes of 4-

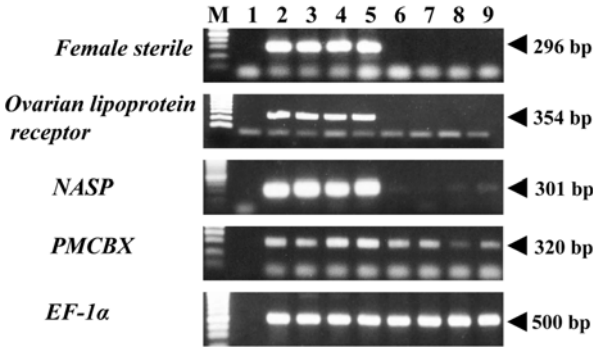

Fig. 5. RT-PCR of a homologue of *female sterile*, *ovarian lipoprotein receptor*, *NASP* and *chromobox protein*, *PMCBX* using the first strand cDNA of ovaries (lanes 2-5) and testtes (lanes 6-9) of broodstock-sized *P. monodon*. *EF-1α* was included as the positive control. Lanes 1 are the negative control (without cDNA template).

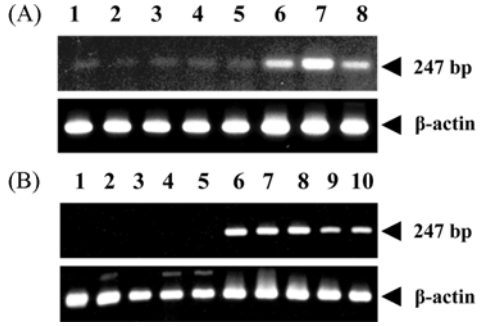

Fig. 6. RT-PCR of a homologue of *Usp9X* using the first strand cDNA of testes (lanes 1-5; panels A and B) and ovaries (lanes 6-8; A and lanes 6-10; B) of broodstock-sized (A) and 4-month-old *P. monodon* (B). Positive control (*β-actin*) was also successfully amplified.

month-old *P. monodon* ($N = 5$ for each sex; Fig. 6).

Usp9X is an X-linked orthologue of Fat facets (*Faf* in *Drosophila* and *Fam* in mice), an ubiquitin-specific protease which is essential for normal development of oocytes in *Drosophila*. Mutations of *Usp9X* lead to abnormal content and the inability of the fertilized eggs to undergo normal embryogenesis. In human, *USP9X* is one of the possible candidate genes responded for the defects of oocyte proliferation and subsequent gonadal degeneration found in Turner syndrome (Jones *et al*., 1996). It contributes in the premature ovarian failure syndrome in POF patients (Fassnacht *et al*., 2006). Moreover, in mice testes, no expression was found in any spermatogenic cells except for weak expression in Sertoli cells. *Usp9X* expression in embryogenic oocytes was reduced at the newborn stage but its expression reappeared in oocytes at the secondary follicle stage (Noma *et al*., 2002).

Yamano *et al*. (2004) illustrated that in most cases ovaries of *M. japonicus* start to develop in the reproductive season but fail to reach full grown requisite for the formation of cortical rods (CRs). Ovaries degenerate without spawning. This is also the major constraint in *P. monodon*. Reduced spawning potential and low degree of maturation of *P. monodon* in captivity crucially prohibits the efficiency of the genetic improvement through domestication and selective breeding programs in this species (B. Withyachumnarnkul, personal communication).

Recently, *cyclin B*, an important regulatory factor in mitosis and meiosis, was recently isolated in *M. japonicus*. Three *cyclin B* transcripts (2.4, 1.9 and 1.7 kb) which share an identical ORF of 1203 bp encoding a putative peptide with different length of 3 UTR, coexisted in ovaries. Quantitative realtime RT-PCR revealed that the short transcript (1.7 kb) was the most abundance, followed by the long (2.4 kb) and the medium (1.9 kb), and the three forms of these transcripts displayed different expression profiles during oogenesis (Qiu and Yamano, 2005). Additionally, differential expression of *cathepsin C* (*dipeptidyl peptidase*) during the final stages of oocyte maturation of *M. japonicus* was also reported (Qiu *et al*., 2005). This suggested that various transcripts possess multifunctions and might perform different roles during oogenesis of *M. japonicus*.

In the present study, a large number of cDNA including sex-related transcripts in ovaries of *P. monodon* were identified. The expression profiles of genes specifically expressed or those preferentially expressed in ovaries (*female sterile*, *ovarian lipoprotein receptor*, *TSP*, *NASP*, and *Usp9X* etc.) of *P. monodon* illustrated in the present study implied that these genes may have contributed in ovarian development in *P. monodon*. Molecular mechanisms and expression patterns of genes controlling each step of oocyte maturation and formation of CRs should be further carried out for better understanding the reproductive maturation of *P. monodon* in captivity.

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