CHAPTER II

MATERIALS AND METHODS

2.1 Sample

Juvenile *P. monodon* (20-30 g body weight, and approximately 4-month-old) were purchased from local farms in Chonburi, eastern Thailand. In addition, male and female broodstock-sized *P. monodon* were live-caught from Angsila, Chonburi and transported back to the Center of Excellence for Marine and Biotechnology (CEMB), Chulalongkorn University. The gender and weight of each *P. monodon* broodstock were recorded

2.2 RNA extraction

Total RNA was extracted from ovaries and testes and other tissues of each the shrimp using TRI-REAGENT (Molecular Research Center). A piece of tissue was immediately placed in mortar containing liquid nitrogen and ground to the fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 µl of TRI REAGENT (1 ml/50-100 mg tissue) and homogenized. Additional 500 µl of TRI REAGENT were added. The homogenate was left at room temperature for 5 minutes before 0.2 ml of chloroform was added. The homogenate was vortexed for at least 15 seconds, left at room temperature for 2 - 15 minutes and centrifuged at 12000 *g* for 15 minutes at 4°C. The mixture was separated into the lower phenol-chloroform phase (red), the interphase, and the upper aqueous phase (colorless).

The aqueous phase (inclusively containing RNA) was carefully transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by an addition of 0.5 ml of isopropanol and mixed thoroughly. The mixture were left at room temperature for 10 - 15 minutes and centrifuged at 12000 *g* for 10 minutes at 4 °C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 7500g for 10 minutes at 4°C. The ethanol was removed. The RNA pellet was air-dried for 5-10 minutes. RNA was dissolved in DEPC-treated H_2O for immediately used. Alternatively, the RNA pellet was kept under absolute ethanol in a -80 °C freezer for long storage.

Total RNA was also extracted from other tissues including eyestalks, gills, heart, hemocytes, hepatopancreases, lymphoid organs, intestine, stomach, pleopods and throracic ganglion of *P. monodon* using the same extraction procedure. The quality of extracted total RNA was examined by electrophoresed through 1.0% agarose gels.

2.3 Spectrophotometrically estimate of extracted total RNA concentration

The concentration of extracted RNA sample is estimated by measuring the optical density at 260 nM (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 40 µg/ml single stranded RNA and 33 µg/ml single stranded DNA (Sambrook and Russell, 2001). Therefore, the concentration of RNA sample were estimated in µg/ml by using the following equation;

[RNA] = OD_{260} x dilution factor x 40 (or 33 for single stranded DNA)

The purity of RNA sample can be evaluated from a ratio of OD_{260}/OD_{280}. The ratios of purified RNA were appropriately 2.0 (Sambrook and Russell, 2001).

2.4 Synthesis of the first strand cDNA

One and a half micrograms of total RNA from various tissues of *P. monodon* were reverse-transcribed to the first strand cDNA using an ImProm- II™ Reverse Transcription System Kit (Promega). Total RNA was combined with 0.5 µg of oligo dT_{12-18} and appropriate DEPC-treated H_2O in final volume of 5 µl. The reaction was incubated at 70 °C for 5 minutes and immediately placed on ice for 5 minutes. Then 5x reaction buffer, $MgCl_2$, dNTP Mix, RNasin were added to final concentrations of 1x, 2.25 mM, 0.5 mM and 20 units, respectively. Finally, 1 µl of ImProm- II™ Reverse transcriptase was add and gently mixed by pipetting. The reaction mixture was incubated at 25 °C for 5 minutes and at 42°C for 90 minutes. The reaction mixture was incubated at 70 °C for 15 minutes to terminate the reverse transcriptase activity. Concentration and rough quality of the newly synthesized first strand cDNA was spectrophotometrically examined (OD_{260}/OD_{280}) and electrophoretically analyzed by 1.2% agarose gels.

2.5 Reverse transcription (RT)-PCR of gene homologues in *P. monodon*

2.5.1 Design of primers from EST of *P.monodon*

In total, 158 primer pairs were designed from EST sequences of homologues of known transcripts from hemocyte, ovarian and testes cDNA libraries of *P. monodon* (Table.2.1). Primers were purchased from Proligo Ltd.

2.5.2 End point RT-PCR

One hundred nanograms of the first strand cDNA of ovaries ($N = 2$) and testes ($N = 2$) of female and male broodstock-sized *P. monodon* were used as the template in a 25 µl RT-PCR reaction composing of 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 100 mM of each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1 unit of Dynazyme^{TM} DNA Polymerase (FINNZYMES). RT-PCR was carried out with the temperature profile of predenaturation at 94°C for 3 minutes followed by 25 cycles of denaturation at 94°C for 45 seconds, annealing at 53°C for 45 seconds and extension at 72°C for 2 minutes. The final extension was carried out at the same temperature for 7 minutes.

Five microliters of the amplification products are electrophoresed though 1.2 - 2.0% agarose gel depended on size of the amplification products. The electrophoresed band was visualized under a UV tramsilluminator after ethidium bromide staining (Sambrook and Russell, 2001). A total of 158 gene homogues were screened. Only gene homologues exhibiting the specific expression pattern in one sex but not the other and those exhibiting differential expression patterns between ovaries and testes were further tested with a larger number of specimens (additional 4 individuals for each sex).

2.5.3 Agarose gel electrophoresis

An appropriate amount of agarose was weighed out and mixed with an desired volume of 1x TBE buffer (89 mM Tris-HCl, 89 mm boric acid and 2 mM EDTA, pH 8.3) The gel slurry was boiled in a microwave oven to complete solubilization and allowed to lower than 60 °C before poured into the gel mold. A comb was inserted.

The agarose gel was left to solidify. When needed, enough amount of 1x TBE buffer covering the gel for approximately 0.5 cm. the comb was removed.

The PCR product was mixed with the one-fourth volume of the 10x loading dye (0.25% bromophenol blue and 25% ficoll in water) and loaded into the well.

Table 2.1 Gene homologue, primer sequences and the expected sizes of the PCR product from EST of *P. monodon*

No	Name	Primer sequence	Expected size (bp)
1	*Guanine nucleotide binding protein* (H)	F:5'AGACCACCCGTCGCTTTGAG3' R:5'AACCTTACGCAGGACACCCA3'	180
2	*Thyroid hormone receptor associated protein complex 230 kDa component* (H)	F:5'GTAGGCTTGGTAGAATGGGC3' R:5'GAGCAGTAGCAGAAGTTGGGAA'	312
3	*Myelodysplasia/Myeloid leukemia factor* (H)	F:5'ATGTCTCCCTTCCCAGCACT3' R:5'GTCTTGTTTCCCTTACACCACC3'	217
4	*NADH dehydrogenase subunit 5* (H)	F:5'GAGGACAGGCTTGATTTATTG3' R:5'ATCCTAAACCATCCCACCCTA3'	250
5	*5-methylcytosine G/T mismatch* (H)	F:5'TTACCACGACCCTTGGAAACT3' R:5'TCTCATGCAGCCCTAGACACT3'	190
6	*MINUTE protein* (H)	F:5'AGGAGAGGGACACCCAGACA3' R:5'TAGCATACCAAACATTAGACC3'	400
7	*Phenylalanine-tRNA synthesis-B-subunit* (H)	F:5'TCAGCCATTCACTGCGGAGC3' R:3'TAGGAGGGACATCGACCACAA3'	221
8	*Cyclin A* (O)[b]	F:5'CAGCAAGTATGAGGTGGATTCT3' R:5'TCTGCCCAACTCTGTAGGTATT3'	256
9	*Leucine-rich repeat protein* (H)	F:5'GGCTCACATCACTCCCACTT3' R:5'CCTCTAGATCCAACATCCGA3'	213
10	*2-cys –thioredoxin peroxidase* (H)	F:5'CGAAGTGGTTGCTTGCTCTA3' R:5'CTGGCAGGTCATTGATTGTT3'	233
11	*Hydroxyacyl-CoA-dehydrogenase* (H)	F:5'CGATTCCCTTCGTAATGTTG3' R:5'ATAGCTCACTGATGTCAAGTCA3'	244
12	*Carbamoyl phosphate synthetase* (H)	F:5'TGTCACTTCACGCAGGATTG3' R:5'ATAGTTGTCATTTGAGGCACC3'	262
13	*3-oxoacid CoA transferase* (O)[a, b]	F:5'CCTCCTACTTTGGCTCTGACG3' R:5'CACTGAATACGCACTTCTCTGT3'	303
14	*Testes development-relate NYD-SP19* (H)	F:5'TTGCCGTTTACAAGCGACAC3' R:5'AGCACTCTCAAGGCTCCGTT3'	193
15	*Prefold in beta subunit 2* (H)	F:5'TCCAGAAGTTCCAGCAGATG3' R:5'TGGTCCCTATTGTTTGTGAGA3'	208
16	*Calcineurin B* (O)[a]	F:5'TGGGTAACGAAGCCTCATTG3' R:5'ATTCGGAAAGCAAAACGCAA3'	319
17	*Phospholipase C* (O)	F:5'GTCTTACCAGAACTAGCAACCA3' R:5'ACATAATCTCCGACCTTCAAAT3'	188
18	*Female sterile* (O)[a, b]	F: 5'GCAATAACGGTGAACAAGGGA3' R: 5'GCAACCACATTAGTAGCCATA3'	296
19	*ATP/GTP binding protein* (O)[b]	F:5'AGGTTGAGGGCAAGCAGGAT3' R:5'GCACAGCATAGTTGAGGAGAA3'	360
20	*Adenosylhomocysteinase* (O)	F:5'CCATCCAGACTGCTGTGCTC3' R:5'TCCCGTGGTGGTTTCTTCCG3'	314

Table 2.1 (cont.)

No.	Name	Primer sequence	Expected size (bp)
21	*Integrin beta 4 binding protein* (O)	F:5'GCACTCGGAAATGTGGTTG3' R:5'CCTGGTTGGAAATGACTGAAT3'	166
22	*Cop 9 constitutive photomorphogenic homolog subunit 6* (O)[a]	F:5'CAGTGATGTTAGCCCAGGAA3' R:5'CCAGCCAAGGAGGTCAAGGT3'	360
23	*Aspartate aminotransferase* (O)[b]	F:5'AGGAATGCCTATGCTGTGCG3' R:5'CTGGATTACTAAGAATGGTGGA3'	334
24	*Dihydropteridine reductase* (O)	F:5'CGTCTACGGAGGTCGTGGAG3' R:5'GAAGCAGCCTGAGCAGTGAT3'	327
25	*Casein kinase II beta chain* (O)	F:5'TGAACCAGATGAGGAGGAAGA3' R:5'GGAAGCCAGTGCCGAAGTAA3'	320
26	*Mitocondrial oxodicarboxylates*(H)	F:5'GGAAGATGGCAAGAAATGAGG3' R:5'AAGGGTTTGACCAGGATAGC3'	320
27	*Proactivator polypeptide precursor* (O)[a, b]	F:5'GCCATAAAGTTCTGCCCCCACC3' R:5'GCCCTCCCAATATCTACATCCA3'	266
28	*Phosphatidyl inosital* (O)	F:5'CAACGCCATCAACTCCATCAC3' R:5' CTTCCAGCACCACACAGTTTTAT 3'	335
29	*COG4122: Predicted O-methyltransferase* (H)[b]	F:5'AGCACCGTAGAGCGGCGATGTT3' R:5'CGAAGGCGATGACTCCACCAGA3'	399
30	*RAB protein 10 CG 17060-PA* (O)[a, b]	F:5'CTATTACAGAGGGGCAATGGGC3' R:5'TTTTCTTTGGCAATGACACGCT3'	177
31	*TATA box binding protein (TBP)-associated factor, 68kDa, isoform CRA_c* (H)[a, b]	F:5' TATGGGCATCACGGACTACGAG3' R:5' GCAGGCAGAGAGGCAATAACGA3'	270
32	*Solute carrier family25 ,member 5;2F1: adenine nucleotide transiocation2 (fibroblast)*(O)	F:5'GTCCGCATCCCAAAGGAACGAG 3' R:5'CGAGCGAAGTCAAGGGGGTAGA3'	239
33	*Ras interacting protein RIPA* (O)	F:5'GTGAGAGTGAGGGGGAAGAGGGTAA3' R:5'GGGAGGTTTGGTTTTTGGGGG3'	263
34	*Carboxyl esterase precursor* (H)	F:5'GCAAGACAACATCCGTGACCTC3' R:5'GGGACATTCCTCAAACAAGCGA3'	282
35	*US small nuclear ribonucleoprotein* (O)	F:5'GCGAGAAAGTGCCAGAACAGATGACG3'R :5'CAGGAGAGGAATTAGGAAGGCCAAAG3'	277
36	*Stromal membrane associated protein* (H)	F: 5'CTTTCTTACCTGACGCTGGATGCTGA3' R: 5'TCGGTGTCCACAAGAGCGGAAGCAGC3'	306
37	*Tissue specific transplantation antigen p35B like protein* (O)	F:5'ATGGTTTTTGGGCTCATTAGTG3' R:5'ATTGAATCGTAGGAAATCGCAG3'	352
38	*Phosphopyruvate hydratase* (O)	F: 5'GGAGAACTGGACCAAGATGACC3' R: 5'CTCACCAGACCTATGGGAAACC3'	233
39	*Fructose 1,6-bisphosphate aldolase* (H)	F: 5'CTCGCTGCTGATGAGTCTGTCT3' R: 5'TTCTGGTAAAGGGTTTCGTGGA3'	170
40	*High mobility group protein DSP1* (O)	F: 5'CGCTGGAAGACGATGACTGATA3' R: 5'TGGTGGTGTGTTACTCCAGGCA3'	285
41	*Domain family member*(O)	F:5'GCTGGCCCTTTTCAATGTCCTA3' R:5'GAAGCAAACATCGTCAGGAACC3'	318
42	*TRAP-like protein precursor*(H)	F:5'CCAATGGTGCCAAGGATGTT3' R:5'TAGTAGATGAGGATAGCGAAGATG3'	198
43	*C-myc binding protein (AMY-1)* (H)	F:5'GTCTGGCGTAATGTCGGCACTG3' R:5'CCTCTGGTGCTGCTTTGGGTTC3'	278
44	*Fus prove protein* ((H)	F:5'GAGATTGGAAGTGCCCTGTGCC3' R:5'GTGTTTCGCCATTCAGGCAGTT3'	353

Table 2.1 (cont.)

No.	Name	Primer sequence	Expected size (bp)
45	*Translocon associated protein gamma* (H)	F:5'CAGGACTTCAGTCGGAGTGTGT3' R:5'ATCTTCTTGTCAGCATCCAGG3'	278
46	*ETS1 protein* (H)	F:5'CGTGGACTGGCGACGGCTGGGA3' R:5'GGGGCGGGGAGGGCAAGGCA3'	305
47	*Cyclophilin* A (O)	F:5'AAAAAGGTGTGGGCGGCTAA3' R:5'ACAACGGCATCTAACTGAAC3'	310
48	*Chromobox protein* (O)[b]	F:5'TGGGAACCTGAATCTCATCTTG3' R:5'ACGAACATTTGCCTGCCTTG3'	303
49	*mRNA splicing factor (deahbox)* (H)	F:5'AATCCTTTTACGAATCTGCCAT3' F5'TTAGGAAAATGCTTAGAGGGTA3'	142
50	*Cdc2 homologue* (O)	F:5'ACTTTCTTCTCGGTATTCTATTGTCG3' R:5'CCCACGTCCGTCGCCTTTGGTTTT3'	
51	*Postsynaptic density protein (citron)* (H)	F:5'TAGAGGAAGCAGGGATGGTC3' R:5'GTCTGAAGCGGCAGCACACA3'	212
52	*Methylcytosine* (H)	F:5'ATGTCTTTACCACGACCCTTG3' R:5'CGGTGATATCCCATAGCCAG3'	380
53	*Kin protein* (H)	F:5'CCAGGCTGTCCTTGACCGTAAC3' R:5'AATCCGTGCGAACCTGGTCTTT3'	188
54	*USO1* (H)	F:5'GCTGACCTATTCCTGCGTCTTTG3' R:5'TCGTGTTTCTTGGCGACCCTTTG3'	314
55	*Small androgen receptor interacting protein* (O)[b]	F:5'ATGACAGACTACAAGGAAGAACAGAA3' R:5'CTCCAATGAGTATGAGACAAGCG3'	345
56	*Broad complex Z4 isoform* (H)[b]	F:5'CTCAGAATTAAGGGCTTGGCAG3' R:5'TGGAGGTGTTACCGATGGCTGC3'	192
57	*Proteosome (proteosome subunit alpha type3)* (H)	F:5'GAGTATGCCCAAAAAGCAGTCG3' R:5'AGGTGCTCACTCGTTCTGCCAA3'	283
58	*Signal recognition particle 72 kDa* (H)	F:5'AGAAGAGGAAGTGGCTGATGAG3' R:5'GCAAGTAAGCAGTGATTGAAGG3'	291
59	*Zonadhesin isoform 4* (O)[b]	F:5'CCTGGGCGTAGCTAATCTTAAC3' R:5'TCGGTAGGGCCATATCCTCTCC3'	177
60	*Microspherule protein 1*(H)	F:5'GGAGGACCAGGTTTCTAAGTGGCAAG3' R:5'ATTATTCATTATCCATTTCCATTGTG3'	317
61	*RUVB like protein2* (O)[b]	F:5'TGGCAGGTCAGGCAGTTCTCAT3' R:5'CCAGTGGCAGGACGGTCAATCT3'	257
62	*Cystathionine gamma lyase* (T)	F:5'CCCAGCAGATTTTAAGGCATTTGA3' R:5'GTGCGTGATGGTGGTTGTCG3'	145
63	*Splicing factor 3A* (H)	F:5'AGTTGAAAGAACGAGGTGGACG3' R:5'TACCCAATACACGCAAATAACC3'	200
64	*Dynectin 4* (H)	F:5'AAGTTATCGTGCTTAGTGGTGTG3' R:5'TCTCGGTGAGGCAAGACTGTTT3'	300
65	*Contractile ring component anillin* (H)	F:5'TGTTTGAGGATGTTGGGGGCT3' R:5'AACTGGAAGGTATGCTGACGGG3'	199
66	*NADPH-ferrihemoprotein reductase; NADPH-cytochroome P450 reductase* (O)	F:5'CCACCCTCAGTCTGGCAAATCT3' R:5'GCTTTATTTAGCCTTCAGCCGAGA3'	189
67	*CG-1681-PA* (O)[b]	F: 5'ATCAAAGCCATTCATTGCGAGC3' R:5'AACCAGACAAAAATAAAACCAAAT3'	243
68	*Nuclear autoantigenic sperm protein* (O)[a, b]	F:5'AGGAAATGGAAACTGATGTCGC3' R:5'TTCTTAGCCATCTCTGGGTTGT3'	301

Table 2.1 (cont.)

No.	Name	Primer sequence	Expected size (bp)
69	*Sperm tail specific protein mst 101* (O)	F: 5'AGCCAAAGATTAGGAAAACGAA3' R:5'GCCAGGGGTGCTTAGAATAGGT3'	301
70	*Ferritin* (O)[a]	F:5'CCAAGTCCGCCAGAACTACCAT3' R:5'GGCTTCCAGACCAGTGCCCCAT3'	280
71	*Rho protein* (H)	F:5'CTGGCTCGTCTCCCTCTTCGTG3' R:5'TCGTAGTCCTCCTGACCTGCTG3'	238
72	*Nm2 protein non-histone chromosome protein 2-like* (H)	F:5'AGGCAACACTCACCCAGAAG3' R:5'TTGATACACCACAGGCACGG3'	365
73	*Nme2 protein* (H)	F:5'TTGCTGACAAGCCCTTCTACCC3' R:5'TGTGCCACTAATCCACCTACTG3'	365
74	*Phosphatidyl serine receptor short form* (O)[b]	F:5'CAAGTGTGGAGAAGATAATGAAGG3' R:5'CCAGCGGTAAGGGGGGCGTC3'	300
75	*Tetratricopeptide repeat domain 5* (H)	F:5'TGCGGCTGTCAAAAAGAACCAA3' R:5'GGCACTCTCCAAGCTCGTTCCA3'	227
76	*Translationally controlled tumor protein* (H)[a]	F:5'AGTATGAGGAGGTGGATGATGC3' R:5'GAAGCCGGTTTCCTGCAGACGC3'	182
77	*Interleukin 1 receptor like 1 ligand precursor* (H)	F:5'CTTCTTTGGTGTTGTGATTGAG3' R:5'AAGCGTGACTTTGTGAGATGGT3'	162
78	*Cardiomyopathy associated 4 sterile muscle* (H)	F:5'CCTGACGCTGGATGCTGATGTT3' R:5'TGTCCACAAGAGCGGAAGCAGC3'	294
79	*Singed protein* (H)	F:5'TGTCCGTGACCGACGACGAGCA3' R:5'ATCTCGGTGCCCTCCTCGTTCA3'	214
80	*Gelsolin, Cytoplasmic (actin depolymerizing factor)*(H)	F:5'GCAGAGACTTACATCAAGACCG'3' R: 5'CTGAAGGAGACACCCCATTTAG3'	253
81	*Wolf hirschorn syndrome candidate 1 protein* (H)[b]	F:5'ATGGCACCACTGTGATGAATGT3' R:5'GGTCAACTGATAACAAAGCCTC3'	208
82	*Ornithine decarboxylase* (H)	F: 5'GCAAACGGCACGCTTCTTTCCA3' R:5'CAGCCAGTCTCCACAAGCCAGC3'	255
83	*Programmed cell death protein6* (H)	F:5'CAGGGTGCCACCGCGACTCTTT3' R:5'GGCTTGCCATTGTCCACCTCAG3'	303
84	*Heterogeneous nuclear RNA protein clone* (H)	F:5'ACTTCCAGGGCAACGGTATG3' R:5'ATGGCGTGCTTTGGCTTTCT3'	350
85	*Poliferating cell nucleolar antigen p120* (H)	F:5'ACCAGTGACCATCCGCACCAAC3' R:5'TCCATTTTTGGGTGCCAGAGCC3'	235
86	*Multicatalytic endopeptidase* (O)	F: 5'GCCGAGTGTTCCAAGTAGAGT3' R: 5'ACATCAACGATAGCACGAGCA3'	217
87	*Ovarian lipoprotein receptor* (O)[a, b]	F:5'CGGGATGAGTGCGAGAAGTGC3' R:5'CAGGGGCTCCGAGTCAAAGA3'	354
88	*Thiolase* (O)[a]	F:5'ATTCCCGAGTTGCCTGCTGT3' R:5'AACCCTGATGCCATTGTCTT3'	231
89	*Carnitine palmitoyl transferase II* (O)	F:5'CAAGAGCAGTGGCATCATCC3' R:5'AGAACTATTCCTAAGGCGGTCAT3'	334
90	*Chaperon subunit 8*(O)	F:5'CTATTCTGGGCTGGAGGAGGC3' R:5'CAACATCTGTGGGAGTGAGGC3'	323
91	*Ferrochelatase* (O)	F:5'GCTGCCATTCCAAGATTACAT3' R:5'TTTCAACTCCATCCTCCTCCA3'	215
92	*Presenilin enhancer* (O)	F:5'CAAGCCAAGCGACCATCCAT3' R:5'TAATCCAACGAGCCATACAA3'	244
93	*Innexin 2*(O)	F:5'CCTTCACGATACCCTCCTTG3' F:5'ACCTTACCACCTTCCCAGAT3'	208
94	*Tetrasparin D 107* (H)[a]	F:5'GTTCTTTGATGGCAAGTTCG3' R:5'CGTTCCACAGCAATGGTAGTTC3'	316

Table 2.1 (cont.)

No.	Name	Primer equence	Expected size (bp)
95	*HLA-B associated transcript 1A, nuclear RNA helicase bat 1*(O)	F:5'AGGCAGGAGTAGGAGAAACG3' R:5'AAGGATGCTCAAAGCCACAG3'	150
96	*Heat shock protein 70(HSP70)*(H)	F:5'TGGTTGCTGTAGGAGAGGG3' R:5'TTTTACTCGTTCTTCATCTTGG3'	176
97	*Semaphorin 2A precursor* (H)	F:5'CTTGGTGGTGATGAATGTAA3' R:5'GGATGGCTGTTCTGCTGGCT3'	256
98	*Profilin* (O)	F:5'GCTGATGGCTCTGGCTATG3' R:5'TCCTGCCTTCCCTTTCTTGC3'	229
99	*Phosphoglucose isomerase* (H)	F:5'TTCTGGGACTGGGTTGGTGG3' R:5'TGGCAATAAGGCATGGGTTT3'	205
100	*Ribophorin I* (O)	F:5'CGACTTCCAGAGAGAGCACA3' R:5'GGTCTTCCATCCTCCAAACA3'	190
101	*AgCP13148* (O)	F:5'CATACCTCGCATCATCAGTG3' R:5'CCTCAGGAGACGATACAAAGC3'	212
102	*Immonophilin FKBP 52* (O)[a]	F:5'GAGAAGAAAGACGGAGGAGTG3' R:5'TATCAGGGTGGCATTTGGCG3'	312
103	*Mapre 1 protein* (H)	F:5'CAGTTGAGAACCTGAGCCGC3' R:5'TGGAGTGCCTTGAAGTTATTGA3'	205
104	*Calcium independent phospholipase A2 isoform1*	F:5'TGTCCCACAGCACCAGTAAT3' R:5'CCAGCCTTCACCTCCTCTTC3'	164
105	*Zeta 1 cop* (H)	F:5'TGGTGTCAGTGTTGAGTTGTCT3' R:5'GCTCTCCTCCAGCCTTAGTGC3'	191
106	*Muskelin 1* (O)	F:5'GTCACGATGGTCTTCCGATTC3' R:5'GTTCCAGTCCTCACAGCCTCC3'	346
107	*Nonclathrin coat protein zeta 2*(H)	F:5'GTGTTGAGTTGTCTCTATGATGC3' R:5'ACAGTCTGGTCGTTGAATGG3'	203
108	*Dendritic cell protein* (H)	F:5'AAGCCAGCAAAGTGATGATT3' R:5'CAAGAGGTCGTGGATGAGTTC3'	185
109	*Calcium regulated heat stable protein*(H)	F:5'CCAAACAAGGGCTTTCTGCTG3' R:5'CGCTTCGGTGGGATGGGACA3'	263
110	*Nit protein 2*(H)	F:5'TTGGTTGCCCTGCCTGAGTG3' R:5'CGGGTGATAGGACATCGGAC3'	292
111	*Endothelial cell growth factor 1*(H)[b]	F:5'GGAGGTCGGCTGCTGTATCG3' R:5'GCCACATCCACCATTTTCTT3'	243
112	*Clatrin adaptor protein AP 50* (H)	F:5'TCCCAACTCCACTGAATACTT3' R:5'TGAGCTTCGACTCTAAACACCTT'	267
113	*Synaptic vesicle-associated integral membrane protein* (H)	F:5'GCCAATGTTATGCCGTCTGC3' R:5'ACCGCTTTCGCTGCTGCTCT3'	187
114	*Carbonic anhydrase* (O)	F:5'TGCGACGGTAACCTAACTGC3' R:5'TTGCCAACAACAGGAAACAT3	332
115	*Peroxinectin* (H)	F:5'TGGACGCCATTACACGAAGC3' R:5'CACCAACCACGCACAGGAAG3'	366
116	*Calponin I* (H)	F:5'GCCGCCGAGTGCTTGGAATG3' R:5'CCTTCCTGCCCAGAGACT3'	316
117	*Defender against cell death 1* (T)	F:5'CGATGCCTACCTCTTCTACG3' R:5'GATGAAATCAGCAAAGCCTC3'	214
118	*Receptor activating protein kinase C* (O)	F:5'AGACCACCCGTCGCTTTGAG3' R:5'AACCTTACGCAGGACACCCA3'	187
119	*Dynein heavy chain* (H)[a]	F:5'CAGTCTGCTGTTACTTGGCTATC3' R:5'GGTCTGTTTCTTCTCACCCTTG3'	192
120	*Glycogen phospholipase* (H)	F:5'GGCTTCCTTGACCGTAACTT3' R:5'CGAAATCCGTGCGAACCTGG3'	188

Table 2.1 (cont.)

No.	Name	Primer equence	Expected size (bp)
121	*L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain* (O)	F:5'GTGGCAGCAGCCGTAGATAG3' R:5'GGAGGAGAGATGTTCAAGATGT3'	221
122	*Pre B cell colony enhancing factor* (O)	F:5'GAACGACGACCCTCTACTCC3' R:5'TCCCTCTGGCACAGCCTTGA3'	239
123	*Diphenoloxidase A2* (H)	F:5'ACAATAATGAGGTAGCACGGT3' R:5'CGGAAAATCTGTCTCTCTGGGA3'	205
124	*Glutathione peroxidase* (H)	F:5'AACTGGCTTCCTCCGCTATC3' R:5'TGAGTTGGTTCATCAGGTGG3'	145
125	*Serine proteinase inhibitor* (O)	F:5'GGAAGAAAGAAGCAAAGTC3' R:5'CCAGTCCTCCAATGTCAGCA3'	293
126	*Aminopeptidase* (O)[b]	F:5'TGGCTCGGCAAGGCTGGCTG3' R:5'CGTGGAGTGCGTGAAGAAAGG3'	174
127	*Vacuolar type H+ ATPase subunit A*(H)	F:5'GACCTCACAGAGTCCATTTACA3' R:5'GGAGTTCTCAGGCACAATACCA3'	150
128	*Asparaginyl tRNA synthetase* (O)[a, b]	F:5'GCTGTCCAACCCTGATGTGC3' R:5'AGATGCCTTCTGTGCGTGA3'	330
129	*Methyl CpG binding protein 2* (H)	F:5'ATTTCCGCTTCAGACACTCCA3' R:5'CATCATTCCATTATGTTTCCTT3'	206
130	*Y-box protein Ct50* (O)[a, b]	F: 5'CGGAGACACAAGCCAAGCCT3' R: 5'GGTGGAACCCAACCAGCAAC3'	435
131	*Hypothetical protein XP 207715 cyclin nucleotide(cyclic nucleotide gated channel beta subunit 1)* (O)	F:5'TCTCCCTTCTATGCCTGTGTCC3' R:5'GCTCGCTTCAACCAAACTGC3'	270
132	*Adipose differentiation related protein* (O)[a, b]	F:5'TCTTGCTCTTGCCTGTGCTT3' R:5'CCGTTGGCTTGGTTATGATG3'	282
133	*NADP-dependent leukotriene B4 12-hydroxydehydrogenase* (O)[b]	F:5'AGAGGGGATGCCCAAGAAGG3' R:5'AGCCAGACGGTGAAACAACG3'	230
134	*Leukemia virus receptor* (H)	F:5'GATGGCGGTCTTCAGGTGTC3' R:5'ATGGAAAGTGCGTGATGGGT3'	230
135	*Myosin regulatory light chain polypeptide 91* (H)	F:5'ATCCCGTAAGGCAGGAAAGA3' R:5'CACCAAACAGAGTGAGGAACAT3'	269
136	*Hepatocarcinogenesis-related transcription factor (X-box protein)* (O)	F: 5'TGATGAACTTCGGGACCTAA3' R: 5'CCTCAACGACAACTGCTGCG3'	230
137	*Cyclic AMP regulated protein* (H)	F:5'AAGGGACCCCGTCAATCCACTG3' R:5'CGTGATTTTATCCGAACGAAGCCG3'	264
138	*Vitellogenin* (O)	F:5'AGCCGAAGAAACGAAGGGA3' R:5'CACTACATCCAAAGCAACTGTCTCG3'	
139	*Phospholipase C* (O)	F: 5'TCTTTGTCCATTTGAAGGTCGG3' R: 5'TCATTGGCTGTCGTTGTAGTGT3'	311
140	*Death box protein 15* (O)	F: 5'AATCCTTTTACGAATCTGCCAT3' R: 5'CGGTCTCTCCAACTAATACAAT3'	
141	*ATP-dependent RNA helicase* (O)	F:5'GAAGGGAAGGCAGCCAGGAG3' R:5'CGACGGATTTCAGCCATTACATAG3'	
142	*splicing factor 3a,subunit 1*(H)	F:5'AGTTGAAAGAACGAGGTGGACG3' R:5'TACCCAATACACGCAAATAACC3'	
143	*DNA primase* (O)	F:5'GAGCCGCAGAGTTGAGGTTG3' R:5'TCGTTTCTTGTCCAGGTTTTTG3'	256
144	*Finger protein* (H)	F:5'CTGTGTGCGGAAGGAAGTTTAC3' R:5'GAGCATCACTCGTGTTTGGGGT3'	378

Table 2.1 (cont.)

No.	Name	Primer sequence	Expected size (bp)
145	*FK 506 binding protein 4* (H)	F:5'ACAGGGAGCGAGGAGGGG3' R:5'AGGGTGGCATTTGGCGGG3'	446
146	*Chloride intracellular channel 6-like protein* (H)	F:5'TCTCTCTGAAGGTCACCACTGTCG3' R:5'CGCAACATCCTTATCCTGAACAAA3'	191
147	*ZZZ3*(H)	F:5'AGGAGCAAGGCAGTTCGGGACA3' R: 5'GTTTGGGGTTTACTCTCATCGT3'	315
148	*ESO 3 protein* (H)[a]	F:5'GTAACAAATGCCATGGGTGAAA3' R:5'GCTGTACTGGAGGTCCAAACTG3'	349
149	*Rasputin CG 9412* (O)	F:5'GGAGGGAGGAAGTGAAGTGG3' R:5'TTGGAGAAAGAGGGGCAGTG3'	248
150	*Survival motor neuron* (O)[a]	F:5'TAACGATAAGGAACTCACCCAT3' R:5'CATCACCTGTGTGCCACTCAAT3'	245
151	*Exocyst complex component sec 6* (H)	F:5'GAACATCATTCACAAGACAGCC3' R:5'AAGTTAGGGTCCAACATTACGG3'	305
152	*Heterotrimeric GTP binding protein* alpha subunit G-alpha-q(H)	F:5'CAAACAGTGAACACGCAGAT3' R:5'GCTCGGTCGGTAAGAAGTCC3'	216
153	*Dolichyl diphosphooligosaccharide protein glycotransferase* (H)[a, b]	F:5'TTCTGGCAACGGCAAAGTAG3' R:5'ATGGGTCAATGCGAACAAAG3'	233
154	*Small ubiquitin-like modifier*(O)	F:5'GGAAGGGAACGAATACATCAAA3' R:5'GCCTGGTCTGTGCGAAAATCTC3'	
155	*Heterogeneous nuclear ribonucleoprotein 87F* (O)	F:5'TTTTCCTACAATGGAGGCAAGTGGCT3' R: 5'AGGCTACAACAACCCTGGTCCTAACA3'	298
156	*Keratinocyte associated protein 2* (O)	F:5'AGGGAAGGGGAGGAGGACCAGT3' R:5'TAGGACGAGACACAGGGCGACC3'	318
157	*PeF protein with along N-terminal hydrophobic domain*(H)	F:5'CAAGGGTATGGGCATCCACAGG3' R:5'GGTCCACAGCACGGAACCAAGAGA3'	314
158	*Prophenoloxidase activating factor* (H)	F:5'TTGGTCTTGCTTCCCTCTAC3' R:5'TTATTTTGTATCTCCTGCTCG3'	154

H= EST from haemocytes, O = EST from ovaries, T = EST from testes ,[a] = the amplification product in shrimp having different GSI was further examined by SSCP analysis, [b] = tissue distribution analysis was carried out

A 100 bp DNA ladder was used as the standard DNA marker. Electrophoresis was carried out at 5 - 6 volts/cm until bromophenol blue move to approximately one-half of gel.

The electrophoresed gel was stained with an ethidium bromide solution (0.5 μg/ml) for 5 min and destained in the water to remove unbound ethidium bromide from the gel. DNA fragments were visualized under a UV transiluminator and photographed through a red filter using Formapan Classic 100 film. The exposure time was 10 - 18 seconds.

2.6 Tissue distribution analysis of genes exhibiting sex-specific or differential expression patterns

2.6.1 Total RNA extraction and the first strand cDNA synthesis

Total RNA was extracted from eyestalk, gills, heart, hemocytes, hepatopancreas, lymphoid organs, intestine, ovaries, pleopods, stomach, testes and thoracic ganglion of broodstock of *P. monodon*. The first strand cDNA was synthesized as described previously.

2.6.2 Tissue distribution analysis by RT-PCR

For the target genes, 150 ng of the first strand cDNA from various tissues was used as the template in 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl and 0.1 % Triton X-100, 2 mM $MgCl_2$, 100 mM each of dATP, dCTP, dGTP and dTTP, 0.2 μM of each primer and 1 unit of Dynazyme^{TM} DNA polymerase (FINNZYMES). *Elongation factor-1α* (F: 5′-ATGGTTGTCAACTTTGCCCC-3′ and R: 5′-TTGACCTCCTTGATCACACC-3′) were also amplified from the same template. The reactions were predenaturation at 94°C for 3 min followed by 25 cycles composing of a 94°C denaturation step for 30s, a 53 °C annealing step for 45s and a 72 °C extension step for 45s. The final extension was carried out at 72 °C for 7 minutes. Five microliters of the amplification product was electrophoretically analyzed though a 1.5% agarose gel.

2.7 Single strand conformational polymorphism (SSCP) analysis

The RT-PCR amplified products which exhibited specific expression or differential expression pattern were further characterized using SSCP to examine whether the SSCP pattern of the amplification product in females having different gonadosomatic index (GSI) was identical or not.

2.7.1 Preparation of glass plates

The long glass plate was thoroughly wiped with 1 ml of 95% commercial glade ethanol with a piece of the tissue paper in one direction. This process was then repeated twice. Afterwards, the long glass plate was coated with 1 ml of freshly prepared Bind silane (10 µl of Bind silane, Amersaham Biosciences, 980 µl of 95% ethanol and 10 µl of 5% glacial acetic acid) and left for approximately 10 - 15 minutes. Excess binding solution was removed with a piece of the tissue paper. The long glass plate was further cleaned with 95% ethanol for 3 times.

The short glass plate was treated as described above with the exception that the binding solution was replaced by the Repel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The cleaned glass plates were assembled with a pair of 0.4 mm spacers.

Different concentrations of low closslink non-denaturing polyacrylamide gels (37.5:1 or 75:1 of acrylamide and bis-acrylamide) were prepared by dilution of a 40% stock solution to required gel concentration. The acrylamide gel solution (30 - 40 ml) was mixed with 240 µl of 10% APS and 24 µl of TEMED. The analytical comb was inserted into the prepared gel and allow for polymerization for at least 4 hours or overnight.

2.7.2 RT-PCR and electrophoresis

RT-PCR was carried out against the first strand cDNA of ovaries of 8 - 12 female broodstock (GSI of 0.65, 0.87, 0.92, 1.10, 1.43, 1.90, 2.02, 2.13, 2.40, 3.02, 4.70 and 5.69%) and that of testes of 8 – 13 male broodstock (GSI of 0.516, 0.518, 0.543, 0.586, 0.605, 0.614, 0.665, 0.666, 0.775, 0.778, 0.844, 0.956 and 1.109%) as the template using identical conditions described previously.

Six microlitres of the amplified PCR products of each of 22 gene homologues were mixed with four volumes of the SSCP loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured in a boiling bath for 5 minutes and immediately cooled on ice for 3 minutes. The denatured products were electrophoretically analyzed in native polyacrylamide gels at 250 - 300 volts for 14 - 18 hours at 4°C.

2.7.3 Silver staining

After electrophoresis, the gel plates were carefully separated apart. The long glass plate with the electrophoresed gel was placed in a plastic tray containing 1.5 liters of the fix/stop solution (1% glacial acetic acid in deionized water) and agitated well for 25 - 30 minutes. The gel was briefly soaked in three times deionized water for 3 minutes with shaking. The gel was lifted out from the tray between each wash and allowed the washed water draining out of the gel for 5 seconds. The gel was transferred to 0.1% silver nitrate solution (1.5 liters) and incubated with agitation at room temperature for 30 minutes. The gel was soaked in 1.5 liters of deionized water with shaking (10 forward and 10 backward steps) and immediately placed in the tray containing 1.5 liters of the chilled developing solution. This step is critical and the time taken to soak the gel in the water and transfer it to chilled developing solution (3% Na_2CO_3 dissolved in 3 litters of deionized water, 0.075% formaldehyde and 1% sodiam triosulfate) should be no longer than 5 - 10 seconds. The gel was well agitated until the first bands are visible (usually 1.5 - 2 minutes) and then transferred to another tray containing 1.5 liters of chilled developer and shaken until bands form every lanes were observed (usually 2 - 3 minutes) One liter of the fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes each. The gel was placed in the plastic bag and air-dried.

2.8 Isolation and characterization of the full length cDNA of functionally important gene homologues of *P. monodon* using Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE – PCR)

2.8.1 Preparation of the 5′ and 3′ RACE template

Total RNA was extracted from ovaries of *P. monodon* broodstock using TRI-REAGENT. The quality of extracted total TNA was determined by agarose gel

electrophoresis. Messenger (m) RNA was purified using a QuickPrep *micro* mRNA Purification Kit (Amercham Phamacia Biotech.). The RACE cDNA template was prepared by combining 1 µg of ovarian mRNA with 1 µl of 5′-CDS primer and 1 µl of 10 µM SMART II A oligonucleotide for 5′-RACE-PCR or 1 µg of ovarian mRNA with 1 µl of 3′ CDS primer A for 3′ RACE-PCR (Table 2.2). The components were mixed and spun briefly. The reaction was incubated at 70°C for 2 minutes and snap-cooled on ice for 2 minutes. The reaction tube was spun briefly. After that, 2 µl of 5x First-Strand buffer, 1 µl of 20 mM DDT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript Reverse Transcriptase were added. The reactions were mixed by gently pipetting and centrifuged briefly to collect the contents at the bottom of the tube.

The tube was incubated at 42 °C for 1.5 hours in a thermocycler. The first strand reaction products were diluted with 125 µl of Tricine-EDTA buffer and heated at 72°C for 7 minutes. The first strand cDNA tenplate was stored at -20.

2.8.2 Primer designed for RACE-PCR and primer walking

Gene-specific p rimers (GSPs) were designed from ovaries and hemocyte libraries. The antisense primer (and nested primer) was designed for 5′ RACE-PCR and the sense primer (and nested primer) for the 3′ RACE-PCR (Table 2.3).

For sequencing of genes that showed the full length from the 5′ direction, the product from colony PCR was considered. If the insert was larger than that of the homologues, the 3′ direction was sequenced. Internal primers were designed for primer walking of the inserted cDNA (Table 2.4).

Table 2.2 Primer sequence for the first strand cDNA synthesis and RACE-PCR

Primers	Sequence
SMART II A Oligonucleotide	5′-AAGCAG TGG TATCAACGCAGAGTACGC GGG-3′
3′ RACE CDS Primer A	5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ N_{-1} N-3′ (N=A, C, G orT; N_{-1}= A,G or C)
5′ RACE CDS Primer	5′-$(T)_{25}$ N_{-1} N-3′ (N=A, C, G orT; N_{-1}= A,G or C)
10X Universal PrimerA Mix (UPM)	Long : 5′–CTAATACGACTCACTATAGGGCAA GCAGTGGTATCAACGCAG AGT-3′
	Short : 5′–CTAATACGACTCACTATAGGG C – 3′
Nested Universal Primer A (NUP)	5 – AAG CAG TGG TAT CAA CGC AGA GT -3′

Table 2.3 Gene-Specific Primers (GSPs) and nested GSP used for isolation of the full length cDNA of functionally important genes in *P. monodon*

Gene specific primer	Sequence	Tm (°C)
Adipose differentiation related protein		
5′ RACE	R: 5′-CCATCTGTGTCGTGAAGGTCG-3′	68.4
Asparaginyl tRNA synthetase		
5′ RACE	R: 5′-GAACGCAGCCGCAGCACCTTAGAG-3′	63.0
Aspartate amino transferase		
5′ RACE	R: 5′-GGCTACATCTCCCAGGGCAAATCC-3′	69.1
Dolichyl diphosphooligosaccharide protein glycotransferase		
5′ RACE	F: 5′-TGTTCAATAGTAACGACACGCAGGAGCC-3′	71.3
Nested DDPG	R: 5′-GCCCTCCTCCCGAAATACCCAGCGA-3′	75.5
Endothelial cell growth factor 1		
5′ RACE	R: 5′-GGACGAGACGATGAGCGGCACAGAT-3′	72.4
3′ RACE	F:5′-TGGAGTGTCTTCGGGGCAACGGA-3′	72.4
Female sterile		
5′ RACE	R: 5′-CTCCCTTGTTCACCGTTATTGC-3′	61.3
Nuclear autoantigenic sperm protein		
5′ RACE	R: 5′-CAGCAAGGACAGACTCCAGAAAGCGGC-3′	74.1
Ovarian lipoprotein receptor		
3′ RACE	F:5′-CGAAAGACGGCGAAGATGTAAGAGG-3′	67.6
3-oxoacid CoA transferase		
5′ RACE	R: 5′-ACCCAAGCCCAGCACCCCGTTCTCAC-3′	66.0

Table 2.4 Internal primers used for primer walking sequencing of the full length cDNA of functionally important genes in *P. monodon*

Internal primer	Sequence	Tm (°C)
ATP/GTP binding protein		
5′ internal primer-I	F:5'TATGCTGTGCGGATGGGACG3'	64.8
Dolichyl diphosphooligosaccharide protein glycotransferase		
5′ internal primer-I	F: 5′-TTACAGTTCCACTCAGGTATCAG-3′	54.1
Endothelial cell growth factor 1		
5′ internal primer-I	F: 5′-GTAACGCACCTGGGCGGAGAAT-3′	57.0
5′ internal primer-II	F: 5′-GCTAAGTGCTCGCTAAACA-3′	45.0
Female sterile		
3′ internal primer-I	F: 5′-CCACATTAGTAGCCATAACATC-3′	53.0
3′ internal primer-II	F: 5′-ACAACTTGGTGGTGCTCTCG-3′	51.0
5′ internal primer-I	F :5′-TGATAGCCTGGAGGATGA-3′	45.0
5′ internal primer-II	F: 5′-TAGTGGTTTGGGAGCCTGTC-3′	51.0
Nuclear autoantigenic sperm protein		
5′ internal primer-I	F: 5′-AGGAAATGGAAACTGATGTCGC-3′	54.0
Ovarian lipoprotein receptor		
3′ internal primer I	F: 5′-CCAAGAGGGTGGATGTGTGTGAT G-3′	62.3
3′ internal primer-II	F: 5′-AGCACAGAAAGCGTCCTCAG-3′	57.8

2.8.3 RACE-PCR

The master mix which is sufficient for 5′ and 3′ RACE-PCR and the control reactions was prepared (Tables 2.5 and 2.6) . For each 25 µl amplification reaction, 14.0 µl sterile deionized H_2O, 2.5 µl of 10x Advantage® 2 PCR buffer, 0.5 µl of 10 uM dNTP mix and 0.5 µl of 50x Advantage® 2 polymerase mix were combined. The reaction was carried out for as described in Table 2.7.

Table 2.5 Compositions for amplification of the 5′ end of gene homologues using 5′ RACE-PCR

Component	5′ RACE-PCR	UPM only (Control)	GSP1 only (Control)
5' RACE–Ready cDNA template	1.5 µl	1.5 µl	1.5 µl
UPM (10x)	5.0 µl	5.0 µl	-
GSP1 (10 uM)	1.0 µl	-	1.0 µl
GSP2 (10 uM)	-	-	-
H_2O	-	1.0 µl	5.0 µl
Master Mix	17.5 µl	17.5 µl	17.5 µl
Final volume	25 µl	25 µl	25 µl

Table 2.6 Compositions for amplification of the 3′ end of gene homologues using 3′ RACE-PCR

Component	3′ RACE-PCR	UPM only (Control)	GSP1 only (Control)
5' RACE–Ready cDNA template	1.5 µl	1.5 µl	1.5 µl
UPM (10x)	5.0 µl	5.0 µl	-
GSP1 (10 uM)	1.0 µl	-	1.0 µl
GSP2 (10 uM)	-	-	-
H_2O	-	1.0 µl	5.0 µl
Master Mix	17.5 µl	17.5 µl	17.5 µl
Final volume	25 µl	25 µl	25 µl

Table 2.7 The amplification conditions for RACE-PCR of various gene homologues of *P. monodon*

Gene homologue	Amplification condition
Adipose differentiation related protein	
5′ RACE	22 cycles of 94 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min and the final extension at 72 °C for 7 min
Aspartase amino transferase	
5′ RACE	22 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
Dolichyl diphosphooligosaccharide protein glycotransferase	
5′ RACE	5 cycles of 94 °C for 30 s and 72 °C for 2 min 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 2 min 20 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 2 min and the final extension at 72 °C for 7 min
Nested DDPG	1 cycles of 94 °C for 3 min 23 cycles of 94 °C for 30 s and 72 °C for 2 min and the final extension at 72 °C for 7 min
Endothelial cell growth factor 1	
5′ RACE	20 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
3′ RACE	22 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min and the final extension at 72 °C for 7 min
Female sterile	
5′ RACE	22 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min and the final extension at 72 °C for 7 min
PCR using primers flank 3' UTR	1 cycle of 94 °C for 30 s 22 cycles of 94 °C for 30 s, 53 °C for 45 s, 72 °C for 45 s and the final extension at 72 °C for 7 min
Nuclear autoantigenic sperm protein	
	5 cycles of 94 °C for 30 s and 72 °C for 3 min 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min 20 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min and the final extension at 72 °C for 7 min
Ovarian lipoprotein receptor	
5′ RACE	22 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min and the final extension at 72 °C for 7 min
3–oxoacid CoA transferase	
5′ RACE	5 cycles of 94 °C for 30 s and 72 °C for 2 min 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 2 min 20 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 2 min and the final extension at 72 °C for 7 min

The primary 5′ and 3′ RACE-PCR product were electrophoretically analyzed through 0.8 – 1.0 agarose gels. If the discrete expected bands were not obtained from the primary amplification, nested PCR was performed using the recipes illustrated in Tables 2.5 and 2.6. The primary PCR product was 50-fold diluted. The primary PCR product was performed using 1 - 5 µl of diluted PCR product as a template using the conditions described in Table 2.7.

2.8.4 Elution DNA fragments from agarose gels

After electrophoresis, the desired DNA fragment was excised from the agarose gel using a sterile scalpel and placed in a pre-weighed microcentrifuge tube and eluted out from the gel using a HiYield™ Gel Elution Kit (RBC). Five hundred microlitres of the DF buffer was added to the sample and mixed by vortexing. The mixture was incubated at 55 °C for 10 - 15 min until the gel slice was completely dissolved. During the incubation period, the tube was inverted every 2-3 min. A DF column was placed in a collection tube and 800 µl of the sample mixture was applied into the DF column and centrifuged at 6,000 *g* (8,000 rpm) for 30s. The flow-through was discarded. The DF column was placed back in the collection tube. The column was washed by the addition of 500 µl of the ethanol-added Wash Buffer and centrifuged at 6,000 *g* for 30s. After discarding the flow-through, the DF column was centrifuged for 2 min at the full speed (14,000 rpm) to dry the column matrix. The dried column was placed in a new microcentrifuge tube and 15 µl of the Elution Buffer or water was added to the center of the column matrix. The column was left at room temperature for 2 min before centrifuged for 2 minutes at the full speed to recover the gel-eluted DNA.

2.9 Cloning of the PCR product

2.9.1 Ligation of the PCR product to the pGEM®-T Easy vector

DNA fragments was ligated to the pGEM®-T Easy vector in a 10 µl reaction volume containing 5 µl of 2x Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DDT, 2 mM ATP and 10% PEG8000), 3 Weiss unit of T4 DNA ligase, 25 ng of the pGEM®-T Easy vector and approximately 50 ng of the DNA

insert. The reaction mixture was incubated overnight at 4 - 8 °C before transformed to *E.coli* JM 109 (or XL1-Blue)

2.9.2 Transformation of the ligation product to *E.coli* host cells

2.9.2.1 Preparation of competent cell

A single colony of *E. coli* JM109 (or XL1-Blue) was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl, pH 7.0) with vigorous shaking at 37 °C overnight. The starting culture was then inoculated into 50 ml of LB broth and continued culture at 37 °C with vigorous shaking to OD_{600} of 0.5 to 0.8. The cells was briefly chilled on ice for 10 minutes and recovered by centrifugation at 2700 *g* for 10 minutes at 4 °C. The pellets were resuspended in 30 ml of ice-cold $MgCl_2/CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged as above. The cell pellet was resuspended with 2 ml of ice-cold 0.1 M $CaCl_2$ and the cell suspension was divided into 200 µl aliquots. These competent cells was either used immediately or stored at -80°C for subsequently used.

2.9.2.2 Transformation

The competent cells were thawed on ice for 5 min. Two to four microlitres of the ligation mixture were added and gently mixed by pipetting. The mixture was left on ice for 30 min. During the incubation period, the ice box was gently moved forward and backward a few times every 5 min. The transformation reaction was heat-shocked in a 42 °C water bath (without shaking) for exactly 45 seconds. The reaction tube was immediately placed on ice for 2 - 3 min. The mixture were removed from the tubes and added to a new tube containing 1 ml of pre-warmed SOC (2% Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). The cell suspension was incubated with shaking at 37 °C for 90 min. The mixture were centrifuged for 20 seconds at room temperature, and resuspended in 100 µl of the SOC medium and spread onto a selective LB agar plates (containing 50 µg/ ml of amplicillin and spread with 20 µl of 25 µg/ml of X-gal and 25 µl of 25 µg/ml of IPTG for approximately 1 hr before using) and further incubated at 37 °C overnight (Sambrook and Russell, 2001). The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.10 Colony PCR and digestion of the amplified inserts by restriction endonucleases

Colony PCR was performed in a 25 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 100 mM of each dNTP, 2 mM $MgCl_2$, 0.1 µM each of pUC1 (5′-CCG GCT CGT ATG TTG TGT GGA-3′) and pUC2 (5′-GTG GTG CAA GGC GAT TAA GTT GG-3′), 0.5 unit of *Taq* DNA polymerase (Fermentas). A colony was picked by a pipette tip, placed in the culture tube and served as the template in the reaction. PCR was carried out in a thermocycler consisting of predenaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30s, annealing at 50°C for 1 min and extension at 72°C for 1.5 min. The final extension was carried out at the same temperature for 7 minutes. The colony PCR products were electrophoresed through a 1.2 % agarose gel and visualized after ethidium bromide staining.

The colony PCR products containing the insert were separately digested with *Alu* I and *Rsa* I (Promega) in a 15 µl reaction volume containing 1x buffer (6 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl and 1 mM DDT, pH 7.5 for *Alu* I and 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DDT, pH 7.9 for *Rsa* I), 0.1 mg/ml BSA, 2 units of each enzyme and 4 µl of the colony PCR product. The reaction mixture was incubated at 37°C overnight. The reaction was analyzed by agarose gel (1.2%) electrophoresis.

2.11 Extraction of recombinant plasmid DNA

Plasmid DNA was isolated using a HiYield™ Plasmid Mini Kit (RBC). A discrete white colony was inoculated into a sterile culture tube containing 3 ml of LB broth supplemented with 50 µg/ml of amplicillin and incubated with shaking (200 rpm) at 37 °C overnight. The culture was transferred into a sterile 1.5 ml microcentrifuge tube and centrifuged at 14,000 rpm for 1 min. The supernatant was discarded. The bacterial pellet was resuspended in 200 µl of the PD 1 buffer containing RNaseA and thoroughly mixed by vortexed. The resuspended cells were lysed by the addition of 200 µl of the PD 2 buffer and mixed gently by inverting the tube for 10 times. The mixture was stood for 2 minutes at room temperature. After that, 300 µl of the PD3 buffer was added to neutralize the alkaline lysis step and

mixed immediately by inverting the tube for 10 times. The mixture was then centrifuged at 14,000 rpm for 15 min.

A PD column was placed in a collection tube and the clear lysate was applied into the PD column and centrifuged at 6,000 *g* (8,000 rpm) for 30s. The flow-through was discarded. The PD column was placed back in the collection tube. The column was washed by the addition of 400 µl of the W1 buffer and centrifuged at 6,000 *g* for 30s. After discarding the flow-through, 600 µl of the ethanol-added Wash buffer was added and centrifuged as above. The PD column was further centrifuged for 2 min at the full speed (14,000 rpm) to dry the column matrix. The dried column was placed in a new microcentrifuge tube and 50 µl of the Elution Buffer or water was added at the center of the column matrix. The column was left at room temperature for 2 min before centrifuged for 2 minutes at the full speed to recover the purified plasmid DNA. The concentration of extracted plasmid DNA was spectrophotometrically measured.

2.12 Semiquantitative RT-PCR of *ADRP, aspartase aminotransferase, female sterile, NASP, OVLP, 3- oxoacid CoA transferase* in ovaries of juvenile *P. monodon* following serotonin administration

2.12.1 Experimental animals

Female juveniles of *P. monodon* (with the average body weight of 33.9 ± 6.40 g) were purchased from a local farm in Chonburi, eastern Thailand. Shrimp were acclimatized for 7 days at the laboratory conditions (25 ppt of seawater and the ambient temperature) and fed daily at approximately 5% of the body weight. Eight shrimp were placed in a glass aquarium (150 L) for each treatment (Appendix A) and were not fed at 24 hr before the treatment.

2.12.2 Preparation of 5-Hydroxy Tryptamine (5-HT) stock solution

The 5-HT was weighed out (0.1123 g) and resuspended with 5 ml of the sterile saline solution (0.85% NaCl) to achieve the final concentration of 22.5 µg/µl. The solution was incubated at 37°C to facilitate dissolution. The stock solution was diluted to the working solution (50 µg g^{-1} body weight with the injection volume of 100 µl per individual) prior to injection

2.12.3 Experimental design

Five groups of shrimp were single injected intramuscularly into the first abdominal segment with serotonin (50 μg g^{-1} body weight) and specimens were collected at 0 hr, 12, 24, 48 and 72 hr post injection (Treatment A). The normal saline control (0.85% at 0 hr, control A) was also included. In addition, other five groups of shrimp were repeatly injected with the same amount of serotonin. The second injection was carried out at 3 days post initial injection and the specimens were collected at 0 hr, 12, 24, 48 and 72 hr after the second injection (Treatment B). The normal saline control (0.85% at 0 hr, control B) was also included.

2.12.4 Total RNA extraction and the first strand cDNA synthesis

Ovaries were dissected from each shrimp immediately after specimens were collected. Total RNA was extracted from ovaries of *P. monodon* (N = 5 for each treatment) and 1.5 μg of total RNA was reverse-transcrbied using an ImProm- II™ Reverse Transcription System Kit (Promega) as described previously.

2.12.5 Optimization of semiquantitative RT-PCR conditions

Initially, RT-PCR of the target genes (Table 2.1) and *elongation factor-1α* (F:5′-ATG GTT GTC AAC TTT GCC CC-3′ and R:5′-TTG ACC TCC TTG ATC ACA CC-3′) were amplified in a 25 μl reaction volume following the standard RT-PCR for screening of gene expression described previously. The reaction contained 10 mM Tris - HCl pH 8.8 at 25°C, 50 mM KCl and 0.1 % Triton X – 100, 2 mM $MgCl_2$, 100 mM each of dATP, dCTP, dGTP and dTTP, 0.2 μM of each primer, 1 μl of 10-fold diluted first strand cDNA and 1 unit of Dynazyme™ DNA polymerase (FINNZYMES). For semiquantitative RT-PCR analysis, primer and $MgCl_2$ and the cycle numbers used for amplification were further optimized.

2.12.5.1 Optimization of primer concentration

The optimal primer concentration for each primer pair (between 0, 0.1, 0.15, 0.2, 0.25 and 0.3 μM) was examined using the standard PCR conditions. The resulting product was electrophoretically analyzed. The primer concentration that gave product

specificity and clear results were selected for further optimization of the PCR conditions.

2.12.5.2. Optimization of $MgCl_2$ concentration

The optimal $MgCl_2$ concentration of each primer pair (0, 0.5, 1, 1.5, 2 and 3 mM of $MgCl_2$) was examined using the standard PCR conditions and the optimized primer concentration. The concentration of $MgCl_2$ that gave the highest yields and specificity for each PCR product was chosen

2.12.5.3. Optimization of the number of amplification cycles

The PCR amplifications were carried out at different cycles (e.g. 18, 20, 23, 25 and 30 cycles) using the optimized concentration of primers and $MgCl_2$. The number of cycles that gave the highest yield before the product reached a plateau phase of amplification was chosen.

2.12. 5.4. Gel electrophoresis, quantitative and data analysis

The amplification product of genes under investigation and *EF-1α* were electrophoretically analyzed by the same gel and photographed by a gel documentation machine (BioRad). The intensity of the amplified target genes and that of *EF-1α* was quantified from the photograph of the gels using the Quantity One programme (BioRad).

The expression level of each gene was normalized by that of *EF-1α*. Expression levels between different groups of *P. monodon* were statistically tested using one way analysis of variance (ANOVA) followed by a Duncan's new multiple range test. Significant comparisons were considered when the *P* value was < 0.05.