การโคลนและลักษณะสมบัติของ cDNA ที่เกี่ยวข้องกับการบ่งบอกเพศของกุ้งกุลาดำ *Penaeus monodon*

นางสาว รุ่งนภา ลีละธนาวิทย์

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CLONING AND CHARACTERIZATION OF cDNA INVOLVED IN SEX DETERMINATION OF THE GIANT TIGER SHRIMP *Penaeus monodon*

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ในการวิเคราะห์หาเครื่องหมายทางพันธุกรรมในระดับ cDNA ที่จำเพาะต่อเพศของกุ้งกุลาดำ (*Penaeus monodon*) ด้วยเทคนิค RT-PCR โดยใช้ไพรเมอร์ที่ออกแบบมาจากส่วน DM domain ของยีน DMRT1, *dsx* และ mab-3, ยีนเพอริโทรฟิน, ยีนซิงฟิงเกอร์ และ จากยีนต่าง ๆ ที่เกี่ยวข้องกับการบ่งชี้เพศ พบว่ายีนเหล่านี้มีการแสดงออกในกุ้งกุลาดำทั้ง 2 เพศ แต่พบแถบดีเอ็นเอจำนวน 5 แถบที่มีขนาดแตกต่างจากผลผลิตเป้าหมาย (PMO720, PMO880, PMO920, PMT700 และ PMT1700) ซึ่งแสดงออกอย่างจำเพาะต่อเพศ จึงทำการโคลนและหาลำดับนิวคลีโอไทด์ของชิ้นดีเอ็นเอดังกล่าว จากนั้นได้ออกแบบไพรเมอร์จาก PMO720, PMO920, และ PMT1700 และทำ RT-PCR พบว่าทั้ง 3 คู่ไพรเมอร์ให้แถบดีเอ็นเอที่มีระดับการแสดงออกที่แตกต่างกันในรังไข่และอัณฑะของกุ้งกุลาดำ และได้ทำการค้นหา single nucleotide polymorphism (SNP) ที่เกี่ยวข้องกับเพศในกุ้งกุลาดำในระดับดีเอ็นเอ โดยการเปรียบเทียบลำดับนิวคลีโอไทด์ของยีนซิงฟิงเกอร์ (450 คู่เบส จากไพรเมอร์ Zincfinger-F + Finger-R) โดยพบความหลากหลายทางพันธุกรรมทั้งภายในเพศเดียวกันและระหว่างเพศ แต่ไม่พบ SNP ที่จะใช้บ่งบอกความแตกต่างระหว่างเพศของกุ้งกุลาดำ

ทำการสร้างห้องสมุด Subtractive cDNA ของรังไข่และอัณฑะในกุ้งกุลาดำ โดยทำการหาลำดับนิวคลีโอไทด์ทั้งหมด 216 โคลน (155 โคลน มาจาก Subtractive cDNAs ของรังไข่ และ 61 โคลน มาจาก Subtractive cDNAs ของอัณฑะ) โดยยีนหลักที่แสดงออกในรังไข่ คือ ทรอมโบสปอนดิน (TSP, 28.7%), เพอริโทรฟิน (10.8%) และ ยีนที่ไม่ทราบหน้าที่ (49.7%) ในขณะที่ยีนหลักที่แสดงออกในอัณฑะของกุ้งกุลาดำเป็นยีนที่ไม่ทราบหน้าที่ (96.7%) จากการทำ Rapid amplification of cDNA ends – polymerase chain reaction (RACE – PCR) เพื่อหาลำดับนิวคลีโอไทด์ที่สมบูรณ์ของ TSP พบลำดับนิวคลีโอไทด์ที่สมบูรณ์ทางด้านปลาย 5′ ของ TSP แต่ไม่พบผลผลิต RACE ที่สมบูรณ์ทางด้านปลาย 3′ และได้ทำการตรวจสอบการแสดงออกอย่างจำเพาะต่อเพศของยีนที่เกี่ยวข้องกับเพศ โดยวิธี RT–PCR พบว่า VCP7, PM233 และ AGESTX มีการแสดงออกอย่างจำเพาะในเพศเมียของกุ้งกุลาดำ ในขณะที่พบการแสดงออกของ TSP ทั้งในเพศผู้และเพศเมียของกุ้งกุลาดำอายุ 4 เดือน แต่จะพบการแสดงออกเฉพาะในเพศเมียในกุ้งขนาดพ่อแม่พันธุ์ และได้ศึกษาการแสดงออกของยีน TSP และ AGESTX โดยใช้เทคนิค semi-quantitative RT-PCR เพื่อวัดระดับของ mRNA ในรังไข่ของกุ้งลาดำขนาดพ่อแม่พันธุ์ และกุ้งกุลาดำอายุ 4 เดือน ซึ่งพบว่าระดับการแสดงออกของ AGESTX ไม่มีความแตกต่างกันในกุ้งทั้ง 2 ขนาด ($P > 0.05$) แต่ พบการแสดงออกแตกต่างกันของ TSP ในกุ้งทั้ง 2 ขนาดอย่างมีนัยสำคัญ ($P<0.05$)

ภาควิชา…….-……..…….. ลายมือชื่อนิสิต……..……..…

สาขาวิชา…….เทคโนโลยีชีวภาพ……..…….. ลายมือชื่ออาจารย์ที่ปรึกษา……..……..…

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RUNGNAPA LEELATANAWIT: CLOING AND CHARACTERIZATION OF cDNA INVOLVED IN SEX DETERMINATION OF THE GIANT TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR: ASSOC. PROF. PADERMSAK JARAYABHAND, Ph. D. THESIS COADVISOR: SIRAWUT KLINBUNGA, Ph. D., 184 pp. ISBN 974-17-5319-5.

Sex-specific cDNA markers of the giant tiger shrimp (*Penaeus mondon*) were identified by reverse transcription-polymerase chain reaction (RT-PCR) using primers designed from conserved regions (DM domain) of DMRT1, *dsx* and mab-3 homologues and peritrophin, zinc finger protein gene homologues and from other sex-related transcripts. Nevertheless, they did not reveal sex-specific expression profiles in this species. Five non-specific RT-PCR fragments (PMO720, PMO880, PMO920, PMT700 and PMT1700) exhibiting sex-specific expression patterns were cloned and sequenced. Primers were designed from PMO720, PMO920 and PMT1700. Expression of these transcripts was examined and displayed differential expression levels between males and females *P. monodon*. Single nucleotide polymorphism (SNP) fixed in each gender of *P. monodon* was examined at the genomic level by comparing DNA sequences of the amplified zinc finger gene segments (450 bp from Zincfinger-F and Finger-R). Polymorphism was found at both within and between genders but did not fix in each sex. Therefore, sex determination SNP markers were still not found in this species.

Additionally, subtractive cDNA libraries of ovaries and testes of *P.* monodon were constructed. A total of 216 clones (155 clones from subtractive cDNAs of ovaries and 61 clones from those of testes) were unidirectionally sequenced. Most of the expressed genes in ovaries encoded thrombospondin (TSP 45 clones, accounting for 28.7%), peritrophin (17 clones, 10.8%) and unknown transcripts (76 clones, 49.7%). Conversely, almost all of the ESTs in *P. monodon* testes were unknown transcripts (59 clones, 96.7%). Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) was carried out for further characterization of TSP. Results indicated successful amplification of 5′ RACE-PCR but not 3′ RACE-PCR. Gender-specific expression of various candidate sex-linked gene homologues was examined by RT-PCR. While XNP-1 and peritrophin were expressed in both ovaries and testes, VCP7, PM233 and AGESTX revealed sex-specific expression in female *P. monodon*. Expression of TSP was found in both male and female of 4-month-old *P. monodon* but temporal sex-specific expression was found in *P. monodon* adults. Expression of TSP and AGESTX homologues was semi-quantitatively estimated. The relative expression level of AGESTX in ovaries of 4-month-old and adults of *P. monodon* was not significantly different ($P>0.05$) but that of TSP was significantly different statistically ($P<0.05$).

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CONTENTS

	Page
THAI ABSTRACT……..…….....	iv
ENGLISH ABSTRACT…….....	v
ACKNOWLEDGMENTS..……..….....	vi
CONTENTS……..…	vii
LIST OF TABLES…….....	xi
LIST OF FIGURES……..…	xiv
LIST OF ABBREVIATIONS……..….	xxi
CHAPTER I INTRODUCTION	
1.1 General introduction……...…..….	1
1.2 Taxonomy of *P. monodon*…….….	4
1.3 Exploitation…….....	4
1.4 Molecular genetic approaches for isolation and characterization of genetic markers involved with sex-determination/differentiation at genomic and cDNA levels……..…	6
1.5 Sex determining systems…….....	21
CHAPTER II MATERIALS AND METHODS	
2.1 Materials…….....	28
2.2 Samples……..…	31
2.3 Nucleic acid extraction……...…	32
2.4 Measuring concentrations of extracted DNA and RNA using spectrophotometry and electrophoresis……...….....	33
2.5 Amplification of conserved sex-specific genes (DMRT-1, *dsx* and mab-3)……...…….....	34
2.5.1 Primer design……..……	34
2.5.2 PCR analysis…….....	35
2.5.3 First strand cDNA synthesis……..….	36
2.5.4 RT-PCR analysis…….....	36
2.5.5 Agarose gel electrophoresis……	40
2.5.6 Elution of DNA fragments……..……	40
2.5.7 Cloning of PCR product……....….	41

CONTENTS (cont.)

Page

2.5.8 Colony PCR and digestion of the amplification product by restriction endonucleases……..... 42
2.5.9 Isolation of recombinant DNA……..... 43
2.5.10 DNA sequencing……..... 43
2.6 Cloning and characterization of Peritrophin homologues in *P. monodon*……..... 44
2.7 Cloning and sequencing of a zinc finger protein gene homologue ……...….. 45
2.8 Identification of sex–specific expression transcripts in the giant tiger shrimp (*P. monodon*) by cDNA subtraction……..... 47
2.8.1 mRNA purification……. 47
2.8.2 First strand cDNA synthesis……... 48
2.8.3 Second strand cDNA synthesis and Purification……..... 48
2.8.4 *Rsa* I Digestion……..... 51
2.8.5 Adaptor Ligation……. 51
2.8.6 First Hybridization……..… 52
2.8.7 Second Hybridization…….. 53
2.8.8 PCR Amplification…….. 54
2.8.9 Cloning…… 55
2.8.10 Sequencing……..… 56
2.9 Characterization of Thrombospondin (TSP) homologue by Rapid amplification of cDNA ends-polymerase chain reaction (RACE - CR)……..... 56
2.9.1 Primer design……... 56
2.9.2 First strand cDNA synthesis…… 58
2.9.3 Rapid Amplification of cDNA Ends (RACE)…….. 59
2.9.4 Cloning and sequencing RACE products……. 61
2.10 Identification of sex-specific/differential expression transcripts using RT-PCR of homospecific primers..... 61

CONTENTS (cont.)

Page

2.11 Identification of sex-specific/differential expression transcripts in ovaries and testes of *P. Monodon* using RT-PCR of hetrospecific primers from the giant freshwater (*Macrobrachium rosenbergii*) and the tropical abalone (*Haliotis asinina*)……...……...….. 65
2.12 Tissue distribution of TSP and AGESTX…...……...……...… 69
2.13 Semi – quantitative Reverse Transcription – Polymerase Chain Reaction of Thrombospondin (TSP) and AGESTX…...…… 69
2.13.1 Primers…… 69
2.13.2 Total RNA extraction and the first stranded cDNA synthesis……...……...….. 70
2.13.3 Determination of PCR condition……..... 70
2.13.3.1 Primer concentration……...….. 70
2.13.3.2 $MgCl_2$ concentration……...….. 70
2.13.3.3 Cycle number…… 70
2.13.3.4 Further characterization to eliminate competition between primer set……...…. 71
2.13.3.5 Gel electrophoresis and quantitative analysis…….. 71
2.13.3.6 Data analysis……. 71

CHAPTER III RESULTS
3.1 Amplification of conserved sex-specific genes (DMRT-1, *dsx* and mab-3)……...……. 72
3.2 Cloning and characterization of Peritrophin homologues in *P. monodon*……..... 98
3.3 Cloning and sequencing of a zinc finger protein gene homologue ……...….. 101
3.4 Identification of sex–specific expression transcripts in the giant tiger shrimp (*P. monodon*) by cDNA subtraction……..... 114
3.5 Characterization of Thrombospondin (TSP) homologue by Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR)……..... 119

CONTENTS (cont.)

Page

3.6 RT – PCR of homospecific primers from *P. Monodon*……..…. 124

3.7 RT-PCR of heterospecific primers from the giant freshwater (*Macrobrachium rosenbergii*) and the tropical abalone (*Haliotis asinina*)……...… 142

3.8 Semi – quantitative Reverse Transcription – Polymerase Chain Reaction of Thrombospondin (TSP) and AGESTX…..……..... 151

CHAPTER IV DISCUSSION…….…….... 157

CHAPTER V CONCLUSION…….. 166

REFERENCES…….. 167

APPENDEICES……..…. 175

Appendix A……... 176

Appendix B……... 183

Biography…….. 184

LIST OF TABLES

		Page
Table 1.1	Total shrimp production. (in 1,000 metric tons) from the aquaculture sector between 1992 – 2001.....……..	2
Table 1.2	Thai Frozen Shrimp Export in 2001…….....……..	3
Table 2.1	Degenerated primer, primer sequences and melting temperature of primers from designed from the conserved DM domain of various species…….....……..	35
Table 2.2	Conditions for amplification of DMRT1, *dsx* and mab-3 homologues in *P. monodon* using genomic DNA as the template…….....….	37
Table 2.3	Conditions for RT-PCR of DMRT1, *dsx* and mab-3 homologues in *P. monodon*…….....	38
Table 2.4	Primer sequences, melting temperature and expected product sizes of PCR products of peritrophin homologues in *P. monodon*……..…	44
Table 2.5	Primers, primer sequences and melting temperature of primers designed from a Zinc Finger gene homologue of the giant tiger shrimp (*Penaeus monodon*)…….....…	45
Table 2.6	Sequences of the PCR-Select cDNA synthesis primer, adaptors and PCR primers…….....…....	49
Table 2.7	Ligation reactions of cDNA of ovaries (tester 1-1 and 1-2) and testes (tester 2-1 and 2-2)…….....…	52
Table 2.8	Composition of the first hybridization reaction of each subtraction.....	53
Table 2.9	Preparation of the primary PCR master mix……...	54
Table 2.10	Preparation of the secondary PCR master mix……..….	55
Table 2.11	Sequences of TSP-specific primers for further characterization of TSP homologues in *P. monodon* using RACE-PCR……...……..……..….	57
Table 2.12	Primer sequences for the first strand cDNA synthesis and RACE – PCR……...	59
Table 2.13	Composition of 5′ RACE–PCR……...…….....	60
Table 2.14	Composition of 3′ RACE–PCR……...…….....	60

LIST OF TABLES (cont.)

		Page
Table 2.15	Primer sequences, melting temperature and expected product sizes of PCR products of primers from the giant tiger shrimp (*Penaeus monodon*)……..….....	63
Table 2.16	Primer, primer sequence and melting temperature of heterospecific primers from the giant freshwater (*Macrobrachium rosenbergii*) and the tropical abalone (*Haliotis asinina*)…….....	66
Table 3.1	Identification of conserved gene homologues encoding for DMRT1, *dsx* and mab-3 in *P. monodon* using genomic DNA as the template…….…..	79
Table 3.2	Identification of conserved gene homologues encoding for DMRT1, *dsx* and mab-3 in *P. monodon* using the first strand cDNA of ovaries and testes as the template……...…….....	82
Table 3.3	Identification of conserved gene homologues encoding for DMRT1, *dsx* and mab-3 in *P. monodon* using PCR products and the first strand cDNA as the template…….....…..	83
Table 3.4	Blast results of RT-PCR products illustrating sex-specific expression in *P. monodon*…….....	96
Table 3.5	Primers, sequence primers and annealing temperature of primers from PMT1700, PMO720 and PMO920……..……....	97
Table 3.6	Amplification of peritrophin homologue in *P. monodon*…….....	99
Table 3.7	Amplification of zinc finger gene homologue in *P. monodon*…….....…	108
Table 3.8	Homologues of genes found in a subtractive library using cDNA of *P. monodon* ovaries as the tester…….…….	116
Table 3.9	Homologues of genes found in a subtractive library using cDNA of *P. monodon* testes as the tester……...	118
Table 3.10	ESTs found from 5′- and 3′-RACE cDNA amplification using mRNA of *P. monodon* as the template……..…	122

LIST OF TABLES (cont.)

Page

Table 3.11 RT-PCR for identification of sex-specific transcripts of *P. monodon* using various homospecific primers (*P. monodon*) derived from normal and subtractive cDNAs of ovaries and candidate female-specific AFLP markers……..……..... 126

Table 3.12 RT-PCR for identification of sex-specific (differentially expressed) transcripts in *P. monodon* using heterospecific primers from *Macrobrachium rosenbergii* and *Haliotis asinina*……..... 143

LIST OF FIGURES

		Page
Figure 1.1	Overall procedures of RAPD – PCR analysis…….……..	8
Figure 1.2	Example of an AFLP procedure using one primer pair…….…...	10
Figure 1.3	Overview of the Genome walking procedure…….....	12
Figure 1.4	Overall concept of the RT-PCR procedure…….…...	14
Figure 1.5	Overview of the Clontech PCR-Select^{TM} procedure…….....	16
Figure 1.6	Overview of the SMART^{TM} RACE cDNA Amplification Kit……..	18
Figure 1.7	Overall concept of the RAP-PCR technique…….….....	20
Figure 2.1	Preparation of adaptor-ligated tester cDNA for hybridization and PCR……...…..	50
Figure 3.1	A 1.0% agarose gel electrophoresis showing the quality of total DNA extracted from pleopod of 13 individuals of *P. monodon*…..	73
Figure 3.2	A 1.0% agarose gel electrophoresis of total RNA extracted from testes and ovaries of *P. monodon*. Lane M = λ *Hind* III……..……	74
Figure 3.3	A 1.0% agarose gel electrophoresis illustrating the first strand cDNA synthesized from total RNA of testes and ovaries of *P. monodon*. Lane M = λ *Hind* III……....….	74
Figure 3.4	Multiple alignments of genes encoding for DMRT1, *dsx* and Mab-3 from several species……..……..…….....….	78
Figure 3.5	An example for amplification of DMRT1/*dsx*-F + DMRT1-R and DMRT1/*dsx*-F + *dsx*-R and DMRT1/*dsx*-F + mab3-R against genomic DNA of male and female *P. monodon*…….....….	80
Figure 3.6	An example for amplification of mab3-F/R against genomic DNA of male and female *P. monodon*……...…	80
Figure 3.7	An example for amplification of DMRT1/*dsx*-F + DMRT1-R and DMRT1/*dsx*-F + mab3-R against genomic DNA of male and female *P. monodon*……...….....	81
Figure 3.8	RT-PCR of DMRT1/*dsx*-F + dT_{15} and mab3-F + dT_{15} using the first strand cDNA synthesized from total RNA of testes and ovaries as the template……....	84

LIST OF FIGURES (cont.)

		Page
Figure 3.9	**A**mplification of DMRT1/*dsx*-F + DMRT1-R, *dsx*-R and mab3-R and mab3-F + DMRT1-R, *dsx*-R and mab3-R using PCR products from DMRT1/*dsx*-F + dT_{15} and mab3-F + dT_{15} male and female *P. monodon* as the template, respectively…….....	85
Figure 3.10	RT-PCR using nDMRT1-F + dT_{15} and n*dsx*-F + dT_{15}…….…….....	86
Figure 3.11	RT-PCR of the first strand cDNA template synthesized from total RNA of testes and ovaries of *P. monodon* using various primer combinations…….....	87
Figure 3.12	RT-PCR of the first strand cDNA template synthesized from total RNA of testes and ovaries of *P. monodon* using various primer combinations…….....….	88
Figure 3.13	Nested RT-PCR of the first strand cDNA template of testes ovaries of *P. monodon* using various primer combinations…….....	89
Figure 3.14	RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries of *P. monodon* using primers DMRT1/dsx-F+ dT_{15} and ndsx-F + dT_{15}.....	90
Figure 3.15	Colony PCR of clones PMO880 and PMO920….....…….....	91
Figure 3.16	Colony PCR of clones PMT700 and PMO920…….	92
Figure 3.17	Colony PCR of PMT1700 and PMO720…….…….....	93
Figure 3.18	Nucleotide sequences of different transcripts obtained from cloning and characterization of PMT700 and PMT1700…….....	94
Figure 3.19	Nucleotide sequences of PMO880 (A), PMO920 (B) and PMO720…….....	95
Figure 3.20	PCR of genomic DNA from males and females using primers PMT1700-F/R, PMO720-F/R and PMO920-F/R……..……..	98
Figure 3.21	RT-PCR of peritrophin homologues in *P. monodon* using the first strand cDNA template from mature and immature ovaries, lymphoid organ, testes and antennal ganglion and primers Peri-F_1/R_1 and Peri-F_2/R_2…….…..….....	100
Figure 3.22	Amplification of genomic DNA isolated from female and male *P. monodon* using primers Peri-F_1/R_1 and Peri-F_2/R_2……...……	101

LIST OF FIGURES (cont.)

Page

Figure 3.23 RT-PCR of zinc finger protein homologues in *P. monodon*. The first strand DNA template from mature and immature ovaries, lymphoid organs, testes and antennal ganglion were examined using primers Zinc-F/R , Finger-F/R……..……..... 103

Figure 3.24 Multiple alignment of nucleotide sequences obtained from the target and the larger amplification bands (Finger-F/R) of RNA from immature and vitellogenic ovaries of *P. mondon*……..….. 104

Figure 3.25 Amplification of genomic DNA isolated from female and male *P. monodon* using primers Zinc-F/R and Finger-F/R……... 105

Figure 3.26 Amplification of zinc finger protein gene homologues in male and female *P. monodon* using primers Finger – F + Finger–R**,** Finger-F + Zincfinger-R and Zinfinger-F + Finger-R……... 106

Figure 3.27 Amplification of a zinc finger protein homologue using genomic DNA of female (*N* = 10) originating from Satun, Phangnga (*N* = 2) and Chumphon (*N* = 4) and male (*N* = 10, panel B) originating from Satun (*N* = 2), Trang (*N* = 2), Phangnga (*N* = 2), Chumphon (*N* = 2) and Trad (*N* = 2) *P. monodon*, respectively as the template……...……..…… 107

Figure 3.28 Colony PCR of 8 randomly selected white colonies from each ligation of the amplified zinc finger protein homologue using Zincfinger-F + Finger-R as the template……..... 109

Figure 3.29 Multiple alignments of a zinc finger gene homologue of males (pZFM) and females (pZFF) of *P. monodon*…….……..…… 113

Figure 3.30 Electrophoresis of the first (A) and the second (B) PCR of subtracted cDNA of *P. monodon*……..... 115

Figure 3.31 5′ RACE-PCR (TSP-1) and ′ RACE-PCR (TSP-2) using the first set (TSP-1 and TSP-2) of TSP-specific primer……...….. 120

Figure 3.32 5′ RACE-PCR (288-1, N288-1, 462-1, N462-1, 371-1 and N371-1) and 3’ RACE-PCR (288-2, N288-2, 462-2 and N462-2) using the second set of TSP-specific primers. RACE-PCR was carried out using 5′ and 3′ RACE first strand cDNA synthesized from mRNA of vitellogenic ovaries of *P. monodon*……..... 121

LIST OF FIGURES (cont.)

		Page
Figure 3.33	BlastX Results of nucleotide sequences of pRACE7155 indicated that 5′ RACE reaction was successful ……...……..	123
Figure 3.34	RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries using combining of AGESTX44-F + AGESTX40-R and Rudimentary-F/R……..…….	127
Figure 3.35	RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries using TSP288-F/R, TSP462-F/R and DSI-F/R for 20, 25, 30 and 35 cycles……...…….....	128
Figure 3.36	RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries using TSP462-2 + TSP371-1 for 20, 25, 30 and 35 cycles…….…....	129
Figure 3.37	RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries using TSP462-F + TSP288-R…..……...….	129
Figure 3.38	Nucleotide sequences of TSPPMT1300 and TSPPMO150 generated from TSP462F + TSP288R…….....	130
Figure 3.39	RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries using TSPPMT1300-F/R…….…….....	130
Figure 3.40	RT-PCR of a EGF-1 homologue (286 bp) of the first strand cDNA of testes and ovaries using EGF-F/R primers…….....	132
Figure 3.41	RT-PCR of the first strand cDNA of testes and ovaries using primer XNP1-F/R for 20, 25 and 30 cycles……..	132
Figure 3.42	RT-PCR resulted from amplification of the first strand cDNA of testes and ovaries using primers FE_{10}-$M_9$520-F/R and ME_4-$M_8$517-F/R……...….	133
Figure 3.43	RT-PCR of the first strand cDNA of testes, ovaries and hemocytes using Sxl-F/R for 20, 25, 30 and 35 cycle……...	133
Figure 3.44	RT-PCR (optimized condition and operated for 35 cycles) illustrating differential expression of the amplified Usp9X homologue in testes and ovaries of *P. monodon*……..	134
Figure 3.45	RT-PCR (optimized 35 cycles) of the first strand cDNA of testes and ovaries of broodstock-sized and 4-month-old *P. monodon* using primers Usp9X-F/R…….……	135

LIST OF FIGURES (cont.)

Page

Figure 3.46 RT-PCR of P AGESTX homologue using primers AGESTX44-F/R and the first strand cDNA of testes and ovaries *P. monodon* as the template……..… 136

Figure 3.47 RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries of broodstock-sized *P. monodon* and 4-month-old *P. monodon* using primers AGESTX44-F/R……..... 136

Figure 3.48 Nucleotide sequence of amplification product of AGESTX homolgue of *P. monodon* from primer AGESTX44-F/R……..... 137

Figure 3.49 Tissue distribution of AGESTX homologue in various tissues……..…..... 137

Figure 3.50 RT-PCR of TSP homologues of male and female for 20 cycles, 25 cycles, 30 cycles, and 35 cycles using primers TSP288-1 + TSP371-1……..….. 138

Figure 3.51 RT-PCR of the first strand cDNA of testes and ovaries of *P. monodon* analyzed by primers TSP288-1 + TSP371-1……..... 139

Figure 3.52 RT-PCR of the first strand cDNA from testes and ovaries of broodstock-sized *P. monodon* and 4-month-old *P. monodon* using primers TSP288-1 + TSP371-1.…….…….... 140

Figure 3.53 Nucleotide sequence of the amplification product of TSP homologue of *P. monodon* from primers TSP288-1 + TSP371-1……..… 140

Figure 3.54 Tissue distribution of TSP homologue in *P. monodon* using TSP288-1 + TSP371-1……..….. 141

Figure 3.55 RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries of *P. monodon* using primers VCP1-F/R, VCP2-F/R, VCP3-F/R, VCP75-F/R and AGESTX44-F/R……..……..…. 144

Figure 3.56 RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries of *P. monodon* using primers Tektin-F/R and Axonemal-F/R……..... 145

Figure 3.57 RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries of *P. monodon* using primers FP-F/R and Lysin-F/R for 25, 25 and 35 cycles……..……..….. 146

LIST OF FIGURES (cont.)

Page

Figure 3.58 RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries of *P.monodon* using primers HSD-F/R for 25, 30 and 35 cycles……..…... 146

Figure 3.59 RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries of *P. monodon* using primers VTG1-F/R and SARIP-F/R for 25, 30 and 35 cycles…….…...….. 147

Figure 3.60 RT-PCR of the first strand cDNA synthesized from total RNA Of testes and ovaries of *P. monodon* using primers VCP75-F/R……...…. 147

Figure 3.61 RT-PCR of the first strand cDNA of testes and ovaries of *P. monodon* using primers VCR7-F/R at an annealing temperature of 60 °C and 58 °C……..... 148

Figure 3.62 Nucleotide sequence of the amplified VCP homologue in *P.* monodon……...… 148

Figure 3.63 RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries of *P. monodon* using primers ME_4-M_8 517-F/R……...… 149

Figure 3.64 Nucleotide sequence of the amplification product of ME_4-M_8 517-F/R……...… 150

Figure 3.65 RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries of broodstrock-sized *P. monodon* for 20, 25, 30 and 35 cycles using primers PM233-F/R……..…..... 150

Figure 3.66 RT-PCR of the first strand cDNA synthesized from total RNA of testes and ovaries of 4-month-old *P. monodon* for 20, 25, 30 and 35 cycles using primers PM233-F/R……...……...….... 151

Figure 3.67 Optimization of primer concentrations for amplification of AGESTX (A) and TSP (B) homologue by varying Concentration of primer from 0.1-0.4 µM……...…….……...…. 153

Figure 3.68 Optimization of $MgCl_2$ concentrations for amplification of AGESTX (A) and TSP (B) homologue by varying concentration of $MgCl_2$ from 1 – 4 mM……..... 153

LIST OF FIGURES (cont.)

Page

Figure 3.69 Optimization of cycling number of PCR for amplification of TSP homologue by varying numbers of amplification cycles from 20-35 cycles……..... 154

Figure 3.70 Optimization of cycling number of PCR for amplification of AGESTX homologue by varying numbers of amplification cycles from 20-35 cycles……..…….....…..... 154

Figure 3.71 Competitive between target (TSP and AGESTX) and internal control primer sets (β-actin). PCR reactions were simultaneously performed in the same tube……..... 155

Figure 3.72 Analysis of expression levels of AGESTX and TSP transcripts between broodstock-sized and 4-month-old *P. monodon*……..... 156

LIST OF ABBREVIATIONS

bp	base pair
°C	degree Celcius
DEPC	Diethylpyrocarbonate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
Kb	kilobase
M	Molar
$MgCl_2$	magnesium chloride
mg	Milligram
ml	Millilitre
mM	Millimolar
ng	Nanogram
OD	optical density
PCR	polymerase chain reaction

RNA	Ribonucleic acid
RNase A	ribonuclease A
rpm	revolution per minute
RT	Reverse transcription
SDS	sodium dodecyl sulfate
Tris	tris (hydroxyl methyl) aminomethane
µg	Microgram
µl	Microlitre
µM	Micromolar
UV	ultraviolet

CHAPTER I

INTRODUCTION

1.1 General introduction

The giant tiger shrimp, *Penaeus monodon* is one of the most important penaeid species widely cultured throughout the Southern Indo-Pacific region. It accounted for 56% of the world cultured shrimp production in 2000 whereas *P. merguiensis*, *P. vannamei* and others contributed for 17%, 16% and 11% of the total production, respectively (Rosenberry, 2001). Farming of *P. monodon* has achieved a considerable economic and social importance particularly in the Southeast Asian region for a long period of time.

In Thailand, *P. monodon* has been intensively cultured for more than two decades and contributed approximately 60% of the total cultivated shrimp production. Farming activity of *P. monodon* in Thailand has rapidly increased reflecting a large annual production. The reasons for this are supported by several factors including the appropriate farming areas without serious disturbing from typhoons or cyclone, small variable of seawater during seasons, and ideal soils for pond construction. Culture of *P. monodon* increases national revenue, therefore *P. monodon* is an economically important species in Thailand.

Shrimp farms and hatcheries are located along the coastal areas of Thailand where Nakorn Sri Thammarat and Surat Thani in Peninsular Thailand are the major parts of *P. monodon* cultivation. In addition, Chanthaburi (eastern Thailand), Samut Sakhon and Samut Songkhran (central region) also significantly contribute on the country production. The intensive farming system (85% of overall) has resulted in consistent production of *P. monodon* of Thailand (Department of Fisheries).

Thailand has been regarded as the leading shrimp producer (mainly *P. monodon*) for over a decade (Table 1.1). The shrimp production from Thailand was estimated to be approximately 280,000 metric tons in 2001 following by other major exporting countries like China (100,000 metric tons), India (100,000 metric tons) and Indonesia (90,000 metric tons). Although the production of each major producer

Table 1.1 Total shrimp production (in 1,000 metric tons) from the aquaculture sector between 1992 – 2001

Year / Country	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Thailand	120.0	150.0	225.0	220.0	160.0	150.0	210.0	220.0	250.0	280.0
China	220.0	55.0	35.0	70.0	80.0	80.0	80.0	85.0	85.0	100.0
Indonesia	150.0	80.0	100.0	130.0	90.0	80.0	80.0	85.0	85.0	90.0
India	42.0	60.0	70.0	70.0	70.0	75.0	70.0	75.0	80.0	100.0
Bangladesh	27.0	29.0	30.0	30.0	35.0	34.0	38.0	45.0	45.0	55.0
Ecuador	110.0	90.0	100.0	100.0	120.0	130.0	155.0	80.0	40.0	20.0
Vietnam	39.0	41.0	50.0	50.0	30.0	30.0	25.0	35.0	35.0	42.0
Mexico	6.0	6.5	12.0	12.0	12.0	16.0	17.0	20.0	25.0	32.0
Philippines	25.0	20.0	18.0	25.0	25.0	10.0	15.0	20.0	20.0	25.0
Columbia	10.0	12.0	18.0	20.0	20.0	18.0	18.0	18.0	20.0	25.0
Taiwan	25.0	20.0	15.0	7.0	6.0	14.0	10.0	9.0	10.0	10.0
Honduras	5.2	5.7	6.5	10.0	10.0	12.0	12.0	10.0	10.0	12.0
Panama	4.2	4.4	4.6	10.0	10.0	10.0	10.0	9.0	8.0	5.5
Guatemala	2.5	2.7	3.0	7.0	7.0	7.0	7.0	6.0	6.0	4.5
Peru	5.6	5.8	6.0	8.0	8.0	6.0	6.0	5.0	5.0	2.5
Japan	3.5	3.5	3.6	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Others	45.0	51.2	50.0	14.0	30.0	35.0	55.0	54.0	50.0	47.0
Total	840.0	636.8	746.7	788.0	718.0	712.0	813.0	781.0	779.0	855.5

Source: Globefish (2002)

Seems to be slightly increased from previous years, outbreaks of infectious diseases (e.g. from white spot syndrome virus, WSSV; yellow head virus, YHV and *Vibrio*) have a great impact on decreasing the expected production in several countries including Thailand, Ecuador, Vietnam and Taiwan. In Ecuador, the production decreased from 155,000 metric tons in 1998 to 80,000, 40,000 and 20,000 metric tons in 1999, 2000 and 2001, respectively. Likewise, Thailand had a severe outbreak of shrimp diseases during 1995 – 1997 causing significant decrease in the shrimp production at that period.

The United States of America (USA) and Japan are major shrimp importers of Thailand (Table 1.2). Approximately 68% of *P. monodon* exported from Thailand are imported to these countries, worthing 38,859 million bahts. The remaining international markets are EU., Australia, Asian countries and others.

Table 1.2 Thai Frozen Shrimp Export in 2001

Country	Quantity (metric tons)	Amount (million baht)
United States	66,990	27,203
Japan	24,837	11,656
Canada	5,758	2,245
Singapore	6,610	2,129
Taiwan	6,308	1,762
Australia	3,638	1,406
Republic of Korea	4,121	1,270
China	3,412	1,051
Hong Kong	2,610	971
United Kingdom	1,587	598
France	1,553	497
Germany	1,242	474
Italy	876	162
New Zealand	337	115
Others	5,031	1,680
Total	134,910	53,219

Source: Globefish

1.2 Taxonomy of *P. monodon*

The giant tiger shrimp (*P. monodon*) is taxonomically classified as (Bailey-Brook and Moss, 1992): a member of Phylum Arthropoda; Subphylum Crustacea; Class Malacostraca; Subclass Eumalacostraca; Order Decapoda; Suborder Natantia; Infraorder Penaeidea; Superfamily Penaeoidea; Family Penaeidae Rafinesque, 1985; Genus *Penaeus* Fabricius, 1798 and Subgenus *Penaeus*.

The scientific name of this shimp is *Penaeus monodon* (Fabricius, 1798) where the English common name is giant tiger shrimp or black tiger prawn.

1.3 Exploitation

Thailand has been regarded as the leader of *P. monodon* production since 1993. The success of *P. monodon* industry in Thailand has resulted in the steadily increased income for the nation annually. Nevertheless, the shrimp industry has encountered several problems including environmental degradation, outbreaks of diseases, and overexploitation of natural female broodstock that are used to produce commercial hatchery stocks for the industry (Browdy, 1998).

Farming of *P. monodon* relies entirely on wild caught broodstock for supply of juveniles because breeding of this species in captivity is extremely difficult. The high demand on female broodstock leads to overexploitation of the natural populations in this species. A research concerning domestication of *P. monodon* has been initiated to overcome this problem by production of high quality pond-reared *P. monodon* broodstock (Withyachumnarnkul et al., 2004).

In addition, appropriate broodstock management and restocking programs should also be applied for maintaining high genetic diversity of natural *P. monodon* stocks in Thailand.

In the future, selective breeding programs to produce broodstock will provide shrimps having desired phenotypes. This requires integrated biological disciplines (including genetics, reproductive biology, hormone regulation and nutrition in *P. monodon*).

An understanding of sexual biology is also important for designing breeding programs in that species. However, studies of sex determining mechanisms in insects and crustaceans are not well advanced. Previous researches have shown that most of the isopod species display chromosomal sex determination in both XX/XY and ZW/ZZ systems but heteromorphism of sex chromosomes were observed in very few cases.

Sex chromosomes in *P. monodon* are not yet cytological identifiable. The effective method for sex determination of *P. monodon* is development of sex-specific DNA markers in this economically important species. Generally, sex markers are present in the genome whenever sex determination are genetically, not enviromentally, controlled and sex determining genes are located on sex chromosomes. Basically, two main forms of sex herterogamy including male heterogamy (XY is male whereas XX is female) and female heterogamy (ZW is female whereas ZZ is male) are reported. Therefore, the Y or W are unique to one sex. Gender can then be indicated by the presence or absence of these markers in genomic (or complementary) DNA of the samples.

In *P. monodon*, females exhibit greater growth rate than do males at all stages of development. Nevertheless, a lack of obvious heteromorphic sex chromosomes in this species causing limited knowledge on sex determination system (XY or ZW) and segregation patterns. This prohibits the possibility to elevate culture efficiency of *P. monodon* through monosex farming. Once sex determination/differentiation system in the tiger prawn is understood, this basic knowledge will allow the possibility to develop a monosex culture of *P. monodon* to increase the productivity of this species successfully. Based on the fact that maturation of *P. monodon* in captivity is quite low, genes expressed in different stages of ovarian development should also be studies.

1.4 Molecular genetic approaches for isolation and characterization of genetic markers involving with sex-determination/differentiation at genomic and cDNA levels

Several molecular genetic approaches can be used to identify sex-identification/differentiation markers. Basically, molecular genetic markers at the DNA level are ideal for sex identification purposes but these markers, in many cases, are not available. Therefore, sex-specific/differential expression markers have been identified in several species.

For species exhibiting well defined sex chromosomes, molecular approaches at the genomic levels for example, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and Genome-walk analysis, are useful for identification of sex-linked markers. The isolated marker can be converted to sequence-characterized amplified region (SCAR) markers by sequencing, cloning and designing of specific primers for direct application for sex determination purposes.

For species that do not exhibit sex chromosomes, both molecular approaches at the DNA and cDNA (reverse transcription-polymerase chain reaction, RT-PCR; cDNA subtraction; rapid amplification of cDNA ends, RACE; differential display-PCR, DD-PCR and RNA arbitrary-primed-PCR, RAP-PCR) levels may be used for isolation and characterization of sex identification DNA markers and/or sex-specific/differential expression markers.

To reduce tedious and time consuming processes, bulked segregrant analysis (BSA) is usually applied during the screening step. This can be carried out by pooling an equal amount of DNA (or RNA before subjected to the first strand cDNA synthesis) of several individuals of males and females of a particular species. After candidate markers are identified, markers can be screened individually to confirm obtained results.

1.4.1 Identification of sex determination markers at the DNA level

1.4.1.1 RAPD analysis

Randomly amplified polymorphic DNA (RAPD) analysis was concurrently developed by Williams et al. (1990) and Welsh and McClelland (1990). It is a conceptually simple and straightforward method to generate genetic markers and DNA fingerprinting patterns without the prior knowledge of the genome which is subjected to be analyzed. The amplification conditions of RAPD differs from that of the standard PCR in that only a single random primer (e.g. 10 mer with GC content usually at least 50%) is employed (Fig. 1.1).

RAPD amplified target DNA on the basis that the nuclear genome contains several priming sites closed to one another that and are located in an inverted sequences resulting in amplification of DNA segments of variable length. The amplification products are separated on agarose gels and detected by staining with ethidium bromide. The advantage of RAPD-PCR is that unlimited numbers of primers are available for screening of sex-related DNA markers.

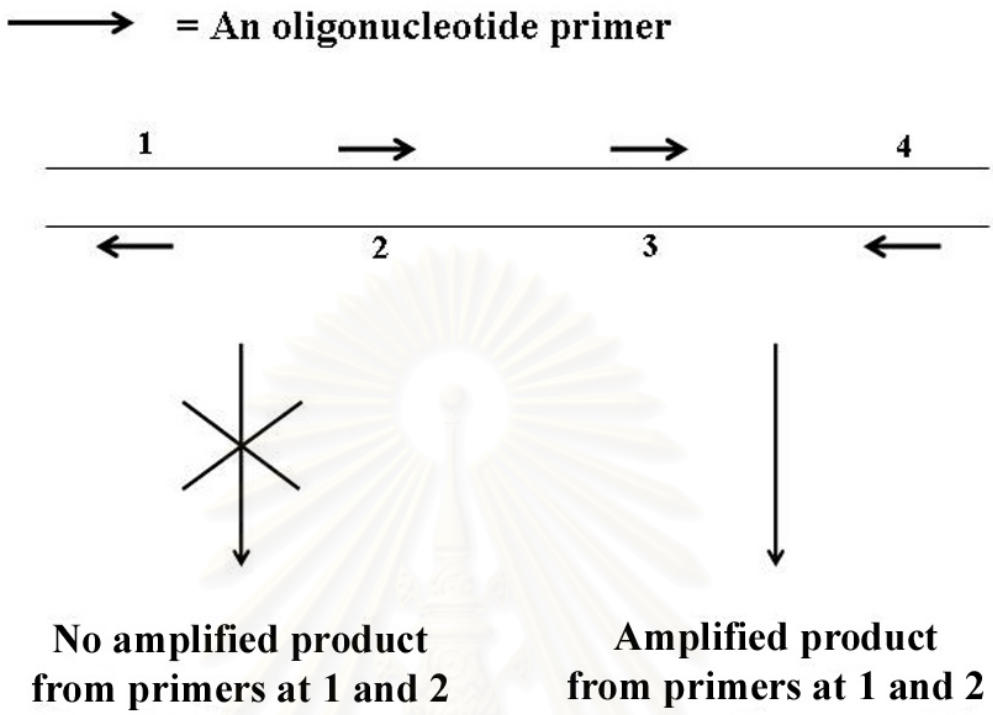

Figure 1.1 Overall procedures of RAPD – PCR analysis. An arbitrary primer (indicated by arrows) is used in the amplification reaction. Notably, the amplification product from the primer at 2 and 4 became the size of the target product is too large to be amplified by PCR.

Source: Seminar of the conference on the use of molecular markers in crop improvement (1992)

1.4.1.2 AFLP analysis

AFLP analysis is a technique for generation of DNA fingerprinting of organisms (Vos et al., 1995). It is a powerful technique, especially when combined with BSA, for isolation of phenotypes affected by (or majority by) single locus markers. In addition, fingerprinting-band AFLP pattern is used to visualize DNA polymorphism between samples.

AFLP is based on the selective amplification of a subset of genomic restriction fragments using PCR (Fig. 1.2). Initially, genomic DNA is simultaneously digested with frequent (usually *Mse* I) and rare (*Eco*RI) cutting restriction endonucleases. Double-stranded DNA adapters are then ligated to the ends of restricted DNA fragments to generate template DNA for amplification. Thus, the sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR.

Selective nucleotides (usually a single base for preamplification and three bases for selective amplification) extending into the restriction fragments are added to the 3′ ends of PCR primers such that only a subset of restriction fragments are recognized. Only restriction fragments in which the nucleotides flanking the restriction site matched the selective nucleotides will be amplified. The subsets of amplified fragments are then analyzed by denaturing polyacrylamide gel electrophoresis followed by radioactive (autoradiography) or non-radioactive (silver staning) detection.

Polymorphism between males and females of investigated species are regarded as candidate sex-specific AFLP markers. After further confirmed by a larger number of pooled DNA, candidate markers should be cloned and converted to SCAR markers and tested against a large numbers of specimens to ensure specificity of the developed markers.

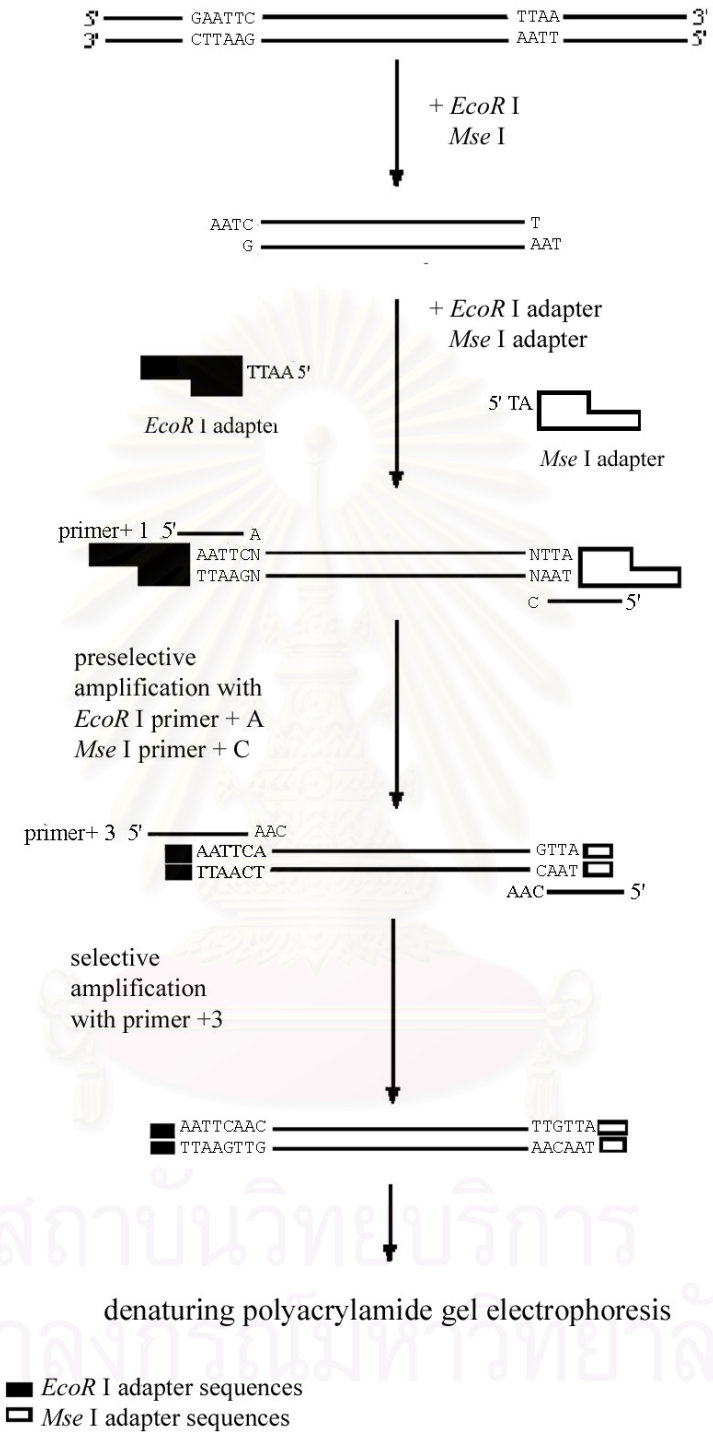

Figure 1.2 Example of an AFLP procedure using one primer pair (AFLP® Analysis System I; GibroBRL, USA).

1.4.1.3 Genome-walk analysis

The primary application of a Genome-walk analysis is the rapid cloning of the promoters and other upstream regulatory elements in genes for which cDNA sequence was previously available. This approach can be applied for identification of single nucleotide polymorphism (SNP) of important sex-related transcripts.

Intially, genomic DNA of interesting species are separately digested with different rare or frequent blunt-end generating restriction enzymes (e.g. *Dra* I, *Eco* RV, *Pvu* II and *Ssp* I). Following digestion, restricted products of each digestion are ligated with the GenomeWalker adaptor (Cato et al., 2001). The first amplification is carried out using the outer adaptor primer (AP1) and an outer gene-specific primer (GSP1) using ligated products as the template.

The primary PCR products are diluted and served as the template for the secondary PCR using the nested adaptor primer (AP2) and a nested gene specific primer (GSP2). The resulting products are electrophoretically analyzed by agarose gel electrophoresis (Fig 1.3). The major PCR product begins with known sequence at the 5′ end of the GSP2 and extends into the unknown adjacent genomic DNA can be cloned and analyzed. A new primer pairs can be designed from characterized sequence. The PCR product of different individuals can be firstly analyze by single strand conformational polymorphism (SSCP) and subsequently by DNA sequencing.

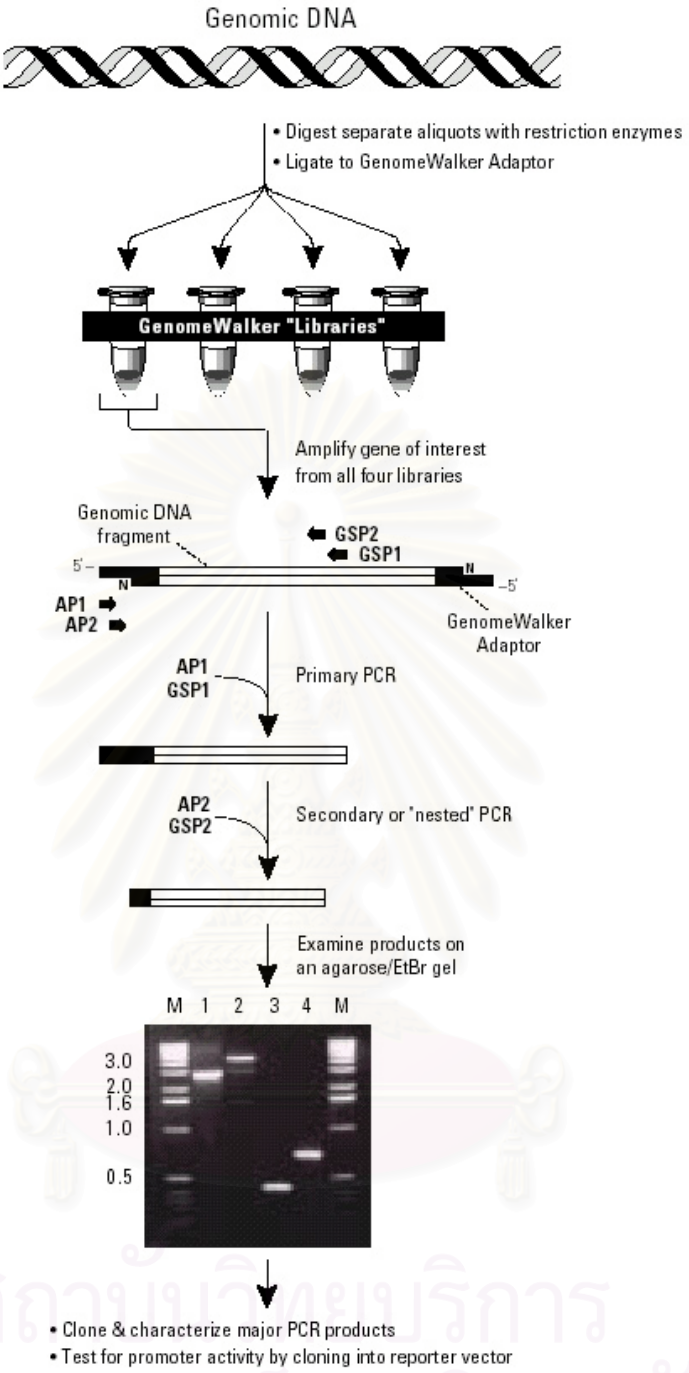

Figure 1.3 Overview of the Genome walking procedure. N: amino group that blocks extension of the 3′ end of the adaptor-ligated genomic fragments. AP1: Adaptor primers. GSP: Gene-specific primers (Universal GenomeWalker^{TM} Kit; CLONTECH, USA).

1.4.2 Identification of sex determination/differentiation markers at the cDNA level

1.4.2.1 RT-PCR

RT-PCR is a comparable method of conventional PCR but the first strand cDNA template rather than genomic DNA was used as the template in the amplification reaction (Fig. 1.4). It is a direct method for examination of gene expression of known sequence transcripts.

For identification of sex-related transcripts, RT-PCR of conserved sex determination/differentiation genes can be carried out using the first strand cDNA of ovaries and testes as the template. Transcripts exhibiting sex-specific expression patterns should also be examined at the genomic level to identification of sex identification DNA markers of the interesting species.

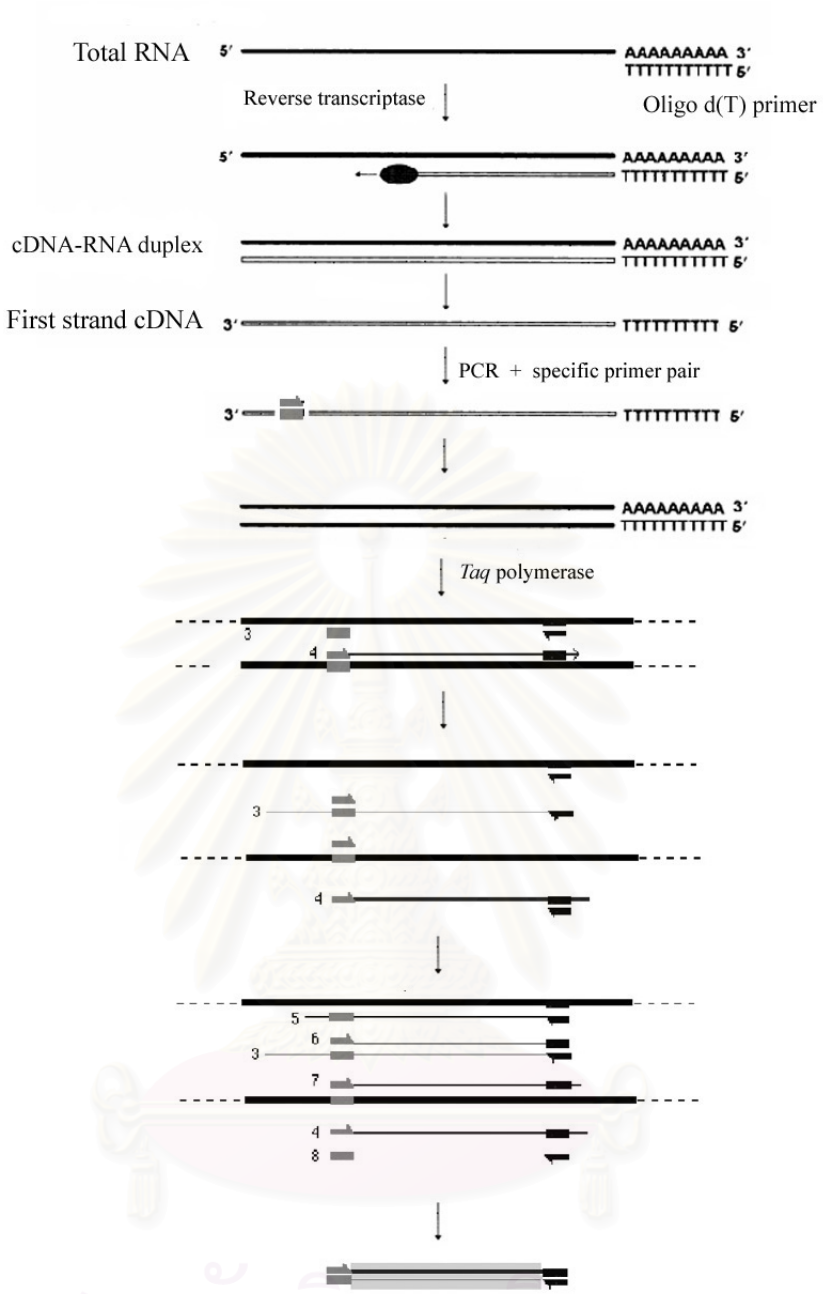

Figure 1.4 Overall concept of the RT-PCR procedure. During first-strand cDNA synthesis an oligo d(T) primer anneals and extends from sites present within the total RNA. Second strand cDNA synthesis primed by the 18 – 25 base specific primer proceeds during a single round of DNA synthesis catalyzed by *Taq* polymerase. These DNA frangments serve as templates for PCR amplification.

1.4.2.2 cDNA subtraction

Subtractive hybridization is a powerful technique to compare two populations of mRNA and obtain clones of genes that are expressed (or differential expressed) in one population but not in the other. Although there are several different methods, a CLONTECH PCR select cDNA subtraction method is convenient for generation of differentially expressed sequences (Fig. 1.5).

First, cDNA is synthesized from two types of mRNA populations and cDNA that contains specific (differentially expressed) transcripts as the tester, and the reference cDNA as the driver.

The tester and driver are digested with *Rsa* I. The tester cDNA is divided to two portions, and each is ligated with different cDNA adaptor. The ends of adaptor do not have a phosphate group, therefore only one strand of each adaptor attachs to the 5′ ends of cDNA. The two adaptors have stretches of identical sequences to allow annealing of the PCR primer once recessed ends have been filled in.

Tester and driver cDNAs are hybridized. In the first hybridization, an excess of driver is added to each tester. The samples are heat-denatured and allow to anneal Differential expressed transcripts are then enriched. During the second hybridization, template for PCR amplification is generated from differentially expressed transcripts. Two rounds of suppression PCR was carried out and only differentially expressed transcripts are amplified exponentially. Subtractive cDNA products are then cloned into the T-vector. Positive clones are characterized by hybridization or sequencing.

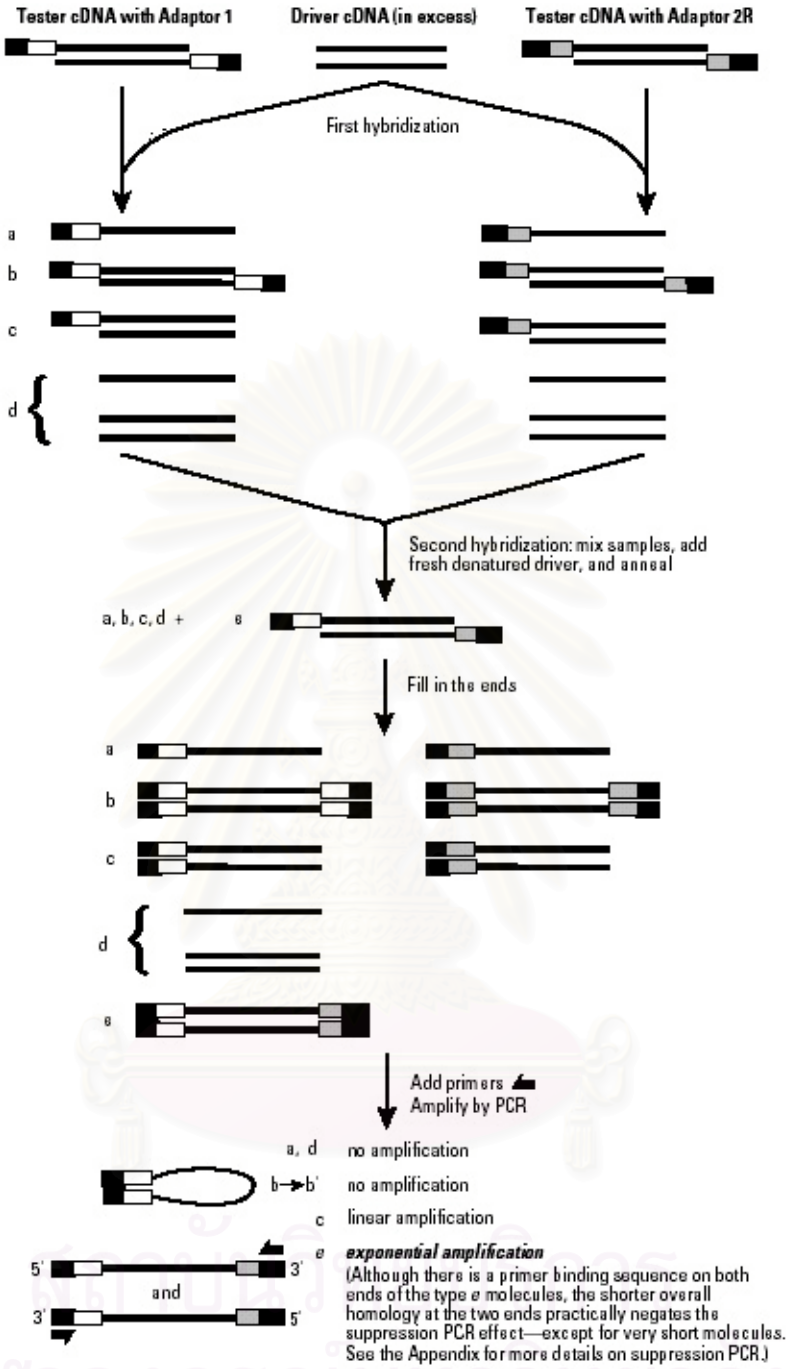

Figure 1.5 Overview of the Clontech PCR-Select^{TM} procedure. The cDNA in which specific transcripts are to be found is called "tester" and the reference cDNA is called "driver".

1.4.2.3 Rapid amplification of cDNA ends-polymerase chain reaction RACE-PCR

RACE-PCR is the common approach used for isolation of the full length of characterized cDNA. Using SMART (**S**witching **M**echanism **A**t 5′ end of RNA **T**ranscript) technology, terminal transferase activity of Powerscript Reverse Transcriptase (RT) adds 3 - 5 nucleotides (predominantly dC) to the 3′ end of the first-strand cDNA. This activity is harnessed by the SMART oligonucleotides whose terminal stretch of dG can anneal to the dC-rich cDNA tail and serve as an extended template for reverse transcriptase. A complete cDNA copy of original mRNA is synthesized with the additional SMART sequence at the end (Fig. 1.6).

The first strand cDNA of 5′ and 3′ RACE is synthesized using a modified oligo (dT) primers and serve as the template for RACE PCR reactions. Gene specific primers (GSPs) are designed from interested gene for 5′- RACE PCR (antisense primer) and 3′-RACE PCR (sense primer) and used with the universal primer (UPM) that recognize the SMART sequence. RACE products are characterized. Finally, the full length cDNA is constructed.

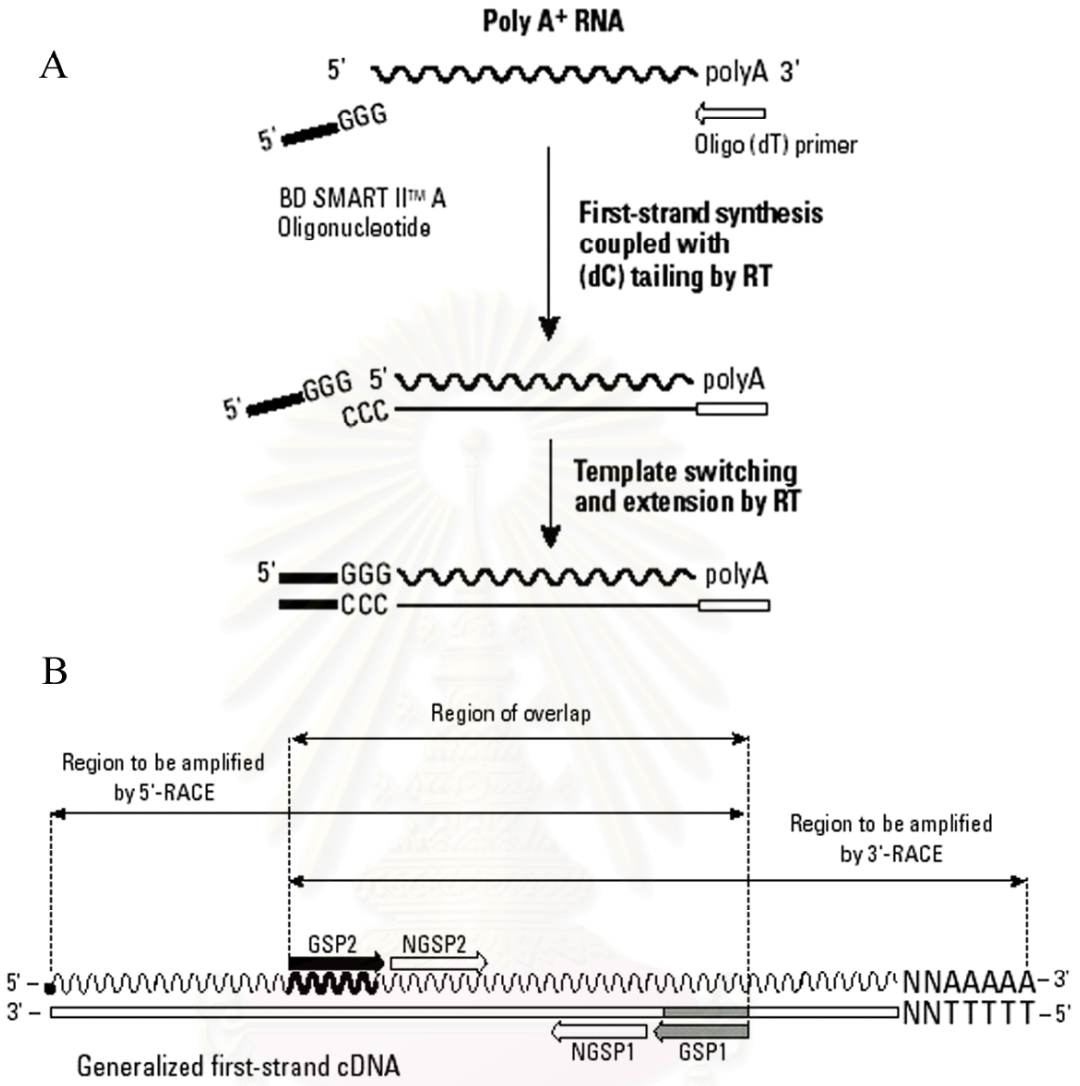

Figure 1.6 Overview of the SMART^{TM} RACE cDNA Amplification Kit.

A. Mechanism of SMART cDNA synthesis. First strand synthesis is primed using a modified oligo (dT) primer. After reverse transcriptase reaches the end of the mRNA template, it added several dC residues. The SMART II A Oligonucleotide annels to the tail of the cDNA and serves as an extended template for PowerScriptRT.

B. Relationships of gene-specific primers to the cDNA template. This diagram shows a generalized first strand cDNA template.

1.4.2.4 Differential display-polymerase chain reaction (DD-PCR) and RNA arbitrarily primed-polymerase chain reaction (RAP-PCR)

DD-PCR is one of the important approaches to identify differentially expressed transcripts in various organisms (Liang and Pardee, 1992). The advantage of this technique is that differentially expressed bands are obtained before further characterized by cloning and sequencing of the target transcripts.

The mRNA is reverse-transcribed using oligo (dT) primers having 4 - 12 additional bases at the 3′ end. The first strand cDNA is amplified by PCR using a short arbitrary primer and anchored primer. The amplified products are size-fractionated through denaturing polyacrylamide electrophoresis. DD-PCR bands are detected by autoradiography.

RNA arbitrarily primed PCR (RAP-PCR; Welsh et al., 1992) is a simple version of DD-PCR. This technique is generally comparable to RAPD-PCR with the exception that the first-strand cDNA synthesized from total RNA (or mRNA) was used as the template (Fig 1.7). It is believed that the first strand cDNA synthesized from a random primer allow better possibility to detect the inner part of the transcripts.

PCR is carried out using combination of two different arbitrary primers. Differentially expressed transcripts are detected after size-fractionated through denaturing polyacylamide gels and silver staining. Fragments from DD-PCR and/or RAP-PCR should be characterized by cloning and sequencing. Primers should be designed and tested for the expression levels of interesting fragments.

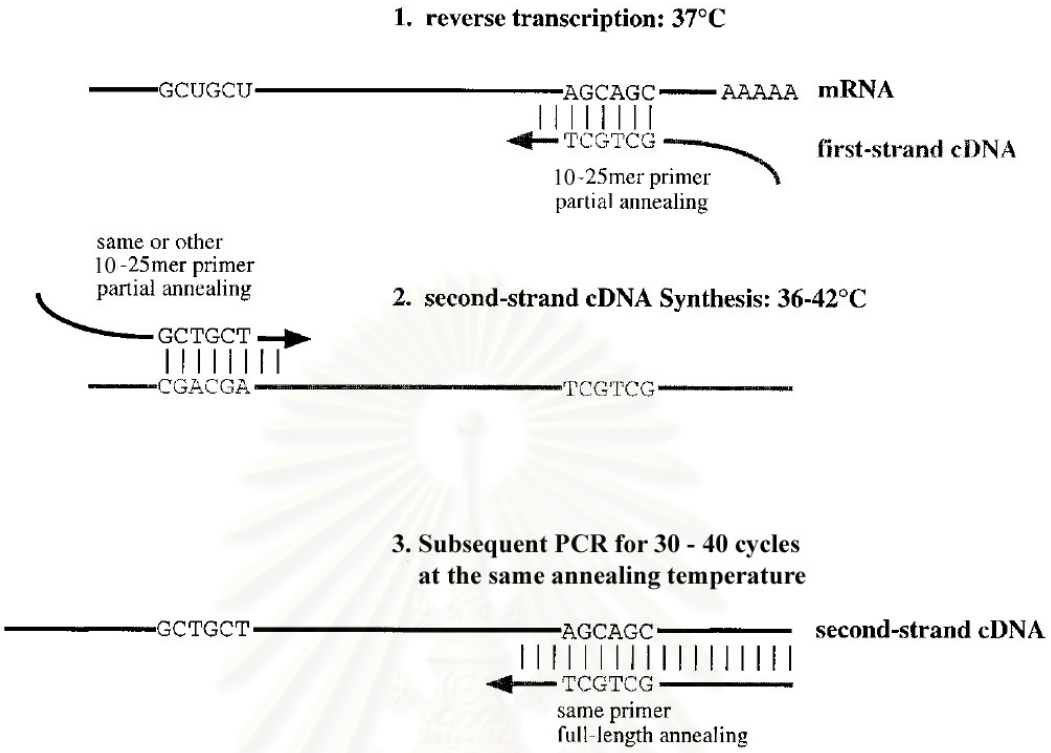

Figure 1.7 Overall concept of the RAP-PCR technique. (1) During the first-strand cDNA synthesis, a single 10 – 25 base arbitrary primer anneals and extends from sites present within the mRNA. (2) Synthesis of the second strand cDNA primed by the same, or another, 10 – 25 base arbitrary primer proceeds during a single round of low-stringency DNA synthesis catalyzed by *Taq* polymerase. The result of the two enzymic steps is the synthesis of a collection of molecules that are flanked at their 3′ and 5′ ends by sequences completely matching the sequences of the arbitrary primers. (3) These DNA frangments serve as templates for PCR amplification.

1.5 Sex determining systems

Vertebrates have sex determination systems but do not conserve across distant related species. Sex determination systems consist of species illustrating sex chromosomes (e.g. XX/XY, XX/XO and ZZ/ZW systems) and those lacking sex chromosomes but sex differentiation of those species is controlled by autosomal genes (Baker et al., 1976). Sex determination systems in invertebrates completely differ from those of vertebrates because most invertebrates do not possess sex chromosomes.

The XX/XY system is found in mammals. Males possess heterogametic sex chromosomes (XY) and females exhibit homogametic sex chromosomes (XX). In contrast, the XX/XO system which is found in grasshoppers, crickets, roaches and some insects, males exhibit a single set of X chromosome (XO) but females possess homogametic sex chromosomes (XX). In addition, ZZ/ZW system is found in birds, some fishes and some insects including butterflies and moths. Sex heterogametic chromosomes (ZW) are found in females but sex homogametic chromosomes (ZZ) are found in males.

During early differentiation of gonads (ovaries and testes) of mammals, the genotype of the zygote determines the nature of gonads, which presumably determines the male or female phenotype through secretion of sex-steroid hormones, estrogen and androgen. In mammals, a gene to initiate male development was isolated in humans and mice (sex-determining region Y, called SRY in human and *Sry* in other species) and localized on the Y chromosome (Sinclair et al., 1990).

The SRY gene is located immediately proximal to the pseudoautosomal boundaries of the Y chromosome and act as a dominant inducer for testicular development in mammals. The product of SRY genes belong to a family of protein that share a region of 79 amino acids that is homologous to part of a high-mobility group (HMG) protein, HMG-1. This protein has a role as a transcription factor in the developing gonads that begins a cascade leading to differentiation of testes. Many genes that encode proteins with SRY-related HMG boxes have been identified and named *SOX*/*sox* genes. SRY is conserved and functional only in mammals but *Sox-9*

gene is widely accepted to be involved in sex determination among mammals, chickens and fish (Zhou et al., 2001).

A multiplex PCR amplification was developed and used to detect *Sry* in mouse using genomic DNA extracted from fetal livers of fetuses (embryonic day 15) as the template. Two sets of primers consisting those specific for *Sry* gene (chromosome Y) and those specific to IL3 (chromosome 11) were used. The 402 bp band (*Sry*) was obtained from male fetuses and the 544 bp product (IL3) was found in both male and female fetuses which confirmed the correct amplification of the template DNA (Lambert et al., 2000).

The Y-linked zinc finger gene (ZFY) is located on Y chromosome near the pseudoautosomal boundary (Page et al., 1987) and its closely related gene, ZFX, is present on the X chromosome (Schneider-Gädicke et al., 1989) of human. The mouse has four zinc finger-related genes. These are two related Y-linked genes (Zfy-1 and Zfy-2), one X-linked gene (Zfx) and an autosomal homologue on chromosome 10 (Zfa) (Ashworth et al., 1989 and 1990; Mardon and Page, 1989; Mardon et al., 1990).

The localization of ZFX and ZFY on X and Y chromosomes suggest that they have evolved from a single ancestral gene located in an ancestral autosomal chromosome pair into heteromorphic sex chromosomes. ZFY-type sequences have also been identified in the channel catfish (Tiersch et al., 1992) and *Xenopus* (Connor and Ashworth, 1992).

Homologues of a human ZFY were isolated from the genome of the American alligator (*Alligator mississippiensis*). ZFY was originally a candidate for the primary testis-determining gene in human, but is now thought to function further down the sex-determinating cascade in several species. Two isolated alligator genes (Zfc and Znc6) coded for zinc finger proteins exhibit amino acid homologies to human ZFY of 91% and 73%, respectively and Znc6 is found on the X chromosome. Analysis of Zfc and Znc6 expression during embryonic development identified two major transcripts of 5.9 kb and 2.7 kb coding for Zfc, in which only one transcript of 4.8 kb was detected for Znc6. Both genes are transcribed at all stages tested. But the expression level of all transcripts appears to decline towards the time of hatching (65 - 72 days). No sex-specific differences in the expression were observed. The expression patterns

indicated that these genes do not play a primary role in temperature-dependent sex-determination.

Subtractive hybridization was used to identify differences in gene expression between medaka (*Oryzias latipes*) males and females during sex differentiation. Fifty female-specific cDNA fragments were cloned. They can be classified into three groups by virtue of whether their earliest expression is at 1, 5, or 30 days after hatching. All 15 near full-length cDNAs belonging to the first two groups were cloned. Many of these female-specific genes are coordinately expressed in oocytes at the earliest stages of oogenesis. Some of the genes that were identified by their sequences include egg envelope proteins, oocyte-specific RNA binding proteins, and a transcription factor containing a basic helix-loop-helix motif (Kanamori, 2000).

Subtractive hybridization and RACE-PCR were also used to identify differentially expressed genes during different stages of oogenesis of the gibel carp (*Carassius auratus gibelio*). A novel SNX gene exhibiting differential transcription between previtellogenic and fully mature oocytes in naturally gynogenetic gibel carp was cloned and characterized. The full length cDNA of SNX was 1392 bp long and coded for a novel SNX protein with 225 amino acids. The 5′ UTR had 72 bp and 3′ UTR had 642 bp. Unlike most of maternal genes that are transcribed after vitellogenesis and stored in oocytes, this gene is expressed at a higher level in the previtellogenic oocytes and at a much lower level in fully matured oocytes. However, RT-PCR analysis of tissue distribution of this gene showed that it was ubiquitously transcribed. Major expression of this gene in the previtellogenic oocytes suggests that it might have an important function in oogenesis of *C. gibelio* (Wen, Xie and Gui, 2003).

In insects, sex determination system has been well studied in *Drosophila melanogaster* because several spontaneous mutants affecting sex determination were found. The ratio of X chromosomes (X) to autosomes (A) is the primary signal for sex determination in this species. The gene *Sex-lethal* (*Sxl*) controls sex determination, dosage compensation, and oogenesis in *D. melanogaster* and can be activated only when the X/A was 1 or more and a female develops. Alternatively, a ratio of 0.5 (X: AA) leaves *sxl* inactivate and male development occurs.

Sxl is an RNA-binding protein that controls splicing of the *transformer* (*tra*) pre-mRNA, its downstream target in sex determination, by binding to and thereby blocking an optimal splice acceptor site (Sosnowski et al., 1989; Inoue et al., 1990). This allows female *D. melanogaster* to produce the functional tra protein through female-specific splicing. The female tra protein induces female-specific splicing of the *doublesex* (*dsx*) pre-mRNA in cooperation with the tra-2 gene product.

The *dsx* gene is known as the final gene of the sex-determining cascade in *D. melanogaster*. Female- or male-specific proteins of *dsx* regulate the expression of sex-specific differentiation gene such as yolk protein genes (Burtis et al., 1991; Jursnich et al., 1993; An et al., 1995a, b). The *dsx* protein binds to a specific site of an enhancer sequence in the 5′ flanking region of the yolk protein genes and regulates their transcription (Garabedian et al., 1986; Coschigano et al., 1993). The *dsx* proteins have a zinc finger-like domain called DM domain (Erdman et al., 1993; Erdman et al., 1996; Raymond et al., 1998).

The *dsx* homologues have been identified in many other species including mab-3 from *Caenorhabditis elegans* (Shen et al., 1998), DMRT1 from human (Raymond et al., 1998; Moniot et al., 2000) and *Dmrt*1 from mouse and chicken (Raymond et al., 1999; De Grandi et al., 2000). These *dsx* homologues were all contain DM domain and considered to regulate sexual differentiation and have been evolutionarily conserved as sex-determining genes.

A homologue of *dsx* was isolated in the silkworm (*Bombyx mori*) which has an epistatic feminizing gene action located on the W chromosome. RT-PCR analysis indicated that *B. mori dsx* (*Bmdsx*) was transcribed in all examined tissues (malpigian tubule, fatbody, testis, antennae, coupulatory organ, ejaculatory cisterna, ovary and pheromone gland), and the size of the amplified products was different between males (226 bp) and females (475 bp). The *Bmdsx* also showed a sex-specific size difference as revealed by Northern blot hybridization. The male-specific cDNA lacked the nucleotide sequence between 713 and 961 nucleotides of female-specific cDNA because the primary *Bmdsx* transcript is alternatively spliced. These results suggest the *Bmdsx* would also regulate sexual differentiation as does the *Drosophila dsx* gene (Ohbayashi et al., 2000).

DMRT1 is a gene encoding a protein with a zinc finger like DNA binding motif (DM domain). The domain contains conserved cysteine and histidine residues that are critical for forming the DNA binding structure (Raymond et al., 1998). DMRT1 has been implicated in vertebrate sexual development by chromosomal location and by embryonic expression. In birds, *Dmrt1* is found on the Z chromosome suggesting that two doses of *Dmrt1* might be necessary for testis development (ZZ = male). In mouse, *Dmrt1* is expressed in the genital ridges of both sex and become testis specific at the end of sex-determining period. Although sex determination is regulated by diverse pathways and upstream signals vary, downstream sex-determining genes may be conserved in part. *Dmrt1* has been suggested to be the first conserved gene involved in sec differentiation found from invertebrates to human (Marchand et al., 2000; Raymond et al., 2000; Zhu et al., 2000).

Marchand et al.(2000) identified a *Dmrt1* homologue in the rainbow trout (*Oncorhynchus mykiss*, rt*Dmrt1*) by RT-PCR using degenerate primers designed against conserved regions of different DM domains including those of *D. melanogaster* dsx, *C. elegans* mab-3 and human DMRT1. Semi-quantitative RT-PCR analysis showed that rtDmrt1 is expressed during testicular differentiation, but not during ovarian differentiation.

After 10 days of steroid treatment, expression was shown to be decreased in estrogen-treated male differentiating gonads but not to be restored in androgen-treated female gonads. This clearly reinforces the hypothesis of an important implication for *Dmrt1* in testicular differentiation in all vertebrates. In the adults, a single 1.5 kb transcript was detected by Northern blot analysis in the testis, and its expression was found to be sustained throughout spermatogenesis and declined at the end of spermatogenesis (stage VI). RT-PCR analysis detected a slight expression of rtDmrt1 in the ovaries of *O. mykiss*.

Drew and Brindley (1995) found two female-specific sequences from *Schistosoma mansoni* by representational difference analysis (RDA), which was a PCR-based modification of the subtractive hybridization process. An excess of male DNA was utilized to remove common sequences of both male and female adult *S. mansoni* from female genomic DNA. Following three rounds of RDA, two major bands (338 bp and 715 bp) were still evident. These fragments were cloned and

characterized. Additional six fragments from the third RDA round ranging from 106 bp – 217 bp in size were also cloned.

Cloned fragments of 338 and 715 bp both strongly hybridized to female amplicon DNA but not male amplicon DNA. Both clones hybridized to two bands within the female amplicon, suggesting that they represented multi-copy DNA sequences. When genomic DNA were probed, the 338- and 715-bp clones hybridized strongly to many bands in female genomic DNA but did not hybridize to the male genomic DNA. In addition, these clones provided positively hybridized bands at approximately multiples of the length of each clone when probed to female genomic DNA indicating a tandem arrangement of each element on the W chromosome of *S. mansoni*. The 338-bp fragment showed 76% identity with members of the SMα family of *S. mansoni* retroposons. In contrast, the 715-bp female-specific fragment did not show significant identity to any previously reported sequence.

Boag et al. (2000) successfully isolated and characterized of sex-specific gene expression from the nodule worm; *Oesophagostomum dentatum* using RNA arbitrarily-primed polymerase chain reaction (RAP-PCR). A total of 31 bands showing differential expression between sexes were cloned and sequenced. Northern blot analysis indicated that ten ESTs were exclusively expressed in males (adults and fourth-stage larvae), while two ESTs were expressed solely in female stages. Three ESTs were expressed in both sexs, but at higher levels in females, and five ESTs could not be detected by Northern blotting analysis suggesting that they were rare transcripts. The remaining 11 ESTs represented false positive results.

Sequence analysis revealed that two male-specific ESTs were significantly matched with *C. elegans* deduced proteins containing EGF-like cysteine motif and a serine/threonine phosphatase and with non-nematode sequences. Two female-specific ESTs were significantly similar to vitellogenin-5 and endonuclease III deduced from *C. elegans* sequences. The remaining ESTs had no similarity to sequences in the GenBank.

Llado et al. (1998) isolated a novel male-specific gene (*Hgm1*) from the plant parasitic nematode, *Heterodera glycines* by differential mRNA display analysis. Several male-specific differential display bands of *H. glycines* were cloned and

sequenced. One recombinant clone, *Hgm1*, contains 279 bp of a cDNA sequence that shows no homology to sequence in available databases. This cDNA was used to screen a *H. glycines* male cDNA library and subsequently by RACE-PCR for identification of the full length cDNA. The full length *Hgm1* contained an 1529 bp cDNA with a predicted open reading frame of 466 amino acid residue corresponding to a 49.5 kDa acidic protein with the pI of 4.72. The expression of *Hgm1* was determined using virtual Northern blot analysis and was not detected in J2 juvenniles but low levels of expression were found in fusiform juveniles with the maximal expression level in males. There is no detectable expression of *Hgm1* in either saccate or enlarged saccate females indicating the male-specific gene expression profile of *Hgm1*.

The objectives of this thesis were identification and partial characterization of sex-related genes/transcripts of *P. monodon* using RT-PCR of conserved sex-determining genes (*Dmrt1*, mab-3 and *dsx*), candidate sex-related expressed sequence tags (ESTs) and candidate sex-specific AFLP markers were also carried out. In addition, Subtractive hybridization of cDNA from ovaries and testes of *P. monodon* was performed. The expression profiles of female-specific/differential expression markers in juvenile and adult *P. monodon* were examined.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

- Autoclave : model HICLAVE, HVE – 50, HIRAYAMA, Japan)
- Automatic micropipettes P2, P10, P20, P200 and P1000 (Gilson Medical Electrical S.A., France)
- Camera Pentax K1000 (Asahi Opt. Co., LTD., Japan)
- -20 ºC Freezer (Songserm, Thailand)
- -30 ºC Freezer (Sanyo, Japan)
- -80 ºC Freezer (Sanyo, Japan)
- Horizontal Gel Electrophoresis Systems (GibcoBRL and BioRad, USA)
- Hybridization Oven : model Midi Dual 14 (Hybaid, England)
- Incubator (Sanyo, Japan)
- Laminar flow Cabinet : Nuaire Class II model NU – 440 – 300E (USA)
- Microcentrifuge tube (Sorenson, USA)
- PCR Thermal cycler
  - : PCR sprint (Hybaid, England)
  - : Omnigene (Hybaid, England)
  - : Perkin Elmer 9700 (USA)

- Pipette tips 10, 20, 200 and 1000 µl
- Power supply (Bio – RAD Laboratories, USA)
  - : Power PAC300
  - : Power PAC Junior
  - : model 200/2.0
- Refrigerated centrifuge 3K18 (Sigma Osterode and Harz, Germany)
- Shaking incubator GFL1083
- Spectrophotometer Spectronic Genesys 5, Milton Roy, Germany)
- UV Transilluminator M – 26 (UVP, USA)
- Vacuum Blotter 785 (Bio – RAD Laboratories)
- Water bath SBS30 (Stuart Scientific, UK)

2.1.2 Chemicals

- Absolute ethanol (Mallinckrodt, Mexico)
- Agarose gel (FMC Bioproducts,USA)
  - : Metaphor agarose
  - : Sekem® LE agarose
- Bacto – agar (Oxoid, England)
- Bacto – tryptone (Oxoid, England)
- Bacto – yeast extract (Oxoid, England)
- Boric acid (Sigma Chemical Co., USA)
- Chloroform (Merck, Germany)

- Diethyl pyrocarbonate sulfoxide (DEPC, Sigma ; USA)
- Lambda DNA (Promega Corporation Medison, Wisconsin, USA)
- 100 base-pair DNA ladder (SibEnzyme)
- 100 mM dATP, dCTP, dGTP, dTTP (SibEnzyme)
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid (EDTA), disodium salt dehydrate (BDH Laboratory Supplies, England)
- Ficoll400 (Sigma Chemical Co., USA)
- Glucose (Sigma Chemical Co., USA)
- Isoamylalcohol (Sigma Chemical Co., USA)
- Isopropanol (LabScan Asia Co., Ireland)
- NucleoSpin Plasmid Extraction Kit (MACHEREY-NAGEL, Germany)
- Phenol, redistilled (Aldrich Chemical Co., USA)
- QIAquick® Gel Extraction Kit (QIAGEN, Germany)
- QIAprep® Spin Miniprep Kit (QIAGEN, Germany)
- QuickPrep *micro* mRNA Purification Kit (Amersham Phamacia Biotech, Sweden)
- Sodium acetate (Merck, Germany)
- Sodium Hydroxide (Merck, Germany)
- Tris-(hydroxyl methyl)-aminomethane (USB, Amersham Life Science, England)
- TRI REAGENT® (Molecular Research Center, Inc., USA)

2.1.3 Enzymes

- Restriction endonucleases ; *Alu*I, *Eco*RI and *Rsa*I (Promega Corporation Medison, Wisconsin, USA)
- DyNAzyme^{TM} DNA Polymerase (Finnzyme, Finland)
- Proteinase K (Sigma Chemical Co., USA)
- RNase A (Sigma Chemical Co., USA)
- T4 DNA ligase (Promega Corporation Medison, Wisconsin, USA)

2.1.4 Bacterial strain

- *Escherichia coli* strain JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* δ*(lac-pro AB) F′ (traD 36 proAB*$^{+}$ *lac*^{q} *lacZ* δ *M15*)
- *Escherichia coli* strain XL-1 Blue (F′:: Tn *10 proA*$^{+}$ *B*$^{+}$ *lacl*^{q} δ*(lacZ) M15 / recA1 endA1 gyrA96 (Nal*^{r}*) thi HsdR17 (*r_h^{-} m_h^{+}*) supE44 relA1 lac*)

2.1.5 Cloning vector

- pGEM® –T easy vector (Promega Corporation Medison, Wisconsin, USA)

2.2 Samples

Juveniles of *P. monodon* (approximately 20 g body weight, 4-month-old) were purchased from local farms in Chachengsao, eastern Thailand. In addition, male and female broodstock-sized *P. monodon* were live-caught from Angsila, Chonburi and transported back to the laboratory at the Marine Biotechnology Research Unit (MBRU), National Center for Genetic Engineering and Biotechnology (BIOTEC) located at Chulalongkorn University. The gender and weight of each *P. monodon* individual were recorded.

Total RNA from mature and immature ovaries and testes were extracted from shrimp for RT – PCR analysis. In addition, DNA from ten male shrimps (2, 2, 2, and

4 from Satun, Trang, Phangnga and Chumphon) and female shrimps (2 individuals each from Satun, Trang, Phangnga, Chumphon and Trad) were used for identification of single nucleotide polymorphisms (SNPs).

2.3 Nucleic acid extraction

2.3.1 DNA extraction

Genomic DNA was extracted from pleopod of each shrimp using a proteinase K / phenol-chloroform method (Klinbunga et al., 1999). After removing from a -80 ºC freezer, a piece of pleopod was placed in a prechilled microcentrifuge tube containing 500 µl of TEN buffer (100 mM Tris-HCl, 100 mM EDTA and 250mM NaCl, pH 8.0) and briefly homogenized with a micropestle. RNase A and 40% SDS solution was added to a final concentration of 100 µg/ml and 1.0% (w/v), respectively. The resulting mixture was then incubated at 65 ºC for 1 hour following by an addition of 5 µl of a proteinase K solution (10 mg/ml). The mixture was further incubated at the same temperature for approxemately 3 – 4 hours. DNA was extracted by an equal volume of buffer equilibrated phenol and mixed gently for 15 minutes. The mixture was centrifuged at 12000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a new sterile microcentrifuge tube and further extracted once with phenol and once with chloroform-isoamyl alcohol (24:1). One-tenth volume of 3 M sodium acetate (pH 5.5) was added. DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and incubated at -80 ºC for 30 minutes. The precipitated DNA was recovered by centrifugation at 12000 rpm for 15 minutes at room temperature and briefly washed twice with 70% ethanol. DNA pellet was air-dried and resuspended in 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). The DNA solution was incubated at 37 ºC for 1 – 2 hours for complete redissolved and kept at 4 ºC until further required.

2.3.2 RNA extraction

Total RNA was extracted from ovaries and testes of each the shrimp using TRI REAGENT®. A piece of tissues 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 and left for 5 minutes, before adding 0.2 ml of chloroform. The homogenate was vortexed for 15 seconds and left at room temperature for 2 – 15 minutes and centrifuged at 12000g for 15 minutes at 4 °C. The mixture was separated into the lower red, phenol-chloroform phase, the interphase, and the colorless upper aqueous phase. The aqueous phase (inclusively containing RNA) was 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 12000g for 10 minutes at 4 - 25 °C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 7500g for 5 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 -86 ° C freezer for long storage.

Total RNA was also extracted from other tissues including antennal gland, eyestalks, gills, heart, hemocytes, hepatopancreases, lymphoid organs, intestine, stomach, pleopod and thoraxic ganglion of *P. monodon* using the same extraction procedure.

2.4 Measuring concentrations of extracted DNA and RNA using spectrophotometry and electrophoresis

The concentration of extracted DNA or RNA samples is estimated by measuring the optical density at 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml double stranded DNA, 40 µg/ml single stranded RNA and 33 µg/ml single stranded DNA (Sambrook et al., 2001). Therefore, the concentration of DNA/RNA samples were estimated in µg / ml by using the following equation,

[DNA] = OD_{260} x dilution factor x 50 (40 or 33 for RNA or single stranded DNA, respectively)

The purity of DNA samples can be evaluated from a ratio of OD_{260} / OD_{280}. The ratios of appropriately purified DNA and RNA were 1.8 and 2.0, respectively (Sambrook et al., 2001).

2.5 Amplification of conserved sex-specific genes (DMRT-1, *dsx* and mab-3)

2.5.1 Primer design

Homologues of genes involving with sex determination were characterized. These included **DMRT1** which is found in various species of vertebrates and invertebrates, ***dsx*** that is found in *Drosophila* and **mab-3** which is found in *C. elegans*. These genes share the conserved DM domain.

Genes encoding for DMRT1, dsx and mab3 from *Bactrocera tryoni* (accession numbers AF029675 and AF029676, http://www.ncbi.nlm.nih.gov), *C. elegans* (NM_063697 and AF022388), *D. melanogaster* (NM_079548 and AY060257), *Homo sapiens* (Al162131 and XM_015427), *Megaselia scalaris* (AF283695 and AF283696), *Mus musculus* (AL133300 and NM_015826), *Fugu rubribes* (AJ295039), *Tetraodon nigroviridis* (AJ295040), *O. mykiss* (AF209095), *O. latipes* (AF319994) and *Sus scrota* (AF216651) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov) and multiple aligned. Primers and nested primers (Table 2.1) were designed and used for PCR and RT-PCR analyses.

Table 2.1 Degenerated primer, primer sequences and melting temperature of primers from designed from the conserved DM domain of various species

Gene/Primer	sequence	Tm (° C)
DMRT1/*dsx* – F	5′- AAN TGC GCN CGV TGC CGN AAY CA -3′	65.4
DMRT1 – R	5′- GTA AGR AAV MDN GAM GGC TGG T -3′	60.2
dsx – R	5′- HAV TAT CAC ATA CAT DAG YGG CA -3′	56.8
nDMRT1 – F	5′- CAG MGV GTB ATG GCS GCB CAG GT -3′	68.6
nDMRT1 – R	5′- GTA GTA GGA GST KTC CAV CAG CAG GT -3′	66.6
n*dsx* – F	5′- ACA GCA GAY CGB CAG MGS GTN ATG GC -3′	69.8
n*dsx* – R	5′- CGA TTG TGC BGG HGT RGG YAC CGT -3′	67.8
mab3 – F	5′- TAN TGC CAN CGV TGT TTN AAT CA -3′	57.4
mab3 – R	5′- GCT NAT CAA YTG YTG RTT CA -3′	53.2

Abbreviations : N = A, G, C and T ; B = G, C and T ; D = A, G and T ; H = A, C and T ; K = G and T ; M = A and C ; R = A and G ; S = C and G ; V = A, G and C ; Y = C and T.

2.5.2 PCR analysis

Generally, PCR was performed in a 25 ul reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X–100 , 100 mM of each dNTP, 2 mM $MgCl_2$, 0.4 uM of each primer, 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland) and 50 ng of genomic DNA. PCR was carried out in a thermocycler consisting of predenaturation at 94 °C for 3 minutes followed by 5 cycles of denaturation at 94 °C for 45 seconds, annealing at 42 °C for 60 seconds and

extension at 72 °C for 2 minutes and additional 35 cycles of higher stringency of the annealing temperature at 50 - 55 °C (Table 2.2). The final extension was carried out at the same temperature for 7 minutes.

2.5.3 First stranded cDNA synthesis

The first strand cDNA was synthesized from 1 μg of total RNA extracted from ovaries and testes of *P. monodon*, using an ImProm – II™ Reverse Transcription System Kit (Promega). Total RNA was combined with 0.5 μg of oligo (dT18) and appropriate DEPC-treated H_2O in a final volume of 5 μl.. The reaction was incubated at 70 ºC 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 unit, respectively. Finally, 1 μl of ImProm – II reverse transcriptase was added and gently mixed by pipetting. The reaction mixture was incubated at 25 ºC for 5 minutes and at 42 ºC for 90 minutes. The reaction was terminated by incubated at 70 ºC for 15 minutes to terminate reverse transcriptase activity. Concentration and rough quality of first stranded cDNA was spectrophotometrically examined (OD_{260}/OD_{280}) and electrophoretically analyzed (1.0% agarose gel).

2.5.4 RT - PCR analysis

One microgram of the first strand cDNA was used as the template in a 25 μl PCR composing of 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X–100, 2 mM $MgCl_2$, 100 mM of each dNTP, 0.5 uM of each primer and 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland). PCR was carried out using the conditions described in Table 2.3. Eight microliters of the amplification products are electrophoresed though 1.2–1.8% agarose gel depended on sizes of the amplification products and visualized under a UV transilluminator after ethidium bromide staining (Sambrook et al., 2001)

Table 2.2 Conditions for amplification of DMRT1, *dsx* and mab-3 homologues in *P. monodon* using genomic DNA as the template

Primer combination	PCR conditions
1. DMRT1/*dsx*-F+DMRT1-R	**Composition:** 100 μM dNTP, 2 mM $MgCl_2$, 0.4 μM of each primer, 1 unit of Taq polymerase and 50 ng of genomic DNA **PCR profile:** I. 1 cycle of 94 °C for 3 minutes II. 5 cycles of 94 °C for 45 seconds, 42 °C for 1 minutes and 72 °C for 2 minutes III. additional 35 cycles at the annealing temperature of 50 °C IV 1 cycle of 72 °C for 7 minutes
2. DMRT1/*dsx*-F + *dsx*-R	Using conditions described in 1
3. DMRT1/*dsx*-F + mab3-R	Using conditions described in 1 except 25 ng of genomic DNA and 0.5μM of each primer were used
4. mab3-F + mab3-R	Using conditions in 3
5. nDMRT1F + dT_{15}	Using conditions described in 1 except 0.5 μM of each primer and PCR products from DMRT1/dsx-F + dT_{15} were used.
6. n*dsx*-F + dT_{15}	Using conditions in 5

Table 2.3 Conditions for RT-PCR of DMRT1, *dsx* and mab-3 homologues in *P. monodon*.

Primer combination	PCR conditions
1. DMRT1/*dsx*-F + dT_{15}	Composition: 100 μM dNTP, 2 mM $MgCl_2$, 0.5 μM of each primer, 1 unit of *Taq* polymerase and 1 μl of the 1st strand cDNA PCR profile: I. 1 cycle of 94 °C for 3 minutes II. 5 cycles of 94 °C for 45 seconds, 42 °C for 1 minutes and 72 °C for 2 minutes III. additional 35 cycles at the annealing temperature of 50 °C IV 1 cycle of 72 °C for 7 minutes
2. DMRT1/*dsx*-F + DMRT1-R	Using conditions in 1 except PCR products from 1 rather than the 1st strand cDNA was used as the template and the initial annealing temperature was 45 °C
3. DMRT1/*dsx*-F + *dsx*-R	Using conditions in 2
4. DMRT1/*dsx*-F+mab3-R	Using conditions in 2
5. mab3-F + dT_{15}	Using conditions described in 1
6. mab3-F+ DMRT1-R	Using conditions in 1 except the template was PCR products from 2 and the initial annealing temperature was 45 °C
7. mab3-F + *dsx*-R	Using conditions in 6
8. mab3-F+mab-R	Using conditions in 6
9. Repeat 3-8	Omitting preamplification (5 cycles) with the annealing temperature was 45 °C and increasing the annealing temperature from 50 °C to 52 °C

Table 2.3 (cont.)

Primer combination	PCR conditions
10. nDMRT1F + dT_{15}	Using conditions described in 1
11. n*dsx*-F + dT_{15}	Using conditions described in 1
12. DMRT1/*dsx*-F+ nDMRT1-R	Using conditions described in 1
13. DMRT1/*dsx*-F + n*dsx*-R	Using conditions described in 1
14. mab3-F + nDMRT1-R	Using conditions described in 1
15. mab3-F + n*dsx*-R	Using conditions described in 1
16. nDMRT1-F + dT_{15}	Using conditions described in 1
17. nDMRT1-F +DMRT1-R	Using conditions described in 1
18. nDMRT1-F + *dsx*-R	Using conditions described in 1
19. nDMRT1-F + mab3-R	Using conditions described in 1
20. nDMRT1-F + nDMRT1-R	Using conditions described in 1
21. nDMRT1-F + n*dsx*-R	Using conditions described in 1
22. n*dsx*-F + dT_{15}	Using conditions described in 1
23. n*dsx*-F + DMRT1-R	Using conditions described in 1
24. n*dsx*-F + *dsx*-R	Using conditions described in 1
25. n*dsx*-F + mab3-R	Using conditions described in 1
26. n*dsx*-F + nDMRT-R	Using conditions described in 1
27. n*dsx*-F + n*dsx*-R	Using conditions described in 1

2.5.5 Agarose gel electrophoresis

An appropriate amount of agarose (or metaphor agarose) was weighed out and mixed with an appropriate volume of 1 x 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 approximately 60 °C before poured into the gel mold. A comb was inserted. The gel was left to solidified. When needed, the comb was carefully removed. The agarose gel was submerged in a chamber containing an enough amount of 1 x TBE buffer covering the gel for approximately 0.5 cm.

Appropriate volumes of PCR products were 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. A 100 bp DNA ladder was used as the standard DNA marker. Electrophoresis was carried out at 5–6 volts/cm until bromophenol blue moved to approximately one-half of the gel**.** The electrophoresed gel was stained with an ethidium bromide solution (2.5 μg/ml) for 5 minutes and destained in running tap water to remove unbound ethidium bromide from the gel. DNA fragments were visualized under a UV transilluminator and photographed through a red filter using Fomapan Classic 100 film. The exposure time was 10 – 18 seconds.

2.5.6 Elution of DNA fragments

After electrophoresis, the desired DNA fragment was excised from the agarose gel using a sterile scalpel and placed in a preweighed microcentrifuge tube. Three volumes of the QG buffer was added and mixed by inversion of the tube. The mixture was incubated at 50 °C for 10 minutes or until the gel slice was completely dissolved. The mixture was transferred into a QIAquick column inserted in a 2 ml collection tube and centrifuged at 12000 rpm for 1 minute. The flow-though solution was discarded. An another 0.5 ml of the QG buffer was added to the QIAquick column and recentrifuged for 1 minute. After this step, 0.75 ml of the PE buffer was added to the QIAquick column and centrifuged as above. The flow-though solution was discarded. The column was recentrifuged to remove the trace amount of the washing solution. The QIAquick column was then placed into a sterile 1.5 ml microcentrifuge tube. DNA was eluted out by an addition of 15 μl of the EB buffer (10 mM Tris-HCl,

pH 8.5) or H_2O to the center of the QIAquick membrane and left for 1 minute, before centrifuged at 12000 rpm for 1 minute.

2.5.7 Cloning of PCR product

2.5.7.1 Ligation of PCR products 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 DTT, 2 mM ATP and 10% polyethylene glycol ; MW 8000), 3 weiss units of T4 DNA ligase, 25 ng of pGEM® –T easy vector and 50 ng of DNA insert. The reaction mixture was incubated overnight at 4 °C before transformed to *E. coli* JM109 (or XL1-Blue).

2.5.7.2 Transformation of ligation products to *E. coli* host cells

2.5.7.2.1 Preparation of competent cells

A single colony of *E. coli* JM109 (or XL1-Blue) was innoculated 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 an OD_{600} of 0.5 to 0.8. The cells were 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. After resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$, the concentrated cell suspension was devided to 200 µl aliquots. These competent cells was either used immediately or stored at –86 °C for subsequently used.

2.5.7.2.2 Transformation

The competent cells were thawed on ice for 5 minutes. Two to four microlitres of the ligation mixture were added and gently mixed by pipetting and left on ice for 30 minutes. The transformation reaction was heat-shocked in a 42 °C water bath for exactly 45 seconds without shocking. The reaction tube was immediately placed in ice for 2-3 minutes. The mixture were removed from the tubes and added to

a new tube containing 1 ml of prewarmed 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 minutes. The mixture were centrifuged for 20 seconds at room temperature, and gently resuspended in 100 µl of SOC medium and spreaded onto a selective LB agar plates containing 50 µg / ml of ampicillin, 25 µg / ml of IPTG and 20 µg / ml of X-gal and further incubated at 37 °C overnight (Sambrook et al., 2001). The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.5.8 Colony PCR and digestion of the amplification product by restriction endonucleases

Colony PCR was performed in a 25 ul reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X - 100, 100 mM of each dNTP, 2 mM $MgCl_2$, 0.1 uM 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 Dynazyme™ DNA Polymerase (FINNZYMES, Finland). An interesting colony was picked by a pipette tip 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 30 seconds, annealing at 50 °C for 1 minute and extension at 72 °C for 1.5 minutes. The final extension was carried out at the same temperature for 7 minutes. The colony PCR products were electrophoresed though 1.2% agarose gel and visualized after ethidium bromide staining.

Colony PCR products containing an insert were separately digested with *Alu* I and *Rsa* I (Promega) in a 15 µl reaction volume containing 10x 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 were electrophoresed though a 1.2% agarose gel and visualized under a UV transilluminator after ethidium bromide staining.

2.5.9 Isolation of recombinant DNA

Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (QIAGEN). A white colony was inoculated into a sterile tube containing 3 ml of LB broth supplemented with 50 μg/ml of ampicillin and incubated with shaking (200 rpm) at 37 °C overnight. The culture was transferred into a new 1.5 ml microcentrifuge tube and centrifuge at 13000 rpm for 1 minute.The supernatant was carefully decanted. The pellets were resuspended in 250 μl of the P1 buffer. A 250 μl of the P2 buffer was added to the tube and gently inverted for 4 – 6 times. Then, 350 μl of the N3 buffer was added. The tube was thoroughly but gently inverted 4 – 6 times and centrifuged at 13000 rpm for 10 minutes. The supernatant was carefully collected and applied to the QIAprep column and centrifuged at 13000 rpm for 1 minute. The flow-though solution was discarded. The QIAprep spin column was washed by adding 500 μl of the PB buffer and centrifuged at 13000 rpm for 1 minute. The flow-though solution was discarded. The PE buffer (750 μl) was added and centrifuged as above. After the flow-though solution was discarded, the QIAquick column was recentrifuged to remove the trace amount of the washing solution. The column was then placed into a sterile 1.5 ml microcentrifuge tube. Plasmid DNA was eluted out by an addition of 50 μl of the EB buffer (10 mM Tris-HCl, pH 8.5) or H_2O to the center of the QIAprep column and left for 1 minute before centrifuged at 13000 rpm for 1 minute. The concentration of extracted plasmid DNA was spectrophotometrically measured.

2.5.10 DNA sequencing

Five recombinant clones (PMO880, PMO920 and PMT700 from primer ndsx-F + dT_{15} and PMO720 and PMT1700 from primers FDMRT1/DSX + dT_{15}) exhibiting 880 bp, 920 bp, 700 bp, 720 bp and 1700 bp insert were unidirectional sequenced using the M13 forward primer (5′- TTT TCC CAG TCA CGA C -3′) on an automatic sequencer at the Bioservice Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC). Nucleotide sequences were blasted against data in the GenBank using Blast*N* (nucleotide similarity) and Blast*X* (translated protein similarity). Significant similarity was considered when the probability (E) value was $<10^{-4}$.

2.6 Cloning and characterization of Peritrophin homologues in *P. monodon*

2.6.1 Primers

Two pairs of primers (Peri-F_1/R_1 and Peri-F_2/R_2) primed at different regions of the template were designed from nucleotide sequence of peritrophin homologues in *P. monodon* previously isolated from a cDNA library of *P. monodon* ovaries (Table 2.4).

Table 2.4 Primer sequences, melting temperature and expected product sizes of PCR products of peritrophin homologues in *P. monodon*

Gene/Primer	Sequence	Tm (° C)	Expected size (bp)
Peri–F_1	5′- TTG AGG ACC ACT TCT GTT CGG -3′	60	269
Peri–R_1	5′- GCT TGG GTT GGC AAA AGT ACC -3′	59	
Peri–F_2	5′- CTT GGC CCA AAA CTC GTT CAA -3′	60	151
Peri–R_2	5′- TCC ACA TGA CTT GGG ATC AGG -3′	60	

2.6.2 RT – PCR analysis

RT-PCR of the first strand cDNA from mature and immature ovaries, testes, lymphoid organ and antennal ganglion of *P. monodon* were performed in a 25 µl PCR composing of 10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl and 0.1% Triton X–100, 100 µM dNTP, 2 mM $MgCl_2$, 0.4 µM of each primer 1 µg of the 1st strand cDNA from various tissues and 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland). PCR was carried out with predenaturation at 94 °C for 3 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 60 seconds and extension at 72 °C for 60 seconds. The final extension was carried out at the same temperature for 7 minutes. The amplification products were electrophoresed though a 1.8% agarose gel and visualized after ethidium bromide staining.

2.6.3 PCR analysis

PCR was performed using conditions described above except 25 ng of genomic was used as the template with primer Peri-F_1/R_1, Peri-F_2/R_2, Peri-F_1 + Peri-R_2 and Peri-F_2 + Peri-R_1. The PCR products were size-fractionated though a 1.8% agarose gel and visualized after ethidium bromide staining.

2.7 Cloning and sequencing of a zinc finger protein gene homologue

2.7.1 Primers

Previously, a plasmid cDNA library of *P. monodon* ovaries was established and a zinc finger protein (X or Y-linked) homologue was isolated. Primers were designed from this EST sequence (Table 2.5). RT-PCR (cDNA template) and typical PCR (genomic DNA template) were carried out. The PCR product was cloned and sequenced for identification of sex-related single nucleotide polymorphism (SNP) of this transcript.

Table 2.5 Primers, primer sequences and melting temperature of primers designed from a zinc finger gene homologue of the giant tiger shrimp (*Penaeus monodon*)

Gene/Primer	Sequence	Tm (° C)
Zinc – F	5′- AGG ACC ATC CAT TCA ACC TGG -3′	60
Zinc – R	5′- AGT GGC ATA CTC TGA CGG TGG -3′	59
Finger – F	5′- AAG AAG CTG ACG ACC CAG ACG -3′	60
Finger – R	5′- TGC TTG AAT CTC CCT CAT CCC -3′	60
Zincfinger – F	5′- TCG AAA CCC TTG CCG CAT AT -3′	60
Zincfinger – R	5′- TCC AGT CCA GTC TGC ATC GTT -3′	64

2.7.2 RT - PCR analysis

The first strand cDNA were synthesized from mature and immature ovaries, testes, lymphoid organ and antennal ganglion. One microgram of the first strand cDNA was used as the template in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl and 0.1% Triton X – 100, 100 μM dNTP, 2 mM $MgCl_2$, 0.4 μM of each primer and 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland). 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 30 seconds, annealing at 55 °C for 60 seconds and extension at 72 °C for 60 seconds. The final extension was carried out at the same temperature for 7 minutes. The amplification products were electrophoresed though 1.0–2.0% agarose gel and visualized under a UV transilluminator after ethidium bromide staining.

RT-PCR products from primers Finger-F/R (201 bp and a large extra band) were cloned using a T-A cloning method. Eight recombinant clones were selected. Plasmid DNA was isolated using a GFX Microplasmid Prep kit (Amersham Pharmacia Biotech) and sequenced using an automated DNA sequencer (LICOR, USA). Nucleotide sequences of these clones were multiple aligned using Clastal X.

2.7.3 PCR analysis

Genomic DNA of *P. monodon* was also used as the template for amplification of a zinc finger gene homologue at the genomic DNA level.

In addition, genomic DNA was extracted from 10 male (N = 2 from Satun, Trang and Chumphon and N = 4 from Phangnga) and 10 female (N = 2 from Satun, Trang, Chumphon, Phangnga and Trad) individuals of *P. monodon*.

A zinc finger protein homologue of each individual was amplified by ZINCFINGER–F + FINGER–R. Amplification reactions were performed in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl and 0.1% Triton X – 100, 2 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP and dTTP, 0.5 μ M of each primer, 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland) and 25 ng of genomic DNA.

The reaction was carried out for predenaturation at 94 °C for 3 minutes followed by 35 cycles composing of a 94 °C denaturation step for 30 seconds, a 55 °C annealing step for 1 minute and a 72 °C extension step for 1 minute. The final extension was carried out at 72 °C for 7 minutes. Eight microliters of the amplification products are electrophoresed though 1.2% agarose gel and visualized by ethidium bromide staining. PCR product from all investigated individuals were eluted out, ligated in pGEM-T Easy vector and transformed to *E. coli* XL–1 Blue. Plasmid DNA was extracted using a NucleoSpin Plasmid Extraction Kit (MACHEREY-NAGEL) and sequenced using an automated DNA sequencer (LICOR, USA).

2.8 Identification of sex–specific expression transcripts in the giant tiger shrimp (*P. monodon*) by cDNA subtraction

Forward and reverse subtractions (Diatchenko et al., 1996) between ovarian and testis cDNAs were carried out using a PCR Select cDNA Subtraction Kit (CLONTECH, USA).

2.8.1 mRNA purification

Total RNA was extracted from ovaries and testes of wild *P. monodon* caught from Chonburi (Gulf of Thailand) using TRI-REAGENT. Messenger (m) RNA was further purified using a QuickPrep *micro* mRNA Purification Kit (Amersham Phamacia Biotech, Sweden).

Extraction buffer (0.4 ml) were added to total RNA (25 µl) of ovaries and testes and mixed. The sample was diluted by adding 0.8 ml of elution buffer, mixed thoroughly and centrifuged at 16,000g for 1 minute. Concurrently, the tube containing 1 ml of oligo(dT)-cellulose for each purification was centrifuged at the same speed for 1 minute. The supernatant was removed. One millilitre of the homogenate was pipetted onto the oligo(dT)-cellulose pellet. The tube was inverted to resuspend the oligo(dT)-cellulose for 3 minutes and centrifuged at 16000xg for 10 seconds at room temperature. The supernatant was carefully removed. The high salt buffer (1 ml) was added to a tube and spun for 10 seconds at 16,000g. The supernatant was carefully removed. The wash was repeated four more times, as described above. The low salt

buffer (1 ml) was added to the oligo(dT)-cellulose pellet. The tube was inverted and spun at 16,000g for 10 seconds. This wash was repeated once. The pellet from the final wash was resuspended in 0.3 ml of the low salt buffer. The slurry was transferred to a MicroSpin column and spun for 5 seconds. The flow-though solution was discarded. The low salt buffer (0.5 ml) was added and further spun for 5 seconds. This step was repeated twice. The column was then placed into a sterile 1.5 ml microcentrifuge tube. The mRNA was eluted out by an addition of 0.2 ml of the prewarmed elution buffer to the top of column and centrifuged at 16000xg for 5 seconds. A second, 0.2 ml of prewarmed elution buffer was added to the top of column to elute residual mRNA and centrifuged as described above.

2.8.2 First strand cDNA Synthesis

One microgram of each tester and driver mRNA was combined with 1 µl of 10 µM of the cDNA synthesis primer in a sterile microcentrifuge tube. Sterile H_2O was added to a final volume of 5 µl. The reaction mixture was mixed by pipetting and centrifuged briefly. The reaction tube was incubated at 70 °C in a thermal cycler for 2 minutes. The tube was cooled on ice for 2 minutes and briefly centrifuged. The first strand cDNA synthesis was synthesized by adding 2 µl of 5x First-Strand Buffer (250 mM Tric-HCl, pH 8.5, 40 mM $MgCl_2$, 150 mM KCl and 5 mM Dithiothreitol), 1 µl of dNTP Mix (10 mM each of dNTPs), 1 µl of sterile H_2O, and 1 µl of (20 units) AMV Reverse Transcriptase. The reaction was gently vortexed and briefly centrifuged. The reaction was incubated at 42 °C for 1.5 hours in an air incubator. The tubes were placed on ice to terminate the first strand cDNA synthesis.

2.8.3 Second strand cDNA Synthesis and purification

The first strand synthesis reaction mixture was combined with 48.4 µl of Sterile H_2O, 16.0 µl of 5x second strand buffer (100 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, 500 mM KCl, 50 mM ammonium sulfate, 0.75 mM β-NAD and 0.25 mg/ml BSA), 1.6 µl of dNTP mix (10 mM) and 4.0 µl of 20x second strand enzyme cocktail (DNA polymerase I, 6 units/µl; RNase H, 0.25 units/µl; and *E. coli* DNA ligase, 1.2 units/µl), mixed and briefly spun. The reaction was incubated at 16 °C for 2 hours. After that, 2 µl of T4 DNA Polymerase (3 units/µl) were added to the second strand reaction mixture and further incubated at 16 °C for 30 minutes. Then, 4 µl of 20x

EDTA / Glycogen (0.2 mM EDTA; 1 mg/ml glycogen) was added to the reaction and mixed thoroughly. Then, 100 µl of phenol : chloroform : isoamyl alcohol (25:24:1) were added and vortexed. The mixture was centrifuged at 14,000 rpm for 10 minutes at room temperature. The top aqueous layer was carefully transferred to a sterile microcentrifuge tube. The phenol extraction was repeated. To precipitate the synthesized cDNA, 40 µl of 4 M NH_4OAc and 300 µl of absolute ethanol were added and thoroughly mixed by vortexing and centrifuged at 14,000 rpm for 20 minutes at room temperature. The supernatant was carefully removed. The pellet was overlaid with 500 µl of 80% ethanol and centrifuged at 14,000 rpm for 10 minutes. The pellet was air dried for 10 minutes to evaporate residual ethanol. The pellet was dissolved in 50 µl of sterile H_2O.

Table 2.6 Sequences of the PCR-Select cDNA synthesis primer, adaptors and PCR primers.

Primer/Adaptor	**Sequence**
cDNA synthesis primer	5′- TTTTGTACAAGCTT$_{30}$N$_1$ n -3′
Adaptor 1	5′- CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT -3′ 3′- GGCCCGTCCA -5′
Adaptor 2R	5′-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3′ 3′-GCCGGCTCCA-5′
PCR primer 1	5′- CTAATACGA CTCCATATAGGGC -3′
Nested PCR primer 1	5′- TCGAGCGGCCGCCCGGGCAGGT -3′
Nested PCR primer 2R	5′- AGCGTGGTCGCGGCCGAGGT -3′
Control Primers :	
G3PDH 5′ Primer	5′- ACCACAGTCCATGCCATCAC -3′
G3PDH 3′ Primer	5′- TCCACCACCCTGTTGCTGTA -3′

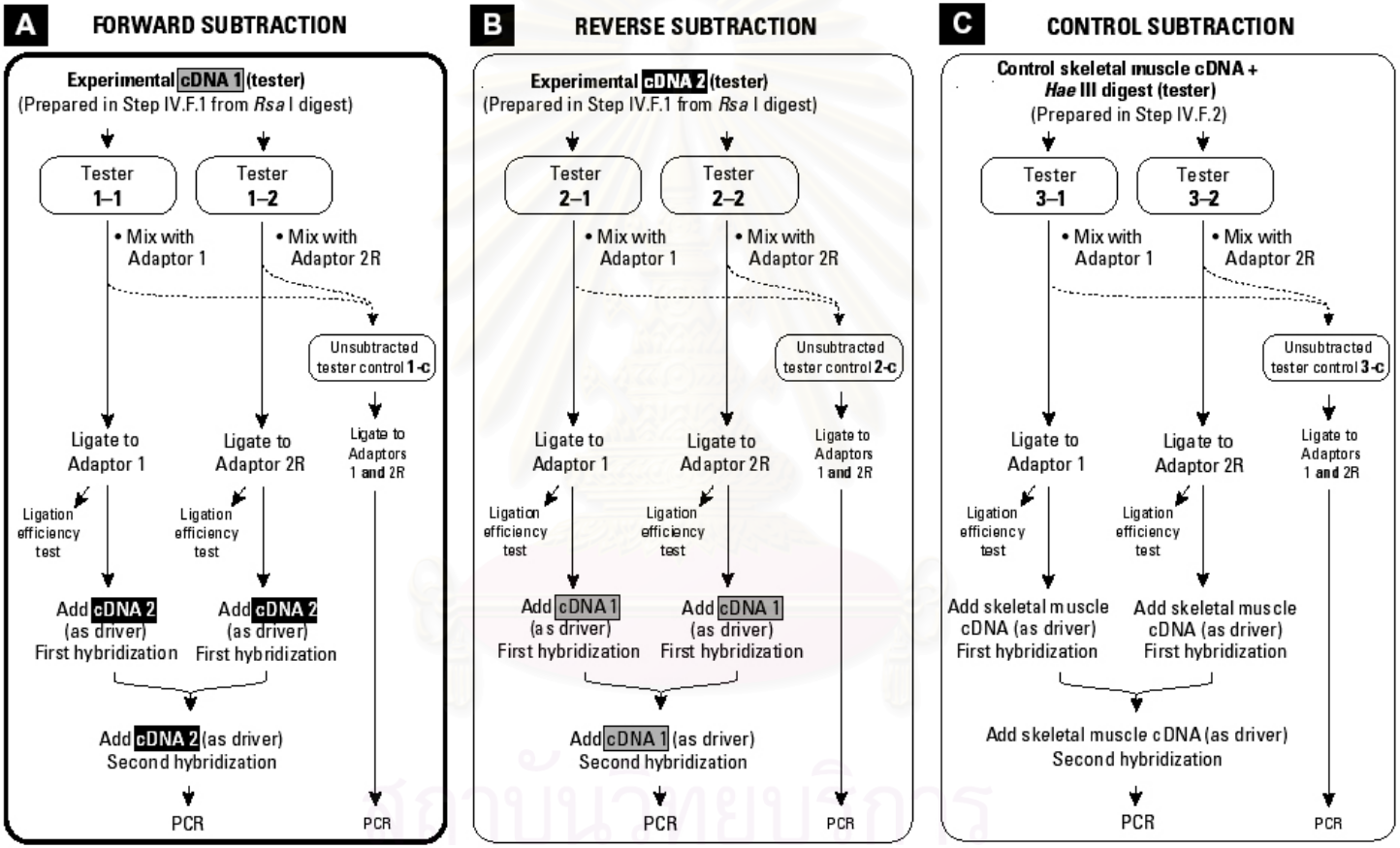

Figure 2.1 Preparation of adaptor-ligated tester cDNA for hybridization and PCR

2.8.4 *Rsa* I Digestion

The restriction mixture containing 43.5 µl of double strand cDNA of tester and driver, 5.0 µl of 10x *Rsa* I restriction buffer (100 mM Bis Tris Propane-HCl (pH 7.0), 100mM $MgCl_2$ and 1 mM DTT) and 1.5 µl of *Rsa* I (10 units/µl) was set up. The digestion was incubated at 37 °C for 1.5 hours. At the end of the incubation time, 5 µl of the digest were collected for analysis of *Rsa* I digestion efficiency by agarose gel electrophoresis. After that, 2.5 µl of 20x EDTA/glycogen mix was added to terminate the reaction. An equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) was added and vortexed. The reaction tube was centrifuged at 14,000 rpm for 10 minutes at room temperature. The top aqueous layer was carefully removed and placed in a sterile microcentrifuge tube. The phenol/chloroform extraction was repeated. To recover the digested cDNA, 25 µl of 4 M NH_4OAc and 187.5 µl of absolute ethanol were added. The tube was thoroughly vortexed and centrifuged at 14,000 rpm for 20 minutes at room temperature. The supernatant was carefully removed. The pellet was briefly washed with 200 µl of 80% ethanol and centrifuged at 14,000 rpm for 5 minutes. The pellet was air dried for about 10 minutes and dissolved in 5.5 µl of sterile H_2O and stored at -20 °C. This digested cDNA served as the experimental driver cDNA. Subsequently, the sample was ligated with adapters to create tester cDNAs for forward and reverse subtraction.

2.8.5 Adaptor Ligation

One microlitre of *Rsa* I – digested tester cDNA was diluted with 5 µl of sterile H_2O. A ligation master mix was prepared by combining 3 µl of sterile H_2O, 2µl of 5x ligation buffer (250 mM Tris-HCl; pH 7.8, 50mM $MgCl_2$, 10 mM DTT and 0.25 mg/ml BSA) and 1 µl of T4 DNA ligase (400 units/µl). For each experimental tester cDNA, the reagents were combined in Table 2.7 in order,

Table 2.7 Ligation reactions of cDNA of ovaries (tester 1-1 and 1-2) and testes (tester 2-1 and 2-2)

	cDNA for ovaries		**cDNA for testes**	
Tube no. :	**1**	**2**	**3**	**4**
	Tester 1 – 1	**Tester 1 – 2**	**Tester 2 – 1**	**Tester 2 – 2**
Component	**(µl)**	**(µl)**	**(µl)**	**(µl)**
Diluted tester cDNA	2	2	2	2
Adaptor 1 (10 µM)	2	-	2	-
Adaptor 2R (10 µM)	-	2	-	2
Master Mix	6	6	6	6
Final volume	10	10	10	10

* tester 1-1 and 1-2 and 2-1 and 2-2 were mixed and subsequently served as unsubtracted tester control 1-C and 2-C, respectively.

The tubes were briefly centrifuged and incubated at 16 °C overnight. The ligation reactions were stopped by adding 1 µl of the EDTA/glycogen mix, heated at 72 °C for 5 minutes to inactivate the ligase activity.

2.8.6 First Hybridization

For each experimental subtraction, The reaction mixture was prepared in order according to Table 2.8. A drop of mineral oil and centrifuged briefly. The samples were incubated in a thermal cycler at 98 °C for 1.5 minutes. The first hybridization was carried out at 68 °C for 8 hours and proceeded immediately to the second hybridization step.

Table 2.8 Composition of the first hybridization reaction of each subtraction

Component*	**Hybridization Sample 1 (µl)**	**Hybridization Sample 2 (µl)**
Rsa I – digested driver cDNA	1.5	1.5
Adaptor 1 – ligated Tester 1 - 1	1.5	-
Adaptor 2R – ligated Tester 1 - 2	-	1.5
4x Hybridization Buffer	1.0	1.0
Final volume	4.0	4.0

* tester 2-1 and 2-2 (testes) were identical

2.8.7 Second Hybridization

To carry out the second hybridization: 1 µl of *Rsa* I – digested driver cDNA, 1 µl of 4x Hybridization Buffer and 2µl of sterile H_2O were added to a 0.5 ml microcentrifuge tube. One microlitre of mixture was placed in another 0.5 ml microcentrifuge tube and overlaid with a drop of mineral oil. The mixture was incubated in a thermal cycler at 98 °C for 1.5 minutes and simultaneously mixed with the two hybridization samples (hybridization samples 1 and 2 from the first hybridization, Table 2.8) by pipetting. The reaction was incubated at 68 °C overnight. After that 200 µl of dilution buffer (20mM HEPES, pH 6.6, 20 mM NaCl and 0.2 mM EDTA, pH 8.0)was added and mixed by pipetting. The reaction mixture was heated in a thermal cycler at 68 °C for 7 minutes and stored at -20 °C.

2.8.8 PCR Amplification

The PCR templates were prepared by aliquot 1 µl of each subtracted cDNA samples or unsubtracted testes control into an appropriately labeled tube. A master mix enough for all of the primary PCR tubes was prepared (Table 2.9).

Table 2.9 Preparation of the primary PCR master mix

Reagent	Amount per reaction (µl)
Sterile H_2O	19.5
10x PCR reaction buffer	2.5
dNTP Mix (10 mM)	0.5
PCR primer 1 (10 mM)	1.0
50x Advantage cDNA Polymerase Mix	0.5
Total volume	24.0

The reaction was mixed well by vortexing, and briefly centrifuged. An aliquot of 24 µ l of the master mix was dispensed to each tube and overlaid with 50 µl of mineral oil. The reaction was incubated in a thermal cycler at 75 °C for 5 minutes to extend the adaptors. The amplification reaction was carried out for 27 cycles composing of a 94 ° C for 30 seconds, 66 °C for 30 seconds and a 72 °C for 1.5 minutes. After amplification, 8 µl from each tube were electrophoretically through a 2.0% agarose gel.

A ten-fold dilution was performed using 3 µl of each primery PCR mixture. One microlitre of each diluted primary PCR product mixture was added into an appropriately labeled tube. A master mix for secondary PCR was prepared (Table 2.10).

Table 2.10 Preparation of the secondary PCR master mix

Reagent	Amount per reaction (µl)
Sterile H_2O	18.5
10x PCR reaction buffer	2.5
Nested PCR primer 1 (10 µM)	1.0
Nested PCR primer 2R (10 µM)	1.0
dNTP Mix (10 mM)	0.5
50x Advantage cDNA Polymerase Mix	0.5
Total volume	24.0

The reaction was mixed well and briefly centrifuged. An aliquot of 24 µl of the second master mix was dispensed to each of tube and overlaid with a drop of mineral oil. The reaction was carried out for 12 cycles composing of a 94 °C for 30 seconds, 68 °C for 30 seconds and a 72 °C for 1.5 minutes. After PCR, 8 µl from each tube were size-fractionated through a 2.0% agarose gel.

2.8.9 Cloning

The resulting products from the forward subtraction (cDNA from ovaries as the tester) and reverse reaction (cDNA from testes as the tester) were separately ligated to pGEM – T Easy vector (Promega, USA) and transformed to *E. coli* JM109. Recombinant clones were selected. Plasmid DNA was extracted as previously described.

2.8.10 Sequencing

Two hundred and seventeen clones (156 and 61 for subtractive ovarian and testis libraries, respectively) were randomly selected and unidirectional sequenced using an automated DNA sequencer (LI-COR, USA). Nucleotide sequence were blasted against data in the GenBank using Blast*N* and Blast*X*. Significant similarity with known transcripts was considered when the probability (E) value was $<10^{-4}$.

2.9 Characterization of Thrombospondin (TSP) homologue by Rapid amplification of cDNA ends-polymerase chain reaction (RACE - PCR)

TSP homologues were further characterized using a SMART RACE cDNA Amplification Kit (Clontech).

2.9.1 Primer design

Gene–Specific Primers (GSPs) were designed from TSP homologues obtained from the subtractive cDNA library of *P.monodon* ovaries. An antisense primer for the 5′ RACE – PCR (TSP-1) and a sense primer for the 3′ RACE – PCR (TSP-2) were designed for 5′ – and 3′ – RACE – PCR, respectively.

After characterization of RACE product, the first set of TSP-specific primers gave rise to many non-TSP products, additional sets of primers; TSP288-1, TSP371-1 and TSP 462-1 and nested primers nTSP288-1, nTSP371-1 and nTSP462-1 were designed for 5′- RACE – PCR and TSP288-2 and TSP462-2 and nTSP288-2 and nTSP462-2 for 3′- RACE – PCR were designed (Table 2.11).

Table 2.11 Sequences of TSP-specific primers for further characterization of TSP homologues in *P. monodon* using RACE-PCR

Primer	Sequence
5′-RACE :	
TSP-1	5′-TCA CAT CAG TAC CTC GGC CGC GAC CAC -3′
TSP288-1	5′-AGT CCA TGA CCA GAT TAG TAC CTC GGC C -3′
TSP371-1	5′-GCT CTC CTT CAG GCT CGT TCT GTG C -3′
TSP462-1	5′-CTG GAG GAG TAG ACG CCG TTT GTG GG -3′
nTSP288-1	5′-ATT AGT ACC TCG GCC GCG ACC ACC G -3′
nTSP371-1:	5′-TCT CCT TCA GGC TCG TTC TGT GCT AGA-3′
nTSP462-1:	5′-AGG AGT AGA CGC CGT TTG TGG GTC TAG T -3′
3′-RACE :	
TSP-2	5′-ATG GGG ATG GTA CCT GCC CGG GCG -3′
TSP288-2	5′-CTG CAA GTG TTT GGG CTC CAA CAA GT -3′
TSP462-2	5′-ATT TCT AAT TCA GGT ACC TCG GCC GC -3′
nTSP288-2	5′-AAA CCT GTA CCT CGG CCG CGA CCA C -3′
nTSP462-2	5′-TAA TTC AGG TAC CTC GGC CGC GAC A -3′

2.9.2 First strand cDNA synthesis

Total RNA was extracted from ovaries of *P. monodon* using TRI-REAGENT. Messenger (m) RNA was purified using a QuickPrep *micro* mRNA Purification Kit (Amersham Phamacia Biotech). RACE – Ready cDNA 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 and 1 µg of ovarian mRNA with 1 µl of 3′– CDS primer A for 5′- RACE – PCR. 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 DTT, 1 µl of dNTP Mix (10 mM) 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. The tubes were incubated at 42 °C for 1.5 hours in an air incubator. The first strand reaction products were diluted with 250 µl of Tricine–EDTA Buffer and heated at 72 °C for 7 minutes. The first strand cDNA template can be stored at -20 °C for up to three months.

Table 2.12 Primer sequences for the first strand cDNA synthesis and RACE - PCR

Primers	Sequence
SMART II A Oligonucleotide	5′– AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG –3′
3′-RACE CDS Primer A	5′– AAG CAG TGG TAT CAA CGC AGA GTA C $(T)_{30}$ N_{-1} N –3′ (N = A, C, G or T; N_{-1} = A, G or C)
5′-RACE CDS Primer	5′– $(T)_{25}$ N_{-1} N -3′ (N = A, C, G or T; N_{-1} = A, G or C)
10X Universal Primer A Mix (UPM)	Long : 5′– CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT –3′ Short : 5′– CTA ATA CGA CTC ACT ATA GGG C – 3′
Nested Universal Primer A (NUP)	5′– AAG CAG TGG TAT CAA CGC AGA GT –3′

2.9.3 Rapid Amplification of cDNA Ends (RACE)

The same master mix for 5′- and 3′-RACE and control reactions was prepared. For each 50 µl amplification reaction, 35.75 µl of PCR-Grade Water, 5 µl of 10x Advantage 2 PCR buffer, 1µl of 10 µM dNTP mix and 1 µl of 50x Advantage 2 polymerase mix were combined. 5′- RACE – PCR and 3′- RACE – PCR were set up according to Table 2.13 and 2.14 respectively.

Table 2.13 Composition of 5′- RACE–PCR

Component	5′-RACE Sample	UPM only (-Control)	GSP1 only (-Control)
5′-RACE-Ready cDNA	1.25 µl	1.25 µl	1.25 µl
UPM (10x)	5.0 µl	5.0 µl	-
GSP1 (10µM)	1.0 µl	-	1.0 µl
GSP2 (10µM)	-	-	-
H_2O	-	1.0 µl	5.0 µl
Master Mix	42.75 µl	42.75 µl	42.75 µl
Final volume	50 µl	50 µl	50 µl

Table2.14 Composition of 3′- RACE–PCR

Component	3′-RACE Sample	UPM only (Control)	GSP2 only (Control)
3′-RACE-Ready cDNA	1.25 µl	1.25 µl	1.25 µl
UPM (10x)	5.0 µl	5.0 µl	-
GSP1 (10µM)	-	-	-
GSP2 (10µM)	1.0 µl	-	1.0 µl
H_2O	-	1.0 µl	5.0 µl
Master Mix	42.75 µl	42.75 µl	42.75 µl
Final volume	50 µl	50 µl	50 µl

The reaction was carried out for 25 cycles composing of a 94 °C for 30 seconds, 68 ° C for 30 seconds and 72 °C for 3 minutes. Nested PCR was performed using primers discribed in Table 2.10. The primary PCR product was 50-fold-diluted (1µl of the product + 49 µl of Tricine-EDTA buffer). The amplification reaction was performed using 5 µl of the diluted PCR product as the template using the same condition for the first PCR for 15 cycles. Products from the first and nested PCR were electrophoretically analyzed.

2.9.4 Cloning and sequencing RACE Products

For the first set of primers, a 2.3 kb 5′-RACE product (TSP1) and 600 bp and 800 bp 3′-RACE products (TSP2) were eluted out and ligated with pGEM-T easy vector. The ligation product was transformed to *E. coli* JM109. Plasmid DNA was extracted and sequenced.

For the subsequent set of primers an approximately 3 kb product from 5′-RACE (462-1 and n462-1 and 371-1 and n371-1) and 800bp (462-2) and 1000 (n462-2) were cloned and sequenced as described previously.

2.10 Identification of sex-specific/differential expression transcripts using RT-PCR of homospecific primers

2.10.1 Primers

Forward and reverse primers of each gene were designed from nucleotide sequence of *Penaeus monodon* using Primer Premier 5 program.(Table 2.15).

TSP primers were designed from TSP homologues obtained from a subtractive cDNA library of *P. monodon* ovaries.

Primers XNP–1 F/R and AGESTX40-F/R and AGESTX44-F/R were designed from a Sex-linked nuclear protein 1 (XNP-1) homologue obtained from 5′ RACE–PCR and hypothetical protein ENSANGP00000010123 (called AGESTX in this thesis) from 3′ RACE –PCR, respectively.

Subsequently, a cDNA of *P. monodon* ovaries was established as part of the large scale cDNA sequencing of *P. monodon* Approximately 1000 EST clones were sequenced. Homologues of sex-related transcripts; Ubiquitin specific protease 9 (X chromosome in *Mus musculus*), Rudimentary protein, and EGF – response factor (Zinc finger protein, X-linked chromosome in *Homo sapiens*) were identified. Primers were then designed from sequence of these gene homologues.

The parallel experiment on isolation and characterization of sex-specific genomic DNA markers of *P. monodon* based on amplified fragment length polymorphism (AFLP) provided several promising sex-specific AFLP markers in this species. Primers were designed from these markers and one of which (FE_{AGA}-M_{CGA} 520 – F/R) were applied to RT-PCR of the first strand cDNA of ovaries and testes of *P. monodon* (S. Thamrungtanakit, personal communication)

2.10.2 RT - PCR analysis

Basically, amplification reactions were performed in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl and 0.1% Triton X – 100, 1.5 – 2 mM $MgCl_2$, 100 – 200 μM each of dATP, dCTP, dGTP and dTTP, 0.2 – 0.4 μ M of each primer, 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland) and 1 μg of first strand cDNA. The reaction was carried out for 35 cycles composing of a 94 °C denaturation step for 30 – 45 seconds, a 55 – 65 °C annealing step for 45 – 60 seconds and a 72 °C extension step for 45 – 60 seconds. The final extension was carried out at 72 °C for 7 minutes. Eight microliters of the amplification products were electrophoretically analyzed though 1.0 - 2.0% agarose gel.

PCR products resulted from TSP 288-1 + TSP 371-1 (350 bp in ovaries), TSP 462-F + TSP 288-R (1300 bp in testes and 150 bp in ovaries) and AGESTX44–F/R (184 bp in ovaries) were cloned and sequenced to confirm whether the amplification products were not non-specifically amplified.

Table 2.15 Primer sequences, melting temperature and expected product sizes of PCR products of primers from the giant tiger shrimp (*Penaeus monodon*)

Gene/Primer	sequence	Tm (° C)
Disulfide isomerase (DSI)		
DSI-F	5′- GCC GTT GCC AAT AAG GAC GA-3′	62
DSI-R	5′- TCA CCC GCC TTG AGA TTG GT -3′	62
Thrombospondin (TSP)		
TSP 288-F	5′- AAC CCT CTC TCG GGA ATC GAA C -3′	68
TSP 288-R	5′- GTC GCG GCC GAG GTA CTA ATC T -3′	70
TSP 462-F	5′- ATG GCT AAT CCG GGG CAG TTA T -3′	66
TSP 462-R	5′- CGG GCA GGT ACA AAC TCC TAC G -3′	70
TSP 288-1	5′- ATT AGT ACC TCG GCC GCG ACC ACC G -3′	82
TSP 371-1	5′- TCT CCT TCA GGC TCG TTC TGT GCT AGA -3′	82
TSP 462-2	5′- ATT TCT AAT TCA GGT ACC TCG GCC GC -3′	78
Sex lethal protein (*Sxl*)		
Sxl-F	5′- GCG AAC AAA CCT TAT CAT CAA CTA C -3′	70
Sxl-R	5′- CTT CAT TTT GCC GTG CTC CT -3′	60
Sex-linked nuclear protein 1 (XNP-1)		
XNP–1 F	5′- TTC CAC CCT TTT GCT TGC TA -3′	66.2
XNP–1 R	5′- GGA TTT GCC TGA GAC ACC TAA -3′	68.8

Table 2.15 (cont.)

Gene/Primer	sequence	Tm (°C)
Hypothetical protein ENSANGP00000010123 (X-linked in *Drosophila melanogater* ; called AGESTX in this thesis)		
AGESTX40 – F	5′- TCA TGT CGG AGG TCG TGA GTA A -3′	71.3
AGESTX40 – R	5′- AGA ACA AGG AAG AGG AGG GAT T -3′	69.5
AGESTX44 – F	5′- GCG TCA TCA AGT TGT CCG AGT C -3′	73.2
AGESTX44 – R	5′- TGA GGG GCA GTG AAG ATA GGT G -3′	73.2
Ubiquitin specific protease 9 (Usp9X; X chromosome in *Mus musculus*)		
Usp9X – F	5′- GGA AAT GGA CCT GGG CGG -3′	60
Usp9X – R	5′- TCT TCT GGA ACT GCT ACC TCT GC -3′	70
Rudimentary protein		
Rudimentary – F	5′- CCA GGT GCG ACT CAC AAG GAA G -3′	70
Rudimentary – R	5′- AGC AGC GGA ACG AGG GGC -3′	62
EGF – response factor (Zinc finger protein, X chromosome, *Homo sapiens*)		
EGF–F	5′- GAA TCG GTG TGT TCT CTG GGC -3′	66
EGF–R	5′- GGC GGC AAG GCT CGG TCT -3′	62
Candidate sex – specific AFLP markers in *Penaeus monodon*		
FE_{AGA} -M_{CGA} 520 – F	5′- AAC CAT AGC GAT GTT GAC GG -3′	60
FE_{AGA} -M_{CGA} 520 – R	5′- TTT GCT TTG CCA TTT CGT AT -3′	54

2.11 Identification of sex-specific/differential expression transcripts in ovaries and testes of *P. Monodon* using RT-PCR of hetrospecific primers from the giant freshwater (*Macrobrachium rosenbergii*) and the tropical abalone (*Haliotis asinina*)

2.11.1 Primers

Primers of each gene were designed from nucleotide sequence of the giant freshwater (*Macrobrachium rosenbergii*) and the tropical abalone (*Haliotis asinina*) using Primer Premier 5 program (Table 2.16).

Primer ME_{ACT}-M_{CTT}517–F/R and ME_{AGG}-M_{CAA}310–F/R were designed from candidate male-specific AFLP markers of the giant freshwater prawn (*Macrobrachium rosenbergii*) (R. Preechaphol, personal communication)

Additionallly, normal and subtractive cDNA libraries was constructed from ovaries and testes of the tropical abalone (*H. asinina*). Sex-related transcripts were found. Expression of these genes were investigated in the original species. Those primers were then applied for examining expression of those gene homologues in *P. monodon* (Table 2.15).

2.11.2 RT - PCR analysis

Amplification reactions of sex-related transcripts from *M. Rosenbergii* and *H. Asinina* were essentially identical to those from *P. monodon*.

Products from VCPg7 – F/R and ME_{ACT}-M_{CTT}517 – F/R were cloned and sequenced as described previously.

Table 2.16 Primer, primer sequence and melting temperature of heterospecific primers from the giant freshwater (*Macrobrachium rosenbergii*) and the tropical abalone (*Haliotis asinina*)

primer	sequence	Tm (° C)
Candidate sex – specific AFLP markers in *Macrobrachium rosenbergii*		
ME_{ACT}- M_{CTT} 517 – F	5′- TGT TAT CCT TCG TTC CCT CC -3′	60
ME_{ACT} –M_{CTT} 517 – R	5′- AAA GTG ACA GTC CTG GCA AA -3′	58
ME_{AGG} –M_{CAA} 310 – F	5′- TTG TCA GAT GGC TAA TAG TGT C -3′	62
ME_{AGG} –M_{CAA} 310 – R	5′- CAA ATG AGA AAT GAA GTG GAA G -3′	60
Sex-related transcripts in *Haliotis asinina*		
Axonemal p66.0		
Axonemal - F	5′- GCA CGA CGA AAC TTC GCC CTG -3′	68
Axonemal - R	5′- AAC GCC CGC TTC GCT CCC CA -3′	68
Tektin A1		
Tektin – F	5′- TGT TGA CAG GGA TGG TGC GG -3′	64
Tektin – R	5′- CTC CTT TGC CTA CAG TTG AGA TTG -3′	70

Table 2.16 (cont.)

primer	sequence	Tm (°C)
Vitelline coat protein (VCP)		
VCP49 – F	5′- ACC CCA CAG GAG GAA CAA ACC -3′	72.8
VCP49 – R	5′- CCG CAG CCA GCT CTA AGG ATA -3′	72.8
VCP75 – F	5′- AGG ATG TGG TGA CGG TAT TGT G -3′	71.3
VCP75 – R	5′- CTT TAT AGT GCG AGC GTT TGG T -3′	69.5
VCP1 – F	5′- GGC TGC ACC AGA CCG ATG AAC GAT ACA C -3′	68
VCP1 –R	5′- TGC TTC AAC ACC ATA CCG TCT CCA CAA C -3′	65
VCP2 – F	5′- ACT GGG CTT TCT ACC ATC AAC GTC CTG T -3′	64
VCP2 – R	5′- CGA CGA CCC CTT GTT CTG GAT AAT CTC A -3′	65
VCP3 – F	5′- ATA CAC AAT CTC GTG CTC GTT CGG TTC A -3′	64
VCP3 – R	5′- CAC TAT TCC GTC TCC GCA ACC TGC TCT T -3′	66
VCP7 – F	5′- TGC TCA GTG CTG GTG GTG CTC TGC TGG G -3′	71
VCP7 – R	5′- TGG CTG GGC GGT GCC TTG TTA CCT TCT A -3′	70
Vitellogenin – 1 (VTG)		
VTG 1 – F	5′- ACA TCA GAA CCG ACG GCA AC -3′	62
VTG 1 – R	5′- TGA GGC AAG GTA GGC GAG G -3′	62

Table 2.16 (cont.)

primer	sequence	Tm (° C)
Small androgenreceptor – interacting protein (SARIP)		
SARIP – F	5′- GGC TTA GTG ACT GAA CGC CTC TA -3′	70
SARIP – R	5′- GCT GCT CTA CTA CGC ACA ACA C -3′	68
Gonadotropin inducible ovarian transcription factor 1 (GIOT1)		
GIOT1 – F	5′- GAC CAC CCA CGC ACA GGA C -3′	64
GIOT1 – R	5′ TAG CAG CAC TAA TAA AGC CCC G -3′	66
Hydroxysteroid dehydrogenase – like protein (HSD)		
HSD – F	5′- GCC GTG GAA GAA GCA GTT GGA -3′	68
HSD – R	5′- CAG GGG GTT GAG GTT GAG TGG -3′	68
Fertilization protein (FP)		
FP – F	5′- CGA CCC ATA GCG GCG TAG TT -3′	64
FP – R	5′- AAG GTC CCA AAG AAA AGC CAG TA -3′	66
Sperm lysin		
Lysin – F	5′- CGA GAA GGC AGA CAG CCA GAC -3′	68
Lysin – R	5′- ATC CAG TGC TTG ATG CTT GAC G -3′	66

2.12 Tissue distribution of TSP and AGESTX homologues

Total RNA was extracted from eyestalks, gills, heart, hemocytes, hepatopancreas, lymphoid organs, intestine, ovaries, pleopods, stomach, testes and thoracic ganglion of *P. monodon* and used for first strand cDNA synthesis.

For primers TSP288-1 + TSP 371-1, 1 μg of the first strand cDNA was used as the template in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl and 0.1% Triton X–100, 2 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP and dTTP, 0.2 μM of each primer and 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland). The reactions were predenaturation at 94 °C for 3 minutes followed by 35 cycles composing of a 94 °C denaturation step for 30 seconds, a 65 °C annealing step for 45 seconds and a 72 °C extension step for 45 seconds. The final extension was carried out at 72 °C for 7 minutes.

For primer AGESTX44-F/R, composition of PCR reactions were identical to those of TSP288-1 + TSP 371-1 except 200 μM each of dNTP and 0.4 μM of each primer were used. The amplification reaction was carried out for predenaturation at 94 °C for 3 minutes followed by 35 cycles of a 94 °C denaturation step for 45 seconds, a 60 °C annealing step for 60 seconds and a 72 °C extension step for 60 seconds. The final extension was carried out at 72 °C for 7 minutes.

Eight microliters of the amplification product was electrophoretically analyzed though 1.8% agarose gels.

2.13 Semi – quantitative Reverse Transcription – Polymerase Chain Reaction of Thrombospondin (TSP) and AGESTX homologues

2.13.1 Primers

Expression levels of 2 female specific primers ; TSD 288 – 1 and TSD 371 – 1 and AGESTX44-F/R were determined. β - actin gene was used as an internal control.

2.13.2 Total RNA extraction and the first strand cDNA synthesis

Total RNA was extracted from 5 individuals of 4-month-old and broodstock-sized *P. monodon* using TRI REAGENT. One microgram of total RNA from each shrimp was subjected to the first strand cDNA synthesis.

2.13.3 Determination of PCR conditions

Amplification was performed in a 25 μl reaction volume containing 1 μg of The first strand cDNA template, 1 x PCR buffer (10 mM Tris-HCl pH 8.8, , 50 mM KCl and 0.1% Triton X – 100) , 200 μM each of dATP, dCTP, dGTP and dTTP and 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland). PCR was carried out using the conditions described in the standard PCR of each gene.

2.13.3.1 Primer concentration

The optimal primer concentration for each primer pair (between 0.1 – 0.4 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 PCR conditions.

2.13.3.2 $MgCl_2$ concentration

The optimal $MgCl_2$ concentration of each primer pair (between 1 – 4 mM $MgCl_2$) was examined using the standard PCR conditions and the optimal primer concentration in 2.13.3.1. The concentration that gave the highest specificity was chosen.

2.13.3.3 Cycle number

The PCR amplifications were carried out at different cycles (e.g. 20, 25, 30 and 35 cycles) using the optimal concentration of primers $MgCl_2$ and analyzed by gel electrophoresis. The number of cycles that still provided the PCR product in the exponential rage and did not reach a plateau level of amplification was chosen.

2.13.3.4 Further characterization to eliminate competition between primer set

To determine effects from competitive between primers of the target transcripts (TSP and AGESTX44-F/R) and β-actin, these primer pairs were used for PCR reaction separately and simultaneously in the same tube. The PCR products were analyzed by agarose gel electrophoresis. The band intensity of each primer set was compared. Decreasing of band intensity indicated competition between the target primer set and β-actin. When competition was detected, reaction conditions were further optimized (e.g. adjusting concentrations of $MgCl_2$).

2.13.3.5 Gel electrophoresis and quantitative analysis

The ratio between the interesting transcript and β-actin in ovaries of juvenile and broodstock-sized shrimps was determined. Eight microlitres of the PCR products were combined with 2 μl of loading dye before loaded to 1.8% agarose gel (AGESTX44-F/R) or 3.5% Metaphor agarose (TSP) and electrophoresed at 5-6 volts/cm. The gel was stained with 2.5 μg / ml EtBr for 5 minutes and destained in the running for 15 minutes. The intensity of target and control bands was quantifiedly from glossy prints of the gels using the Gel Pro programme.

2.13.3.6 Data analysis

The expression level of each transcript in ovaries of 4-month-old and broodstock-sized shrimps was normalized by that of β-actin. Significantly different expression levels between different groups of *P. monodon* were tested using one way analysis of variance (ANOVA).

CHAPTER III

RESULTS

3.1 Amplification of conserved sex-specific genes (DMRT-1, *dsx* and mab3)

Total DNA was extracted from pleopods of *P. monodon* using a proteinase K/phenol-chloroform method. Slightly sheared total DNA was obtained. However, high molecular weight total DNA (> 23.1 kb) was also observed (Fig. 3.1) and the ratio of OD_{260} / OD_{280} was approximately 1.6 – 2.0 suggesting that the quality of extracted genomic DNA of *P. monodon* was acceptable for further used.

The ratio of OD_{260}/OD_{280} of total RNA extracted from ovaries and testes of each *P. monodon* was 1.7 – 2.1 indicating accepted quality of total RNA used in this study. An ethidium bromide stained 1% agarose gel of total RNA from ovaries and testes revealed predominant discrete bands along with smeared high molecular weight RNA (Fig. 3.2). The first strand cDNA synthesized from those total RNA covered 0.1 – 7 kb products (Fig. 3.3).

DMRT1 is one of the conserved genes involved with sex differentiation found from invertebrates to vertebrates and human. DMRT1 has been fully characterized in human and shows similarity with doublesex (*dsx*) and mab-3 genes in *D. melanogaster* and *C. elegans*, respectively. These genes also share a number of properties and contain the conserved DM domain (a zinc finger-like DNA binding motif). Amino acid sequences of these genes in various species deposited in the GenBank (www.ncbi.nlm.nih.gov) were then retrieved and aligned (Fig. 3.4).

Several conserved sites were found and used to design degenerated primers (and nested primers) for further characterization of DMRT-1/*dsx*/mab-3 homologues in *P. monodon*. Initially, genomic DNA of two males (from Chumphon and Trang) and two females (from Satun and Trang) of *P. monodon* were tested against different combinations of designed primers (Table 2.1). The amplification products were electrophoretically analyzed. Generally, smear patterns or non-specific bands (Figs. 3.5 - 3.7) were obtained. Therefore, PCR conditions were further optimized (Table 3.1). Although various components of PCR were carefully calibrated, amplification of

genomic DNA of *P. monodon* with primers designed from DMRT1, *dsx* and mab3 were still not successful.

Amplification results from using genomic DNA of male and female *P. monodon* as the template of nested PCR also provided non-specific amplification products or smear patterns. Although primers DMRT1/*dsx*-F + DMRT1-R and DMRT1/*dsx*-F + mab3-R provided discrete amplification bands, these bands were found in both male and female *P. monodon* (Fig. 3.7).

The first strand cDNA was then used as the template for identification of DMRT-1/*dsx*/mab-3 homologues in *P. monodon* by RT-PCR. Several different amplification conditions were carried out but a single specific fragment was not obtained from any condition (Fig. 3.8 – 3.13). Moreover, DMRT1, *dsx* and mab3 in different reference species exhibit large differences in size. The expected product sizes could not be precisely estimated in *P. monodon*. Therefore, bands specifically found in one sex but not in the other were further characterized by DNA cloning and sequencing (Table 3.2).

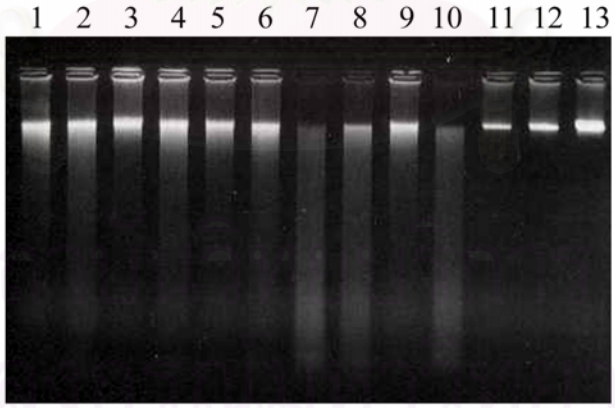

Figure 3.1 A 1.0% agarose gel electrophoresis showing the quality of total DNA extracted from pleopod of 10 individuals of *P. monodon* (lanes 1 – 10). Lanes 11 – 13 = 50, 100 and 200 ng of undigested λ DNA, respectively.

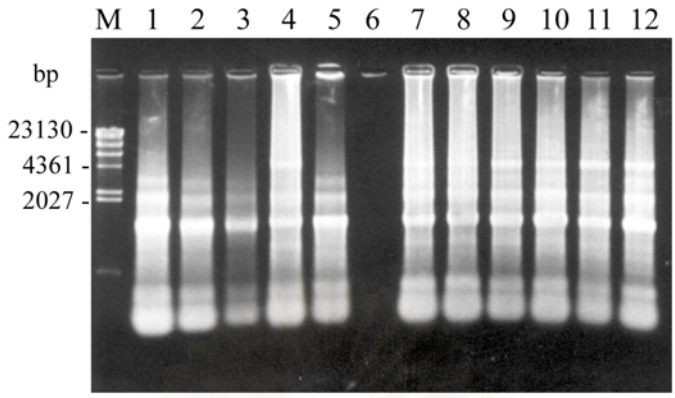

Figure 3.2 A 1.0% agarose gel electrophoresis of total RNA extracted from testes (lanes 1 – 5) and ovaries (lanes 7 – 12) of *P. monodon*. Lane M = a λ-*Hind* III DNA marker.

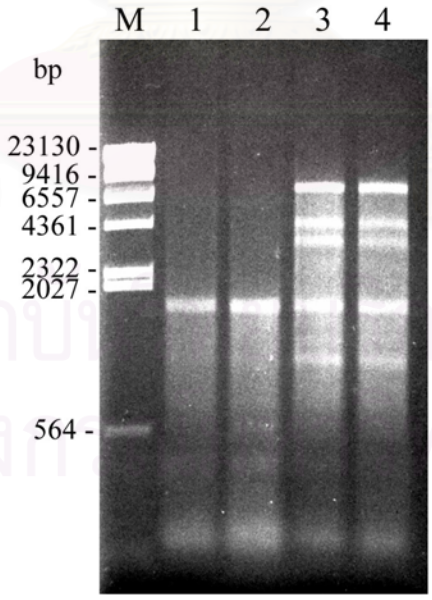

Figure 3.3 A 1.0% agarose gel electrophoresis illustrating the first strand cDNA synthesized from total RNA of testes (lane 1 – 2) and ovaries (lane 3 – 4) of *P. monodon*. Lane M = a λ-*Hind* III DNA marker.

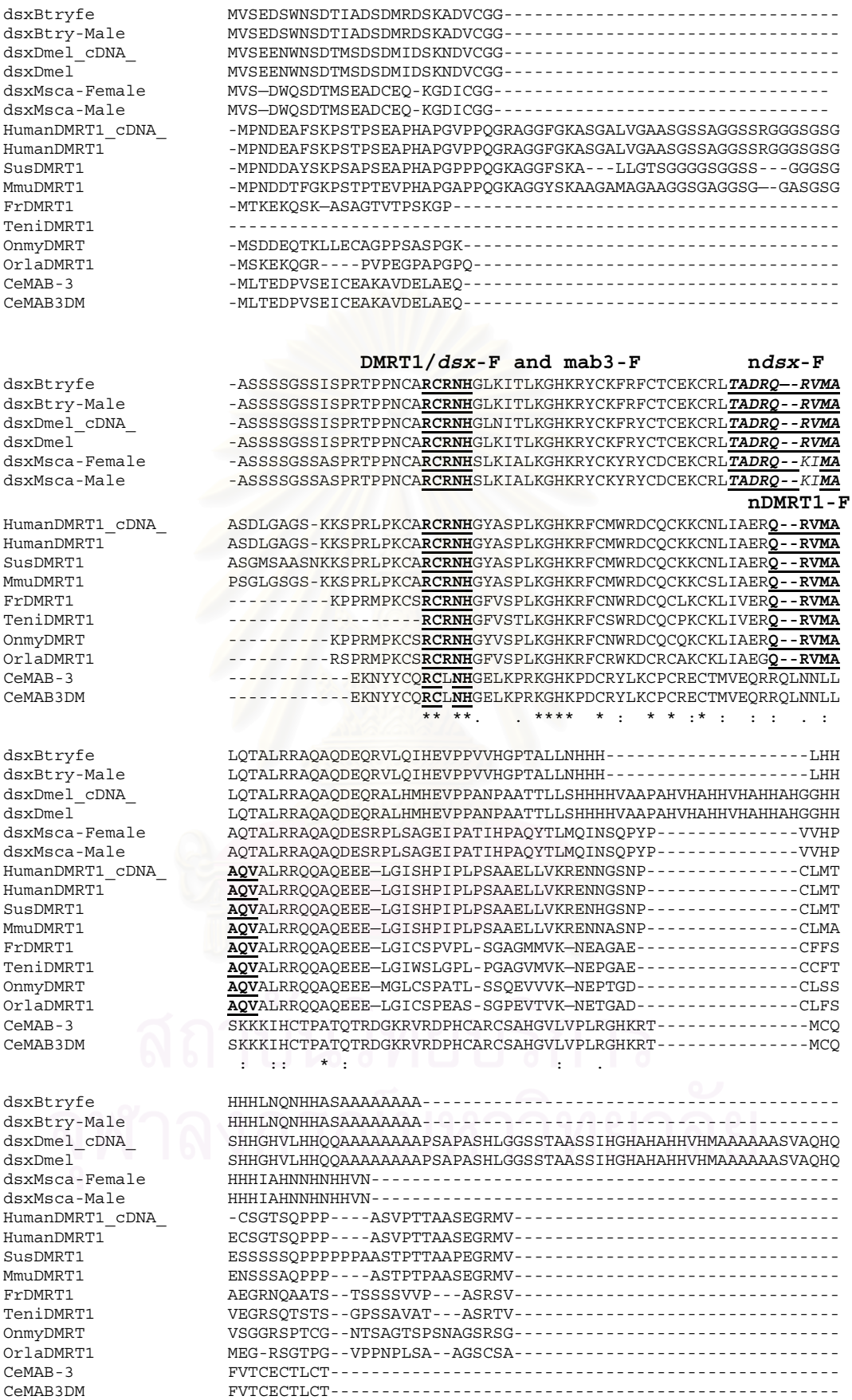
DMRT1/dsx-F and mab3-F
ndsx-F
nDMRT1-F
dsxBtryfe
dsxBtry-Male
dsxDmel_cDNA_
dsxDmel
dsxMsca-Female
dsxMsca-Male
HumanDMRT1_cDNA_
HumanDMRT1
SusDMRT1
MmuDMRT1
FrDMRT1
TeniDMRT1
OnmyDMRT
OrlaDMRT1
CeMAB-3
CeMAB3DM

Figure 3.4

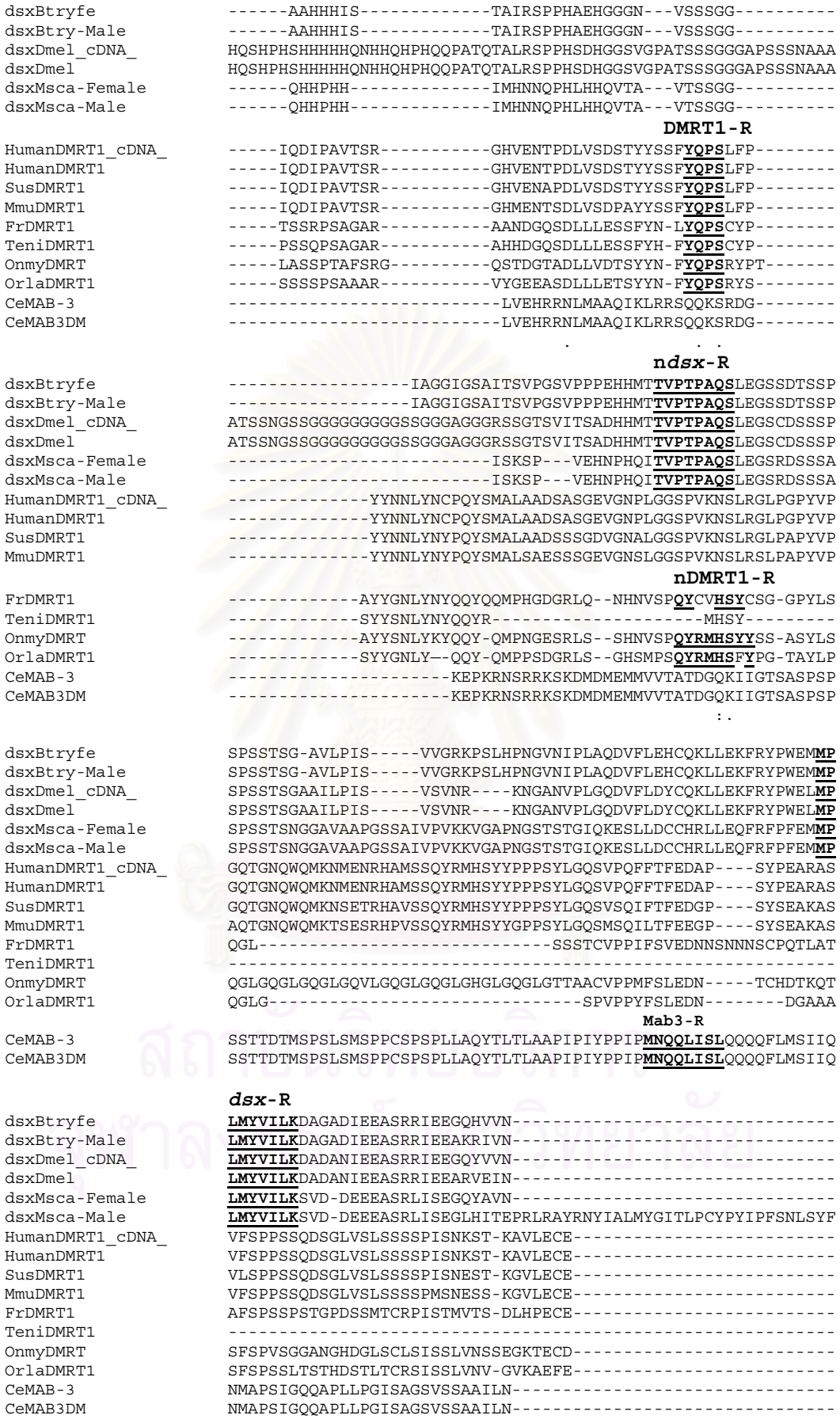

Figure 3.4 (cont.)

```
dsxBtryfe           ------------------------------------------------------------
dsxBtry-Male        ------------------------------------------------------------
dsxDmel_cDNA_       ------------------------------------------------------------
dsxDmel             ------------------------------------------------------------
dsxMsca-Female      ------------------------------------------------------------
dsxMsca-Male        GLTSNTSGPITDSPTNLSVSNNNDSNPVAIMNSTPSTMISHNNTSSRGSPPPSLLPPTAN
HumanDMRT1_cDNA_    ------------------------------------------------------------
HumanDMRT1          ------------------------------------------------------------
SusDMRT1            ------------------------------------------------------------
MmuDMRT1            ------------------------------------------------------------
FrDMRT1             ------------------------------------------------------------
TeniDMRT1           ------------------------------------------------------------
OnmyDMRT            ------------------------------------------------------------
OrlaDMRT1           ------------------------------------------------------------
CeMAB-3             ------------------------------------------------------------
CeMAB3DM            ------------------------------------------------------------


dsxBtryfe           ----------------------------EYS------RQHNLNIYDGGELRST-------
dsxBtry-Male        ----------------------------QTISLHWMDRQLYYNYYSSAALVNTPPTYFPY
dsxDmel_cDNA_       ----------------------------EYS------RQHNLNIYDGGELRN--------
dsxDmel             ----------------------------RTV------AQIYYNYYTPMALVNGAPMYLTY
dsxMsca-Female      ----------------------------EYSR-----QHNLNIFDGGE-----------
dsxMsca-Male        RSHSPIFDLSAHRQSLQ--------LSQEDSRK---EVEVNVHRFHRNDQEKLAFNRELS
HumanDMRT1_cDNA_    ----------------------------PAS------EPSSFTVTPVIEEDE--------
HumanDMRT1          ----------------------------PAS------EPSSFTVTPVIEEDE--------
SusDMRT1            ----------------------------SAS------EPSSFPVTPVIEERTK-------
MmuDMRT1            ----------------------------SASS-----EPSSYAVSQVLEEDEDE------
FrDMRT1             ----------------------------ATG------ETANFTISSIMDGDAGN------
TeniDMRT1           ------------------------------------------------------------
OnmyDMRT            ----------------------------GQD-----QGQGFTVDSIIEGNHK-------
OrlaDMRT1           ----------------------------SGG------QPSVFPADSMSSESK--------
CeMAB-3             ----------------------------------------EFWSMYLKNYGLQA-------
CeMAB3DM            ----------------------------------------EFWSMYLKNYGLQA-------


dsxBtryfe           ----------------TRQCG-------------------------------------
dsxBtry-Male        PI-------AIGSNGLLTSHFSHLTASMRPPSPEQPTLSRTPPSPSKPSRPGSILSETMS
dsxDmel_cDNA_       ---------------TTRQCG-------------------------------------
dsxDmel             PSIEQGRYGAHFTHLPLTQICPPTPEPLALSRSPSSPSGPSAVHNQKPSRPGSSNGTVHS
dsxMsca-Female      ------------------------------------------------------------LRS
dsxMsca-Male        PDHKRLLDSQVTINHEHEGSRKRRLESRSPSIEEQPQFLKRMYGFQPVYDLSTHRPPLRS
HumanDMRT1_cDNA_    ------------------------------------------------------------
HumanDMRT1          ------------------------------------------------------------
SusDMRT1            ------------------------------------------------------------
MmuDMRT1            ------------------------------------------------------------
FrDMRT1             ------------------------------------------------------------
TeniDMRT1           ------------------------------------------------------------
OnmyDMRT            ------------------------------------------------------------
OrlaDMRT1           ------------------------------------------------------------
CeMAB-3             ------------------------------------------------------------
CeMAB3DM            ------------------------------------------------------------


dsxBtryfe           ------------------------------------------------------------
dsxBtry-Male        PPAAATNLPSSATAAAAT--------------------------------------------
dsxDmel_cDNA_       ------------------------------------------------------------
dsxDmel             AASPTMVTTMATTSSTPTLSRRQRSRSATPTTPPPPPPAHSSSNGAYHHGHHLVSSTAAT
dsxMsca-Female      QSRQCG--------------------------------------------------------
dsxMsca-Male        SQEECRKEEEELNVHRFRRYAQEKLAFNGQETQAAINHEHELKMRESRKRHHESRSPSID
HumanDMRT1_cDNA_    ------------------------------------------------------------
HumanDMRT1          ------------------------------------------------------------
SusDMRT1            ------------------------------------------------------------
MmuDMRT1            ------------------------------------------------------------
FrDMRT1             ------------------------------------------------------------
TeniDMRT1           ------------------------------------------------------------
OnmyDMRT            ------------------------------------------------------------
OrlaDMRT1           ------------------------------------------------------------
CeMAB-3             ------------------------------------------------------------
CeMAB3DM            ------------------------------------------------------------
```

Figure 3.4 (cont.)

```
dsxBtryfe             ---------------------------
dsxBtry-Male          ---------------------------
dsxDmel_cDNA_         ---------------------------
dsxDmel               ---------------------------
dsxMsca-Female        ---------------------------
dsxMsca-Male          EQSQKKICLSPPVIRSDSTDVERGSP-
HumanDMRT1_cDNA_      ---------------------------
HumanDMRT1            ---------------------------
SusDMRT1              ---------------------------
MmuDMRT1              ---------------------------
FrDMRT1               ---------------------------
TeniDMRT1             ---------------------------
OnmyDMRT              ---------------------------
OrlaDMRT1             ---------------------------
CeMAB-3               ---------------------------
CeMAB3DM              ---------------------------
```

Figure 3.4 Multiple alignment of genes encoding DMRT1, *dsx* and mab-3 from *B. tryoni* (Btry, AF029675 and AF029676), *D. melanogaster* (Dmel, NM_079548 and AY060257), *M. scalaris* (Msca, AF283695 and AF283696), *H. sapiens* (Human, A1162131 and XM_015427), *S. scrota* (Sus, AF216651), *M. musculus* (Mmu, AL133300 and NM_015826), *F. rubribes* (Fr, AJ295039), *T. nigroviridis* (Teni, AJ295040), *O. mykiss* (Onmy, AF209095), *O. latipes* (Orla, AF319994) and *C. elegans* (Ce, NM_063697 and AF022388). Conserved amino acid sequences used for designation of primers and nested primers were illustrated in boldface and underlined. Primer sequences are shown in Table 2.1.

Table 3.1 Identification of conserved gene homologues encoding for DMRT1, *dsx* and mab-3 in *P. monodon* using genomic DNA as the template

Primer combination	Amplification results	PCR conditions
1. DMRT1/*dsx*-F+DMRT1-R	Smear products (Fig. 3.5)	Composition: 100 μM dNTP, 2 mM $MgCl_2$, 0.4 μM of each primer, 1 U. of Taq polymerase and 50 ng of genomic DNA PCR profile: I. 1 cycle of 94 °C for 3 min II. 5 cycles of 94 °C for 45 sec, 42 °C for 1 min and 72 °C for 2 min III. 35 cycles at the annealing temperature of 50 °C IV 1 cycle of 72 °C for 7 min
2. DMRT1/*dsx*-F+DMRT1-R	No products	Using conditions in 1 except 25 ng of genomic DNA and 0.5μM of each primer were used. The annealing temperature was increased from 50 °C to 55 °C
3. DMRT1/*dsx*-F+DMRT1-R	No products	Using conditions in 2 but the annealing temperature was decreased from 55 °C to 53 °C
4.DMRT1/*dsx*-F+DMRT1-R	Smear products	Using conditions in 2 but the annealing temperature was decreased from 53 °C to 52 °C and *Taq* polymerase was decreased from 1 U to 0.5 U
5 DMRT1/*dsx*-F+DMRT1-R	Faint bands (550bp and 850 bp in size, Fig. 3.7)	Using conditions in 1 but the 1st annealing temperature was decreased from 42 °C to 40 °C
6. DMRT1/*dsx*-F + *dsx*-R	Smear products (Fig. 3.5)	Using conditions described in 1
7. DMRT1/*dsx*-F+ *dsx*-R	No product	Using conditions in 2
8. DMRT1/*dsx*-F+ *dsx*-R	Non-specific product at 120 bp in size	Using conditions in 3
9. DMRT1/*dsx*-F + dsx-R	Smear products	Using conditions in 4
10. DMRT1/*dsx*-F + *dsx*-R	Smear products	Using conditions in 5
11. DMRT1/*dsx*-F + mab3-R	Smear with non-specific amplification fragments (500, 600, 800 and 900 bp) (Fig. 3.5)	Using conditions in 1 except 25 ng of genomic DNA and 0.5μM of each primer were used.
12. DMRT1/*dsx*-F + mab3-R	No products	Using conditions in 2
13. DMRT1/*dsx*-F + mab3-R	500 bp fragment but did not find in all individuals and did not show sex-specific nature	Using conditions in 3
14. DMRT1/*dsx*-F + mab-R3	Smear products	Using conditions in 4
15. DMRT1/*dsx*-F + mab-R3	Faint bands at 550, 850, and 100 bp in length (Fig. 3.7)	Using conditions in 5
16. mab3-F + mab3-R	Smear products (Fig. 3.6)	Using conditions in 11

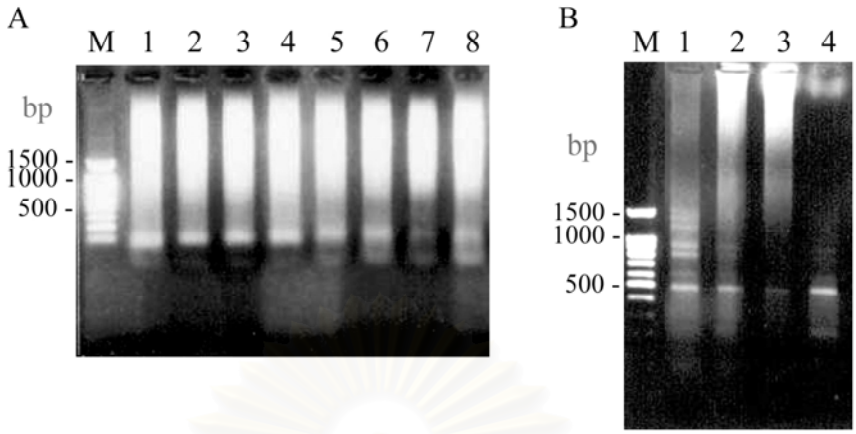

Figure 3.5 An example for amplification of DMRT1/*dsx*-F + DMRT1-R (panel A : lanes 1 – 4) and DMRT1/*dsx*-F + *dsx*-R (panel A : lane 5 – 8) and DMRT1/*dsx*-F + mab3-R (panel B : lane 1 – 4) against genomic DNA of male (lanes 1 – 2 and 5 – 6; panel A and 1 – 2; panel B) and female (lanes 3 – 4 and 7 – 8; panel A and 3 – 4; panel B) *P. monodon*

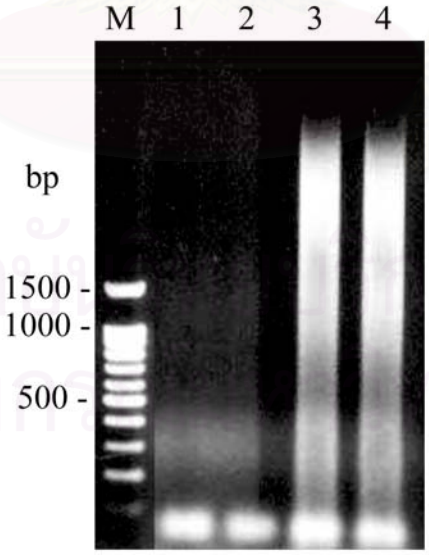

Figure 3.6 An example for amplification of mab3-F/R (lanes 1 – 4) against genomic DNA of male (lanes 1 – 2) and female (3 – 4) *P. monodon*.

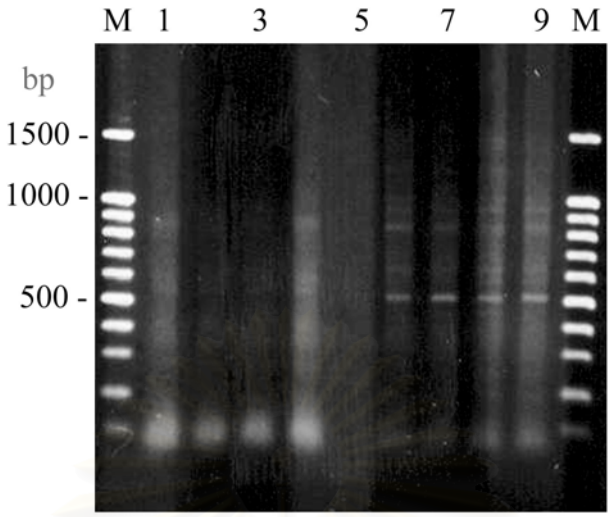

Figure 3.7 An example for amplification of DMRT1/*dsx*-F + DMRT1-R (lanes 1 – 4) and DMRT1/*dsx*-F + mab3-R (lane 6 – 9) against genomic DNA of male (lanes 1 – 2, and 6 – 7) and female (3 – 4 and 8 – 9) *P. monodon*

Table 3.2 Identification of conserved gene homologues encoding for DMRT1, *dsx* and mab-3 in *P. monodon* using the first strand cDNA of ovaries and testes as the template

Primer combination	Amplification results	PCR conditions
1. DMRT1/*dsx*-F + dT_{15}	**-A 1700 bp** fragment from the first strand cDNA of testes **-A 720 bp** fragment from the first strand cDNA of ovaries (Fig. 3.8)	Composition: 100 μM dNTP, 2 mM $MgCl_2$, 0.5 μM of each primer, 1 U. of *Taq* polymerase and 1 μl of the 1st strand cDNA PCR profile: I. 1 cycle of 94 °C for 3 min II. 5 cycles of 94 °C for 45 sec, 42 °C for 1 min and 72 °C for 2 min III. 35 cycles at the annealing temperature of 50 °C IV 1 cycle of 72 °C for 7 min
2. DMRT1/*dsx*-F + DMRT1-R	Non-specific products (Fig. 3.9)	Using conditions in 1 except the template was PCR products from 1 rather than the first strand cDNA and the initial annealing temperature was 45 °C
3. DMRT1/*dsx*-F + *dsx*-R	Non-specific products (Fig. 3.9)	Using conditions in 2
4. DMRT1/*dsx*-F+mab3-R	Non-specific products (Fig. 3.9)	Using conditions in 2
5. mab3-F + dT_{15}	Smear products (Fig. 3.8)	Using conditions described in 1
6. mab3-F+ DMRT1-R	Non-specific products (Fig. 3.9)	Using conditions in 1 except the template was PCR products from 2 rather than the first strand cDNA and the initial annealing temperature was 45 °C
7. mab3-F + *dsx*-R	Non-specific products (Fig. 3.9)	Using conditions in 6
8. mab3-F+mab3-R	Non-specific products (Fig. 3.9)	Using conditions in 6
9. Repeat 3-8	Extensive non-specific products	Omit preamplification (5 cycles) with the annealing temperature was 45 °C and increase the annealing temperature from 50 ° C to 52 °C

Table 3.3 Identification of conserved gene homologues encoding for DMRT1, *dsx* and mab-3 in *P. monodon* using PCR products and the first strand cDNA of ovaries and testes as the template

Primer combination	Amplification results	PCR conditions
1.nDMRT1F + dT_{15}	Band patterns were not different between male and female *P. monodon* (Fig. 3.10)	**Composition:** 100 μM dNTP, 2 mM $MgCl_2$, 0.5 μM of each primer, 1 U. of *Taq* polymerase and 1 μl of the 1st strand cDNA or PCR products from DMRT1/dsx-F + dT_{15} **PCR profile:** I. 1 cycle of 94 °C for 3 min II. 5 cycles of 94 °C for 45 sec, 42 °C for 1 min and 72 °C for 2 min III. 35 cycles at the annealing temperature of 50 °C IV 1 cycle of 72 °C for 7 min
2.n*dsx*-F + dT_{15}	**-880, 920 and 1300 bp** were found in ovaries for both the first strand cDNA and PCR product template **-700 bp** fragments were found in testis for both types of template (Fig. 3.10)	Using conditions described in 1
3.DMRT1/*dsx*-F+nDMRT1-R	Smear patterns (Fig. 3.11)	Using conditions in 1 but the 1st strand cDNA was used as the template
4.DMRT1/*dsx*-F + n*dsx*-R	Smear and non-specific patterns (Fig. 3.11)	Using conditions in 3
6.mab3-F + nDMRT1-R	Smear and non-specific patterns (Fig. 3.12)	Using conditions in 3
7 mab3-F + n*dsx*-R	Smear and non-specific patterns (Fig. 3.12)	Using conditions in 3
8. nDMRT1-F + dT_{15}	Different band patterns between ovaries and testis cDNA (Fig. 3.13, panel A)	Using conditions in 3
9. nDMRT1-F +DMRT1-R		Using conditions in 3
10. nDMRT1-F + *dsx*-R		Using conditions in 3
11. nDMRT1-F + mab3-R		Using conditions in 3
12. nDMRT1-F + nDMRT1-R		Using conditions in 3
13. nDMRT1-F + n*dsx*-R		Using conditions in 3
14. n*dsx*-F + dT_{15}	Different band patterns between ovaries and testis cDNA (Fig. 3.13, panel B)	Using conditions in 3
15. n*dsx*-F + DMRT1-R		Using conditions in 3
16. n*dsx*-F + *dsx*-R		Using conditions in 3
17. n*dsx*-F + mab3-R		Using conditions in 3
18. n*dsx*-F + nDMRT-R		Using conditions in 3
19. n*dsx*-F + n*dsx*-R		Using conditions in 3

DMRT1/*dsx*-F + dT_{15} yielded a 1700 band in male and a 720 bp band in female *P. monodon* but not in the opposite sex whereas n*dsx*-F + dT_{15} generated a 700 bp fragment specifically found in male and 1300 bp, 920 bp and 880 bp specifically found in female *P. monodon* (Figs. 3.8, 3.13 and 3.14). These bands (called PMO720, PMO920, PMO880, PMO1300, PMT1700 and PMT700) were consistently obtained when amplification reactions were repeated.

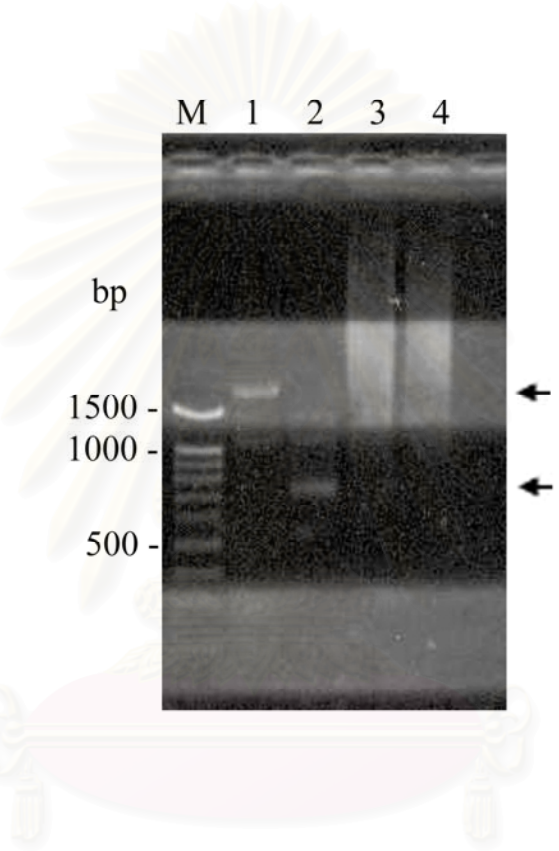

Figure 3.8 RT-PCR of DMRT1/*dsx*-F + dT_{15} (lanes 1 – 2) and. mab3-F + dT_{15} (lanes 3 – 4) using the first strand cDNA synthesized from total RNA of testes (lanes 1 and 3) and ovaries (lanes 2 and 4) of *P. monodon* as the template. The former set of primers generated a 720 bp and a 1700 bp bands (arrows) specifically found in ovaries and testes of *P. monodon*, respectively.

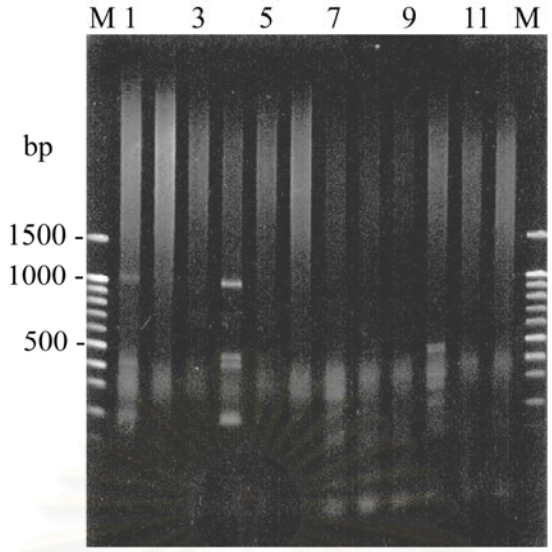

Figure 3.9 Amplification of DMRT1/*dsx*-F + DMRT1-R (lanes 1 and 4), DMRT1/*dsx*-F + *dsx*-R (lanes 2 and 5) and DMRT1/*dsx*-F + mab3-R (lanes 3 and 6) and mab3-F + DMRT1-R (lanes 7 and 10), mab3-F + dsx-R (lanes 8 and 11) and mab3-F + mab3-R (lanes 9 and 12) using PCR products from DMRT1/ *dsx*-F + dT_{15} (lanes 1 – 6) and mab3-F + dT_{15} (lanes 7 – 12) of male (lanes 1 – 3 and 7 – 9) and female (lanes 4 – 6 and 10 – 12) *P. monodon* as the template, respectively.

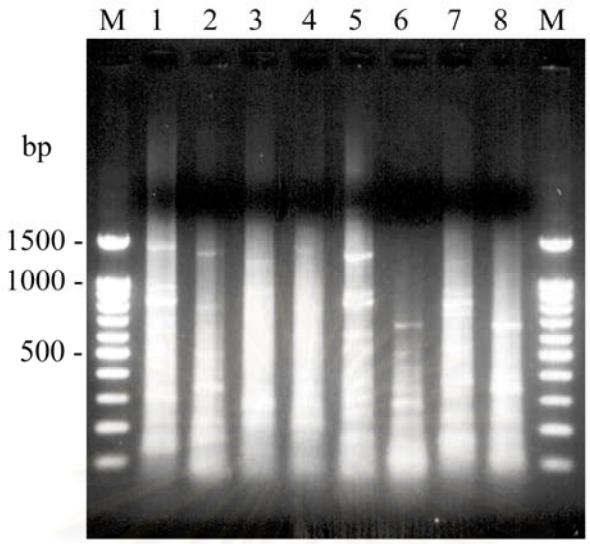

Figure 3.10 RT-PCR using nDMRT1-F + dT_{15} (lanes 1 - 4) and n*dsx*-F + dT_{15} (lanes 5 – 8) as primers, the first strand cDNA from ovaries and testes of *P. monodon* (lanes 1 – 2 and lanes 5 – 6) and PCR products of ovaries and testes from primers DMRT1/*dsx*-F + dT_{15} (lanes 3 – 4 and lanes 7 – 8) as the template, respectively.

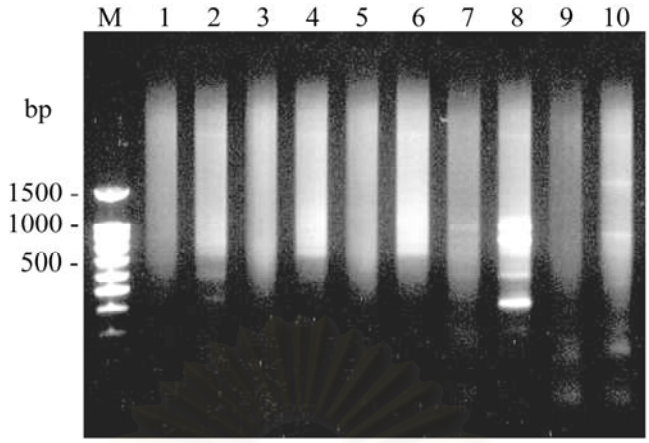

Figure 3.11 RT-PCR of the first strand cDNA template synthesized from total RNA of testes (lanes 1, 3, 5, 7 and 9) and ovaries (lanes 2, 4, 6, 8 and 10) of *P. monodon* using various primer combinations.

lanes 1 – 2 = DMRT/*dsx*-F + DMRT1-R

lanes 3 – 4 = DMRT/ *dsx*-F + *dsx*-R

lanes 5 – 6 = DMRT/ *dsx*-F + mab3-R

lanes 7 – 8 = DMRT/*dsx*-F + nDMRT1-R

lanes 9 – 10 = DMRT/*dsx*-F + n*dsx*-R

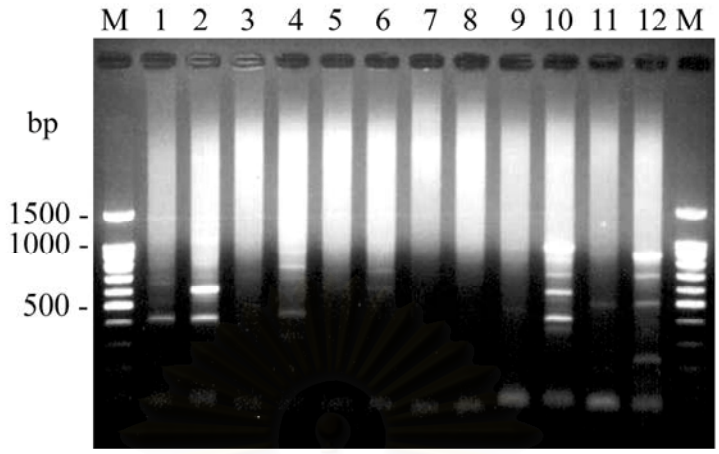

Figure 3.12 RT-PCR of the first strand cDNA template synthesized from total RNA of testes (lanes 1, 3, 5, 7 and 9) and ovaries (lanes 2, 4, 6, 8 and 10) of *P. monodon* using various primer combinations.

lanes 1 – 2 = mab3-F + dT_{15}

lanes 3 – 4 = mab3-F + DMRT1-R

lanes 5 – 6 = mab3-F + *dsx*-R

lanes 7 – 8 = mab3-F + mab3-R

lanes 9 – 10 = mab3-F + nDMRT1-R

lanes 11 – 12 = mab3-F + n*dsx*-R

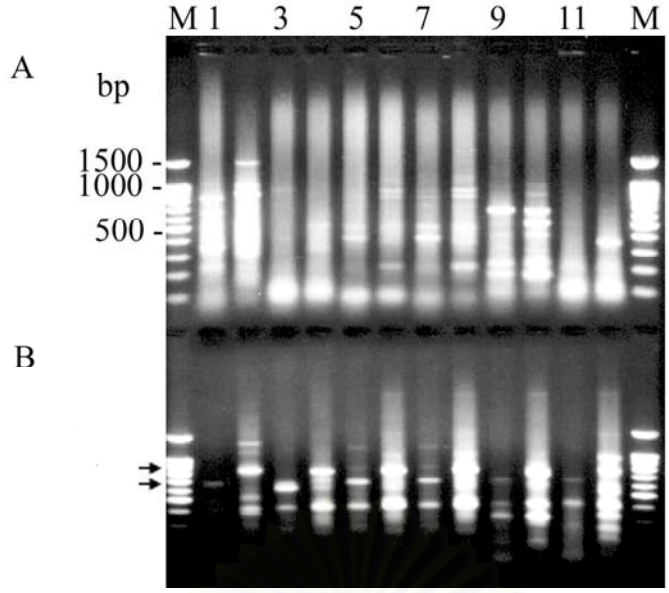

Figure 3.13 Nested RT-PCR of the first strand cDNA template of testes (panels A and B; lanes 1, 3, 5, 7, 9 and 11) and ovaries (panels A and B; lanes 2, 4, 6, 8 ,10, and 12) of *P. monodon* using various primer combinations. Arrows indicate RT-PCR bands which were eluted out from the gel and cloned.

Panel A,

lanes 1 – 2 = nDMRT1-F + dT_{15}
lanes 3 – 4 = nDMRT1-F+ DMRT1-R
lanes 5 – 6 = nDMRT1-F + *dsx*-R
lanes 7 – 8 = nDMRT1-F + mab3-R
lanes 9 – 10 = nDMRT1-F + nDMRT1R
lanes 11 – 12 = nDMRT1-F + n*dsx*-R

Panel B,

lanes 1 – 2 = n*dsx*-F + dT_{15}
lanes 3 – 4 = n*dsx*-F + DMRT1-R
lanes 5 – 6 = n*dsx*-F + *dsx*-R
lanes 7 – 8 = n*dsx*-F + mab3-R
lanes 9 – 10 = n*dsx*-F + nDMRT1-R
lanes 11 – 12 = n*dsx*-F + n*dsx*-R

All bands except PMO1300 from ndsx-F + dT_{15} were gel-eluted, cloned and sequenced. Due to small size differences, PMO880 and PMO920 fragments were simultaneously eluted out from the gel and cloned together. Colony PCR was carried out to verify insert sizes (Figs. 3.15 - 3.17). Notably, the colony PCR product was also digested with *Alu* I and *Rsa* I to determine whether more than one insert type was obtained from a particular fragment (data not shown). For recombinant clones showing more than two digestion patterns (not from insert directions), the most common insert type was sent out to the BioService Unit (BSU), National Center for Genetic Engineering and Biotechnology (BIOTEC) for sequencing.

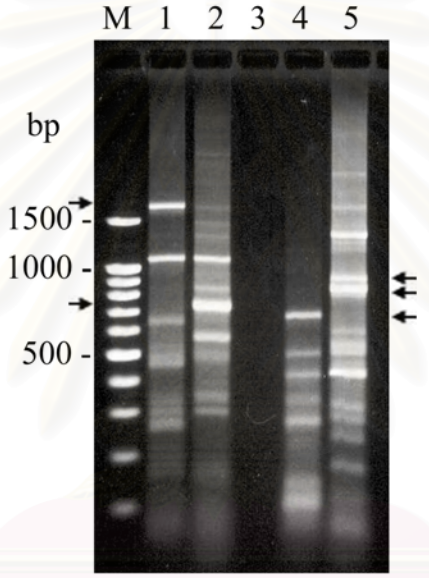

Figure 3.14 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 and 4) and ovaries (lanes 2 and 5) of *P. monodon* using primers DMRT1/*dsx*-F + dT_{15} (lanes 1 and 2) and n*dsx*-F + dT_{15} (lanes 4 and 5). Lane M is a 100 bp DNA marker. Arrows indicate candidate sex-specific expression RT-PCR fragments (1700 bp and 700 bp in testes and 920 bp, 880 bp and 720 bp in ovaries, respectively) that was eluted out from the gel and cloned.

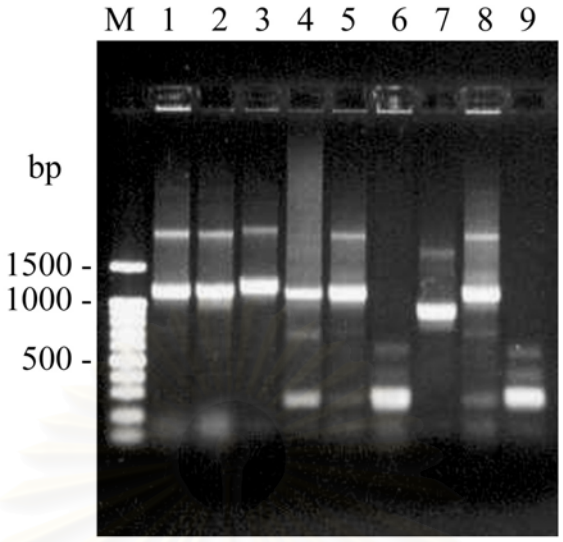

Figure 3.15 Colony PCR of clones PMO880 and PMO920. Results indicated that both PMO880 (880 bp + 334 bp; lanes 1, 2, 4, 5 and 8) and PMO920 (920 bp + 334 bp; lane 3) were found. A slightly smaller amplification fragment (lane 7) and colonies that contained no insert (lanes 6 and 9) were also found.

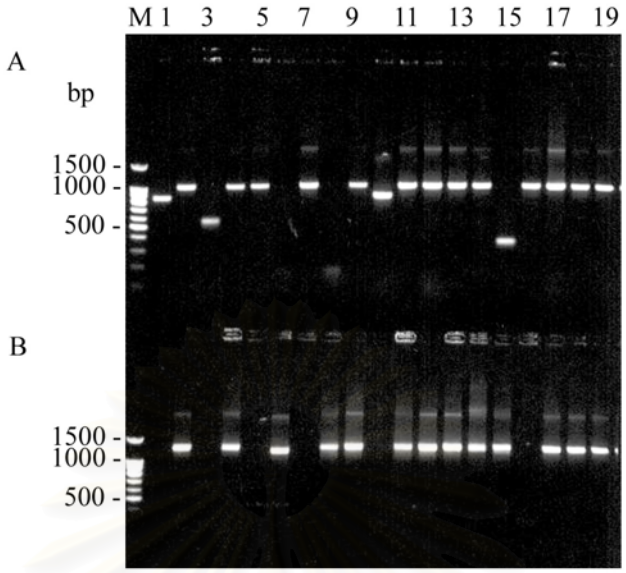

Figure 3.16 Colony PCR of clones PMT700 (panel A) and PMO920 (panel B). Results indicated that clones containing PMT700 (700 bp + 334 bp; lane 2, 4, 5, 7, 9, 11 – 14 and 16 – 19; panel A) and PMO920 (920 bp + 334 bp; lane 2, 4, 8, 9, 11 – 15 and 17 – 19; panel B) were found. Clones containing smaller amplification fragments (lanes 1, 3 and 15; A and 6; B) and those containing no insert (6 and 8; A and 1, 3, 5, 7, 10 and 16; B) were also observed. A 100 bp ladder (lanes M) was used as the DNA marker.

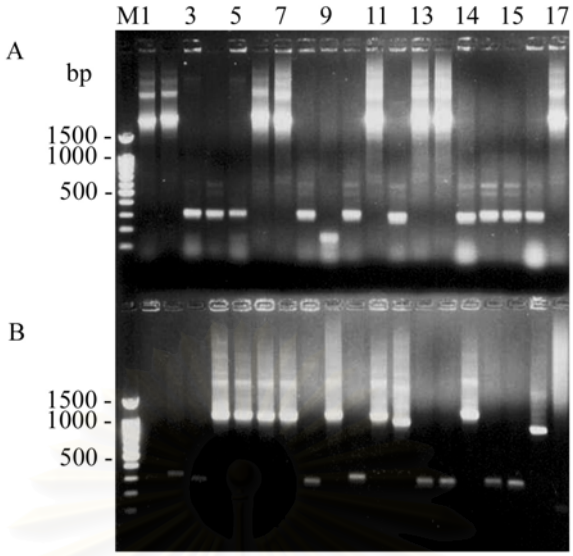

Figure 3.17 Colony PCR of PMT1700 (panel A) and PMO720 (panel B). Results indicated the existence of PMT1700 (1700 bp + 334 bp; lanes 1, 2, 7, 11, 13, 14 and 19; panel A) and PMO720 (720 bp + 334 bp; lane 4 – 7, 9, 11 and 15; panel B). Clones containing smaller amplification products (lanes 6; A and 12 and 18; B) and those having no inserts (lanes 3 – 5, 8 – 10, 12 and 15 – 18; A and 1 – 3, 8, 10, 13 – 14 and 16 – 17; B) were also observed. A 100 bp ladder (lanes M) was used as the DNA marker.

A .

ACAGCAGATCGCCAGCGCGTAATGGCGAGTCAAAGGCCCTCGATGCCAAGGTCCTGGAGATGCCCTATCGAGGAGA
AAGCGCCTCCATGTACGTTTTTCTCCCGAAGGCCAACGAGACAGGGCAAGAGGTGGACGACATGGTAAAGGGAGTC
ACGCCAGAGATCCTCCAAACTGCCATGAAGGACGAGATAAACACCGCTAATGTTGTAGTTCAAATGCCCAAGTTCA
AGTCGGAGTACACGCTTCGGGACAGTTTGAAAATGGCTCTGACCCGCATGGGCCTTCGCGACCTCTTTTCCGCGGC
GGCTGACCTGACGGGTTTCTCCCCTGCCGGAGGTCTGAGAGTCACCAACGGCGTCCACAAGGCGGTCATCGAAGTG
AACGAAGAAGGCGCAGAAGCAGCGGCCGCCACAGCCATAGTGTCGGGAAGGTCGTCGATTTTACCGGACATCAAAC
ATTTCACTTGCAATCGTCCGTTTTTCTACTTCATTCGCGACAACTCGAGTGAAAACATCCTCTTCATGGGAATTTA
TAGGAAGCCCTAGATATGTCTTGCATCTTTGCAATTGAAAAATGGAGAATGTTAATCTGG

B.

AAATGCGCACGGTGC**CGTAACCAGTAAGAGATCGGGAG**CTTTAGAAGTATTGCTCCCAAAAGATACGATCGAGGAA
GTAAGGGATGTAAAAAGAAATCCTAATGAATTGCTTGCAAAACACGAAATCAACAAAATCAGCGATGTAGAGGGAG
TTTTAAAAGAACTGATCCCAAAAGATAATATCATGAAACGAAGTGATGTGCAAGTTTTAGGGAAACTGCTTGCAGA
AGATGAAATAGTAAAGATAAAAAGAGCAGATGAGCCTATAGAAACGGAGGGAGTTTTAGAATCACTTCCTAAACAA
ATCAGCAAAAGAAGTGAAGTGAAGGAAGTCCATCAGGAACTACTCCCAGGAGGTAAGCTCACCAATGTAGGTGAGA
CAGACGAGTTTTTAGAACTAATTGCAGAAGGTAAGCTTATCAAA**GACTCTCTTAAACTGCCAAAAAGC**ATGGGATT
CATTACCCGACGAAGAAGAGGTAGTGAAAGAACTAACAGAACTTTTGCAGTCATCAAAGGACAGACATAAGAAGGA
GCTCCTGCCAGACCCAGA

Figure 3.18 Nucleotide sequences of different transcripts obtained from cloning and characterization of PMT700 (A) and PMT1700 (B). Positions of a forward and that complementary to a reverse primer were illustrated in boldface and underlined.

A

ACAGCAGACCGGCAGCGCGTTATGGCAGACGAAAGCTTGCGGAGTGAACGTAGTGTTACAACAGATAACCATCCCT
TCTCGCATCTGTGCGAGTCCCAGCCTGATAAATTTTTGTGCGCCAACTGCAAAACCATGGTCCAATGTGTAAAGGG
GCAAGCCTTTACTCGTCACTGCATCGAGAGCCACTACTGTTCGGAGAAGAGCGAGTTTGGAGGTGCTGTCTGCTAC
CCGGATGAACCTACAGACTGCACCTGTACGAAGGCTAATGATTTCCGTGTGGATCCCTATGATCCCCAGAGATTCT
TCTCTTGCAAGAATATTGGATCTCTACCAGAGAGTTACAAGTGCCCAGACGGCATGGAGTTCGATGAGGCTTCGGC
GCAGTGTCGGAACGTA

B

ACAGCAGACCGTCAGCGGGTAATGGCTGCAACAAGTGAAGGGAATGCCTGCACTAAACAAAATAGTGTTGTCTGGA
AAATGTCTAATGGTGCACTATCGGTGTACCAAGATCTGCTTCC**TGGAACCAGTGTTTCTGCAAGT**AATGGTATTCT
ATATGTAACGGCTCCAAATGCTACCCACTCTTATAGCTTTGATAGTAAAACTTTGCTCTTCAACCTAATTAGTACT
GATGCTGCTGAAATGGATTCTGCATTGCTATCTAAAACTGGACACCTTGTGACTGCAATGAAAAAGCAGGACCAAC
TTGTCCTAATGGTACAAGGAAATGAAGTCTCATCCTTAAGAGTAAAAG**TTGATGACTTGGTGCTATTCGA**ACAGTA
TGGCAAAATACTATTACTGGCAGCAGTAACCAGGAACCCTCTCTCGGGAATCGAACATCACCTGGAGCTGTACAGC
ATGGATCCACATGCAATGACTCTGAATTGGTTGGACTCGAAGCCATTGTATGAAGCAGCTACACTAACTACCTTCT
ACAGTGGAAATGCAGCTAATGGAAAAACCATGGCTGTA

C

ACTAGTGATTAATTGCGCGCGCTGCCGAAATCAAGAGAGAGTGAAAATATCGCCAATATGCTTTCCTTGACCCAGT
TTCTAGTGACTTTATCCCTGATCTCAGCATCGGTCCATGCTGCCTCGCCTCCGCTTTCAGTACTCGGTAGGAACAA
TCGTGTAGGTAGTGCAAGTGGTTCTGCCGCCGGTA**ATTTGTACGCTGCCCCTGCC**GTCCAACCGGTCACGATCACG
GTCACCCAGACTACCACCATCGACCGGTCCGCCATCATCACGGACACTGCCTACAACCGCGTTCCCGTCACCATCA
CGGACTTCGTACTGTGGACCTCCACGTTACCTTCACGAGTGGTTGTACAGACGCCTGTGGACGATGACGTGGCCAT
CCAAACCAGAGAGGTGTCAAAACCCACAACCACAACAGTCGTAGACCAAGTGTCTAACATAAGGGTCGTGACTGAC
GTCTCCGTCGAACACTTTACCATCACGCACAGCTACTATAACATCATGCAGTCGACCTA**CACCAGAACATCAACAC**
AGGGGAATTACTATCTCTTCAACGTTGGTGAGG

Figure 3.19 Nucleotide sequences of PMO880 (A), PMO920 (B) and PMO720 (C). Positions of forward primers and those complementary to reverse primers were illustrated in boldface and underlined.

Nucleotide sequences of these clones (Figs. 3.18 and 3.19) were blasted against data in the GenBank using Blast*N* (comparisons of nucleotide sequences) and Blast*X* (comparisons of translated nucleotide sequences of queries). Results indicated that PMO880 and PMT700 were homologues of peritrophin and serine proteinase inhibitor, respectively. The remaining clones (PMO720, PMO920 and PMT1700) did not show significant similarity with any deposited data in the GenBank and were, therefore, regarded as unknown genes.

Table 3.4 Blast results of RT-PCR products illustrating sex-specific expression in *P. monodon*

Clone	Gene homologue	Closest species	E-value	Length of nucleotide sequenced (bp)
PMO720	Unknown	-	$>10^{-4}$	565
PMO880	Peritrophin	Green tiger shrimp, *P. semisulcatus*	10^{-75}	396
PMO920	Unknown	-	$>10^{-4}$	570
PMT700	Serine proteinase inhibitor	Crayfish, *Pacifastacus leniusculus*	2×10^{-37}	592
PMT1700	Unknown	-	$>10^{-4}$	550

Primer pairs of PMT1700, PMO720 and PMO920 were designed and used for both PCR (genomic DNA as the template) and RT-PCR (the first strand cDNA as the template) analyses (Table 3.5). Using genomic DNA as the template, PMT1700-F/R provided the expected 433 bp product in both male and female *P. monodon*. In contrast, the expected PCR product (351 bp) and two bands of approximately 600 bp in size were found from PMO720-F/R. Polymorphic amplification results were observed from PMO920-F/R. A 350 bp band (expected product was 255 bp) was found in male while females revealed an additional 400 bp band. This possibly indicated allelic variation of this marker. Larger numbers of specimens should be examined for sex-specificity of PMO920 in *P. monodon*. Using the first strand cDNA as the template, differential expression levels were found with all primer pairs. These was regarded as sex differential expression markers

Table 3.5 Primers, sequence primers and annealing temperature of primers from PMT1700, PMO720 and PMO920

Primer	**sequence**	**Tm (°C)**
PMO720 - F	5′- ATT TGT ACG CTG CCC CTG CC -3′	64
PMO720 - R	5′- TCC CCT GTG TTG ATG TTC TGG TG -3′	58
PMO920 - F	5′- TGG AAC CAG TGT TTC TGC AAG T -3′	54
PMT1700 - F	5′- CGT AAC CAG TAA GAG ATC GGG AG -3′	54
PMT1700 - R	5′- GCT TTT TGG CAG TTT AAG AGA GTC -3′	53
PMO920 - R	5′- TCG AAT AGC ACC AAG TCA TCA A -3′	52

.

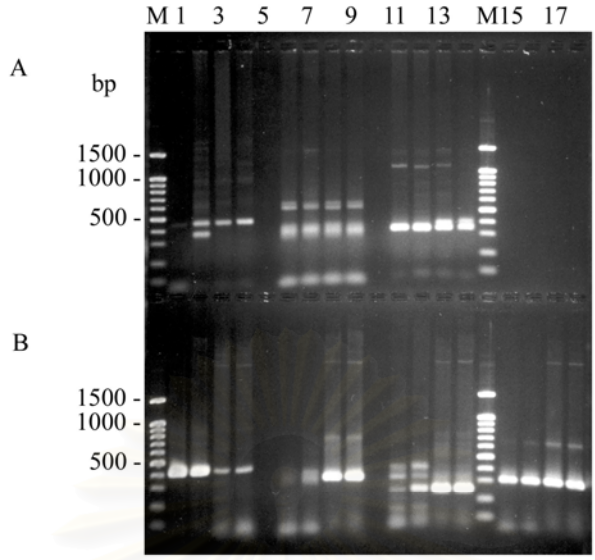

Figure 3.20 PCR of genomic DNA (panel A) from males (lanes 1 – 2, 6 – 7 and 11 – 12) and females (lanes 3 – 4, 8 – 9 and 13 – 14) using primers PMT1700-F/R (lanes 1 – 4), PMO720-F/R (lanes 6 – 9) and PMO920-F/R (lanes 11 – 14). β-actin (lanes 15 – 18, B) was included as the positive control for RT-PCR.

3.2 Cloning and characterization of Peritrophin homologues in *P. monodon*

Two pairs of primers primed at different regions of the template were designed from a peritrophin homologue (Peri-F_1/R_1 and Peri- F_2/R_2). RT-PCR of the first strand cDNA of immature and vitellogenic ovaries, testes, lymphoid organ and antennal ganglion of *P. monodon* using primers Peri-F_1/R_1 and Peri- F_2/R_2 did not show tissue-specific expression in *P. monodon* (Fig. 3.21 and Table 3.6). A single band (269 bp) was amplified from the former primer pair whereas two amplification products were oatained from Peri- F_2/R_2. The suggested that more than one type of peritrophim homologues are available in *P. monodon*.

Using genomic DNA of different *P. monodon* individuals as the template, polymorphism of peritrophin was found (Fig. 3.22) from Peri- F_2/R_2. The primer pair revealed two amplification fragments as previously observed when the first strand cDNA was used as the template suggesting the availability of large introns in this gene segment. Nevertheless, no length variation of peritrophin was observed between male and female *P. monodon*.

Table 3.6 Amplification of peritrophin homologue in *P. monodon*

Primer combination	Results	PCR conditions
1.Peri-F_1/R_1	An expected 269 bp band (Fig. 3.21)	**Composition:** 100 μM dNTP, 2 mM $MgCl_2$, 0.4 μM of each primer, 1 U. of *Taq* polymerase and 1 μl of the first strand cDNA from various tissues **PCR profile:** I. 1 cycle of 94 °C for 3 min II. 35 cycles of 94 °C for 30 sec, 55 °C for 1 min and 72 °C for 1 min III. 1 cycle of 72 °C for 7 min
2.Peri-F_1/R_1	A 269 bp fragment (Fig. 3.22)	Using conditions in 1 except 25 ng of genomic was used as the template
3.Peri-F_2/R_2	Two bands at the expexted size (151 bp) and a larger band (Fig. 3.21)	Using conditions in 1
4.Peri-F_2/R_2	Two bands at about 900 bp and 950 bp indicating the existence of a large introns (Fig. 3.22)	Using conditions in 2
5.Peri-F_1 + Peri-R_2	Non specific amplification products	Using conditions in 2
6.Peri-F_2 + Peri-R_1	Non specific amplification products	Using conditions in 2

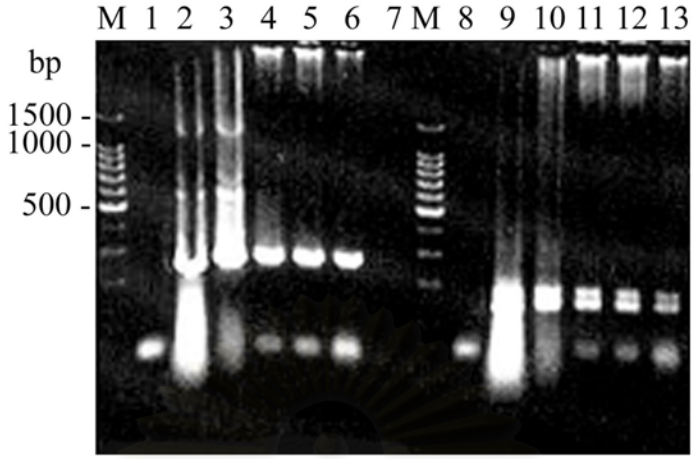

Figure 3.21 RT-PCR of peritrophin homologues in *P. monodon* using the first strand cDNA template from mature (lanes 2 and 9) and immature ovaries (lanes 3 and 10), lymphoid organ (lanes 4 and 11) testes (lanes 5 and 12) and antennal ganglion (lanes 6 and 13) and primers Peri-F_1/R_1 (lanes 1 – 7), Peri-F_2/R_2 (lanes 8 – 13), respectively. Lanes M is a 100 bp DNA marker. Lanes 1 and 8 are negative control (without cDNA template).

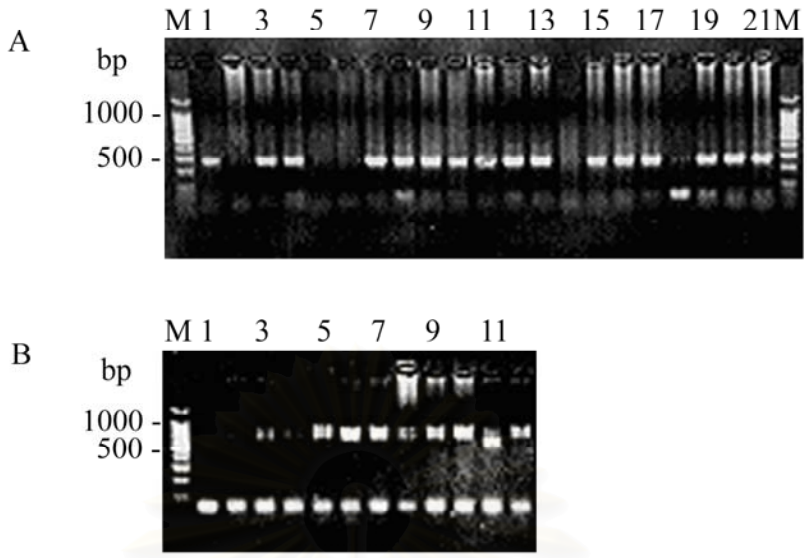

Figure 3.22 Amplification of genomic DNA isolated from female (lanes 1 – 9, panel A and lanes 2 – 7, panel B) and male (lanes 10 – 21, panel A and lanes 8 – 12, panel B) *P. monodon* using primers Peri-F_1/R_1 (panel A) and Peri-F_2/R_2 (panel B). Lanes M were a 100 bp DNA marker. Lane 1 (panel B) was negative control (without genomic DNA template). The expected product of Peri-F_1/R_1 and Peri-F_2/R_2 when the first strand cDNA was used as the template were 269 bp and 151 bp, respectively.

3.3 Cloning and sequencing of a zinc finger protein gene homologue

Two pairs of primers primed at different regions of the template were designed from a homologue of X- and Y-related zinc finger gene (Zinc-F/R and Finger-F/R). RT-PCR of the first strand cDNA from immature and vitellogenic ovaries, testes, lymphoid organ and antennal ganglion of *P. monodon* using primers these primers revealed the expected products in both primer set. Expression of zinc finger protein homologue in *P. monodon* was not tissue-specific in this species Nevertheless, an additional larger band (approximately 275 bp) was observed in ovaries but not in testes of *P. monodon* (Fig. 3.23 and Table 3.7).

It is interesting to verify that the extra amplification fragment of the zinc finger gene homologue (Finger-F/R) from immature and vitellogenic ovaries were sex-specific and was not found in the testes. The RT-PCR products from ovaries was then electrophoresed, gel-eluted (Fig. 3.23), cloned and sequenced.

Eight recombinant clones were selected. Plasmid DNA was extracted from each clone and sequenced. DNA sequences of these clones revealed that 201 bp and 277 bp fragments were homologous (Fig. 3.24). The larger fragment possessed an extra 76 bp at the 3′ terminus. Comparing this with the sequence of the original EST clone indicate that a 277 bp fragment was unfortunately generated from mispairing of CCC at the 3′ of the primer with the cDNA template. As a result, a 277 bp fragment was not sex-specific transcript.

Amplification of a zinc finger protein homologue using Zinc-F/R and the first strand cDNA from ovaries, testes, lymphoid organ, and antennal ganglion was successful but Zinc-F/R did not show the amplification product when genomic DNA was used as the template.

Amplification of Finger-F/R product using genomic DNA as the template exhibited a 350 bp fragment suggesting the existence of a small intron (approximately 150 bp) within the amplification product (Fig. 3.25). A new pair of primers (Zincfinger-F/R) was designed and used for RT-PCR. The expected product (308 bp) was obtained with no size differences between the PCR product of male (testes) and female (ovaries) *P. monodon*.

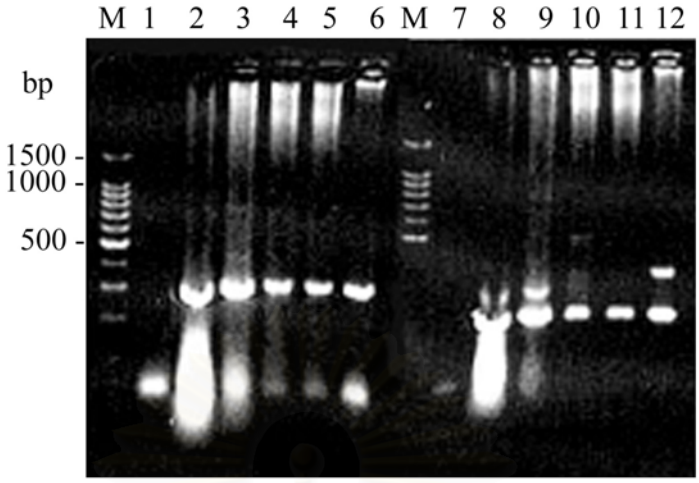

Figure 3.23 RT-PCR of zinc finger protein homologues in *P. monodon*. The first strand DNA template from mature (lanes 2 and 9) and immature ovaries (lanes 3 and 10), lymphoid organs (lanes 4 and 11) testes (lanes 5 and 12) and antennal ganglion (lanes 6 and 13) were examined using primers Zinc-F/R (lanes 1 – 6), Finger-F/R (lanes 7 – 12). The expected 201 bp fragment and an accompanying larger fragment was gel-eluted (lanes 8 and 9) and cloned into pGEM-Teasy

Lanes M is a 100 bp DNA marker. Lanes 1 and 8 are negative control (without cDNA template).


```
F12             AAGAAGCTGACGACCCAGACGACTAC-AGAAAGGACAACAGTTATGAAGGTGGATCAGTC
F33             AAGAAGCTGACGACCCAGACGACTACGAGAAAGGACAACAGTTATGAAGGTGGATCAGTC
F11             AAGAAGCTGACAACCCAGACGACTAC-AGAAAGGACAACAGTTATGAAGGTGGATCAGTC
F13             AAGAAGCTGACGACCCAGACGACTAC-AGARAGGACAACAGTTATGAAGGTGGATCAGTC
F41             AAGAAGCTGACGACCCAGACGACTAC-AGAAAGGACAACAGTTATGAAGGTGGATCAGTC
F14             AAGAAGCTGACGACCCAGACGACTAC-AGAAAGGACAACAGTTATGAAGGTGGATCAGTC
F24             AAGAAGCTGACGACCCAGACGACTAC-AGAAAGGACAACAGTTATGAAGGTGGATCAGTC
F32             AAGAAGCTGACGACCCAGACGACTAC-AGAAAGGACAACAGTTATGAAGGTGGATCAGTC
F34             AAGAAGCTGACGACCCAGACGACTAC-AGAAAGGACAACAGTTATGAAGGTGGATCAGTC
                ************************** *********************************
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```
F12             AACGAAGGCGACATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCT
F33             AACGAAGGCGACATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCT
```

```
F11             AACGAAGGCGACATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCT
F13             AACGAAGGCGACATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCT
F41             AACGAAGGCGACATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCT
F14             AACGAAGGCGACATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCT
F24             AACGAAGGCGACATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCT
F32             AACGAAGGCGACATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCT
F34             AACGAAGGCGACATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCT
                ************************************************************

F12             GACAGTTACGGCAGTGGATCATACGCAGGACCATCCATTCAACCTGGGGGTGACCTCCCT
F33             GACAGTTACGGCAGTGGATCATACGCAGGACCATCCATTCAACCTGGGGGTGACCTCCCT
F11             GACAGTTACGGCAGTGGATCATACGCAGGACCATCCATTCAACCTGGGGGTGACCTCCCT
F13             GACAGTTACGGCAGTGGATCATACGCAGGACCATCCATTCAACCTGGGGGTGACCTCCCT
F41             GACAGTTACGGCAGTGGATCATACGCAGGACCATCCATTCAACCTGGGGGTGACCTCCCT
F14             GACAGTTACGGCAGTGGATCATACGCAGGACCATCCATTCAACCTGGGGGTGACCTCCCT
F24             GACAGTTACGGCAGTGGATCATACGCAGGACCATCCATTCAACCTGGGG-TGACCTCCCT
F32             GACAGTTACGGCAGTGGATCATACGCAGGACCATCCATTCAACCTGGGGGTGACCTCCCT
F34             GACAGTTACGGCAGTGGATCATACGCAGGACCATCCATTCAACCTGGGGGTGACCTCCCT
                ************************************************************

F12             TGGGATGAGGGAGATTCAAGCAGTTTTCCACAAGAAGGCTTCTCAGGGGACTTACCAGCA
F33             TGGGATGAGGGAGATTCAAGCAGTTTTCCACAAGAAGGCTTCTCAGGGGACTTACCAGCA
F11             TGGGATGAGGGAGATTCAAGCA--------------------------------------
F13             TGGGATGAGGGAGATTCAAGCA--------------------------------------
F41             TGGGATGAGGGAGATTCAAGCA--------------------------------------
F14             TGGGATGAGGGAGATTCAAGCAGTTTTCCACAAGAAGGCTTCTCAGGGGACTTACCAGCA
F24             TGGGATGAGGGAGATTCAAGCA--------------------------------------
F32             TGGGATGAGGGAGATTCAAGCA--------------------------------------
F34             TGGGATGAGGGAGATTCAAGCAGTTTTCCACAAGAAGGCTTCTCAGGGGACTTACCAGCA
                **********************

F12             GGCCAGCAACCTCAAGGGGGATGAGGGAGATTCAAGCA
F33             GGCCAGCAACCTCAAGGGGGATGAGGGAGATTCAAGCA
F11                          ---------------------------------------
F13                          ---------------------------------------
F41             -------------------------------------
F14             GGCCAGCAACCTCAAGGGGGGATGAGGGAGTTCAAGCA
F24             -------------------------------------
F32             -------------------------------------
F34             GGCCAGCAACCTCAAGGGGATGAGGGAGATTCAAGCA
```

Figure 3.24 Multiple alignments of nucleotide sequences obtained from the target and the larger amplification bands (Finger-F/R) of total RNA from immature and vitellogenic ovaries of *P. mondon*.

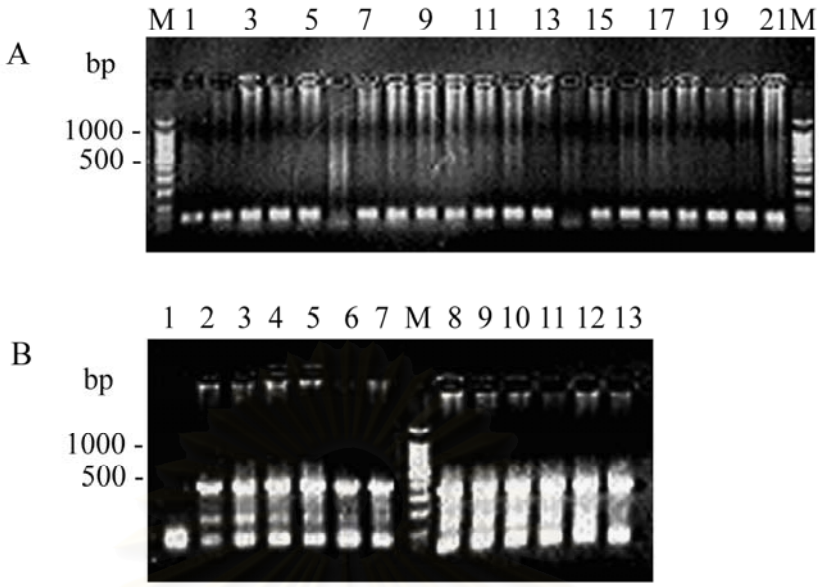

Figure 3.25 Amplification of genomic DNA isolated from female (lanes 1 – 9, panel A and lanes 2 – 7, panel B) and male (lanes 10 – 21, panel A and lanes 8 – 13, panel B) *P. monodon* using primers Zinc-F/R (panel A) and Finger-F/R (panel B). Lanes M were a 100 bp DNA marker. Lane 1 (panel B) was negative control (without genomic DNA template). The expected product of Zinc-F/R and Finger-F/R when the first strand cDNA was used as the template were 309 bp and 201 bp, respectively.

Using different primer combination; Zinc-F+ Zincfinger-R (expected size from the cDNA sequence = 170 bp) and Zincfinger-F + Zinc-R (expected size = 550 bp), the former pair of primers did not give the amplification product when genomic DNA was used as the template. The latter did not generate the expected product for both cDNA and genomic DNA template suggesting that primers Zinc-F and/or Zinc-R may have primed both exon and intron regions.

A larger 450 bp fragment obtained from Zincfinger-F + Finger-R when genomic DNA was used as the template (Figs. 3.26 and 3.27). Based on the previous characterization in various mammalian species, X-linked zinc finger protein gene (Zfx) and Y-linked zinc finger protein gene (Zfy) usually showed fixed single nucleotide polymorphism (SNP) between males and females of various vertebrates. The PCR product of these primers was selected for identification of sex-specific single nucleotide polymorphism (SNP) in *P. monodon*. Therefore, ten individuals of male (*N* = 2, 2, 2, and 4 from Trang, Satun, Phangnga and Chumphon, respeectively) and ten individuals of female (2 individuals each from Satun, Trang, Phangnga, Chumphon and Trad) was separately cloned and sequenced.

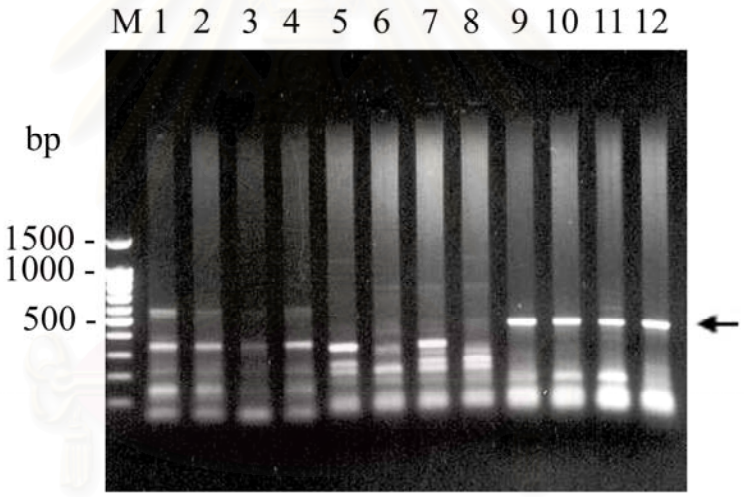

Figure 3.26 Amplification of zinc finger protein gene homologues in male (lanes 1 – 2, 5 – 6 and 9 – 10) and female (lanes 3 – 4, 7 – 8 and 11 – 12) *P. monodon* using primers Finger–F + Finger–R (lane 1 – 4)**,** Finger-F + Zincfinger-R (lane 5 – 8) and Zinfinger-F + Finger-R (lanes 9 – 12). An arrow indicated a 450 bp fragment that was individually cloned from genomic DNA of male (*N* = 8) and female (*N* = 10) *P. monodon* A 100 bp ladder (lane M) was included as the DNA marker.

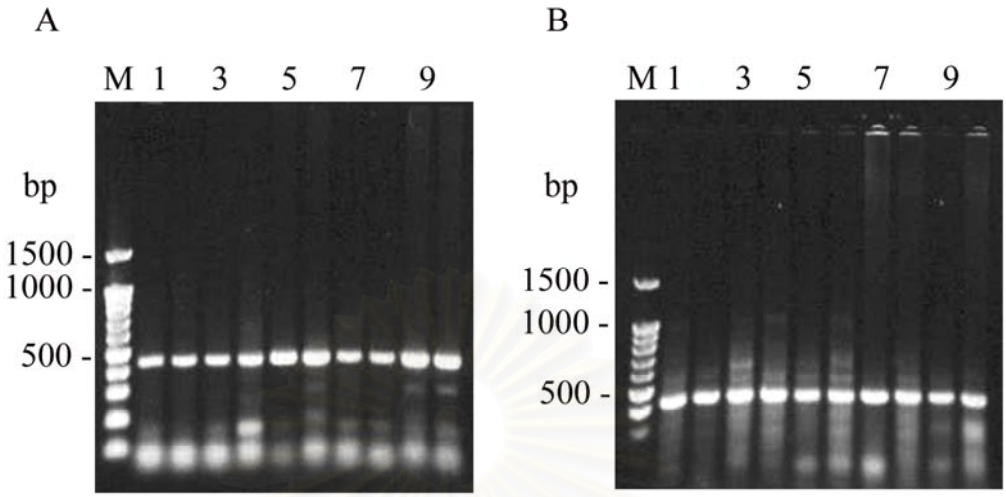

Figure 3.27 Amplification of a zinc finger protein homologue (Zincfinger-F + Finger-R) using genomic DNA of female (*N* = 10, panel A) originating from Satun (*N* = 2), Trang (*N* = 2), Phangnga (*N* = 2) and Chumphon (*N* = 4) and male (*N* = 10, panel B) originating from Satun (*N* = 2), Trang (*N* = 2), Phangnga (*N* = 2), Chumphon (*N* = 2) and Trad (*N* = 2) *P. monodon*, respectively as the template. A 100 bp ladder was used as the marker. Notably, the amplified PCR product of male *P. monodon* from Trang was not successfully cloned.

Table 3.7 Amplification of zinc finger gene homologue in *P. monodon*

Primer combination	Results	PCR conditions
1. Zinc-F/R	An expected 309 bp band (Fig. 3.23)	**Composition:** 100 μM dNTP, 2 mM $MgCl_2$, 0.4 μM of each primer, 1 U. of *Taq* polymerase and 1 μl of the 1st strand cDNA from various tissues **PCR profile:** I. 1 cycle of 94 °C for 3 min II. 35 cycles of 94 °C for 30 sec, 55 °C for 1 min and 72 °C for 1 min III. 1 cycle of 72 °C for 7 min
2. Zinc-F/R	No amplification product (Fig. 3.25)	Using conditions in 1 except 25 ng of genomic was used as the template
3. Zinc-F + Finger-R	Non specific amplification products	Using conditions in 2
4. Zinc-F + Zincfinger-R	No aplification products	Using conditions in 2
5. Finger F/R	An expected 201 bp band (Fig. 3.23)	Using conditions in 1
6. Finger F/R	Approximately 350 bp fragment indicating the existence of small intron within the amplification region (Fig. 3.25)	Using conditions in 2
7. Finger-F + Zinc-R	Non specific amplification products	Using conditions in 2
8. Finger-F + Zincfinger R	Non-specific products (Fig. 3.26)	Using conditions in 2
9. Zincfinger-F/R	No amplification products	Using conditions in 2
10. Zincfinger-F + Zinc-R	No amplification products	Using conditions in 2
11. Zincfinger-F + Finger-R	An expected 450 bp fragment (Fig. 3.26)	Using conditions in 2

Colony PCR was carried out to identify insert sizes of positive clones (Fig. 3.28). Two recombinant clones from each insert were unidirectionally sequenced. The nucleotide sequence of each clone was blasted against those of the GenBank. Six of those (pZFF1/1, pZFF1/2, pZFF8/2, pZFM2/1, pZFM2/2 and pZFM10/2) were unknown genes and were not included in the analysis. Multiple sequence alignments of a zinc finger gene homologue indicated substitutional polymorphism within male and female and between genders of *P. monodon* but did not fix for each gender. Therefore, sex-specific markers at the genomic DNA level still could not identify in *P. monodon.*

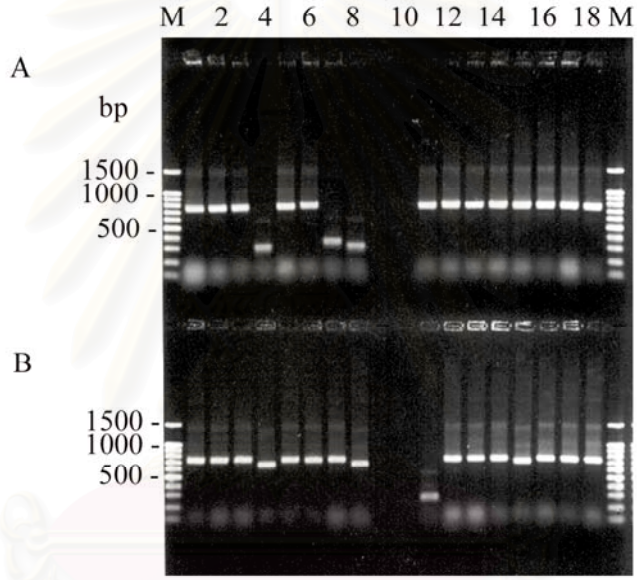

Figure 3.28 Colony PCR of 8 randomly selected white colonies from each ligation of the amplified zinc finger protein homologue using Zincfinger-F + Finger-R as the template.

Panel A; lanes 1 – 8 and 11 – 18 = clones originating from two females *P. monodon* from Satun

Panel B; lanes 1 – 8 and 11 – 18 = clones originating from two females *P. monodon* from Trang

Colonies possessing slightly smaller inserts (lanes 4, panel B) and no insert (lanes 4, 7 – 8; panel A and lanes 11; panel B) were also found. A 100 bp ladder was used as the DNA marker

```
pZFF3/2         CCAGTCCAGTCTGCATCGTTGCCCGGATCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF6/2         CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF9/1         CCAGTCCAGTCTGCATCGTTGCCCGGATCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM10/2        CCAGTCCAGTCTGCATCGTTGCCCGGATCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM5/1         CCAGTCCAGTCTGCATCGTTGCCCGGATCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM10/1        CCAGTCCAGTCTGCATCGTTGCCCGGATCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM9/2         CCAGTCCAGTCTGCATCGTTGCCCGGATCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM5/2         CCAGTCCAGTCTGCATCGTTGCCCGGATCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM7/2         CCAGTCCAGTCTGCATCGTTGCCCGGATCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF10/1        CCAGTCCAGTCTGCATCGTTGCCCGGATCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM2/1         CAAGTCCAGTCTGCATCGTTGCCCGGATCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF3/1         CCAGTCCAGTCTGCATCGTTGCCCGGCTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF5/1c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM8/1c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF8/1         CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF4/2         CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF7/2         CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF6/1         CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF5/2         CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM6/2c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM6/1c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM8/2c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM7/1c        CCAGTCCAGTCTGCATCGTTGCCCGGTTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF9/2c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF4/1c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFF7/1c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM1/1c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCCCA
pZFM1/2c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
pZFM9/1c        CCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCA
                * ************************ ****************************** **

pZFF3/2         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF6/2         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF9/1         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM10/2        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAAACGACTAC
pZFM5/1         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM10/1        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM9/2         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM5/2         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM7/2         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF10/1        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM2/1         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF3/1         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF5/1c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM8/1c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF8/1         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF4/2         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF7/2         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF6/1         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF5/2         TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM6/2c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM6/1c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM8/2c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM7/1c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF9/2c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF4/1c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFF7/1c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM1/1c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM1/2c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
pZFM9/1c        TTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTAC
                ************************************************** ********
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Figure 3.29

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pZFF3/2         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF6/2         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF9/1         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM10/2        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAACGCGACATGGGATCAGACTTT
pZFM5/1         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM10/1        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM9/2         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM5/2         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM7/2         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF10/1        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM2/1         AGAAAGGACAACAGTTACGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF3/1         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF5/1c        AGAAAGGACAACAGTATTGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM8/1c        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF8/1         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF4/2         AGAGAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF7/2         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF6/1         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF5/2         AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
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pZFM8/2c        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM7/1c        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF9/2c        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF4/1c        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFF7/1c        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM1/1c        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM1/2c        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
pZFM9/1c        AGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTT
                *** ***********   ********************** **********************

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pZFF6/2         GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFF9/1         GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFM10/2        GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFM5/1         GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFM10/1        GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFM9/2         GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFM5/2         GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFM7/2         GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFF10/1        GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFM2/1         GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFF3/1         GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFF5/1c        GGCGCGGAATTATCTTAAAGCGGGACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFM8/1c        GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFF8/1         GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFF4/2         GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
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pZFM6/1c        GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
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pZFM1/2c        GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
pZFM9/1c        GGCGCGGAATTATCT-AAAGCGGAACACGACCCTGACAGTTACGGCAGTGGATCATACGC
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Figure 3.29 (cont.)

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pZFF3/2         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFF6/2         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFF9/1         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM10/2        AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM5/1         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM10/1        AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM9/2         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGTTTTATGGTATAAAATGGTACAT
pZFM5/2         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM7/2         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
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pZFM2/1         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFF3/1         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFF5/1c        AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM8/1c        AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFF8/1         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFF4/2         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFF7/2         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFF6/1         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFF5/2         AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM6/2c        AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM6/1c        AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
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pZFF7/1c        AGGACCATCCATTCAGCCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM1/1c        AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM1/2c        AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
pZFM9/1c        AGGACCATCCATTCAACCTGGGGTAAGTTACTCTCTGCTTTATGGTATAAAATGGTACAT
                *********** *** ********************** ******** *************

pZFF3/2         CATGTCATGTCAATGCAATTAAGAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFF6/2         CATGTCATGTCAATGCAATTAAGAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFF9/1         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFM10/2        CATGTCATGTCAATGCAATTAAAAATTGTCTCACCTTCAAGCAT-CATTGTTGTTGCATC
pZFM5/1         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFM10/1        CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFM9/2         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFM5/2         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFM7/2         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFF10/1        CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
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pZFF3/1         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
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pZFF8/1         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFF4/2         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFF7/2         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFF6/1         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFF5/2         CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFM6/2c        CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFM6/1c        CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATT
pZFM8/2c        CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATT
pZFM7/1c        CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATA
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pZFF4/1c        CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATC
pZFF7/1c        CATGTCATGTCAATACAATTAAAAGTTGTCTCACCTTCAAGCATCCATTGTTGTTGCATT
pZFM1/1c        CATGTCATGTCAATACAATTAAAAGTTGTCTCACCTTCAAGCATCCATCGTTGTTGCATA
pZFM1/2c        CATGTCATGTCAATACAATTAAAAGTTGTCTCACCTTCAAGCATCCATTGTTGTTGCATT
pZFM9/1c        CATGTCATGTCAATGCAATTAAAAGTTGTCTCACCTTCAAGCATGCATTGTTGTTGCATT
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Figure 3.29 (cont.)

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pZFF3/2         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFF6/2         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFF9/1         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFM10/2        ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFM5/1         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFM10/1        ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFM9/2         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFM5/2         ACAGACAGTCACTGACATTTTACTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFM7/2         ACAGACAGTCACTGACATTTTACTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFF10/1        ACAGACAGTCACTGACATTTTACTTTGTCTAAWCTCATAGGGTGACCTCCCTTGGGATGA
pZFM2/1         ACAGACAGTCACTGACATTTTTCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFF3/1         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFF5/1c        ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFM8/1c        ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFF8/1         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCCCATAGGGTGACCTCCCTTGGGATGA
pZFF4/2         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFF7/2         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFF6/1         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFF5/2         ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFM6/2c        ACAGACAGTCACTGACATTTTGCTTTGTCTAATTTCATAGGGTGACCTCCCTTGGGATGA
pZFM6/1c        ACAGACAGTCACTGACATTTTACTTTGTCTAATCTCATAGGGTGACCTGCCTTGGGATGA
pZFM8/2c        ACAGACAGTCACTGACATTTTACTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFM7/1c        ACAGACAGTCACTGACATTTTACTTTGTCTAATCTCATAGGGTGACCTCCCTTGGGATGA
pZFF9/2c        ACAGACAGTCACTGACATTTTGCTTTGTCTAACCTCATAGGGTGACCTCCCTTGGGATGA
pZFF4/1c        ACAGACAGTCACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTTCCTTGGGATGA
pZFF7/1c        ACAGACAGTAACTGACATTTTGCTTTGTCTAATATCATAGGGTGACCTTCCTTGGGATGA
pZFM1/1c        ACAGACAGTAACTGACATTTTGCTTTGTCTAATATCATAGGGTGACCTTCCTTGGGATGA
pZFM1/2c        ACAGACAGTTACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTTCCTTGGGATGA
pZFM9/1c        ACAGACAGTTACTGACATTTTGCTTTGTCTAATCTCATAGGGTGACCTTCCTTGGGATGA
                ********* ********** **********   ************* ***********

pZFF3/2         GGGAGATTCAAGCA
pZFF6/2         GGGAGATTCAAGCA
pZFF9/1         GGGAGATTCAAGCA
pZFM10/2        GGGAGATTCAAGCA
pZFM5/1         GGGAGATTCAAGCA
pZFM10/1        GGGAGATTCAAGCA
pZFM9/2         GGGAGATTCAAGCA
pZFM5/2         GGGAGATTCAAGCA
pZFM7/2         GGGAGATTCAAGCA
pZFF10/1        GGGAGATTCAAGCA
pZFM2/1         GGGAGATTCAAGCA
pZFF3/1         GGGAGATTCAAGCA
pZFF5/1c        GGGAGATTCAAGCA
pZFM8/1c        GGGAGATTCAAGCA
pZFF8/1         GGGAGATTCAAGCA
pZFF4/2         GGGAGATTCAAGCA
pZFF7/2         GGGAGATTCAAGCA
pZFF6/1         GGGAGATTCAAGCA
pZFF5/2         GGGAGATTCAAGCA
pZFM6/2c        GGGAGATTCAAGCA
pZFM6/1c        GGGAGATTCAAGCA
pZFM8/2c        GGGAGATTCAAGCA
pZFM7/1c        GGGAGATTCAAGCA
pZFF9/2c        GGGAGATTCAAGCA
pZFF4/1c        GGGAGATTCAAGCA
pZFF7/1c        GGGAGATTCAAGCA
pZFM1/1c        GGGAGATTCAAGCA
pZFM1/2c        GGGAGAGTCAAGCA
pZFM9/1c        GG------------
```

Figure 3.29 Multiple alignments of a zinc finger gene homologue of males (pZFM) and females (pZFF) of *P. monodon*.

3.4 Identification of sex–specific expression transcripts in the giant tiger shrimp (*P. monodon*) by cDNA subtraction

Subtractive cDNA libraries were constructed using cDNA from ovaries as the tester and cDNA from testes as the driver and *vice versa*. After hybridization, products of cDNA subtraction were amplified by PCR to identify differentially expressed transcripts. Nested PCR was also performed to further reduce background PCR products and to enrich differentially expressed transcripts selectively (Fig. 3.30). Results from subtracted and unsubstracted controls (skaeletal muscle cDNA) were successful. As a result, forward (cDNA of ovaries as the tester) and reverse (cDNA of testes as the tester) subtracted cDNA fragments were cloned and further characterized. The major amplification products from subtractive cDNA of ovaries were more than 500 bp in size but those of testes were slightly greater than 200 bp.

A total of 216 clones (155 clones from subtractive cDNA of ovaries and 61 from that of testes) were unidirectionally sequenced. Nucleotide sequences of these transcripts were compared with those previously deposited in the GenBank using BLAST*N* and BLAST*X*. Significant probabilities of matched nucleotides/protein were considered when the E-value was $< 10^{-4}$.

Among 155 recombinant clones from the forward subtraction, transcripts of ovaries of *P. monodon* could be categorized to 3 major groups, thrombospondin families (TSP, 28.7%), peritrophin (10.8%) and unknown (49.7%) gene products (Table 3.8). Besides major groups, others including a homologue of sex-related CG4790 gene product in *D. melanogaster* were found in subtraction of cDNA from ovaries. In contrast, unknown transcripts (96.7%) were abundantly expressed in testes of *P. monodon*. Only 2 clones representing anti-LPS and serine protease HTRA3 homologues were isolated (Table 3.9).

Unknown cDNA from subtracted cDNA libraries indicated different groups of transcripts of ovaries and testes. From the forward subtraction, unknown transcripts (49.7%) were classified to 15 clusters and 33 singletons (Table 3.8). In contrast, unknown transcripts (96.7%) obtained from reverse subtraction were allocated to 9 different clusters and 14 singletons.

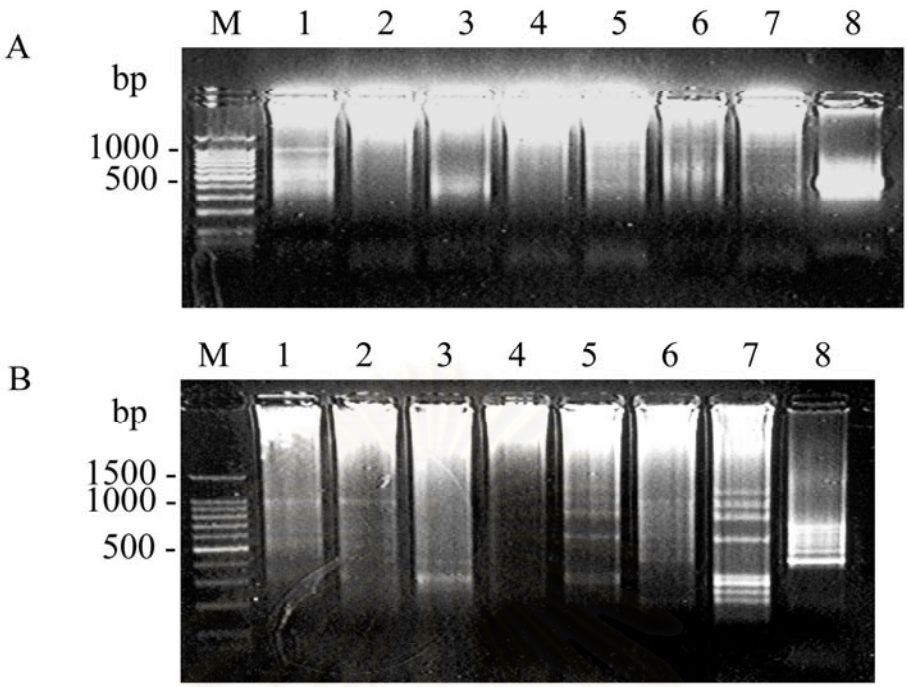

Figure 3.30 Electrophoresis of the first (A) and the second (B) PCR of subtracted cDNA of *P. monodon*

Lane 1 : subtracted cDNA of ovaries

Lane 2 : unsubtracted tester control for cDNA of ovaries

Lane 3 : subtracted cDNA of testes

Lane 4 : unsubtracted tester control for cDNA of testes

Lane 5 : subtracted control (skeletal muscle cDNA)

Lane 6 : unsubtracted tester control for the control subtraction

Lane 7 : The PCR control of subtracted cDNA of skeletal muscle (previded by the kit)

Lane 8 : G3PDH (PCR control)

Table 3.8 Homologues of genes found in a subtractive library using cDNA of *P. monodon* ovaries as the tester

Gene Identity	No. of Clones	Closest species	Clone
Thrombospondin family	45	*Penaeus monodon* and *Marsupenaeus japonicus*	pO16, pO18, pO118, pO123, pO136, pO137, pO143, pO152, pO157, pO159, pO219, pO228, pO230, pO231, pO240, pO246, pO253, pO162, pO163, pO25, pO279, pO280, pO282, pO287, pO288, pO294, pO39, pO314, pO336, pO344, pO363, pO366, pO371, pO379, pO384, pO137, pO437, pO447, pO456, pO462, pO470, pO474, pO475, pO477, pO487
Peritrophin	17	*Penaeus monodon*	PO110, pO111, pO115, pO125, pO133, pO150, pO222, pO239, pO265, pO313, pO329, pO330, pO331, pO385, pO393, pO446, pO449
Prostaglandin synthase	1	*Homo sapiens*	pO13
ATP synthase β	1	*Homo sapiens*	pO15
H. sapiens Mrna	1	*Homo sapiens*	pO112
RAPD genomic fragment	1	*Leilemur dorsalis*	pO124
NF_kB	1	*Rattus norvegicus*	pO126
Proteosome subunit 9	1	*Homo sapiens*	pO129
60S ribosomal protein L6	1	*Rattus nornegicus*	pO114
Disulfide isomerase	2	*Drosophila melanogaster*	pO134, pO448
Leucocyte surface antigen CD53	2	*Homo sapiens*	pO243, pO259
Sex-linked CG4790 gene product	4	*Drosophila melanogaster*	pO212, pO244, pO271, pO281

Table 3.8 (cont)

Gene Identity	No. of Clones	Closest species	Clone
Putative oncoprotein	1	*Ictalurus punctatus*	pO322
Elongation factor α	1	*Armadillidium vulgare*	pO421
Unknown genes	76	-	**Cluster 1** : pO227, pO236, pO272, pO317, pO365 **Cluster 2** : pO130, pO335, pO389, pO438 **Cluster 3** : pO132, pO142, pO276, pO328 **Cluster 4** : pO138, pO151, pO261 **Cluster 5** : pO148, pO220, pO368 **Cluster 6** : pO226, pO229, pO342 **Cluster 7** : pO286, pO453, pO467 **Cluster 8** : pO131, pO145 **Cluster 9** : pO28, pO345 **Cluster 10** : pO214, pO257 **Cluster 11** : pO49, pO350 **Cluster 12** : pO42, pO355 **Cluster 13** : pO420, pO461 **Cluster 14** : pO17, pO273 **Cluster 15** : pO354,pO357,pO362, pO364 **Unknown singletons** : pO14, pO13, pO19, pO128, pO140, pO144, pO155, pO160, pO26, pO29, pO213, pO235, pO250, pO283, pO290, pO34, pO332, pO337, pO340, pO347, pO348, pO349, pO351, pO358, pO359, pO360, pO383, pO391, pO392, pO46, pO419, pO440, pO486

Table 3.9 Homologues of genes found in a subtractive library using cDNA of *P. monodon* testes as the tester

Gene Identity	No. of Clones	Closest species	Clone
Serine protease HTRA3	1	*Homo sapiens*	pT221
Anti-LPS	1	*Limulus polyphemus*	pT151
Unknown genes	59	-	**Cluster 1** : pT14 Pt16,, pT112, pT115, pT140, pT146, pT312, pT333 **Cluster 2** : pT15, p19, pT110, pT111, pT116, pT134, pT135, pT316, pT317, pT327 **Cluster 3** : pT18, pT143, pT147, pT152, pT34, pT318, pT323, pT326 **Cluster 4** : pT114, pT145, pT222, pT328 **Cluster 5** : pT122, pT128, pT139, pT142, pT148, pT38, pT322 **Cluster 6** : pT137, pT321 **Cluster 7** : pT311, pT335 **Cluster 8** : pT149, pT324 **Cluster 9** : pT119, pT325 **Unknown singletons** : pT13, pT17, pT113, pT117, pT124, pT125, pT127, pT136, pT150, pT158, pT23, pT220, pT32, pT232

3.5 Characterization of Thrombospondin (TSP) homologue by rapid amplification of cDNA ends-polymerase chain reaction (RACE - PCR)

RACE-PCR was carried out using primers designed from TSP transcripts (pO228, a homologue of mammalian cartilage oligomeric matrix protein, COMP; E-value = 3 x 10^{-33}). Fragments of approximately 2.3 kb, 600 bp and 800 bp ffrom 5′-RACE-PCR and that of 700 bp frrom 3′- RACE-PCR were cloned and sequenced (Fig. 3.31).

After characterization of RACE product, the first set of TSP-specific primers gave rise to many non-TSP products and most of the clones obtained were homologues of elongation factor 2-α (EF2-α) in *Armadillidium vulgare*. Other transcripts including sex-linked nuclear protein, XNP-1 in *C. elegans*, hypothetical proteins FLJ23251 in *Homo sapiens* and transketolase in *Danio rerio* were also obtained from 5′- RACE products. Peritrophin homologues were obtained from 3′-RACE product (Table 3.10).

New primers and nested primers were designed from homologues of *D. melanogaster* TSP (clone pO288, E-value = 3 x 10^{-12}; pO371, E-value = 3 x 10^{-13} and pO462, E-value = 6 x 10^{-38}) and used to identified 5′ and 3′ TSP homologues in *P. monodon*. Amplification results indicated the 3 kb fragments of 5′- RACE products from primer 462-1 and 371-1 and 800 bp and 1000 bp fragments of 3′- RACE products from primer 462-2 and N462-2. These fragments were cloned and sequenced (Fig. 3.32).

All clones obtained from 5′- RACE product were TSP homologues. Blast results on nucleotide sequences indicated successful 5′ RACE reaction (Fig. 3.33). However, 3′ RACE-PCR was still not successful as other transcripts including XNP-1 (*Caenorhabditis elegans*) and ENSANGP00000010123 (*Anopheles gambiae*), which are sex-linked in the original species and oxidoreductase and unknown transcripts were found rather than the 3′ end of TSP (Table 3.10).

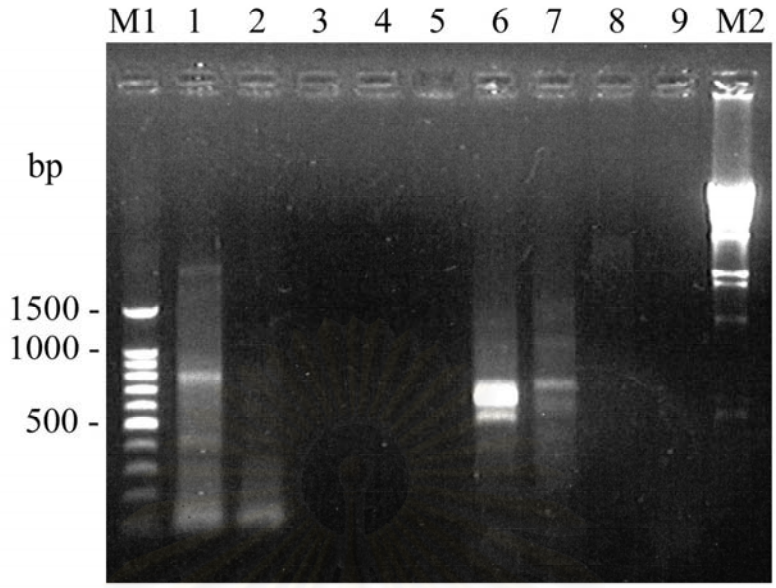

Figure 3.31 5′ RACE-PCR (TSP-1 : lanes 1 – 4) and 3’ RACE-PCR (TSP-2 : lanes 6 – 9) using the first set (TSP-1 and TSP-2) of TSP-specific primer.

Lane 1 : 5′ RACE 1 (immature ovaries)

Lane 2 : 5′ RACE 2 (mature ovaries)

Lane 3 : 5′ UPM control

Lane 4 : 5′ GSP1 control (TSP-1)

Lane M1 : 100 bp DNA ladder

Lane 6 : 3′ RACE 1 (immature ovaries)

Lane 7 : 3′ RACE 2 (mature ovaries)

Lane 8 : 3′ UPM control

Lane 9 : 3′ GSP2 control (TSP-2)

Lane M2 : λ-*Hind* III

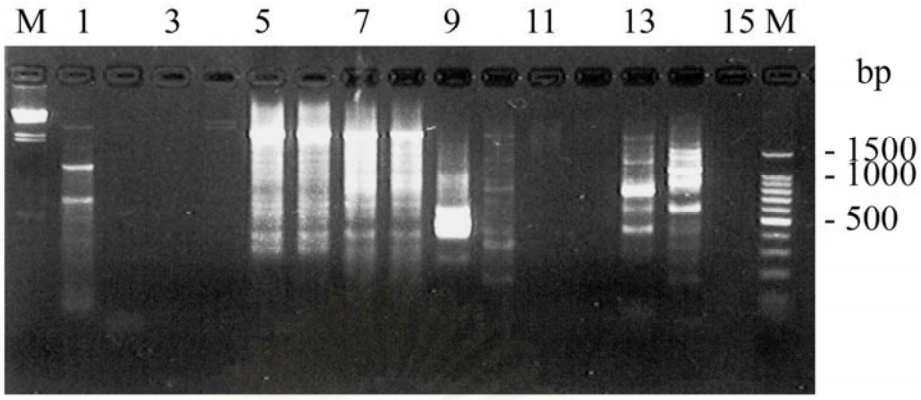

Figure 3.32 5′ RACE-PCR (288-1, N288-1, 462-1, N462-1, 371-1 and N371-1 : lanes1 – 8) and 3′ RACE-PCR (288-2, N288-2, 462-2 and N462-2 : lanes 9 – 14) using the second set of TSP-specific primers. RACE-PCR was carried out using 5′ and 3′ RACE first strand cDNA synthesized from mRNA of vitellogenic ovaries of *P. monodon*.

Lane 1 : 288-1

Lane 2 : N288-1

Lane 3 : 5′ UPM control

Lane 4 : 5′ GSP1 (288-1) control

Lane 5 : 462-1

Lane 6 : N462-1

Lane 7 : 371-1

Lane 8 : N371-1

Lane 9 : 288-2

Lane 10 : N288-2

Lane 11 : 3′ UPM control

Lane 12 : 3′ GSP2 (288-2) control

Lane 13 : 462-2

Lane 14 : N462-2

Lane M : 100 bp DNA ladder

Table 3.10 ESTs found from 5′- and 3′-RACE cDNA amplification using mRNA of *P. monodon* as the template**.** Sex-related transcripts in matched species are illustrated in boldface.

	Gene Identity*	Closest Species	No. of clone
5′-RACE products			
	Thrombospondin	***Penaeus monodon and Marsupenaeus japonicus***	19
	Elongation factor-2	*Armadillidium vulgare*	10
	Sex-linked nuclear protein, XNP-1	***Caenorhabditis elegans***	3
	Hypothetical proteins FLJ23251	*Homo sapiens*	3
	Transketolase	*Danio rerio*	1
3′-RACE products			
	Peritrophin	*Penaeus monodon*	5
	Sex-linked ENSANGP00000010123[a]	***Anopheles gambiae***	4
	Oxidoreductase	*Homo sapiens*	1
	Unknown	-	6

*Accession numbers CF805568 – CF805612

[a] called AGESTX in this thesis

```
Query: 186 GYECSGEGRFPDPSTCGVFIDCITKENGGYFVTQDNCNGFAYNATSRICSDQL-CASRHE 362
           G  C GEGRFP+PSTCG F DCI   +GGY     +CNGFAY+ATSR CS ++ C SR
Sbjct: 5   GPRCLGEGRFPNPSTCGEFFDCIPDGSGGYESLLSDCNGFAYDATSRTCSGEIECHSRSS 64

Query: 363 RSITNEYHPFSQICESQPDRFMCANCKTLVHCVKGQAFVRQCTEDFYCTEKLGFGGAVCY 542
           RS+T  YH +S +CE QPD F+CA+C+T+V CVKGQAFVRQCT    C     FGG VCY
Sbjct: 65  RSVTTAYHLYSYLCEGQPDAFVCADCRTMVMCVKGQAFVRQCTSGKACYVSATFGGGVCY 124

Query: 543 PNEPAECMCLRPNEFRVDPYDPQRFFSCENAGSQPESYKCPDGKKFDENSAQCVNQIGFP 722
           P  PAEC C + + F  D YDPQ+FFSC   G+ P+++ CPDG  FDE++ +C NQ   P
Sbjct: 125 PGLPAECTCQKADVFVRDMYDPQKFFSCSATGAVPDNHVCPDGMVFDESTVRCRNQASVP 184

Query: 723 ECVKSGTFANTQNCGEYYSCIPLRHGWLQKFFMCSGNHLFNERTKTCEDPCTLKFVCHRR 902
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Sbjct: 185 ACSRPGTFANLDNCAEYYSCMPLKHGWLQRLFMCSNGTLYNEVSGACEDPCASQMVCYEE 244

Query: 903 GDF 911
           G F
Sbjct: 245 GRF 247
```

Figure 3.33 BlastX Results of nucleotide sequences of pRACE7155 indicated that 5′ RACE-PCR was successful.

3.6 RT – PCR of homospecific primers from *P. monodon*

Sex specific/differential expression markers were also examined in transcripts previously isolated from subtractive (this thesis) and normal cDNA libraries of ovaries of *P. monodon* using RT-PCR. Primers from the former library included homologues of TSP, XNP-1, DSI and ENSANGP00000010123 (X-linked in *Drosophila melanogater* which is subsequently called AGESTX in this thesis) whereas those of the latter included homologues of EGF1, rudimentary protein and Ubiquitin X-specific pretease 9 (Usp9X).

An additional primer pair was designed from a homologues of *Sxl* previously found in hemocyte cDNA library of *P. monodon* and *P. japonicus*. In contrast, FE_{AGA}-M_{CGA}520 was a candidate female-specific AFLP marker of *P. monodon*. A primer pair designed from the nucleotide sequence of this AFLP marker was tested using the first strand cDNA template.

Amplification of AGESTX using AGESTX40-F/R did not reveal the amplification product. Combining of AGESTX44-F + AGESTX40-R generated the target (314 bp) fragment along with other non-specific fragments (Fig. 3.34). The expect product (234 bp) accompanying with non-specific amplification products were observed when a homologue of *D. melanogaster* rudimentary protein transcript was amplified using Rudimentary-F/R (Fig. 3.34).

Non-specific amplification products were observed when the first strand cDNA of ovaries and testes was amplified by primers TSP288-F/R (Fig. 3.35). TSP462-2 + TSP371-1 (Fig. 3.36) generated a 800 bp fragment in both ovaries and testes. Additional amplification fragments of 350 bp and 450 bp in length were only found in ovaries but not in testes of *P. monodon*. These bands were consistently found when RT-PCR was carried out at 55 °C against larger sample sizes (N = 11 for males and N = 9 for females). Nevertheless, increasing an annealing temperature to 65 °C eliminated these bands. Amplification of the first strand cDNA at 55 °C using a single primer (only TSP462-2 or TSP371-1 in the reaction tube; data not shown) also generated these fragments. Therefore, amplification products of 350 bp and 450 bp in length were regarded as non-specific products and were not further characterized.

RT-PCR of the first strand cDNA of testes and ovaries using primers TSP462-F + TSP288-R (Fig. 3.37) consistently generated 3 non-specific bands (150 bp and 1400 bp in ovaries and 1300 bp in testes) at the annealing temperature of 58 °C. A 150 bp fragment was still found even the annealing temperature was increased to 65 ° C. Although these amplified bands should have considered as non-specific amplification products, they showed sex-specificity in ovaries and testes of *P. monodon.* Therefore these bands (called TSPPMT1300 and TSPPMO150) were cloned and sequenced for further development of sex-specific expression markers in *P. monodon*.

Nucleotide sequences of TSPPMT1300 and TSPPMO150 (Fig. 3.38) did not show significant similarity with any sequence previously deposited in the GenBank and, therefore, were recognized as unknown transcripts. Amplification of TSPPMT1300 using the first strand cDNA of testes and ovaries as the template by TSPPMT1300-F: 5′- AGT ACG CTC GGG AAA GTG AAA A -3′ and TSPPMT1300-R: 5′- CGA ACA AGA TAG AAG TCC AAC ATT -3′ revealed the expected product of 211 bp in length (Fig. 3.39) and did not exhibit sex-specific or sex differential expression in ovaries and testes of *P. monodon*.

Table 3.11 RT-PCR for identification of sex-specific transcripts of *P. monodon* using various homospecific primers (*P. monodon*) derived from normal and subtractive cDNAs of ovaries and candidate female-specific AFLP markers

Gene homologues/primer	Closest species	Chromosome location in the closest species/original type of marker	Amplification results (figure number)
1. TSP288-F/R	*P. monodon and Marsupeneus japonicus*	Not known/subtractive cDNA	NS (Fig. 3.35)
2. TSP462-F/R	*P. monodon and M. japonicus*	Not known/subtractive cDNA	Expressed in both ovaries and testes
3. TSP288-1/371-1	*P. monodon and M. japonicus*	Not known/subtractive cDNA	380 bp fragment in ovaries (Fig. 3.50)
4. TSP462-2/371-1	*P. monodon and M. japonicus*	Not known/subtractive cDNA	NS (Fig. 3.36)
5. TSP462-F/288-R	*P. monodon and M. japonicus*	Not known/subtractive cDNA	NS (Fig. 3.37)
6. AGESTX40-F/R (ENSANGP00000010123)	*Drosophila melanogaster*	X/subtractive cDNA	-
7. AGESTX44 -F/R (ENSANGP00000010123)	*D. melanogaster*	X/subtractive cDNA	184 bp fragment in ovaries (Fig. 3.46)
8. AGESTX44-F/ AGESTX40-R (ENSANGP00000010123)	*D. melanogaster*	X/subtractive cDNA	NS (Fig. 3.34)
9. DSI-F/R	*D. melanogaster*	3L/subtractive cDNA	180 bp fragment in both ovaries and testes
10.EGF-1 (Zinc finger protein)	*Homo sapiens*	X/cDNA	NS (Fig. 3.40)
11.Rudimentary protein	*D. melanogaster*	X/cDNA	NS (Fig. 3.34)
12.XNP1-F/R	*C. elegans*	X/subtractive cDNA	Expressed in both ovaries and testes (224 bp) (Fig. 3.41)
13.*Sxl*-F/R	*D. melanogaster*	X/cDNA	2 amplified fragments (560 bp and 600 bp) in both ovaries and testes (Fig. 3.43)
14.Ubiquitin X-specific protease 9	*Mus musculus*	X/cDNA	a 247 bp fragment specific expression in juvenile females but differentially expressed in adults (Fig. 3.44)
15. FE_{AGA} –M_{CGA}520-F/R	*P. monodon*	Female/AFLP	- (Fig. 3.42)

NS = non-specific amplification; - = amplification was not successful.

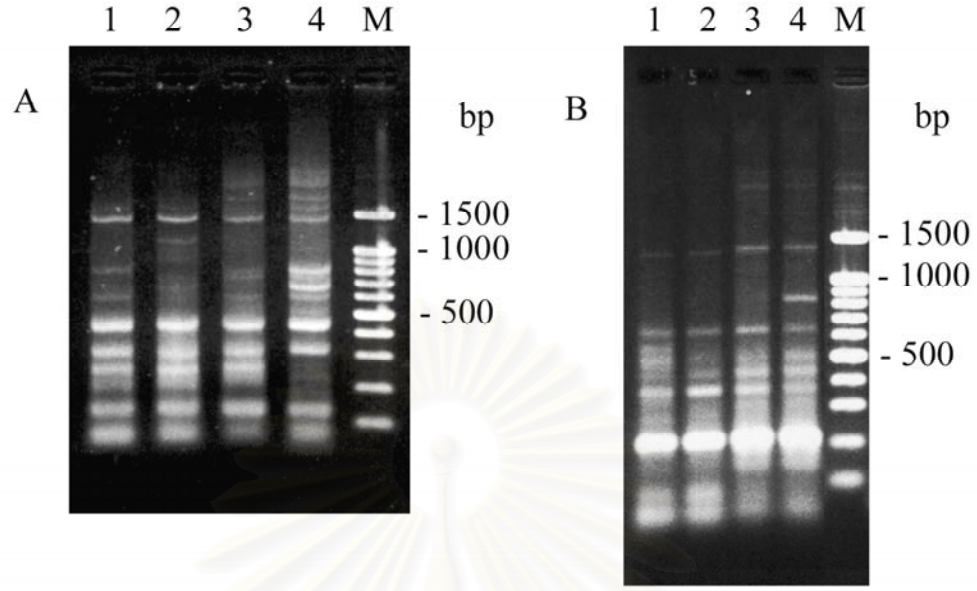

Figure 3.34 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2; panels A and B) and ovaries (lanes 3 – 4; panels A and B) using combining of AGESTX44-F + AGESTX40-R (panel A) and Rudimentary-F/R (panel B), respectively. Lanes M are a 100 bp DNA ladder.

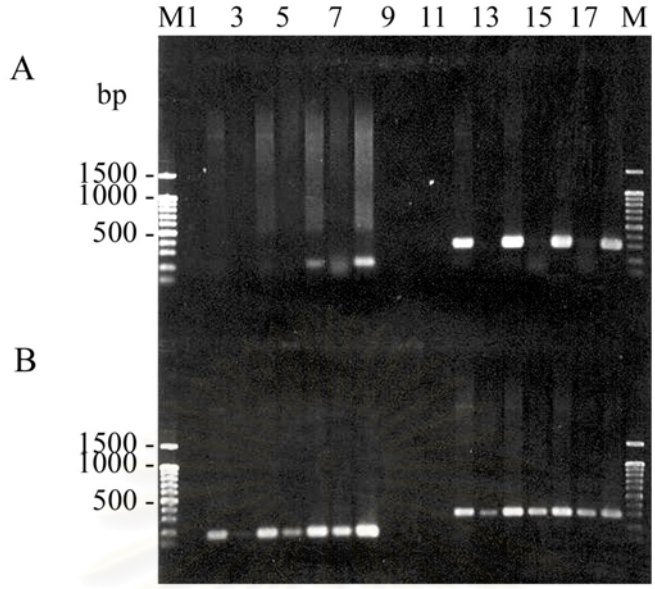

Figure 3.35 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1, 3, 5, 7, 11, 13, 15 and 17 ; panel A and lanes 1, 3, 5 and 7 ; panel B) and ovaries (lanes 2, 4, 6, 8, 12, 14, 16 and 18 ; panel A and lanes 2, 4, 6 and 8 ; panel B) using TSP288-F/R (lanes 1 – 8, A), TSP462-F/R (lanes 11 – 18, A) and DSI-F/R (lanes 1 – 8, B) for 20 (lanes 1 – 2 and 11 – 12, A and 1 – 2, B), 25 (lanes 3 – 4 and 13 – 14, A and 3 – 4, B), 30 (lanes 5 – 6 and 15 – 16, A and 5 – 6, B) and 35 cycles (lanes 7 – 8 and 17 – 18, A and 7 – 8, B), respectively. Positive control (β-actin, 327 bp) was also included (lanes 11 – 18; B). Lanes M are a 100 bp ladder.

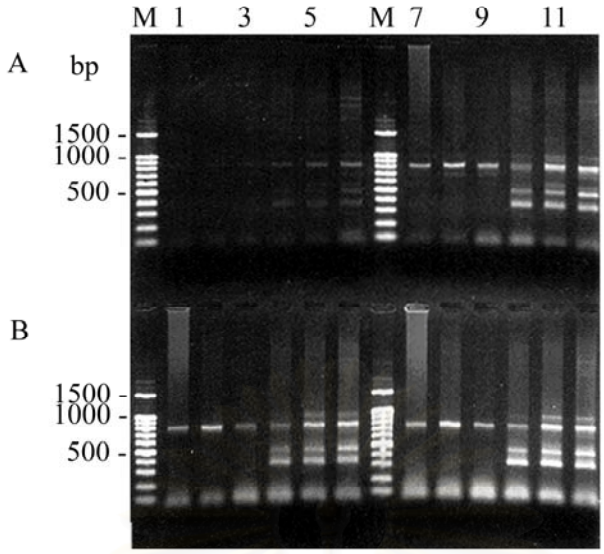

Figure 3.36 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 3, 7 – 9; panels A and B) and ovaries (lanes 4 – 6 and 10 – 12; panel A and B) using TSP462-2 + TSP371-1 for 20 (lanes 1 – 6, A), 25 (lanes 7 – 12, A), 30 (lanes 1 – 6, B) and 35 cycles (lanes 7 – 12, B), respectively. Positive control (β-actin, 327 bp, not shown) was also successfully amplified. Lanes M are a 100 bp DNA ladder.

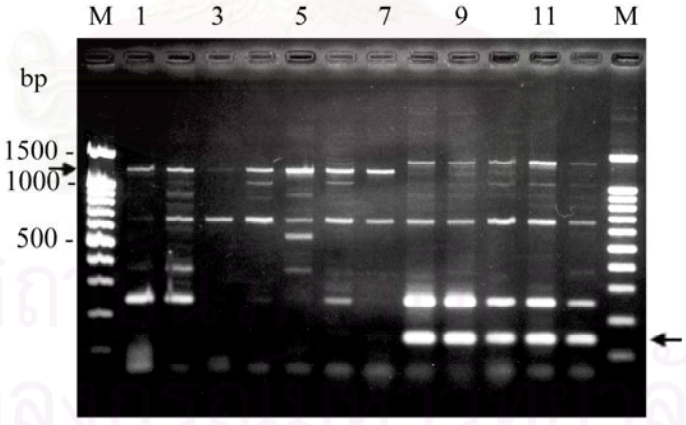

Figure 3.37 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 7) and ovaries (lanes 8 – 12) using TSP462-F + TSP288-R. Positive control (β-actin, 327 bp, not shown) was also successfully amplified. Arrows indicate the amplified 1300 bp and 150 bp fragments that were cloned and characterized. Lanes M are a 100 bp ladder.

A

GAGCAGCTCGAAAAAGCCGACGACTAATTATAAGAACGANATCAAAATGGTGTAGAC
TTACCATAAATACCATAAATAAACCGCATTTTACAATCCTTAGATATTAGTTTCCTC

TSPPMT1300-F

TTGGCTGCAGTAGACGTTGGCCTACCCTTCCTCGGGTGCA**AGTACGCTCGGGAAAGT**
GAAAAAAAGAAAAAGAGAGACTGGGAAAAAAATTAGAAAGAAATAATAATAGTAAAA
AAAAGCGCTACTAAACCCGCGCTCCTCGATATAATCCTTCTGTTTCCGCGGACCAAG
TAATAGTATAACTACTTTATTTTTTTTATTACGGTTATAGTAACATATGGTGTACA**A**

TSPPMT1300-R

ATGTTGGACTTCTATCTTGTTCGAATAATGAACGACGAATGCAATTGGGAACGGGCG
TAGGGACCTAGTCCAGCGAATGTGTGGGTATGTAGTGCAAAATCTAATCATGGAGCC
GGCGCTG

B

GTCGCGGCCGAGGTACTAATCTGCGCTTCATCTAATGCTTCGTTCGCAGACCGCTTA
GCCGGTTTCCCAAGGCAAAGAACTACAAGTAACAGCAGCAAGACGCACCGGAACCCC
ATAACTGCCCCGGATTAGCCATA

Figure 3.38 Nucleotide sequences of TSPPMT1300 (A) and TSPPMO150 (B) generated from TSP462F + TSP288R. Positions of a reverse primer and that complementary to a reverse primer of the former transcript are illustrated in boldface and underlined.

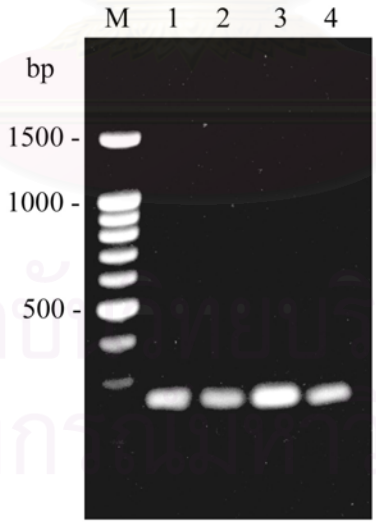

Figure 3.39 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2) and ovaries (lanes 3 – 4) using TSPPMT1300-F/R. Lane M is a 100 bp DNA ladder.

EGF-1 contains zinc finger protein motifs responds for growth factors. In *P. monodon*, factors or neurohormones controlling growth and development are not understood. Although RT-PCR of the gene homologue was not sex-specific, differential expression of this transcript was observed. Nevertheless, the expected amplification product (286 bp) was observed along with with other non-specific amplification fragments (Fig. 3.40). Increasing the annealing temperature from 55 °C to 58 °C or performing the touchdown PCR (68 °C - 1 °C every other cycle for 12 cycles following by 25 cycles of the annealing temperature at 60 °C) did not eliminate these non-specific bands.

A homologue of XNP1 (224 bp, Fig. 3.41) and disulfide isomerase (150 bp. not shown) was successfully amplified in both ovaries and testes of *P. monodon*. Neither sex-specific nor differential expression was observed. The *C. elegans* XNP-1 is orthologous to the humans gene, ATR-X which when mutated leads to ATR-X mental retardation syndrome and Nigmegen breakage syndrome. Polymorphism of *P. monodon* XNP-1 was found at the genomic level (Supaporn Thamrungtanakit, personal communication) analyzed by single strand conformation polymorphism (SSCP). As a result, correlation between base substitutions of XNP-1 in *P. monodon* and phenotypes should be further investigated.

Primers designed from candidate female-specific AFLP markers (FE_{AGA}-M_{CGA}520-F/R) in *P. monodon* generated the amplification product in both ovaries and testes (Fig. 3.42)

A homologue of sex lethal protein (*Sxl*) transcripts were found in normal EST libraries previously established from hemocytes of *P. monodon* (A. Tassanakajon, personal communication) and *M. japonicus* (T. Aoki, personal communication). This protein plays an important rule for sex determination cascades in *Drosophila*. Therefore, RT-PCR of Sxl was carried out to identity whether this transcript was specifically expressed in female (ovaries) *P. monodon*. Results indicated two amplification bands (560 bp and 600 bp) in testes, ovaries and hemocytes. This indicated that expression of Sxl is not gender-specific in *P. monodon* and at least 2 isoforms of *Sxl* are existent in *P. monodon* (Fig. 3.43).

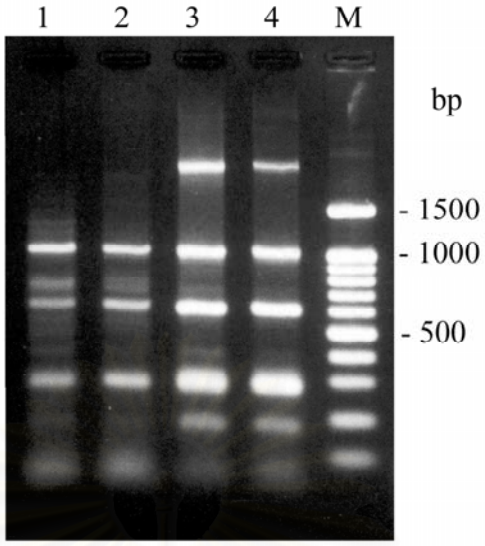

Figure 3.40 RT-PCR of a EGF-1 homologue (286 bp) of the first strand cDNA of testes (lane 1 – 2) and ovaries (lane 3 – 4) using EGF-F/R primers. A 100 bp DNA ladder was used as a marker.

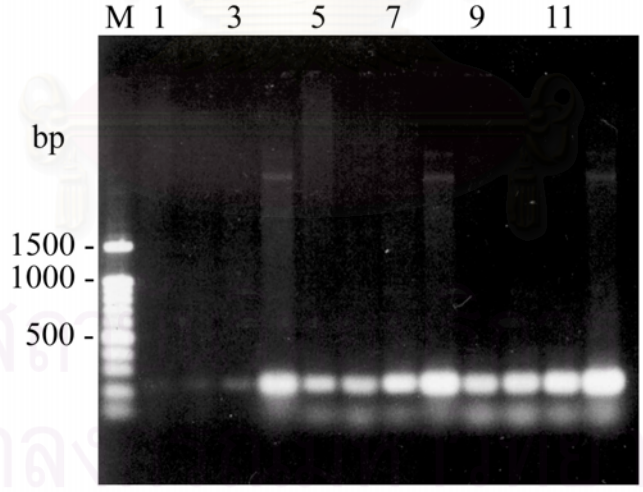

Figure 3.41 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2, 5 – 6 and 9 – 10) and ovaries (lanes 3 – 4, 7 – 8 and 11 – 12) using primer XNP1-F/R for 20 (lanes 1 – 4), 25 (lanes 5 – 8) and 30 cycles (lanes 9 – 12). Lane M is a 100 bp DNA marker.

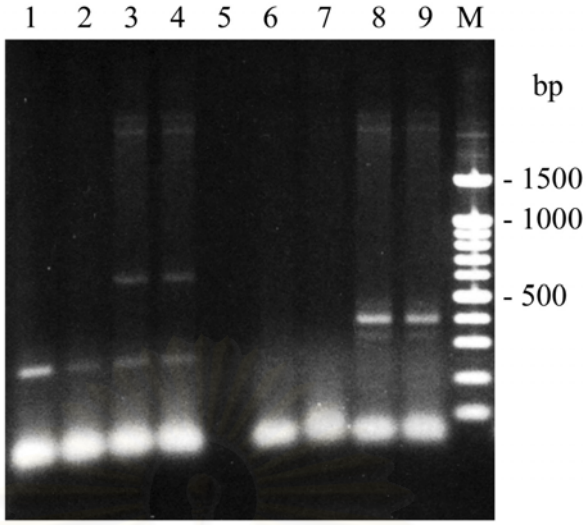

Figure 3.42 RT-PCR resulted from amplification of the first strand cDNA of testes (lanes 1 – 2 and 6 – 7) and ovaries (lanes 3 – 4 and 8 – 9) using primers FE_{AGA}-M_{CGA}520-F/R (lanes 1 – 4) and ME_{ACT}-M_{CTT} 517-F/R (lanes 6 – 9)

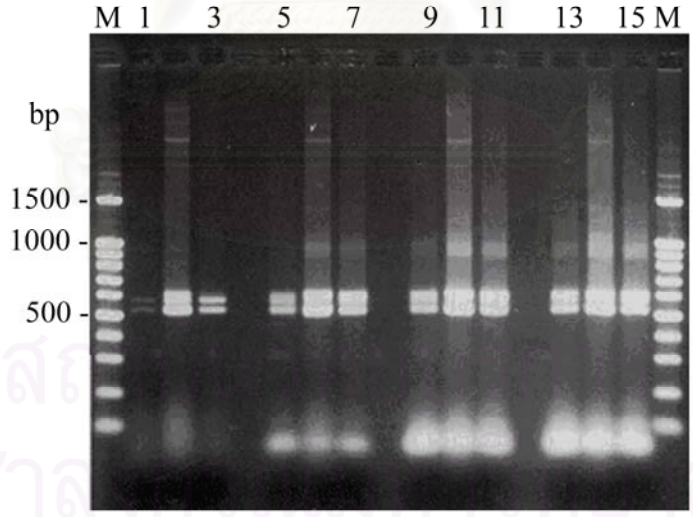

Figure 3.43 RT-PCR of the first strand cDNA of testes (lanes 1, 5, 9 and 13), ovaries (lanes 2, 6, 10 and 14) and hemocytes (lanes 3, 7, 11 and 15) using Sxl-F/R for 20 (lanes 1 – 3), 25 (lanes 5 – 7), 30 (lanes 9 – 11) and 35 cycles (lanes 13 – 15), respectively. Positive control (β-actin, 327 bp, not shown) was also successfully amplified. Lanes M are a 100 bp DNA ladder.

Significant differential expression levels of Usp9X homologue (247 bp, Fig. 3.44) were observed between ovaries and testes of broodstock-sized *P. monodon* ($p < 0.05$). Results were consistent when samples sizes of *P. monodon* were increased ($N = 13$ and 11 for broodstock-sized females and males, respectively). Temporal sex-specific expression of an Usp9X-F/R was observed in 4-month-old *P. monodon* where positive amplification results were only found in ovaries (Fig. 3.45)

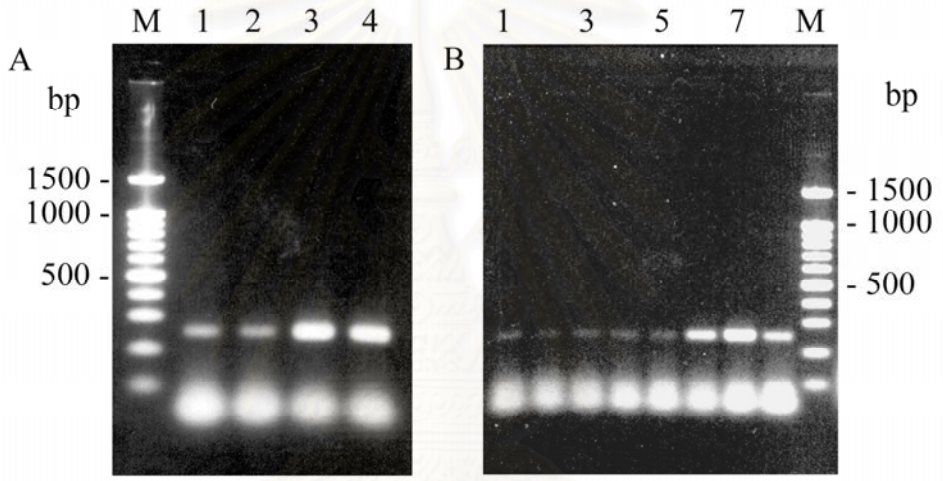

Figure 3.44 RT-PCR (optimized condition and operated for 35 cycles) illustrating differential expression of the amplified Usp9X homologue in testes (lanes 1 – 2; panel A and lanes 1 – 5; panel B) and ovaries (lanes 3 – 4; panel A and lanes 6 – 8; panel B) of *P. monodon*. A 100 bp DNA ladder was included as the markers.

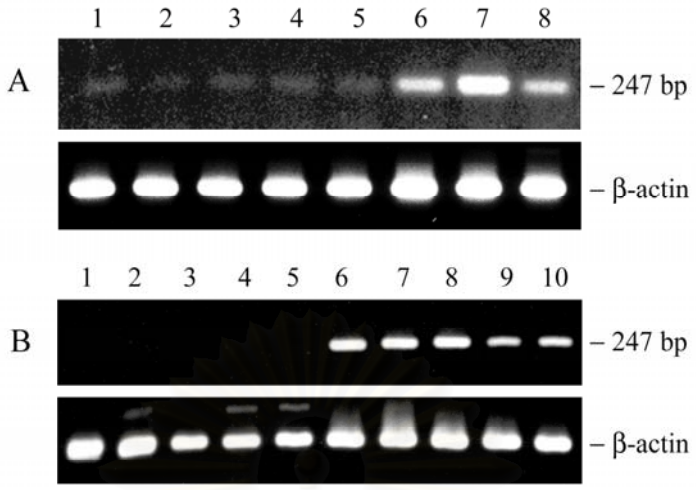

Figure 3.45 RT-PCR (optimized and carried out for 35 cycles) of the first strand cDNA of testes (lanes 1 – 5; panel A and B) and ovaries (lanes 6 – 8; panel A and lanes 6 – 10; panel B) of broodstock-sized *P. monodon* (panel A) and 4-month-old *P. monodon* (panel B) using primers Usp9X-F/R. Positive control (β-actin, 327 bp) was also successfully amplified.

Amplification of AGESTX using AGESTX44-F/R indicated specific expression of this transcript in ovaries but not in testes. Results were consistent when sample sizes of *P. monodon* were increased (N = 13 and 11 for broodstock-sized and N = 5 and 3 for 4-month-old females and males, respectively) (Figs. 3.46 and 3.47). Therefore this primer showed sex-specific expression in female *P. monodon* (Fig. 3.47).

The amplification product of AGESTX44-F/R was verified by cloning and sequencing. A 184 bp fragment was eluted and cloned. Colony PCR was carried out to verify insert sizes. The product was also digested with *Alu* I and *Hae* III to determine whether more than one insert type were obtained from a particular fragment (data not shown). Nucleotide sequences of recombinant clones were aligned with the original sequence used to design primer AGESTX44-F/R, two recombinant clones were AGESTX homologue (Fig. 3.48)

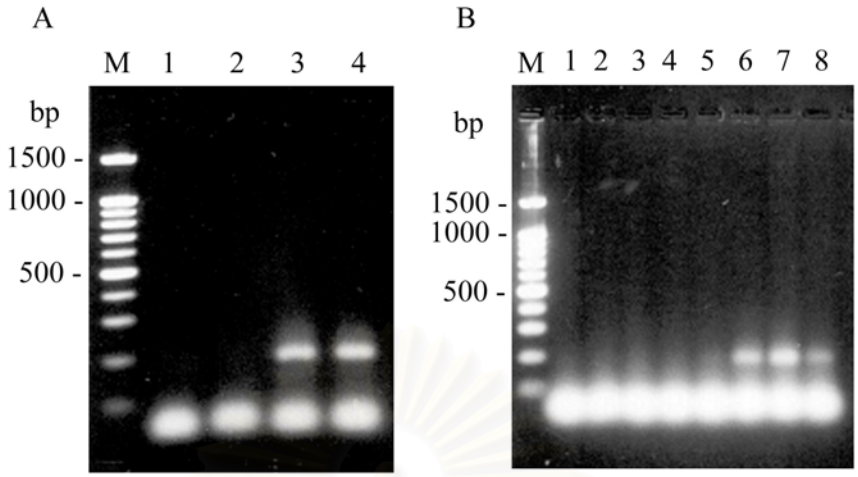

Figure 3.46 RT-PCR of AGESTX homologue using primers AGESTX44-F/R and the first strand cDNA of testes (lanes 1 – 2; panel A and lanes 1 – 5; panel B) and ovaries (lanes 3 – 4; panel A and lanes 6 – 8; panel B) *P. monodon* as the template.

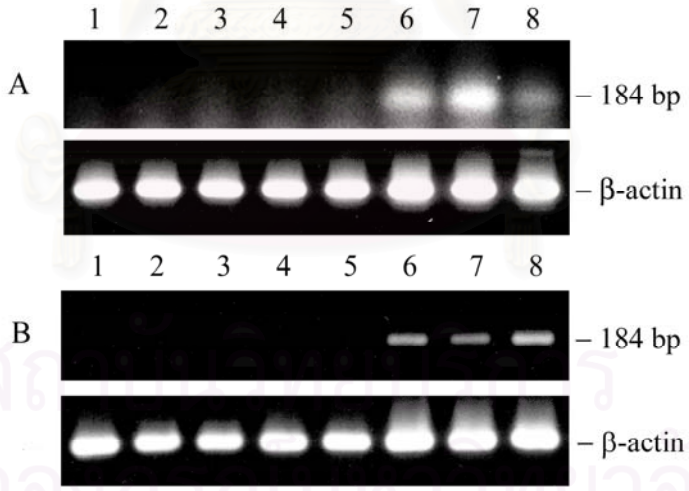

Figure 3.47 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 5) and ovaries (lanes 6 – 8) of broodstock-sized *P. monodon* (panel A; upper) and 4-month-old *P. monodon* (panel B; upper) using primers AGESTX44-F/R. Positive control (β-actin, 327 bp: panel A; lower and B; lower) was also successfully amplified.

GCGTCATCAAGTTGTCCGGAGTCTCTGGCATACCGGCCTGCGTGGCCAGATAGAAGG
TCAGCAGGCTGCGCCAGAAGTTCCAGAGCCAGCAGTAGCTGCAGAACCAGCCCCTCC
CCAGATTCCCCTTGCCTGTGG

Figure 3.48 Nucleotide sequence of the amplification product of AGESTX homologue of *P. monodon* from primer AGESTX44-F/R.

Tissue distribution of AGESTX was determined by using RT-PCR (AGESTX44-F/R). Total RNA was extracted from eyestalks, gills, heart, hemocytes, hepatopancreas, lymphoid organs, intestine, ovaries, pleopods, stomach, testes and thoracic ganglion of *P. monodon* and used for synthesis of the first strand cDNA.

RT-PCR illustrated that AGESTX was expressed in ovaries, hemocytes, hepatopancreas, lymphoid organs, stomach and thoracic ganglion but did not in eyestalks, gills, heart, intestine, pleopods and testes but the most abundant of this transcripts was found in ovaries (Fig. 3.49).

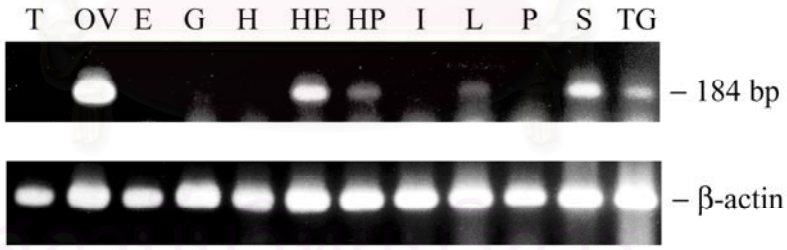

Figure 3.49 Tissue distribution of AGESTX homologue in various tissues ; T = testis, OV = ovaries, E = eyestalks, G = gills, H = heart, HE = hemocytes, HP = hepatopancreas, I = intestine, L = lymphoid organs, P = pleopods, S = stomach and TG = thoracic ganglion. Positive control was β-actin (327 bp).

Moreover, TSP288-1 + TSP371-1 yielded a female-specific cDNA marker in broodstock-sized *P. monodon* (Figs. 3.50 and 3.51). A 380 bp amplified TSP was found in ovaries but not in testes of *P. monodon* since the 20th amplification cycles. No amplicon was observed in cDNA from testes of male *P. monodon* after 35 cycles. Results were consistent when sample sizes of *P. monodon* were increased (N = 13 and 11 for broodstock-sized females and males, respectively) (Fig. 3.51).

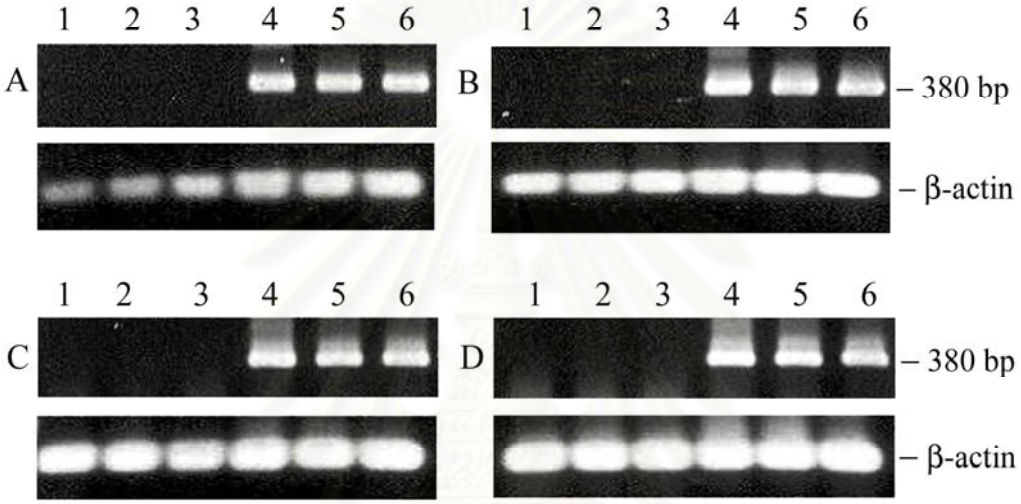

Figure 3.50 RT-PCR of TSP (A-D) homologues of male (lanes 1 – 3; A – D) and female (lanes 4 – 6; A – D) for 20 cycles (A), 25 cycles (B), 30 cycles (C), and 35 cycles (D) using primers TSP288-1 + TSP371-1. Positive amplification of β-actin (327 bp) of the same template was observed.

Figure 3.51 RT-PCR of the first strand cDNA of testes (panels A and B; lanes 1 – 7) and ovaries (panels A and B; lanes 8 – 11) of *P. monodon* analyzed by primers TSP288-1 + TSP371-1. Lanes M are a 100 bp DNA ladder.

The 380 bp amplification product of TSP288-1 + TSP371-1 was cloned and sequenced as did AGESTX. Results of BLAST*N* and BLAST*X* of recombinant clones indicated that they were TSP homologues (E-value = 4e-28) (Fig. 3.53).

Expression level of TSP homologues was tested in ovaries and testes of broodstock-sized and 4-month-old *P. monodon* by RT-PCR using TSP288-1 + TSP371-1 as the primers. Sex-specific expression of TSP homologues were observed in ovaries of broodstock-sized *P. monodon* but differential expression of TSP was found in both ovaries and testes of 4-month-old *P. monodon* but significant greater expression level was found in ovaries ($P < 0.05$). Therefore, TSP was differentially expressed at the juvenile stage and temporally female-specific expressed in adults of *P. monodon* (Fig. 3.52).

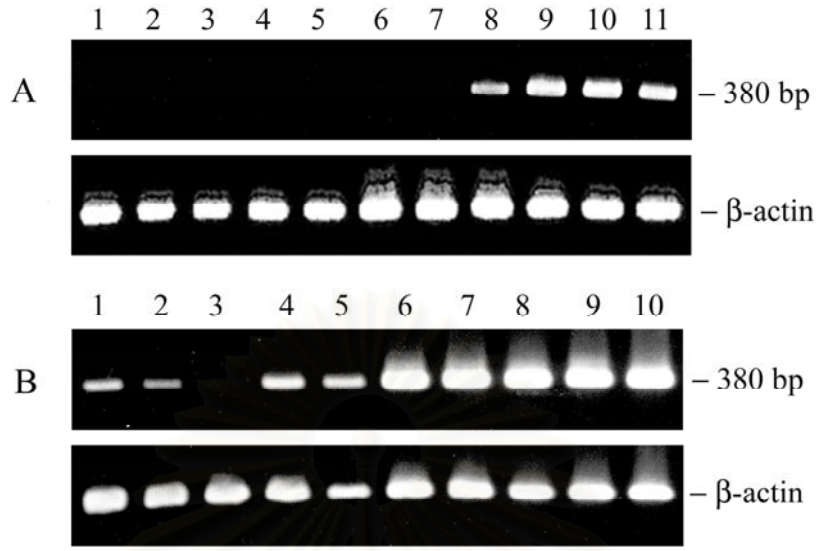

Figure 3.52 RT-PCR of the first strand cDNA from testes (lanes 1-7; panel A and lanes 1-5; panel B) and ovaries (lanes 8-11; panel A and lanes 6-10; panel B) of broodstock-sized *P. monodon* (panel A; upper) and 4-month-old *P. monodon* (panel B; lower) using primers TSP288-1 + TSP371-1 and positive control; β-actin, 327 bp was also successfully amplified.

TCTCCTTCAGGCTCGTTCTGTGCTAGAATACCAGGCAGAGAACTCCTTGTAGCCATA
TTAAAGAGGCTTCTTGTGCTAATACCTGTAGTATTCAGGACTTGGTCTTTAAGGTTC
TGTGGCAAGTCATCAAATATGGCTTGGGGAACACCATCTTCACAAGTATACTTGACA
TTTGACCAAATAATCTCTTCTTGAGAGAAGCAGTAGAGTCCCAATCTTCCTCCTTTC
AGGGCATCATTATAAATGTTTCCAGAGTCCATGACCAGATTAGTACCCCTGGTACAA
GTAGAACCTAATGGTGCCAATATTAGGACGGTGGTCGCGGCCGAGGTACTAATA

Figure 3.53 Nucleotide sequence of the amplification product of TSP homologue of *P. monodon* from primers TSP288-1 + TSP371-1.

Like AGESTX, tissue distribution of TSP homologues (TSP288-1 + TSP371-1) was also determined. TSP homologues were expressed in almost tissues except heart and testis where ovaries and intestine were exhibited higher expression levels than that of pleopods, eyestalks and thoracic ganglion (Fig. 3.54).

Figure 3.54 Tissue distribution of TSP homologue in *P. monodon* using TSP288-1 + TSP371-1; T = testes, OV = ovaries, E = eyestalks, G = gills, H = heart, HE = hemocytes, HP = hepatopancreas, I = intestine, L = lymphoid organs, P = pleopods, S = stomach and TG = thoracic ganglion. Positive control (β-actin, 327 bp)

3.7 RT-PCR of heterospecific primers from the giant freshwater (*Macrobrachium rosenbergii*) and the tropical abalone (*Haliotis asinina*)

Primers ME_{ACT}-M_{CTT}517–F/R and ME_{AGG}-M_{CAA}310–F/R were designed from sequences of candidate male-specific AFLP markers of the giant freshwater prawn (*M. rosenbergii*) (R. Preechaphol, personal communication). Additionallly, normal and subtractive cDNA libraries was constructed from ovaries and testes of the tropical abalone (*H. asinina*). Sex-related transcripts (VCP, VTG, Axomemal protein, FP, GIOT-1, HSD, Lysin, Tektin and SARIP) were found and primers were designed from those transcripts and used to examine expression of those gene homologues in *P. monodon* (Table 2.15). Notably, a large number of VCP ESTs were found in *H. asinina* and could be gathered into 9 clusters and several other unique sequences. Primers used in this thesis were designed from different forms of VCP (clusters 1, 2, 3, 7 and VCP49 and VCP75)

Amplification of GIOT1 and vitelline coat protein (VCP) using GIOT1-F/R, VCP49-F/R and VCP49-F + VCP75-R and ME_{AGG}-M_{CAA}310–F/R did not yield the amplification products. VCP1-F/R generated a 340 bp fragments found in both ovaries and testes of *P. monodon* along with non-specific fragments. Likewise, VCP2-F/R and VCP3-F/R, Axonemal-F/R, Tektin-F/R, FP-F/R and Lysin-F/R also provided non-specific amplification results (Figs. 3.55 - 3.57).

Amplification of the first strand cDNA using HSD-F/R, VTG1-F/R and SARIP-F/R yielded positive amplification products in ovaries but not in testes (Figs. 3.58 and 3.59). Originally, HSD and SARIP were positively amplified in both ovaries and testes of *H. asinina*. Characterization of these gene homologues in *P. monodon* should be carried out further.

Female-specific RT-PCR fragment was found from VCP75-F/R (189 bp in size, Fig. 3.60) and VCP7-F/R (400 bp in size, Fig. 3.61). Results from the former was inconsistent owing to the existence of non-specific products when sample sizes of *P. monodon* were increased. In contrast, the latter yielded consistent results when sample sizes of broodstock-sized of females and males *P. monodon* were increased (N = 13 and 11, respectively; Fig. 3.61).

Table 3.12 RT-PCR for identification of sex-specific (or differentially expressed) transcripts in *P. monodon* using heterospecific primers from *M. rosenbergii* and *H. asinina*.

Gene homologues/ primer	Original species	Sex specificity/original type of marker	Amplification results
1. ME_{ACT}-M_{CTT}517-F/R	*Macrobrachium rosenbergii*	Male/AFLP	400 bp in ovaries*
2. ME_{AGG}-M_{CAA}310-F/R	*M. rosenbergii*	Male/AFLP	-
3. VCP1-F/R	*Haliotis asinina*	Female/cDNA	NS
4. VCP2-F/R	*H. asinina*	Female/cDNA	NS
5. VCP3-F/R	*H. asinina*	Female/cDNA	NS
6. VCP7-F/R	*H. asinina*	Female/cDNA	400 bp in ovaries*
7. VCP49-F/R	*H. asinina*	Female/cDNA	-
8. VCP75-F/R	*H. asinina*	Female/cDNA	189 bp in ovaries
9. VTG1-F/R	*H. asinina*	Female/cDNA	Two bands in ovaries
10. Axonemal-F/R	*H. asinina*	Male/cDNA	NS
11. FP-F/R	*H. asinina*	Male/cDNA	NS
12. GIOT1-F/R	*H. asinina*	Male/cDNA	-
13. HSD-F/R	*H. asinina*	Male/cDNA	Two bands in ovaries
14. Lysin-F/R	*H. asinina*	Male/cDNA	NS
15. Tektin-F/R	*H. asinina*	Male/cDNA	NS
16. SARIP-F/R	*H. asinina*	Male/cDNA	Two bands in ovaries

*The amplification products were cloned and sequenced. NS = non-specific amplification; - = amplification was not successful.

The intensity of a 400 bp fragment from VCP7-F/R varied greatly with different annealing temperature, therefore this fragment was cloned and sequenced Nucleotide sequences of positive clones were blasted against data in the GenBank and revealed that this transcript was a homologue of TSP (E-value = 4 x 10^{-66}, Fig. 3.62). This implied that VCP genes of *H. asinina* and TSP genes of *P. monodon* may originally evolve from the same gene family.

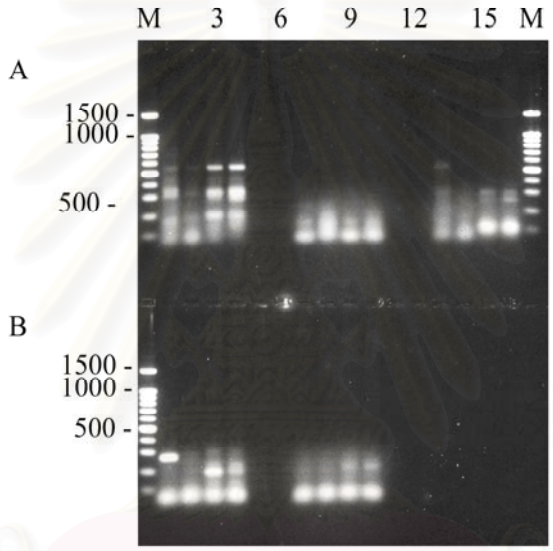

Figure 3.55 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2, 7 – 8 and 13 – 14; panel A and lanes 1 – 2; panel B) and ovaries (lanes 3 – 4, 9 – 10 and 15 – 16; panel A and lanes 3 – 4; panel B) of *P. monodon* using primers VCP1-F/R (lanes 1 – 4; panel A), VCP2-F/R (lanes 7 – 10; panel A), VCP3-F/R (lanes 13 – 16; panel A), VCP75-F/R (lanes 1 – 4; panel B) and AGESTX44-F/R (lanes 7 – 10; panel B). Lanes M is a 100 bp DNA marker.

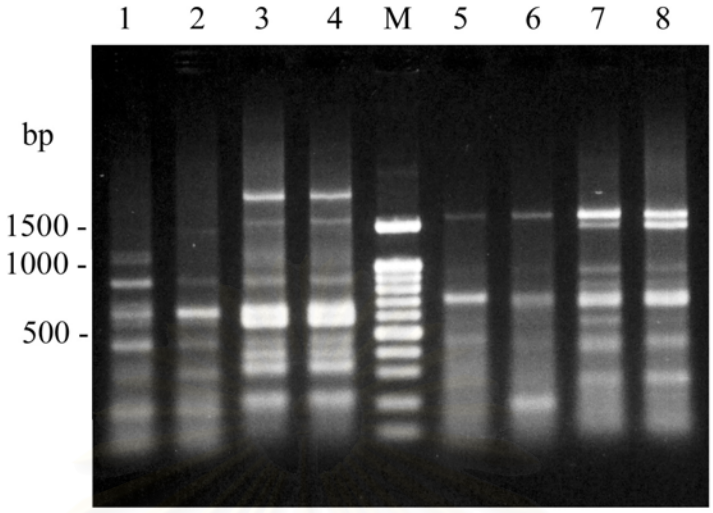

Figure 3.56 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2 and 5 – 6) and ovaries (lanes 3 – 4 and 7 – 8) of *P. monodon* using primers Tektin-F/R (lanes 1 – 4) and Axonemal-F/R (lanes 5 – 8). Lanes M is a 100 bp DNA marker**.**

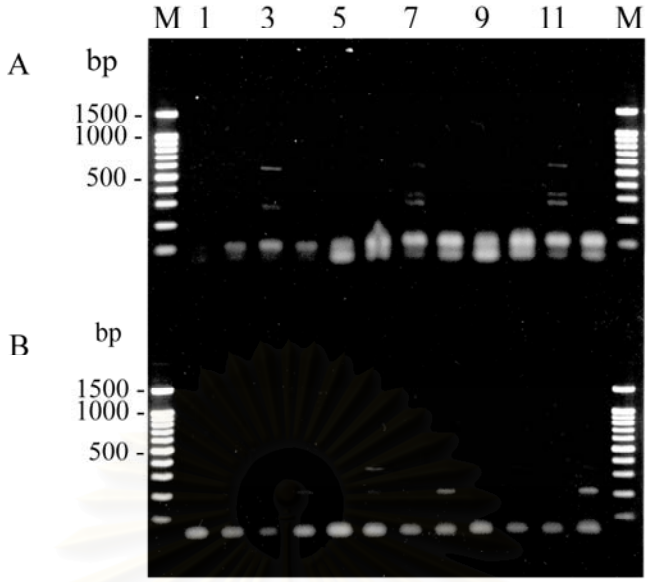

Figure 3.57 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2, 5 – 6 and 9 – 10; panel A and B) and ovaries (lanes 3 – 4, 7 – 8 and 11 – 12; panel A and B) of *P. monodon* using primers FP-F/R (panel A) and Lysin-F/R (panel B) for 25 (lanes 1 – 4), 25 (lanes 5 – 8) and 35 cycles (lanes 9 – 12). Lanes M is a 100 bp DNA marker**.**

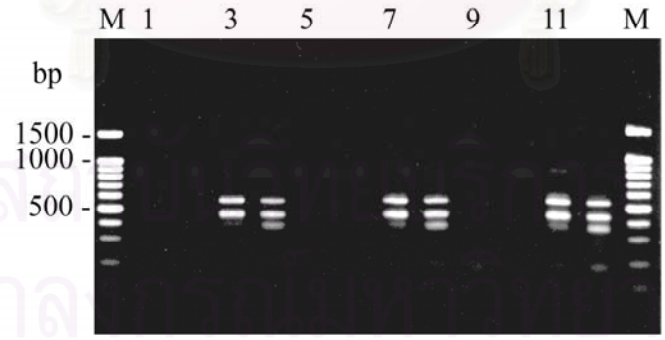

Figure 3.58 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2, 5 – 6 and 9 – 10) and ovaries (lanes 3 – 4, 7 – 8 and 11 – 12) of *P. monodon* using primers HSD-F/R for 25 (lanes 1 – 4), 30 (lanes 5 – 8) and 35 cycles (lanes 9 – 12). Lanes M is a 100 bp DNA marker**.**

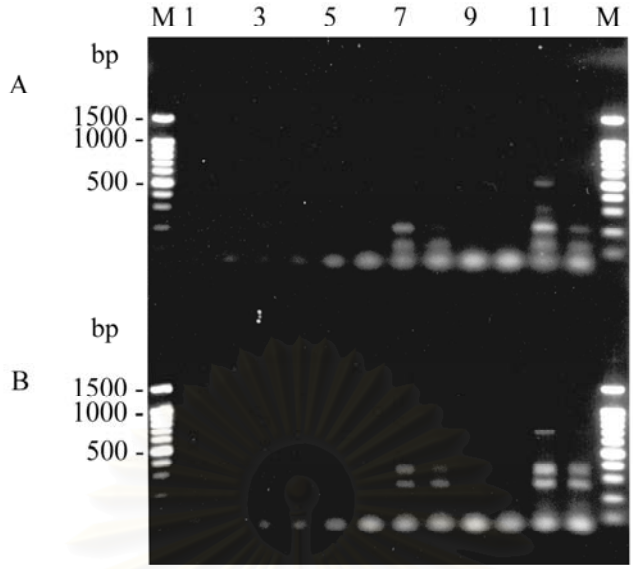

Figure 3.59 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2, 5 – 6 and 9 – 10; panel A and B) and ovaries (lanes 3 – 4, 7 – 8 and 11 – 12; panel A and B) of *P. monodon* using primers VTG1-F/R (panel A) and SARIP-F/R (panel B) for 25 (lanes 1 – 4), 30 (lanes 5 – 8) and 35 cycles (lanes 9 – 12). Lanes M are a 100 bp DNA marker**.**

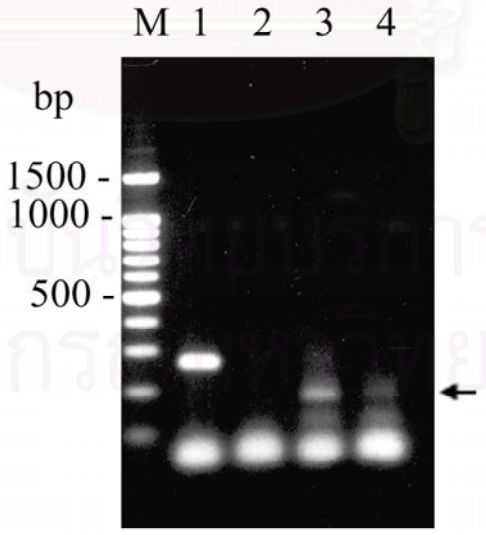

Figure 3.60 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2) and ovaries (lanes 3 – 4) of *P. monodon* using primers VCP75-F/R (lanes 1 – 4). Lane M is a 100 bp DNA marker.

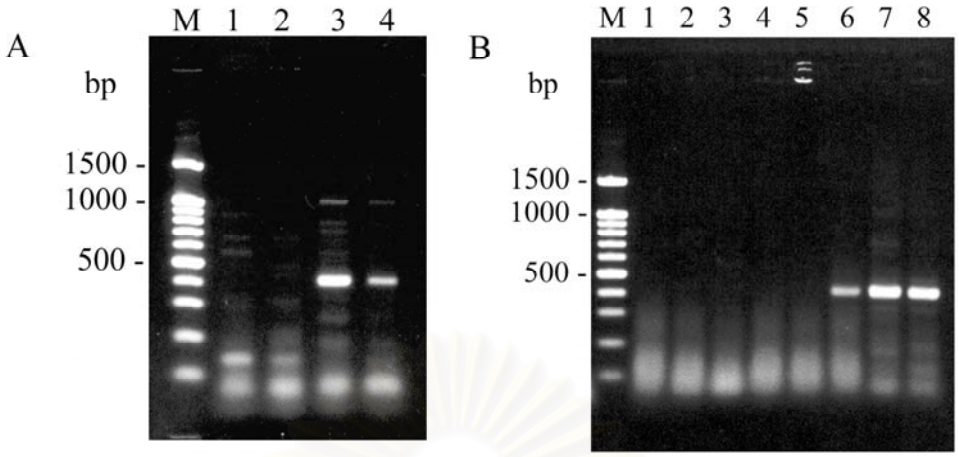

Figure 3.61 RT-PCR of the first strand cDNA of testes (lanes 1 – 2; panel A and 1 – 5; panel B) and ovaries (lanes 3 – 4; panel A and 6 – 8; panel B) of *P. monodon* using primers VCR7-F/R at an annealing temperature of 60 °C (A) and 58 °C (B). Sex-specific expression was found with this primer pair.

TGCTCAGTGCTGGTGGTGCTCTGCTTGGCCAAACCAGCTTTGTGGTCCCTTCTTCCACATCATTACATA
GAACTTGGAATTGCTTTGGTAGCTGAAGATAAAGCCAACAAAGTCATCATCACTGGTGTCCTCTATGTA
GAAGGTCCCTTCAAAGTCGACATCCACAAGAGTATGGTCACCCACTGCTATAGCTGGGTCCGAGTTCAA
TGTCTGGTGAATTTCTGCACCATTGTCGTAGACAACCCATACTGGAGGAGTAGACGCCGTTTGTGGGTC
TAGTGCCACCATTTGGAGTTGCCTGAAGTCGGTGGCATGCACATTTGGGTTGGCAATGCAGTTATCATC
CGAGTCTACAATTTTGTCACCATCAAAGTAGAAGGTAACAAGGACCGCCAGCCAA

Figure 3.62 Nucleotide sequence of the amplified VCP homologue in *P.* monodon using primers VCP7-F/R

Primers initially developed from male-specific AFLP markers of *M. rosenbergii*, ME_{ACT}-M_{CTT} 517-F/R generated a 400 bp product specifically found in ovaries but not in testes of *P. monodon* (Fig. 3.63). Results were consistent when sample sizes of *P. monodon* were increased (N = 13 and 11 for broodstock-sized females and males, respectively).

Based on the fact that the original primers were designed from nucleotide sequence of *M. rosenbergii,* the amplified fragment was then cloned and sequenced. Therefore new primers (PM233-F: 5′- ACC GCT GGG GTA GAC AAC AAA -3′ and PM233-R: 5′- GCA CGA AGG AAG ACT GAC TGG -3′) of newly isolated unknown transcript was designed (Fig. 3.64) and tested with first strand cDNA of pooled testes (N = 6) and pooled ovaries (N = 6) by RT-PCR. Positive amplification product was only observed in ovaries (Fig. 3.65). Expression of PM233-F/R was tested against the first strand cDNA of ovaries and testes of broodstock-sized and 4-month-old *P. monodon* and found that PM233-F/R was specifically provided the amplification product only in ovaries of both broodstock-sized and 4-month-old *P. monodon* since 20 amplification cycles (Figs. 3.65 and 3.66). Non-specific amplification products were observed at 35 amplification cycles. A non-specific band in testes that exhibits an identical size (233 bp) with the target product should be characterized prior to the unambiguous conclusion that this primer pair illustrated a

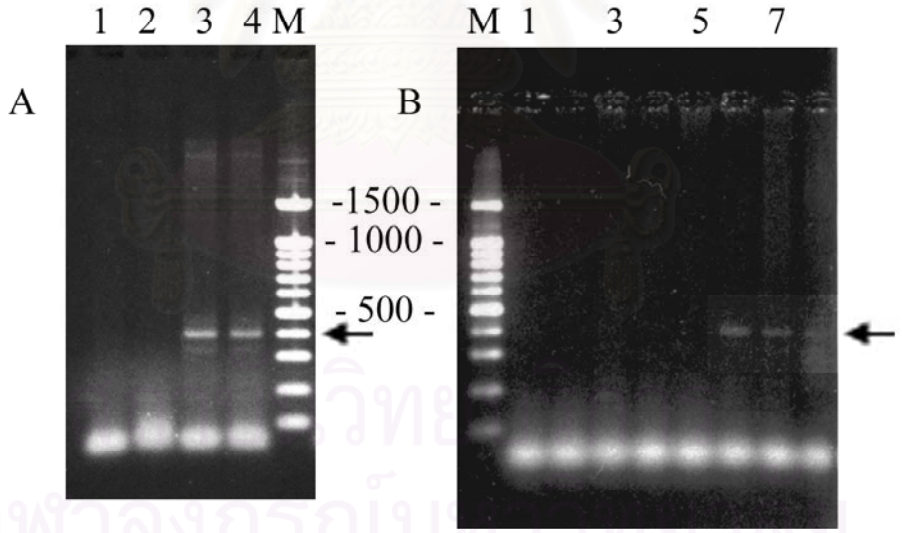

Figure 3.63 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2; panel A and lanes 1 – 5; panel B) and ovaries (lanes 3 – 4; panel A and lanes 6 – 8; panel B) of *P. monodon* using primers ME_{ACT}-M_{CTT} 517-F/R (panel A and B). Lanes M is a 100 bp DNA marker**.** Sex-specific expression (indicated by a arrowhead) was found with this primer pair.

AAAGTGACAGTCCTGGCAAAACGGTGGCAGAGTAATTATTGGAGTACAAGATGGTAC

PM233-F

CAACCTCAATATTCACGAGCTCACGAGTG**ACCGCTGGGGTAGACAACAAA**TCTCACT

GCAACAAGTGTTGAGCCTCCCAGCAGCGGTAACCTCCTGGAGAAGCTATGCAACGGG

CTTTGACAACTTCCTCATAGCCACAACGCCAACCTTTGCTCGTGTGTACATGGAGAA

TAGGGGAGAGTACCAAATTCTTCAGAACCTGTTGCCTACTGGTAGCCTTGCAAGCTT

PM233-R

CACTGAAATGGTG**CCAGTCAGTCTTCCTTCGTGC**AAGGATGAAGTCATCCTAATGAC

AGGATTTGGAAGTCTTGTGCAGATCTTCGTCTGGGAGGGAACGAAGGATAACAA

Figure 3.64 Nucleotide sequence of the amplification product of ME_{ACT}-M_{CTT} 517-F/R. A primer pair were designed (hereafter called PM233-F/R) as illustrated in boldface and underlined.

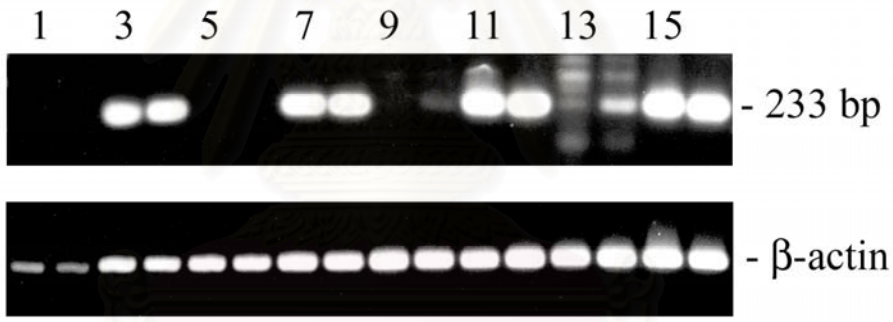

Figure 3.65 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 2, 5 – 6, 9 – 10 and 13 – 14) and ovaries (lanes 3 – 4, 7 – 8, 11 – 12 and 15 – 16) of broodstrock-sized *P. monodon* for 20 cycles (lanes 1 – 4), 25 cycles (lanes 5 – 8), 30 cycles (lanes 9 – 12), and 35 cycles (lanes 13 – 16) using primers PM233-F/R. Positive amplification of β-actin (327 bp) was observed from the same template.

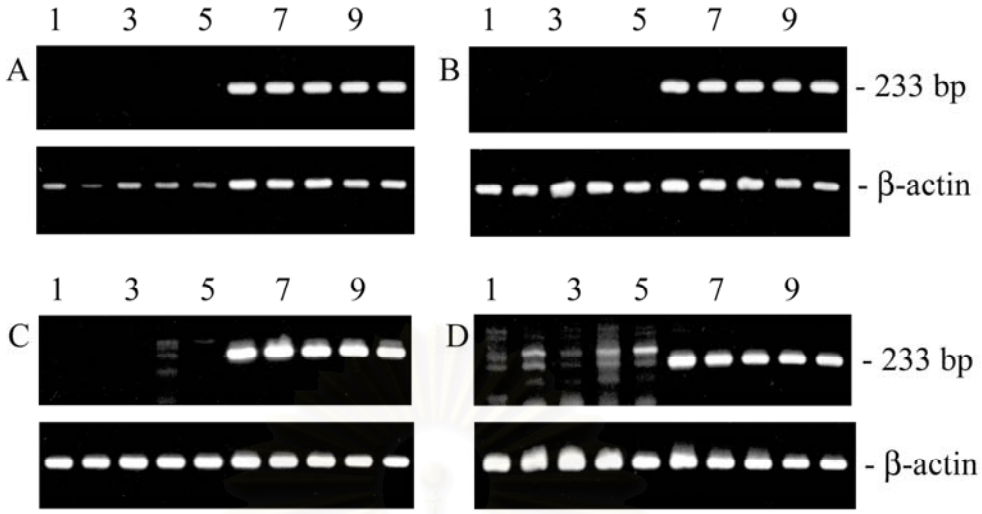

Figure 3.66 RT-PCR of the first strand cDNA synthesized from total RNA of testes (lanes 1 – 5; A – D) and ovaries (lanes 6 – 10; A – D) of 4-month-old *P. monodon* for 20 cycles (A), 25 cycles (B), 30 cycles (C), and 35 cycles (D) using primers PM233-F/R. Positive amplification of β-actin (327 bp) was observed from the same template.

3.8 Semi – quantitative Reverse Transcription – Polymerase Chain Reaction of Thrombospondin (TSP) and AGESTX

A homologue of AGESTX (AGESTX44-F/R) exhibited female-specific expression patterns in both 4-month-old and broodstrock-sized of *P. monodon* whereas a homologue of TSP (TSP 288 – 1 + TSP 371 - 1) was temporally specific to female adults of *P. monodon*. Expression levels of these transcripts were then semi-quantitively examined by comparing with that of the control transcript (β-actin; PMACTIN-F: 5′-GGT ATC CTC ACC CTC AAG TA -3′, Tm 60 °C and PMACTIN-R: 5′- AAG AGC GAA ACC TTC ATA GA -3′, Tm 56 °C).

A single tube semi-quantitative RT-PCR is an approach that the target and the internal control transcripts were simultaneously amplified. Therefore, the appropriate amplification conditions of the target product and the internal control should be first examined separately. The conditions usually require further optimization when the product and the control were simultaneously amplified.

The optimal concentration of primers for amplification of the target and the control was examined by varying primer concentrations (0.10, 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40 μM) under the standard PCR reaction of each transcript. The most optimal concentration of each primer pair was considered from that provided specific and clear amplification results and the band intensity did not reach a plateau of intensity. As a result, the primer concentration for TSP 288-1 + TSP 371-1 and AGESTX44-F/R was chosen at 0.15 μM and 0.10 μM, respectively. The concentration of PMACTIN-F/R was selected at 0.10 μM and 0.15 μM (Fig. 3.67).

The optimal concentration of $MgCl_2$ ((1.0, 1.5, 2.0, 3.0 and 4 mM) was also calibrated in the same manner. The most optimal concentration for TSP and AGESTX and PMACTIN-F/R was 1.5 mM (Fig. 3.68)

Finally, the optimal number of amplification cycles for TSP and AGESTX were calibrated and found that the number of cycles before reaching the plateau amplification phase was 20 cycles (Fig. 3.69) and 22 cycles (Fig. 3.70).

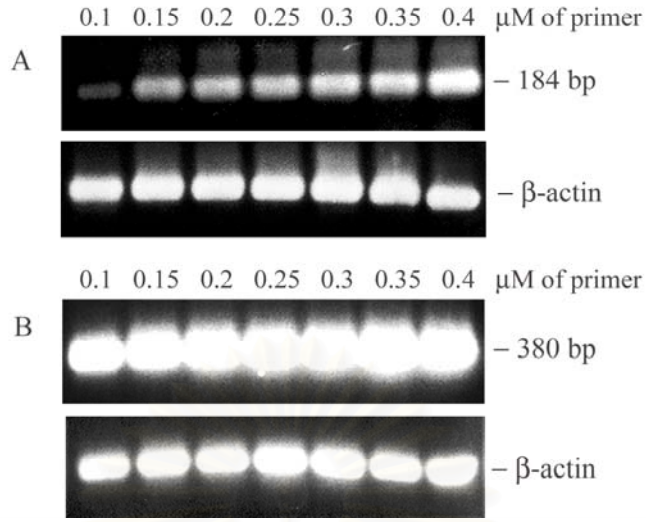

Figure 3.67 Optimization of primer concentrations for amplification of AGESTX (A) and TSP (B) homologue by varying concentration of primer from 0.1-0.4 µM.

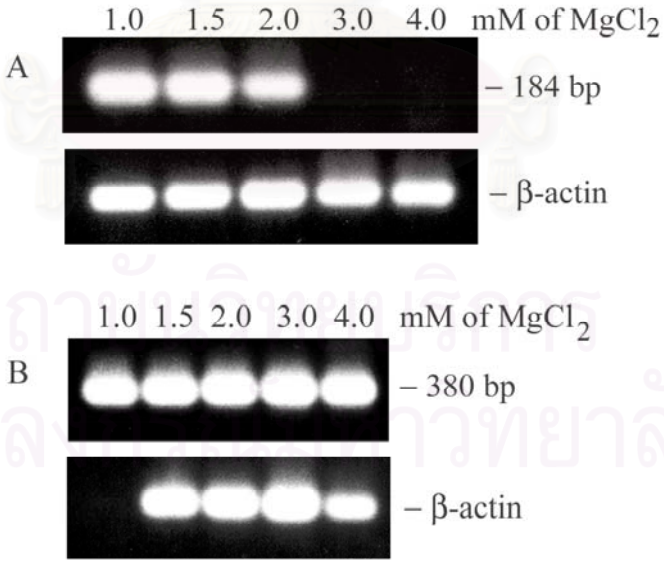

Figure 3.68 Optimization of $MgCl_2$ concentrations for amplification of AGESTX (A) and TSP (B) homologue by varying concentration of $MgCl_2$ from 1 – 4 mM.

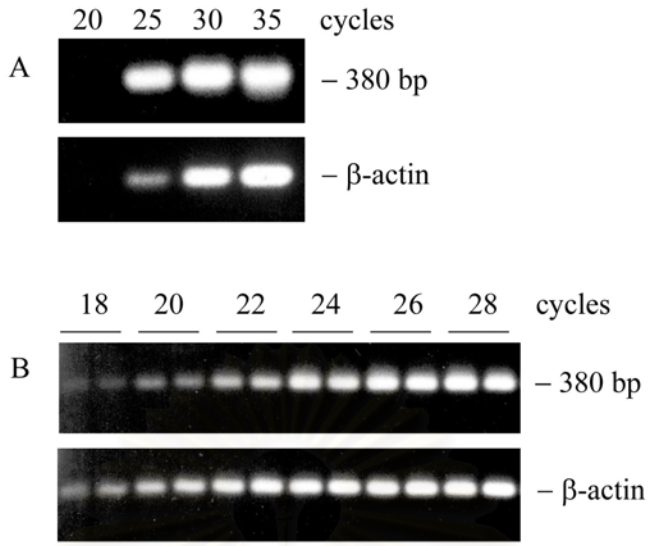

Figure 3.69 Optimization of number of PCR cycles for amplification of TSP homologue by varying numbers of amplification cycles from 20 – 35 cycles.

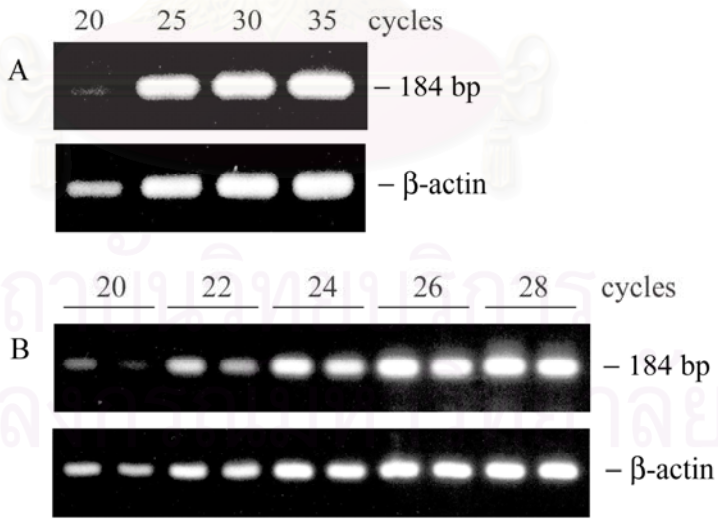

Figure 3.70 Optimization of number of PCR cycles for amplification of AGESTX homologue by varying numbers of amplification cycles from 20 – 35 cycles.

Using the optimized conditions described above, competitive effects between target and control primers occurred resulted in decreases of the intensity of the target and/or the control products compared with those that amplified separately. As a result, $MgCl_2$ concentration for AGESTX44-F/R was further adjusted to 2.0 mM which eliminated effects from primer competition successfully (Fig. 3.71).

Semi-quantitative PCR for TSP and AGESTX was carried out against the first strand cDNA synthesized from ovaries of broodstock-sized (N = 5) and 4-month-old *P. monodon* (N = 5). The amplification products were size-fractionated through a 1.8% agarose (PAGESTX44-F/R) or a 3.5% Metaphor agarose (TSP) (Fig. 3.72) gels. A ratio of band intensity between the target and β-actin was analyzed. Results indicated that expression of AGESTX homologue in ovaries of 4-month-old and broodstrock-sized *P. monodon* were not significantly differences statistically ($P > 0.05$) where as the significant expression level of TSP was observed between 4-month-old and adult *P. monodon*.

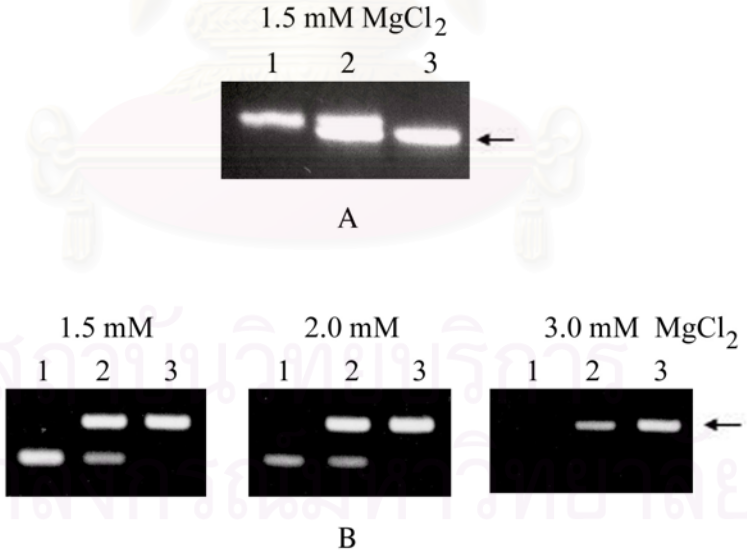

Figure 3.71 Competitive between target (TSP; panel A and AGESTX; panel B) and internal control primer sets (β-actin). PCR reactions were simultaneously performed in the same tube. Lanes 1; An amplicon from the target gene primer set, lanes 3; An amplicon from the internal control primer set, and lanes 2; Amplicons from both primer sets. Arrows indicate PCR product of β-actin (327 bp).

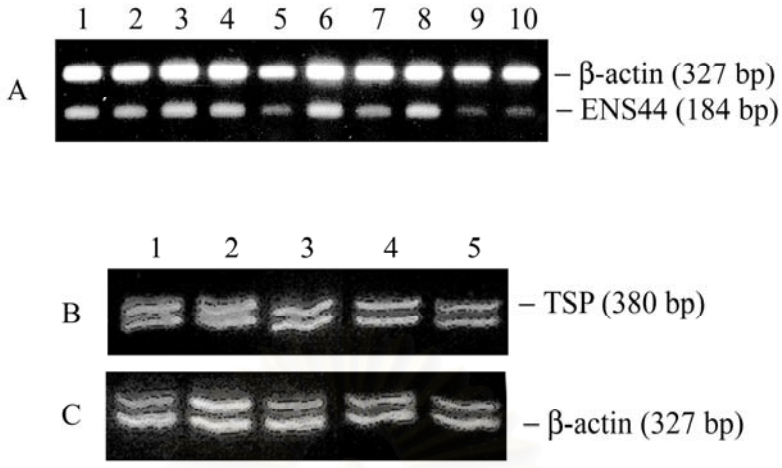

Figure 3.72 Analysis of expression levels of AGESTX (panel A) and TSP (panel B and C) transcripts between broodstock-sized (lanes 1 – 5; panel A and B) and 4-month-old (lanes 6 – 10; panel A and lanes 1 – 5 panel C) *P. monodon*.

CHAPTER IV

DISCUSSION

Sex determination markers of *P. monodon* cannot be isolated at the genomic DNA levels

Female *P. monodon* attains relatively larger sizes than do males. This reflects gender-different growth rates in *P. monodon.* An understanding of a sex determination system in *P. monodon* would provide the opportunity to develop a monosex culture of *P. monodon* through chromosome manipulation (gynogenesis of androgenesis) to elevate culture efficiency of this important species.

The chromosome numbers of penaeid shrimps have been reported in *P. esculentus, P. monodon, Farfantepenaeus aztecus, Fenneropenaeus chinensis*, *Fenneropenaeus merguiensis*, *Fenneropenaeus penicillatus* and *Marsupenaeus japonicus*, and (2*N* = 88), *P. semisulcatus* and *Litopenaeus setiferus* (2*N* = 90) and *Farfantepenaeus californiensis* and *Litopenaeus occidentalis* (2*N* = 92) (Benzie, 1998). Neither sex chromosomes nor environmental sex determination have been reported in penaeid shrimps.

Sex determination is problematic in researches of many species. This can usually be solved by the application of DNA-based technology but this is only possible if a sex-specific (located on unique sex chromosomes) marker is available. The lack of sex chromosomes reported in *P. monodon* and other penaeid shrimps implied that development of genomic DNA-based sex determination markers in *P. monnodon* may not be possible.

Identification of the DMRT1/*dsx*/mab-3 homologue in *P. monodon* at the genomic DNA level was not successful. Replacement of the template with the first strand cDNA of ovaries and testes resulted in the ability to identify several RT-PCR fragments exhibiting sex-specific expression profile in *P. monodon*. After characterization, 3 primer pairs were designed from PMO720, PMO920 and PMT1700 and further tested. PMO720 displayed a greater expression level in males whereas PMO920 and PMT1700 revealed higher expression levels in females,

respectively. PMT1700 and PMO920 exhibited polymorphism at the genomic DNA level. The latter showed heterozygotes in females (N = 2) and homozygotes in males (N = 2). Increasing the number of shrimps indicated that this marker was polymorphic but did not relate to sex-specificity.

Sexual identification has long been studied in a variety of species. Sex-specific markers should be developed from fixed polymorphism in genomic DNA of male and female of a particular species to avoid destruction of specimens.

Form the parallel study of this thesis, sex-specific DNA markers in *P. monodon* are being identified by AFLP analysis (S. Thumrungtanakit, personal communication). A total of 256 primer combinations were completely screened. 5 candidate female-specific and 1 male-specific AFLP markers were observed from screening of various DNA bulks. These AFLP markers still showed male- and femal-specific nature. Therefore, they were cloned and sequenced. Specific primers were designed and tested against different individuals of males and females from natural populations (Satun, Trang, Phangnga and Chumphon). The amplification product of each AFLP-derived marker was found in both genders. Further characterization of the PCR product using single strand conformational polymorphism (SSCP) did not reveal sex-specificity of these developed markers.

Manipulation of sex ratio and sexual maturation are important tools for crustacean aquaculture. Although sex determination in crustaceans has been reviewed and interested during the last two decades, the genetic basis for sex determination in decapods has not been studied.

In *M. rosenbergii*, implantation of androgenic glands in sexually undifferentiated females produced reproductively competent neomales. Mating of neomales with normal females suggested a complex heterogametic (ZW) system of females in this species.

Sex determination markers were then studied in *M. rosenbergii* (R. Preechaphol) using AFLP analysis. Bulked segregrant analysis (BSA) was carried out for genomic DNA of small (SM1B and SM2, 2 bulks), large orange-claw (OC1, 1 bulk), blue-claw (BC1 and BC2, 2 bulks) males and females (F1 and F2, 2 bulks). Morphotype-specific AFLP markers were not found in each male morphotypes.

Nevertheless, 5 male-specific and 4 female-specific AFLP markers were identified from screening of 64 primer combinations (8 *Eco* RI+3 primers +8 *Mse* I+3 primers). These markers were cloned and sequenced. Sex-specific PCR for male and female *M. rosenbergii* was developed. Polymorphism of developed SCAR markers were observed but these SCAR markers did not reveal sex specificity.

In mammalian species, X-linked zinc figer proten gene (Zfx) and Y-linked zinc finger protein gene (Zfy) usually showed fixed single nucleotide polymorphism (SNP) between males and females. Previously, a homologue of zinc finger protein gene was isolated in *P. monodon* (S. Klinbunga personal communication). The amplification product of this gene in females and males of *P. monodon* was characterized by sequencing. Unlike mammalian species, this gene homologue did not exhibit fixed polymorphism between genders.

Sex chromosomes have not been cytologically identified in penaeid species. Recently, Li et al. (2003) constructed the genetic linkage map of the kuruma prawn (*M. japonicus*) by AFLP analysis in a two-way pseudo-testcross strategy. Genotyping was carried out against 56 progeny (8% top and bottom) with 54 primer combinations.

A total of 502 scorable bands in 56 progeny, 359 segregated in a 1:1 ratio and 138 in a 3:1 ratio. Markers with a 1:1 segregating ratio were combined with those of the first stage of mapping and were ordered into 43 and 31 linkage groups in paternal and maternal maps, respectively. Sex-linked markers were placed on the maternal as the linkage group 28 of the female map but not the paternal map leading to an interesting argument that female is the heterogametic sex in *M. japonicus*. Nevertheless, the female linked markers have not been cloned and characterized for direct application for sex determination in that species.

Wilson et al. (2002) used an identical approach to construct the male and female genetic linkage maps in *P. monodon*. A total of 673 polymorphic AFLP loci that confirmed to Mendelian segregation ratios were scored in three families and used to constructed separate male and female linkage maps for each family. Common markers found in two or more reference families were used to construct a common linkage map across three families. Nevertheless, sex-linked AFLP markers were not

found in *P. monodon*. This indirectly implied that female-linked markers found in *M. japonicus* placed in the female map may be resulted artifact AFLP bands.

Sex-specific/differential expression markers were found in *P. monodon*

The most important step towards understanding molecular mechanisms of sex determination in *P. monodon* is the identification and characterization of sex-specific/differential expression markers. Such molecular markers could be used to verify the genetic system of sex chromosomes in *P. monodon.* In the present study, subtractive cDNA libraries were constructed to identify sex-related transcripts in *P. monodon*. Expression of interesting ESTs in ovaries and testis of adult *P. monodon* was further examined using RT-PCR.

Gender-specific gene expression has been recently reported in a mosquito-borne filarial nematode (*B. malayi*) isolated by differential display (DD) PCR and *In silico* subtraction of EST cluster database and further confirmed by RT-PCR. Six of 12 (27%) and seven of 15 (47%) initially identified by DD-PCR and *In silico* subtraction revealed gender-specific expression in that species (Michalski and Weil, 1999).

In the silkworm, sex-specific mRNA isoforms were found in double sex (*dsx*) gene where the male-specific cDNA lacked the sequence between 713 – 961 nucleotides of the female-specific cDNA (Ohbayashi et al., 2001).

Based on RT-PCR, expression of peritrophin (found in a subtracted cDNA libraries of ovaries but not that of testes) was found in both ovaries and testes of *P. monodon*. In insects, several forms of peritrophin were isolated and have been demonstrated as potential vaccine antigens (Barry et al., 1999; Tellam et al., 2000).

Interestingly, a number of peritrophin ESTs were found in the lymphoid organ cDNA library of *P. monodon* infected with *Vibrio harveyi* but not in the normal library (A. Tassanakajon, unpublished data). Therefore, expression levels of different isoforms of peritrophin during ovarian development in response to infection by shrimp pathogenic agents (e.g. *Vibrio harveyi*; white spot syndrome virus, WSSV and/or yellow head virus, YHV) should be further examined.

Peritrophin is highly expressed during oocyte development of marine shrimps and 2 forms of transcripts (1 and 2 with the identical ORFs of 834 bp encoding proteins of 277 amino acids, accession numbers AF510331 and AF510332) were recently isolated from ovaries of *P. monodon*. Peritrophin is a component of cortical rods and is the precursor of the jelly layer of the shrimp eggs (Khayat et al., 2001). Synthesis of peritrophin in ovaries of *P. semisulcutus* is inhibited by crustacean hyperglycemic hormone purified from the sinus gland extract of Kuruma shrimp, *M. japonicus* (Avarre et al., 2001). In *D. melanogaster*, several types of peritrophin were isolated and the peritrophin A-encoded gene (NC004354) is X-linked.

A homologue of *C. elegans* XNP-1 was also found in this study. A homologue of XNP-1 in human is called an ATRX protein. Mutations on the human ATRX gene result in several mental retardation syndromes. Villard et al. (1999) isolated and characterized of XNP-1 from *C. elegans* and found that the open reading frame of XNP-1 was 4077 nucleotides encoding a protein of 1359 amino acids. Comparing of ATRX (also called human XNP) and XNP-1 at the genomic level revealed that the structure of these gene homologues is partially conserved. The former is composed of 35 exons whereas the latter is composed of 10 exons. Several exon-intron boundaries are present in the same nucleotide for both genes.

Sex-specific expression of *P. monodon* XNP-1 was examined by RT-PCR. An XNP-1 homologue of *P. monodon* did not reveal sex-specific or sex-differential expression between male and female *P. monodon*. The PCR product (224 bp) of XNP-1 from different individuals was further characterized using single-stranded conformation polymorphism (SSCP) analysis (15% polyacrylamide, 37.5:1 supplemented with 5% glycerol) to determine whether fixed single nucleotide polymorphism (SNP) between genders of *P. monodon* was existent. Only a single SSCP pattern was observed. Therefore, sex-related SNP was not found in an XNP-1 homologue of *P. monodon*.

Ubiquitin X-specific protease 9 exhibited a female specific expression profile in ovaries of 4-month-old shrimps but showed differential expression between male and female *P. monodon* In contrast, TSP was temporally expressed in females in adults but commonly expressed in both genders in 4-month-old *P. monodon*.

In addition, female-specific expression profiles were observed in a homologue of AGESTX and PM233 (unknown transcript) in different stages of *P. monodon*. VCP7 which was subsequently confirmed to be a homologue of TSP was only examined in the bloodstock-sized *P. monodon* and illustrated female-specific expression pattern.

TSP in vertebrates is a family of at least 5 proteins (TSP1 - 4 and cartilage oligomeric matrix protein, COMP). TSP1 and TSP2 are involved with embryonic development, tissue differentiation and blood coagulation. The biological role of TSP3 and TSP4 are not well understood but mutations in the COMP gene cause skeletal disorders in human (Carron et al., 2000).

A TSP homologue in invertebrates was first isolated in *D. melanogaster* (open reading frame; ORF, of 3183 nucleotides encoding a protein of 1060 amino acids) (Adolph, 2001). Recently, complete sequences of 3 closely related TSP homologues (ORFs of 3345, 3099 and 2976 nucleotides coding for 1114, 1032 and 991 amino acids, accession numbers AB121209, AB121210 and AB121211) with unknown functions were isolated and characterized in *M. japonicus*.

Therefore, sex-specific markers of *P. monodon* were successfully developed at the cDNA level but not the genomic DNA level. New primer pairs of cDNAs showing sex-specific expression in *P. monodon* should be designed and further characterized to identify the existence of single nucleotide polymorphism (SNP) between males and females of *P. monodon*.

Large numbers of unknown transcripts were found in our subtractive cDNA libraries. Genes of cDNA clones showing sex-specific/differential expression will be characterized to identify fixed SNP in genomic DNA of male and female *P. monodon*. Long-term benefits of the present study include the possibility to improve reproductive performance of domesticated broodstock, to induce high quality egg development in captive females without eyestalk ablation and to produce monosex culture of *P. monodon*.

Candidate transcripts involving with oocyte developments of *P. monodon*: possible application for studies on maturation of female *P. monodon* in captivity

Ovarian maturation in penaeid shrimps involves formation of cortical rods (CRs) in the peripheral crypts of oocytes at final stages of oocyte development. CRs first microscopically appear as spherical structures in fully yolk-invested oocytes one to two days before spawning. They then extend toward the center of the oocytes, forming a rod-like shape. At the time of spawning the contact of eggs with seawater stimulates the release of CRs outward, which results in the formation of a jelly layer that surrounds the fertilized eggs within 15 minutes after spawning (Wallis et al., 1990). The jelly layer is thought to function as a barrier against polyspermy and between the egg and the external environment.

Recently, shrimp ovarian peritrophin (SOP) have been isolated in *P. semisulacatus* (Khayat et al., 2001) and *P. monodon*. The ORF of shrimp peritrophin was 834 nucleotides coding for 277 amino acids in both species. Deduced amino acids revealed the presence of repeated cysteine-rich domains that are related to the chitin binding domains of insect intestinal peritrophins and mucin and crustacean tachycitin and invertebrate chitinase. Peritrophin is a major protein of CRs and the jelly layer of the eggs. In *P. semisulcatus*, this protein was immunologically detected in ovaries, purified CRs, fertilized eggs and in the cloudy, whitish flocculent material appearing in seawater immediately after spawning.

Peritrophin transcripts were detected in all stages of ovarian development but were more abundant in previtellogenic ovaries than vitellogenic and mature ovaries. *De novo* synthesis of pertrophin in *P. semisulcatus* was inhibited by sinus gland extract of the same shrimp species and crustacean hyperglycemic (CHH) peptides that were purified from sinus glands of *M. japonicus*.

More recently, three CR proteins displayed similar immunological characteristics were isolated. The cDNA sequences and developmental expression profiles at both transcriptional and translational levels were studied and found that they involve with CR formation processes. Deduced amino acids of these transcripts revealed significant similarities to TSP3, 4/cartilage oligomeric matrix protein family. Nucleotide sequences of these cDNA indicated that all three forms of CR-related

proteins were transcribed from a single locus but polymorphism occurred through alternative splicing of exons. Semi-quantitative analysis, in situ hybridization and western blot analysis indicated that transcription, translation of TSP and formation of the CR structure in *M. japonicus* occurred at different stages of ovarian development (Yamano et al., 2004).

In the present study, several candidate transcripts related with ovarian development of *P. monodon* were found. Homologues of peritrophin of *P. semisulcatus* and TSP of *M. japonicus* were identified. In addition, homologues of TSP1 from mammals and *D. melanogaster* TSP were also identified.

Usp9X in mice is an X-linked orthologue of Fat facets (Faf) an ubiquitin-specific protease essential for normal development of oocytes in *Drosophila*. Mutations of this gene in *Drosophila* lead to abnormal content and the inability of the fertilized egg to undergo normal embryogenesis. It was also found that this protein is important for development primordial germ cells. In mice, expression of Usp9X rapidly decreased to an undetected level by 15.5 days post coitum (dpc) and after birth to adult. No expression was found in any spermatogenic cells except for weak expression in Sertoli cells. Usp9X expresses in embryogenic oocytes and was also reduced at the newborn stage. Its expression reappeared in oocytes at secondary follicle stage (Noma et al., 2002).

Apart from those previously reported transcripts, ubiquitin X-specific protease 9 (Usp9X) and AGESTX that showed sex-related expression in *P. monodon* were also isolated. The expression profiles of these transcripts implied that they may have contributed in ovarian development in *P. monodon*. Nevertheless, these transcripts were not the full-length cDNAs, therefore, isolation and characterization of the full length of Usp9X and AGESTX cDNAs and genes should be carried out further.

Reduced spawning potential of *P. monodon* in captivity and degree of maturation of *P. monodon* in captivity crucially prohibits several applications to improve efficiency of culture and management including the development of effective selective breeding programs of this species.

Yamano et al. (2004) pointed out that ovaries of *M. japonicus*, in most cases, start to develop in the reproductive season but fail to reach the necessary stage required by the formation of CRs. Accordingly, ovaries are degenerate without spawning. Ovarian development of penaeid shrimps may nott pass through to the accumulation of yolk substances or in some cases to the stage after yolk accumulation is achieved. Further studies about mechanisms controlling formation of CRs is, therefore, required to control maturation of *P. monodon* in captivity successfully.

CHAPTER V

CONCLUSION

1. Primers designed from conserved sex-specific genes (DMRT-1, *dsx* and mab-3) generated cDNA markers (PMT1700, PMO720 and PMO920) showing differential expression between male and female *P. monodon*.

2. Subtraction of cDNA from ovaries (forward) and testes (reverse) indicate different groups of transcripts.

3. TSP (28.7%), peritrophin (10.8%) and unknown transcripts (49.7%) are abundantly expressed in the forward subtractive library, and unknown transcripts (96.7%) are found in the reverse subtractive library.

4. Five sex-specific/differential expression markers (TSP, AGESTX, PM233, Usp9X and VCP7) were identified in *P. monodon.*

5. Semi-quantitative analysis of TSP and AGESTX in 4-month-old and adult *P. monodon* was developed. Results indicated the significantly different expression level of TSP (but not AGESTX) in ovaries of 4-month-old and broodstock-sized *P. monodon.*

6. Expression of TSP (primers TSP288-1 + TSP371-1) was found in ovaries, eyestalks, gills, hemocytes, hepatopancreas, intestine, lymphoid organs, pleopods, stomach and thoracic ganglion but not found in testes and heart whereas that AGESTX (primers AGRESTX44-F/R) was found in ovaries, hemocytes, hepatopancreas, lymphoid organs, stomach and thoracic ganglion but did not express in eyestalks, gills, heart, intestine, pleopods and testes of *P. monodon*.

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APPENDICES

Appendix A

1. LB Broth (per Liter)

10 g of NaCl

10 g of tryptone

5 g of yeast extract

Add deionized H_2O to a final volume of 1 liter. Adjust to pH 7.0 with 5 N NaOH and autoclave.

2. LB Agar (per Liter)

- 10 g of NaCl
- 10 g of tryptone
- 5 g of yeast extract
- 20 g of agar

Add deionized H_2O to a final volume of 1 liter. Adjust to pH 7.0 with 5 N NaOH and autoclave. After, pour into petri dishes (~25 ml/100-mm plate)

3. LB-Ampicillin Agar (per Liter)

- Prepare 1 liter of LB agar. Autoclave and cool to 55 °C
- Add 50 ml of filter-sterilized ampicillin
- Pour into petri dishes (~25 ml/100-mm plate)

4. 1x TAE Buffer

- 40 mM Tris-acetate
- 1 mM EDTA

5. SOB Medium (Per liter) :

- Bacto-tryptone 20 g
- Yeast extract 5 g
- NaCl 0.5 g

6. SM buffer

- This medium is used for phage storage and dilution.

Per liter:

- NaCl 5.8 g
- $MgSO_4 \cdot 7\ H_2O$ 2 g
- 1 M Tris · Cl (pH 7.5) 50 ml
- 2% gelatin 5 ml

7. Ampicillin

Stock solution. 25 mg/ml of the sodium salt of ampicillin in water. Sterilize by filtration and store in aliquots at –20 °C

8. Working concentration. 35-50 μg/ml.

- 0.5 M EDTA (pH 8.0)

Add 186.1 g of disodium ethylene diamine tetraacetate · $2H_2O$ to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving.

9. 5 M NaCl

Dissolve 292.2 g of NaCl in 800 ml of H_2O. Adjust volume to 1 liter. Dispense into aliquots and sterilize by autoclaving.

10. 1 M $MgCl_2$

Dissolve 203.3 g of $MgCl_2 \cdot 6H_2O$ in 800 ml of H_2O. Adjust volume to 1 liter. Dispense into aliquots and sterilize by autoclaving.

11. 3 M Sodium acetate (pH 5.2)

Dissolve 408.1 g of sodium acetate · $3H_2O$ in 800 ml of H_2O. Adjust pH to 5.2 with glacial acetic acid. Adjust volume to 1 liter. Dispense into aliquots and sterilize by autoclaving.

12. 10% Sodium dodecyl sulfate (SDS) (also called sodium lauryl sulfate)

Dissolve 100 g of electrophoresis-grade SDS in 900 ml of H_2O. Heat to 68 °C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust volume to 1 liter. Dispense into aliquots.

13. Ethidium bromide 10 mg/ml

Add 1 g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer to a dark bottle and store at 4 °C.

14. TE pH 7.4

- 10 mM Tris · Cl (pH 7.4)
- 1 mM EDTA (pH 8.0)

15. TE pH 7.6

- 10 mM Tris · Cl (pH 7.6)
- 1 mM EDTA (pH 8.0)

16. TE pH 8.0

- 10 mM Tris · Cl (pH 8.0)
- 1 mM EDTA (pH 8.0)

17. Tris-Borate (TBE)

-Working solution

- 0.089 M Tris-borate
- 0.089 M boric acid
- 0.002 M EDTA

- Concentrated stock solution (5x)

Per liter:

- Tris base 54 g
- Boric acid 27.5 g
- 0.5 M EDTA (pH 8.0) 20 ml

18. Gel-Loading Buffer Type II

- 10x buffer
- 0.25% bromophenol blue
- 0.25% xylene cyanol
- 25% Ficoll (type 400) in H_2O
- Store at room temperature.

19. 10x MOPS Electrophoresis Buffer

- 0.2 M MOPS (pH 7.0) <!>
- 20 mM sodium acetate
- 10 mM EDTA (pH 8.0)

Dissolve 41.8 of MOPS in 700 ml of sterile DEPC-treated <!> H_2O. Adjust the pH to 7.0 with 2 N NaOH. Add 2 ml of DEPC-treated 1 M sodium acetate and 20 ml of DEPC-treated 0.5 M EDTA (pH 8.0). Adjust the volume of the solution to 1 liter with DEPC-treated H_2O. Sterilize the solution by passing it through a 0.45-μm Millipore filter, and store it at room temperature protected from light. The buffer yellows with age if it is exposed to light or is autoclaved. Straw-colored buffer works well, but darker buffer does not.

20. 10x TEN buffer

- 0.1 M Tris-Cl (pH 8.0)
- 0.01 M EDTA (pH 8.0)
- 0.1 M NaCl

21. Equilibration of Phenol

Before use, phenol must be equilibrated to a pH of > 7.8 because the DNA partitions into the organic phase at acid pH. Wear gloves, full face protection, and a lab coat when carrying out this procedure.

1. Store liquefied phenol at –20°C. As needed, remove the phenol from the freezer, allow it to warm to room temperature, and the then melt it at 68°C. Add hydroxyquinoline to a final concentration of 0.1 %. This compound is an antioxidant, a partial inhibitor of RNase, and a weak chelator of metal ions (Kirby 1956). In addition, its yellow color provides a convenient way to identify the organic phase.

2. To the melted phenol, add an equal volume of buffer (usually 0.5 M Tris-Cl [pH 8.0] at room temperature). Stir the mixture on a magnetic stirrer for 15 minutes. Turn off the stirrer, and when the phases have separated, aspirate as much as possible of the upper (aqueous) phase using a glass pipette attached to a vacuum line equipped with appropriate traps (please see Appendix 8, Figure A8-2).

3. Add an equal volume of 0.1 M Tris-Cl (pH 8.0) to the phenol. Stir the mixture on a magnetic stirrer for 15 minutes. Turn off the stirrer and remove the upper aqueous phase as described in Step 2. Repeat the extractions the pH of the phenolic phase is >7.8 (as measured with pH paper)

4. After the phenol is equilibrated and the final aqueous phase has been removed, add 0.1 volume of 0.1 M Tris-Cl (pH 8.0) containing 0.2% β-mercaptoethanol<!>. The phenol solution may be stored in this form under 100 mM Tris-Cl (pH 8.0) in a light-tight bottle at 4°C for periods of up to 1 month.

22. Phenol: Chloroform: Isoamyl Alcohol (25:24:1)

A mixture consisting of equal parts of equilibrated phenol and chloroform: isoamyl alcohol<!> (24:1) is frequency used to remove proteins from preparation of nucleic acids. The chloroform denatures protein and facilitates the seperation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction. Neither chloroform nor isoamyl alcohol requires treatment before use. The phenol: isoamyl alcohol mixture may be stored under 100 mM Tris-Cl (pH8.0) in a light-tight bottle at 4 ^{0}C for period of up to 1 month.

23. Glycerol (10% v/v)

Dilute 1 volume of molecular-biology-grade glycerol in 9 volume of sterile pure H_2O. Sterilize the solution by passing it through a prerinsed 0.22 uM filter. Store in 200-ml aliquots at 4 ^{0}C

24. IPTG (20% w/v, 0.8 M)

IPTG is isopropylthio-B-D-galactoside. Make a 20% solution of IPTG by dissolving 2 g of IPTG in 8 ml of distilled H_2O. Adjust the volume of the solution to 10 ml with H2O and sterilize by passing it through a 0.22 uM disposable filter. Dispense the solution into 1-ml aliquots and store them at -20 ^{0}C

25. X-gal solution (2% w/v)

X-gal is 5-bromo-4-chloro-3-indolyl-B-d-galactoside. Make a stock solution by dissolving X-gal in dimethylformamide at a concentration of 20 mg/ml solution. Use a glass or polypropylene tube. Wrap the tube containing the solution in aluminum foil to prevent damage by light and store at -20 ^{0}C .It is not necessary to sterilize X-gal solution by filtration.

Appendix B

Publications from this thesis

1. **Leelatanawit, R.**, Klinbunga, S.,Tassanakajon, A., Jarayabhand, P., Hirono, I. Aoki, T. and Menasveta, P. 2004. Isolation and Characterization of Differentially Expressed Genes in Ovaries and Testes of the Giant Tiger Shrimp (*Penaeus monodon*). Mar Biotechnol 6: (in press).

2. **Leelatanawit, R.**, Klinbunga, S., Tassanakajon, A., Jarayabhand, P. and Menasveta, P. 2003. Isolation and Characterization of Differentially Expressed Genes in Ovaries and Testes of the Giant Tiger Shrimp (*Penaeus monodon*) **BioThailand 2003**, 17-20 July 2003, Chonburi, Thailand.

3. **Leelatanawit, R.**, Klinbunga, S., Tassanakajon, A., Jarayabhand, P. and Menasveta, P. 2003. Cloning and characterization of a sex-linked Zinc Finger X/Y homologue in the giant tiger shrimp (*Penaeus monodon*). 29th Congress on Science and Technology of Thailand.

4. **Leelatanawit, R.**, Klinbunga, S., Puanglarp, N., Tassanakajon, A., Jarayabhand, P., Hirono, I., Aoki, T. and Menasveta, P. 2004. Differentially Expressed Genes in Ovaries and Testes of the Giant Tiger Shrimp (*Penaeus monodon*). **5th National Symposium on Marine Shrimps.** 29 – 30 March 2004. Bangkok (oral presentation).

Biography

Miss Rungnapa leelatanawit was born on November 7, 1978 in Bangkok. She graduated with the degree of Bachelor of Science from the Department of Industrial Technology at Silapakorn University in 2000. In 2001, she has studied in Master degree of Science at the Biotechnology, Chulalongkorn University.