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*Repro*Array^{GTS}: A cDNA microarray for identification of reproduction-related genes in the giant tiger shrimp *Penaeus monodon* and characterization of a novel *nuclear autoantigenic sperm protein* (*NASP*) gene

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ABSTRACT

Expressed sequence tags (ESTs) were established from various tissues of the giant tiger shrimp (*Penaeus monodon*). To simultaneously examine expression patterns of a large number of transcripts in ovaries and testes of *P. monodon*, a cDNA microarray (*Repro*Array^{GTS}) containing 4992 features amplified from cDNAs of ovary (1920) and testis (3072) EST libraries was constructed and subjected to high-throughput gene expression analysis in four different stages of ovarian development (previtellogenesis, vitellogenesis, early cortical rod and late cortical rod stages). Several transcripts were found to be differentially expressed during *P. monodon* ovarian development. Among many important reproduction-related genes with differential expression from microarray data, *nuclear autoantigenic sperm protein* (*NASP*) was further characterized by RACE-PCR. The full-length cDNA of *P. monodon NASP* (*PmNASP*) was 2126 bp in length containing an open reading frame (ORF) of 1812 bp corresponding to a deduced protein of 603 amino acids with 5′ and 3′UTRs of 93 and 202 bp (excluding the poly A tail), respectively. Higher *PmNASP* transcript levels at later stages of ovarian development was consistently confirmed by quantitative real-time PCR. This study indicated that *Repro*Array^{GTS} is effective for high-throughput screening of genes that play important roles in ovarian development of *P. monodon*.

1. Introduction

The giant tiger shrimp (*Penaeus monodon*) is one of the most economically important marine species for aquaculture. In Thailand, an annual production of cultured *P. monodon* during the nineties was at least 200,000 metric tons, generating an income of over 2 billion USD (Rosenberry, 2000). However, due to poor reproductive maturation of closed thelycum species, unilateral eyestalk ablation is used in practice to induce ovarian development and ovulation of *P. monodon*. Although the technique gives predictable peaks of maturation and spawning, an eventual loss in egg quality and high mortality are undesirable consequences (Benzie, 1998). Therefore, an ultimate goal for the shrimp industry is to be able to predict maturation and spawning without the use of eyestalk ablation in captive shrimp (Quackenbush, 1992).

Selective breeding programs of *P. monodon* will undoubtedly be the key to generate shrimp with commercially desired traits such as high growth rate and ability to induce high quality eggs in domesticated females without the irreversible side-effects of eyestalk ablation (Lyons and Li, 2002). However, breeding of pond-reared *P. monodon* is rather difficult and rarely produces the amount of high quality larvae desired by the industry (Leelatanawit et al., 2004). These problems impede the domestication and a selective breeding program for genetic improvement of this species (Preechaphol et al., 2007). The ability to use selectively bred stocks with improved culture performance is a major determinant of sustainability of the shrimp industry (Clifford and Preston, 2006). Currently, an effort to domesticate *P. monodon* is being carried out in Thailand for production of high quality pond-reared *P. monodon* broodstock (B. Withyachumnarnkul, personal communication).

Gonad development and maturation require precise coordination of gene expression (von Schalburg et al., 2008). Currently, basic knowledge on shrimp reproduction is still limited and not well

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understood. It is therefore important to obtain fundamental knowledge on ovarian and/or testicular development of shrimp in order to improve reproductive maturation in captivity of closed thelycum species like *P. monodon*.

Recently, many studies successfully employed an EST approach (single-pass sequencing of randomly selected clones from cDNA libraries) to discover important genes in *P. monodon* (Lehnert et al., 1999; Supungul et al., 2002, 2004; Tassanakajon et al., 2006). For instance, Tassanakajon et al. (2006) reported a total of 10,100 clones sequenced from the 5′ end resulting in 4845 unique sequences (917 contigs and 3928 singletons). Approximately half of investigated ESTs displayed significant homology to known genes in the GenBank.

Additionally, both conventional and suppression subtractive hybridization (SSH) cDNA libraries of ovaries and testes of *P. monodon* were established (Leelatanawit et al., 2004; Preechaphol et al., 2007; Leelatanawit et al., in press). Gene homologues related to reproduction and gonad development (e.g. *progesterone receptor*, *progestin membrane receptor component 1*; *PGMRC1*, *ovarian lipoprotein receptor*, *polehole* and *nuclear autoantigenic sperm protein*) were identified. Moreover, expression levels of 15 ovary and 50 testis gene homologues during reproductive maturation stages of *P. monodon* were examined by semiquantitative RT-PCR (Preechaphol et al., 2007) or quantitative real-time PCR (Leelatanawit et al., in press). Sex-specific and sex-differential expression markers were also reported. Nevertheless, the gene-by-gene screening approach is tedious and time-consuming, particularly when a large number of genes are of interest.

The use of microarrays to simultaneously measure expression levels of a large number of genes can expedite gene discovery process. Recently, Pongsomboon et al. (2008) identified yellow head virus (YHV) responsive genes in hemocytes of *P. monodon* by using a cDNA microarray composed of 2,028 different ESTs from *P. monodon* and *Marsupenaeus japonicus*. A total of 105 differentially expressed genes were identified and grouped into five different expression clusters. One of the clusters which included *cathepsin L-like*, *cysteine peptidase*, *hypothetical proteins* and unknown genes exhibited a rapid increase in expression levels within 15 min of YHV injection. Microarray-based data were further validated by quantitative real-time PCR analyses of selected differentially expressed transcripts.

Although microarrays for screening immune-related genes in *P. monodon* were available, no arrays specific to gonad development and maturation existed for this species. In the present study, the first in-house version of a cDNA microarray (*Repro*Array^{GTS}) to identify reproduction-related genes in *P. monodon* was constructed from our previously established ovary and testis EST libraries (Leelatanawit et al., 2004; Preechaphol et al., 2007; Leelatanawit et al., in press). *Repro*Array^{GTS} was used to screen differentially expressed transcripts during ovarian development of *P. monodon*. One of the more highly expressed transcripts, a homologue of *nuclear autoantigenic sperm protein* (*NASP*), was further validated and characterized. The full-length cDNA of *PmNASP* was successfully determined by RACE-PCR. Expression patterns of this transcript were confirmed by quantitative real-time PCR.

2. Materials and methods

2.1. EST libraries

Conventional and SSH cDNA libraries were established from ovaries and testes of *P. monodon* broodstock (Leelatanawit et al., 2004; Preechaphol et al., 2007; Leelatanawit et al., in press). Recombinant clones were randomly selected and unidirectionally sequenced. Nucleotide sequences were analyzed using ESTplus, an integrative system for comprehensive and customized EST analysis and proteomic data matching (Pacharawongsakda et al., 2008). An *E-value*<1e-5 was taken to indicate a significant probability of homology for both nucleotides and amino acids. The sequences were grouped in different gene ontology categories according to Ashburner et al. (2000).

2.2. Amplification of cDNAs and microarray construction

Inserts from the testis library were colony PCR-amplified using overnight cultures of recombinant clones as templates whereas those from the ovary libraries were amplified using recombinant plasmid (approximately 10 ng) as templates. PCR was carried out in a 96-well plate using a GeneAmp PCR 9700 thermocycler (ABI). The reaction mixture contained 0.2 mM dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM each of LM13-F (5′-CAC GAC GTT GTA AAA CGA CGG CCA G-3′) and LM13R (5′-CAT GGT CAT AGC TGT TTC CTG T-3′), and 4.5 U *Taq* polymerase (Center for Molecular Genetics, Russia). PCR reactions were carried out by pre-denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 25 s, 60 °C for 25 s and 72 °C for 3 min, and a final extension of 72 °C for 7 min. Five microliters of the amplicons were electrophoretically analyzed in a 1% agarose gel prepared in 1× TAE buffer. The PCR products were purified, air-dried and resuspended with 8 μL 3×SSC giving approximately 250–500 ng/μL. The plates were covered tightly and left at 4 °C overnight. The resuspended products were transferred to 384-well plates and left at 4 °C one day prior to microarray fabrication.

2.3. Microarray fabrication

PCR products of 4992 ESTs (1920 and 3072 amplified products from the ovary and testis libraries, respectively) were spotted onto aminosilane UltraGAPS slides (Corning) using an automatic spotter, *SpotArray 72* (PerkinElmer) equipped with 16 SMP3 pins (4×4 configuration, TeleChem International). Each spot printed by these pins contained about 0.7–1.0 nL with an average spot diameter of 125 μm. Each block had 15 rows and 21 columns. The fabricated slides were stored in a humidity-controlled cabinet until used.

2.4. Post-processing and hybridization test

The arrays were post-processed according to the protocol recommended for UltraGAPS slides (Corning). Briefly, the slide was rehydrated by water steam for 3 s and snap-dried on a 70 °C heat block. The arrays were immobilized using a UV cross-linker before blocking the unused surfaces. The arrays were immersed in the blocking solution (5×SSC, 0.1% SDS, and 0.1 mg/mL BSA) at 42 °C for 45–60 min before washing twice with 0.1×SSC at room temperature and final rinsing with MilliQ-water for 30 s. The arrays were centrifuged at 600 *g* for 2 min at room temperature. Quality and spot morphology of arrays from different locations of the same and different print-runs were tested using an AlexaFluor® 546-labled DNA probe amplified from pBluescript using LM13F and LM13R primers (Invitrogen, Carlsbad, CA, USA). The probe was resuspended in hybridization solution (50% formamide; 5×SSC; 0.1% SDS; 1 mg/ml human placental DNA; 10 mM Tris, pH 7.5). Post-processed slides were hybridized at 42 °C for 16 h and post-hybridization washed according to the recommended protocol described by Corning before scanning.

2.5. Microarray experiments for large scale identification of differentially expressed transcripts in ovaries of P. monodon

RNA samples were extracted from wild *P. monodon* broodstock ovaries using TRI-REAGENT according to manufacturer's instruction (Molecular Research Center, USA). According to gonadosomatic indices (GSI: ovarian weight/body weight×100) and conventional histology, total RNA samples from ovaries with GSI values of 1.46, 4.62, 6.23 and 12.55% were extracted to represent Stages I–IV of shrimp ovarian development (previtellogenesis (I), vitellogenesis (II), cortical rod (III), and late cortical rod (IV), respectively). Contaminating genomic DNA was removed by treatment with DNase I at 0.15 U/μg total RNA at 37 °C for 30 min. The quality and

quantity of the RNA was assessed on gel electrophoresis and NanoDrop (ND-8000). The first-strand cDNA was synthesized and labeled with aminoallyl-dUTP (aa-dUTP; Sigma) using a LabelStar Array kit (Qiagen). The resulting cDNA samples were cleaned up using a Microcon YM-50 filter (Millipore) and resuspended in 6 L of 0.1 M sodium borate buffer (pH 8.7). The aa-dUTP cDNA was fluorescently labeled with Cy3- or Cy5- dyes (GE Healthcare) at room temperature for 1 h. The unincorporated dye was removed using a Microcon YM-50 filter and the purified probe was resuspended in 6 μL TE. The Cy3- and Cy5- samples were mixed together for each experiment and hybridized onto the arrays as described earlier. In this study, RNA sample from Stage I was labeled with Cy3-dye (green) and used as a reference whereas RNA samples from other stages were labeled with Cy5-dye (red). Two technical replicates were conducted for all sample comparisons.

2.6. Microarray imaging and data analysis

The hybridized slides were scanned with either a ScanArray Express (PerkinElmer, Waltham, MA) or GenePix 4000B (Molecular Devices, Sunnyvale, CA, USA). Microarray spots on scanned images were located using GenePix Pro version 6.1. All "empty" (no PCR product) and "bad" (flagged by visual inspection of the spot images) spots were removed from the analysis. Only the spots with intensities greater than one standard deviation above the background intensity were further analyzed. The processed data were normalized within each array by the scaled print-tip (Lowess) method, and across arrays, using the Aroma package (Bengtsson, 2004; available from: http://www.maths.lth.se/help/R/aroma/) run in the R project environment (http://cran.r-project.org). The microarray data have been deposited in NCBI's Gene Expression Omnibus with GEO accession number GSE12125 at http://www.ncbi.nlm.nih.gov/geo/ (Edgar et al., 2002). The average logarithmic base 2 values of relative intensities between Cy3- and Cy5- samples (*M* values) were subjected to hierarchical clustering analysis and illustrated using the Treeview software (Eisen et al., 1998).

2.7. Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR)

A gene specific primer for 5′ RACE-PCR of *P. monodon NASP* was designed (Table 1). RACE-PCR of *NASP* was carried out using a BD SMART RACE cDNA Amplification kit following the protocol recommended by the manufacturer (BD Clontech). The amplified fragment was electrophoretically analyzed, eluted from an agarose gel before cloning into pGEM-T Easy vector and sequenced (Sambrook and Russell, 2001). The full-length cDNA was assembled and hereafter called *PmNASP*. The protein domain of deduced amino acids of PmNASP was analyzed using SMART (http://smart.embl-heidelberg.de/). The p*I* and molecular weight of the deduced protein were estimated using ProtParam (http://www.expasy.org/tools/protparam.html).

Table 1
Nucleotide sequences of primers used for the isolation, characterization and expression analysis of *NASP* in *P. monodon*.

Primer	Sequence
5′RACE-PCR	R: 5′-CAG CAA GGA CAG ACT CCA GAA AGC GGC-3′
Primer walking	
Internal primer-I	F: 5′-AGG AAA TGG AAA CTG ATG TCG C-3′
RT-PCR	
NASP-RT_{301}	F: 5′-AGG AAA TGG AAA CTG ATG TCG C-3′
	R: 5′-TTC TTA GCC ATC TCT GGG TTG T-3′
EF1-α-RT_{500}	F: 5′-ATG GTT GTC AAC TTT GCC CC-3′
	R: 5′-TTG ACC TCC TTG ATC ACA CC-3′
Real-time PCR	
$NASP_{186}$	F-5′-GCC GTC CAA GAA AGA GAT TGA TAC-3′
	R: 5′-CGG CCA TAG TAG AAA TAA GCA TCA C-3′
EF1-α_{214}	F: 5′-TCC GTC TTC CCC TTC AGG ACG TC-3′
	R: 5′-CTT TAC AGA CAC GTT CTT CAC GTT-3′

2.8. Real-time quantitative PCR and tissue distribution analysis of PmNASP

Ovaries were dissected out from female juveniles (4-month old, $N=4$) and broodstock ($N=19$) and weighed. According to GSI and conventional histology, each adult shrimp was classified into one of the four ovarian developmental stages ($N=5$, 6, 3 and 5 for Stages I–IV, respectively). The first-strand cDNAs were synthesized from 1 μg DNase I-treated total RNA of each shrimp using the QuantiTech Reverse® Transcription kit (Qiagen). Expression levels of the target ($PmNASP_{186}$) and control ($EF\text{-}1\alpha_{214}$) transcripts during the ovarian development of *P. monodon* were examined. For each amplification, 50 ng of cDNA were used as template in a 20 L reaction containing 10 L of 2× QuantiTech™ SYBR Green PCR (Qiagen) and 0.3 μM each primer (Table 1). The thermal profile was 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min. Each sample was run in duplicate using the Mx3000 QPCR system (Stratagene). Relative expression levels of *PmNASP* transcript (copy number of *PmNASP*/copy number of *EF-1α*) in all experimental groups were statistically analyzed using one way analysis of variance (ANOVA) followed by Duncan's new multiple range test. Differences were considered significant if the *P* values were <0.05.

For tissue distribution analysis, end point RT-PCR (30 cycles) was performed against the first-strand cDNA from various tissues of a female and from testes of a male broodstock of *P. monodon* using the conditions described in Preechaphol et al. (2007).

3. Results

3.1. Features of ReproArray^{GTS}

A total of 1920 ESTs from ovary cDNA libraries and 3072 ESTs from a testis cDNA library were chosen for construction of *Repro*Array^{GTS} (GE613296–GE616370). The majority of known ESTs (54 and 41%) were placed in the Gene Ontology functional category of "cellular components" after annotation. The remaining annotated ESTs were placed in the "molecular function" (19 and 22%) and "biological processes" (27 and 37%) categories (Fig. 1).

Microarray data sets were normalized using the scaled print-tip (Lowess) normalization for each slide according to Yang et al. (2002). The intensities before and after normalization using the Lowess method is illustrated in Fig. 2A. This normalization compensated for unequal efficiency of each print-tip (Fig. 2B). This feature allowed a more consistent intensity distribution of *Repro*Array^{GTS}. All slides in this study were further normalized together enabling unbiased comparisons (Fig. 2C).

To validate the quality of *Repro*Array^{GTS}, a vector probe amplified from the region flanked by M13F and M13R of the pBluescript vector was fluorescently labeled and hybridized onto three slides (two from different locations of the same print-run and one from a different print-run). Since this labeled probe contained common sequences for all spots on the arrays, spot morphology and signals can be analyzed. From all the slides tested, consistent spot morphology and strong signals were obtained (data not shown). The R^2 correlation values of >0.8 for the tested slides indicate consistency of printing (0.89 and 0.90 for a comparison of slides from the same print-run and those from different print-runs, respectively).

3.2. Differential expression patterns of genes in various reproductive maturation stages of P. monodon

Using the *Repro*Array^{GTS}, differentially expressed transcripts during the ovarian development of *P. monodon* were identified (Fig. 3).

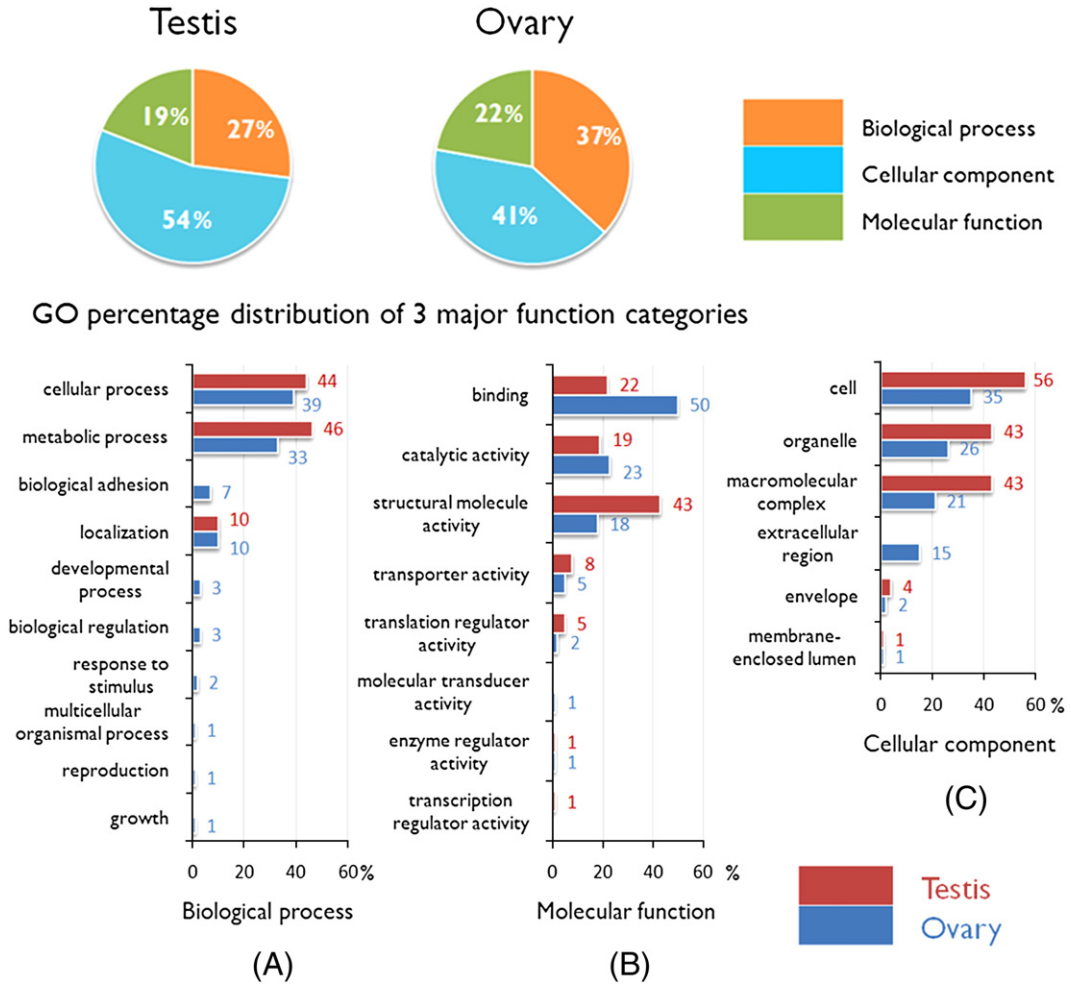


Fig. 1. Gene ontology annotation of gene features on *Repro*Array^{GTS}. Pie diagrams (upper panel: left, testis ESTs; right, ovary ESTs) show the percentage distribution of sequences among the three principal GO categories: biological processes (lower panel; A), molecular functions (B) and cellular components (C). The percentage of sequences in each category was calculated from the total number of sequences with GO assignment.

Several clusters of genes exhibiting similar expression profiles were observed (Fig. 3A). Relative to Stage I, genes in Clusters i–iii (e.g. *inhibitor of tyrosine kinase*, *neutralized protein*, *cyclin A* and *NADH-dependent leucotriene B4 12-hydroxydehydrogenase*; *LTB4DH*) had lower expression levels at particular stages during ovarian development. *Calreticulin* and *bystin*, members of Cluster iv, showed lower expression in Stages II–IV when compared to Stage I. In contrast, genes in Clusters v–viii had higher expression levels during ovarian development of *P. monodon*. Examples of these genes were *cyclin B, cell division cycle 25* (*Cdc25*), *Cdc16, mitogen-activated protein kinase binding protein 1* (*MAPKBP1*), *receptor for activated protein kinase C* (*RACK*), *cortical rod protein*, *polehole*, *hepatocarcinogenesis-related transcription factor* and *nuclear autoantigenic sperm protein (NASP)*. Of these, the expression levels of *NASP* analyzed by microarrays were positively correlated with the GSI values (Fig. 3B).

3.3. Sequence characterization of *P. monodon NASP (PmNASP)*

The full-length cDNA of *PmNASP* was 2126 bp in length with 5′ and 3′ UTRs of 93 and 202 bp (excluding the poly A tail), respectively. The ORF was 1812 bp corresponding to a polypeptide of 603 amino acids (Fig. 4; GenBank accession number FJ040859). The estimated p*I* of the deduced protein was 5.16 with a molecular weight of 67.04 kDa. The tetratrico peptide repeat (TPR) is found at positions 386–419 (*E*-value = 8.50e-02) of the deduced protein. *PmNASP* showed the highest similarity with that of the zebrafish (*Danio rerio*) with an *E*-value of 3e-78.

3.4. Expression levels of *PmNASP* confirmed by quantitative real-time PCR

The increased expression levels of the *PmNASP* mRNA during ovarian development of *P. monodon* revealed by microarrays were confirmed by real-time PCR. Results from both techniques were concordant (Fig. 5). The expression of *PmNASP* in ovaries of broodstock was greater than that of juveniles ($P<0.05$). The expression levels of *PmNASP* increased significantly in the late cortical rod stage ($P<0.05$). The transcript was preferentially expressed in ovaries relative to testes of both *P. monodon* juveniles and broodstock (Fig. 6). Tissue distribution analysis indicated that *PmNASP* was expressed abundantly in ovaries, weakly in testes and intestine, but was absent in other tissues (pleopods, stomach, hemocytes, hepatopancreas, gill, heart, lymphoid organs, thoracic ganglion, and eyestalk; Fig. 6).

4. Discussion

4.1. Identification and characterization of reproduction-related genes

The development and maturation of ovaries and oocytes require coordinated expression of specific sets of genes to ensure the proper development of oocytes and to store transcripts and proteins as maternal factors for early embryogenesis (Qiu and Yamano, 2005; von Schalburg et al., 2008). An initial step toward understanding molecular mechanisms of ovarian (and oocyte)

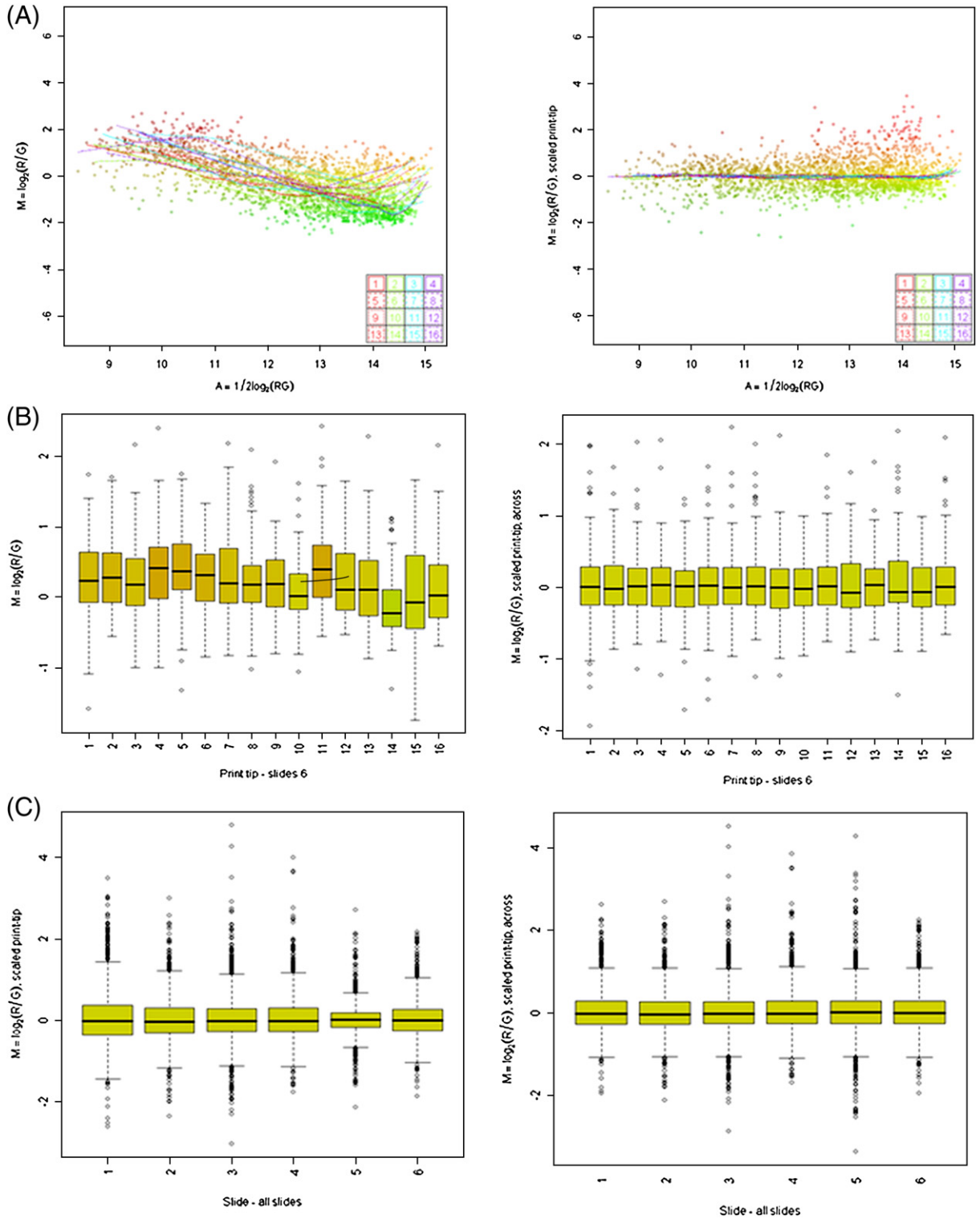
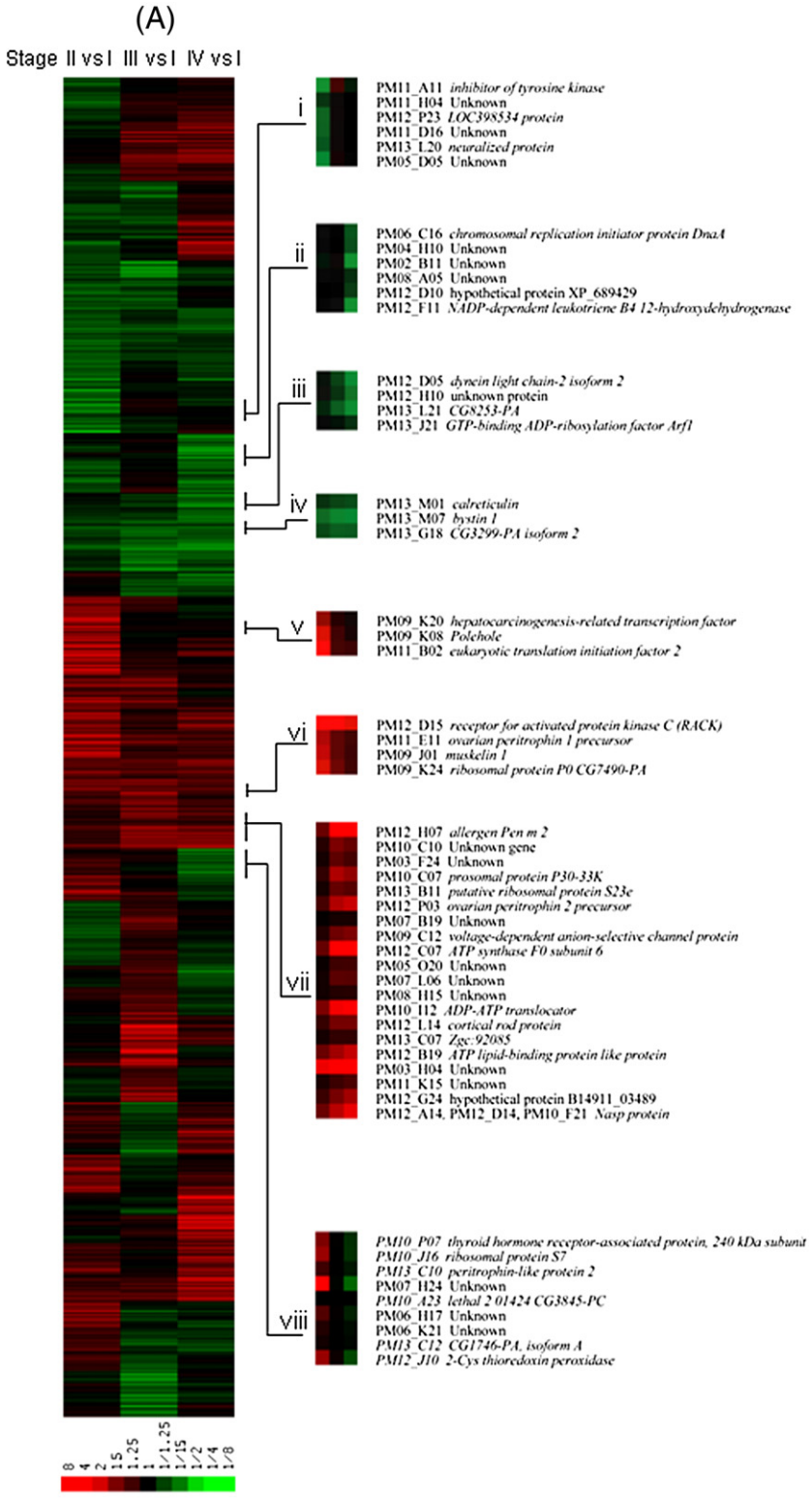


Fig. 2. Hybridization intensity before (upper panel: left) and after normalization (right). (A) MA plot represents log ratios of Cy5 to Cy3 intensities over average intensities. (B) Boxplots of 16 print-tips before and after normalization using the scaled print-tip (Lowess) method. (C) Boxplots of all the slides conducted in this study before and after normalization between slides.

maturation in *P. monodon* is to identify and characterize reproduction-related genes expressed in ovaries of this economically important species.

Previously, cDNA microarrays have been applied for large scale gene discovery and expression analysis in several aquatic organisms including oysters (Jenny et al., 2007) and salmonids (von Schalburg et al., 2005). To provide an insight into molecular aspects governing reproductive processes of *P. monodon*, the first reproduction cDNA microarray (*Repro*Array^{GTS}) was constructed to examine a large number of expression profiles of *P. monodon* reproduction-related genes.

Fig. 3. Microarray expression data of *P. monodon* transcripts during ovarian development. (A) Clustered gene expression patterns during the ovarian development of female broodstock. (B) The expression profile of the *PmNASP* transcript during ovarian developmental stages.


(A)
Stage II vs I III vs I IV vs I
i
PM11_A11 inhibitor of tyrosine kinase
PM11_H04 Unknown
PM12_P23 LOC398534 protein
PM11_D16 Unknown
PM13_L20 neuralized protein
PM05_D05 Unknown
ii
PM06_C16 chromosomal replication initiator protein DnaA
PM04_H10 Unknown
PM02_B11 Unknown
PM08_A05 Unknown
PM12_D10 hypothetical protein XP_689429
PM12_F11 NADP-dependent leukotriene B4 12-hydroxydehydrogenase
iii
PM12_D05 dynein light chain-2 isoform 2
PM12_H10 unknown protein
PM13_L21 CG8253-PA
PM13_J21 GTP-binding ADP-ribosylation factor Arf1
iv
PM13_M01 calreticulin
PM13_M07 bystin 1
PM13_G18 CG3299-PA isoform 2
v
PM09_K20 hepatocarcinogenesis-related transcription factor
PM09_K08 Polehole
PM11_B02 eukaryotic translation initiation factor 2
vi
PM12_D15 receptor for activated protein kinase C (RACK)
PM11_E11 ovarian peritrophin 1 precursor
PM09_J01 muskelin 1
PM09_K24 ribosomal protein P0 CG7490-PA
vii
PM12_H07 allergen Pen m 2
PM10_C10 Unknown gene
PM03_F24 Unknown
PM10_C07 prosomal protein P30-33K
PM13_B11 putative ribosomal protein S23e
PM12_P03 ovarian peritrophin 2 precursor
PM07_B19 Unknown
PM09_C12 voltage-dependent anion-selective channel protein
PM12_C07 ATP synthase F0 subunit 6
PM05_O20 Unknown
PM07_L06 Unknown
PM08_H15 Unknown
PM10_I12 ADP-ATP translocator
PM12_L14 cortical rod protein
PM13_C07 Zgc:92085
PM12_B19 ATP lipid-binding protein like protein
PM03_H04 Unknown
PM11_K15 Unknown
PM12_G24 hypothetical protein B14911_03489
PM12_A14, PM12_D14, PM10_F21 Nasp protein
viii
PM10_P07 thyroid hormone receptor-associated protein, 240 kDa subunit
PM10_J16 ribosomal protein S7
PM13_C10 peritrophin-like protein 2
PM07_H24 Unknown
PM10_A23 lethal 2 01424 CG3845-PC
PM06_H17 Unknown
PM06_K21 Unknown
PM13_C12 CG1746-PA, isoform A
PM12_J10 2-Cys thioredoxin peroxidase
8
4
2.5
1.25
1
1/1.25
1/1.5
1/2
1/4
1/8
(B)
Expression level of NASP transcript
fold change
2.50
2.00
1.50
1.00
0.50
0.00
Stage
II vs I
III vs I
IV vs I

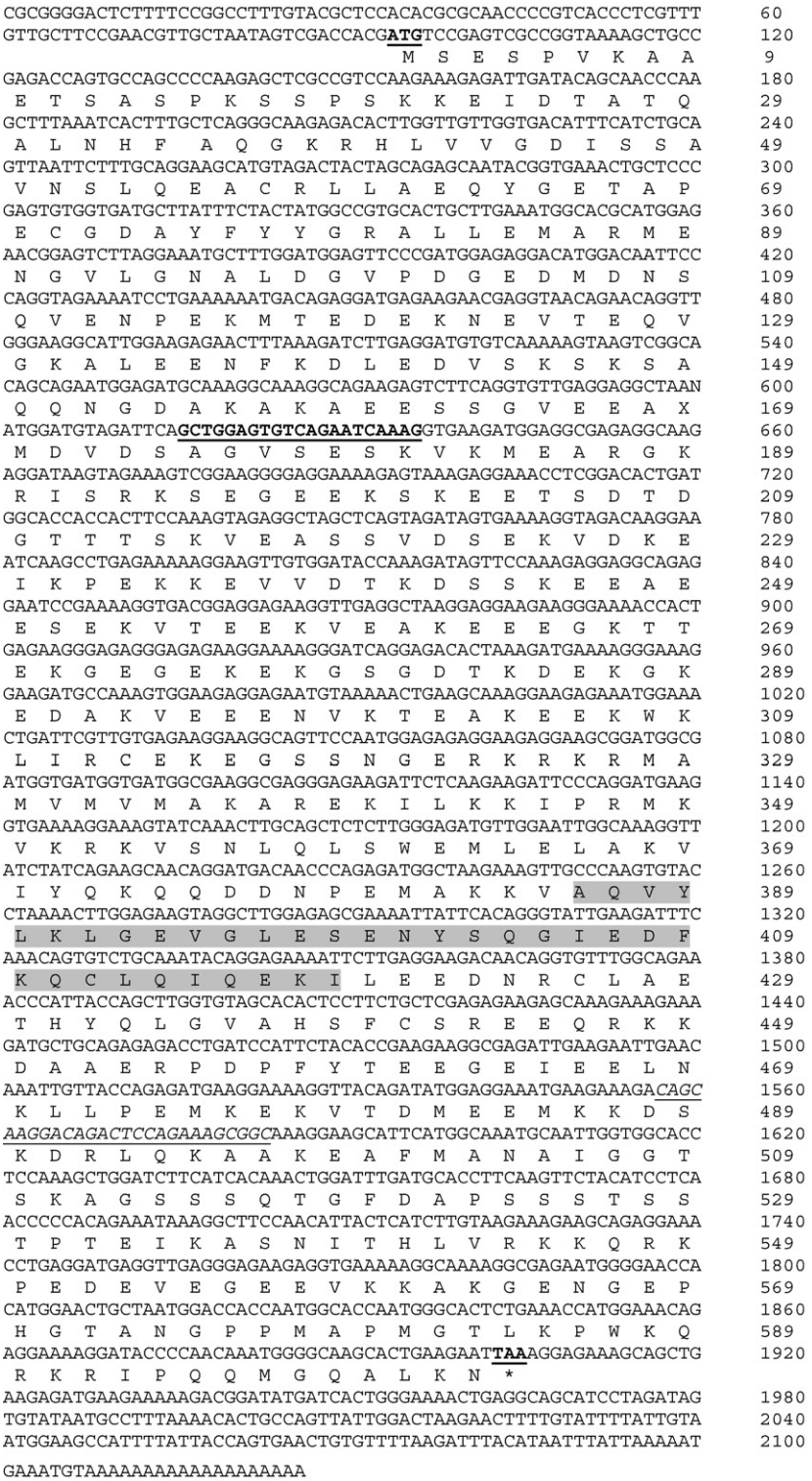


Fig. 4. The full-length cDNA sequence of *PmNASP*. Start and stop codons are illustrated in boldface and underlined. The 5′RACE-PCR primer is underlined and italicized whereas the internal sequencing primer is boldfaced and underlined. The tetratrico peptide repeat (TPR, positions 386–419, *E*-value = 8.50e-02) domains of the deduced PmNASP protein are highlighted.

In penaeid shrimps, ovarian development is characterized by the accumulation of a major yolk protein (vitellogenin and its derived protein, vitellin) and the formation of cortical rods in the oocytes (Meusy and Payen, 1988). In *M. japonicus*, several genes including *thrombospondin* (*TSP*), *peritrophin* (also called *cortical rod protein*, *CRP* and *shrimp ovarian peritrophin*, *SOP*), and *cathepsin C* (*dipeptidyl peptidase*) exhibited differential expression patterns during the final stages of oocyte maturation (Qiu et al., 2005; Qiu and Yamano, 2005). In addition, *peritrophin* was also abundantly expressed during oogenesis of *P. semisulcatus* (Khayat et al., 2001).

Differentially expressed transcripts were functionally implicated in the maturation and development of *P. monodon* ovaries (and oocytes) were identified though microarray analysis. Higher expression levels of *TSP* and *peritrophin* during *P. monodon* ovarian development

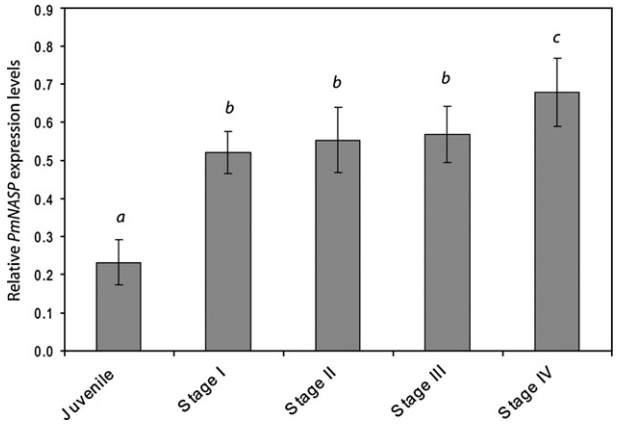


Fig. 5. Relative expression levels of the *PmNASP* transcript during ovarian development in juvenile and broodstock (Stages I–IV) *P. monodon* determined by quantitative real-time PCR. *Elongation factor* (*EF-1α*) was included as the reference transcript.

determined by microarray analysis were concordant with those previously reported in *M. japonicus* examined by real-time quantitative RT-PCR (Yamano et al., 2004). Moreover, relative to previtellogenic stage, *cyclin B* and *MAPKBP1* were expressed higher from vitellogenesis until late cortical rod stages whereas *Cdc25* mRNA expression was higher at the vitellogenesis stage but lower thereafter. Cyclin B is a regulatory protein that plays an important role in eukaryotic cell division cycles (Chapman and Wolgemuth, 1993). Signal transduction pathways that activate the cyclinB/Cdc2 complex (also called "maturation-promoting factor," MPF) and related processes (e.g. the MAPK pathway) are crucial for oocyte maturation in all animals studied so far (Kishimoto, 2003; Qiu and Yamano, 2005). Basically, the maturation process of oocytes in various organisms is regulated by relative levels of Cdc25 and Myt1 proteins through activation of MPF. The dominance of Cdc25 over Myt1 proteins initiates the activation of MPF leading to oocyte maturation (Kishimoto, 2003). Quantitative real-time PCR illustrated that *cyclins B* expression was higher during the final stage of ovarian development in *P. monodon* broodstock (S. Klinbunga, unpublished data) but not in *M. japonicus* ($P>0.05$, Qiu and Yamano, 2005).

Several differentially expressed transcripts were found during the ovarian development in *P. monodon* such as *LTB4DH, cyclin A, thyroid hormone receptor-associated protein* (*240 kDa*) and *thioredoxin peroxidase*. LTB4DH is a key enzyme responsible for biological inactivation of prostaglandins and related eicosanoids (Tai et al., 2002). Katsu et al. (1995) has suggested that cyclin A was involved in cell cycle only after the completion of meiosis I. However, shrimp oocytes are ovulated at meiosis I (Yano, 1995). Therefore, a role of cyclin A and other proteins with lower expression during meiotic maturation of shrimp oocytes should be further examined at both transcriptional and translational levels.

In this study, increased levels of the *PmNASP* transcript during development were consistently confirmed by real-time PCR analysis ($P<0.05$) and semiquantitative RT-PCR ($N=6$, 3, 8 and 13 for previtellogenic, vitellogenic, early cortical rod and mature ovaries; data not shown). Expression profiles and tissue distribution analysis suggested that *PmNASP* should play an important role at the final stage of ovarian development of *P. monodon*.

The full-length cDNA of *PmNASP* (ORF of 1812 bp, 603 amino acids) was successfully isolated by RACE-PCR and reported for the first time in crustaceans. The deduced PmNASP protein contains a TPR domain, which functionally mediates protein-protein interactions and assembly of multiprotein complexes. Proteins containing TPRs are involved in a variety of biological processes, such as cell cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport, neurogenesis and protein folding (D'Andrea and Regan, 2003). Examples of important TPR-containing proteins are the anaphase promoting complex (APC) subunits, Cdc16, Cdc23, Cdc27 and HSP90-binding immunophilins (inactive progesterone receptor) (Das et al., 1998).

NASP was found in two major forms: somatic (*sNASP*) or testis (*tNASP*) (Richardson et al., 2000). The *sNASP* was found all dividing somatic cells in mouse and HeLa cells whereas *tNASP* was found in gametes, embryonic cells and transformed cells (Alekseev et al., 2003). Overexpression of *tNASP* affects progression through the cell cycle. In the nematode *Caenorhabditis elegans*, NASP forms a protein complex with a zinc finger transcription factor, TRA-4, and a histone deacetylase, HDA-1, to promote female development by repressing male-specific genes (Grote and Conradt, 2006). Typically, *NASP* mRNA levels in testis are at least an order of magnitude higher than in other adult tissues (Richardson et al., 2006). The higher expression levels of *PmNASP* in ovaries compared to testes of *P. monodon* broodstock ($P<0.05$, Preechaphol et al., 2007) and phylogenetic analysis (data not shown) suggested that this gene is a new isoform of the NASP protein family.

The involvement of *PmNASP* in *P. monodon* female reproductive system development was previously examined after serotonin injection (Sittikankeaw, 2006). Serotonin (5-HT) is one of the biogenic amines (e.g. epinephrine and dopamine) known to modulate the release of neuropeptide hormones from the sinus gland (Meusy and Payen, 1988). In captive *P. monodon*, injection of exogenous 5-HT resulted in comparable rates of ovarian maturation and spawning to those of unilateral eyestalk-ablated shrimp (Wongprasert et al., 2006). Also, hatching rate and the amount of nauplii produced per spawner were significantly higher in the 5-HT injected shrimp, compared to the eyestalk-ablated shrimp. The mRNA expression levels of *PmNASP* were found to be higher in juvenile *P. monodon* at 12 h after serotonin injection (50 μg g^{-1} body weight; $P<0.05$) before returning to normal levels at 48 h post-injection ($P>0.05$). A booster injection of serotonin at 72 h after the initial injection also induced *PmNASP* expression 24 h later ($P<0.05$), where it remained significantly different from the control 48 and 72 h after the booster ($P<0.05$) (Sittikankeaw, 2006). The increases in the *PmNASP* expression upon introduction of serotonin strengthened the argument that *PmNASP* plays an important role in reproductive maturation in females of this species.

4.2. Future of specialized microarrays

Microarrays of all ORFs in various organisms are commonly employed for genome-wide expression studies (Richmond et al., 1999; Gasch et al., 2000; Gregorio et al., 2001; Karoonuthaisiri et al.,

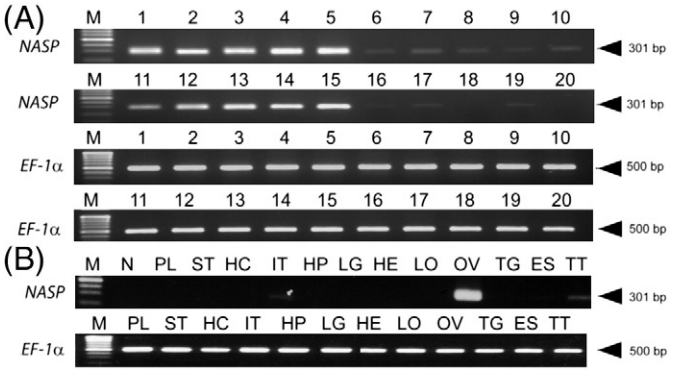


Fig. 6. (A) RT-PCR of *PmNASP* and *EF-1α* in ovaries (lanes 1–5 and 11–15) and testes (lanes 6–10 and 16–20) of juvenile (top) and broodstock shrimp (bottom). (B) Expression of the *PmNASP* transcript in 12 tissues of female (pleopods, PL; stomach, ST; hemocytes, HC; intestine, IT; hepatopancreas, HP; gills, GL; heart, HE; lymphoid organs, LO; ovaries, OV; thoracic ganglion, TG; eyestalk, ES) and testes (TT) of male *P. monodon* broodstock. *EF-1α* was successfully amplified from the same template. Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

2005). In this study, the specialized-tissue microarray, *Repro*Array^{GTS}, was constructed to identify differentially expressed genes during reproductive maturation of *P. monodon*. An advantage of the specialized arrays is the reduction of cost and labor for probe synthesis and purification, especially for an organism with many available EST libraries. However, there should be large enough number of genes on the arrays to make this high-throughput technique worthwhile and to ensure valid normalization. If only a few sets of genes are included on the array, it is necessary to include various control spots for normalization propose. Besides limited number of genes presented on the specialized microarray, the redundancy of cDNA sequences from EST libraries is another major disadvantage of this type of arrays. To reduce the redundancy, normalized cDNA libraries can be used as templates for microarray probe amplification, or probes synthesized from unique contigs can be assembled from the EST sequences.

Even though the *Repro*Array^{GTS} described here represents limited numbers of *P. monodon* genes, it has proven to be useful for high-throughput gene expression studies and gene discovery as demonstrated for the *NASP* gene. Through this specialized microarray, the ability to identify expression of functionally important genes involved in ovarian development in *P. monodon* will allow more rapid and focused studies on molecular mechanisms of genes controlling each step of oocyte maturation and formation of cortical rods (CRs), leading to a better understanding of the reproductive maturation of *P. monodon* in captivity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbd.2008.11.003.

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