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Suppression subtractive hybridization (SSH) for isolation and characterization of genes related to testicular development in the giant tiger shrimp *Penaeus monodon*

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Suppression subtractive hybridization (SSH) cDNA libraries of the giant tiger shrimp, *Penaeus monodon*, were constructed. In total, 178 and 187 clones from the forward and reverse SSH libraries, respectively, of *P. monodon* were unidirectionally sequenced. From these, 37.1% and 53.5% Expressed Sequence Tags (ESTs) significantly matched known genes (E-value < 1e-04). Three isoforms of *P. monodon progestin membrane receptor component 1: PM-PGMRC1-s* (1980 bp), *PM-PGMRC1-m* (2848 bp), and *PM-PGMRC1-l* (2971 bp), with an identical ORF of 573 bp corresponding to a deduced polypeptide of 190 amino acids, were successfully identified by RACE-PCR. Interestingly, *PM-PGMRC1* showed a greater expression level in testes of juvenile than broodstock *P. monodon* (P < 0.05). Dopamine administration (10^{-6} mol/shrimp) resulted in up-regulation of *PM-PGMRC1* in testes of juveniles at 3 hrs post treatment (P < 0.05), but had no effect on *PM-Dmc1* (P > 0.05). [BMB reports 2008; 41(11): 796-802]

INTRODUCTION

A total of 343 economically important shrimp species have been reported by the FAO (1). Among cultured marine shrimp, one of the most economically important species is the giant tiger shrimp, *Penaeus monodon*. Annual production of farmed *P. monodon* in Thailand alone has reached or exceeded 200,000 metric tons since 1993 (2). However, a range of shrimp diseases and the lack of high quality wild and/or domesticated broodstock has lead to recent production declines (3,4).

Low reproductive maturation rates in cultured *P. monodon* has limited the ability to selectively improve or maintain important genetic traits (4). Accordingly, the domestication of *P. monodon* has been remarkably slow in Thailand (5). Breeding of this shrimp species using spermatozoa from captive males has yielded low quality offspring. However, the use of spermatozoa from wild males on the eggs of either wild or domesticated females successfully resolves this problem (B. Withyachumnarnkul, personal communication).

Baseline information related to testicular development and sperm quality in penaeid shrimp is rather limited (6). An initial step towards understanding molecular mechanisms of testicular and spermatozoa development in *P. monodon* should be to identify and characterize differentially expressed genes in various stages of testicular development.

Suppression subtractive hybridization (SSH), a technique combining suppression Polymerase Chain Reaction (PCR) with hybridization technology, is widely used to isolate differentially expressed genes in two closely related samples/specimens/species (7,8).

Accordingly, SSH should facilitate the identification of genes involved in testicular development. These genes may be candidates for molecular markers, which could assist the domestication and selective breeding programs of *P. monodon*.

In this study we carried out SSH of genes expressed in testes of broodstock and juvenile *P. monodon*. The full length cDNAs of sex-related transcripts, *PM-PGMRC1*, were further characterized by RACE-PCR and reported for the first time in crustaceans. Upon dopamine administration, the expression profiles of *PM-PGMRC1* and *PM-Dmc1* in testis of *P. monodon* were examined using real-time PCR.

RESULTS AND DISCUSSION

SSH libraries and Expressed Sequence Tag (EST) analysis

A total of 367 ESTs from testes (178 and 187 clones from the forward and reverse SSH libraries, respectively) of *P. monodon* were unidirectionally sequenced. The positive recombinant clones of both libraries were 95.1% and 96.4%, of which

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82.4% and 86.2% had insert sizes > 250 bp in length. The percentage of ESTs which significantly matched known genes in respective libraries were 37.1% (66 ESTs) and 53.5% (100 ESTs). Seven known transcripts: *allergen Pen m 2*, *COI*, *EF-1α*, *GTP-binding protein, 26S proteasome non-ATPase subunit 12, receptor for activated protein kinase C (RACK)* and *myelodysplasia/myeloid leukemia factor* were found with low frequencies in both libraries, suggesting that the cDNA subtraction was successful (Tables 1 and 2).

Unknown transcripts were predominant in both forward and reverse SSH libraries (112 and 87 ESTs accounting for 62.9% and 46.5%, respectively; E-value > 1e-04). The percentage of unknown transcripts in SSH libraries was greater than that in the conventional library of *P. monodon* broodstock testis (290/889 clones, 32.6%, S. Klinbunga, unpublished data), but lower than those found in the SSH library of tropical abalone (*Haliotis asinina*) testes (125/160, 65%) (9).

Disregarding ribosomal and hypothetical protein homologues, ESTs categorized as members of gene expression and protein synthesis (14 and 22 ESTs accounting for 7.9% and 11.8% in the forward and reverse SSH libraries, respectively), and metabolism (18 and 13 ESTs accounting for 10.1% and 7.0%) were predominant among known transcripts in both SSH libraries. Numbers of transcripts belonging to internal/external structure and stress response/cell defense groups of the forward SSH library (2 and 4 ESTs accounting for 1.1 and 2.2%) were lower than those of the reverse SSH library (7 and 13 ESTs accounting for 3.7 and 7.0%). Highly redundant ESTs were not observed and a relatively large number of known gene homologues were found in these libraries (Tables 1 and

Table 1. Known transcripts (excluding ribosomal and functionally unidentified transcripts) found in the forward SSH library of testes of *P. monodon*

Homologue*	Species	Size (bp)	E-value	Redundancy
Actin-depolymerizing factor 1	*Bombyx mori*	316	8e-21	1
Activated protein kinase C receptor	*Toxoptera citricida*	410	4e-70	1
Allergen Pen m 2	*Penaeus monodon*	309	5e-22	1
Antimicrobial peptide	*Fenneropenaeus chinensis*	389	2e-22	1
ATP-dependent RNA helicase	*Aedes aegypti*	472	3e-76	1
Basic leucine zipper and W2 domain-containing protein 2	*Danio rerio*	466	2e-09	1
Bmsqd-2	*Apis mellifera*	713	1e-102	1
C-1-tetrahydrofolate synthase, cytoplasmic (C1-THF synthase)	*Pongo pygmaeus*	714	7e-85	1
C2 domain containing protein	*Tetrahymena thermophila SB210*	592	2e-19	1
Cytochrome c oxidase subunit 6a polypeptide 1	*Xenopus tropicalis*	345	2e-08	1
Cytochrome c oxidase subunit I	*Fenneropenaeus chinensis*	467	4e-76	2
	Marsupenaeus japonicus	712	1e-112	
Cytochrome c oxidase subunit III	*Penaeus monodon*	439	1e-70	1
Cytosolic manganese superoxide dismutase	*Penaeus monodon*	343	2e-12	2
		346	6e-13	
DEAD (Asp-Glu-Ala-Asp) box polypeptide 54 isoform 3	*Pan troglodytes*	562	1e-19	1
Dolichyl-diphosphooligosaccharide-protein-glycotransferase	*Branchiostoma belcheri tsingtaunese*	427	4e-19	1
Elongation factor-1 alpha	*Penaeus monodon*	353	7e-62	2
	Armadillidium vulgare	557	2e-95	
Eukaryotic translation initiation factor 2 subunit 2	*Bombyx mori*	600	7e-36	1
GTP-binding protein	*Bombyx mori*	373	5e-49	1
Malate dehydrogenase 1, isoform CRA_d	*Homo sapiens*	374	4e-43	1
Meiotic recombination protein DMC1/LIM15 homolog isoform 1	*Canis familiaris*	506	1e-24	1
Myelodysplasia/myeloid leukemia factor CG8295-PD, isoform D	*Drosophila melanogaster*	709	1e-33	1
Myosin	*Dictyostelium discoideum AX4*	596	6e-23	1
NTF2-related export protein (p15)	*Tribolium castaneum*	420	4e-19	1
Oncoprotein nm23	*Ictalurus punctatus*	436	6e-34	1
26S proteasome regulatory complex ATPase RPT4	*Aedes aegypti*	490	2e-81	1
Proteasome (prosome, macropain) 26S subunit, ATPase, 5, isoform CRA_a	*Homo sapiens*	199	3e-12	1
Proteasome 26S non-ATPase subunit 12	*Tribolium castaneum*	209	4e-18	1
Proteasome subunit alpha type 2 (Proteasome component C3, Macropain subunit C3)	*Strongylocentrotus purpuratus*	401	4e-40	1
Proteasome subunit, alpha type, 5	*Apis mellifera*	707	4e-23	1
Ras-related nuclear protein	*Marsupenaeus japonicus*	602	4e-50	1
Receptor for activated protein kinase C (RACK) 1 isoform 1	*Bombyx mori*	644	1e-113	2
		702	7e-17	
Sensitized chromosome inheritance modifier 19 CG9241-PA	*Drosophila melanogaster*	368	3e-15	1
Signal peptidase complex subunit 2	*Tribolium castaneum*	352	3e-28	1
Signal sequence receptor	*Bombyx mori*	279	9e-14	1
Transmembrane protein	*Pan troglodytes*	540	2e-64	1

*GenBank accession numbers FD663981-FD664019.

Table 2. Known transcripts (excluding ribosomal and functionally unidentified transcripts) found in the reverse SSH library of testes of *P. monodon*

Homologue*	Species	E-value	Size (bp)	Redundancy
Alcohol dehydrogenase	*Bombyx mori*	3e-35	323	3
		2e-90	680	
		2e-90	680	
Allergen Pen m 2	*Penaeus monodon*	2e-21	309	2
		5e-22	309	
Calcitonin gene-related peptide-receptor component protein isoform a	*Homo sapiens*	8e-21	510	1
Calcium-dependent chloride channel-1	*Homo sapiens*	4e-11	443	1
Cathepsin B	*Hippoglossus hippoglossus*	1e-26	238	1
Cement precursor protein 3B variant 2	*Phragmatopoma californica*	3e-08	437	1
Cement precursor protein 3B variant 3	*Phragmatopoma californica*	2e-11	620	2
		3e-07	270	
Centromere/kinetochore protein zw10 homolog	*Apis mellifera*	1e-20	423	1
Cytochrome b	*Penaeus monodon*	3e-80	494	1
Cytochrome c oxidase subunit I	*Fenneropenaeus chinensis*	5e-68	403	3
		1e-66	405	
		7e-68	405	
Drosophila melanogaster eEF1delta	*Drosophila yakuba*	8e-16	146	1
Elongation factor-1 alpha	*Armadillidium vulgare*	1e-113	660	2
		1e-113	660	
Eukaryotic translation initiation factor 3 subunit 4	*Danio rerio*	1e-55	521	2
		1e-55	521	
F-box protein 22	*Gallus gallus*	6e-08	329	1
Ferric reductase-like protein	*Aedes aegypti*	1e-28	452	1
Gelsolin, cytoplasmic (Actin-depolymerizing factor) (ADF)	*Homarus americanus*	3e-05	396	2
		3e-05	396	
GTP binding protein	*Bombyx mori*	2e-70	503	1
Heat shock protein gp96	*Strongylocentrotus purpuratus*	1e-21	498	1
Helicase, lymphoid-specific isoform 2	*Danio rerio*	1e-43	408	1
Innexin 2	*Penaeus monodon*	1e-62	360	1
Intracellular fatty acid binding protein	*Penaeus monodon*	1e-156	303	1
Karyopherin (importin) alpha 4	*Rattus norvegicus*	8e-08	383	1
Kinesin heavy chain	*Loligo pealei*	1e-20	570	1
Mcm3-prov protein (minichromosome maintenance protein 3)	*Xenopus laevis*	3e-09	240	1
Myeloid leukemia factor 2 (Myelodysplasia-myeloid leukemia factor 2)	*Danio rerio*	2e-06	464	1
Niemann-Pick disease type C2	*Oreochromis mossambicus*	5e-06	456	2
		5e-06	456	
Nop56 CG13849-PA, isoform A (nucleolar KKE/D repeat protein; DmNOP56)	*Drosophila melanogaster*	2e-49	348	1
Nucleolin	*Xenopus laevis*	3e-04	518	1
Peptidylprolyl isomerase A	*Ictalurus punctatus*	8e-15	309	1
Progestin membrane receptor component 1	*Oryzias latipes*	1e-47	574	1
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 13	*Tribolium castaneum*	2e-78	706	1
Proteasome 26S non-ATPase subunit 12	*Tribolium castaneum*	1e-71	560	2
		1e-71	560	
Proteasome 26S subunit 4 ATPase CG5289-PA	*Drosophila melanogaster*	1e-89	647	1
Receptor for activated protein kinase C (RACK) 1 isoform 1	*Bombyx mori*	1e-107	628	1
Ribosomal RNA methyltransferase	*Aedes aegypti*	6e-10	505	1
RNA polymerase 1-1	*Mus musculus*	2e-25	326	1
Small nuclear ribonucleoprotein D2 polypeptide 16.5kDa, isoform CRA_b	*Homo sapiens*	2e-34	313	1
Small nuclear ribonucleoprotein E (snRNP-E)	*Drosophila melanogaster*	2e-14	227	1
Small optic lobes CG1391-PB, isoform B (Calpain)	*Apis mellifera*	7e-81	580	2
		7e-81	580	
Tetraspanin 3, isoform CRA_a	*Homo sapiens*	4e-10	590	1
Tetraspanin 96F CG6120-PA	*Drosophila melanogaster*	5e-14	438	1
Transcription initiation factor TFIID subunit 12	*Xenopus laevis*	1e-21	519	1

*GenBank accession numbers FD663981-FD664019.

2). The diversity of genes found in these libraries gives rise to the potential of discovering genes with functional importance.

Full length cDNA and sequence analysis of *P. monodon progestin receptor membrane component 1 (PM-PGMRC1)*

Progestins are sex steroid hormones that play important roles in gametogenesis. In fish, progestin also plays an important role in spermiation and sperm maturation (10). The effects of 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) in the male Japanese eel were found to induce DNA replication in spermatogonia, but prevent DNA replication at the initiation of meiosis (10).

Two totally distinct classes of putative membrane-bound progestin receptors have been reported in vertebrates; progestin membrane receptor component (PGMRC; subtypes 1 and 2) and membrane progestin receptors (mPR; subtypes α, β, γ). Both have never been studied in any crustacean (11).

Full length cDNA of *PM-PGMRC1*, initially found in the reverse SSH library, was successfully identified by RACE-PCR (Fig. 1 and Supplementary material 1) and reported for the first time in crustaceans. Three full length cDNAs of *PGMRC1, PM-PGMRC1-s* (1980 bp), *PM-PGMRC1-m* (2848 bp), and *PM-PGMRC1-l* (2971 bp) were found in *P. monodon* (GenBank accession numbers EU440763-EU440765). They shared an identical ORF of 573 bp deducing to a 190 aa polypeptide, but differed in length of the 3' UTR region. The predicted p*I* and MW of the protein encoded by this cDNA were 4.60 and 20.98 kDa, respectively. A cytochrome b-5 like heme/steroid binding domain (Cyt-b5 domain; positions 68th-166th, E-value = 1.3e-19), functionally important for ubiquitous electron transportation (12) in heme-binding protein and progesterone receptor (13), was found in the deduced PM-PGMRC1. The closest similarity of these transcripts was *PGMRC1* of the medaka, *Oryzias latipes* (E-value = 1e-41, 2e-41, and 2e-41, respectively).

Phylogenetic analysis indicated that different subtypes of *PGMRC* (*PGMRC1* and *PGMRC2*) arose from gene duplication process. *PM-PGMRC1* was clustered with *PGMRC1* of the sea urchin (*Strongylocentrotus purpuratus*), but distantly related to *PGMRC1* of vertebrates and fish. Accordingly, it should be regarded as a new member of the invertebrate *PGMRC* (Fig. 1). It would also be interesting to establish whether different isoforms of *PM-PGMRC1* are transcribed from a single locus through the alternative splicing process or encoded from different loci.

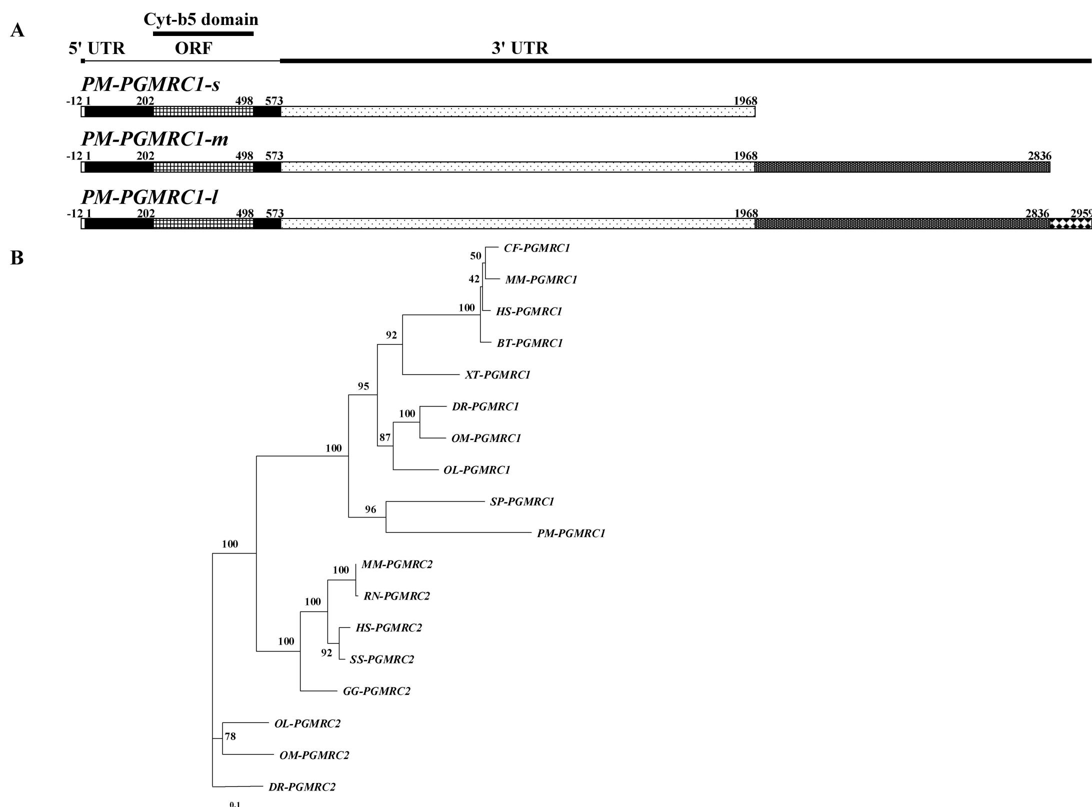

Fig. 1. (A) A schematic diagram illustrating three different isoforms of *PM-PGMRC1* (*PM-PGMRC1-s, PM-PGMRC1-m* and *PM-PGMRC1-l)* which share an identical ORF of 573 bp (190 amino acids), but different length of the 3' UTR. The Cyt-b5 domain (E-value = 1.3e-19) is also illustrated. (B) A bootstrapped neighbor-joining tree illustrating relationships between *PGMRC1* and *PGMRC2* 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.

RT-PCR and tissue distribution analysis

PM-Inx2 and *PM-PGMRC1* did not reveal differential expression between ovaries and testes of *P. monodon* ($P > 0.05$). In contrast, *PM-Dmc1* was expressed differentially in gonads (testes > ovaries) of broodstock ($P < 0.05$, Fig. 2). More importantly, *PM-PGMRC1* was more abundantly expressed in testes of juveniles than broodstock ($P < 0.05$). *PM-Inx2* and *PM-Dmc1* were preferentially expressed in ovaries of juveniles compared to broodstock ($P < 0.05$).

Tissue distribution analysis indicated that *PM-Inx2* and *PM-Dmc1* were more abundantly expressed in testes than ovaries, while *PM-PGMRC1*, which was constitutively expressed in all examined tissues, showed comparable expression levels in testes and ovaries of *P. monodon* broodstock (Fig. 2). Therefore, *PM-Inx2* and *PM-Dmc1* may play the important role in spermatogenesis but not in oogenesis, whereas *PM-PGMRC1* is functionally important for both spermatogenesis and oogenesis of *P. monodon*.

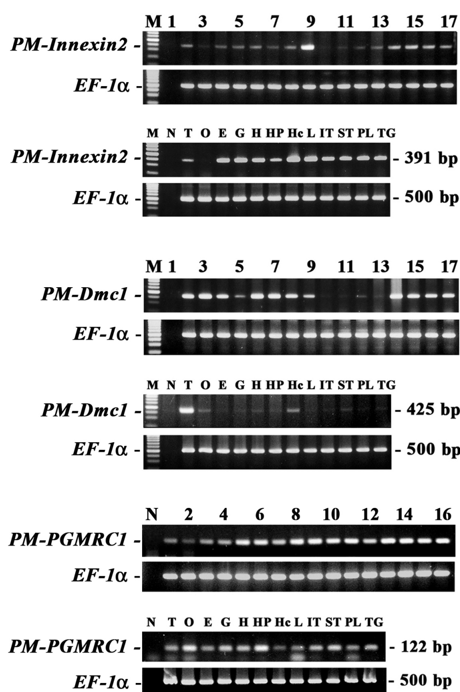

Fig. 2. RT-PCR of *PM-Inx2, PM-Dmc1* and *PM-PGMRC1* using the first strand cDNA of testes of broodstock (lanes 2-5 for *PM-Inx2* and *PM-Dmc1* or 1-4 for *PM-PGMRC1*) and juveniles (lanes 6-9 for *PM-Inx2* and *PM-Dmc1* or 5-8 for *PM-PGMRC1*), and ovaries of broodstock (lanes 10-13 for *PM-Inx2* and *PM-Dmc1* or 9-12 for *PM-PGMRC1*) and juveniles (lanes 14-17 for *PM-Inx2* and *PM-Dmc1* or 13-16 for *PM-PGMRC1*) of *P. monodon*. Lane 1 is the negative control (without the cDNA template). *EF-1α* was included as the positive control. The first strand cDNA of testes (T), eyestalks (E), gills (G), heart (H), hepatopancreas (HP), hemocytes (Hc), lymphoid organs (LO), intestine (IT), stomach (ST), pleopods (PL), and thoracic ganglion (TG) of a male broodstock, and ovaries (O) of a female broodstock was used for tissue distribution analysis. Lane N is the negative control.

Invertebrate gap-junction proteins, Inxs, were originally identified in *Drosophila* and *Caenorhabditis* (14). In *Bombyx mori*, northern blotting and *in situ* hybridization revealed that *Bm-Inx2* was expressed across all developmental stages and in various tissues, with high expression observed in the nervous system during embryogenesis. In contrast, *Bm-Inx4* was transiently expressed at the germ-band formation stage of embryogenesis, and was specifically expressed in ovaries and testes during the larval and pupal stages (15).

Spermatogenesis is an essential process for production of haploid gametes. During meiosis, a single round of DNA replication is followed by two successive rounds of nuclear divisions (16). Dmc1, involved in meiotic recombination, occurs during the meiotic prophase (17). A study carried out on mice has shown that RNA interference (RNAi) against endogenous *Dmc1* induces defects in spermatocytes, indicating its important roles in spermatogenesis (18).

Recently, the full length *Dmc1* cDNA was cloned from the testis of the Japanese eel (*Anguilla japonica*) (19). In that study, *Dmc1* mRNA was abundantly expressed in the testes and ovaries, and lower expressed in the brain. *In situ* hybridization revealed that *Dmc1* of *A. japonica* was localized only in primary spermatocytes, implying its important role during the initial stages of spermatogenesis (19).

Expression of *PM-Dmc1* and *PM-PGMRC1* upon dopamine treatment

In the red swamp crayfish, *Procambarus clarkia*, dopamine inhibited testicular maturation dose-dependently, whereas its antagonist, spiperone, induced testicular maturation (20). Nevertheless, in *P. monodon*, effects of dopamine on spermatogenesis are reported for the first time in this study. The results from real-time quantitative PCR revealed that expression levels of *PM-Dmc1* in testes were not significantly altered after dopamine treatment ($P > 0.05$), whereas *PM-PGMRC1* was up-regulated at 3 hrs post treatment ($P < 0.05$) (Supplementary material 2). These preliminary results may suggest that dopamine might not inhibit spermatogenesis in *P. monodon*. However, further studies need to be conducted on broodstock at both mRNA and protein levels.

In the present study, genes expressed in testes of *P. monodon* were identified by SSH analysis. The expression profiles of *PM-Inx2, PM-Dmc1* and *PM-PGMRC1* implied that these genes may contribute to testicular development and/or spermatogenesis. The analysis of baseline information acquired as part of this study will address the paucity of data and better understanding of reproductive maturation in cultured male *P. monodon*.

MATERIALS AND METHODS

Specimens

Juveniles (approximately 4-month-old) and broodstock *P. mono-*

don were purchased from a commercial farm in Chachoengsao (eastern Thailand) and wild-caught from Chonburi (Gulf of east Thailand), respectively.

In order to examine the effects of dopamine on expression of *PM-Dmc1* and *PM-PGMRC1*, male juveniles (approximately 20 g body weight) were acclimatized in laboratory conditions for 7 days. One and four groups of shrimp (*N* = *5* for each group) were intramuscularly injected with saline (0.85% NaCl) or dopamine (10^{-6} mol/shrimp), while a group of non-injected shrimps (*N* = *5*) was also maintained as the control.

Specimens were collected at 3, 6, 12 and 24 hrs post injection. Tissues were dissected out from each specimen and stored at −70°C until used.

Total RNA and mRNA Isolation

Total RNA was extracted from different tissues of each shrimp using TRI-REAGENT (Molecular Research Center). Messenger (m) RNA was further purified using a QuickPrep Micro mRNA Purification Kit (GE Healthcare). Total RNA and mRNA were kept under absolute ethanol at -70°C prior to reverse transcription.

Construction of suppression subtractive hybridization (SSH) cDNA libraries and EST analysis

Two micrograms of mRNA from broodstock and juvenile *P. monodon* testes were reverse transcribed. SSH between cDNA from testes of broodstock (tester) and juvenile (driver) shrimp, and *vice versa*, were carried out using a PCR Select cDNA Subtraction Kit (BD Clontech) and ligated to pGEM-T Easy vector. The resulting products were transformed into *E. coli* JM109. Plasmid DNA was extracted from recombinant clones with insert > 250 bp in size and unidirectionally sequenced. Nucleotide sequences of ESTs were compared with data in the GenBank using BLAST*N* and BLAST*X* (21). Significant matched nucleotides/proteins were considered when the E-value was < 1e-04.

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

RACE-PCR of *PM-PGMRC1* was carried out for both 5' and 3' directions (PGMRC1-5' RACE; 5'-TGTCGTTTCATCTTGGGCA CAGGAGGTT-3' and PGMRC1-3' RACE; 5'-GCAAAGGACAC CAAAGCGAAGACGGATG-3') using a BD SMART RACE cDNA Amplification Kit following 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 (22, 23). The protein domain of *PM-PGMRC1* deduced amino acids 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).

Phylogenetic analysis of *PM-PGMRC1*

The deduced amino acid sequence of *PM-PGMRC1* was phylogenetically compared to *PGMRC1* and *PGMRC2* sequences retrieved from the GenBank: *Oryzias latipes* (*OL-PGMRC1*, BAE47967.1), *Danio rerio* (*DR-PGMRC1*, NP_001007393.1), *Oncorhynchus mykiss* (*OM-PGMRC1*, AAL49963.1), *Strongylocentrotus purpuratus* (*XP-PGMRC1*, XP_783332.1), *Canis familiaris* (*CF-PGMRC1*, XP_538151.1), *Homo sapiens* (*HS-PGMRC1*, NP_006658.1), *Mus musculus* (*MM-PGMRC1*, AAB97466.1), *Bos taurus* (*BT-PGMRC1*, NP_001068601.1), *Xenopus tropicalis* (*XT-PGMRC1*, NP_001006842.1); and *Rattus norvegicus* (*RN-PGMRC2*, NP_001008375.1), *Mus musculus* (*MM-PGMRC2*, AAH44759.1), *Sus scrofa* (*SS-PGMRC2*, ABX45132.1) *Homo sapiens* (*HS-PGMRC2*, NP_006311.1), *Gallus gallus* (*GG-PGMRC2*, NP_001006441.1), *Oryzias latipes* (*OL-PGMRC2*, NP_001098 199.1), *Oncorhynchus mykiss* (*OM-PGMRC2*, ABD58973.1), *Danio rerio* (*DR-PGMRC2*, NP_998269.1). Multiple alignments were carried out using ClustalW (24). The bootstrapped neighbor-joining tree (25) was constructed to illustrate phylogenetic relationships among sequences of *PGMRC* using PHYLIP (26).

RT-PCR and tissue distribution analysis

RT-PCR of *PM-Inx2* (F: 5'-AAGATGTGGGAAGGAGGCAAGA-3' and R: 5'-TGAGCGGGAGAACGCAGAGT-3'), *PM-PGMRC1* (F: 5'-GCCCAAGATGAAACGACAGG-3' and R: 5'-TGGAGCCTC GGGTGACATC-3') and *PM-Dmc1* (F: 5'-ATGGAAGATCAGGC TTTAGATGC-3' and R: 5'-GTGACGCAGAGAGTGTGGGAG-3') was carried out using the conditions described previously (4). *EF-1α* (F: 5'-ATGGTTGTCAACTTTGCCCC-3' and R: 5'-TTGAC CTCCTTGATCACACC-3') was included as the positive control. Amplicons were electrophoretically analyzed in 1.5% agarose gels (22). Relative expression levels of investigated transcripts (intensity of the target /intensity of *EF-1α*) from *P. monodon* testes and ovaries were quantified using the Quantity One software (BioRad) and statistically analyzed. Significant differences were considered if the P values were < 0.05.

Real-time quantitative RT-PCR of *PM-Dmc1* and *PM-PGMRC1* in testes of juvenile *P. monodon* treated with dopamine

The first strand cDNA from testes of juvenile shrimp treated with dopamine was used as the template for real-time PCR analysis. The target and control transcripts were examined: *PM-PGMRC1*$_{122}$ (F: 5'-GCCCAAGATGAAACGACAGG-3' and R: 5'-TGGAGCCTCGGGTGACATC-3'), *PM-Dmc1*$_{150}$ (F: 5'-AT GTGCGAGAAGCGAAGGC-3' and R: 5'-GCAGAGAGTGTGG GAGATTTGTG-3'), the control *EF-1*$_{122}$ (5'-TTCCGACTCCAAG AACGACC-3' and 5'-GAGCAGTGTGGCAATCAAGC-3'). For each amplification, a 25 μl reaction contained 12.5 μl of 2x SYBR Green Master Mix (Qiagen). Gene-specific primers were used at a final concentration of 0.3 μM. The thermal profile was 95°C for 15 min followed by 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 45 s. Each sample was run in duplicate. Relative expression levels of investigated genes (log copy number of the target genes/log copy number of *EF-1α*) in all experimental groups (control, saline-injected and dopamine-injected shrimp) were statistically analyzed using one way analysis of variance (ANOVA) followed by Duncan's new multiple range test.

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