เครื่องหมายจำแนกเพศและการแสดงออกของยีนในต่อมแอนโดรเจนิกของกุ้งก้ามกราม *Macrobrachium rosenbergii* เพศผู้ที่มีลักษณะทางสัณฐานวิทยาที่แตกต่างกัน

นางสาว รชนิมุข ปรีชาผล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SEX DETERMINATION MARKERS AND GENE EXPRESSION IN ANDROGENIC GLAND OF DIFFERENT MALE MORPHOTYPES OF THE GIANT FRESHWATER PRAWN *Macrobrachium rosenbergii*

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รชนิมุข ปรีชาผล : เครื่องหมายจำแนกเพศและการแสดงออกของยีนในต่อมแอนโดรเจนิกของกุ้งก้ามกราม *Macrobrachiumn rosenbergii* เพศผู้ที่มีลักษณะทางสัณฐานวิทยาที่แตกต่างกัน (SEX DETERMINATION MARKERS AND GENE EXPRESSION IN ANDROGENIC GLAND OF DIFFERENT MALE MORPHOTYPES OF THE GIANT FRESHWATER PRAWN *Macrobrachium rosenbergii*) อ. ที่ปรึกษา : รศ.ดร. เผดิมศักดิ์ จารยะพันธุ์, อ. ที่ปรึกษาร่วม : ดร. ศิราวุธ กลิ่นบุหงา 195 หน้า. ISBN 974-17-6100-7.

ในการวิเคราะห์หาเครื่องหมายทางพันธุกรรมระดับดีเอ็นเอที่จำเพาะต่อเพศของกุ้งก้ามกราม (*Macrobrachium rosenbergii*) ด้วยวิธี AFLP โดยใช้ไพรเมอร์ทั้งหมดจำนวน 64 ไพรเมอร์คู่ผสม และใช้ดีเอ็นเอต้นแบบโดยการรวมดีเอ็นเอด้วยวิธี BSA จากกุ้งก้ามกราม 3 กลุ่ม คือ กุ้งก้ามทองเล็ก (SOC1, $N = 10$), กุ้งก้ามใหญ่ (BC1, $N = 5$) และกุ้งเพศเมีย (F1, $N = 10$) พบเครื่องหมายที่แสดงออกจำเพาะต่อเพศผู้ และเพศเมียจำนวน 90 และ 42 แถบ ตามลำดับ ในไพรเมอร์ 40 ไพรเมอร์คู่ผสม จากนั้นจึงใช้ดีเอ็นเอต้นแบบทั้งชุดแรกและชุดที่สอง (BC2, $N = 10$; OC1, $N = 15$; SOC2, $N = 5$ และ F2, $N = 20$) เพื่อตรวจสอบผลที่ได้อีกครั้ง และพบเครื่องหมายที่แสดงออกจำเพาะต่อเพศผู้และเพศเมียจำนวน 5 และ 4 แถบ ตามลำดับ ซึ่งลำดับนิวคลีโอไทด์ทั้งหมดเป็น Unknown ($E > 10^{-4}$) จึงออกแบบไพรเมอร์จากนิวคลีโอไทด์ดังกล่าวทั้งหมด 13 คู่ พบว่ามีไพรเมอร์ 9 คู่ ที่ให้แถบดีเอ็นเอตามที่คาดหมายในกุ้งก้ามกรามทั้งสองเพศ จึงนำผลิตภัณฑ์ลูกโซ่โพลีเมอเรสดังกล่าวมาตรวจสอบ single nucleotide polymorphism (SNP) ด้วยวิธี SSCP พบความแตกต่างของผลิตภัณฑ์ลูกโซ่โพลีเมอเรสทั้งในเพศเดียวกันและระหว่างเพศ (ยกเว้นผลิตภัณฑ์จากไพรเมอร์ $FE_{+3}8M_{+3}270.2$) แต่ไม่พบ SNP ที่ใช้บ่งบอกความแตกต่างระหว่างเพศในกุ้งก้ามกราม

จากการใช้วิธี RT-PCR ตรวจสอบการแสดงออกของยีนที่ควบคุมการสร้างฮอร์โมนแอนโดรเจนิกในต่อมแอนโดรเจนิกของกุ้งก้ามกราม พบว่าไพรเมอร์ที่ออกแบบไม่ให้ผลแถบดีเอ็นเอที่คาดไว้ จึงทำการตรวจสอบการแสดงออกของยีนอื่นๆ ด้วยวิธี RT-PCR โดยใช้ไพรเมอร์ที่จำเพาะต่อเพศที่ได้จากวิธี AFLP และไพรเมอร์ที่ออกแบบจากยีนที่เกี่ยวข้องกับเพศในกุ้งกุลาดำ (*P.monodon*) และ หอยเป๋าฮื้อ (*H. asinina*) พบเครื่องหมายที่ให้ผลผลิตที่แสดงออกเฉพาะในต่อมแอนโดรเจนิก จากการใช้ไพรเมอร์ $ME_{+3}8M_{+3}1310.1$ โดยลำดับนิวคลีโอไทด์ของเครื่องหมายดังกล่าวคล้ายคลึงกับยีน Sarco / endoplasmic reticulum Ca^{2} + ATPase C (SERCA) ($E = 1 \times 10^{-103}$) จึงทำการออกแบบไพรเมอร์และตรวจสอบการแสดงออกของยีน SERCA (ขนาด 279 คู่เบส) และพบว่ามีการแสดงออกแตกต่างกันระหว่างกุ้งก้ามกรามเพศผู้และเพศเมีย นอกจากนี้ยังพบว่ามีการแสดงออกที่จำเพาะในกุ้งก้ามกรามเพศเมียจากไพรเมอร์ peritrophin ส่วนไพรเมอร์ DSI ให้ผลการแสดงออกของยีนที่สูงมากในกุ้งก้ามใหญ่

นอกจากนี้ ได้วิเคราะห์หาเครื่องหมายทางพันธุกรรมระดับ cDNA ที่จำเพาะต่อเพศ และ/หรือ ที่มีการแสดงออกแตกต่างกันระหว่างเพศในกุ้งก้ามกรามด้วยวิธี RAP-PCR พบเครื่องหมายที่จำเพาะต่อเพศผู้และเพศเมียจำนวน 43 และ 24 เครื่องหมาย และพบเครื่องหมายที่แสดงออกแตกต่างกันในกุ้งก้ามกรามเพศผู้และเพศเมียจำนวน 29 เครื่องหมาย นอกจากนี้ยังพบเครื่องหมายที่แสดงออกในกุ้งก้ามกรามเพศผู้แต่ละแบบ (กุ้งก้ามใหญ่, กุ้งก้ามทอง และกุ้งก้ามทองเล็ก) จำนวน 10, 12 และ 13 เครื่องหมาย ตามลำดับ ซึ่งเครื่องหมายที่จำเพาะต่อกุ้งก้ามทองเล็ก (ขนาด 340 คู่เบส) และกุ้งก้ามกรามเพศเมีย (ขนาด 315 คู่เบส) สามารถพบได้จากการใช้ไพรเมอร์ UBC428 และ UBC122 ร่วมกับไพรเมอร์ที่ใช้ทั้งหมด (30 ไพรเมอร์) อย่างไรก็ตาม ไพรเมอร์ที่ออกแบบใหม่ไม่แสดงเครื่องหมาย SCAR ที่คาดไว้ นอกจากนี้ ได้ออกแบบไพรเมอร์จากเครื่องหมายที่จำเพาะต่อกุ้งก้ามกรามเพศผู้, กุ้งก้ามใหญ่ และกุ้งก้ามทองเล็กอีก 5, 2 และ 2 เครื่องหมาย ตามลำดับ พบว่ามีไพรเมอร์ 3 คู่บ่งบอกระดับการแสดงออกของยีนที่แตกต่างกัน (M122/159RAP.2, SOC268/273RAP และ BC428/273RAP) โดยระดับการแสดงออกของยีนจาก M122/159RAP.2 (คล้ายคลึงกับ I–connectin mRNA ใน crayfish, *P. clarkii*) ให้ผลแสดงออกแตกต่างกันระหว่างต่อมแอนโดรเจนิกและท่อนำไข่ของกุ้งก้ามกราม แต่ไม่แสดงออกแตกต่างกันในกุ้งก้ามกรามเพศผู้แต่ละแบบ และรูปแบบการแสดงออกของเครื่องหมายที่พัฒนาจาก SOC268/273RAP และ BC428/273RAP นั้นมีแนวโน้มของการเกิดจากกระบวนการ alternative splicing นอกจากนี้ ยังพบรูปแบบความแตกต่างทางพันธุกรรมที่ไม่เกี่ยวข้องกับเพศจากการใช้จีโนมิกดีเอ็นเอในกระบวนการลูกโซ่โพลีเมอเรสของเครื่องหมายที่พัฒนาจากแถบ RAP-PCR อีกด้วย

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....

ปีการศึกษา.....2547..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4472379223 : MAJOR BIOTECHNOLOGY

KEY WORD: *Macrobrachium rosenbergii* / GIANT FRESHWATER PRAWN / SEX – SPECIFIC MARKER / ANDROGENIC GLAND

RACHANIMUK PREECHAPHOL: SEX DETERMINATION MARKERS AND GENE EXPRESSION IN ANDROGENIC GLAND OF DIFFERENT MALE MORPHOTYPES OF THE GIANT FRESHWATER PRAWN *Macrobrachium rosenbergii*. THESIS ADVISOR: ASSOC. PROF. PADERMSAK JARAYABHAND, Ph. D., THESIS CO-ADVISOR: SIRAWUT KLINBUNGA, Ph. D., 195 pp. ISBN 974-17-6100-7.

Sex-specific DNA markers of the giant freshwater prawn (*Macrobrachium rosenbergii*) were identified by AFLP analysis. A total of 64 primer combinations were primary screened against bulked genomic DNA of the first sample set including small orange claw (SOC1, $N = 10$) and blue claw (BC1, $N = 5$) males and female (F1, $N = 10$). Ninety candidate male- and forty-two candidate female-specific markers from 40 informative primers were secondary tested with both the first and the second (BC2, $N = 10$; OC1, $N = 15$; SOC2, $N = 5$ and F2, $N = 20$) sample sets. Only 5 and 4 male- and female-specific AFLP markers were consistently found and regarded as the unknown DNA segments after characterization ($E > 10^{-4}$). SCAR markers were developed from candidate sex-specific markers. Among 13 primer pairs tested, 9 pairs of primers generate the expected amplification product in both male and female *M. rosenbergii*. Further analysis of the product by SSCP analysis revealed polymorphism of all markers except $FE_{+3}8M_{+3}270.2$–F/R but polymorphic patterns did not show sex-specificity in *M. rosenbergii*. RT-PCR of an AGH homologue in *M. rosenbergii* was not successful. Accordingly, primers designed from AFLP of this study and sex-related transcripts from previous studies in the black tiger shrimp (*Penaeus monodon*) and the tropical abalone (*Haliotis asinina*) were applied for identification of sex-specific/differential expression markers in *M. rosenbergii*. A homologue of Sarco / endoplasmic reticulum Ca^2 + ATPase C (SERCA) were isolated from $ME_{+3}8M_{+3}1310.1$ primers ($E = 1 \times 10^{-103}$). An amplified SERCA (279 bp) revealed differential expression patterns between male and female *M. rosenbergii*. In addition, a female-specific expression marker was observed from a peritrophin homologue. Likewise, DSI was regarded as differential expression marker for the BC morphotype. Additionally, RAP-PCR analysis was also carried out to isolate various types of expression markers in different morphotype of males and between males and females of *M. rosenbergii*. In a total, 43 and 24 of male- and female-specific and 29 differential expression RAP-PCR markers were found. Moreover, 10, 12 and 13 RAP-PCR fragments exhibiting morphotype-specific expression in BC, OC and SOC males were also isolated. Interestingly, SOC- (340 bp) and female-specific (315 bp) RAP-PCR markers were successfully identified from combination of the first primers and all investigated second primers (30 primer combination) but conversion of these to SCAR markers was not successful. Additionally, 9 primer pairs were designed and tested. Three developed markers (M122/159RAP.2, SOC268/273RAP and BC428/273RAP) illustrated interesting results at the transcriptional level. The expression levels of M122/159RAP.2 (significantly similar to I–connectin mRNA of the crayfish, *P. clarkii*) clearly showed differential expression patterns in AG of males and oviducts of female *M. rosenbergii* but no differences were found between different male morphotypes. Conversely, the amplification product of SOC268/273RAP and BC428/273RAP implied possible length polymorphism through alternative splicing. They also provided polymorphic patterns when genomic DNA of different male and female individuals of *M. rosenbergii* were genotyped.

Field of study……Biotechnology……..… Student's signature..…….……

Academic year……..2004……...…. Advisor's signature……...

Co-advisor's signature…...……...

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LIST OF ABBREVIATIONS

bp	base pair
^{o}C	degree Celsius
DEPC	Diethylpyrocarbonate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid (disodium salt)
EtBr	ethidium bromide
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
kb	kilobase pair
M	Molar
mg	milligram
min	minute
ml	millilitre
mM	millimolar
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
RNA	Ribonucleic acid

rpm	revolution per minute
Rnase A	Ribonuclease A
SDS	sodium dodecyl sulfate
Sec	second
Tm	melting temperature
Tris	Tris (hydroxy methyl) aminomethane
U	unit
UV	ultraviolet
w/v	weight/volume
μg	Microgram
μl	Microlitre
μM	Micromolar

CHAPTER I

INTRODUCTION

The giant freshwater prawn (*Macrobrachium rosenbergii*) is widely distributed in fresh and brackish waters in most of tropical and subtropical areas of the Indo-Pacific region. The first recognizable illustration of *M. rosenbergii* appeared in 1705s and, therefore, regarded as one of the first crustacean species described scientifically. The external morphology of *M. rosenbergii* is illustrated in Fig. 1.1.

Ling et al. (1961) succeeded to disclosing the life cycle of *M. rosenbergii* and this species had been initially cultured commercially. Farming of *M. rosenbergii* has commented in Asian, as well as in Europe, the United States of America and Africa. In Thailand, commercial farming of *M. rosenbergii* began in middle-1970s. The total production of *M. rosenbergii*, including captures and harvest from inland farming, in each country is shows in Table 1.1.

Two forms of *M. rosenbergii* have been described independently based on external morphology (De Man, 1879). These included the western sub-species which are found in the waters of the East Coast of India, Bay of Bengal, Gulf of Thailand, Malaysia, and the Indonesian regions of Sumatra, Java and Kalimantan and the eastern sub-species which inhabits the Philippines, the Indonesian regions of Sulawesi and Irian Jaya, Papua New Guinea and northern Australia.

Hedgecock et al. (1979) recognized three 'geographical races' of *M. rosenbergii*; an eastern, a western, and an Australian 'race,' based on allozyme and morphological data. More recently, Bruyn et al. (2004) examined the evolutionary relationships among wild *M. rosenbergii* stocks at a regional scale using 16S ribosomal (r) RNA gene of mitochondrial DNA (mtDNA) sequences, and related the findings to the biogeographical history of the region. Significant mtDNA divergences between eastern and western *M. rosenbergii* forms supporting previous studies were found.

Table 1.1 The total production quantity and values of giant freshwater prawn (in metric tons) 1950-2002[a] (Available at http://www.fao.org/fi/statist/FISOFT/FISHPLUS.asp)

Inland waters	1950	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971
Africa																						
Senegal	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mauritius	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	<0.5	<0.5
America, North																						
United States of America	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Puerto Rico	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Martinique	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-
Mexico	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	10
Jamaica	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Guatemala	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Honduras	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
Guadeloupe	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-
El Salvador	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Dominican Republic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-
Costa Rica	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
America, South																						
Peru	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
French Guiana	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Colombia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-
Ecuador	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-
Brazil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asia																						
Thailand	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Malaysia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indonesia	2,000	2,000	2,200	2,600	2,400	2,800	3,200	3,400	2,800	3,800	3,700	4,100	4,300	4,000	4,400	4,300	5,200	5,400	4,700	4,600	4,800	4,800
Taiwan Province of China	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-
India	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
China	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Brunei Darussalam	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	<0.5	<0.5
Bangladesh	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Atlantic, Western Central																						
Venezuela	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Oceania																						
French Polynesia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-
Fiji Islands	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-
Other	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

General notes: [a] ... = Data not available; unobtainable; data not separately available but included in another category, - = Nil or zero, 0 = More than zero but less than half the unit used, nei = Not elsewhere included, F = FAO estimate from available sources of information, R = Repetition of data previously reported by the country.

Table 1.1 (cont.)

Inland waters	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991
Africa																				
Senegal	.	.	.	.	.	.	.	.	.	.	.	.	1	5	6	7	3	3	2	2
Mauritius	<0.5	<0.5	<0.5	<0.5	<0.5	1	5	15	16	25	30	24	23	25	27	48	48	50	42	47
America, North																				
United States of America	-	-	10	30	67	70	51	110	136	150	182	182	144	131	81	91	130	159	184	184
Puerto Rico	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	97	76	102	110	78
Martinique	-	-	-	-	-	-	-	-	-	-	6	13	30	35	50	60	52	57	49	52
Mexico	59	104	146	183	216	246	271	293	310	324	334	340	325	325	350	361	410	353	201	139
Jamaica	.	.	.	.	.	.	.	.	.	.	.	.	15	15	19	20	30	30	30	30
Guatemala	.	.	.	.	.	.	.	.	.	.	.	.	.	5	4	12	10	8	10	7
Honduras	-	-	-	-	-	19	30	35	42	197	33	35	46	50	84	18	5	<0.5	<0.5	<0.5
Guadeloupe	-	-	-	-	-	-	-	-	.	.	8	10	40	31	46	46	63	44	42	44
El Salvador	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	<0.5	1	4	7
Dominican Republic	-	-	-	-	-	-	-	-	-	-	-	3	50	100	100	120	100	50	50	43
Costa Rica	.	.	.	.	.	.	.	.	.	.	.	.	2	4	4	6	7	7	7	7
America, South																				
Peru	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	18	15	10	5	4
French Guiana	.	.	.	.	.	.	.	.	.	.	.	.	2	15	36	71	63	89	83	83
Colombia	-	-	-	-	-	-	-	-	-	.	.	.	5	1	1	1	10	50	60	30
Ecuador	-	8	20	41	55	146	232	311	385	454	516	573	625	671	711	745	764	764	849	870
Brazil	15	47	80	112	144	176	209	241	273	305	338	370	400	400	400	500	600	700	600	550
Asia																				
Thailand	-	-	-	<0.5	1	2	100	100	113	157	280	1,153	3,102	2,456	4,513	11,837	10,912	7,949	6,503	7,766
Malaysia	-	-	-	6	15	24	33	41	48	55	87	51	67	84	79	5	68	128	137	145
Indonesia	4,900	4,300	3,065	2,516	1,940	2,759	4,193	3,691	3,746	3,139	3,427	3,649	4,320	3,483	3,747	4,010	5,086	6,196	5,442	5,888
Taiwan Province of China	-	-	-	-	-	-	-	-	-	-	1,477	706	1,315	697	1,382	1,354	4,355	6,725	11,607	16,196
India	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	150	198	196
China	.	.	.	.	.	.	.	.	.	.	.	.	-	-	-	-	-	-	-	-
Brunei Darussalam	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	17	31	29	20	73	89	65	31	19	44	20	20
Bangladesh	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Atlantic, Western Central																				
Venezuela	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	<0.5	1	2	23	31
Oceania																				
French Polynesia	-	-	-	-	2	3	3	3	6	6	13	7	13	14	13	17	20	20	18	15
Fiji Islands	-	-	-	-	-	-	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1	2	4
Other	0	0	0	0	0	1	2	4	7	8	13	19	75	58	64	44	42	31	27	24

General notes: [a] ... = Data not available; unobtainable; data not separately available but included in another category, - = Nil or zero, 0 = More than zero but less than half the unit used, nei = Not elsewhere included, F = FAO estimate from available sources of information, R = Repetition of data previously reported by the country.

Table 1.1 (cont.)

Inland waters	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002
Africa											
Senegal	6	10	11	20	25	21	20	50	-	-	-
Mauritius	55	42	63	53	62	44	28	25	31	19	27
America, North											
United States of America	147	159	159	-	-	-	-	-	-	44	54
Puerto Rico	199	112	100	20	1	2	.	7	12	13	5
Martinique	38	45	40	40	35	44	25	20	19	19	19
Mexico	167	175	124	92	90	112	37	32	45	48	29
Jamaica	30	30	30	40	40	40	40	40	10	10	.
Guatemala	35	32	76	99	5	75	52	43	46	30	8
Honduras	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Guadeloupe	35	40	26	30	30	20	14	20	14	14	14
El Salvador	8	9	9	10	21	14	16	17	9	3	4
Dominican Republic	164	739	961	86	90	27	87	16	82	62	78
Costa Rica	7	10	13	15	78	78	87	90	8	10	5
America, South											
Peru	1	1	212	100	31	23	45	16	13	6	7
French Guiana	-	-	-	-	-	7	18	25	25	25	25
Colombia	55	10	10	-	-	-	-	-	-	-	-
Ecuador	850	.	.	.	.	.	.	.	.	.	.
Brazil	650	700	700	341	486	446	279	227	4,531	4,250	4,043
Asia											
Thailand	10,306	9,204	10,124	7,792	8,000	7,856	4,764	8,494	9,917	12,067	31,174
Malaysia	94	52	46	79	114	143	281	653	1,338	752	535
Indonesia	6,163	4,923	5,393	5,524	6,126	5,208	5,362	5,937	5,449	5,185	5,640
Taiwan Province of China	7,665	5,475	6,556	8,467	7,354	7,551	8,165	7,223	8,149	6,859	7,026
India	455	178	311	.	178	1,500	3,900	7,000	16,600	24,230	30,500
China	-	-	-	-	37,363	42,851	61,868	79,055	97,420	128,338	113,743
Brunei Darussalam	16	11	3	5	14	17	35	26	23	16	13
Bangladesh	.	.	.	1,596	4,185	4,534	5,751	5,394	5,504	9,471	7,998
Atlantic, Western Central											
Venezuela	22	25	13	31	30	30	.	.	.	.	.
Oceania											
French Polynesia	11	8	8	6	4	7	5	5	4	4	4
Fiji Islands	6	85	87	91	93	40	20	2	5	5	5
Other	23	26	28	27	29	34	7	6	10	27	54

General notes: [a] ... = Data not available; unobtainable; data not separately available but included in another category, - = Nil or zero, 0 = More than zero but less than half the unit used, nei = Not elsewhere included, F = FAO estimate from available sources of information, R = Repetition of data previously reported by the country.

1.1 Taxonomy

The taxonomic definition of the giant freshwater prawn, *M. rosenbergii* is shown below

Phylum Arthropoda

Class Crustacea

Order Decapoda

Suborder Natantia

Family Palaemonidae

Genus *Macrobrachium*

Species *rosenbergii*

Scientific name: *Macrobrachium rosenbergii* (De Man, 1879)

Common name: Giant freshwater prawn, giant river prawn

1.2 Habitat and life cycle

M. rosenbergii lives in tropical freshwater environments that are influenced by adjacent brackishwater areas, due to the fact that its larval development must take place in the brackishwater (Sandifer et al., 1975). Some species of *Macrobrachium* prefer clear water rivers while others, including *M. rosenbergii,* are often found in turbid conditions (New and Singholka, 1985). Successful mating can only take place between ripe females, which have just completed their pre-mating moult and hard-shelled males. All types of males are capable to fertilize females but their behavior is different (Ling, 1969). After the pre-mating moult process, courting and mating commence. Courting behavior involves stroking, movements of the antennae and male chelipeds touching the female, and raising the body, which lasts a few minutes until the female accepts the male. Mating is preceded by a cleaning act, in which the male holds the female and carefully cleans her ventral thoracic region (Ling et al., 1961). The copulation of adults results in the deposition of a

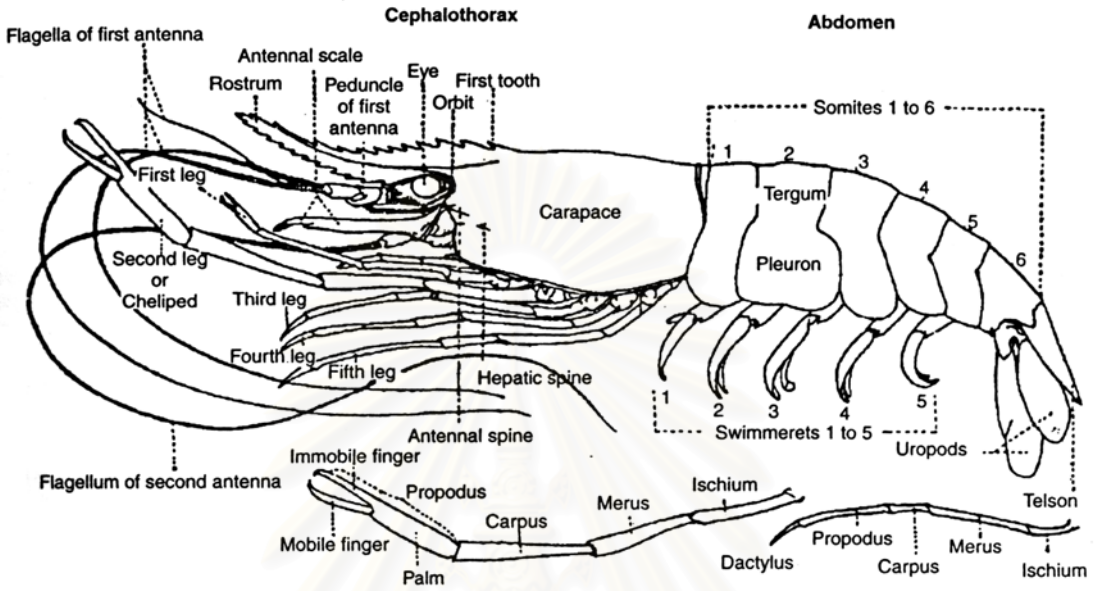

Figure 1.1 The external features of *Macrobrachium rosenbergii* (Source: Cowles, 1914).

spermatophore on pre-cleaned area underside of the thoracic region of the female's body (between the walking legs).

Fully mature females migrate downstream from freshwater into estuarine areas, satisfying for survival and early development, where the eggs hatch as free-swimming larvae (Fig. 1.2). From egg hatching until metamorphosis into postlarvae (PL), the planktonic larvae pass through several zoeal stages (Ling, 1969). The diet of larvae is principally zooplankton (mainly minute crustaceans), very small worms, and the larval stages of mollusks and other crustaceans (New and Singholka, 1985).

After metamorphosis PL exhibit a more benthic life style and begin to migrate upstream towards the freshwater. Postlarvae are also able to walk, not only on the substratum but also crawling over stones at the shallow edges of rivers. The juveniles exhibit nocturnal swimming activity, probably to take advantage of pelagic food resources, whereas during the day they settle on the bottom and crawl (Scudder et al., 1981). Postlarval and adult *M. rosenbergii* are omnivorous, eating algae, aquatic plants, mollusks, aquatic insects, worms, and other crustaceans (Ling, 1969). Cannibalistic behavior may occur if food becomes insufficient, and/or in overpopulated ponds. Adult prawns are active at night (Ling, 1969; Nakamura, 1975), while during the day prawn rearrange their positions to remain in shaded areas (Karplus and Harpaz, 1990).

1.3 Sexual dimorphism

Nagamine and Knight (1980) reported that *M. rosenbergii* can be sexually distinguished with the first appearance of gonopores in juveniles, at 5.9 mm (carapace length) for males and 7.6 mm for females. Male gonopores are situated at the base of the coxae of the fifth pereiopods while female gonopores appear as oval apertures on the coxae of the third pereiopods. New and Singholka (1985) illustrated that the ventral side of the first abdominal somite in male *M. rosenbergii* has a central lump or point, which can be felt with the finger. This feature is absent in females. Mature females have proportionally smaller heads and claws than males (Sandifer and Smith, 1985). They exhibit a typical brood chamber, formed by the first, second and third abdominal pleurae.

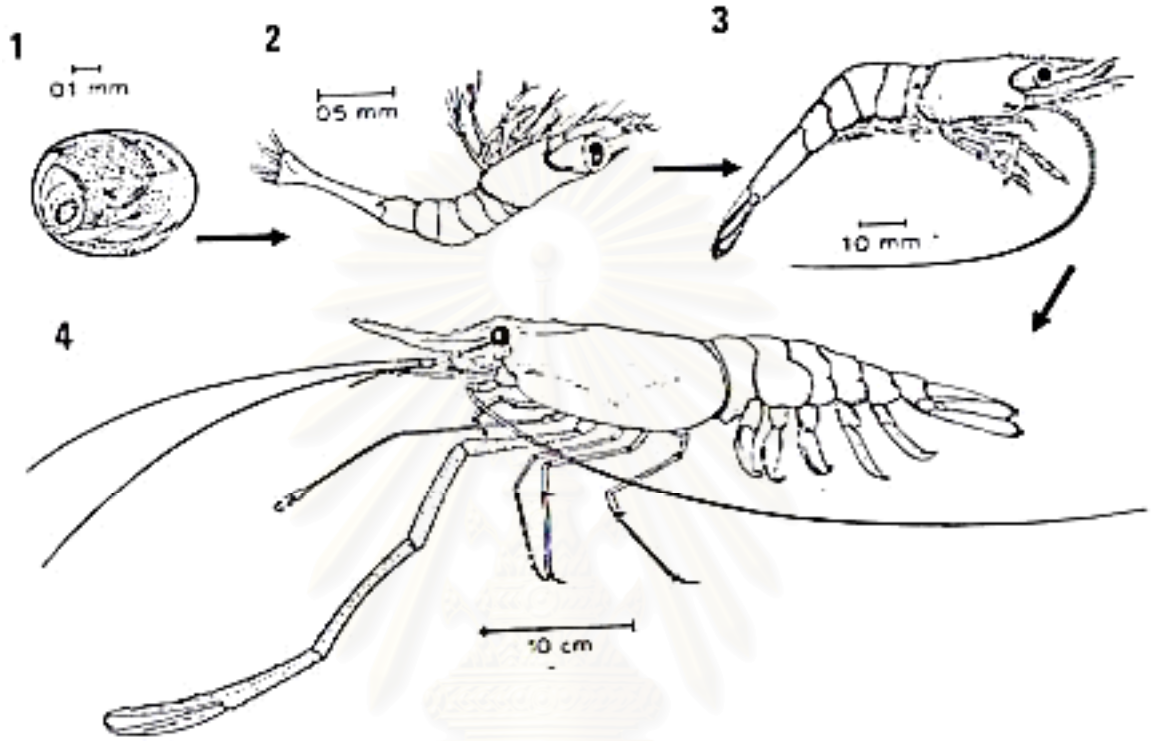

Figure 1.2 Life cycle of *Macrobrachium rosenbergii* (New and Singholka, 1985).

Macrobrachium also exhibit reproductive setae on the pleopods and thorax, which are functionally distinct. The ovipositing setae, which are mostly permanent, on the coxae of the last three pairs of pereiopods and pleopods guide the eggs during spawning, and the ovigerous setae, which only occur following a pre-spawning moult and are used to secure the eggs to the pleopods for brooding (Nagamine and Knight, 1980).

Females *M. rosenbergii* when fully mature can lay between 80,000 and 100,000 eggs in each spawning. However, their first broods are often not more than 5,000 - 20,000 (New and Singholka, 1985). Ovaries frequently ripen again while eggs are being carried in the brood chamber. Male prawns, except small males (SM), are easily recognised by longer and stronger chelipeds with larger spines than the females. Apart from these characteristics, and the presence of gonopores, males may also be recognized by the appendix masculina, a spinous process adjacent to the appendix interna on the endopod of the second pleopod (Sandifer and Smith, 1985).

1.4 Major male morphological characteristics of *M. rosenbergii*

1.4.1 External morphology

Male populations are composed of a large fraction of small prawns and a fraction of large prawns. Prawn size variation actually reflects a complex population structure of this species. Three sexually mature male morphotypes (small male, orange claw male and blue claw male), that differ in their morphology, physiology and behavior are found.

The blue claw (BC) males are relatively large and possess extremely long blue claws, covered with longer and stronger spines, with high claw to body length ratio. The BC male was the only male type of this species described during the early studies of its biology (Ling, 1969).

The second type of male possesses short delicate claws, often with little pigment and translucent, was first described among pond populations (Fujimura and Okamoto, 1972). These stunted small males (SM), with the lowest ratio of claw to body length (Kuris et al., 1987) were initially differentiated from the younger juveniles by their greater age.

The third male type, the orange claw (OC) male, was first described by Sandifer and Smith (1977), who emphasised the golden colour of its claws and their length apprimately 30 to 70% shorter than those of BC males). These OC males are spineless and often orange-coloured claws, with lower claw to body length ratio than that of BC male.

Kuris et al. (1987) provided an operational definition of male morphotypes by means of allometric growth techniques supplemented with observations of colour and spination. Transition from the SM to the OC morphotype was gradual, and a recognizable intermediate form, the weak orange claw (WOC), was identified and differentiated from the SM and the strong orange claw (SOC). All four male morphotypes exhibited linear and highly correlated associations of the logarithmically transformed relationship of propodus and carpus length to carapace length. Spination was found to be a reliable discriminator between BC and OC males.

Another important physiological characteristic of each male morphotype is its growth rate. Different male morphotypes in a monitored developing population had significantly different growth rates (Ra'anan et al. 1991). The OC males showed the highest growth rates (relative growth rate, R = 1.22 ± 0.4% per day) which decreased with the increase in the natural logarithm of body weight. The BC males had the lowest growth rate (R = 0.07 ± 0.04% per day) which was relatively constant over size. Small males had an intermediate growth rate (R = 0.82 ± 0.33 per day), which relatively constant over size.

1.4.2 Internal morphology and physiology

Most comparative studies on the anatomy and physiology of the male morphotypes of *M. rosenbergii* focused on the reproductive system because different reproductive strategies are adopted by different male morphotypes (Telecky, 1984; Ra'anan and Sagi, 1985; Sagi, 1990).

The male reproductive system in *M. rosenbergii* consists of a pair of testes, vasa deferentia, ejaculatory ampullae and genital pores. The testes are whitish-paired structures united at their anterior ends that lie mid-dorsally in the cephalothorax and are composed of numerous branching spermatogenic lobules. The spermatogenic zone is located along the wall of each lobule and its lumen is filled with developing and mature spermatozoa

that are non-flagellated, non-motile and resemble everted umbrellas (Nagamine et al., 1980a). The vasa deferentia are two symmetrical elongated tubes, which consist of a short proximal portion attached to the testes, a long convoluted portion and a straight distal portion ending with the ejaculatory ampullae. This structure consists of an enlargement of the vas deferens encircled by a thick layer of transverse and longitudinal muscles, and opening at the base of the fifth pereiopod. The androgenic gland is attached to each ampulla.

There are also a number of internal morphological and physiological differences, as well as differences in moult frequencies. SMs have relatively large testes that both produce and store sperm. The testes of BC males serve mainly as sperm reservoirs. The orange claw male forms (further divided to WOC, SOC and TOC in some literatures) represent a series of gradual changes between SM and BC.

The abundance of mature sperm found in the testes of SM declines and almost disappears in the early OC males stages. At the same time, the rate of production of spermatocytes (cells from which spermatozoa arise) increases as the SM moults into the OC males phase. The OC males phase is also characterized by frequent moulting, a process has shown to be strongly correlated with spertmatogenesis (i.e. monitored by *in vivo* incorporation of [^{3}H]-thymidine, Sagi et al., 1991). Orange claw males in the premoult phase had a rate of [^{3}H]-thymidine incorporation about four times higher than that in the intermoult phase. In the more advanced OC male phases, the spermatogenic zone becomes thinner as the testicular cylinders become filled with spermatocytes (Sagi et al., 1988; Sagi, 1990).

Since the male morphotypes differ in their growth rates (Ra'anan and Cohen 1985), comparative studies have been carried out on the relative weights of their midgut glands and hepatopancreas (Sagi and Ra'anan, 1988). These organs play a major role in food assimilation (Dall and Moriarty, 1983) and mobilization of energy during moulting (Skinner, 1985). Sagi and Ra'anan (1988) suggested that the slow-growing SM and BC males have the lowest relative midgut gland weight, while the OC males have intermediate values.

1.4.3 Mating behavior

Sandifer and Smith (1977) reported that the OC male morphotype of *M. rosenbergii* was less aggressive than the BC male. Additionally, Ra'anan and Sagi (1985) concluded in their study on male alternative mating strategies that the BC males were aggressive, dominant and territorial whereas the OC males were aggressive, sub-dominant while non- territorial and SM males were submissive and non-territorial.

Mating behavior in the three male morphotypes has been described. Chow et al. (1982) divided the mating behavior of BC males into four phases: contact, seizure, mounting and copulation. The male guards following mating the female for 2 to 3 days, but during that time the female gradually moves away from his close vicinity. The OC males are capable to fertilize females but having an incomplete sexual behavior, never grooming or protecting a female. The SM mates with females by sneaking between a receptive female and her guarding BC male (Ra'anan and Sagi, 1985). The SM moves from one side of the female, over her back to the other side and finally'slipping their abdomen beneath her to apply their spermatophore. Following this act, the SM rapidly retreated before the return of the BC male (Telecky, 1984). In a recent study, only the SM but not the BC or the OC males were found to be chemically attracted to females shortly after their pre-mating moult (Thomas, 1998).

Ra'anan and Cohen (1985) represented the normal male developmental pathway from SM to BC through OC. Early studies on the effect of prawn density on male morphotypes which were carried out over a relatively narrow range (0.5 - 1.5/m^2), suggested fixed proportions of male morphotypes (SM:OC:BC = 5:4:1) irrespective of prawn density. Subsequently, Karplus et al. (1986) studied such effects covering a wider density range (1-4/m^2) and revealed that the proportions of the male morphotypes change significantly with density. The changes reflected the correlation between stunted prawns and density. At high densities, large fractions of the population are in close contact with BC males, which inhibit their growth. An inverse relationship between prawn density and mean size of the various male morphotypes has been described in prawn populations covering a wide range of stocking densities (Cohen et al., 1981, 1983, and Karplus et al., 1986). The only male morphotype that showed no effect of density on its mean weight

was the SM. The growth of SM was negligible suppressed at all densities (Cohen et al., 1981; Karplus et al., 1986).

1.5 Sex ratio

Higher proportions of female than male were found in *M. rosenbergii* populations when prawns were raised in different geographical areas at a wide range of densities, in earthen ponds and tanks (D'Abramo et al. 1986; Karplus et al., 1986; Lin and Boonyaratpalin, 1988; Siddiqui et al., 1997). Three different mechanisms were suggested for this circumstance. Firstly, the environmental conditions may affect sex determination and favour the development of females. Secondly, females may already outnumber males at stocking. Thirdly, selective male mortality may occur (Smith et al., 1978).

Malecha et al. (1992) reported a higher frequency of females in the F_1 progeny resulted from mating between six neomales and normal females (1:3.20 of male:female). Moreover, six crosses between F_1 sibling resulted in F_2 broods with a cumulative sex ratio of 1:3.34. These results support a hypothesis that sexes of *M. rosenbergii* is determined chromosomally with females heterogamous (ZW) and males homogamous (ZZ). Nevertheless, the environmental effects may cause biased sex ratio or alternatively, sex determination is more complex than the simple ZW/ZZ system in this species.

Karyotyping of *M. rosenbergii* chromosome was studied from the second meiotic metaphase of the fertilized egg. The diploid chromosome number is 118 and no marked distinction of the sex chromosomes was observed (Damrongphol et al., 1991).

1.6 The role of androgenic glands on sex differentiation of *M. rosenbergii*

Cronin (1947) described that sexual differentiation in crustaceans is largely regulated by a gland called the androgenic gland (Charniaux-Cotton, 1954). In decapods this gland is usually located at the sub-terminal portion of the sperm duct. The cells may be arranged as thin, parallel and anastomosing cords (Carpenter and DeRoos 1970) or in a compact, lobed structure (Kleinholz and Keller, 1979). A combination of the two structures was found in *M. rosenbergii,* in which the androgenic gland is composed of strands of cells surrounded by a thin layer of connective tissue, forming a pyramidal cluster loosely associated with the posterior portion of the ejaculatory duct (Veilh and Malecha, 1983).

Charniaux-Cotton (1954) suggested a regulatory role for the androgenic gland and showed that bilateral androgenic gland ablation (andrectomy) in the amphipod, *Orchestia gammarella* blocked differentiation of secondary male characteristics and resulted in decreased spermatogenesis. Once the external male sexual characteristics are formed in gonochoristic shrimp, the androgenic gland is not required for their maintenance (Touir, 1977). Further studies have revealed that the androgenic gland has played a role in the development and maintenance of male characteristics in many malacostracan crustaceans.

Nagamine et al. (1980a and 1980b) demonstrated that the presence of the androgenic glands in *M. rosenbergii* is necessary for the development and regeneration of male secondary sexual characteristics. Their studies were, carried out before the existence of the different male morphotypes of *M. rosenbergii* had been recognized.

The important roles of androgenic glands in the male developmental pathway and growth of *M. rosenbergii* were further analyzed (Sagi et al., 1990). The androgenic gland was essential for the continuation of the morphotypic differentiation process (Fig. 1.3). When andrectomy is performed on SOC males, differentiation continues and leads to the BC morphotype. In contrast, andrectomized SMs differentiate into SOC males but further differentiation into BC males is prevented. When this was performed on juveniles, a wide range of abnormalities in gonadal development was observed, which depended on the age at which the andrectomy had been performed.

Reduced testicular development was observed in *M. rosenbergii* male andrectomised at a relatively old age. In males andrectomised when younger, partly testicular and partly ovarian gonads ('ovotestes') or abnormally lobulated ovaries developed (Snir, 1992).

A high degree of feminization, which included initiation of oogenesis and development of oviducts and female gonopores, occurred in mature *M. rosenbergii* males that had been andrectomized in the youngest developmental stage. Males andrectomized in later developmental stages were either partially feminized or not feminized (Nagamine et al., 1980a).

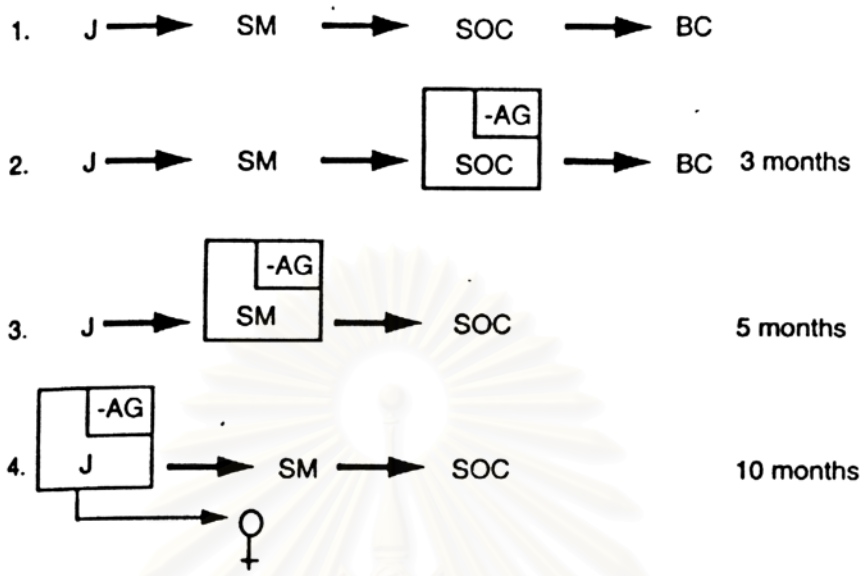

Figure 1.3 The effect of andrectomy on the development pathway of male *M. rosenbergii* (Sagi, 1990).

Abbreviations: J = juvenile; SM = small male; SOC = strong orange claw; BC = blue claw male; AG = androgenic gland ablation. The number of months between the andrectomy and the end of the experiment are indicated.

Reimplantation of the androgenic gland into andrectomized *M. rosenbergii* males reversed the effect of the andrectomy. Androgenic gland implantation masculinized female recipients, as manifested by the development of the *appendices masculina,* the male gonopore complex, mature masculine chelipeds and initiation of spermatogenesis in the ovaries (Nagamine et al., 1980a and 1980b).

The androgenic gland also has a role in the growth regulation of *M. rosenbergii,* as indicated by the reduction in growth rates of andrectomised SM and OC males compared with untreated shrimps. In general, andrectomised males exhibited a reduced growth rate with the similar level of normal females (Sagi et al., 1997).

Sexual differentiation in decapods is controlled by the presence of the androgenic hormone (AGH) which induces the male characteristics of the genital tract. AGH promotes the development of not only the primary, but also the secondary male sex characteristics (Charniaux-Cotton and Payen, 1985).

Recently, AGH was extracted and purified from the androgenic glands (AGs) of the isopod (*Armadillidium vulgare*) by high-performance liquid chromatography (Okuno et al., 1999). AGH consisted of two peptide chains and their *N*-terminal amino acid sequences were determined. A cDNA encoding AGH was cloned by PCR and sequenced. The cDNA had an open reading frame of 432 bp, which encoded a preproAGH consisting of a signal peptide (21 residues), B chain (44 residues), C peptide (46 residues), and A chain (29 residues). Upon processing, A and B chains might form a heterodimer interlinked by disulfide bonds. A chain possessed a putative N-linked glycosylation site. A Northern blot analysis using the AGH cDNA as a probe detected a hybridization signal of 0.8 kb in the RNA of the AGs.

Three kinds of polyclonal antibodies were raised against the different components of the pro-Arv-AGH. These included the whole molecule of the recombinant pro-Arv-AGH expressed in *Escherichia coli,* the *N*-terminal nanopeptide of the B chain, and the *N*-terminal octapeptide of the A chain. All of these antibodies illustrated AGH activity in AG extracts (Okuno et al., 2001).

Ohira et al. (2003) cloned cDNAs encoding AGH precursors from two additional terrestrial isopods, *Porcellio scaber* and *P. dilatatus.* Partial cDNA fragments encoding AGH in the former (Pos-AGH) and the latter (Pod-AGH) were amplified by RT-PCR using degenerate oligonucleotide primers designed from the conserved amino acid sequence of *A. vulgare* AGH (Arv-AGH). Full-length cDNAs were obtained by 5'- and 3'-RACE-PCR. Both AGH precursors consisted of a signal peptide, B chain, C peptide and A chain, and exhibited the same organization as that of Arv-AGH. The amino acid sequences of the A and B chains, which comprise mature AGH peptide, were highly conserved among the three species, while that of the C peptide showed only low sequence similarity. Northern blot analysis indicated that each cDNA fragment used as a probe specifically hybridized with a single band (0.75 kb) in mRNA extracted from whole male reproductive organs. Moreover, tissue-specific gene expression by RT-PCR revealed that

both AGH transcripts were detected only in the testes that carry the androgenic glands, but not in that from testis, seminal vesicle, vas deferens, or hepatopancreas.

M. rosenbergii is one of the important aquaculture species in Asia. Genetic improvement to elevate the culture efficiency and the production of this species is of interest. Although the wide range of size differences in prawn populations is a typical for *M. rosenbergii* culture, this is a major obstacle to increase profitability. As shrimp price is size - dependent. Therefore, understanding sexual biological system of *M. rosenbergii* is necessary for genetic improvement and breeding programs of this species. Application of molecular markers is probably useful for primary understanding of sex determination/differentiation system of *M. rosenbergii*.

1.7 Molecular biological approaches used in this thesis

The introduction of the polymerase chain reaction (PCR) has revolutionized molecular biological researches. PCR is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA (Fig. 1.4). The use of PCR for molecular genetic studies in aquatic organisms is increased dramatically. This technique has facilitated the analysis of sequence variation and has enabled a new PCR-based technique to develop for wider applications. The basic knowledge of a particular region from a few taxa (conserved but, however specific sequences) permits the amplification of the same DNA sequences from distant related species.

The PCR technique involves three steps

(a) Denaturation of double stranded DNA by heating.

(b) Annealing of extension primers to the target sites.

(c) Primer extension, in which strands complementary to the region between the flanking primers are synthesised under the influence of a thermostable DNA polymerase (usually *Taq* polymerase).

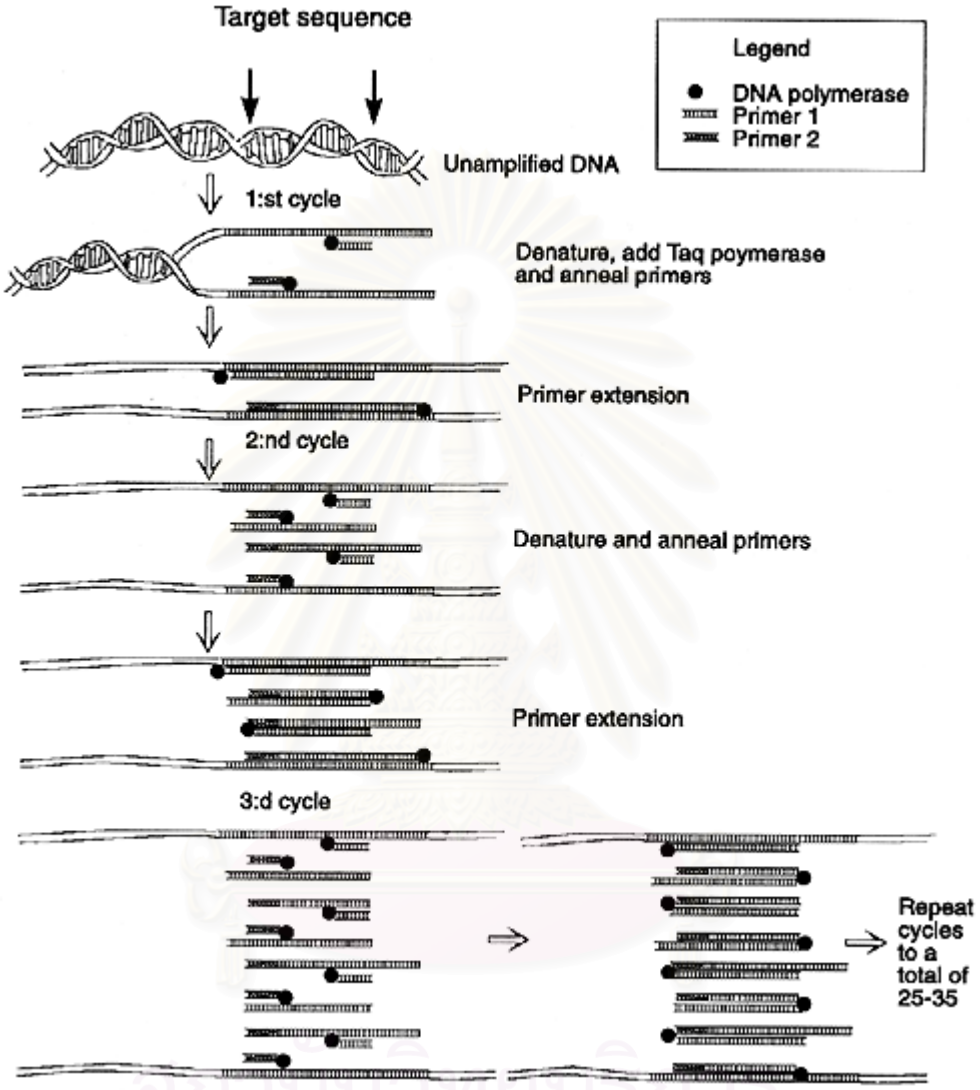

Figure 1.4 General illustration of the polymerase chain reaction for amplifying DNA.

The reaction is carried out repeatedly through step a-c. Initially, synthesis of amplified products will go beyond the sequence complementary to the other primer binding site. This happens at both primers annealing sites on both DNA strands resulting in a synthesis of longer but completely products (product with primer at only one end).

In the subsequent cycle, the sample and newly synthesized strands serve as DNA template. The amount of DNA in the amplified region (which has its end defined by the position of primers) will increase exponentially whereas longer sequence and the starting DNA will accumulate in a linear fashion. The required products are obtained within only a few hours.

1.7.1 Amplified Fragments Length Polymorphism (AFLP)

Amplified Restriction Fragment Polymorphism (AFLP) technology is initially developed and used for fingerprinting genomic DNA of various organisms (Vos et al., 1995). DNA fingerprinting is used for determining the identity of a specific DNA sample or to assess the relatedness between samples. Fingerprints are also used as the source for genetic markers to generate linkage maps or to identify molecular markers linked to phenotypic traits and/or genetic loci.

AFLP was developed to increase the potential of restriction fragment length polymorphism (RFLP) and increase reproducibility of randomly amplified polymorphic DNA (RAPD) by selective amplification of a random array of restriction fragments ligated to linkers of known sequence. Initially, genomic DNA is digested with a rare-cut restriction enzymes (usually *Eco* RI) and a frequent-cut restriction (usually *Mse* I) enzymes and ligated with double-stranded DNA adaptors to generate template DNA for amplification and used for the priming sites of PCR amplification.

PCR amplification was carried out for 2 times; preselective and selective amplification. The former was carried out by adding a single known base to the 3’ end of the primer complementary to either adaptor. The product from the primary amplification is diluted and further amplified by primers having 3 added nucleotides of the 3’ end. Since these bases extend pass the ligated sites into the DNA fragment, the numbers of amplified fragments are significantly reduced and can be simply analyzed by polyacrylamide gel electrophoresis (Fig. 1.5).

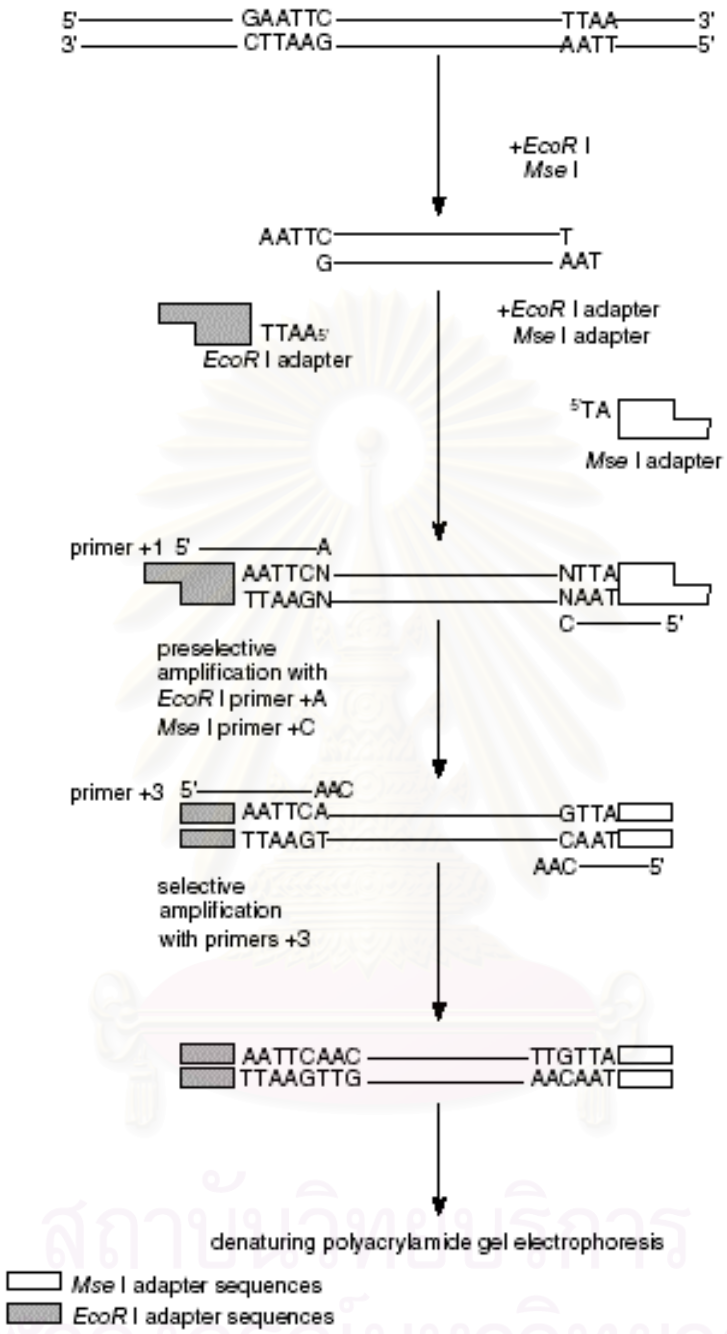

Figure 1.5 A schematic diagram illustrating principles of AFLP analysis (AFLP® Analysis System I, USA).

The main advantages of AFLP are its reproducibility due to specificity of the PCR primer and high stringency of the amplification reaction. Like RAPD-PCR, AFLP analysis does not require the prior knowledge about genome sequences of species under investigation. The high number of potential polymorphic fragments detected in a single AFLP reaction make this technique ideal for various application including construction the genetic linkage maps of species that their genome are not well studied.

1.7.2 DNA sequencing

Polymorphism at the DNA level can be studied by several methods but the direct strategy is determination of nucleotide sequences of a defined region. There are two general methods for sequencing of DNA segments: the “chemical cleavage” procedure (Maxam and Gilbert, 1977) and the “chain termination” procedure (Sanger, 1977). Nevertheless, the latter method is more popular because chemical cleavage procedure requires the use of several hazardous substances.

DNA sequencing is the most optimal method for several genetic applications. This technique provides high resolution and facilitating interpretation. DNA fragments generated from PCR can be directly sequenced or alternatively, those fragments can be cloned and sequenced. This eliminates the need to establish a genome library and searching of a particular gene in the library.

The enzymatic sequencing approach has presently been developed to automated method (Fig. 1.6). DNA sequences can be detected using a fluorescence-based system following labeling with a fluorescence dye. PCR allow the possibility to isolate homologous DNA sequences from any organism of interest with unprecedented speed. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

DNA sequencing is particularly suitable for population genetic studies of various species. The DNA sequence of each individual is directly compared. Levels of genetic diversity between individuals within geographic samples, between geographic samples and species can be reliably examined. In addition, DNA sequencing can be used in coupling with RAPD and AFLP markers to convert dominant markers to sequence-characterized amplified region (SCAR) markers that are co-dominantly segregated.

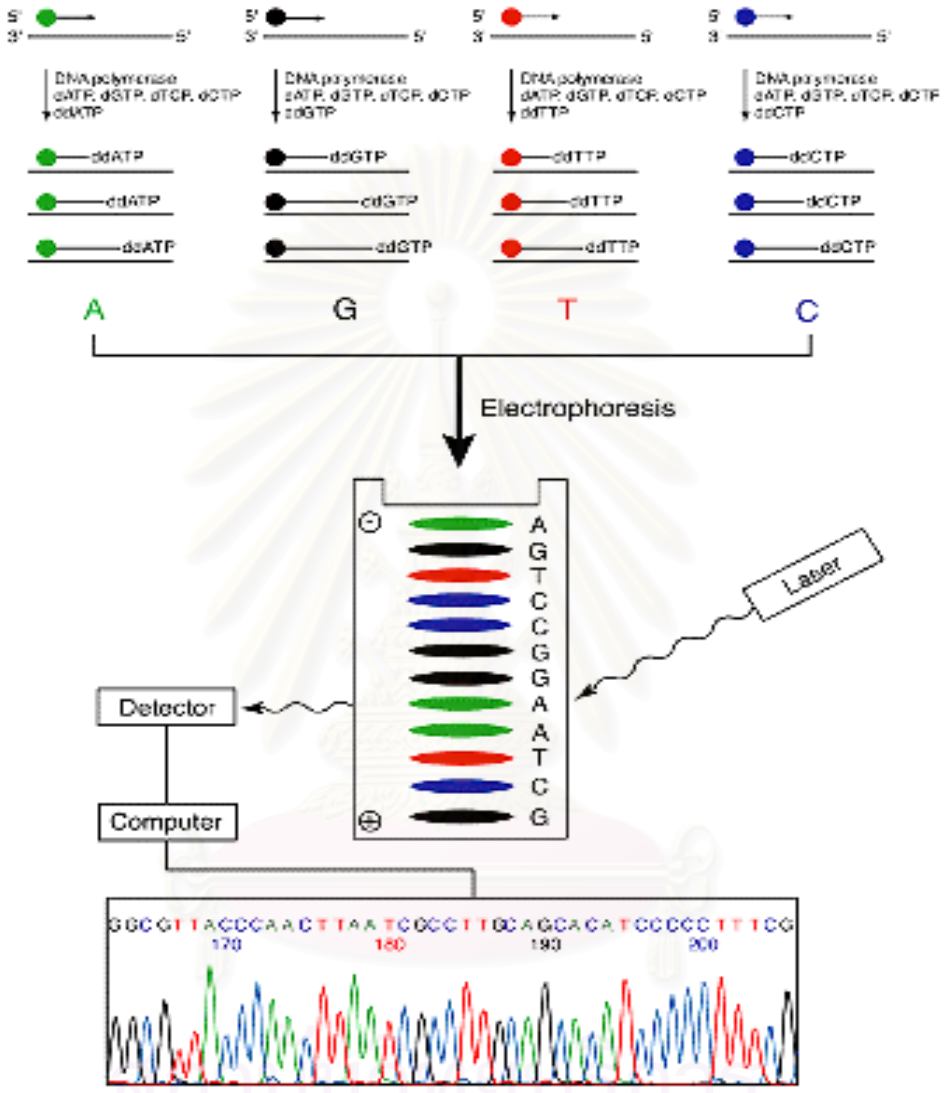

Figure 1.6 A schematic diagram illustrating principles of Automated DNA sequencing.

1.7.3 Reverse transcription-polymerase chain reaction (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. 1.7). It is a direct method for examination of gene expression of known sequence transcripts in the target species. Alternatively, RT-PCR can also be used to identify homologues of interesting genes by using degenerate primers and/or conserved gene-specific primers from the original species and the first strand cDNA of the interesting species as the template. The amplified product is further characterized by cloning and sequencing.

1.7.4 RNA arbitrary-primed (RAP)-PCR

RAP-PCR is a comparable method of conventional randomly amplified polymorphic DNA (RAPD) but the first strand cDNA template rather than genomic DNA was used as the template in the amplification reaction. RAPD-PCR established in 1990 (Williams et al., 1990 and Welsh and McClelland, 1990).

The amplification conditions in RAPD differ from the standard PCR in that only single random primer (usually 10 mer with GC content usually at least 50%) is employed. RAPD is utilized to amplified target DNA on the basis that the nuclear genome may contain several priming sites closed to one another that is located in an inverted orientation. Accordingly, the primer is utilized to scan genome for the small inverted sequences resulting in amplification of DNA segments of variable length.

Subsequently, RNA fingerprinting by arbitrary primed PCR (RAP-PCR) (Welsh et al., 1992) was introduced. The technique required reverse transcription of the target total RNA (or mRNA) to the first strand cDNA (by oligo dT or short and long random nucleotides). The synthesized cDNA was included as the template in the PCR reaction composing of the single primer or a combination of primers. The amplification products are size-fragtionated through agarose or denaturing polyacylamide gels and detected by either radiolabeled or non-radiolabeled (Etbr or silver staining) detection methods.

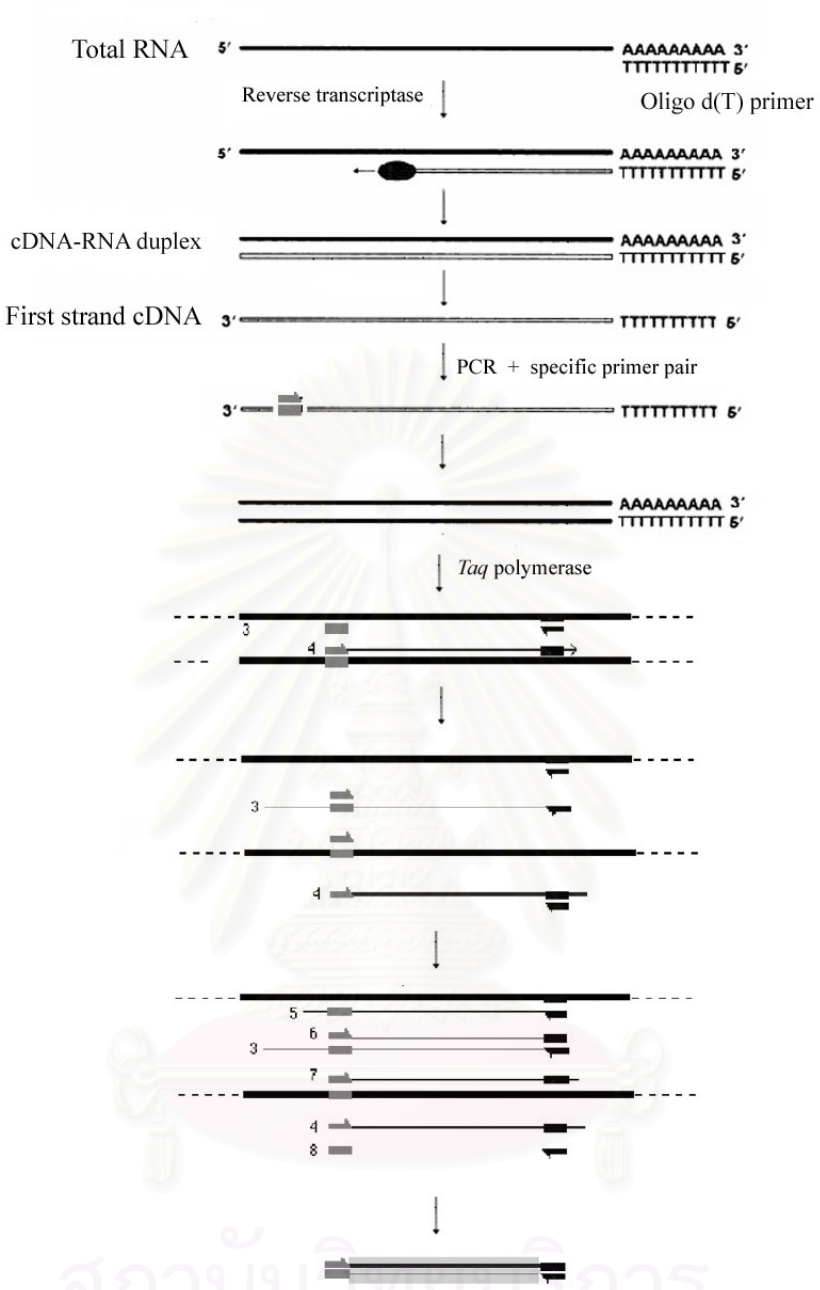

Figure 1.7 Overall concepts of the RT-PCR procedure. During first-strand cDNA synthesis an oligo d(T) primer anneals and extends from sites present within the total RNA. Second strand cDNA synthesis primed by the 18 – 25 base specific primer proceeds during a single round of DNA synthesis catalyzed by *Taq* polymerase. These DNA fragments serve as templates for PCR amplification.

The intensity of RAP-PCR bands produced from different experimental samples is considered. Bands that are present in one sample and absent in another or bands that exhibit large differences in the intensity across the experimental treatments should represent potentially differentially expressed mRNA transcripts and required further characterization. The fragments can be cloned and sequenced. The expression levels of interesting bands are then examined using specific primers.

1.7.5 Single-stranded conformation polymorphism (SSCP) analysis

Single-stranded conformation polymorphism (SSCP) analysis was originally described by Orita et al (1989). SSCP is one of the most widely used for the detection of mutations and variation of the DNA (deletions, insertions and single nucleotide polymorphism, SNP). The amplified PCR product (usually less than 400 bp in length) is denatured and loaded into low cross-link non-denaturing polyacrylamide (with or without glycerol supplementation). The principle of this technique relies on different mobility due to differential folding of the single stranded DNA (Fig. 1.8).

The major advantage of SSCP is that many individual PCR products may be screened for variation simultaneously. Heteroduplexes can occationally resolved from homoduplexes and give additional information on the presence of variants. Therefore, SSCP is regarded as one of the potential techniques that can be used to detect low polymorphism in various species prior to confirmation of the results by nucleotide sequencing. The other advantage of SSCP is that small PCR amplicons are required. This small sizes of PCR products are relative easy to amplify.

Nevertheless, the disadvantages of SSCP are reproducibility of the technique because SSCP patterns are affected by temperature and degree of cross-linking. Additionally, multiallelic patterns of some nuclear DNA markers may the SSCP patterns complicate for precise estimation of allele frequencies precise.

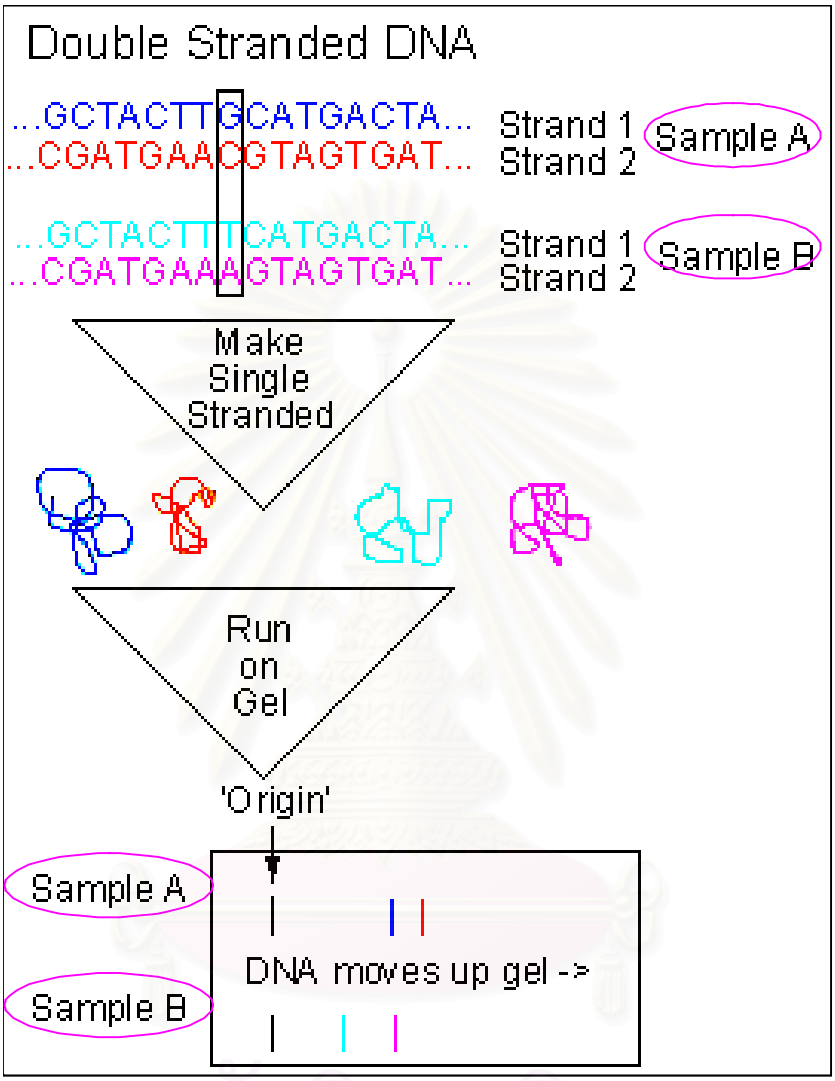

Figure 1.8 A schematic diagram illustrating principles of SSCP analysis

1.8 Molecular studies of sex-related markers at the DNA and cDNA markers

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-

determining 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.

Amplified fragment length polymorphism (AFLPs) is a popular approach to identify DNA markers related phenotypic characters and for genomic mapping in agricultural species particularly for which microsatellites are unavailable. AFLP have been used to construct the genetic linkage maps in several aquatic species, including catfish (Liu et al, 1998), rainbow trout (Young et al., 1998), tilapia (Kocher et al., 1998; Agresti et al., 2000), medaka (Naruse et al., 2000), eastern oyster (Yu and Guo, 2002), Pacific oyster (Li and Guo, 2004), Kumura shrimp (Moore et al., 1999 and Li et al, 2003), giant tiger shrimp (Wilson et al., 2002) and Japanese flounder (Coimbra et al., 2003).

A genetic linkage map was constructed from an intraspecific cross of the Colorado potato beetle, *Leptinotarsa decemlineata.* The map was made with 172 AFLP markers and 10 anonymous codominant markers segregating among 74 backcross (BC_1) individuals. A pyrethroid-resistance candidate gene, *LdVsscI* was placed onto the linkage map. The sex chromosome was identified by exploiting the XO nature of sex determination in this species based on patterns of *LdVsscI* variation and the codominant markers derived from AFLP markers (Hawthorne, 2001).

Beatty (1964) carried out breeding experiments and suggested that the three-spined stickleback (*Gasterostus aculeatus*) had heterogametic females (ZZ male; ZW female). Klinkhardt and Buuk (1990) karyotyped chromosome of *G. aculeatus* and concluded that no heterochromatic sex chromosomes were observed in this species

Griffiths et al. (2000) isolated sex-specific markers of (*Gasterostus aculeatus*) by AFLP. Pre-sexed specimens from geographically different locations were collected from Edinburgh, Scotland (N = 16), Milngavie, Scotland (N = 5), Silverdale, England (N = 8)

west coast of Canada (N = 6) and varioud population in Japan (N = 10). The nine-spined *Pungitus pungitus* (N = 8) and 15-spined sticklebacks *Spinachia spinachia* (N = 6) collected from Sweden were included as the control. Three males and female individuals were screened for sex-specific AFLP markers. Primers produced bands in one sex were re-screened with additional 6 individuals. Two candidate male-specific markers were found from primers E_{AAG}/M_{CAA} and E_{AAG}/M_{CAG}, respectively. Sequence-characterized amplified region (SCAR) markers were developed. Primers GalF/R yielded a 600 bp fragment in females and both 600 bp and 371 bp fragments in males. Therefore, it was concluded that this species possesses the XX/XY sex differentiation system rather than the ZW/ZZ system previously reported by Beatty (1964).

At the cDNA level, 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 et al., 2003).

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*.

The objectives of this thesis were isolation of sex-specific markers at the genomic level using AFLP analysis. Candidate markers were cloned and characterized. In addition, RT-PCR was carried out to examine the expression levels of homologues of sex-related transcripts from related aquatic species (*P. monodon* and *H. asinina*). In addition, sex-differential expression markers were also identified by RAP-PCR. Candidate RAP-PCR markers were also cloned and characterized. The expression profiles of candidate markers were examined. Polymorphism of identified genomic DNA and cDNA markers were examined by SSCP analysis for further applications (e.g. identification of single nucleotide polymorphism and genetic linkage analysis).

CHAPTER II

MATERIALS AND METHODS

2.1 Samples

The giant freshwater prawn (*M. rosenbergii*) was collected from commercial farms in Chachoengsao and Ayuthaya provinces and from Samyan market, Bangkok. Colors of the 2nd periopod (claw) were used to preliminary differentiate different male morphotypes. The body weight and a ratio of the claw length to the body length and of each shrimp (relative claw length) were measured. Classification of the male morphotypes was carried out using the relative claw length and colour of the 2nd periopods (Ra'anan and Sagi, 1985). Small orange-claw (SOC) orange-claw male (OC) and blue-claw male (BC) individuals showing the ratios of ≤ 1.5, 1.60 – 2.20 and ≥ 2.20 individuals were used in the experiments (See Appendix C). **Notably, SOC in some publications are referred to the strong orange-claw male morphotype whereas others referred SOC as the small orange claw male as consistently used in this thesis.**

A piece of pleopod tissue of each shrimp was used for genomic DNA extraction. The terminal portion of vas deferens containing androgenic glands (males) and oviducts (females), testes and ovaries were used for total RNA extraction.

2.2 Nucleic acid extraction

2.2.1 DNA extraction

Genomic DNA was extracted from a piece of pleopod of each *P. monodon* individual using a phenol-chloroform-proteinase K method (Klinbunga et al., 1999). A piece of pleopod tissue was dissected out from a frozen pleopod and placed in a prechilled microcentrifuge tube containing 500 µl of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl; pH 8.0) and briefly homogenized with a micropestle. SDS (10%) and RNase A (10

mg.ml^{-1}) solutions were added to a final concentration of 1.0 % (w/v) and 100 μg.ml^{-1}, respectively. of The resulting mixture was then incubated at 37 ^{o}C for 1 hour. At the end of the incubation period, a proteinase K solution (10 mg.ml^{-1}) was added to the final concentration of 100 μg.ml^{-1} and further incubated at 55 ^{o}C for 3 – 4 hours. An equal volume of buffer-equilibrated phenol was added and gently mixed for 15 minutes. The solution was centrifuged at 10000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a newly sterile microcentrifuge tube. This extraction process was then repeated once with phenol:chloroform:isoamylalcohol (25:24:1) and twice with chloroform:isoamylalcohol (24:1). The aqueous phase was transferred into a sterile microcentrifuge. One-tenth volume of 3 M sodium acetate, pH 5.2 was added. DNA was precipitated by an addition of two volume of prechilled absolute ethanol and mixed thoroughly. The mixture was incubated at -20 ^{o}C for 2 hours. The precipitated DNA was recovered by centrifugation at 12000 rpm for 10 minutes at room temperature and washed twice with 1 ml of 70% ethanol (5 minutes and brief washes, respectively). After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 100 μl of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The DNA solution was incubated at 37 ^{o}C for 1 - 2 hours and kept at 4 ^{o}C until further needed.

2.2.2 RNA extraction

Total RNA was extracted from androgenic glands and testes of each male, and ovaries and oviducts of each female using TRI REAGENT®. The tissue was transferred to a microcentrifuge tube containing 500 μ1 of TRI REAGENT® (1 ml / 50-100 mg tissue and 2 ml for an androgenic gland (AG) of BC and OC and 1 ml for AG of SOC and oviduct, respectively) and homogenized. Additional 500 μl of TRI REAGENT® were added. The homogenate were 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 12000 g 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 12000 g 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 7500 g 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.

To extract total RNA from testes and ovaries of *M. rosenbergii*, a piece of tissues was immediately placed in a mortar containing liquid nitrogen and ground with the pestle to the fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 µ1 of TRI REAGENT® (1 ml / 50-100 mg tissue) and homogenized. The extraction procedure was then carried out as above.

2.3 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 $\mu g.ml^{-1}$ of double stranded DNA, 40 $\mu g.ml^{-1}$ of single stranded RNA and 33 $\mu g.ml^{-1}$ of single stranded (ss) DNA (Sambrook et al., 2001). Therefore, the concentration of DNA/RNA samples were estimated in $\mu g.ml^{-1}$ by using the following equation,

[DNA] = OD_{260} x dilution factor x 50 (40 or 33 for RNA or ssDNA, 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 approximately 1.8 and 2.0, respectively (Sambrook et al., 2001).

2.4 Synthesis of the first strand cDNA

The first strand cDNA was synthesized from 1.5 µg of total RNA extracted from androgenic glands, oviducts, ovaries and testes of *M. rosenbergii,* using an ImProm-II™ Reverse Transcription System Kit (Promega). Total RNA was combined with 0.5 µg of oligo (dT_{18}) and appropriate DEPC-treated H_2O in a final volume of 5 µl. The reaction was

incubated at 70 ^{o}C for 5 minutes and immediately placed on ice for at least 5 minutes. Then 5x-reaction buffer, $MgCl_2$, dNTP Mix, RNasin was added to final concentrations of 1x, 2.25 mM, 0.5 mM and 20 units, respectively. Finally, 1 µl of ImProm-II^{TM} reverse transcriptase was added and gently mixed by pipetting. The reaction mixture was incubated at 25 ^{o}C for 5 minutes and at 42 ^{o}C for 90 minutes. The reaction was terminated by incubated at 70 ^{o}C for 15 minutes to terminate the 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). The first stranded cDNA was diluted to 500 ng/µl and kept at 20 °C until required.

The first strand cDNA synthesis for RNA-arbitrarily primed (RAP) PCR analysis carried out essentially similar to the conditions described above with the exception that 0.5 µ M oligo (dT_{18}) was replaced with 1 µM of each RAPD primer.

2.5 Agarose gel electrophoresis

An appropriate amount of agarose (or metaphor agarose) was weighed out and mixed with an appropriate volume of 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.3). The gel slurry was boiled in a microwave oven to complete solubilization, and allowed to approximately 60 ^{o}C before poured into the gel mold. A comb was inserted. The gel was left to solidify. When needed, the comb was carefully removed. The agarose gel was submerged in a chamber containing an enough amount of 1x TBE buffer covering the gel for approximately 0.5 cm. Appropriate volumes of PCR products were mixed with the one-fifth 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 (0.5 $\mu g.ml^{-1}$) for 5 - 15 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 - 20 seconds.

2.6 Bulk segregant analysis

For bulk segregant analysis, BSA (Michelmore et al., 1991) was used for rapidly identifying markers linked to any specific gene or genomic region. This technique involves screening for differences pooled samples derived from a segregating characteristic or gender. Each pool, or bulk, contains individuals selected to have identical genotypes for a particular genomic region but random genotypes at loci unlinked to selected region.

The concentration of extracted DNA was spectrophotometrically examined and further adjusted for only the high molecular weight DNA using agarose gel elctrophoresis. The pooled genomic DNA (250 ng) of the first sample set (BC1, $N = 5$; SOC1, $N = 10$ and F1, $N = 10$) was prepare for initial screening of candidate sex-specific AFLP markers. Candidate AFLP markers from the first sample set were screened with the second sample set (BC2, $N = 10$; OC1, $N = 15$ SOC2, $N = 5$ and F2, $N = 20$) of *M. rosenbergii*.

The first strand cDNA template used for RAP-PCR analysis was synthesized from pooled total RNA of BC ($N = 3$), OC ($N = 3$) and SOC ($N = 3$) males and females ($N = 3$).

Detailed information of specimens used in the experiments (e.g.. total length, head length, body length, weight, collected site and ratio of claw length/ body length) is shown in Appendix.

2.7 Amplified fragment length polymorphism (AFLP) analysis (Vos et al., 1995)

2.7.1 Restriction enzyme digestion and adapter ligation

Each bulked DNA (250 ng) was simultaneously digested with 2.5 units of *Eco* RI and *Mse* I in a 25 μl reaction mixture containing 10 mM Tris-HCl, pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate at 37°C for approximately 4 hours. The reaction was terminated by incubation at 70°C for 15 minutes. The *Eco* RI and *Mse* I adaptors (Table 2.1) were ligated to restricted genomic DNA by adding 24 ml of the adaptor ligation solution (*Eco* RI and *Mse* I adaptors, 0.4 mM ATP, 10 mM Tris-HCl, pH 7.5, 10 mM Mg-acetate and 50 mM K-acetate) and 1 U of T4 DNA ligase. The reaction was incubated at 16°C for approximately 16 hours.

2.7.2 Preamplification

An aliquot of the ligation product was ten-fold diluted with TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The preamplification reaction was carried out in a 50 µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µ M of each dNTP, 1.5 mM $MgCl_2$, 30 ng of E_{+A} (5'-GAC TGC GTA CCA ATT CA-3') M_{+C} (5'-GAT GAG TCC TGA GTA AC-3') primers, 1.5 units of DyNAzyme^{TM} II DNA Polymerase (Finnzymes) and 5 µl of the diluted ligation product (or 0.5 - 1.0 undiluted product). PCR was performed in a Perkin Elmer 9700 thermocycler consisting of denaturation at 94°C for 30 seconds, annealing at 56°C for 1 minute and extension at 72°C for 1 minute. The preamplification product was then diluted for 12.5 fold.

2.7.3 Selective amplification

Selective amplification was carried out in a 20 (or 25) µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 30 ng of a combination of E_{+3} and M_{+3} primer (Table 2.1), 1.5 units of DyNAzyme^{TM} II DNA Polymerase (Finnzymes) and 5 µl of diluted preamplification product. The amplification reaction was carried out by denaturation at 94°C for 30 seconds, annealing at 65°C for 45 seconds and extension at 72°C for 90 sec for 2 cycles followed by 12 cycles of a touch down phase with lowering of the annealing temperature for 0.7 in every cycle. The amplification consisting of 94°C for 30 seconds, 56 °C (or 53 °C) for 45 seconds and 72°C for 90 sec was performed for additional 23-25 cycles. The final extension was carried out at 72°C for 5 minutes.

2.8 Denaturing Polyacrylamide Gel Electrophoresis

2.8.1 Preparation of Glass Plate

The long glass plate was thoroughly wiped with 2 ml of 95% commercial grade ethanol in one direction with a tissue. This process was then repeated twice. Afterwards, the long glass plate was coated with 1 ml of freshly prepared Bind silane (10 µl of Bind silane; Pharmacia, USA, 995 µl of 95% ethanol and 10 µl of 5% glacial acetic acid) and left for

approximately 10 -15 minutes. Excess binding solution was removed with a piece of tissue. The long glass plate was further cleaned with 95% ethanol for 3 times. The short glass plate was treated as described above with the exception that the binding solution was replaced by the Repel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The cleaned glass plates were assembled with a pair of 0.4 mM spacer. The bottom and both sides of assembled glass plates were sealed with the plastic tape.

Table 2.1 AFLP primers and their sequences used for identification of sex-specific markers in *M. rosenbergii*

Primer	Sequences
Adator sequences	
Eco RI adaptor	5'-CTC GTA GAC TGC GTA CC-3'
	5'-AAT TGG TAC GCA GTC TAC-3'
Mse I adaptor	5'-GAC GAT GAG TCC TGA G-3'
	5'-TAC TCA GGA CTC AT-3'
Preamplification primers	
E_{+A}	5'-GAC TGC GTA CCA ATT CA-3'
M_{+C}	5'-GAT GAG TCC TGA GTA AC-3'
Selective amplification primers	
E_{+3}-1	E_{+A}AC
E_{+3}-2	E_{+A}AG
E_{+3}-3	E_{+A}CA
E_{+3}-4	E_{+A}CT
E_{+3}-5	E_{+A}CC
E_{+3}-6	E_{+A}CG
E_{+3}-7	E_{+A}GC
E_{+3}-8	E_{+A}GG
M_{+3}-1	M_{+C}AA
M_{+3}-2	M_{+C}AC
M_{+3}-3	M_{+C}AG
M_{+3}-4	M_{+C}AT
M_{+3}-5	M_{+C}TA
M_{+3}-6	M_{+C}TC
M_{+3}-7	M_{+C}TG
M_{+3}-8	M_{+C}TT

2.8.2 Preparation of denaturing polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels are used for the separation of single-stranded AFLP and RAP-PCR fragments. These gels are polymerized in the presence of an agent (urea) that suppresses base pairing in nucleic acids. Denatured DNA migrates through these gels at a rate that is almost completely independent of its base composition and sequence. 4.5 or 6% denaturing polyacrylamide gels were prepared by combining 40 ml of the degassed acrylamide solution (19:1 acrylamide: bisacrylamide with 7 M urea in TBE buffer) with 240 µl of freshly prepared 10% ammonium persulphate and 24 µl of TEMED. The acrylamide solution was gently swirled and degassed for 20 minutes. The assembled plate sandwich was hold at a 45-degree angle on the bottom corner. The acrylamide solution was then gently injected into one side of the assembled plates using a 50 ml syringe. The filled plate sandwich was left in the horizontal position. The flat edge of the shark-tooth comb was then inserted. The gel was left at room temperature for 1 hour. After that, the polymerized gel was covered by the water-soaked tissue paper and left at room temperature for 4 hours (or overnight) for complete polymerization. When required, the spring clips and the sealing tapes were carefully removed. The top of the gel was rinsed with 1x TBE. The sharkstooth comb was rinsed with water.

2.8.3 Electrophoresis

The gel sandwich was place in the vertical sequencing apparatus with the short glass plate inward. The gel sandwich was securely clamped with the integral gel clamps along the sides of the sequencing apparatus. The upper and lower buffer chambers were filled with approximately 300 ml of 1x TBE. The sharkstooth comb was reinserted into the gel until the teeth just touched the surface of the gel. Six microlitres of the acrylamide gel loading dye (98% formamide, 200 µl EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol) was loaded into each well. The gel was prerun at 30 -40 W for 20 minutes.

Six microlitres of the amplification products were mixed with 3 µl of the loading buffer and heated at 95 °C for 5 minutes before snapped cooled on ice for 3 minutes. The sample

was carefully loaded into the well. Electrophoresis was carried out at 35 – 40 W for approximately 2.5 hours (XC move out from the gel for approximately 30 minutes).

2.9 Silver staining

The gel plates were carefully separated using a plastic wedge. The long glass plate with the gel was placed in a plastic tray containing 1.5 litres of the fix/stop solution and agitates well for 40 minutes (25-30 minutes for SSCP gels). The gel was soaked with shaking 3 times for 2 minutes with deionized water. The gel was lifted out from the tray between each wash and allowed the washed water draining out of the gel for 4 seconds. The gel was transferred to 0.1% silver nitrate (1.5 litres) and incubated with agitation at room temperature for 30 minutes. The gel was soaked in 1.5 litres off deionized water with shaking (10 forward and 10 backward agitation) and immediately placed in the tray containing 1.5 litres of the chilled developing solution. This step is crucial and the time taken to soak the gel in the water and transfer it to chilled developing solution should be no longer than 5 - 10 seconds. The gel was well agitated until the first bands are visible (usually 1.5 - 2 minutes). The gel was then transferred to another tray containing 1.5 litres of chilled developer and shaked until bands from every lane were observed (usually 2 – 3 minutes). One litre of the fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was left at room temperature (SSCP gels) or at 80 °C for 2-3 hour (AFLP gels).

2.10 Cloning of AFLP fragments

2.10.1 Elution of DNA from polyacrylamide gels

Candidate sex-specific AFLP fragments were excised from the gel using a sterile razor blade and washed 3 times for 30 minutes each at room temperature with 200 µl of sterile deionized H_2O. Twenty microlitres of H_2O was then added and incubated overnight at 37 °C. Reamplification of the target fragment was carried out using the same PCR recipes as those for selective amplification with the exception that 100 ng of each primer and 5 µl of the eluted AFLP product were used. The amplification conditions were composed of 5 cycles of 94 °C for 30 seconds, 42 °C for 1 minute and 72 °C for 1 minute followed by additional 35

cycles at a higher stringent annealing temperature at 50 °C. The final extension was performed at 72 °C for 7 minutes. The reamplified product was electrophoretically analysed through a 1.5 - 1.75 % agarose gel at 7.5 V/cm for approximately 1 hour.

2.10.2 Elution of DNA from agarose gels

The required DNA fragment was fractionated through agarose gels in duplication. One was run side-by-side with a 100 bp DNA markers and the other was loaded into the distal well of the gel. After electrophoresis, lanes representing the DNA standard and its proximal DNA sample were cut and stainined with ethidium bromide (0.5 $\mu g.ml^{-1}$) for 5 minutes. Positions of the DNA markers and the EtBr-stained reamplified fragment were used to align the position of the non-stained target DNA fragment.

The DNA fragment was excised from the gel with a sterile razor blade. DNA was eluted out from the agarose gels using a QIAquick gel Extraction kit (QIAGEN) according to the protocol recommended by the manufacture. The excised gel was transferred into a microcentrifuge tube and weighed. Three gel volumes of the QG buffer were added. The mixture was incubated at 50 °C for 10 minutes with briefly vortexing every 2 - 3 minutes. After the gel was completely dissolved, 1 gel volume of isopropanol was added and gently mixed. The mixture was applied to the QIAquick spin column placed on a 2 ml collection tube and centrifuged at 13000 rpm for 1 minute at room temperature. The flow-through was discarded and 0.75 ml of the PE buffer was added. The QIAquick spin column was centrifuged at 13000 rpm for 1 minute at room temperature. The flow-through was discarded. The column was further centrifuged at room temperature for an additional 1 minute at 13,000 rpm to remove trance amount of the washing buffer. The column was then placed in a new microcentrifuge tube and 30 µl of the EB buffer (10 mM Tris-Cl, pH 8.5) was added to the center of the QIAquick membrane. The column was incubated at room temperature for 1 minute before centrifuged at 13000 rpm for 1 minute. The eluted sample was stored at -20 °C until further required.

2.10.3 Ligation of PCR product to vector

The ligation reaction was set up in the total volume of 10 µl containing of 3 µl of the gel-eluted PCR product, 25 ng of pGEM®-T easy vector, 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 % PEG 8000) and 3 Weiss units of T4 DNA ligase. The ligation mixture was gently mixed by pipetting and incubated at 4 °C overnight.

2.10.4 Preparation of competent cells

A single colony of *E. coli* JM109 was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) with vigorous shaking at 37 °C overnight. The starting culture was inoculated into 50 ml of LB broth and continued culture at 37 °C with vigorous shaking to the OD_{600} of 0.5 - 0.8. The cells were chilled on ice for 10 minutes before centrifuged at 3000 g for 10 minutes at 4 °C. The pellets were resuspended in 30 ml of ice-cold $MgCl_2$ – $CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged as above. The supernatant was discarded and the pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ and divided into 200 µl aliquots. These competent cells could be used immediately or stored at –70°C for subsequent used.

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

The competent cells were thawed on ice for 5 minutes and divided to aliquots of 100 µ l. Two microlitres of the ligation mixture was added and gently mixed by pipetting. The mixture was incubated on ice for 30 minutes. The reaction tube was then placed in a 42 °C water bath for 45 seconds without shaking. The tube was then immediately snapped on ice for 2 - 3 minutes. One microlitre of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM M $MgSO_4$ and 20 mM glucose) was added to the tube. The cell suspension was incubated with shaking at 37 °C for 1.5 hours. At the end on the incubation period, the cultured cell suspension was centrifuged at 12000 rpm for 20 seconds at room temperature. The pellet was gently resuspended in 100 µl of SOC and spread on a LB agar plate containing 50 $\mu g.ml^{-1}$ of ampicillin, 25 $\mu g.ml^{-1}$ of

IPTG and 20 μg.ml^{-1} of of X-gal. The plate was left until the cell suspension was absorbed and further incubated at 37 °C overnight. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.10.6 Detection of recombinant clone by colony PCR

Colony PCR was performed in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 uM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 0.2 μM of pUC1 (5'-TCCGGCTCGTATGT TGTGTGGA-3') and pUC2 (5'-GTGGTGCAAGGCGATTAAGTTGG-3') primers and 1 unit of DyNAzyme^{TM} II DNA Polymerase. A recombinant colony was scraped by the micropipette tip and mixed well in the amplification reaction. The PCR profiles was predenaturing at 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 60 seconds and 72 °C for 90 seconds. The final extension was carried out at 72 °C for 7 minutes. The resulting PCR products were electrophoretically analyzed through agarose gels.

2.10.7 Isolation and digestion of recombinant plasmid DNA

A recombinant clone was inoculated into 3 ml of LB broth (1% tryptone, 0.5% yeast extract, 1.0 % NaCl) containing 50 μg/ml of ampicillin and incubated at 37 °C with constant shaking at 250 rpm overnight. The culture was transferred into 1.5-ml microcentrifuge tube and centrifuged at 12000 g for 1 min. The cell pellet was collected and resuspended with 250 μl of the buffer P1. The mixture was completely dispersed by vortexing. The mixture was then treated with 250 μl of the buffer P2, gently mixed and placed on ice for 10 min. Additionally, 350 μl of the buffer N3 was added and gently mixed.

To separate the cell debris, the mixture was centrifuged at 12000 g for 10 minutes. The supernatant was transferred into the QIAprep column and centrifuged at 12000 g for 30 - 60 seconds. The flow-through was discarded. The QIAprep spin column was washed by adding 0.75 ml of the buffer PE and centrifuged for 30 - 60 seconds. The flow-through was discarded. The spin tube was centrifuge for an additional 1 minute to remove the residual wash buffer. The QIAprep column was placed in a new 1.5 ml microcentrifuge tube and 40 μ l of the buffer EB (10 mM Tris-Cl, pH 8.5) was added to elute the extracted plasmid DNA.

The column was left at room temperature for 1 minute and centrifuge at 12000 g for 1 minute.

The insert size of each recombinant plasmid was examined by digestion of the plasmid with *Eco* RI. The digest was carried out in a 15 µl containing 1x restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl and 50 mM $MgCl_2$), 1 µg of recombinant plasmid and 2 - 3 unit *Eco* RI and incubated at 37 °C for 3 hours before elctrophoretically analyzed by agarose gel elctrophoresis.

2.10.8 DNA sequencing

The recombinant plasmid was unidirectional sequenced using a Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Biosciences, Sweden) with the M13 reverse or M13 forward primers on an automated DNA sequencer (MegaBace1000, Amersham BioSciences). Nucleotide sequences were blasted against data in the GenBank (http://www.ncbi.nlm.nih.gov/blast) using Blast*N* (nucleotide similarity) and Blast*X* (translated protein similarity). Significant similarity was considered when the probability (E) value was $<10^{-4}$.

2.11 Primer design

PCR primers were designed from sequence of candidate sex maker from AFLP analysis using Primer Premier 5.0. The criteria for primer designing were the primer length of 18 - 25 bases, the melting temperature of 55 – 70 °C, random base distribution of the primers to avoid polypurine and polypyrimidine tracts, less than 5 °C differences of the melting temperature of a primer pair.

2.12 Polymerase chain reaction (PCR)

The PCR reactions were performed in a 50 µl reaction volume containing 10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl, 0.1% Triton X - 100, 1.5 – 2.0 mM $MgCl_2$, 1 unit of Dynazyme^{TM} DNA Polymerase (FINNZYMES, Finland), 200 µM of each dATP, dCTP, dGTP and dTTP, 0.25 µM of each primer (Tables 2.2 and 2.3) and 50 ng of genomic DNA. The amplification condition was carried out as described in Table 2.3.

Table 2.2 Sequences and melting temperature of primers and sizes of the expected amplification product of candidate sex-specific AFLP marker of *M. rosenbergii*

Gene/Primer	Sequence	Tm (°C)	Expected sizes (bp)
1. $ME_{+3}3M_{+3}7425.1$ - F	5′- GGA TGT CTG ACG GCT TGC TG -3′	64	202
$ME_{+3}3M_{+3}7425.1$ - R	5′- GAG GCG GAA AAG GAT ATG TG -3′	60	
2. $ME_{+3}3M_{+3}7425.2$ - F	5′- AAG CAC GCA CAT ACT CAC ACA T -3′	64	262
$ME_{+3}3M_{+3}7425.2$ - R	5′- GCA TCT TTC CAT CCT CCA AC -3′	60	
3. $ME_{+3}7M_{+3}6425$ - F	5′- AGA CCA CAT CCC TGA AAC CT -3′	60	333
$ME_{+3}7M_{+3}6425$ - R	5′- CAT ATC TTG CCC ATC CTG CT -3′	60	
4. $ME_{+3}4M_{+3}5570$ - F	5′- TCA ACC ATA GCC TGT ACC TTT C -3′	64	384
$ME_{+3}4M_{+3}5570$ - R	5′- ACT TCA CCC CTG GAG AGA CTG T -3′	68	
5. $ME_{+3}8M_{+3}1310.1$ - F	5′- AGT GAG GTA GGT TAC TGA CA -3′	58	210
$ME_{+3}8M_{+3}1310.1$ - R	5′- AGG ATG GAC TGA ATC GGG CA -3′	62	
6. $ME_{+3}8M_{+3}1310.2$ - F	5′- GTA CAT TAT GAA CTA AGA CAA G -3′	58	235
$ME_{+3}8M_{+3}1310.2$ - R	5′- TGT ATT GCT GTT TCC ATA GGG C -3′	64	
7. $ME_{+3}8M_{+3}1310.3$ - F	5′- TTG TCA GAT GGC TAA TAG TGT C -3′	62	219
$ME_{+3}8M_{+3}1310.3$ - R	5′- CAA ATG AGA AAT GAA GTG GAA G -3′	60	
8. $ME_{+3}4M_{+3}8517$ - F	5′- TGT TAT CCT TCG TTC CCT CC -3′	60	277
$ME_{+3}4M_{+3}8517$ - R	5′- AAA GTG ACA GTC CTG GCA AA -3′	58	
9. $FE_{+3}1M_{+3}7290$ - F	5′- GCT TAG GGT TCT GGT CTT GTG A -3′	66	143
$FE_{+3}1M_{+3}7290$ - R	5′- AAA AGG GAG GGA GCA AAG AGT G -3′	66	
10. $FE_{+3}5M_{+3}1390$ - F	5′- ACC CTC TTT TCT GGA TGG CA -3′	60	277
$FE_{+3}5M_{+3}1390$ - R	5′- CGG CAA ACC TCA AAT CAC TC -3′	60	
11. $FE_{+3}8M_{+3}3270.1$ - F	5′- ACT ATT ATT TCT TAT TTT TCT CTC -3′	58	152
$FE_{+3}8M_{+3}3270.1$ - R	5′- CAA GCC TTT ACA AAT AGG GAA T -3′	60	
12. $FE_{+3}8M_{+3}3270.2$ - F	5′- CGC CTA TTC CTC AAT CGG TCA A -3′	64	147
$FE_{+3}8M_{+3}3270.2$ - R	5′- ATG CTT CCA TTC CTC CGT CCT T -3′	66	
13. $FE_{+3}8M_{+3}6710$ - F	5′- GCT TTA TTT CCT GCG TTA TTG G -3′	62	385
$FE_{+3}8M_{+3}6710$ - R	5′- ACG ATT GGC TCA TTC GCT CCT A -3′	66	

Table 2.3 PCR profiles and composition (dNTPs and $MgCl_2$) for specificity test of SCAR markers derived from candidate sex-specific AFLP primers of *M. rosenbergii*

Primer	dNTPs (μM)	$MgCl_2$ (mM)	PCR condition
1 $ME_{+3}3M_{+3}7425.1$	200	2.0	94 °C, 3 min, 1 cycle followed by 94 °C, 30 s; 64 °C, 45 s and 72 °C, 45 s for 12 cycles where the annealing temperature were lowered for 2 °C in every 3 cycles and 94 °C, 30 s; 56 °C, 45 s and 72 °C, 1 min for additional 28 cycles and 72 °C, 7 min; 1 cycle
2. $ME_{+3}3M_{+3}7425.2$	200	2.0	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 55 °C, 1 min and 72 °C, 1 min for 35 cycles and
3. $ME_{+3}7M_{+3}6425$	200	2.0	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 65 °C, 45 s and 72 °C, 45 s for 10 cycles where the annealing temperature were lowered for 2 °C in every other cycles and 94 °C, 30 s; 56 °C, 45 s and 72 °C, 1 min for additional 25 cycles and 72 °C, 7 min; 1 cycle
4. $ME_{+3}4M_{+3}5570$	200	1.5	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 55 °C, 45 s and 72 °C, 45 s; for 30 cycles and 72 °C, 7 min; 1 cycle
5. $ME_{+3}8M_{+3}1310.1$	200	1.5	As described in 2.
6. $ME_{+3}8M_{+3}1310.2$	200	2.0	As described in 2.
7. $ME_{+3}8M_{+3}1310.3$	200	1.5	As described in 2.
8. $ME_{+3}4M_{+3}8517$	200	2.0	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 55 °C, 45 s and 72 °C, 45 s; for 35 cycles and 72 °C, 7 min; 1 cycle
9. $FE_{+3}1M_{+3}7290$	200	2.0	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 45 °C, 1 min and 72 °C, 30 s; 5 cycles and 94 °C, 45 s; 55 °C, 1 min and 72 °C, 30 s; for additional 30 cycles and 72 °C, 7 min; 1 cycle

Table 2.3 (cont.)

Primer	dNTPs (μM)	$MgCl_2$ (mM)	PCR condition
10. $FE_{+3}5M_{+3}1390$	200	2.0	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 55 °C, 1 min and 72 °C, 1 min for 35 cycles and 72 °C, 7 min; 1 cycle
11. $FE_{+3}8M_{+3}3270.1$	200	1.5	As described in 10.
12. $FE_{+3}8M_{+3}3270.2$	200	1.5	As described in 10.
13. $FE_{+3}8M_{+3}6710$	200	1.5	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 66 °C, 1 min and 72 °C, 30 s for 12 cycles where the annealing temperature was lowered 2 °C in every other cycles and 94 °C, 45 s; 55 °C, 1 min and 72 °C, 30 s for 23 cycles and 72 °C, 7 min; 1 cycle

Five microliters of the amplification products were electrophoretically analyzed though 1.0 - 2.0% agarose gel to verify whether the amplification was successful. Polymorphism of the markers was further analyzed using single-stranded conformation polymorphism (SSCP, see below).

2.13 RT-PCR analysis

Degenerated primers were designed from cDNAs encoding androgenic gland hormone precursors of Terrestrial Isopod, *Armadilidium vulgare* (Okuno et al., 1999) and *Porcellio scaber* and *Porcellio dilatatus* (Ohira et al., 2003). In addition, RT-PCR was carried out for identifying candidate sex-specific AFLP marker of *M. rosenbergii* homologues of sex-related transcripts originally identified in other commercially important aquatic species including the giant tiger shrimp (*Penaeus monodon*) and the tropical abalone (*Haliotis asinina*).

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, 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland), 0.25 - 0.4 µM of each primer, and 500 - 1,000 ng of the first strand cDNA. PCR was carried out as described in Tables 2.5, 2.7, 2.9 and 2.11, respectively.

Five microliters of the amplification product were electrophoretically analyzed though 1.0 - 2.0% agarose gels. The interesting PCR product was excised out and purified from the agarose gel using QIAquick® Gel Extraction Kit (QIAGEN). Eluted DNA was cloned as previously described. Recombinant plasmid was extracted and nucleotide sequence of the clone was examined using automated DNA sequencer.

Table 2.4 Sequences of degenerated primers designing from nucleotide sequences of cDNAs encoding androgenic gland hormone of the terrestrial isopods; *P. scaber*, *P. dilatatus* and *A. vulgare*

Gene/Primer	Sequence
Mar-AGH – F1	5′- ATH MGN TTT ACN GTG CAN TGY AT-3′
Mar-AGH – F2	5′- GTG CAN TGT ATH TGY AAY GAR YT -3′
Mar-AGH – R1	5′- TC TTS GTG RAA GGC KAT NTC AC -3′

Table 2.5 PCR profiles and composition (dNTPs and $MgCl_2$) for expression analysis of cDNA markers derived from nucleotide sequences of cDNAs encoding androgenic gland hormone of terrestrial isopod; *P. scaber*, *P. dilatatus* and *A. vulgare*

Gene	Template (ng)	$MgCl_2$ (mM)	Primer (µM)	PCR conditions
1. Mar-AGH – F1 Mar-AGH – R1	500	2.0	0.4	94 °C, 3 min; 1 cycle 94 °C, 45 s 48 °C, 1 min 72 °C, 45 s; 35 cycles 72 °C, 7 min; 1 cycle
2. Mar-AGH – F2 Mar-AGH – R1	500	2.0	0.4	As described in 1.

Table 2.6 Sequences and melting temperature of primers and sizes of the expected amplification product of candidate sex-specific AFLP marker of *M. rosenbergii*

Primer	Sequence	Tm (°C)
Candidate sex-specific primers of *M. rosenbergii* from AFLP analysis		
1. $ME_{+3}3M_{+3}7425.1$ - F	5′- GGA TGT CTG ACG GCT TGC TG -3′	64
$ME_{+3}3M_{+3}7425.1$ - R	5′- GAG GCG GAA AAG GAT ATG TG -3′	60
2. $ME_{+3}3M_{+3}7425.2$ - F	5′- AAG CAC GCA CAT ACT CAC ACA T -3′	64
$ME_{+3}3M_{+3}7425.2$ - R	5′- GCA TCT TTC CAT CCT CCA AC -3′	60
3. $ME_{+3}7M_{+3}6425$ - F	5′- AGA CCA CAT CCC TGA AAC CT -3′	60
$ME_{+3}7M_{+3}6425$ - R	5′- CAT ATC TTG CCC ATC CTG CT -3′	60
4. $ME_{+3}4M_{+3}5570$ - F	5′- TCA ACC ATA GCC TGT ACC TTT C -3′	64
$ME_{+3}4M_{+3}5570$ - R	5′- ACT TCA CCC CTG GAG AGA CTG T -3′	68
5. $ME_{+3}8M_{+3}1310.1$ - F	5′- AGT GAG GTA GGT TAC TGA CA -3′	58
$ME_{+3}8M_{+3}1310.1$ - R	5′- AGG ATG GAC TGA ATC GGG CA -3′	62
6. $ME_{+3}8M_{+3}1310.2$ - F	5′- GTA CAT TAT GAA CTA AGA CAA G -3′	58
$ME_{+3}8M_{+3}1310.2$ - R	5′- TGT ATT GCT GTT TCC ATA GGG C -3′	64
7. $ME_{+3}8M_{+3}1310.3$ - F	5′- TTG TCA GAT GGC TAA TAG TGT C -3′	62
$ME_{+3}8M_{+3}1310.3$ - R	5′- CAA ATG AGA AAT GAA GTG GAA G -3′	60
8. $ME_{+3}4M_{+3}8517$ - F	5′- TGT TAT CCT TCG TTC CCT CC -3′	60
$ME_{+3}4M_{+3}8517$ - R	5′- AAA GTG ACA GTC CTG GCA AA -3′	58

Table 2.7 PCR profiles and composition (dNTPs and $MgCl_2$) for specificity test of SCAR markers derived from candidate sex-specific AFLP primers of *M. rosenbergii*

Marker	Template (ng)	$MgCl_2$ (mM)	Primer (μM)	PCR conditions
1. $ME_{+3}3M_{+3}7425.1$	1,000	2.0	0.4	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 53 °C, 1 min and 72 °C, 45 s for 35 cycles and 72 °C, 7 min; 1 cycle
2. $ME_{+3}3M_{+3}7425.2$	1,000	2.0	0.4	As described in1.
3. $ME_{+3}7M_{+3}6425$	1,000	2.0	0.4	As described in1.
4. $ME_{+3}4M_{+3}5570$	1,000	2.0	0.4	As described in1.
5. $ME_{+3}8M_{+3}1310.1$	1,000	2.0	0.4	As described in1.
6. $ME_{+3}8M_{+3}1310.2$	1,000	2.0	0.4	As described in1.
7. $ME_{+3}8M_{+3}1310.3$	1,000	2.0	0.4	As described in1.
8. $ME_{+3}4M_{+3}8517$	1,000	2.0	0.4	As described in1.

Table 2.8 Sequences and melting temperature of primers and sizes of the expected amplification product of sex-related transcripts of *P. monodon*

Gene/Primer	Sequence	Tm (°C)
Difulfide 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
Sex-linked nuclear protein 1 (XNP-1)		
XNP - 1 F	5'- TTC CAC CCT TTT GCT TGC TA -3'	58
XNP - 1 R	5'- GGA TTT GCC TGA GAC ACC TAA -3'	62
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
Hypothetical protein ENSANGP00000010123 (X-linked in *Drosophila melanogater*)		
ENSANGP40 - F	5'- TCA TGT CGG AGG TCG TGA GTA A -3'	66
ENSANGP40 - R	5'- AGA ACA AGG AAG AGG AGG GAT T -3'	64
ENSANGP44 - F	5'- GCG TCA TCA AGT TGT CCG AGT C -3'	68
ENSANGP44 - R	5'- TGA GGG GCA GTG AAG ATA GGT G -3'	68
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
Rudimentary protein		
Rudimentary – F	5'- CCA GGT GCG ACT CAC AAG GAA G -3'	70
Rudimentary – R	5'- AGC AGC GGAACGAGG GGC -3'	62
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
Sex-related transcripts in *Penaeus monodon*		
Peritrophin		
Peritrophin - F_1	5'- TTG AGG ACC ACT TCT GTT CGG -3'	60
Peritrophin - R_1	5'- GCT TGG GTT GGC AAA AGT ACC -3'	59
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

Table 2.9 PCR profiles and composition (dNTPs and $MgCl_2$) for expression analysis of homologues of sex-related transcripts of *P. monodon*

Gene/primer	Template (ng)	$MgCl_2$ (mM)	Primer (μM)	PCR conditions
1. DSI	1,000	2.0	0.4	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 53 °C, 1 min and 72 °C, 45 s; 35 cycles and 72 °C, 7 min; 1 cycle
2. XNP-1	1,000	2.0	0.4	As described in 1.
3. Sxl	1,000	2.0	0.4	94 °C, 3 min; 1 cycle 94 °C, 30 s; 55 °C, 1 min and 72 °C, 45 s for 35 cycles and 72 °C, 7 min; 1 cycle
4. ENSANGP40	1,000	2.0	0.4	As described in 3.
5. ENSANGP44	1,000	2.0	0.4	As described in 3.
6. EGF	1,000	2.0	0.4	As described in 3.
7. Rudimentary	1,000	2.0	0.4	As described in 3.
8. Usp9X	1,000	2.0	0.4	As described in 3.
9. Peritrophin	500	2.0	0.25	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 66 °C, 1 min and 72 °C, 1 min for 10 cycles where the annealing temperature was lowered for 2°C in every other cycles and 94 °C, 30 s; 56 °C, 1 min and 72 °C, 1 min for 28 cycles and 72 °C, 7 min; 1 cycle
10. TSP288 - F 288 - R	1,000	2.0	0.4	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 53 °C, 1 min and 72 °C, 1 min for 35 cycles and 72 °C, 7 min; 1 cycle
11. TSP 462 - F TSP 462 - R	1,000	2.0	0.4	As described in 10.
12. TSP 462 - 2 371 - 1	1,000	2.0	0.4	As described in 10.
13. TSP 462-F 288 – R	1,000	2.0	0.4	As described in 10.
14. TSP 288-1/ 371 – 1	1,000	2.0	0.4	As described in 10.

Table 2.10 Sequences and melting temperature of primers and sizes of the expected amplification product of sex-related transcripts of *H. asinina*

Gene/Primer	Sequence	Tm (°C)
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 Al		
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
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 2.11 PCR profiles and composition (dNTPs and $MgCl_2$) for expression analysis of sex-related transcripts of *H. asinina*

Gene	Template (ng)	$MgCl_2$ (mM)	Primer (μM)	PCR conditions
1. Axonemal	1,000	2.0	0.4	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 53 °C, 1 min and 72 °C, 1 min for 35 cycles and 72 °C, 7 min; 1 cycle
2. Tektin	1,000	2.0	0.4	As described in 1
3. VTG 1	1,000	2.0	0.4	As described in 1

2.14 RNA arbitrary primed (RAP)-PCR

The first strand cDNA was synthesized from 1.5 µg of pooled total RNA extracted from androgenic glands (*N* = 3) and oviducts (*N* = 3) of male and female *M. rosenbergii,* respectively. Five separate primers (1 µM each of UBC 268, UBC 428, UBC 122, OPA 02 and OPA 16) were used for the first strand cDNA synthesis.

One microgram of the first stranded cDNA as a template for PCR amplification 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, 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland), 1 µM each of the first arbitrary primer (that used for the first strand cDNA synthesis) and the second arbitrary primers (the first primer or one of those in Table 2.10)

PCR was performed by predenaturation at 94 °C for 3 min, follow by 40 cycles of denaturation at 94 °C for 30 s, annealing at 36 ^{o}C for 60 s, and extension at 72 °C for 90 s. The final extension was carried out for 7 minutes at 72 °C. Five microlitres of the amplification product were electrophoretically analyzed to verify whether the amplification was successful. Six microlitres of the PCR product were mixed with one-half volume of formamide loading buffer and denatured at 95 °C for 10 min prior to electrophoresis through a 4.5% denaturing polyacrylamide gel. Size-fractionated bands were visualized by silver staining. The gel was dried overnight and photograph under fluorescence light using Camera Pentax K1000.

2.15 Cloning and characterization of candidate sex/morphotype differential expression markers in *M. rosenbergii* resulted from RAP-PCR analysis

Fragments showing presence/absence and differential expression (at least 2-3 times differences in band intensity) were considered. Interesting bands were eluted out from the gel and reamplification steps were described previously exception that 0.2 µM of each primer and 0.2 µM of dNTPs were used. The amplification conditions were composed of 1 cycle of 94 °C for 3 min, follow by additional 25 - 35 cycles at 94 °C for 30 seconds, 36 °C for 1

minute and 72 °C for 1.30 minute. The final extension was performed at 72 °C for 7 minutes. The reamplified product was electrophoretically analyzed through a 1.5 - 1.75 % agarose gel at 7.5 V/cm for approximately 1 hour.

The DNA fragment was excised from the agarose gel, cloned and sequenced. Primer pairs were designed and analyzed against the first strand cDNA of female and different male morphotypes using the conditions described in Table 2.14.

Table 2.12 Sequences of arbitrary primers used for screening of sex-differential expression marker in *M. rosenbergii* using RAP-PCR analysis

Primer no.	Primer name	Sequence
1.	UBC 101	GCGCCTGGAG
2.	UBC 119	ATTGGGCGAT
3.	UBC 122	GTAGACGAGC
4.	UBC 128	GCATATTCCG
5.	UBC 135	AAGCTGCGAG
6.	UBC 138	GCTTCCCCTT
7.	UBC 158	TAGCCGTGGC
8.	UBC 159	GAGCCCGTAG
9.	UBC 169	ACGACGTAGG
10.	UBC 174	AACGGGCAGC
11.	UBC 191	CGATGGCTTT
12.	UBC 217	ACAGGTAGAC
13.	UBC 222	AAGCCTCCCC
14.	UBC 228	GCTGGGCCGA
15.	UBC 263	TTAGAGACGG
16.	UBC 268	AGGCCGCTTA
17.	UBC 273	AATGTCGCCA
18.	UBC 299	TGTCAGCGGT
19.	UBC 428	GGCTGCGGTA
20.	UBC 456	GCGGAGGTCC
21.	UBC 457	CGACGCCCTG
22.	UBC 459	GCGTCGAGGG
23.	OPA 01	CAGGCCCTTC
24.	OPA 02	TGCCGAGCTG
25.	OPA 07	GAAACGGGTG
26.	OPA 09	GGGTAACGCC
27.	OPA 16	AGCCAGCGAA
28.	OPA 17	GACCGCTTGT
29.	OPZ 09	CACCCCAGTC
30.	OPB 10	CTGCTGGGAC

Table 2.13 Sequences and melting temperature of primers and sizes of the expected amplification product of candidate sex-specific/differential expression marker from RAP-PCR analysis

Gene/Primer	Sequence	Tm (°C)	Expected sizes (bp)
Candidate sex-specific expression makers			
Female-specific expression makers			
1. FeRAP315.1 - F	5'- CAA CGT ACT ACT CTT GTT CAT C -3'	**62**	**203**
FeRAP315.1 - R	5'- ATT GTT CCA AAG TGC CTA TTA -3'	**56**	
2. FeRAP315.2 - F	5'- TCC TAC TAC TAG CCA AGA CGA TT -3'	**66**	**209**
FeRAP315.2 - R	5'- CGA CCA GGC AGA TGA CCC AAG -3'	**72**	
Male-specific expression makers			
1. M268/128RAP - F	5'- GTG GGG GGA GTG CCG TCA GT -3'	**68**	**194**
M268/128RAP - R	5'- ACA AAG GTG TAA CAG GAG AAA AAC -3'	66	
2. M428/228RAP - F	5'- AAC TCG TTT GCT CCG AAG AA -3'	58	240
M428/228RAP - R	5'- AAA GAA CCT CAA GTA ATG CCT -3'	58	
3. M122/135RAP - F	5'- TCA GAC TAC ATA CCC CTT CAA T -3'	62	284
M122/135RAP - R	5'- TGG GCG ACC AAA ACA GCA G -3'	60	
4. MA16/222RAP - F	5'- CAT AAG AGC GAA TGA ACG AAG CA -3'	66	147
MA16/222RAP - R	5'- TCC TTA TTT GTA ATG TTT CGT CT -3'	60	
5. M122/159RAP.1 - F	5'- CTG CTG CTA CCT TTG GCT ATT A -3'	**64**	**261**
M122/159RAP.1 - R	5'- GCG TCA GTT ACA GTT GTC ACC -3'	**64**	
6. M122/159RAP.2 - F	5'- CGA CGG TAA TGT ATG AAG CAG T -3'	**64**	**292**
M122/159RAP.2 - R	5'- CCC GAG GAG AAC CAC CAA AGT -3'	**70**	
Candidate differential expression makers			
1. SOCRAP340.1 - F	5'- GAG TTT CTT TTA CCT GTG CGT GG -3'	68	206
SOCRAP340.1 - R	5'- CCC TGA TGT TGC GAG CAT TGG A -3'	68	
2. SOCRAP340.2 - F	5'- GTT TAG CAA CCA AGT ATT TAG TG -3'	62	265
SOCRAP340.2 - R	5'- TTG ACC GTC CTC GCA ATC CGT -3'	66	
3. SOC268/273RAP - F	5'- ATA TTT GGT GCT CCA TCG TA -3'	56	172
SOC268/273RAP - R	5'- TGA AGT CCT CAC TCC ATT GC -3'	60	
4. SOC122/228RAP - F	5'- ACG ACG ACG GCG ACG AAC G -3'	64	166
SOC122/228RAP - R	5'- TTG CAG TCC ATA CTT GCT GAA T -3'	62	
5. BC428/228RAP - F	5'- TAC ACT GCG GAA AGT AAT GCT -3'	60	184
BC428/228RAP - R	5'- AGG ACT TAG ACT TAT TTT ACG -3'	56	
6. BC428/273RAP - F	5'- CAA GAC TGT GGG AAA TCG TGT -3'	62	247
BC428/273RAP - R	5'- TAA TGA GAT GCT GAA GAA AAG A –3'	58	

Table 2.14 PCR profiles and conditions for testing of primers from candidate sex-specific/differential expression maker from RAP-PCR analysis

Primer	cDNA template (ng)	$MgCl_2$ (mM)	Primer (μM)	PCR condition
Candidate female-specific expression makers				
1. FeRAP315.1	500	1.5	0.2	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 50 °C, 1 min and 72 °C, 45 s for 35 cycles and 72 °C, 7 min; 1 cycle
2. FeRAP315.2	1,000	2.0	0.4	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 53 °C, 1 min and 72 °C, 45 s for 35 cycles and 72 °C, 7 min; 1 cycle
Candidate male-specific expression makers				
1. M268/128RAP	1,000	2.0	0.25	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 60 °C, 1 min and 72 °C, 30 s for 35 cycles and 72 °C, 7 min; 1 cycle
2. M428/228RAP	500	2.0	0.2	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 48 °C, 1 min and 72 °C, 45 s; 35 cycles and 72 °C, 7 min; 1 cycle
3. M122/135RAP	1,000	2.0	0.25	As described for 1.
4. MA16/222RAP	500	2.0	0.25	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 53 °C, 1 min and 72 °C, 45 s for 35 cycles and 72 °C, 7 min; 1 cycle
5. M122/159RAP.1	500	2.0	0.2	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 64 °C, 1 min and 72 °C, 45 s for 12 cycles where the annealing temperature was lowered for 2 °C in every other cycles and 94 °C, 30 s; 53 °C, 1 min and 72 °C, 45 s for additional 25 cycles and 72 °C, 7 min; 1 cycle

Table 2.14 (cont.)

Primer	cDNA template (ng)	$MgCl_2$ (mM)	Primer (μM)	PCR condition
6. M122/159RAP.2	1,500	2.0	0.2	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 53 °C, 1 min and 72 °C, 45 s for 35 cycles and 72 °C, 7 min; 1 cycle
Candidate differential expression markers				
1. SOCRAP340.1	500	1.5	0.2	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 50 °C, 1 min and 72 °C, 45 s for 35 cycles and 72 °C, 7 min; 1 cycle
2. SOCRAP340.2	1,000	2.0	0.4	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 53 °C, 1 min and 72 °C, 45 s; 40 cycles and 72 °C, 7 min; 1 cycle
3. SOC268/273RAP	1,000	2.0	0.25	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 53 °C, 1 min and 72 °C, 30 s for 35 cycles and 72 °C, 7 min; 1 cycle
4. SOC122/228RAP	500	2.0	0.25	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 53 °C, 1 min and 72 °C, 45 s for 35 cycles and 72 °C, 7 min; 1 cycle
5. BC428/228RAP	500	2.0	0.2	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 48 °C, 1 min and 72 °C, 45 s for 35 cycles and 72 °C, 7 min; 1 cycle
6. BC428/273RAP	1,500	2.0	0.2	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 53 °C, 1 min and 72 °C, 45 s for 35 cycles and 72 °C, 7 min; 1 cycle

2.16 SSCP analysis

2.16.1 Non - Denaturating Polyacrylamide gel electrophoresis

Non- denaturating polyacrylamide gels are used for size-fractionation of both single- and double-stranded DNA. As a general rule, double-stranded DNAs migrate through these gels at rates that are inversely proportional to the $\log_{10}$ of their size. However, their base composition and sequence also affect electrophoretic mobility, so that duplex DNAs of exactly the same size can differ in mobility by up to 10%.

The glass plates (PROTEAN II xi Cell) were cleaned and prepared as described previously. Different concentration of low crosslink non-denaturing polyacrylamide gels (37.5:1 or 75:1 of acrylamide and bis-acrylamide) were prepared by dilution of a 40% stock solution to required gen concentration. The acrylamide gel solution (30 - 40 ml) may be mixed with glycerol (5% or 10% concentration), if desired, and 240 µl of 10% APS and 24 µl of TEMED. The analytical comb was inserted into the prepared gel and allow for polymerization for 4 hours or overnight.

For SSCP analysis, 6 µl of the amplified products were mixed with 24 µl of the SSCP loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured in a boiling bath for 5 minutes and immediately cooled on ice for 3 minutes. The denatured PCR products were electrophoretically analyzed in native polyacrylamide gels (different gel concentration of 37.5:1 or 75:1 crosslink with and/or witout glycerol) at 250 – 300 volts for 16 – 24 hours at 4 °C. The electrophoresed bands were visualized by silver staining described previously with the exception that the gel was rinsed for 3 times for 3 minutes each after the fix/stop step.

CHAPTER III

RESULTS

3.1 Genomic DNA samples

Genomic DNA was extracted from pleopods of *M. rosenbergii* using a proteinase K / phenol / chloroform extraction method (Klinbunga et al., 1999). The quality of extracted genomic DNA was examined by agarose gel elctrophoresis (Fig. 3.1). Generally, high molecular weight DNA was observed along with partial degraded DNA. The quantity of genomic DNA was spectrophotometrically estimated. The ratio between OD_{260} to OD_{280} ratio was approximately 1.8 indicating the acceptable quality of extracted DNA.

3.2 Amplified fragment length polymorphism (AFLP) analysis

3.2.1 First screening of candidate sex-specific markers in *M. rosenbergii* using AFLP analysis

Pooled genomic DNA of BC (N = 5) and SOC (N = 10) males and females (N = 10) was subjected to AFLP analysis. The preamplification products indicated successful digestion/ligation of *M. rosenbergii* genomic DNA following an AFLP approaches (Fig. 3.2).

Selective amplification products were carried out for 64 primer combinations. The amplification success of the selective amplification products were electrophoretically analyzed by agarose gels (Fig. 3.3). The AFLP products were then analyzed by 4.5% denaturing polyacrylamide gel electrophoresis. Sex-specific markers were considered from those clearly found in males but absent from female and *vice versa* (Fig. 3.4 – 3.8). A total of 90 male- and 42 female-specific AFLP markers from 40 polymorphic primer combinations were found from screening of 3 bulked DNA (BC1, SOC1 and FE1) of *M. rosenbergii* (Table 3.1 and Appendix D).

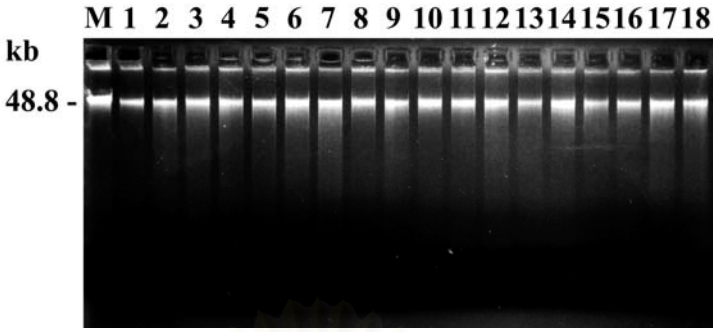

Figure 3.1 A 1.0% ethidium bromide stained agarose gel showing genomic DNA extracted from pleopods of *M. rosenbergii* (lanes 1 - 18) and 100 ng of λ DNA (lane M).

3.2.2 Second screening of candidate sex-specific markers in *M. rosenbergii* using AFLP analysis

To eliminate false positive AFLP markers from the first screening, polymorphic AFLP primer combinations yielded candidate male- or female-specific markers were further screened with the first sample set (BC1, SOC1 and FE1) and the second sample set (BC2 ,*N* =10; OC1 males, *N* = 15; SOC2 males, *N* = 5 and FE2, *N* = 20).

On the one hand, a large number of positive markers from the first screening were reduced. On the other hand, a 270 bp fragment from *Eco* RI_{+3}-8/ *Mse* I_{+3}-3 which was not found from the first screening against 3 bulked DNA was observed in the second screening against both sample sets (Fig 3.6 and 3.15). Conversely, a 290 bp AFLP fragment from *Eco* RI_{+3}-1/*Mse* I_{+3}-7 which were specifically found with FE1 still retained its female specificity when screened with more bulked DNA (Fig 3.4 and 3.9). Finally, the number of candidate primers was significantly reduced to only 5 and 4 candidate markers for male and female, respectively (Fig 3.9 – 3.16 and Table 3.2).

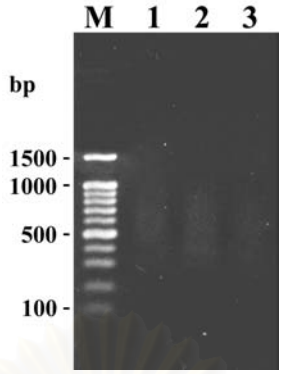

Figure 3.2 A 1.5% ethidium bromide stained agarose gel showing preamplification products of bulked genomic DNA of BC1 (lane 1) and SOC1 (lane 2) males and FE1 (lane 3). A 100 bp DNA ladder (lane M) was used as the DNA standard.

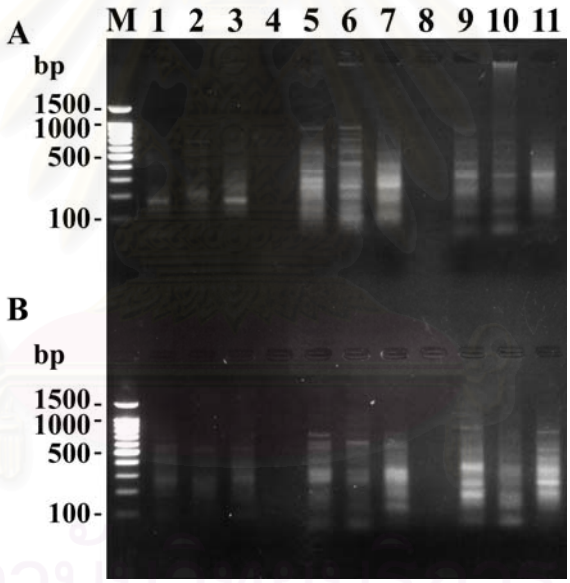

Figure 3.3 A 1.8% ethidium bromide stained agarose gel showing the selective amplification products using various primer combinations.

Lanes 1 – 3; A = *Eco* RI_{+3}-1/*Mse* I_{+3}-7 Lanes 5 – 7; A = *Eco* RI_{+3}-2/*Mse* I_{+3}-7

Lanes 9 - 11; A = *Eco* $RI+_{3}$-3/*Mse* I_{+3}-7 Lanes 1 - 3; B = *Eco* RI_{+3}-4/*Mse* I_{+3}-7

Lanes 5 - 7; B = *Eco* RI_{+3}-5/*Mse* I_{+3}-7 Lanes 9 - 11; B = *Eco* RI_{+3}-6/*Mse* I_{+3} -7

Lane M = A 100 bp DNA ladder.

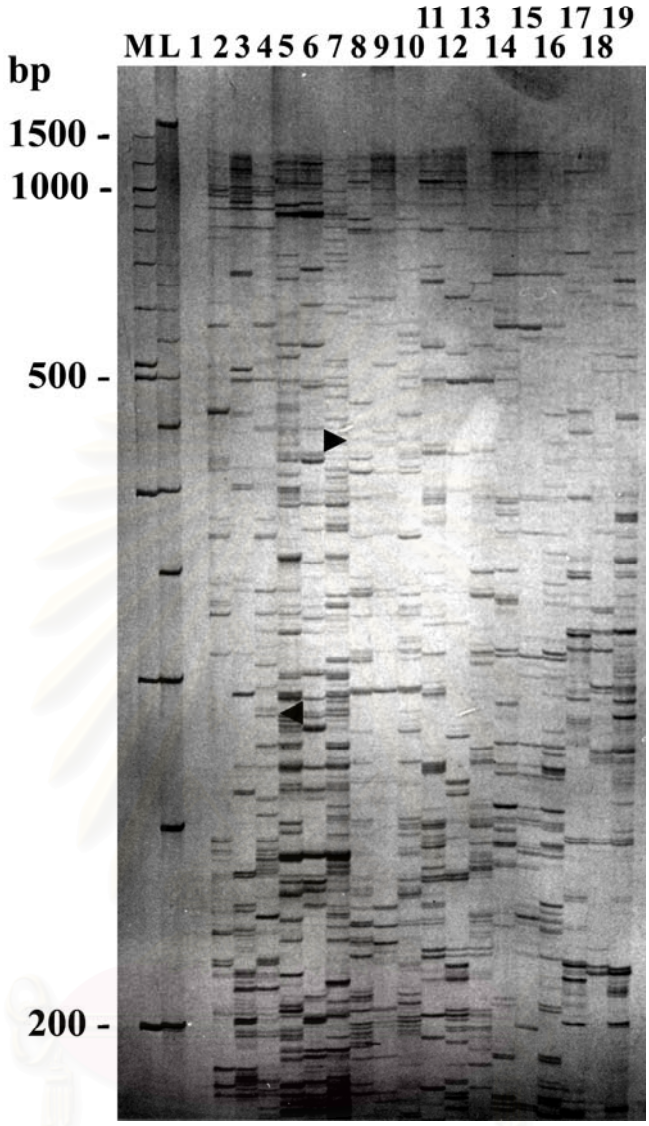

Figure 3.4 A 4.5% denaturing polyacrylamide showing AFLP patterns of BC1 (lanes 2, 5, 8, 11, 14 and 17) and OC1 (lanes 3, 6, 9, 12, 15 and 18) and FE1 (lanes 4, 7, 10, 13, 16 and 19) of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-1/*Mse* I_{+3}-7 (lanes 2-4), *Eco* RI_{+3}-2/*Mse* I_{+3}-7 (lanes 5-7), *Eco* RI_{+3}-3/*Mse* I_{+3}-7 (lanes 8-10), *Eco* RI_{+3}-4/*Mse* I_{+3}-7 (lanes 11-13), *Eco* RI_{+3}-5/*Mse* I_{+3}-7 (lanes 14 – 16), *Eco* RI_{+3}-6/*Mse* I_{+3}-7 (lanes 17-19). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific markers.

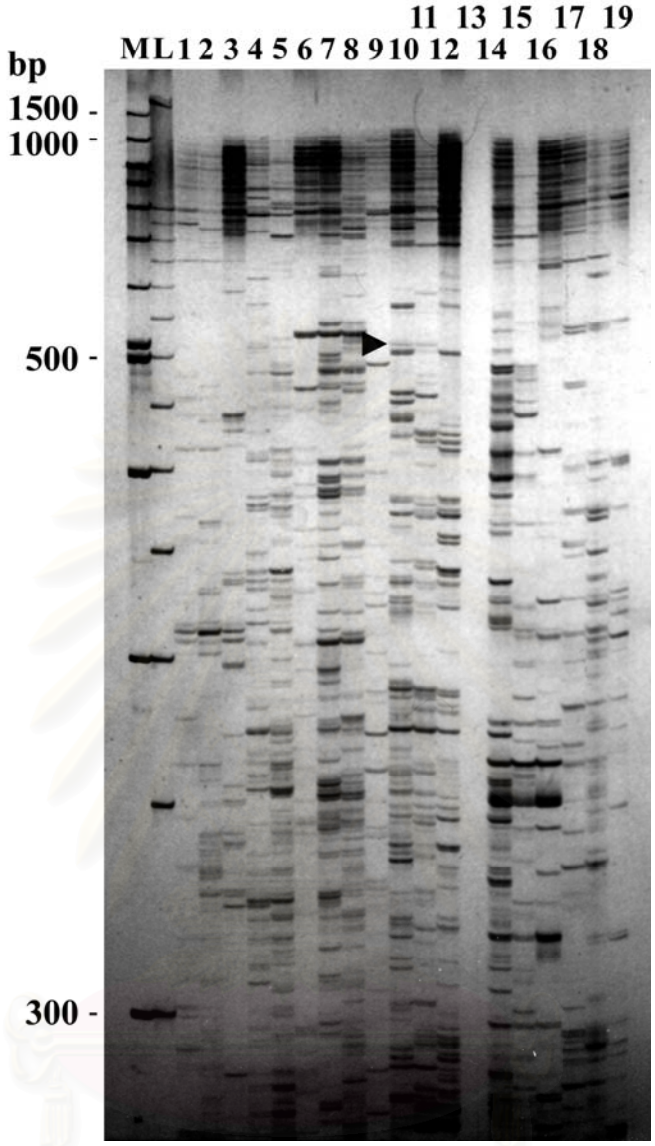

Figure 3.5 A 4.5% denaturing polyacrylamide showing AFLP patterns of BC1 (lanes 1, 4, 7, 10, 14 and 17) and OC1 (lanes 2, 5, 8, 11, 15 and 18) and FE1 (lanes 3, 6, 9, 12, 15 and 19) of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-1/*Mse* I_{+3}-8 (lanes 1 - 3), *Eco* RI_{+3}-2/*Mse* I_{+3}-8 (lanes 4 - 6), *Eco* RI_{+3}-3/*Mse* I_{+3}-8 (lanes 7 - 9), *Eco* RI_{+3}-4/*Mse* I_{+3}-8 (lanes 10 - 12), *Eco* RI_{+3}-5/*Mse* I_{+3}-8 (lanes 14 – 16), *Eco* RI_{+3}-6/*Mse* I_{+3}-8 (lanes 17 - 19). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific markers.

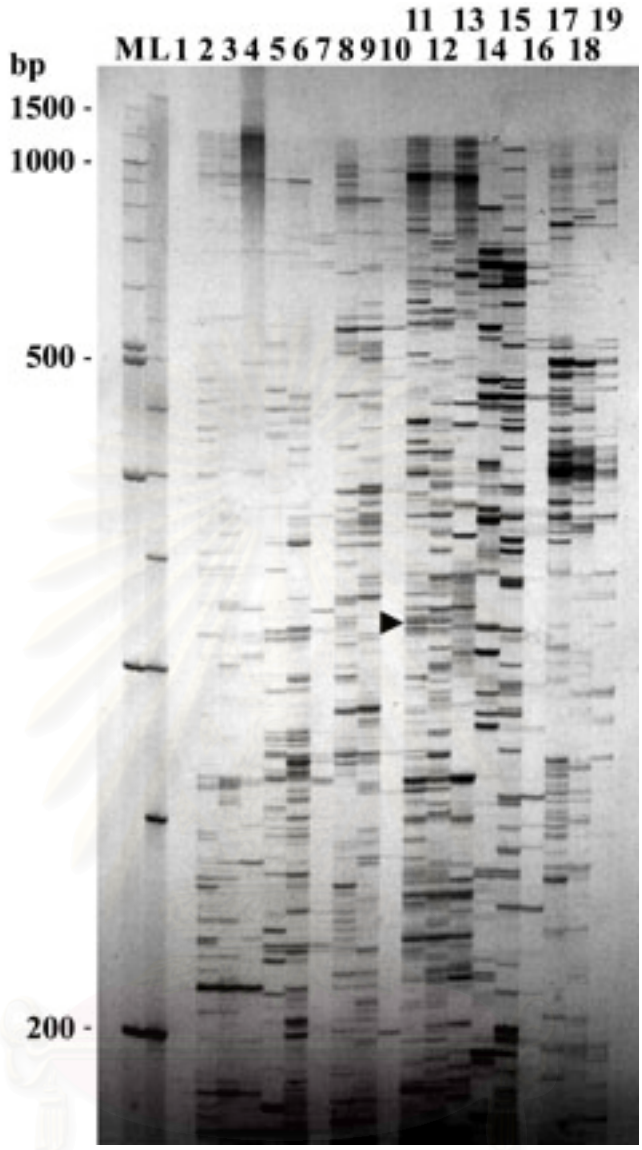

Figure 3.6 A 4.5% denaturing polyacrylamide showing AFLP patterns of BC1 (lanes 2, 5, 8, 11, 14 and 17) and OC1 (lanes 3, 6, 9, 12, 15 and 18) and FE1 (lanes 4, 7, 10, 13, 16 and 19) of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-7/*Mse* I_{+3}-1 (lanes 2 - 4), *Eco* RI_{+3}-7/*Mse* I_{+3}-2 (lanes 5 - 7), *Eco* RI_{+3}-8/*Mse* I_{+3}-1 (lanes 8 - 10), *Eco* RI_{+3}-8/*Mse* I_{+3}-2 (lanes 11 - 13), *Eco* RI_{+3}-8/*Mse* I_{+3}-3 (lanes 14 - 16), *Eco* RI_{+3}-8/*Mse* I_{+3}-3 (lanes 17 - 19). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific markers.

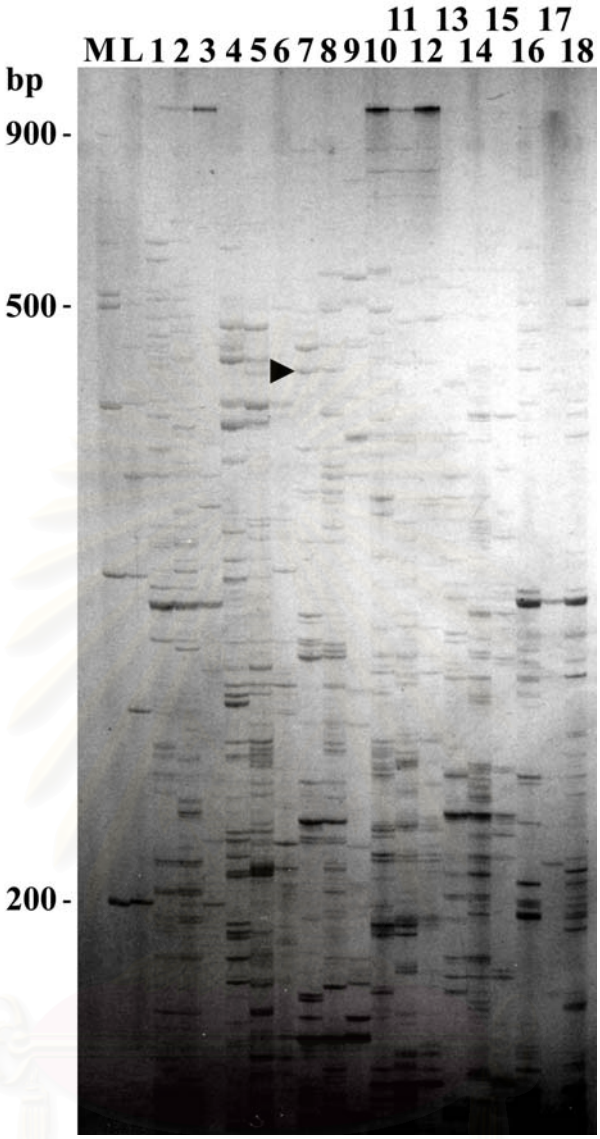

Figure 3.7 A 4.5% denaturing polyacrylamide showing AFLP patterns of BC1 (lanes 1, 4, 7, 10, 13 and 16) and OC1 (lanes 2 5, 8, 11, 14 and 17) and FE1 (lanes 3, 6, 9, 12, 15 and 18) of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-7/*Mse* I_{+3}-4 (lanes 1 - 3), *Eco* RI_{+3}-7/*Mse* I_{+3}-5 (lanes 4 - 6), *Eco* RI_{+3}-7/*Mse* I_{+3}-6 (lanes 7 - 9), *Eco* RI_{+3}-8/*Mse* I_{+3}-8 (lanes 10 - 12), *Eco* RI_{+3}-8/*Mse* I_{+3}-5 (lanes 14 – 16), *Eco* RI_{+3}-8/*Mse* I_{+3}-6 (lanes 17 - 19). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific markers.

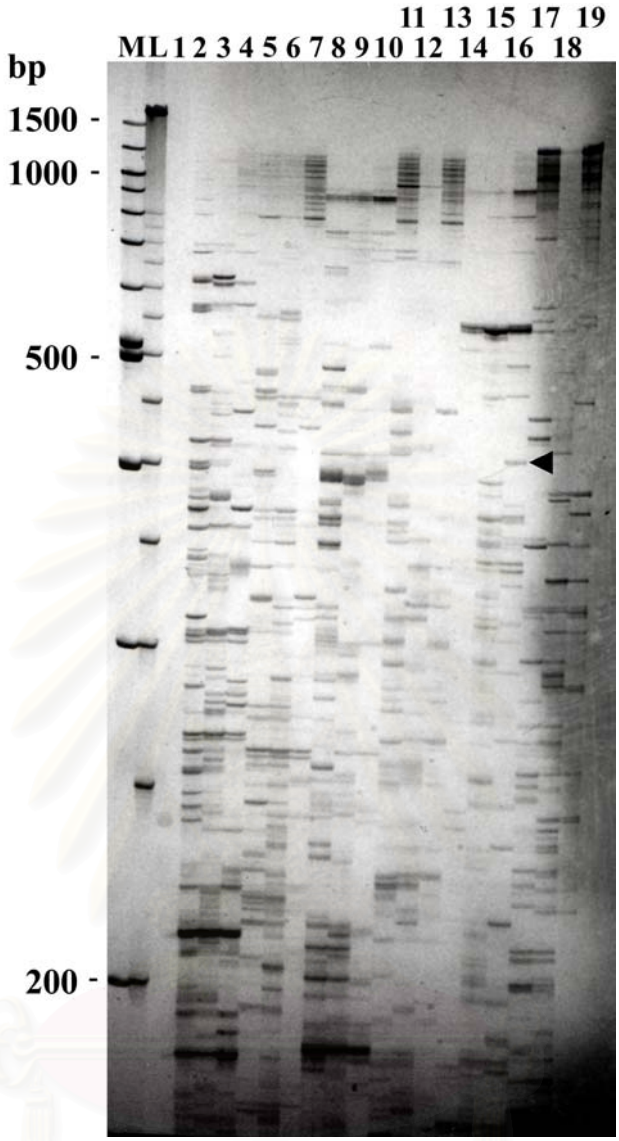

Figure 3.8 A 4.5% denaturing polyacrylamide showing AFLP patterns of BC1 (lanes 2, 5, 8, 11, 14 and 17) and OC1 (lanes 3, 6, 9, 12, 15 and 18) and FE1 (lanes 4, 7, 10, 13, 16 and 19) of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-7/*Mse* I_{+3}-7 (lanes 2 - 4), *Eco* RI_{+3}-7/*Mse* I_{+3}-8 (lanes 5 - 7), *Eco* RI_{+3}-8/*Mse* I_{+3}-7 (lanes 8 - 10), *Eco* RI_{+3}-8/*Mse* I_{+3}-8 (lanes 11 - 13), *Eco* RI_{+3}-5/*Mse* I_{+3}-1 (lanes 14 - 16), *Eco* RI_{+3}-5/*Mse* I_{+3}-4 (lanes 17 - 19). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific markers.

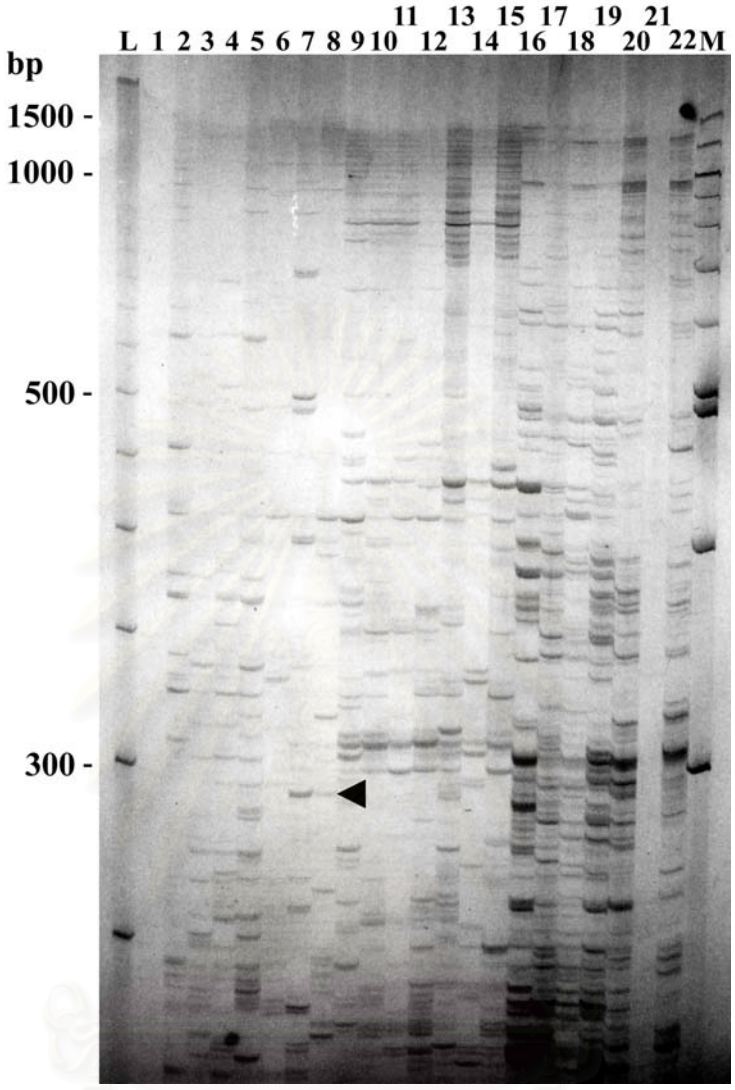

Figure 3.9 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of BC1 (lanes 2, 9 and 16), BC2 (lanes 3, 10 and 17), OC2 (lanes 4, 11 and 18), SOC1 (lanes 5, 12 and 19) and SOC2 (lanes 6, 13 and 20) males and FE1 (lanes 7, 14 and 21) and FE2 (lanes 8, 15 and 22) females of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-1/*Mse* I_{+3}-7 (lanes 2 - 8), *Eco* RI_{+3}-1/*Mse* I_{+3}-8 (lanes 9 - 15) and *Eco* RI_{+3}-2/*Mse* I_{+3}-1 (lanes 16 - 23). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific AFLP markers.

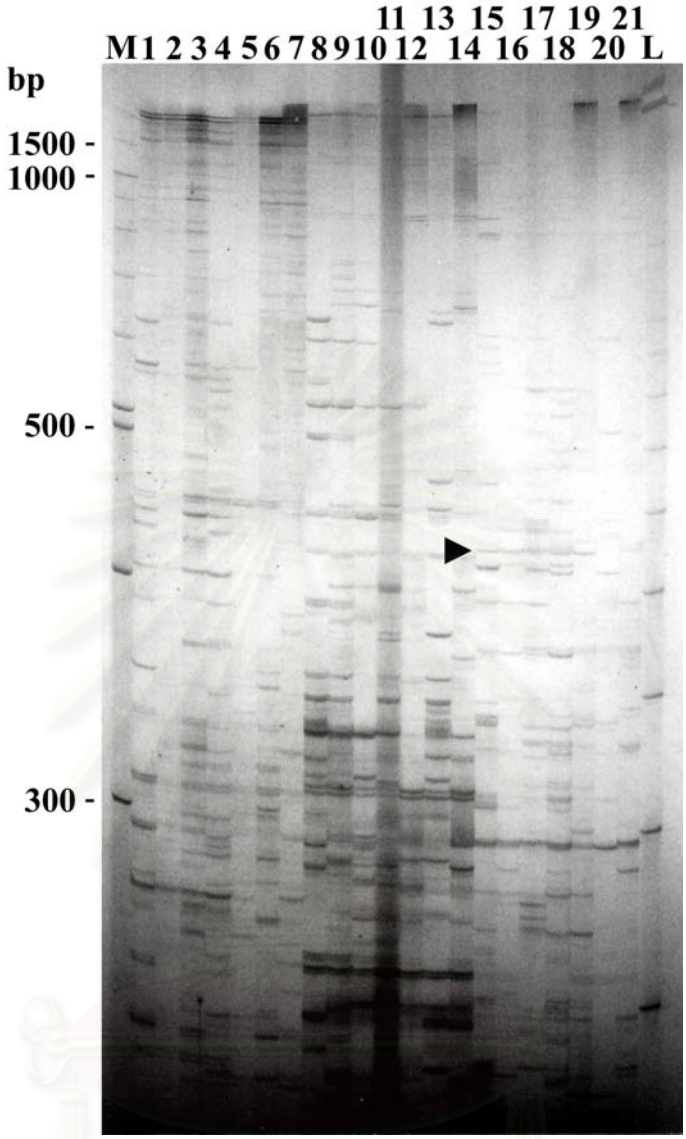

Figure 3.10 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of BC1 (lanes 1, 8 and 15), BC2 (lanes 2, 9 and 16), OC2 (lanes 3, 10 and 17), SOC1 (lanes 4, 11 and 18) and SOC2 (lanes 5, 12 and 19) males and FE1 (lanes 6, 13 and 20) and FE2 (lanes 7, 14 and 21) females of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-3/*Mse* I_{+3}-5 (lanes 1 - 7), *Eco* RI_{+3}-3/*Mse* I_{+3}-6 (lanes 8 - 14) and *Eco* RI_{+3}-3/*Mse* I_{+3}-7 (lanes 15 - 21). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific AFLP markers.

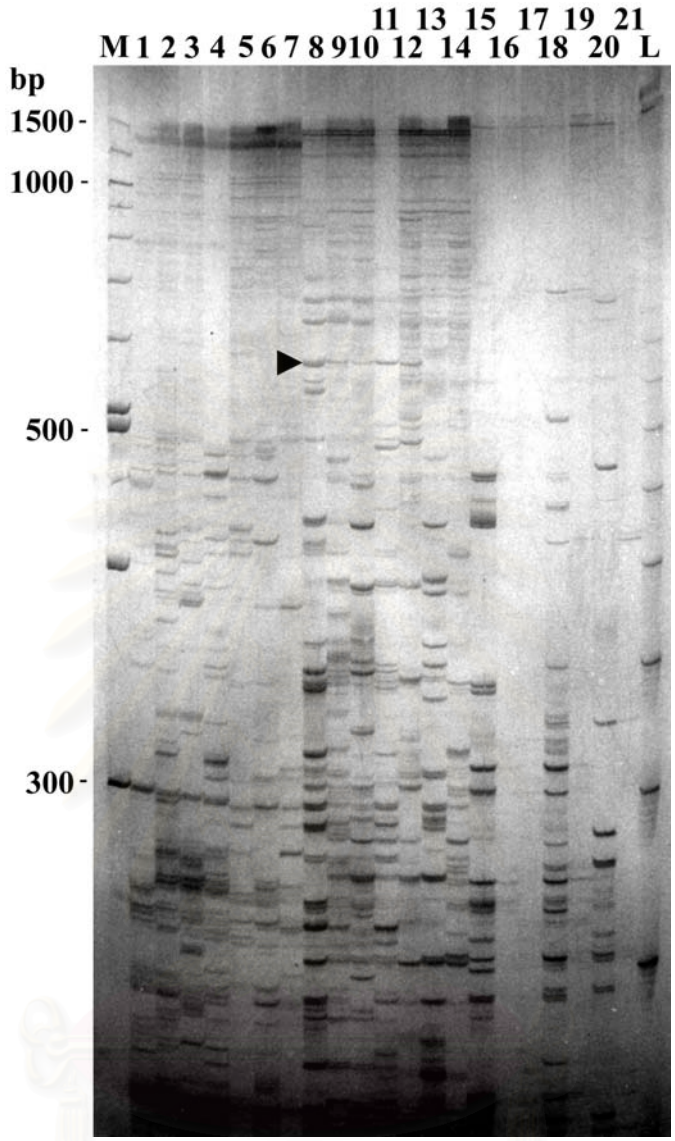

Figure 3.11 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of BC1 (lanes 1, 8 and 15), BC2 (lanes 2, 9 and 16), OC2 (lanes 3, 10 and 17), SOC1 (lanes 4, 11 and 18) and SOC2 (lanes 5, 12 and 19) males and FE1 (lanes 6, 13 and 20) and FE2 (lanes 7, 14 and 21) females of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-4/*Mse* I_{+3}-4 (lanes 1 - 7), *Eco* RI_{+3}-4/*Mse* I_{+3}-5 (lanes 8 - 14) and *Eco* RI_{+3}-4/*Mse* I_{+3}-6 (lanes 15 - 21). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific AFLP markers.

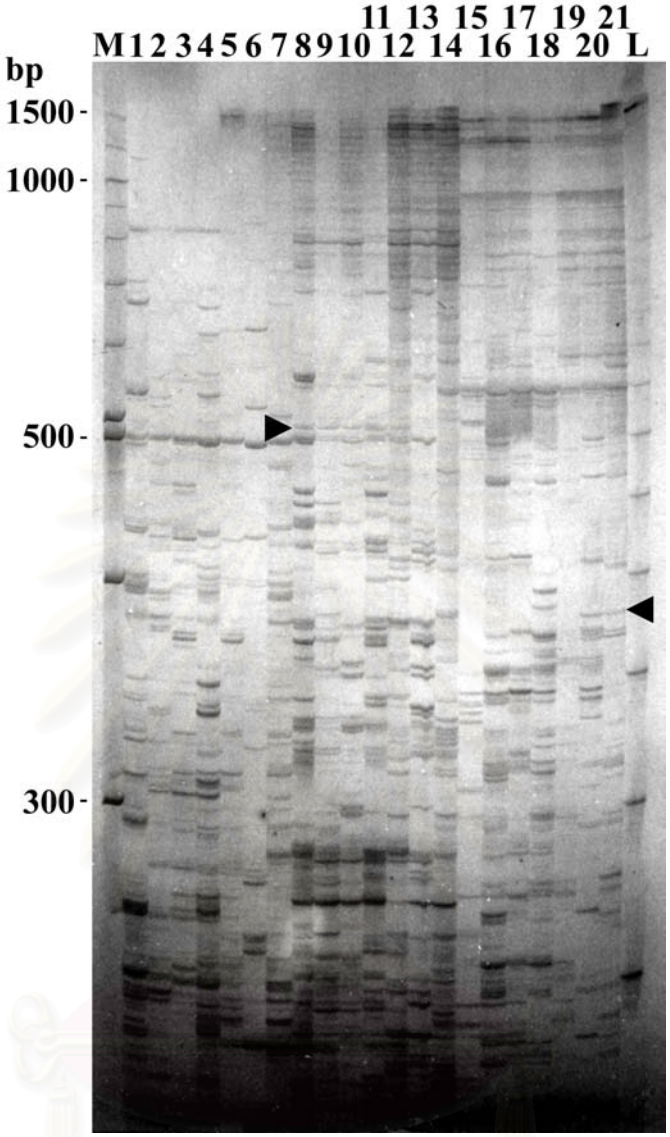

Figure 3.12 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of BC1 (lanes 1, 8 and 15), BC2 (lanes 2, 9 and 16), OC2 (lanes 3, 10 and 17), SOC1 (lanes 4, 11 and 18) and SOC2 (lanes 5, 12 and 19) males and FE1 (lanes 6, 13 and 20) and FE2 (lanes 7, 14 and 21) females of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-4/*Mse* I_{+3}-7 (lanes 1 - 7), *Eco* RI_{+3}-4/*Mse* I_{+3}-8 (lanes 8 - 14) and *Eco* RI_{+3}-5/*Mse* I_{+3}-1 (lanes 15 - 21). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific AFLP markers.

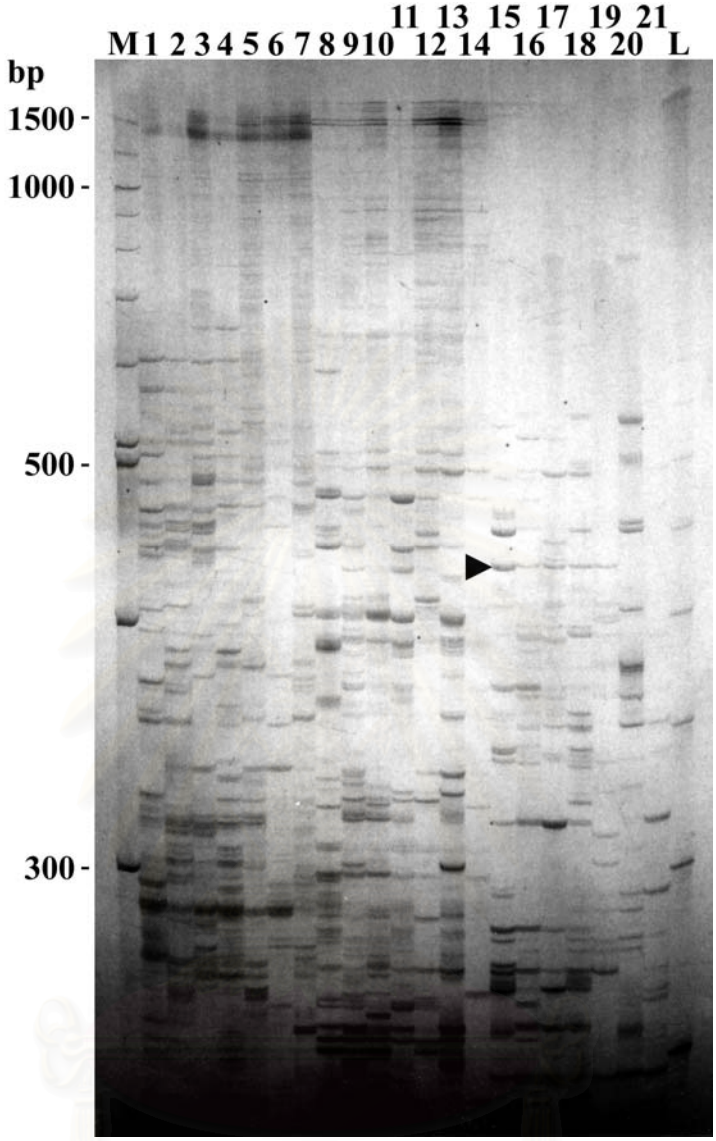

Figure 3.13 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of BC1 (lanes 1, 8 and 15), BC2 (lanes 2, 9 and 16), OC2 (lanes 3, 10 and 17), SOC1 (lanes 4, 11 and 18) and SOC2 (lanes 5, 12 and 19) males and FE1 (lanes 6, 13 and 20) and FE2 (lanes 7, 14 and 21) females of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-7/*Mse* I_{+3}-4 (lanes 1 - 7), *Eco* RI_{+3}-8/*Mse* I_{+3}-14 (lanes 8 - 14) and *Eco* RI_{+3}-7/*Mse* I_{+3}-6 (lanes 15 - 21). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific AFLP markers.

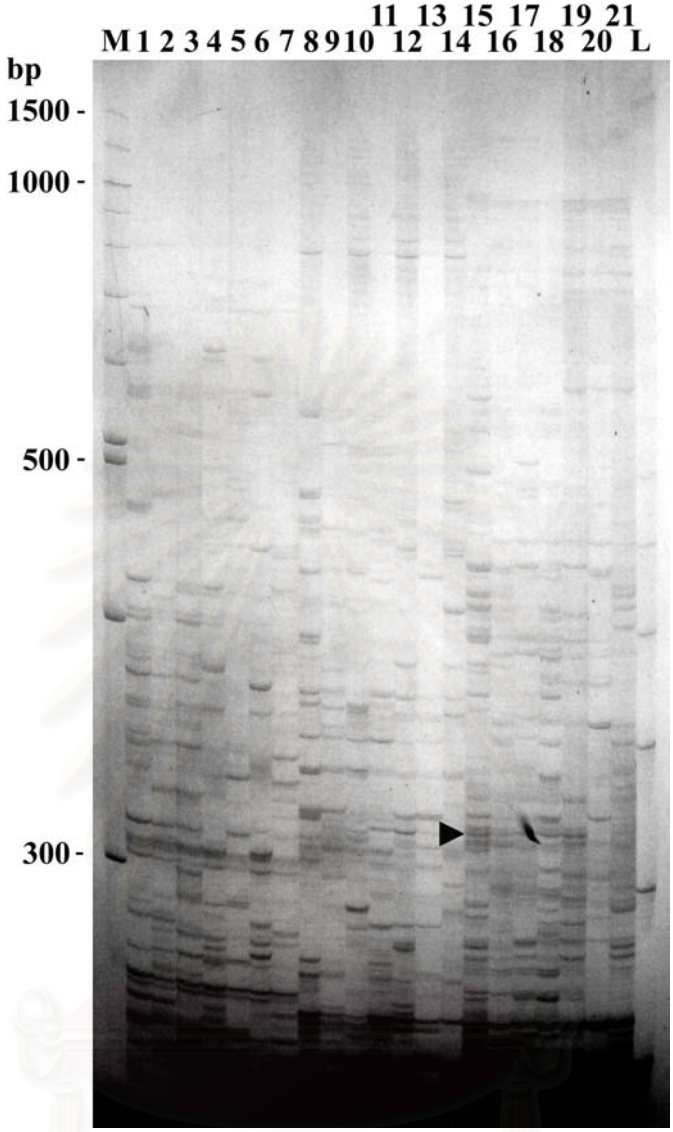

Figure 3.14 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of BC1 (lanes 1, 8 and 15), BC2 (lanes 2, 9 and 16), OC2 (lanes 3, 10 and 17), SOC1 (lanes 4, 11 and 18) and SOC2 (lanes 5, 12 and 19) males and FE1 (lanes 6, 13 and 20) and FE2 (lanes 7, 14 and 21) females of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-7/*Mse* I_{+3}-7 (lanes 1 - 7), *Eco* RI_{+3}-8/*Mse* I_{+3}-14 (lanes 8 - 14) and *Eco* RI_{+3}-8/*Mse* I_{+3}-1 (lanes 15 - 21). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific AFLP markers.

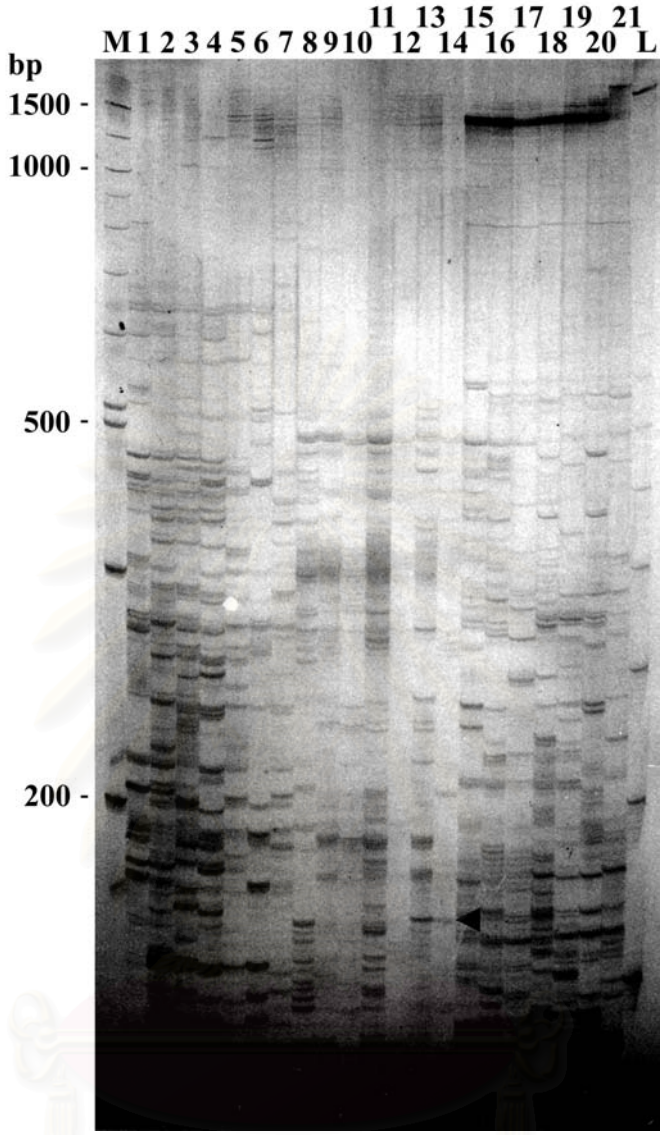

Figure 3.15 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of BC1 (lanes 1, 8 and 15), BC2 (lanes 2, 9 and 16), OC2 (lanes 3, 10 and 17), SOC1 (lanes 4, 11 and 18) and SOC2 (lanes 5, 12 and 19) males and FE1 (lanes 6, 13 and 20) and FE2 (lanes 7, 14 and 21) females of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-8/*Mse* I_{+3}-2 (lanes 1 - 7), *Eco* RI_{+3}-8/*Mse* I_{+3}-3 (lanes 8 - 14) and *Eco* RI_{+3}-8/*Mse* I_{+3}-4 (lanes 15 - 21). Lanes M and L are 100 bp and 50 bp DNA standards, respectively. Arrowheads indicated candidate sex-specific AFLP markers.

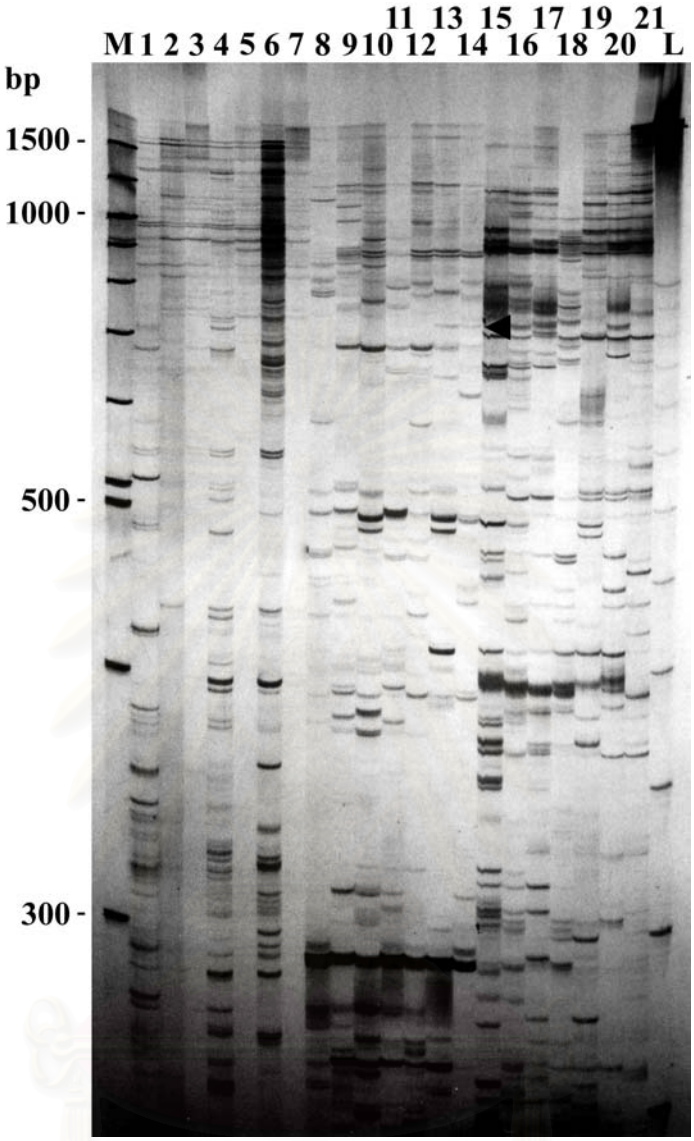

Figure 3.16 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of BC1 (lanes 1, 8 and 15), BC2 (lanes 2, 9 and 16), OC2 (lanes 3, 10 and 17), SOC1 (lanes 4, 11 and 18) and SOC2 (lanes 5, 12 and 19) males and FE1 (lanes 6, 13 and 20) and FE2 (lanes 7, 14 and 21) females of *M. rosenbergii* using primer combinations *Eco* RI_{+3}-8/*Mse* I_{+3}-5 (lanes 1 - 7), *Eco* RI_{+3}-8/*Mse* I_{+3}-6 (lanes 8 - 14) and *Eco* RI_{+3}-8/*Mse* I_{+3}-7 (lanes 15 - 21). Lanes M and L are 100 bp and 50 bp DNA markers, respectively. Arrowheads indicated candidate sex-specific AFLP markers.

Table 3.1 Number of AFLP bands showing candidate sex-specific AFLP markers across primer combinations when screened with the first sample set (BC1, SOC1, and FE1)

	Mse I_{+3} - 1	*Mse* I_{+3} - 2	*Mse* I_{+3} - 3	*Mse* I_{+3} - 4	*Mse* I_{+3} - 5	*Mse* I_{+3} - 6	*Mse* I_{+3} - 7	*Mse* I_{+3} - 8
Eco RI_{+3}-1	2 (0)*	-	-	-	-	-	0 (3)	2 (2)
Eco RI_{+3}-2	-	-	-	2 (0)	-	-	1 (0)	8 (1)
Eco RI_{+3}-3	-	1 (3)	1 (0)	-	2 (2)	1 (0)	1 (8)	5 (1)
Eco RI_{+3}-4	1 (0)	-	1 (1)	2 (0)	1 (0)	1 (0)	1 (2)	1 (4)
Eco RI_{+3}-5	0 (4)	-	-	4 (3)	2 (0)	-	-	2 (2)
Eco RI_{+3}-6	-	-	-	-	-	-	-	5 (1)
Eco RI_{+3}-7	1 (0)	5 (1)	2 (0)	3 (1)	1 (1)	5 (0)	2 (0)	5 (1)
Eco RI_{+3}-8	2 (0)	2 (0)	3 (0)	1 (1)	2 (0)	1 (0)	3 (0)	5 (0)

* Numbers in parentheses indicated candidate female-specific AFLP marker.

Table 3.2 Number of AFLP bands showing candidate sex-specific AFLP markers across primer combinations when screened with both the first sample set (BC1, SOC1, and FE1) and the second sample set (BC2, OC1, SOC2 and FE2)

Candidate AFLP marker	**Primer combination**	**Size (bp)**
Male-specific	*Eco* RI_{+3}-3/*Mse* I_{+3}-7	425
	Eco RI_{+3}-7/*Mse* I_{+3}-6	425
	Eco RI_{+3}-4/*Mse* I_{+3}-5	570
	Eco RI_{+3}-8/*Mse* I_{+3}-1	310
	Eco RI_{+3}-4/*Mse* I_{+3}-8	517
Female-specific	*Eco* RI_{+3}-1/*Mse* I_{+3}-7	290
	Eco RI_{+3}-5/*Mse* I_{+3}-1	390
	Eco RI_{+3}-8/*Mse* I_{+3}-3	270
	Eco RI_{+3}-8/*Mse* I_{+3}-6	710

3.2.3 Cloning and characterization of candidate sex-specific markers

All AFLP fragments shown sex-specificity from the second screening constituting of a 425 bp fragment from E_{+3}-3/M_{+3}-7 (called $ME_{+3}3M_{+3}7425$), a 425 bp fragment from E_{+3}-7/M_{+3}-6 (called $ME_{+3}7M_{+3}6425$), a 570 bp fragment from E_{+3}-4/M_{+3}-5 (called $ME_{+3}4M_{+3}5570$), a 310 bp fragment from E_{+3}-8/M_{+3}-1 (called $ME_{+3}8M_{+3}1310$), a 517 bp fragment from E_{+3}-4/M_{+3}-8 (called $ME_{+3}4M_{+3}8517$), a 290 bp fragment from E_{+3}-1/M_{+3}-7 (called $FE_{+3}1M_{+3}7290$), a 390 bp fragment from E_{+3}-5/M_{+3}-1 (called $FE_{+3}5M_{+3}1390$), a 270 bp fragment from E_{+3}-8/M_{+3}-3 (called $FE_{+3}8M_{+3}3270$) and a 710 bp fragment from E_{+3}-8/M_{+3}-6 (called $FE_{+3}8/M_{+3}6$ 710) were gel - eluted and successfully reamplified (Fig. 3.17).

The reamplified PCR product was successfully cloned. Colony PCR was performed to evaluation of the insert sizes (insert size + 344 bp of the vector, Fig. 3.18). The colony PCR products (Fig. 3.19) or alternatively the recombinant plasmid (Fig. 3.20) were digested with restriction enzymes (usually *Hin*d III and *Rsa* I for colony PCR products and *Eco* RI for recombinant plasmid DNA) and revealed that more than one type of sequences were found from a single insert (Fig. 3.19 and 3.20 and Table 3.3). In a total, 13 clone types were found from 9 inserts.

Recombinant plasmid was then extracted from representative of different clone types and subjected to DNA sequencing unidirectionally (Fig 3.21 – 3.33). The original AFLP primer combinations were found in all sequences indicating that the insert was not generated by non-specific amplification from only one primer. Comparing of nucleotide sequences of these characterized AFLP markers did not revealed significant matching with any sequence previously deposited in the GenBank ($P > 10^{-4}$).

3.2.4 Primer design and development of sex-specific PCR in *M. rosenbergii*

Primer pairs were designed from nucleotide sequences to convert the dominant marker like AFLP to co-dominant SCAR marker. Sequences and positions of primers are shown in Fig. 3.21 – 3.33.

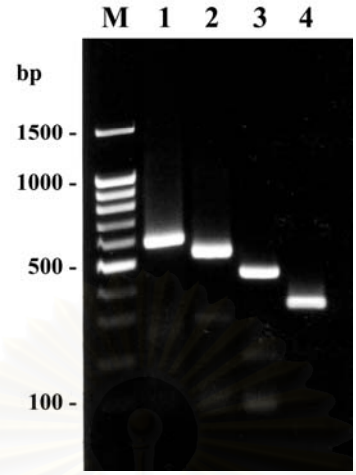

Figure 3.17 A 1.5% ethidium bromide stained agarose gel showing the reamplification products of 4 candidate male-specific AFLP markers resulted from primer combinations *Eco* RI_{+3}-4/*Mse* I_{+3}-5 (lane 1), *Eco* RI_{+3}-4/*Mse* I_{+3}-8 (lane 2), *Eco* RI_{+3}-7/*Mse* I_{+3}-6 (lane 3) and *Eco* RI_{+3}-8/*Mse* I_{+3}-1 (lanes 4). A 100 bp ladder (lane M) was used as the DNA marker.

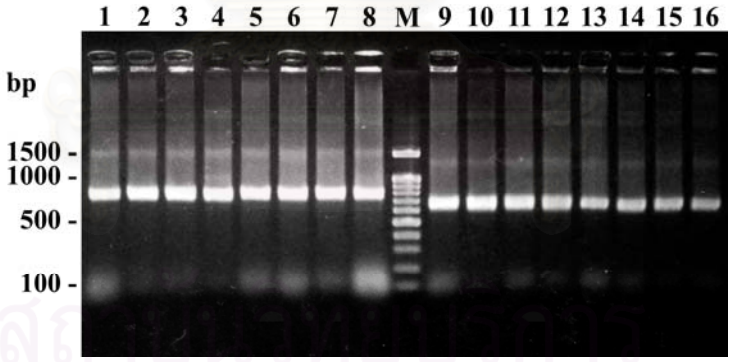

Figure 3.18 A 1.5% ethidium bromide stained agarose gel showing colony PCR products of 2 candidate male-specific AFLP markers from *Eco* RI_{+3}-7/*Mse* I_{+3}-6 (425 bp of an insert + 334 bp of the vector, lane 1 - 8) and *Eco* RI_{+3}-8/*Mse* I_{+3}-1 (310 bp of an insert + 334 bp of the vector, lanes 9 - 16). A 100 bp ladder (lane M) was used as the DNA marker.

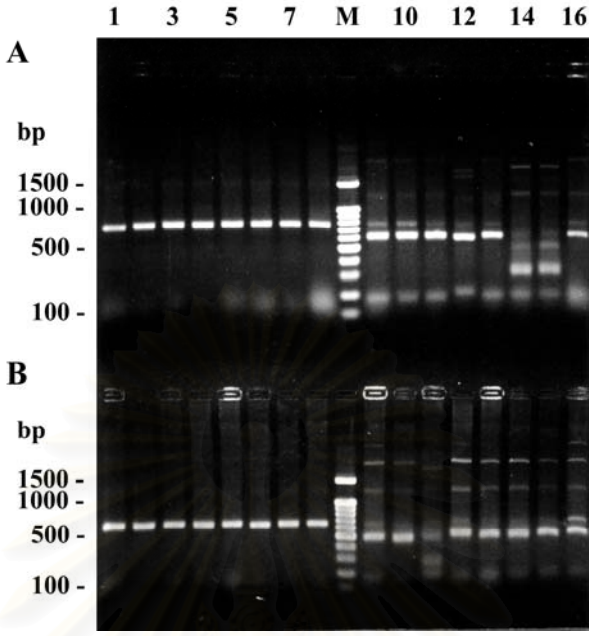

Figure 3.19 A 1.2% ethidium bromide stained agarose gel showing digestion patterns of colony PCR products of cloned AFLP fragments from *Eco* RI_{+3}-7/*Mse* I_{+3}-6 (425 bp insert; lanes 1 - 8, A) and *Eco* RI_{+3}-8/*Mse* I_{+3}-1 (310 bp insert; lanes 1- 8, B) with *Hin*d III and *Eco* RI_{+3}-7/*Mse* I_{+3}-6 (lanes 9 – 16, A), and *Eco* RI_{+3}-8/*Mse* I_{+3}-1 (lanes 9 – 16, B) with *Rsa* I. A 100 bp ladder (lane M) was used as the DNA marker.

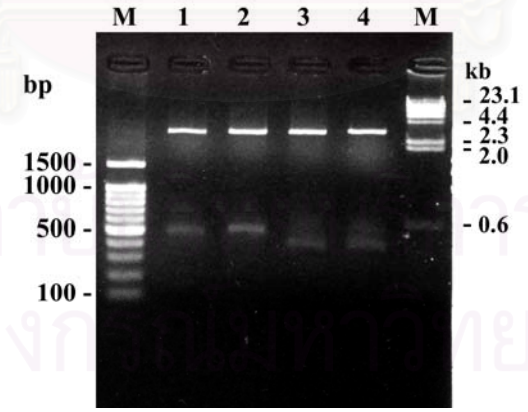

Figure 3.20 A 1.5% ethidium bromide stained gel showing the digestion product of the recombinant clone carrying a 425 bp insert from *Eco* RI_{+3}-7/*Mse* I_{+3}-6 (lanes 1 - 2) and a 310 bp insert from *Eco* RI_{+3}-8/*Mse* I_{+3}-1 (lanes 3 - 4) with *Eco* RI. A 100 bp ladder (lanes M) and λ–*Hin*d III was used as the DNA marker.

Table 3.3 Number of primers designing from each AFLP marker

Candidate Sex-specific AFLP marker	Size (bp)	No. of clones types from digestion (no. of designed primer pairs)	Name of primers
Male			
Eco RI_{+3}-3/*Mse* I_{+3}-7	425	3 (2)	$ME_{+3}3M_{+3}7425.1$
			$ME_{+3}3M_{+3}7425.2$
Eco RI_{+3}-7/*Mse* I_{+3}-6	425	2 (1)	$ME_{+3}7M_{+3}6425$
Eco RI_{+3}-4/*Mse* I_{+3}-5	570	1 (1)	$ME_{+3}4M_{+3}5570$
Eco RI_{+3}-8/*Mse* I_{+3}-1	310	3 (3)	$ME_{+3}8M_{+3}1310.1$
			$ME_{+3}8M_{+3}1310.2$
			$ME_{+3}8M_{+3}1310.3$
Eco RI_{+3}-4/*Mse* I_{+3}-8	517	2 (1)	$ME_{+3}4M_{+3}8517$
Female			
Eco RI_{+3}-1/*Mse* I_{+3}-7	290	1 (1)	$FE_{+3}1M_{+3}7290$
Eco RI_{+3}-5/*Mse* I_{+3}-1	390	1 (1)	$FE_{+3}5M_{+3}1390$
Eco RI_{+3}-8/*Mse* I_{+3}-3	270	3 (2)	$FE_{+3}8M_{+3}3270.1$
			$FE_{+3}8M_{+3}3270.2$
Eco RI_{+3}-8/*Mse* I_{+3}-6	710	2 (1)	$FE_{+3}8M_{+3}6710$

GACTGCGTACCAATTCACAAAGTCTTCTGATCATTACCTGACATAGATTTGGTTATGTCATC
AGAGGCCTGGAAGTTACAAAGTCTTCTGATCATTAGCTGATACAGATTTGGTTGTCATCAAA
GGTCTGGAAGTTACAAAGGCATCTGATGGTTTAGCTGGAAGCTACAA***GGATGTCTGACGGCT***
TGCTGGAAGCTGCAAGGGCAACAGCTTTCCAGCTGCCCTGTGAAGCTACTGTGCAGATATGC
AAAGGAGTCTGATAAAGGCTTCAGCAGGATAAATGCGGCGCCTAAACTCAGGAACCTCACCG
AGGCATTTCTGGGCGGAGTTTCTCCACCACTTTCTGACAGGAG***CACATATCCTTTTCCGCCT***
CCCAGATGCGATCTCCATTCCAGCTTTCAACC**CAGTTACTCAGGACTCATC**

Figure 3.21 Nucleotide sequence of candidate male-specific marker, $ME_{+3}3M_{+3}7425.1$ (actual size = 423 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GACTGCGTACCAATTCACACTATGCATGCCTAAAGAGATATATCGGACCAATGCA***AAGCACG***
CACATACTCACACATCCGTGGAAAGGATATCATATAGCAAAAGGTTCTAAAATATTCCAGCA
TATGAATAACCACTCGTTACAGTTCTAGCAAAGGCGTGAGTAAGAAGCTGCGTCTACAGACT
TACTGATTTATTCATACAACAAACACACAGGTCAATAGCTCTTGCGAGCGGAAATGTAGTGC
CTGACACGGGGCGAACAAAACAGAGATCCGAGTACAATCAATTGTGTTT***GTTGGAGGATGGA***
AAGATGCACATAAATCAATATGCAAGACACGCATGAAGTTATGATACATATAGCTGGAATGC
TGGCATTGTAATGCTTGAGCTAGGAATGGTA**CAGTTACTCAGGACTCATC**

Figure 3.22 Nucleotide sequence of candidate male-specific marker, $ME_{+3}3M_{+3}7425.2$ (actual size = 422 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACTCTTTTACATTGATATGAAGGTTCCTTTCGGCTGG***AGACCACATC***
CCTGAAACCTACATGTCGTTGAGGAGAGCCTCCAACCCAGATCTGAAGCATCTTGAGTACAA
CATTAGGTTGGGGCTCATAGGAGACAGCGACTTCCCTGTCACAAATCTGTCTTCCACCAGCC
ACCACCAAAGGTCCTCCTTGATTTCGGGGGTGATGGTGAAGACGAACAAGTCCGGGAAGACT
TTCCTTCACTAGTTGGCCCTGAGGTAAAACTGCAACACTCTGGTGTATAACCTGCCCAACAG
AACAAACTTCTCGATGGAAGACATTGTTCCCAGAAGGCTCATCATGCGTTGGCTG***AGCAGGA***
TGGGCAAGATATGAAGTCCCAGACTTTCCTGAGGCAAT**GCTGAATTGGTACGCAGTC**

Figure 3.23 Nucleotide sequence of candidate male-specific marker, $ME_{+3}7M_{+3}6425$ (actual size = 429 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACTATAGGTGCATTGAAGAGGGAGTCCTTGGGATCCTCTAAACAAAG
C***TCAACCATAGCCTGTACCTTTC***CCAGTGGAAAGCATAGAGGTAGTCTAAAAAGAAATGTTC
CGCAGTGAGAAACTACTCTCATGAGCCACCTGACAAGAAAACGAAGCGTATTCTCTACTTTG
AAGGACCAGTTTTGCCCACTGGTTGGATACCTGGTGTGCCTGGTAAGTAAGGGGTCCCTAGC
TCCTGACACCAACAACCTACTGTGCATTGCTCTCTCATCTGAGGACGTGATACCTGTCCAGG
CGTAATCTGCTAGAGAGTTCATCCACAGGTTTGGTCATGAAACAGCCTGCAGTGAAACCACT
GCTATGGATTCAAAGGCATTGGCCTCCACAAGACTGATGGAGAAGCCCTCCAG***ACAGTCTCT***
CCAGGGGTGAAGTTAGCTGGCAGGCTTGATGCATCCAACCAGAGAGAAACCTCTGCATCCTC
CAGAATGTAAAACCTCCACTGTTGTGAAGTTGGCAAGGGTAGGAGTTTGCTTAGTCTGCTGT
AGGCA**AGTGAATTGGTACGCAGTC**

Figure 3.24 Nucleotide sequence of candidate male-specific marker, $ME_{+3}4M_{+3}5570$ (actual size = 582 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACAATTCTGTAGCGATCAAATGGTAATTGCTCCCGGGTC***AGTGAGGT***
AGGTTACTGACATGCACTTTTTACCTAGTCGTTGTGACAGGAAGCGCTCAGTTCCTTTCGAA
AACGAAAGGTTCGCTCCCCCAGATACTGTTTTATAATTACTGTCATTTACCATTAGGCCCTG
GTAAAGTTATCTAAATATAAGATTATTCAATTGAATAATCTTTCTAAAATGTGTCATA***TGCC***
CGATTCAGTCCATCCTCTTTTCTATTTTATCATGTGCGAGCGGCACTGGGTT**CCTGAATTGG**
TACGCAGTC

Figure 3.25 Nucleotide sequence of candidate male-specific marker, $ME_{+3}8M_{+3}1310.1$ $ME_8M_1310.1$ (actual size = 319 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACAACAGTGTTATGGCAACAT***GTACATTATGAACTAAGACAAG***TGCA
TACCTTTTACAAGGGAGAAATATGGTATTCCAAAGGGAAAATATGGGAGCTACATTCCTTCC
CCCCCGCCCGGCCGCATATTCCTGAAGTCTGGAAGAAAATAAAGCTCTCATATATTAGAACC
AATCAGAGCGTGATTGGGAGCAGTGACGTCGTGACACTCCGCCCTTCTAGTAAGAGTCATTA
T***GCCCTATGGAAACAGCAATACA***GTCCTAGCACTGAAGAAGTTCCTCAAGAG**CCTGAATTGG**
TACGCAGTC

Figure 3.26 Nucleotide sequence of candidate male-specific marker, $ME_{+3}8M_{+3}1310.2$ (actual size = 319 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACAAGTTGATTGATTGATTGATGATTGATTGTCT***TTGTCAGATGGCT AATAGTGTC***TGTGTTCACACGAGATTGTAGAAATTGTTCATGATTTACTTGCTTTTGTGGTT TCATTGTGTCGTGGTTGGCTTTATGCCAGCACATTGGCTCACCTGGGGACTTTCGTATGAGA GAGAGAGAGAGAGAGAGAGAGAGAGCGAAAGATGATCTTCACTCGGATATCGTTTATTTT***CT TCCACTTCATTTCTCATTTG***TTATTTGGTCTTTCTCTTTCTTTCGTTCGCTC**CCTGAATTGG TACGCAGTC**

Figure 3.27 Nucleotide sequence of candidate male-specific marker, $ME_{+3}8M_{+3}1310.3$ (actual size = 319 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACTTCCTTACTTTCCGCTAACTCTTTCGCCAAAACTGATCAGTGTTT CTTCTGTCTCATCAAACTTCTAAACAAAAAGCATTTNCTATGGTCC***TGTTATCCTTCGTTCC CTCC***ACATGACGAGATCACCTCAAAACTAACTATCACATGACATCTGTATGTTGACCTTTCA ATAACGTTCTTTATATGTCTATGTTCTGCAGGATTCCATCCGTACCCAGTCTACATTATAGG AAAAAAACACATATCAATAACTTCAGTCTATTTTGACTCACATTCACCACTATTACTTCATT TCCGAGAAGTCGAGTTGGCACAGTAACACCTTCGAGAATTTTTTTACCTAAATGA***TTTGCCA GGACTGTCACTTT***CTGCTCATCCTTTATTCAATATTTCACCTATTCTCTCACATTACCATCA TCTATTATTGCTATCCTGATACACCTCTACCTTGGAGTGTGGTGTCTCTCTGGAATAGTGAA TTGGTACGCAGTC**AGTGAATTGGTACGCAGTC**

Figure 3.28 Nucleotide sequence of candidate male-specific marker, $ME_{+3}4M_{+3}8517$ (actual size = 528 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACTGACA***GCTTAGGGTTCTGGTCTTGTGA***TAAGTAATGAAATCCATT AGACATCAAAGTCCTGAGACCTTATAGCACAGGCTACGCATAACTCAGAGCCATTGTAGAAA TTGTGTGAAAATCTTATCT***CACTCTTTGCTCCCTCCCTTTT***TATATAGCATTATTTCATTGT AAGTTGAAGAAGCATCTTGCAAGCTGTTGTTTCCTCACTTTTTATATAGCATTATTTCACTG TAAGTTGTAAGAAGCATCTTGCAAGCT**GTTGAATTGGTACGCAGTC**

Figure 3.29 Nucleotide sequence of candidate male-specific marker, $FE_{+3}1M_{+3}7290$ (actual size = 294 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACAATGTCAGCCTAGAAGAAGGCCGACTTTGGCGTGTTTCATGTGTG
AAT***ACCCTCTTTTCTGGATGGCA***ATCATGAGTCTGCAGGCCTCGTCATGACCCGCTGCCCTT
TGCATCAGACCTCTCGAGTTTGTTACAAGTTCCACGAACTCTTTTTCATCTGAGACTGGGAC
TCAAATGATCGACTATGATATGAATAGATAACAAATAACGAGGAAAAATATTCGGTAAGTTC
TTTGTAACCAACGTCATTGTAATCACAAAGAAGAGAAAGATATGATAAATCACGACATCGCA
CTGTCATGAATG***GAGTGATTTGAGGTTTGCCG***AAGCTAACCATTTCCTCT**GGTGAATTGGTA**
CGCAGTC

Figure 3.30 Nucleotide sequence of candidate male-specific marker, $FE_{+3}5M_{+3}1390$ (actual size = 379 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACAGCGCGGAAAGCGTCACTATCGCTGTC***ACTATTATTTCTTATTTT***
TCTCTCCTTGTTTCTAATTTTCCTGATAATCATCAGCATTGTGATTCATTTCTCCCCAATTT
TCTATTTTCCTATTTCCTCCCAGTTTTCCTTTCGCCCTCAATCCTATAAG***ATTCCCTATTTG***
TAAAGGCTTGTTATTCATTCTCCCCAATTTTCTTTTCTCCTATTTTTTCCCAGTTTCCTTTT
CC**CCTGAATTGGTACGCAGTC**

Figure 3.31 Nucleotide sequence of candidate male-specific marker, $FE_{+3}8M_{+3}3270.1$ (actual size = 269 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACAGTGTG***CGCCTATTCCTCAATCGGTCAA***TCCGTAAGTAGACACAA
GATGTGAGGGGAATATGCAAGCATATTCGAAGGTTCCTTCAATCAAGCATTAGTCTAACTCC
TTCCTCATACTTCCAAGAGGAAAG***AAGGACGGAGGAATGGAAGCAT***ACTTCCATATTTCACC
ATGGGGAAGCTTCTGCAAGATGACAGTGTACCGAGCCAATTAGCTCTAGCCGGGCTGGCATT
T**CCTGAATTGGTACGCAGTC**

Figure 3.32 Nucleotide sequence of candidate male-specific marker, $ME_{+3}8M_{+3}3270.2$ (actual size = 268 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

GATGAGTCCTGAGTAACTCCTGTTACATCAATAGGTCACATCTTTGACTTGGAGTCCAAAGG
CTGGAGACTTCCTTGTCTCCCAGGAGAGCTCCCCAACCTAGGTCTGATGCATCTGAATAAAA
CACTAGGTTGGGGCTCAGAGGCAGGAGGGACTTTCCTTGCGAGAGTTTGTCTCCTTGATCTC
CACTAAAGCAGATCCT***GCTTTATTTCCTGCGTTATTGG***AAAAACGAAAGTGTCGGGTATTTC
TTTCTGTTCCAACAGACTGTTTAGAAAGAATTGCAAGTTCCTCACATGTAGCCTTCCCAGGG
ATACCAACTGTTCCATGGACGGTAGGGTTCGCAGAACAAGACTGAAGGGAGAGGAATCTGTC
CACTTTCTGAAGGCACAAGTCTATACTTTGGGGGGAGGGAAAAACCTGAAAAATCAGTGAGT
TTGTCCTCATCTCCAAATAAGCTATCTCCTGAGAAGATGTCAGCTGCAACTTCTAAAAGGTT
ATTAGTAGGCCCAATTCCTGGACTAGAAGAAGGGTCTTGTGAAGGTCCTCCACACATTTGTC
TTTCGAC***TAGGAGCGAATGAGCCAATCGT***CGGGTTACAGCGAAATGTTGATCCCCATCAGGT
GTAACCATTTCACTCAGGAGCCTGGNACTCGCGCGAAAACCTGCGGACCTGTGGACAGGCCT
NAAGCAAAGGC**CCTGAATTGGTACGCAGTC**

Figure 3.33 Nucleotide sequence of candidate male-specific marker, $FE_{+3}8M_{+3}6710$ (actual size = 712 bp). Positions of original AFLP primers were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

PCR was carried out against genomic DNA of male and female individuals of *M. rosenbergii* and analyzed by agarose gel elctrophoresis. Among 13 primer pairs screened, 4 pairs of primers ($ME_{+3}8M_{+3}1310.2$-F/R, $ME_{+3}8M_{+3}1310.3$-F/R, $FE_{+3}1M_{+3}7290$-F/R and $FE_{+3}8M_{+3}3270$) did not yield the expected amplification product. Positive amplification was found in the remaining SCAR markers. Nevertheless, they did not retain the original sex specificity as all SCAR markers generate positive amplification fragment in both males and females of *M. rosenbergii* (Fig. 3.34 and 3.42). Although the amplification products from $ME_{+3}7M_{+3}6425$-F/R (Fig. 3.36) and $ME_{+3}8M_{+3}1310.1$-F/R (Fig. 3.38) clearly provided bands representing homozygotic and heterozygotic states, they were not fixed in each sex of *M. rosenbergii*.

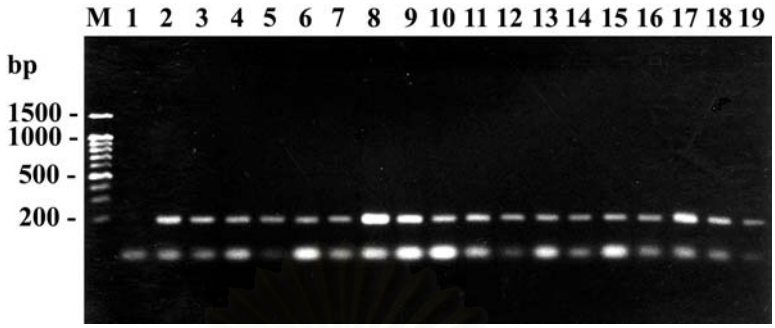

Figure 3.34 A 1.5% ethidium bromide stained agarose gel showing PCR products of a SCAR markers derived from $ME_{+3}3M_{+3}7425.1$ tested against genomic DNA of male (lanes 2 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes 1 and M are a negative control (without DNA template) and a 100 bp DNA marker, respectively.

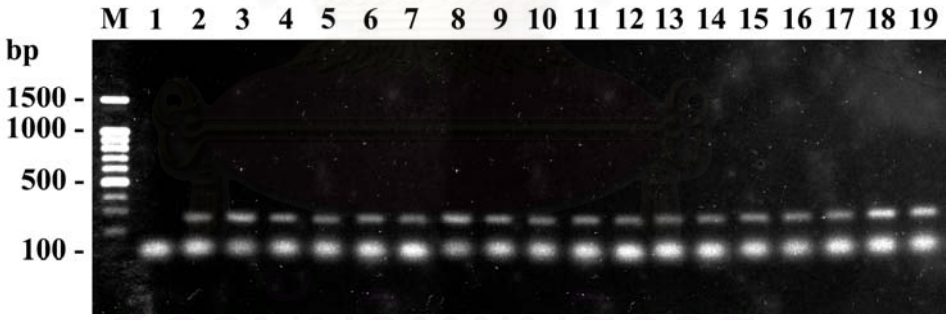

Figure 3.35 A 1.5% ethidium bromide stained agarose gel showing PCR products of a SCAR markers derived from $ME_{+3}3M_{+3}7425.2$ tested against genomic DNA of male (lanes 2 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes 1 and M are a negative control (without DNA template) and a 100 bp DNA marker, respectively.

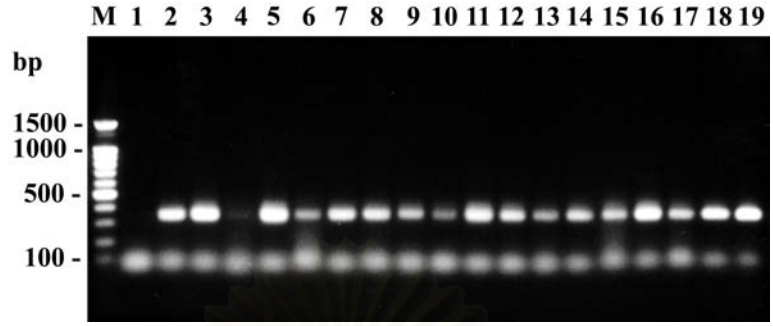

Figure 3.36 A 1.2% ethidium bromide stained agarose gel showing PCR products of a SCAR markers derived from $ME_{+3}7M_{+3}6425$ tested against genomic DNA of male (lanes 2 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes 1 and M are a negative control (without DNA template) and a 100 bp DNA marker, respectively.

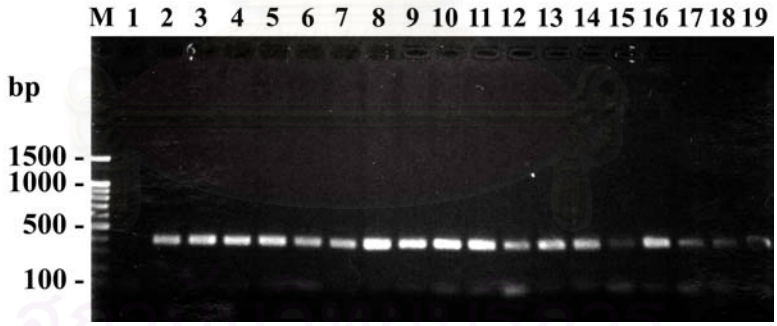

Figure 3.37 A 1.2% ethidium bromide stained agarose gel showing PCR products of a SCAR markers derived from $ME_{+3}4M_{+3}5570$ tested against genomic DNA of male (lanes 2 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes 1 and M are a negative control (without DNA template) and a 100 bp DNA marker, respectively.

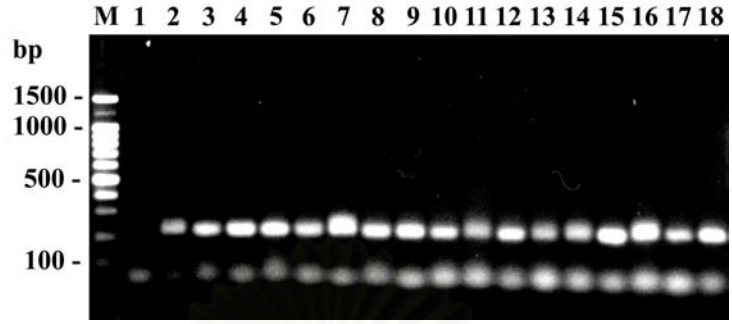

Figure 3.38 A 1.5% ethidium bromide stained agarose gel showing PCR products of a SCAR markers derived from $ME_{+3}8M_{+3}1310.1$ tested against genomic DNA of male (lanes 2 – 9) and female (lanes 10 – 18) *M. rosenbergii*. Lanes 1 and M are a negative control (without DNA template) and a 100 bp DNA marker, respectively.

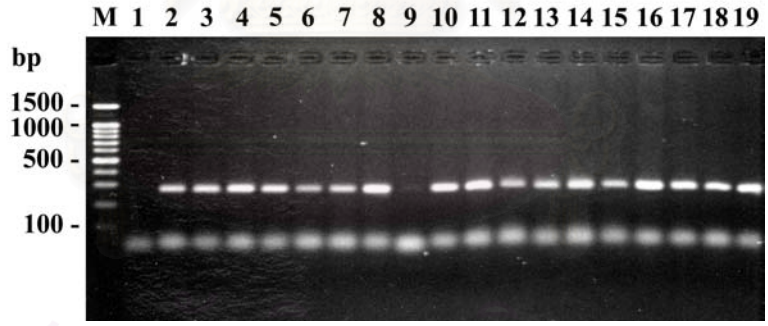

Figure 3.39 A 1.2% ethidium bromide stained agarose gel showing PCR products of a SCAR markers derived from $ME_{+3}4M_{+3}8517$ tested against genomic DNA of male (lanes 2 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes 1 and M are a negative control (without DNA template) and a 100 bp DNA marker, respectively.

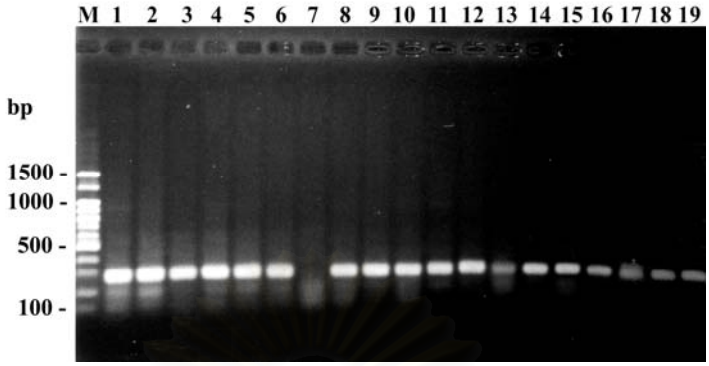

Figure 3.40 A 1.5% ethidium bromide stained agarose gel showing PCR products of a SCAR markers derived from $FE_{+3}5M_{+3}1390$ tested against genomic DNA of male (lanes 2 – 9) and female (lanes 10 – 18) *M. rosenbergii*. Lanes 1 and M are a negative control (without DNA template) and a 100 bp DNA marker, respectively.

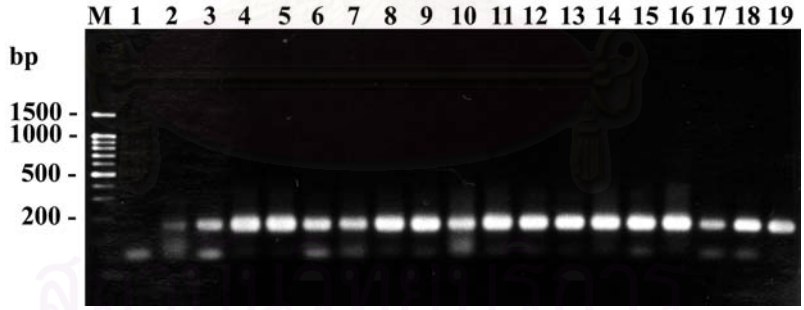

Figure 3.41 A 1.8% ethidium bromide stained agarose gel showing PCR products of a SCAR markers derived from $FE_{+3}8M_{+3}3270.2$ tested against genomic DNA of male (lanes 2 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes 1 and M are a negative control (without DNA template) and a 100 bp DNA marker, respectively.

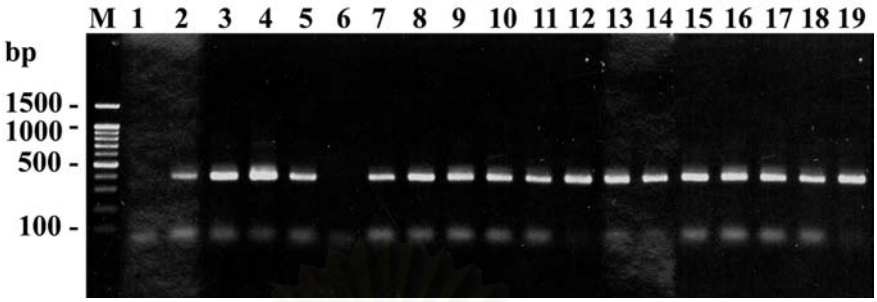

Figure 3.42 A 1.2% ethidium bromide stained agarose gel showing PCR products of a SCAR markers derived from $FE_{+3}8M_{+3}6710$ tested against genomic DNA of male (lanes 2 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes 1 and M are a negative control (without DNA template) and a 100 bp DNA marker, respectively.

SSCP analysis was then performed to identify whether the amplification product of developed SCAR markers contained single nucleotide polymorphism (SNP) for each sex of *M. rosenbergii*. All except that from $FE_{+3}8M_{+3}3270.2$-F/R were polymorphic but not sex – linked. However, these polymorphic markers can further be used for other applications for example, population genetic studies of *M. rosenbergii* over vast geographic locations.

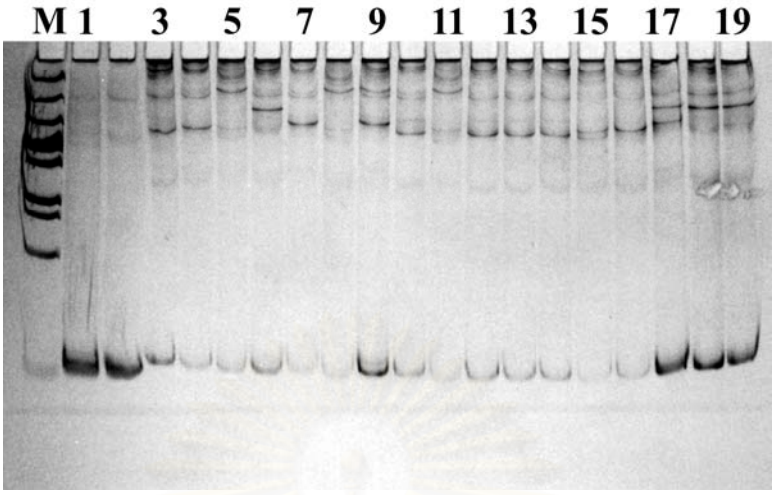

Figure 3.43 A 17.5% silver stained non-denaturing polyacrylamide gel (37.5:1) showing SSCP patterns of the amplification product of a SCAR marker derived from $ME_{+3}3M_{+3}7425.1$ tested against genomic DNA of male (lanes 3 – 11) and female (lanes 12 – 19) *M. rosenbergii*. Lanes M and 1 and 2 were a 100 bp DNA ladder and the non-denatured PCR product (double stranded DNA control), respectively.

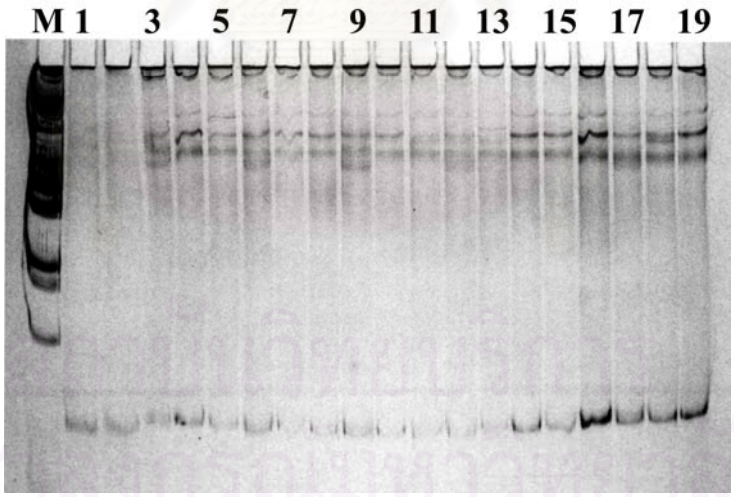

Figure 3.44 A 15% silver stained non-denaturing polyacrylamide gel (37.5:1) showing SSCP patterns of the amplification product of a SCAR marker derived from $ME_{+3}3M_{+3}7425.2$ tested against genomic DNA of male (lanes 3 – 11) and female (lanes 12 – 19) *M. rosenbergii*. Lanes M and 1 and 2 were a 100 bp DNA ladder and the non-denatured PCR product (double stranded DNA control), respectively.

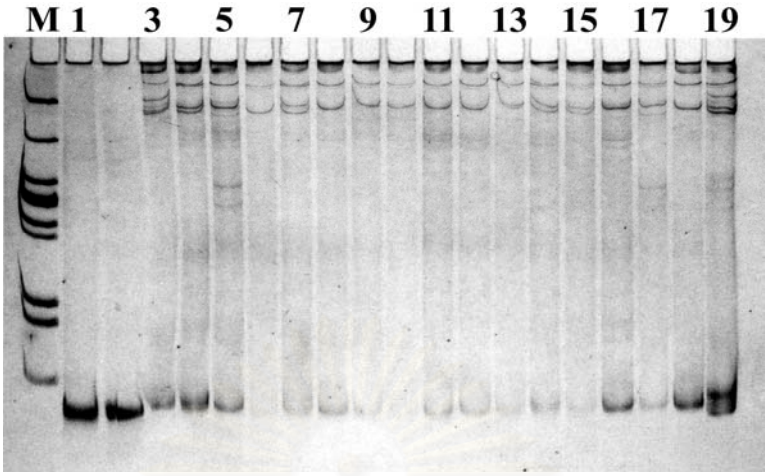

Figure 3.45 A 12.5% silver stained non-denaturing polyacrylamide gel (37.5:1) showing SSCP patterns of the amplification product of a SCAR marker derived from $ME_{+3}7M_{+3}6425$ tested against genomic DNA of male (lanes 3 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes M and 1 and 2 were a 100 bp DNA ladder and the non-denatured PCR product (double stranded DNA control), respectively.

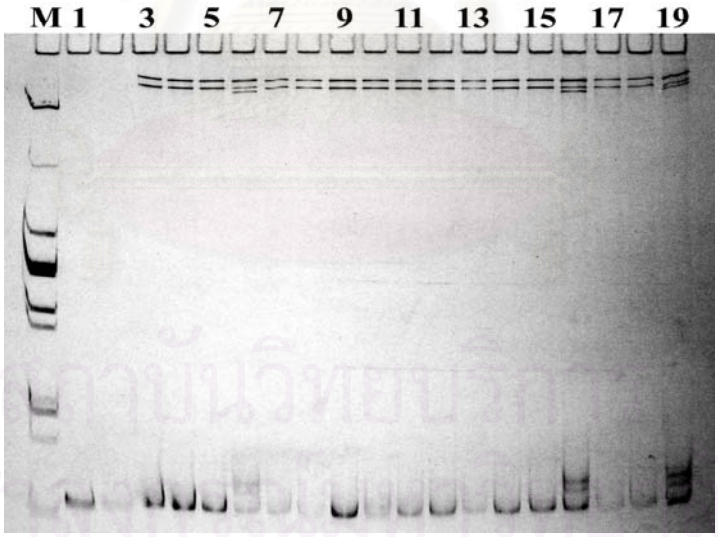

Figure 3.46 A 12.5% silver stained non-denaturing polyacrylamide gel (75:1) showing SSCP patterns of the amplification product of a SCAR marker derived from $ME_{+3}4M_{+3}5570$ tested against genomic DNA of male (lanes 3 – 11) and female (lanes 12 – 19) *M. rosenbergii*. Lanes M and 1 and 2 were a 100 bp DNA ladder and the non-denatured PCR product (double stranded DNA control), respectively.

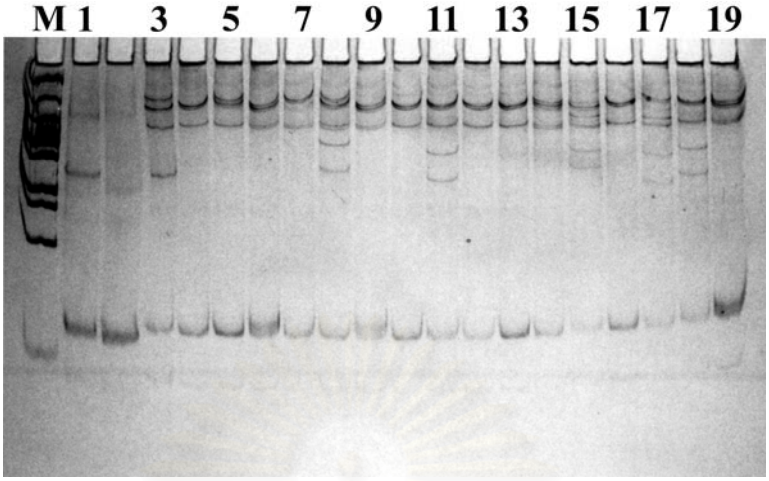

Figure 3.47 A 17.5% silver stained non-denaturing polyacrylamide gel (37.5:1) showing SSCP patterns of the amplification product of a SCAR marker derived from $ME_{+3}8M_{+3}1310.1$ tested against genomic DNA of male (lanes 3 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes M and 1 and 2 were a 100 bp DNA ladder and the non-denatured PCR product (double stranded DNA control), respectively.

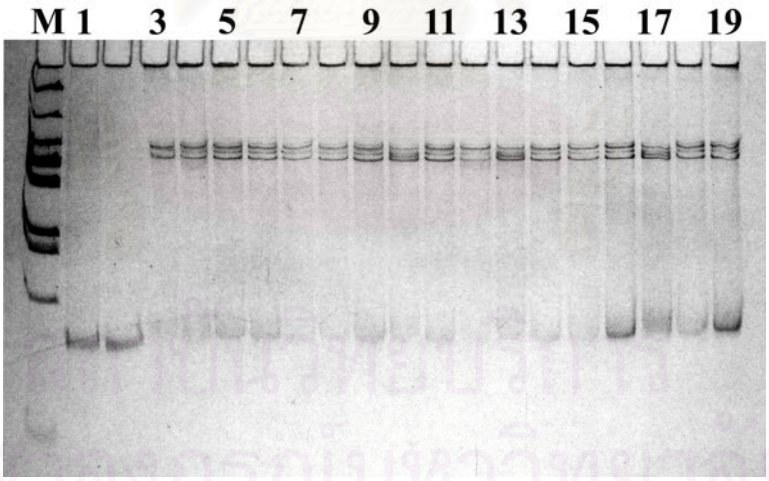

Figure 3.48 A 15% silver stained non-denaturing polyacrylamide gel (37.5:1) showing SSCP patterns of the amplification product of a SCAR marker derived from $ME_{+3}4M_{+3}8517$ tested against genomic DNA of male (lanes 3 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes M and 1 and 2 were a 100 bp DNA ladder and the non-denatured PCR product (double stranded DNA control), respectively.

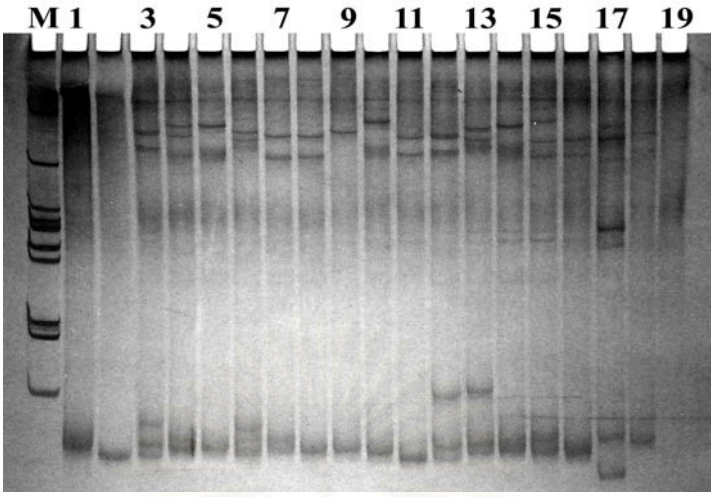

Figure 3.49 A 17.5% silver stained non-denaturing polyacrylamide gel (37.5:1) showing SSCP patterns of the amplification product of a SCAR marker derived from $FE_{+3}5M_{+3}1390$ tested against genomic DNA of male (lanes 3 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes M and 1 and 2 were a 100 bp DNA ladder and the non-denatured PCR product (double stranded DNA control), respectively.

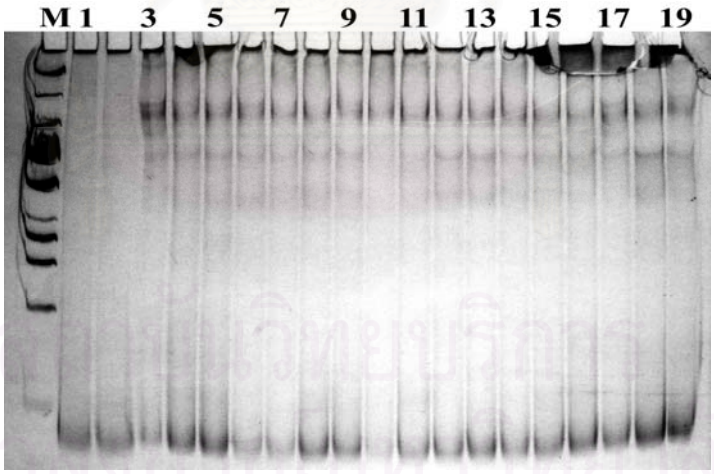

Figure 3.50 A 20% silver stained non-denaturing polyacrylamide gel (37.5:1) showing SSCP patterns of the amplification product of a SCAR marker derived from $FE_{+3}8M_{+3}3270.2$ tested against genomic DNA of male (lanes 3 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes M and 1 and 2 were a 100 bp DNA ladder and the non-denatured PCR product (double stranded DNA control), respectively.

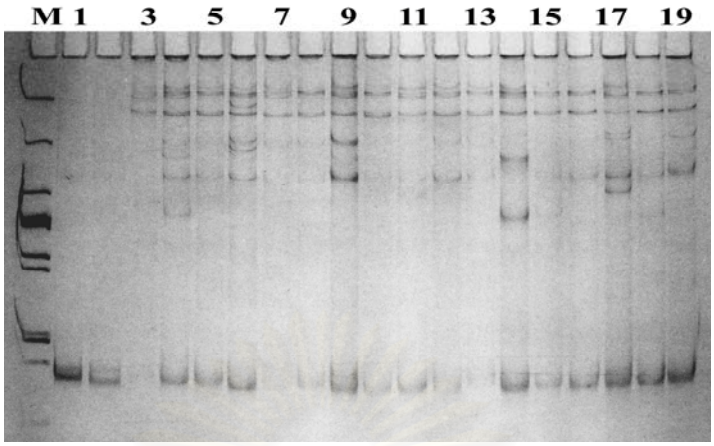

Figure 3.51 A 15% silver stained non-denaturing polyacrylamide gel (37.5:1) showing SSCP patterns of the amplification product of a SCAR marker derived from $FE_{+3}8M_{+3}6710$ tested against genomic DNA of male (lanes 3 – 10) and female (lanes 11 – 19) *M. rosenbergii*. Lanes M and 1 and 2 were a 100 bp DNA ladder and the non-denatured PCR product (double stranded DNA control), respectively.

Table 3.4 The amplification results and SSCP analysis of developed SCAR markers

Markers	Agarose gel electrophoresis		SSCP analysis
	Male	Female	
$ME_{+3}3M_{+3}7425.1$	+	+	Polymorphism
$ME_{+3}3M_{+3}7425.2$	+	+	Polymorphism
$ME_{+3}7M_{+3}6425$	+	+	Polymorphism
$ME_{+3}4M_{+3}5570$	+	+	Polymorphism
$ME_{+3}8M_{+3}1310.1$	+	+	Polymorphism
$ME_{+3}8M_{+3}1310.2$	-	-	ND
$ME_{+3}8M_{+3}1310.3$	-	-	ND
$ME_{+3}4M_{+3}8517$	+	+	Polymorphism
$FE_{+3}1M_{+3}7290$	-	-	ND
$FE_{+3}5M_{+3}1390$	+	+	Polymorphism
$FE_{+3}8M_{+3}3270.1$	-	-	ND
$FE_{+3}8M_{+3}3270.2$	+	+	SSCP patterns were unclear
$FE_{+3}8M_{+3}6710$	+	+	Polymorphism

ND = not determined

3.3 RT-PCR analysis

3.3.1 RNA extraction

Total RNA was extracted from androgenic glands (AG) and oviducts of male and female *M. rosenbergii* and electrophoretically examined (Fig. 3.52). In addition, total RNA was also extracted from testes and ovaries for further examination of the expression patterns of interesting genes (Fig 3.53). An ethidium bromide stained 1% agarose gel of total RNA revealed predominant discrete ribosomal RNA bands along with smeared high molecular weight RNA The ratio of OD_{260} / OD_{280} of extracted total RNA was 1.7 - 2.1 indicating the acceptable quality of total RNA used in this study.

Initially, pooled total RNA of BC, OC, SOC males (N = 2 for each morphotypes) and females (N = 3) were subjected to the first strand cDNA synthesis and RT-PCR. Genes of interest were further examined using the first strand cDNA from ovaries and testes if they are regarded as sex-related transcripts.

3.3.2 Identification of an AGH homologue in *M. rosenbergii*

High sequence similarity was found between cDNAs encoding AGH precursors of Isopods *A. vulgare* (Okuno et al., 1999) and *P. scaber* and *P. dilatatus* (Ohira et al., 2003) (Fig. 3.54). Degenerated primers were designed from AGH of these species. Nevertheless, designed primers did not provide the expected amplification product in *M. rosenbergii* (Fig. 3.55)

3.3.3 Identification of transcripts in AG and oviducts of *M. rosenbergii* using candidate sex-specific AFLP marker

Primers for identification of sex-specific SCAR marker described previously were applied for RT-PCR analysis using the first strand cDNA from AG and oviducts of *M. rosenbergii* as the template. Primers from $ME_{+3}4M_{+3}8517$, $ME_{+3}8M_{+3}1310.2$, $ME_{+3}8M_{+3}1310.3$ and $ME_{+3}4M_{+3}5570$ did not provide the amplification product in both AG and oviducts of *M. rosenbergii* (Fig. 3.56).

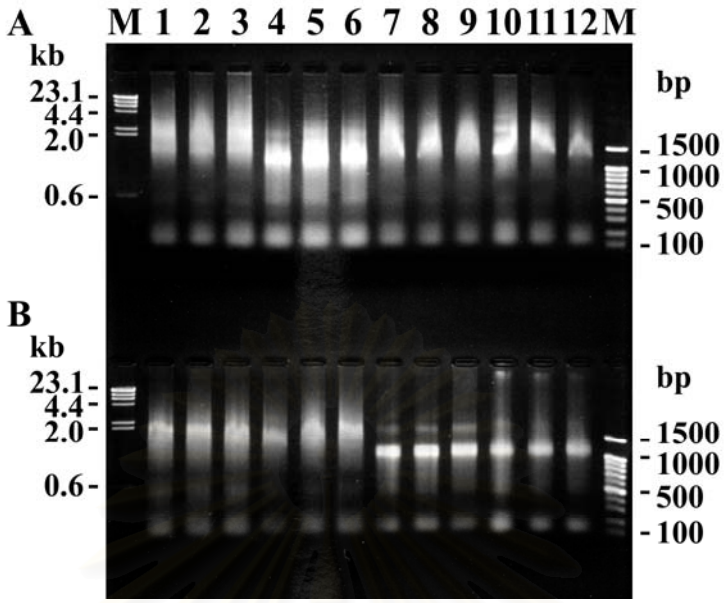

Figure 3.52 A 1.0% ethidium bromide stained agarose gel showing total RNA extracted from androgenic glands of BC (lanes 1 – 6, panel A), OC (lanes 7 – 12 panel A) and SOC (lanes 1 – 6, panel B) male and oviducts (lanes 7 - 12 panel B) of female *M. rosenbergii* individuals. Lanes L and M are a 100 bp DNA ladder and λ-*Hin*d III DNA marker, respectively.

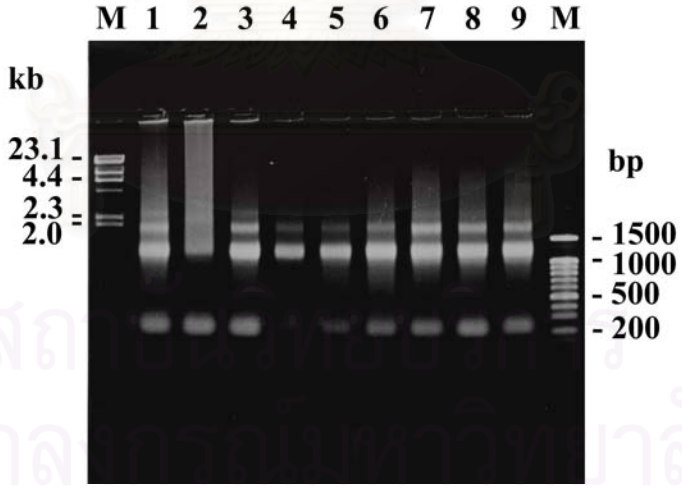

Figure 3.53 A 1.0% ethidium bromide stained agarose gel showing total RNA extracted from testes of BC (lanes 1 - 2), OC (lanes 3 – 4) and SOC (lanes 5 – 6) male and ovaries (lanes 7 - 9) of female *M. rosenbergii*. Lanes L and M are a 100 bp DNA ladder and λ-*Hin*d III DNA marker, respectively.

$ME_{+3}3M_{+3}7425.1$ primers yielded the smear amplification results. Primers designed from $ME_{+3}3M_{+3}7425.2$ and $ME_{+3}7M_{+3}6425$ were successfully amplified the first strand cDNA of AG and oviducts of *M. rosenbergii*. Only $ME_{+3}8M_{+3}1310.1$ primers provided two amplification bands at approximately 500 bp and 700 bp in length in AG but not in oviducts. Sizes of the amplification product were larger than 210 bp expected from the original DNA sequence. Therefore, they were not regarded as the primary target product (Table 3.5).

The first strand cDNA from testes and ovaries were also screened with $ME_{+3}8M_{+3}1310.1$ primers. Results indicated the occurrence of 500 bp and 700 bp fragments in AG but not in testes, oviducts and ovaries (Fig. 3.57). Further characterization of the bands was then carried out. Using a single forward and a reverse primers and combination of both primers to amplify the first strand cDNA from AG by PCR, indicated that a 500 bp fragment should be a product generated from a reverse primer (Fig. 3.58).

Nevertheless, both 500 bp and 700 bp fragments were cloned and sequenced. Blast analysis revealed that the former was truly generated from the reverse primer and significantly matched with β - actin of *P. monodon* (E value = 1×10^{-46}) whereas a 700 bp fragment was a homologue of Sarco / endoplasmic-reticulum Ca^2 + ATPase C (SERCA) of the red swamp crayfish, *Procambarus clarkii* (1×10^{-103}) (Table 3.6). A primer pair was designed from homologues of *P. clarkii* SERCA (Fig. 3.59 and Table 3.7).

The expect 279 bp fragment was found in both male and female *M. rosenbergii* but the expression levels of SERCA in BC, OC and SOC males was greater than that in oviduct at 25 and 30 amplification cycles. The positive control reveals approximately identical intensity among different samples suggesting differential expression of SERCA in AG and oviducts of *M. rosenbergii* (Fig. 3.60).

```
P.scaber     ATGAAAGGTCTCCTCTTCATAGTTAGCCTGCTCTGCTTGACCCTCCACCAGCGTGTATGG
P.dilatatus  ATGAAAGGTCTCCTCTTCATAATTAGCCTGCTCTTCTTGACCCTCCACCAGCGTGTATGG
A.vulgare    ATGAAGGGTCTCGTCATCTTAGTCTCTCTTATGTGCTTAGCTCTCTACAATCGCATATGT
             ***** ****** ** ** ** *    **  * * ***  * *** ** * **  ****

                                                          Mar-AGH – F1→
P.scaber     GCCTACCAGGTAATAGGTATGAAATCGGATGTCATATGTGCGGACATTAGGTTTACTGTG
P.dilatatus  GCCTACCAGGTAGAAGGTATGAAATCGGATGTCATATGTGCGGACATTAGGTTTACTGTG
A.vulgare    GCCTACCAGGTACGAGGTATGAGATCCGATGTGTTATGTGGAGATATAAGATTTACAGTG
             ************  ******** *** *****  ******  ** ** ** ***** ***

             Mar-AGH – F2→
P.scaber     CATTGTATATGCAACGAATTAGGACTTTTCCCTACGTCGAGACTTTCGAAGCCTTGCCCA
P.dilatatus  CATTGTATATGCAACGAATTAGGACGTTTCCCTACGGCGAGACTTACGAAACCTTGCCCT
A.vulgare    CAGTGTATATGCAACGAATTGGGATATTTCCCAACAGAGAGACTGGACAAGCCTTGTCCT
             ** ****************** ***  ******  **   ******    ** ***** **

P.scaber     TGGCCTAACAGGGGAAGAAGATCTGCTGATGATGAAGATTATCTATTTGAAGAAGATGAA
P.dilatatus  TGGCCTAACAGAGAAAGAAGGTCTACTGACGATGAAGATTATCTATTTGAAGAAGATGAA
A.vulgare    TGGCCAAACAGAGAAAAAAGATCTGCTCCTGAAGATGAATTGGCATTTGAAGACTACGAA
             ***** ***** * ** *** *** **   ** ** ** *    ********  * ***

P.scaber     GACGATGAATTTTTCCATCCTCGAGCCCTCAGTC---CCCCTGCGGCTAAAAATGGTGAT
P.dilatatus  GACGAGGAATTTTTCCATCCTCGAGCCCTCAGTCGTCCCACTGCGGCTAAATATGATGAT
A.vulgare    GATCAAGACTATTTCCATCCACGGGCACTGAGTATTCCCTCTGAAATTGAACATGACAAC
             **  * ** * ********* ** ** ** ***    ** ***    * ** ***   *

P.scaber     GAGAGATTAGAAGATGAAGTTTCTTTTCATAGTCGCTCGAAACGTGACATCGCCTTCCAC
P.dilatatus  GAGATATTAGAAGATGAAGTTTCCTTTCATAGTCGCACGAAACGTGATATCGCCTTCCAC
A.vulgare    GAGAAAGAGAGTGATGCTTTTTCTATTCTGAGTAGAGGGAAACGTGAGATAGCCTTTTAC
             **** *      ****  ****  ***  *** *   ********* ** *****  **

             ←Mar-AGH – R1
P.scaber     GAAGAATGTTGCAACATTCGAACCGAACATAAATGCAATAAAACAACAGTGGAACTCTAT
P.dilatatus  GAAGAATGTTGCAACATTCGAACCGAACATAAATGCAATAGAACAACAGTGGAACTCTAT
A.vulgare    CAAGAGTGTTGCAATATTAGGACAGAGCACAAATGCAATCGAACCACTGTTTCCTTGTAC
              **** ******** *** * ** ** ** *********  *** ** **    *  **

P.scaber     TGTCGAAGATACACACGTTGAAATGGTATTAATTATGAAGACTTTTTGGCAGGAATTTCG
P.dilatatus  TGTCGAAGATATTCACCTTGAAATGATATTAATCACGAAGGCTTTTTGGCAGGGTTTTCA
A.vulgare    TGTCGAACATACTAGACGTTGGAATTTCACTTAACGCGAGGAATTTTGGCATTTTTTGGG
             ******* ***      *  *   *          **   ********    **

P.scaber     GAAGTCATAATCTTCTTTGTTAGAACAAAAATGGAATTATTTTTTTTTATCTTCTTTTCA
P.dilatatus  GAAGTTATAATCTTCTTTGTTAGAACAAAAAGGAATTA--TTTTTTTTATCTTCCTTTCA
A.vulgare    GCAATTTAAGAAAATTAAATTTAAATTTTAAAATTTTGTTATTAATAAATTCTCTTGCAG
             * * *  *    *  **  **   **    *    **  *  ** *** *

P.scaber     AATAAGTTTGAAAATTGTCATTGTTCAAACATCAAAATTTTGTTATCAATAAATAATCTT
P.dilatatus  AAAAAGTTTGAAAATTGTCAATGTTCAAACCTCAAAATTTTGTAATCAATAAATTATCTT
A.vulgare    TTTAAAAAAAAAAAAAAAAAA----------------------------------------
                **     ****     *
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Figure 3.54 Multiple sequence alignment of androgenic hormone precursors of Terrestrial Isopod, *A. vulgare*, *P. scaber* and *P. dilatatus.* Positions and sequences of the forward primers and those complementary to the reverse primer are boldfaced.

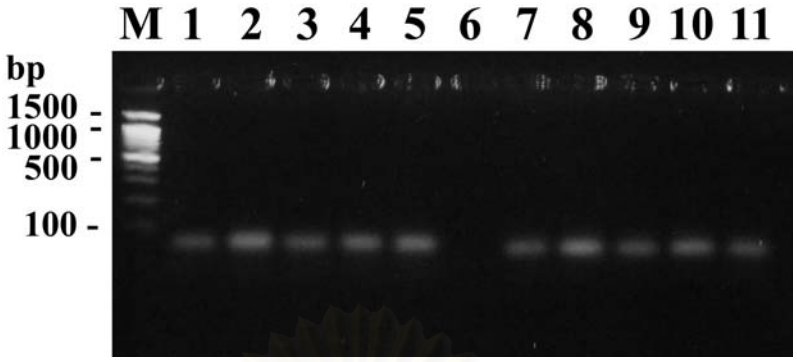

Figure 3.55 A 1.75% ethidium bromide stained agarose gel showing the amplification results using primer Mar-AGH-F1/R1 (lanes 1 – 5) and Mar-AGH-F2/R1 (lanes 7 – 11) using the first strand cDNA template synthesized from AG total RNA of BC (lanes 2 and 8), OC (lanes 3 and 9) and SOC (lanes 4 and 10) and oviducts total RNA of females (lanes 5 and 11). A 100 bp ladder was included as the DNA standard.

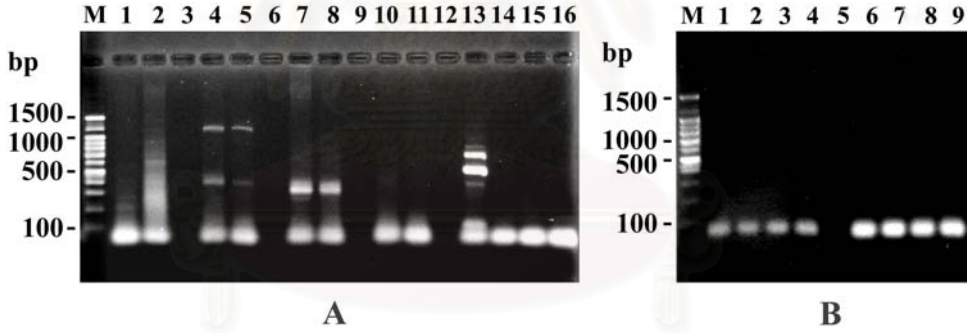

Figure 3.56 RT-PCR products amplified from the first strand cDNA of pooled AG total RNA of BC males (lanes 1, 4, 7, 10, 13 and 15, panel A) and pooled oviduct total RNA of female (lanes 2, 5, 8, 11, 14 and 16, panel A) *M. rosenbergii* using primers designed from $ME_{+3}3M_{+3}7425.1$ (lanes 1 – 2) $ME_{+3}3M_{+3}7425.2$ (lanes 4 – 5) $ME_{+3}7M_{+3}6425$ (lanes 7 – 8) $ME_{+3}4M_{+3}5570$ (lanes 10 – 11) $ME_{+3}8M_{+3}1310.1$ (lanes 13 – 14) and $ME_{+3}8M_{+3}1310.2$ (lanes 15 – 16). Two additional primer pairs designed from $ME_{+3}4M_{+3}8517$ (lanes 1 – 4, panel B) and $ME_{+3}8M_{+3}1310.3$ (lanes 6 – 9, panel B) were used to amplify the first strand cDNA of pooled BC (lanes 1 and 6, B), OC (lanes 2 and 7, B) males and FE1 (lanes 3 and 8, B) and FE2 (lanes 4 and 9, B) of *M. rosenbergii*.

Table 3.5 A summary of RT-PCR results using primers derived from candidate sex-specific AFLP marker

Primer combination	Amplification results
1. $ME_{+3}3M_{+3}7425.1$	Non-specific amplification products
2. $ME_{+3}3M_{+3}7425.2$	Non-specific products at 400 and 1,250 bp in size, found in both sexes
3. $ME_{+3}7M_{+3}6425$	Non-specific products at 300 bp in size, found in both sexes
4. $ME_{+3}4M_{+3}5570$	No products
5. $ME_{+3}8M_{+3}1310.1$	**Non-specific products at 500 and 700 bp in size, found in only males**
6. $ME_{+3}8M_{+3}1310.2$	No products
7. $ME_{+3}8M_{+3}1310.3$	No products
8. $ME_{+3}4M_{+3}8517$	No products

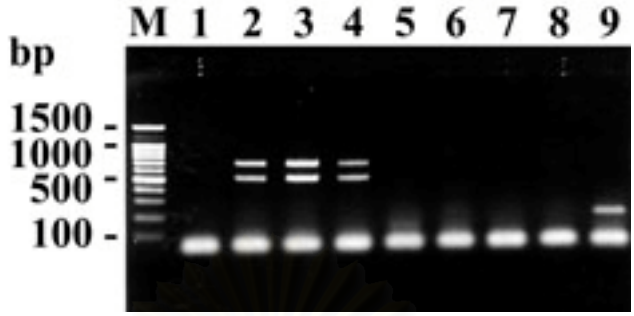

Figure 3.57 Primers from $ME_{+3}8M_{+3}1310.1$ were tested against the first strand cDNA template from AG and testes of BC, OC and SOC male (lanes 2 – 4 and 5 – 7, respectively) and from oviducts and ovaries of female *M. rosenbergii* (lanes 8 and 9, respectively). Lanes M and 1 are a 100 bp DNA marker and the negative control (without the first strand cDNA template).

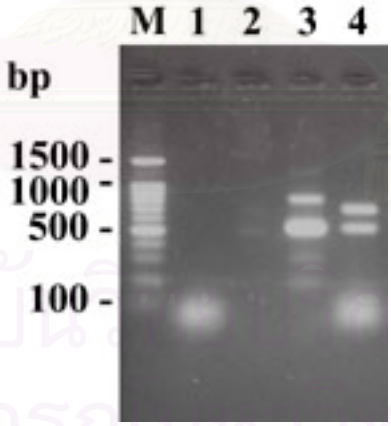

Figure 3.58 The first strand cDNA from AG of the BC male was amplified by the $ME_{+3}8M_{+3}1310.1$ forward primer (lane 2), reverse primer (lane 3) and both primers lane 4). Lanes M and 1 is a 100 bp DNA marker and the negative control.

A.

AGGATGGACTGAATCGGGCATCATACTTTTTACAATGAGCTCAGAGTGGCCCCGGAAGAGTCTCCGGTGCTCTTG
ACAGAAGCCCCTCTCAATCCAAAAGCAAACCGCGAGAAAATGACCCAAATTATGTTCGAAACTTTTGTAACACCT
GCCATGTGCGTTGCAATTCAGGCCGTTCTTTCCTTGNTATGCATCTGGGCGAACAACGGGTATTGTACTGGACTC
AGGTGATGGTGTGACCCACACTGTCCCTATCTACGAAGGTTACGCTCTTCCTCATGCCATCCTTCGGTTGGACTT
GGCTGGCCGTGATTTGACCGCTTACCTAATGAAGATCACGACTGAGAGAGGCTACTCGTTCACAACTACGGCTGA
GCGTGAAATTGTTCGAGACATTAAGGAAAAGCTATGTTACGTCGCCCTTGACTTCGAAAATGAAATGAATATTGC
TGCTGCATCCTCATCACTTGAAAAATCCTATGAAT**TGCCCGATTCAGTCCATCCT**

B.

AGTGAGGTAGGTTACTGACAGCTGCTCTTGGCTTGCCAGAGGCTCTCATTCCTGTCCAGCTTCTGTGGGTTAACC
TTGNTGACTGATGGTCTTCCTGCTACTGCCCTTGNGTTTCAATCCTCCTGATCTTGACATTATGGAGAAGCCCCC
ACGTAAAGCTGATGAGTCTCTGATCTCTGG***CTGGCTCTTCTTCCGTTACAT***GGCCATTGGAGGATATGTAGGCTG
TGCAACAGTATTTGCTGCAACCTGGTGGTTCATGTATGACCCAACTGGTCCTCAACTCAGCTACTATCAGCTGTC
CCACCATCTTTCCTGTCTTGGTGATCCTGAAAACTTTGAGGGATTGGACTGTGGTATCTTCAACCACCCTGCCCC
CATGACCATGGCTTTGTCTGTGTTGGTCACCATTGAGATGCTCAACGCCTTGAACAGCTTGTC***TGAGAACCAGTC***
GCTGCTTGTGATGCCTCCCTGGGGTCAACTTCTGGCTGCTGGCGGCTATGGCCCTCTCCATGACCCTTCACTTCA
TCATTCTCTACGTTGAAATCCTTGGTACTGTGTTCCAGGTGATGCCTCTTACTTTAGCTCAGTGGATAGCAGTAA
TGAAGATTCTCTTCCTGTTGTGTNGCTGGATGAAAACTCAATNCGTGNCCGACATAACCGATGTGCCGATCAGTC
ATCTAATCACTANTGATNCNNGNCGCTGCAGGTCAACATATGGAAAGTCCACGCGTGGATCTACTGATATCTNAN
TGTACTAAANCTGGGTANCAGGCAACTTNCTGGTAATGTNCNNCAATCCAAANNANCGAATAAGAACCGGNCAAA
GANACATATGTGCCGCNTCNCGACTNGCNNTAATGACCGAAGGGGTGGCTCCCCCACNCGNNGGAGNCAGATNAG
AAANANANAAGNGTNCCAAAAGAAAACCCCCCNCCGTNTGGCCAAAAAAAA

Figure 3.59 Nucleotide sequences of the amplification 500 bp (A) and 700 bp (B) products from a primer pair designed from $ME_{+3}8M_{+3}1310$. Position of forward primers and those complementary to the reverse primer were illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were bold-italicized and underlined.

Table 3.6 Blast results of RT-PCR products generated by $ME_{+3}8M_{+3}1310$ primers

Clones	Gene homologue	Closest species	E – value	Length of nucleotide sequenced (bp)
$ME_{+3}8M_{+3}1$ - 500bp	Actin	Giant tiger shrimp, *Penaeus monodon*	1×10^{-46}	505
$ME_{+3}8M_{+3}1$- 700bp	Sarco / endoplasmic-reticulum Ca^{2} + ATPase C (SERCA)	Red swamp crayfish, *Procambarus clarkii*	1×10^{-103}	700

Table 3.7 Sequences, melting temperature and the expected size of primer designed from a homologue of SERCA in *P. clarkii*

Primer	Sequence	Tm (°C)	Size (bp)
PCSERCA-F	5′ - CTG GCT CTT CTT CCG TTA CAT - 3′	62	279
PCSERCA-R	5′ - ACA AGC AGC GAC TGG TTC TCA - 3′	64	

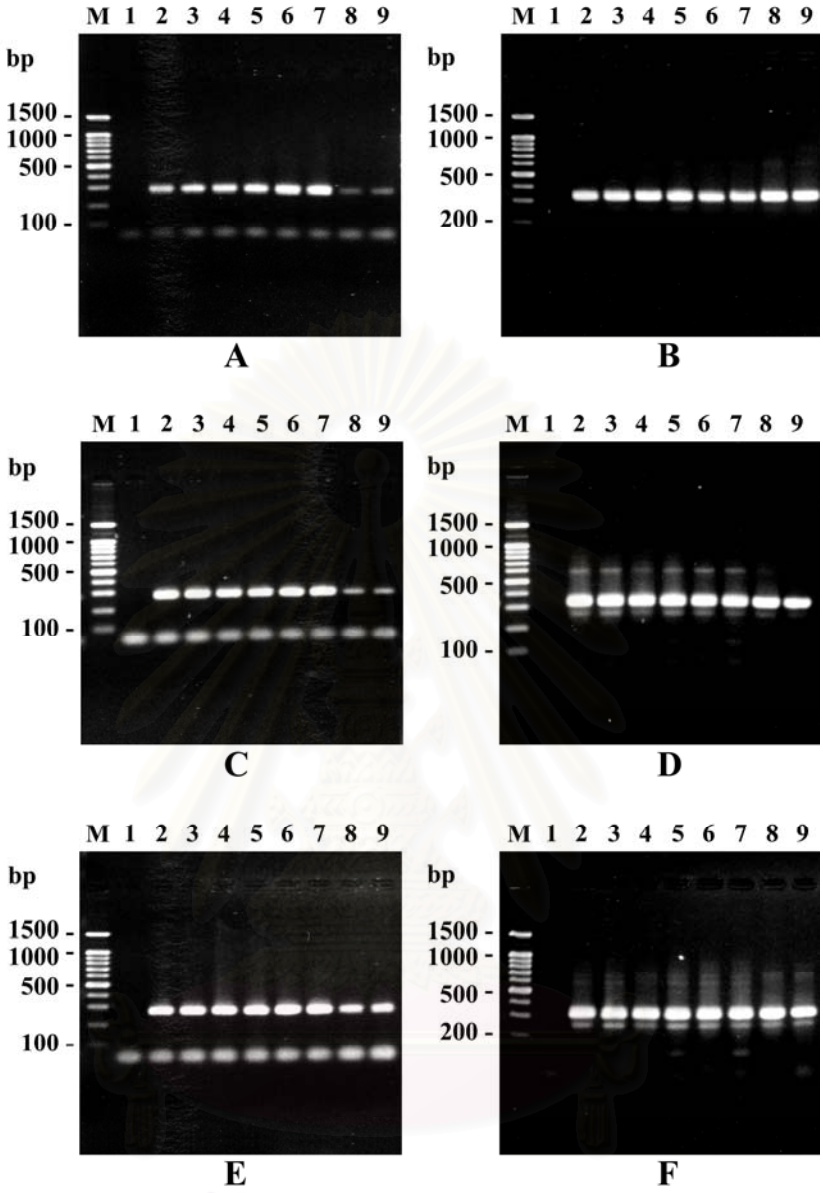

Figure 3.60 RT-PCR of the first strand cDNA of AG of BC (lanes 2 – 3, panels A - D), OC (lanes 4 – 5, panel A - D) and SOC (lanes 6 – 7, panel A - D) male and oviducts of female (lanes 8 – 9, panels A - D) of *M. rosenbergii* using PCSERCA-F/R for 25 (A), 30 (C) and 35 cycles (E). Positive (β - actin of *M. rosenbergii*, panel B, D and F) and negative control (without cDNA template, lanes 1 in panels A, C and E) at the corresponding cycles was also included. A 100 bp ladder (lanes M) was used as the DNA standard.

3.3.4 RT-PCR using hetrospecific primers from *P. monodon* and *H. asinina*

Primers originally developed from sex-related transcripts in *P. monodon* were applied for identification of homologues in *M. rosenbergii*. These included DSI, XNP-1, Sxl, ENSANGP40, ENSANGP44, EGF, rudimentary protein, Usp9X, peritrophin and TSP. In addition, homologues of axonemal protein, tektin and VTG-1 originally identified in *H. asinina* were also analyzed.

Primers for DSI and EGF generated positive amplification product in *M. rosenbergii*. The former clearly indicated high expressed level in AG of BC and lower levels in that of OC, SOC and oviducts of female *M. rosenbergii*. The latter also showed differential expression between males and females of *M. rosenbergii* (Fig. 3.61). TSP288-F/R and TSP 462-F + TSP 288–R gave positive amplification product in both AG and oviducts of *M. rosenbergii* (Fig. 3.62).

Female-specific expression was found when amplification of the first strand cDNA of AG, testes, oviduct and ovaries with peritrophin primers (a touchdown PCR; 1 cycle of 94 °C for 3 min followed by 10 cycles of 94 °C for 30 s, 66 °C for 1 min and lowering for 2°C in every 2 cycles and 72 °C, 1 min and 28 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min followed by 1 cycle of 72 °C for 7 min). The amplification product (600 bp) was only observed in ovaries but not in AG, testes and oviduct of male and female *M. rosenbergii* (Fig. 3.63).

The number of specimens used for RT-PCR of EGF was then increased. The first sample set (BC, OC, SOC and female, $N = 2$ for each type) showed a greater expression level in all male morphotypes than female. Nevertheless, the template from this sample set gave problematic amplification with other transcripts. The new set of the first strand cDNA template was prepared from other specimens (BC, OC, SOC and female, $N = 2$ for each type) and RT-PCR did not reveal sex-differential expression of EGF in *M. rosenbergii* (Fig. 3.64).

Using primers from sex-related transcript of *H. asinina*, positive amplification was found with axonemal protein gene (290 bp and 1250 bp in the BC male) and tektin (150 bp fragment in female) but not VTG-1 (Fig. 3.65).

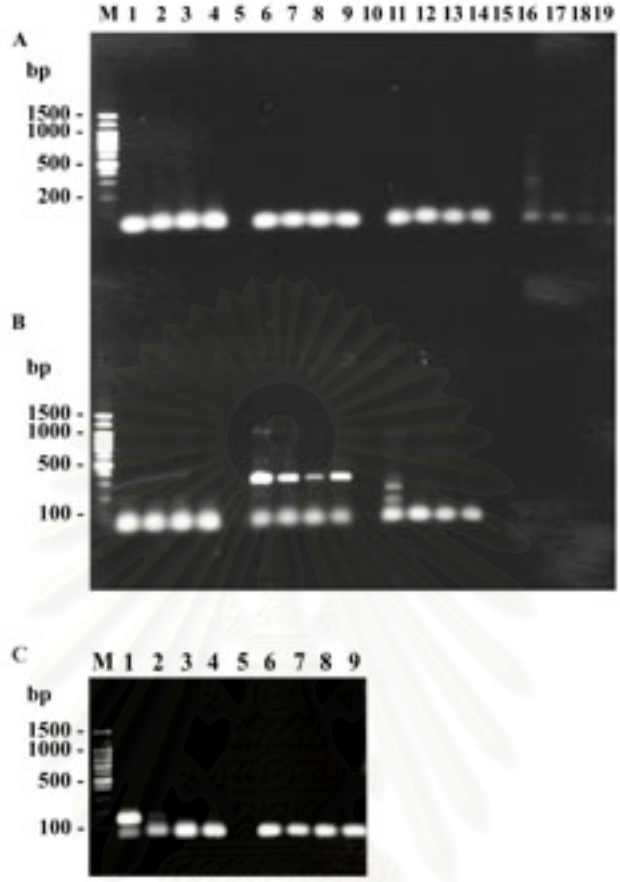

Figure 3.61 RT-PCR of the first strand cDNA of pooled AG total RNA of BC (lanes 1, 6, 11 and 16, panel A, lanes 1, 6 and 11, panel B and Lanes 1 and 6, panel C) and OC; (lanes 2, 7, 12 and 17, panel A; lanes 2, 7 and 12 panel B and 2 and 7 panel C) males and pooled oviduct total RNA of 2 females (FEI; lanes 3, 8, 13 and 18, panel A, lanes 3, 8 and 13 panel B and lanes 3 and 8 panel C and FEII; lanes 4, 9, 14 and 19, panel A) using primers from *P. monodon* including Sxl-F/R (lanes 1 – 4, A), ENSANGP40-F/R (lanes 6 – 9, A), ENSANGP44-F/R (lanes 11 – 14, A), Rudimentary-F/R (lanes 16 – 19, A), Usp9x-F/R (lanes 1 – 4, B), EGF-F/R (lanes 6 – 9, B), TSP 288-1 + TSP 371-1 (lanes 11 – 14, B), DSI-F/R (lanes 1 – 4, C) and XNP1-F/R (lanes 6 – 9, C). A 100 bp ladder (lanes M) was used as the DNA marker.

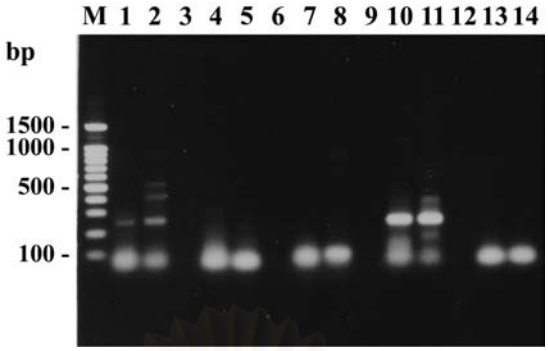

Figure 3.62 RT-PCR of the first strand cDNA of testes of the BC male (lanes 1, 4, 7, 10 and 13) and ovaries of female (lanes 2, 5, 8, 11 and 14) using primers from *P. monodon* including TSP288-F/R (lanes 1 - 2), TSP462-F/R (lanes 3 – 4), TSP 462–2 + TSP 371-1 (lanes 7 – 8), TSP 462-F + TSP 288–R (lanes 10 – 11) and TSP 288-1 + TSP 371-1 (lanes 13 – 14).

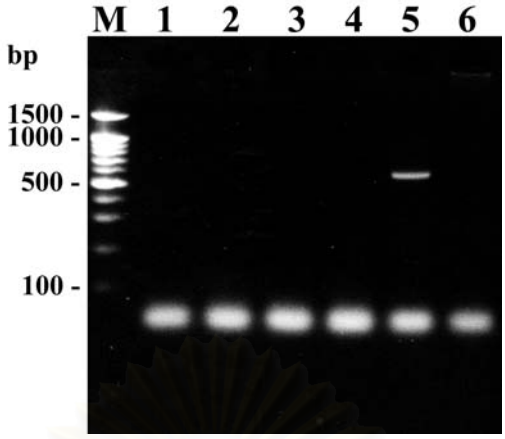

Figure 3.63 High stringency amplification of peritrophin against the first strand cDNA of testes of BC, OC and SOC (lanes 2 – 4), and ovaries (lane 5) using a touchdown PCR. Lanes M and 1 were a 100 bp DNA ladder and the negative control (without cDNA template), respectively. Amplification was also carried out against genomic DNA of *M. rosenbergii* (lane 6).

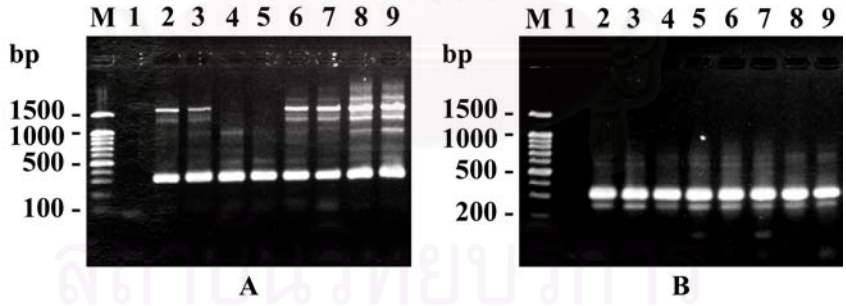

Figure 3.64 RT-PCR of the first strand cDNA of AG of BC (lanes 2 – 3, panels A and B), OC (lanes 4 and 5, A and B), and SOC (lanes 6 and 7, panel A and B) male and oviducts (lanes 8 and 9, panel A and B) of female *M. rosenbergii* using primers EGF-F/R (A) and β - actin (positive control, B). Lanes M and 1 were a 100 bp DNA ladder and the negative control (without cDNA template).

Table 3.8 A summary of RT-PCR for identification of sex-related transcripts in *M. rosenbergii* using primers from *P. monodon*

Primer	Amplification results
Transcripts in androgenic glands and oviducts	
1. DSI	Differentially expressed 150 bp fragment
2. XNP-1	No products
3. Sxl	No products
4. ENSANGP 40	No products
5. ENSANGP 44	No products
6. EGF	Non-differentially expressed 300 bp fragment
7. Rudimentary	Smear products
8. Usp9X	No products
9. TSP 288 - 1 + TSP 371 – 1	150 and 250 bp fragment found the BC male sample
Transcripts in testes and ovaries	
1. Peritrophin	600 fragment in ovaries
2. TSP 288 – F + TSP 288 – R	250 bp, 400 bp and 500 bp fragments in testes and ovaries
3. TSP 462 – F + TSP 462 – R	No products
4. TSP 462 – 2 + TSP 371 – 1	No products
5. TSP 462 – F + TSP 288 – R	250 bp fragment in ovaries and testes
6. TSP 288 – 1 + TSP 371 – 1	No products

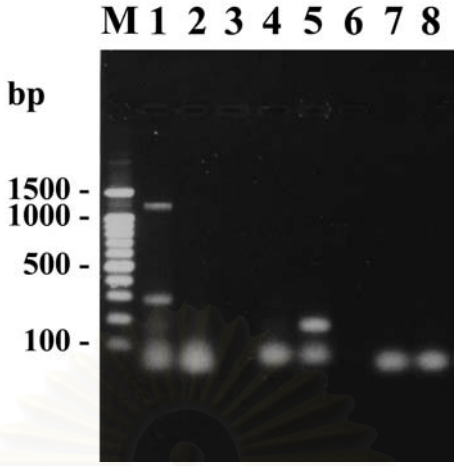

Figure 3.65 RT-PCR of the first strand cDNA of testes of BC (lanes 1, 4 and 7) and ovaries (lanes 2, 5 and 8) of female *M. rosenbergii* using primers Axonemal F/R (lanes 1 -2), Tektin-F/R and VTG1 (lanes 7 -8) originally from *H. asinina*. Lanes M is a 100 bp DNA ladder.

3.4 RAP-PCR analysis

3.4.1 Identification of sex-specific/differential, morphotype specific/differential expression markers using RAP-PCR

RAP-PCR was carried out to isolate various types of expression markers in different morphotype of males and in female of *M. rosenbergii*. Total RNA extracted from AG and oviducts each individual was reverse-transcribed to the first strand cDNA by a decanucleotide primer (UBC 268, UBC 428, UBC 122, OPA 02 or OPA 16) and subject to low-stringency PCR using the single synthesizing primer or combined with the other primers (29 RAPD primers).

Accordingly, 150 primer combinations were screened across the first strand cDNA of AG and oviducts from BA, OC and SOC males and females of *M. rosenbergii*. The amplification products were analyzed by agarose gel electrophoresis (Fig. 3.66). Successful amplification products were further analyzed by denaturing polyacylamide electrophoresis (Fig. 3.67 – 3.69).

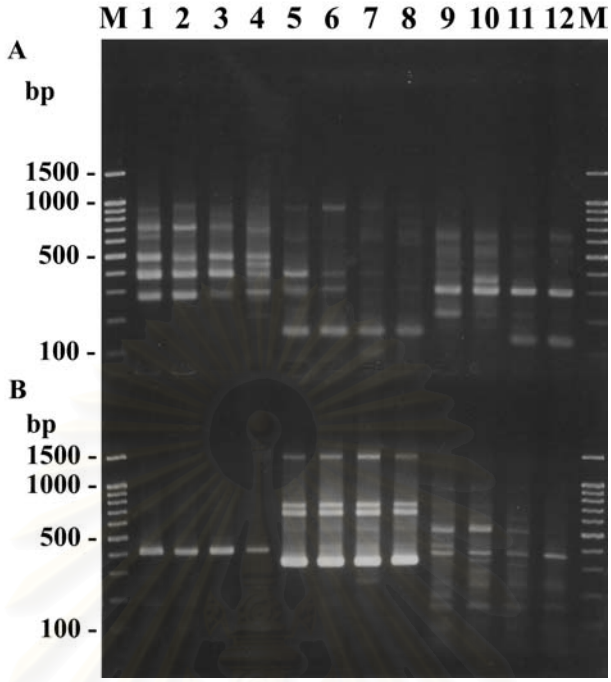

Figure 3.66 A 2.0% ethidium bromide stained agarose gel showing RAP-PCR products generated from the first strand cDNA (using UBC268 as the synthesizing primer) of AG of BC, OC and SOC (lanes 1 – 3, 5 – 7 and 9 – 11 panel A and B, respectively) male and oviducts (lanes 4, 8 and 12 panel A and B) of female *M. rosenbergii* using primers UBC268/UBC268 (lanes 1 – 4, panel A), UBC268/UBC273 (lanes 5 – 8, A), UBC268/UBC299 (lanes (9 – 12, A), UBC268/UBC428 (lanes 1 – 4, B), UBC268/UBC456 (lanes 5 – 8, B) and UBC268/UBC457 (lanes 9 -12, B). A 100 bp ladder (lane M) was used as the DNA marker.

In a total, 43 and 24 RAP-PCR markers revealed sex-specific expression between male and female *M. rosenbergii* whereas 29 markers indicated differential expression between genders. Additionally, 10, 12 and 13 RAP-PCR fragments exhibit morphotype-specific expression against a small sample set (one pooled BC, OC, SOC and female total RNA) (Appendix E).

Screening the first strand cDNA of BC, OC and SOC males and females yielded a 340 bp fragment across UBC428 combined with all prime in the SOC male (called

SOC340RAP, Fig. 3.70). Likewise, a 315 bp fragment was found across UBC122 combined with all primers (FE315RAP, Fig. 3.71). The 340 bp and 315 bp showing SOC-specific and female-specific expression patterns were excised, eluted out from the gel and reamplified with various combinations of UBC 428 and other primers (Fig. 3.72 A) and UBC122 (Fig. 3.72B, odd lanes) and combination of UBC122 and other primers (Fig. 3.72B even lanes). The product of each reamplification was analyzed by SSCP analysis. Results clearly indicated that several different transcripts are composed in a single insert (Fig. 3.72).

3.4.2 Cloning and characterization of candidate sex/morphotype-specific expression markers in *M. rosenbergii*

One candidate female-specific (FERAP315), male-specific (M268/128RAP, M428/228RAP, MA16/222RAP, M122/135RAP, and M122/159RAP), 3 SOC-specific (SOC340RAP, SOC268/273RAP and SOC122/228RAP) and 2 BC-specific (BC428/228RAP BC428/273RAP) were cloned and sequenced. A single insert may provide more than one type of sequences (Appendix F).

Based on the fact that SOC340RAP and FE315RAP could be amplified when combined UBC428 and UBC122 with all investigated primers, the cloning strategy was different from that of other RAP-PCR markers. The former fragment generated from seven different primer combinations was separately eluted out from the gel and reamplified. The reamplified product was pooled (set I, UBC428/OPA101, UBC428/UBC119, UBC428/UBC122 and UBC428/UBC128 and set II, UBC428/UBC158, UBC428/UBC169 and UBC428/UBC191) and cloned. The colony PCR products from different pooled insert were digested with *Hin*d III and *Rsa* I for the set I (Fig. 3.73, lanes 1 - 10, A and B) and set II (Fig. 3.73, lanes 11 – 17, A and B) inserts.

Likewise, FE315RAP generated from UBC122/UBC159, UBC122/OPZ09, UBC122/OPA01 and UBC122/OPB10 was separately eluted out from the gel and reamplified. The eluted DNA of each tube was then reamplified using the single primers (UBC122) or combined primers (UBC122 and each of UBC159, OPZ09, OPB01 and UBC222) resulting in 8 different reaction tubes. The reamplified 315 bp fragment was pooled to generate the sample set I (i.e. from 4 tubes using UBC122 for reamplification)

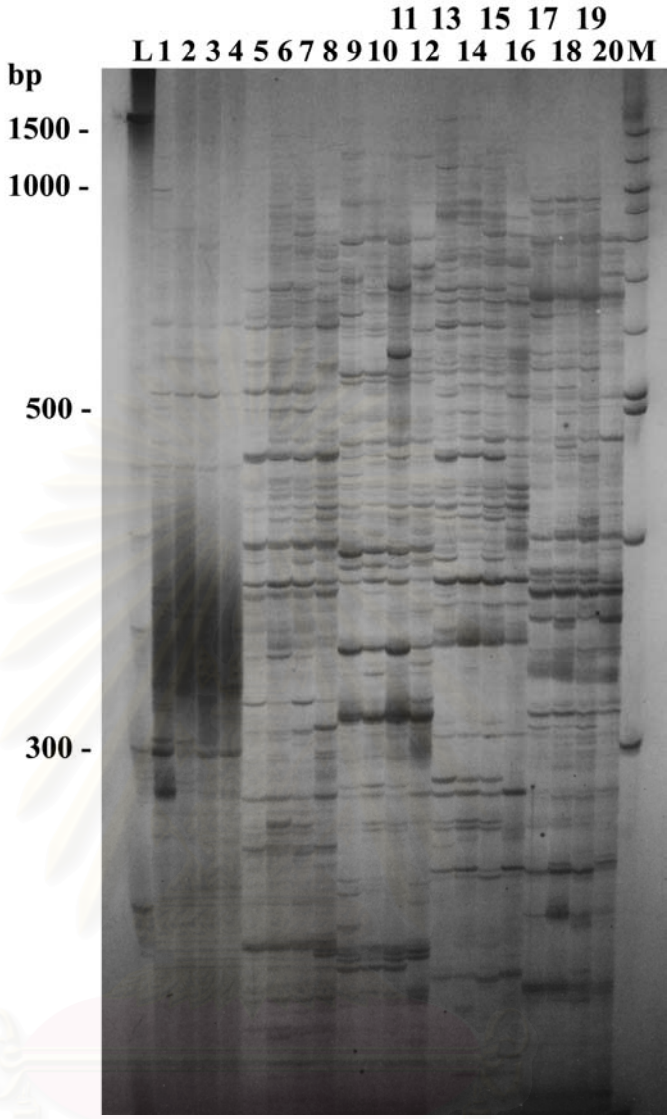

Figure 3.67 A 4.5% denaturing polyacrylamide gel showing RAP-PCR patterns from pooled AG total RNA of BC (lanes 1, 5, 9, 13 and 17), OC (lanes 2, 6, 10, 14 and 18), and SOC (lanes 3, 7, 11, 15 and 19) males and pooled oviducts total RNA of female (lanes 4, 8, 12, 16 and 20) *M. rosenbergii*.

Lanes 1 – 4 = UBC 268/UBC 101, lanes 5 – 8 = UBC 268/UBC 119, lanes 9 – 12 = UBC 268/UBC 122, lanes 13 – 16 = UBC 268 - UBC 128, lanes 17 – 20 = UBC 268/UBC 135, lane M = 100 bp DNA ladder; lane L = 50 bp DNA ladder.

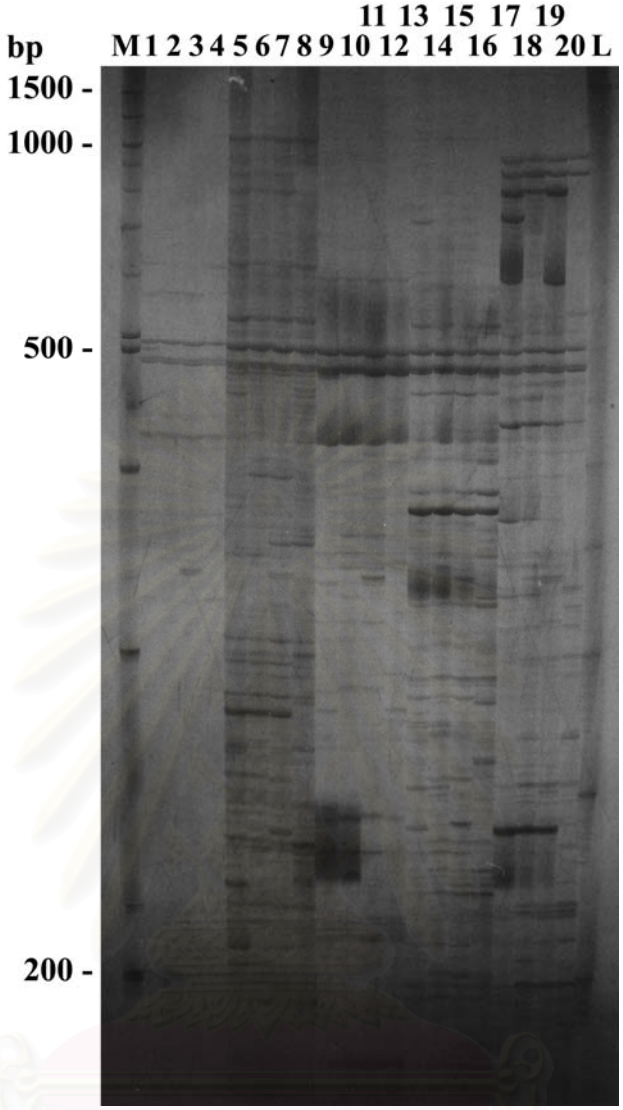

Figure 3.68 A 4.5% denaturing polyacrylamide gel showing RAP-PCR patterns from pooled AG total RNA of BC (lanes 1, 5, 9, 13 and 17), OC (lanes 2, 6, 10, 14 and 18), and SOC (lanes 3, 7, 11, 15 and 19) males and pooled oviducts total RNA of female (lanes 4, 8, 12, 16 and 20) *M. rosenbergii*.
Lanes 1 – 4 = UBC 428/UBC 217, lanes 5 – 8 = UBC 428/UBC 228, lanes 9 – 12 = UBC 428/UBC 263, lanes 13 – 16 = UBC 428/UBC 268, lanes 17 – 20 = UBC 428/UBC 273, lane M = 100 bp DNA ladder; lane L = 50 bp DNA ladder.

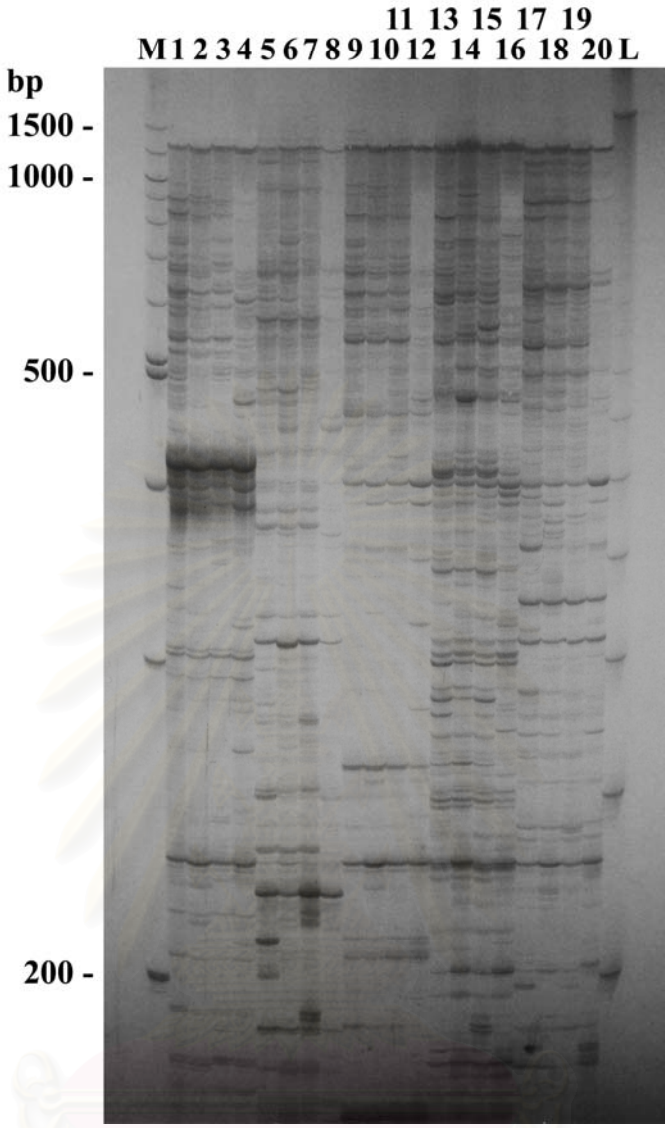

Figure 3.69 A 4.5% denaturing polyacrylamide gel showing RAP-PCR patterns from pooled AG total RNA of BC (lanes 1, 5, 9, 13 and 17), OC (lanes 2, 6, 10, 14 and 18), and SOC (lanes 3, 7, 11, 15 and 19) males and pooled oviducts total RNA of female (lanes 4, 8, 12, 16 and 20) *M. rosenbergii*.
Lanes 1 – 4 = UBC 122/UBC 217, lanes 5 – 8 = UBC 122/UBC 228, lanes 9 – 12 = UBC 122/UBC 263, lanes 13 – 16 = UBC 122/UBC 268, lanes 17 – 20 = UBC 122/UBC 273, lane M = 100 bp DNA ladder; lane L = 50 bp DNA ladder.

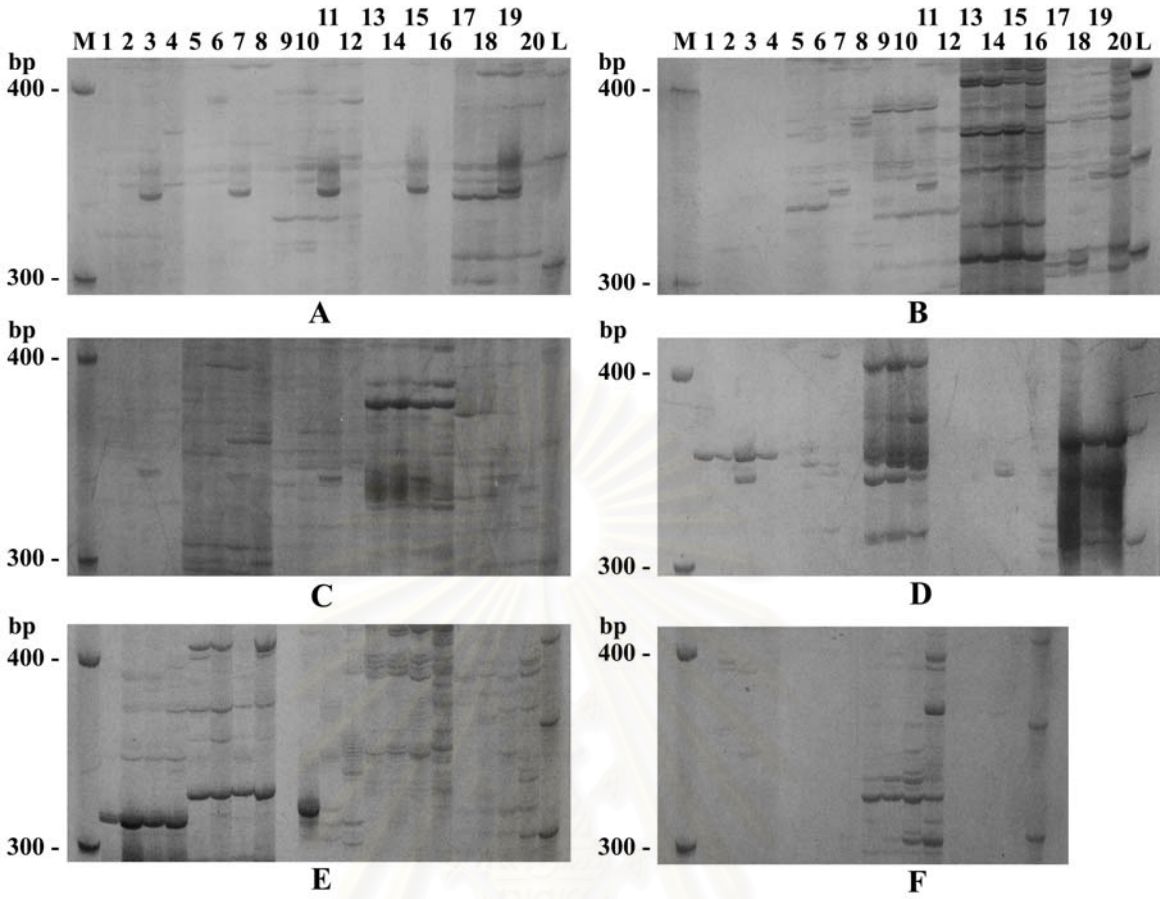

Figure 3.70 A 4.5% silver stained gel denaturing polyacrylamide gel showing SOC - specific RAP-PCR bands derived from primer UBC428 combined with other 30 second primer using the first strand cDNA from pooled AG total RNA of BC (lanes 1, 5, 9, 13 and 17, A - F), OC (lanes 2, 6, 10, 14 and 18, A - F) and SOC (lanes 3, 7, 11, 15 and 19, A - F) male and female (lanes 4, 8, 12, 16 and 20, A - F) *M. rosenbergii*. Notably, lanes 17 – 20 were not available for the panel F.

Lanes 1 – 4 A, B, C, D, E and F = UBC 428/UBC 101, 138, 217, 299, OPA 02 and UBC 159, respectively; lanes 5 – 8 A, B, C, D, E and F = UBC 428/UBC 119, 158, 228, 428, OPA 07 and OPZ 09, respectively; lanes 9 – 12 A, B, C, D, E and F = UBC 428/UBC 122, 169, 263, 456, OPA 09 and OPB 10, respectively; lanes 13 – 16 A, B, C, D, E and F = UBC 428/UBC 128, 174, 268, 457, OPA 16 and UBC 222, respectively; lanes 17 – 20 A, B, C, D and E = UBC 428/UBC 135, 191, 273, 459 and OPA 01, respectively; lane M = 100 bp DNA ladder; lane L = 50 bp DNA ladder.

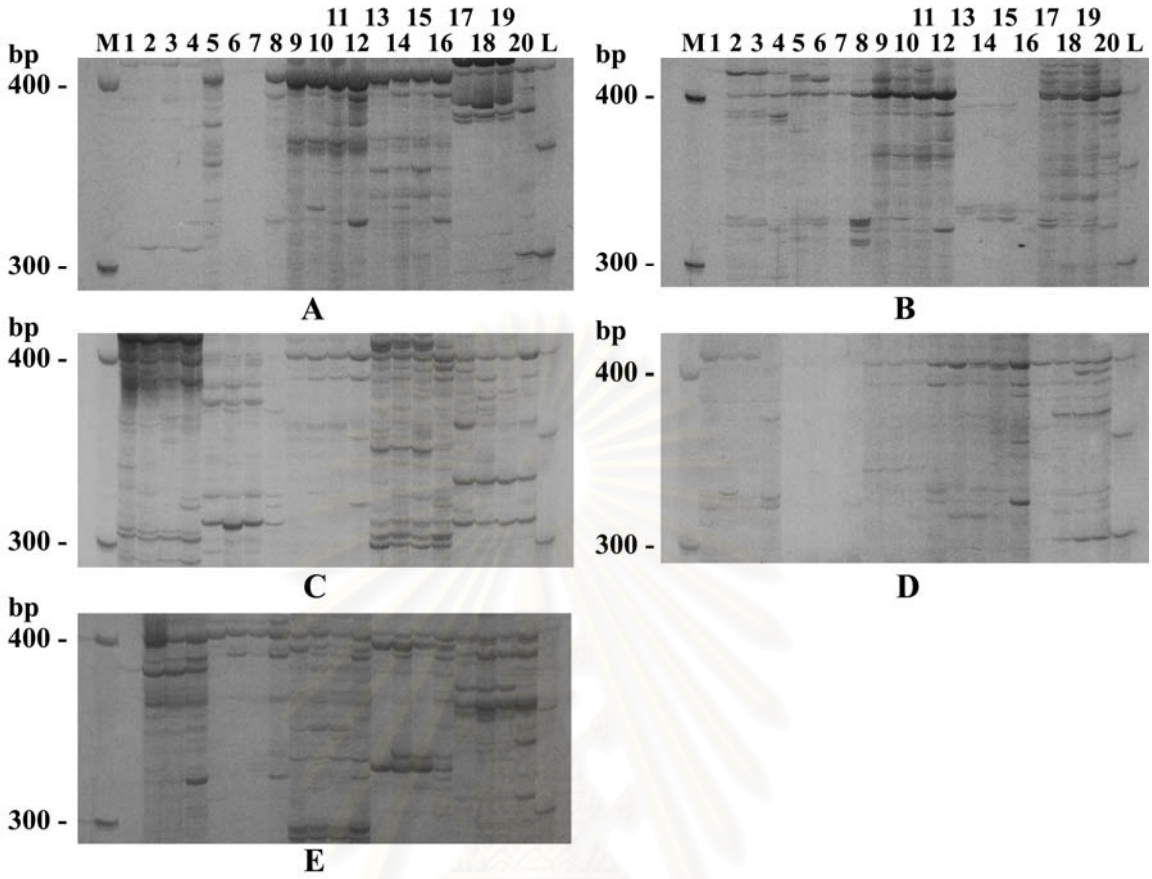

Figure 3.71 A 4.5% silver stained gel denaturing polyacrylamide gel showing SOC - specific RAP-PCR bands derived from primer UBC122 combined with other 30 second primer using the first strand cDNA from pooled AG total RNA of BC (lanes 1, 5, 9, 13 and 17, A - E), OC (lanes 2, 6, 10, 14 and 18, A - E) and SOC (lanes 3, 7, 11, 15 and 19, A - E) male and female (lanes 4, 8, 12, 16 and 20, A - E) *M. rosenbergii*.

Lanes 1 – 4 A, B, C, D and E = UBC 122/UBC 101, 138, 217, 299 and UBC 159, respectively; lanes 5 – 8 A, B, C, D and E = UBC 122/UBC 119, 158, 228, 428 and OPZ 09, respectively; lanes 9 – 12 A, B, C, D and E = UBC 122/UBC 122, 169, 263, 456 and OPA 01, respectively; lanes 13 – 16 A, B, C, D and E = UBC 122/UBC 128, 174, 268, 457 and OPB 10, respectively; lanes 17 – 20 A, B, C, D and E = UBC 428/UBC 135, 191, 273, 459 and UBC 222, respectively; lane M = 100 bp DNA ladder; lane L = 50 bp DNA ladder.

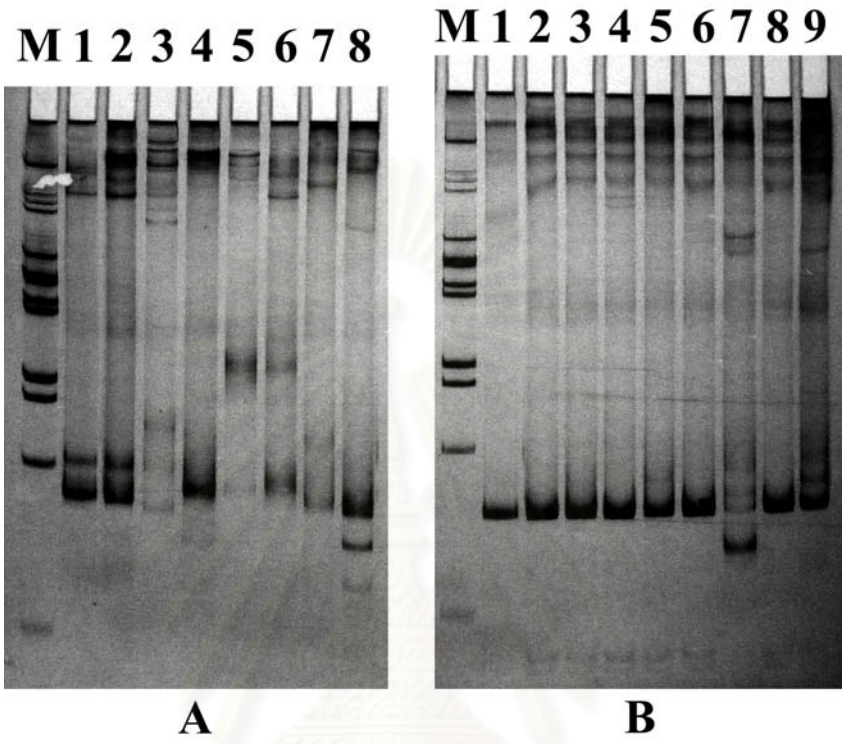

Figure 3.72 A 15% silver stained polyacrylamide gel (37.5:1) supplemented with 5% glycerol showing SSCP patterns of the reamplification products of candidate SOC–specific (340 bp, A) and female-specific (315 bp, B) RAP-PCR markers. A 100 bp ladder (lanes M) was used as the DNA marker.

and the sample set II (i.e. from 4 tubes using UBC122 and each of UBC159, OPZ09, OPB01 and UBC222 for reamplification). Both sample sets were cloned. The colony PCR products from different pooled insert were digested with *Hin*d III and *Rsa* I for the set I (Fig. 3.74A) and set II (3.74B) inserts.

A total of 10 and 6 composite digestion patterns were found from SOC340RAP and FE315RAP. Recombinant plasmid was extracted from a representative of each clone type and sequenced. Four clones from SOC340RAP exhibited significant matching with ribosomal RNA (18S rRNA for 340-6 and 340-7 and 28S rRNA for 340-2 and 340-15).

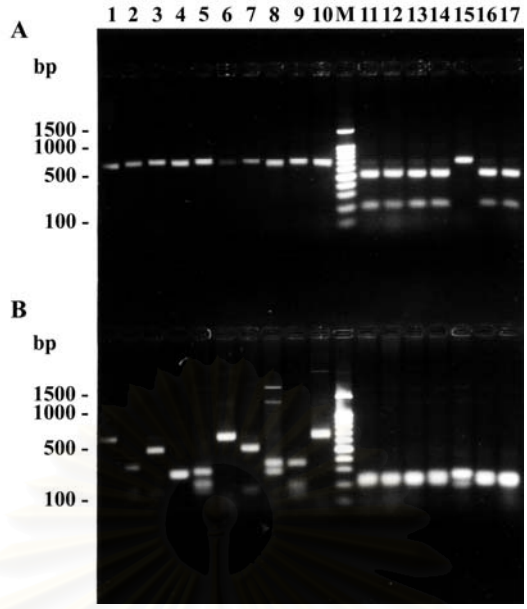

Figure 3.73 A 1.2% ethidium bromide stained agarose gel showing digestion patterns of colony PCR products of the candidate SOC-specific RAP-PCR insert (SOC340RAP; 340 bp) with *Hin*d III (sample set I and II for lanes 1 – 10 and 11 – 17, A) and *Rsa* I (sample set I and II for lanes 1 – 10 and 11 – 17, B). A 100 bp ladder was used as the DNA standard.

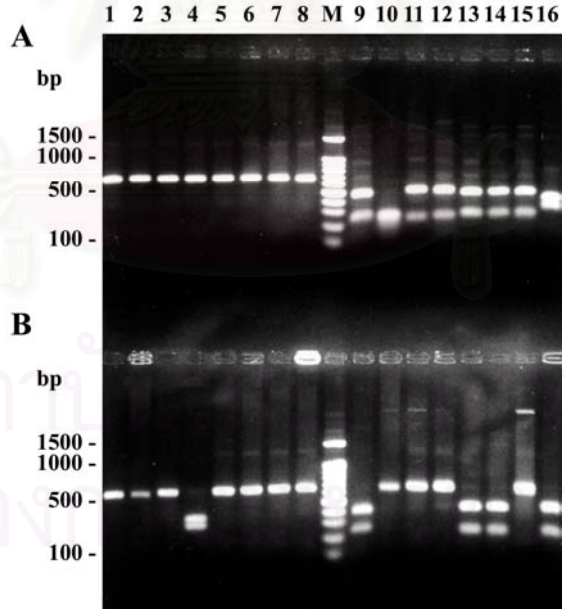

Figure 3.74 A 1.2% ethidium bromide stained agarose gel showing digestion patterns of colony PCR products of the candidate female-specific RAP-PCR insert (FE315RAP; 315 bp) with *Hin*d III (lanes 1 – 10, A for the sample set I and lanes 1 – 8, B for the sample set II) and *Rsa* I (lanes 11 – 17, A for the sample set I and lanes 9 – 16, B for the sample set II). A 100 bp ladder was used as the DNA standard.

A.

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SOC340-l    GTAGACGAGCAAGCAACTTGGAACGGGGAAGAAATAGCTTTCTTTCCAATCGGCATGACA
SOC340-4    GTAGACGAGCAAGCAACTTGGAACGAGGAAGAAATAGCTTTCTTTCCAATCGGCATGACA
            ************************ **********************************

SOC340-l    TTACACTCGGTGAAAGTAGCAGCCGTTAGACCTGTTGTTCGGATGCCTTTTTTTCTCTTT
SOC340-4    TTACACTCGGTGAAAGTAGCAGCCGTTAGACCTGTTGTTCGGATGCCTTTTTTTCTCTTT
            ************************************************************

SOC340-l    TTTTT-GTACAGATTTCTGCAGGCGAGAAACTTTTCTTTTCAGAGATGACGTAGCATGGC
SOC340-4    TTTTTTGTTCAGATTTCTGCAGGCGAGAAACTTTTCTTTTCAGAGATGACGTAGCATGGC
            ***** ** **************************************************

SOC340-l    AGAATGCTTTCCCTTTCGTTTCCACTTTCATGTATCGTCATATCGGCCCCGTAAGGAGGT
SOC340-4    AGAATGCTTTCCCTTTCGTTTCCACTTTCATGTATCGTCATATCGGCCCCGTAAGGAGGT
            ************************************************************
SOC340-l    AGTGCCGTCAGTGCACCTTACGCGGAGCACTGTAGGCATTACTTGAGGTTCTTTGCAGCG
SOC340-4    AGTGCCGTCAGTGCACCTTACGCGGAGCACTGTAGGCATTACTTGAGGTTCTTTGCAGCG
            ************************************************************

SOC340-l    TCCCTTCGGTCCCTAGCTGGACCCCTTTTCATTTCTTTTCCTGTACCGCAGCCA
SOC340-4    TCCCTTCGGTCCCTAGCTGGACCCCTTTTCATTTCTTTTCCTGTACCGCAGCCA
            ********************************************************
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B.

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SOC340-31   TTAGCCGTGGCGCGGAAAAGAAGCTTCTCTTTTCTCGTTAAGGTGTGATAAAACTGGCAG
SOC340-41   TTAGCCGTGGCGCGGAAAAGAAGCTTCTCTTTTCTCGTTAAAGTGTGATAAAACTGGCAG
            ************************************** ******************

SOC340-31   GAGCGTTAGGCAAGGTACTGATAATGTACATTGACAGCATACAGTAAGTACGATACTCAG
SOC340-41   GAGCGTTAGGCAAGGTGCTGATAATGTACATTGACAGCACACAGTAAGTACGATACTCAG
            **************** ********************** ********************

SOC340-31   AGATTTAAGAACTTCTGGAGGTAAACTGAGAGAATATAAAAAGTTTCGTTGAACTGAGCG
SOC340-41   AGATTTAAGAACTTCTGGAGGTAAACTGAGAGAATATAAAAAGTTTCGTTGAACTGAGCG
            ************************************************************

SOC340-31   TCTATACGCATCGTGTCAAATGTCGACTCAAGGACACTAGTCGAGATTATTGTGAAGGCT
SOC340-41   TCTATACGCATCGTGTCAAATGTCGACTCAAGGACACTAGTCGAGATTATTGTGAAGGCT
            ************************************************************

SOC340-31   TACAAATCCAACAAAGCAAATTGAATTCTACCAAGAGATTAGCGATTAATTTCGTGATTT
SOC340-41   TACAAATCCAACAAAGCAAATTGAATTCTACCAAGAGATTAGCGATTAATTTCGTGATTT
            ************************************************************

SOC340-31   ATGAGCCAACGATTTGTACTTACCGCAGCC
SOC340-41   ATGAACCAACGATTTGTACTTACCGCAGCC
            **** *************************
```

Figure 3.75 Nucleotide sequence alignment illustrating identical sequences of recombinant clones from a candidate SOC-specific RAP marker.

The remaining clones were regarded as unknown sequences as they were not significantly matched with any sequence in the GenBank ($P > 10^{-4}$). Among this clones 340-1 and 340-4 and 340-31 and 340-41 showed identical sequenced. Therefore, 4 different sequences were found from a SOC340RAP insert excluding clones representing 18S and 28S rRNA. Conversely, all clones from a FE315RAP insert were unique and did not match with sequences in the GenBank. As a result, 6 different unknown sequences were found from this insert. Blast results of clones from SOC340RAP and FE315RAP inserts are illustrated by Table 3.9.

Apart from those RAP-PCR markers, almost all of the cloned fragment with the exception of M122/159RAP.1, and M122/159RAP.2 generate only one insert sequence (Table 3.10). All of the remaining inserts (except M122/159RAP.2 which was significantly similar to I–connectin mRNA of the crayfish, *P. clarkii*) also represented unknown transcript.

Two primer pairs were designed from sequences of candidate SOC-specific and female-specific RAP-PCR markers (SOC340RAP.1, SOC340RAP.2, FE315RAP.1 and FE315RAP.2). Nevertheless, the amplification product was not obtained from these primer pairs (Table 3.11). Three developed markers (M122/159RAP.2, SOC268/273RAP and BC428/273RAP) illustrated interesting results at the transcriptional level.

The expression levels of M122/159RAP.2 clearly showed differential expression patterns in AG of males and oviducts of female *M. rosenbergii* but no differences were found between different male morphotypes (Fig. 3.81 and Table 3.11)

Two amplified fragments were generated from SOC268/273RAP primer. These included the expected 194 bp fragment and a 150 bp fragment. Both bands were equally expressed in AG of OC and SOC males whereas the expression level of a 194 bp band predominated in AG of the BC males. The expression level of the expected fragment also predominated in oviducts of female (Fig. 3.82). The expression patterns of this transcript implied the possible existence of alternative spicing and different isoforms of this transcript in different tissues of *M. rosenbergii*.

Table 3.9 Blast results of sequences obtained from a SOC-specific and female-specific expression markers from RAP-PCR analysis

Clones	Gene homologue	Closest species	E - value	Length of nucleotide sequenced (bp)
Candidate SOC-specific -specific expression makers				
1. SOCRAP340-1	Unknown	-	$> 10^{-4}$	352
2. SOCRAP340-2	28s ribosomal RNA	Caribbean spiny lobster, *Panulirus argus*	4×10^{-32}	351
3. SOCRAP340-4	Unknown	-	$> 10^{-4}$	353
4. SOCRAP340-5	Unknown	-	$> 10^{-4}$	335
5. SOCRAP340-6	18s ribosomal RNA	*South coast rock lobster, Palinurus gilchristi*	8×10^{-22}	336
6. SOCRAP340-7	18s ribosomal RNA	*South coast rock lobster, Palinurus gilchristi*	8×10^{-22}	335
7. SOCRAP340-15	28s ribosomal RNA	Caribbean spiny lobster, *Panulirus argus*	4×10^{-33}	351
8. SOCRAP340-31	Unknown	-	$> 10^{-4}$	329
9. SOCRAP340-38*	Unknown	-	$> 10^{-4}$	333
10. SOCRAP340-41*	Unknown	-	$> 10^{-4}$	329
Candidate female-specific expression makers				
1. Fe315RAP-5*	Unknown	-	$> 10^{-4}$	317
2. Fe315RAP-9	Unknown	-	$> 10^{-4}$	316
3. Fe315RAP-47	Unknown	-	$> 10^{-4}$	319
4. Fe315RAP-50	Unknown	-	$> 10^{-4}$	307
5. Fe315RAP-55*	Unknown	-	$> 10^{-4}$	313
6. Fe315RAP-69	Unknown	-	$> 10^{-4}$	3185

* = Primers were designed.

Table 3.10 Blast results of sequences from sex-specific/differential expression markers identified by RAP-PCR analysis

Clones	Gene homologue	Closest species	E - value	Length of nucleotide sequenced (bp)
Candidate male-specific expression makers				
1. M268/128RAP	Unknown	-	$> 10^{-4}$	289
2. M428/228RAP	Unknown	-	$> 10^{-4}$	276
3. M122/135RAP	Unknown	-	$> 10^{-4}$	414
4. MA16/222RAP	Unknown	-	$> 10^{-4}$	301
5. M122/159RAP.1	Unknown	-	$> 10^{-4}$	428
6. M122/159RAP.2	I – connectin mRNA	Red swamp crayfish, *Procambarus clarkii*	5×10^{-54}	426
Candidate morphotype-specific expression markers				
1. SOC268/273RAP	Unknown	-	$> 10^{-4}$	248
2. SOC122/228RAP	Unknown	-	$> 10^{-4}$	191
3. BC428/228RAP	Unknown	-	$> 10^{-4}$	224
4. BC428/273RAP	Unknown	-	$> 10^{-4}$	710

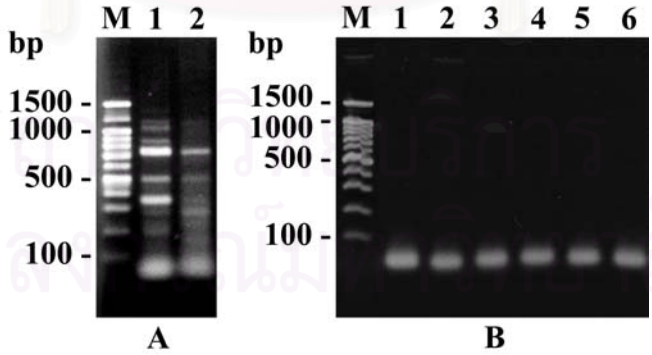

Figure 3.76 RT-PCR of the first strand cDNA of AG of BC (lanes 1, panels A and lane 3, panel B), OC (lanes 4, B), and SOC (lanes 5, B) male and oviducts (lanes 2, A and 6, B) of female *M. rosenbergii* using Fe315RAP.2 primers. Lanes M and 1 and 2 (B) were a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

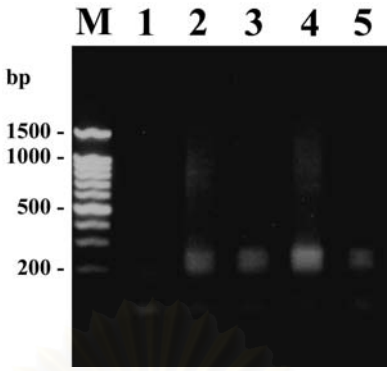

Figure 3.77 RT-PCR of the first strand cDNA of AG of BC (lanes 2), OC (lanes 3), and SOC (lanes 4) male and oviducts (lanes 5) of female *M. rosenbergii* using M268/128RAP primers. Lanes M and 1 were a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

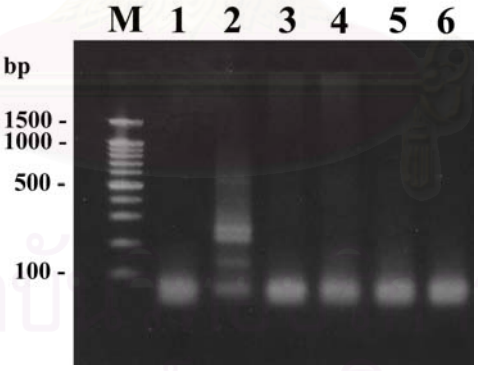

Figure 3.78 RT-PCR of the first strand cDNA of AG of BC (lanes 3), OC (lanes 4), and SOC (lanes 5) male and oviducts (lanes 6) of female *M. rosenbergii* using M428/228RAP primers. Lanes M and 1 were a 100 bp DNA ladder and the negative control (without cDNA template), respectively. Amplification was also carried out against genomic DNA of *M. rosenbergii* (lane 2).

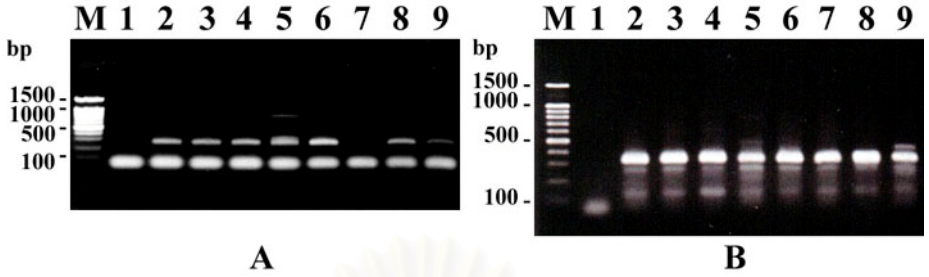

Figure 3.79 RT-PCR of the first strand cDNA of AG and testes of BC (lanes 2 and 5, panel A), OC (lanes 3 and 6, A), and SOC (lanes 4 and 7, A) male and oviducts (lanes 8) and ovaries (lane 9) of female *M. rosenbergii* using M122/135RAP primers. The positive control (β - actin) was amplified from the same template (B). Lanes M and 1 were a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

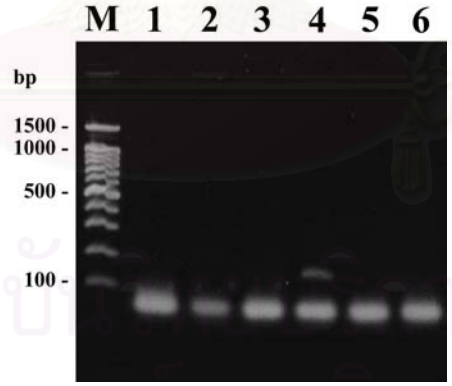

Figure 3.80 RT-PCR of the first strand cDNA of AG of BC (lanes 3), OC (lanes 4), and SOC (lanes 5) male and oviducts (lanes 6) of female *M. rosenbergii* using M122/159RAP.1 primers. Lanes M and 1 and 2 were a 100 bp DNA ladder and the negative control (without cDNA template), respectively. Amplification was also carried out against genomic DNA of *M. rosenbergii* (lane 2).

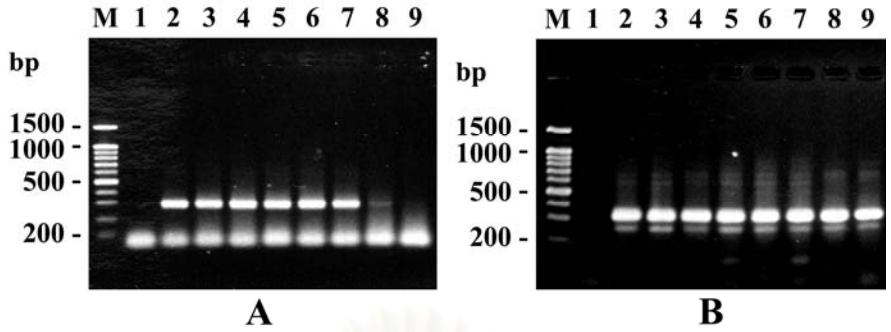

Figure 3.81 RT-PCR of the first strand cDNA of AG of BC (lanes 2 and 3, panel A), OC (lanes 4 and 5, A), and SOC (lanes 6 and 7, A) male and oviducts (lanes 8 and 9) of female *M. rosenbergii* using M122/159RAP.2 primers. The positive control (β - actin) was amplified from the same template (B). Lanes M and 1 were a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

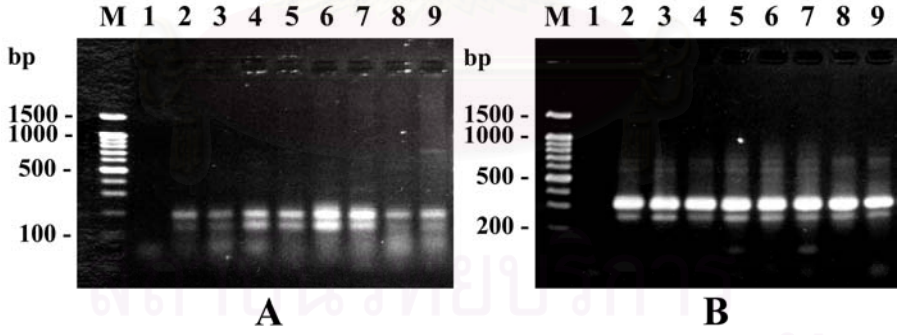

Figure 3.82 RT-PCR of the first strand cDNA of AG of BC (lanes 2 and 3, panel A), OC (lanes 4 and 5, A), and SOC (lanes 6 and 7, A) male and oviducts (lanes 8 and 9) of female *M. rosenbergii* using SOC268/273RAP primers. The positive control (β - actin) was amplified from the same template (B). Lanes M and 1 were a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

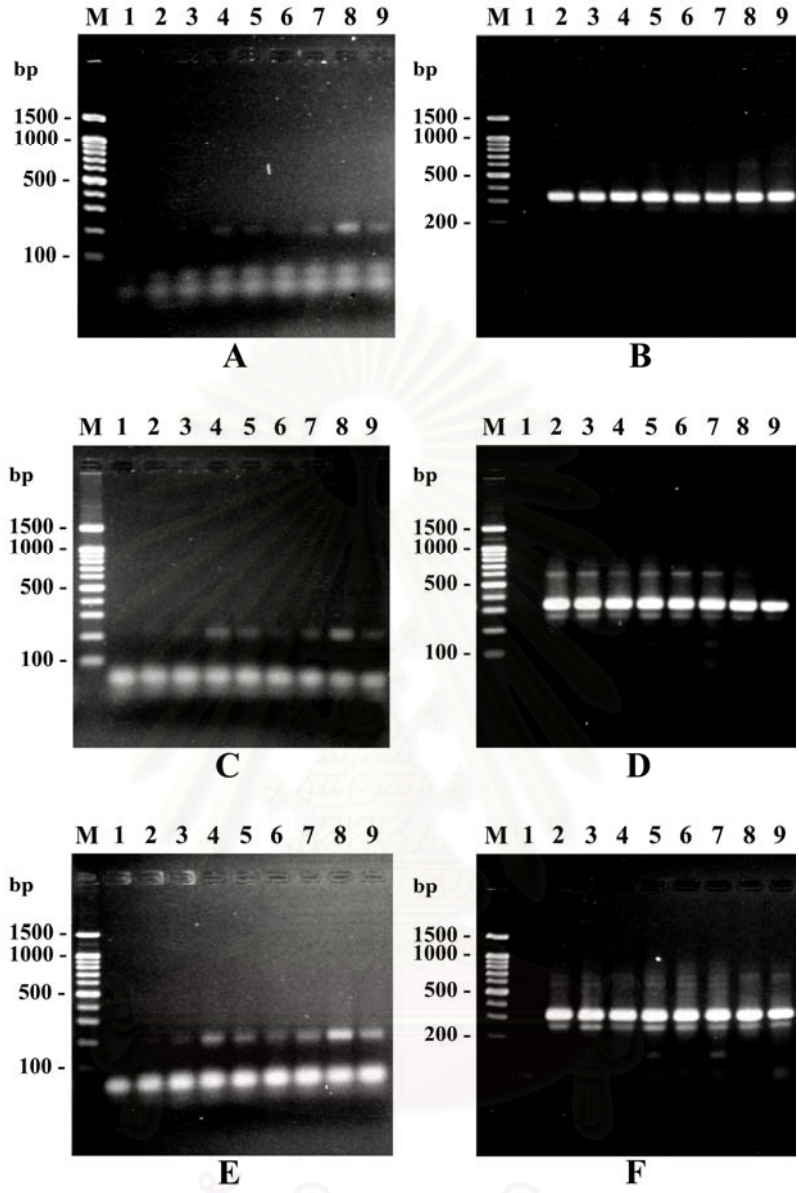

Figure 3.83 RT-PCR of the first strand cDNA of AG of BC (lanes 2 – 3, panels A - D), OC (lanes 4 – 5, panel A - D) and SOC (lanes 6 – 7, panel A - D) male and oviduct of females (lanes 8 – 9, panels A - D) of *M. rosenbergii* using SOC122/228RAP primers for 25 (A), 30 (C) and 35 cycles (E). Positive (β - actin of *M. rosenbergii*, panel B, D and F) and negative control (without cDNA template, lanes 1 in panels A, C and E) at the corresponding cycles was also included. A 100 bp ladder (lanes M) was used as the DNA standard.

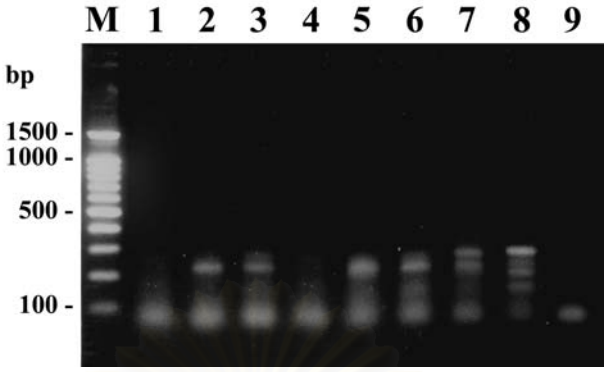

Figure 3.84 RT-PCR of the first strand cDNA of AG and testes of BC (lanes 2 and 5, panel A), OC (lanes 3 and 6, A), and SOC (lanes 4 and 7, A) male and oviducts (lanes 8) and ovaries (lane 9) of female *M. rosenbergii* using BC428/273RAP primers. Lanes M and 1 were a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

Differentially expressed patterns were also found from BC428/273RAP. Surprisingly, the expression level of this transcript in the BC male was much lower than that in OC and SOC males and females. An additional amplified product (approximately 260 bp in length) was observed in oviducts and ovaries but not in AG.

3.4.3 SSCP analysis

SSCP analysis was used for further characterization of derived RAP-PCR markers providing the positive amplification product when genomic DNA was used as the template. Notably, only markers exhibiting differentially expressed patterns (M122/159RAP.2, SOC268/273RAP and BC428/273RAP) were analyzed against 9 individuals of female and 3 individuals each of BC, OC and SOC males of *M. rosenbergii* (Fig. 3.85 – 3.90).

Monomorphic SSCP patterns were found from M122/159RAP.2 I (Fig. 3.86). In contrast, polymorphic SSCP patterns were existent from SOC268/273RAP and BC428/273RAP but fixed sex-specific (or morphotype-specific) patterns were not observed in these markers.

Table 3.11 A summary of amplification results of cDNA and genomic DNA of *M. rosenbergii* using primers developed from RAP-PCR analysis

Primer	Amplification results		SSCP
	cDNA	Genomic	
Candidate female-specific expression makers			
1. Fe315RAP.1	-	NS	-
2. Fe315RAP.2	NS (Fig. 3.76)	-	-
Candidate male-specific expression makers			
1. M268/128RAP	2 fragments at 194 (expected) and 225 bp in both males and females (Fig. 3.77	NS	-
2. M428/228RAP	NS (Fig. 3.78)	240 bp band along with other fragments	ND
3. M122/135RAP	284 bp fragment in AG, ovaries and oviducts of shrimps except in testes of SOC males, (Fig. 3.79)		-
4. MA16/222RAP	-	-	-
5. M122/159RAP.1	150 bp fragment in AG of OC male (Fig. 3.80)	-	-
6. M122/159RAP.2	Differentially expressed 241 bp fragment between males and females (Fig 3.81)	241 bp fragment (Fig 3.85)	Monomorphism (Fig 3.86)
Candidate differential expression markers			
1. SOC340RAP.1	-	-	-
2. SOC340RAP.2	-	-	-
3. SOC268/273RAP	Differentially expressed 150 bp and 194 bp fragments between morphotypes and sexes (Fig. 3.82)	194 bp fragment (Fig 3.87)	Polymorphism (Fig 3.88)
4. SOC122/228RAP	166 bp fragment (Fig 3.83)	166 bp fragment	ND
5. BC428/228RAP	-	Smear products	-
6. BC428/273RAP	Differentially expressed 247 bp fragment between the BC male and OC and SOC males and females (Fig 3.84)	247 bp fragment and NS (Fig 3.89)	Polymorphism (Fig 3.90)

- = amplification was not successful; NS = PCR generated non – specific amplification product, ND – not determined.

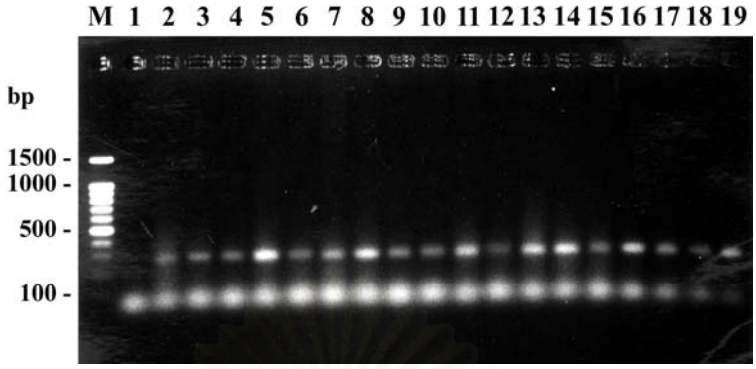

Figure 3.85 A 1.2% ethidium bromide stained agarose gel showing the PCR products of male (lanes 2 – 10) and female (lane 11 – 19) *M. rosenbergii* generated by M122/159RAP.2 primers. Lanes M and 1 are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

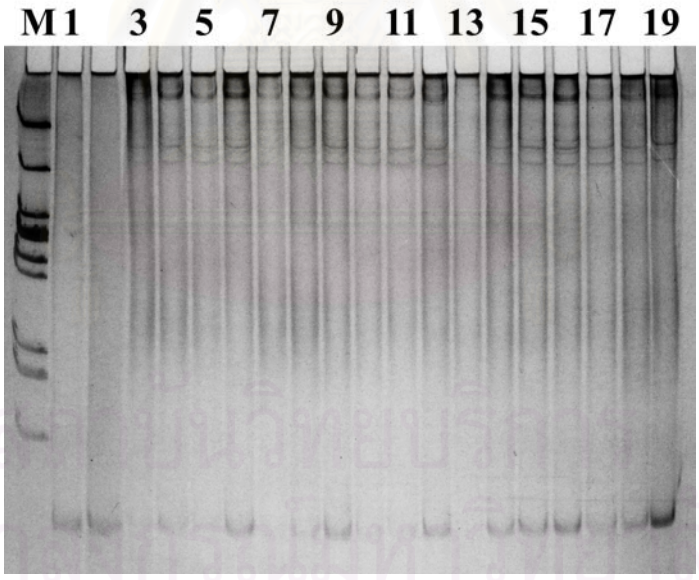

Figure 3.86 A 12.5% silver stained non-denaturing polyacylamide gel (37.5:1) showing SSCP patterns of male (lanes 3 – 11) and female (lanes 12 – 19) *M. rosenbergii* generated from M122/159RAP.2 primer. Lanes M and 1 and 2 are a 100 bp DNA marker and the non-denatured PCR product (double strand DNA control), respectively.

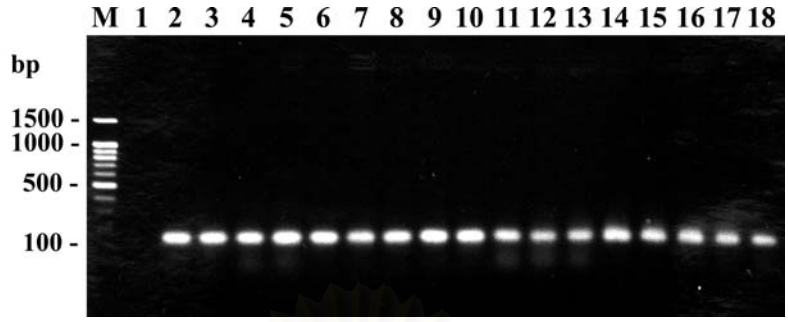

Figure 3.87 A 1.5% ethidium bromide stained agarose gel showing the PCR products of male (lanes 2 – 10) and female (lane 11 – 19) *M. rosenbergii* generated by SOC268/273RAP primers. Lanes M and 1 are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

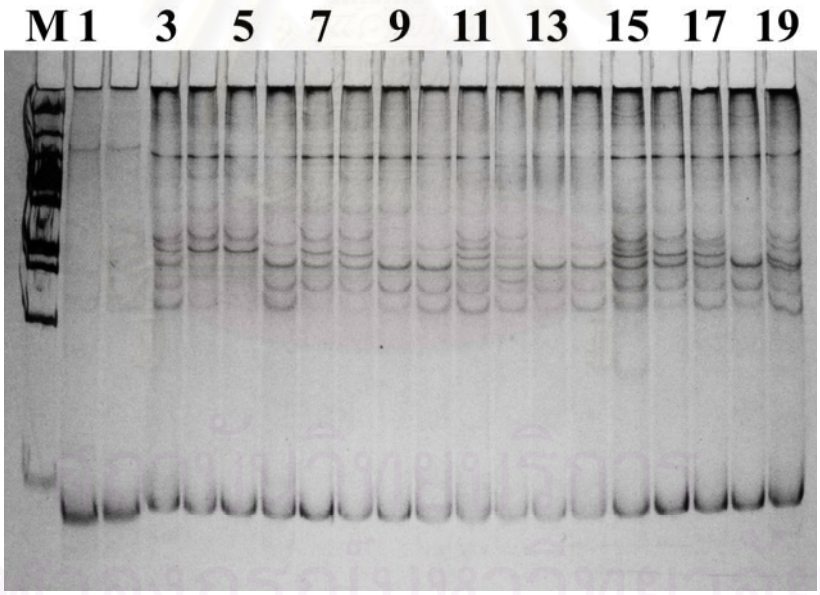

Figure 3.88 A 12.5% silver stained non-denaturing polyacylamide gel (37.5:1) showing SSCP patterns of male (lanes 3 – 11) and female (lanes 12 – 19) *M. rosenbergii* generated from SOC268/273RAP primer. Lanes M and 1 and 2 are a 100 bp DNA marker and the non-denatured PCR product (double strand DNA control), respectively.

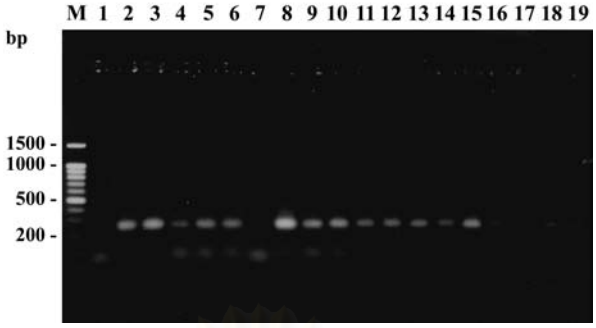

Figure 3.89 A 1.5% ethidium bromide stained agarose gel showing the PCR products of male (lanes 2 – 10) and female (lane 11 – 19) *M. rosenbergii* generated by BC428/273RAP primers. Lanes M and 1 are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

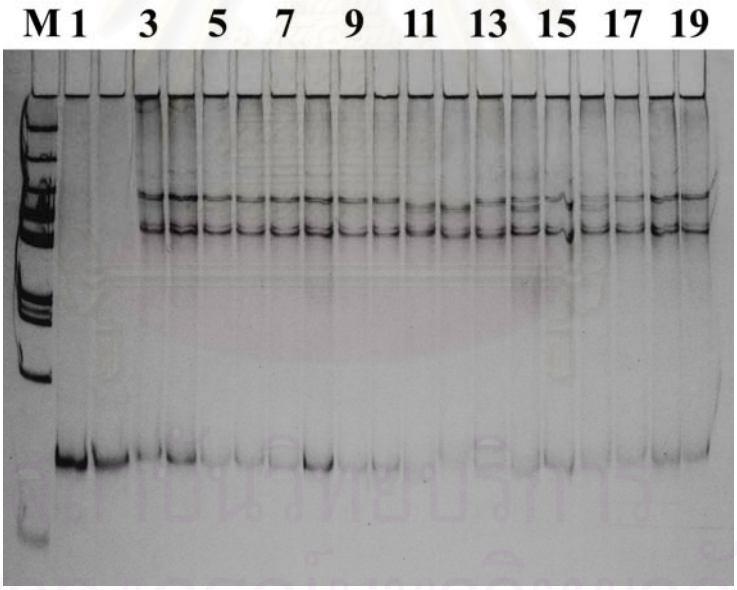

Figure 3.90 A 12.5% silver stained non-denaturing polyacylamide gel (37.5:1) showing SSCP patterns of male (lanes 3 – 10) and female (lanes 11 – 19) *M. rosenbergii* generated from BC428/273RAP primer. Lanes M and 1 and 2 are a 100 bp DNA marker and the non-denatured PCR product (double strand DNA control), respectively.

CHAPTER IV

DISCUSSION

Sex determination markers of *M. rosenbergii* cannot be isolated at the genomic DNA levels

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 markers located on the unique sex chromosomes is available. 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. When these markers are not available, sex-specific/differential expression markers have alternatively been studies in several species.

For species exhibiting well defined sex chromosomes, molecular approaches at the genomic levels for example, RAPD, AFLP and Genome-walk analysis, are useful for identification of sex-linked markers. The isolated marker can be converted to SCAR markers for direct application for sex determination purpose.

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

The giant freshwater prawn (*M. rosenbergii*) is particularly interesting cultured species with respect to variation in growth and the role of size in the social structure of populations. Both sexes have similar size frequency distributions in populations of immature prawns (Ra'anan and Cohen, 1985). As individuals mature, the size distribution becomes quite different for males and females. Mature females grow more slowly than males of similar sizes. Therefore, the basic knowledge about the sex determination/differentiation system in this species, if discovered, allow further applications leading to significant increase the production through all male (or the majority of male) culture.

To reduce tedious and time consuming processes, BSA is applied during the screening step. This was carried out by pooling an equal amount of DNA (or total RNA before subjected to the first strand cDNA synthesis for identification of sex-specific/differential expression markers) of several individuals of males and females of a particular species. After candidate markers are identified, markers can be screened individually or may be screened against more number of bulked DNA to confirm obtained results.

Several candidate male and female-specific AFLP markers were found in this study. According to the previous study on mating of neomales with normal females, the sex ratio of the offspring from each cross was biased towards the ZW/ZZ system. It was then expected that W-linked AFLP markers were of interest. Nevertheless, relatively large numbers of candidate sex-linked markers were found in both male and female *M. rosenbergii*. Therefore, five and four candidate male- and female-specific markers were chosen for subsequent characterization.

Generally, different clone types were obtained from a single insert. The number of designed primers varied between 1 – 3 primer pairs depending on different sequences found. No sex-specific SCAR markers were successfully developed as the markers generated the amplification product in both genders. Further characterization of SNP in the amplified gene segment revealed polymorphism in several AFLP-derived markers but none of them revealed sex-linked nature. Therefore, development of sex-specific SCAR markers at the gonomic DNA level of *M. rosenbergii* was not successful.

Polymorphic SSCP markers originally developed for sex differentiation purposes can be used for studies of genetic diversity and the construction of genetic linkage maps in this economically important species. $ME_{+3}3M_{+3}7425.1$ primers generated a single pattern in 3 female individuals from Chachoengsao province whereas this SSCP pattern was not found in the other female group originating from Ayuthaya province.

Currently, isolation and characterization of sex-diagnostic markers in *M. rosenbergii* are also carried out by the other research groups in Thailand. Mekdang (unpublished data) used 99 primer combinations of AFLP to isolate sex-linked AFLP markers in *M. rosenbergii*. Six AFLP fragments showing sex-specific nature in male and female *M. rosenbergii* identified. The markers were converted to SCAR markers by

cloning and sequencing and tested against genomic DNA of male and female *M. rosenbergii* individuals. However, sex-specificity did not retain in the converted markers.

In the parallel of this study, Thamrungtanakit (2004) combined bulked segregant analysis (BSA) and AFLP for isolation of sex-specific AFLP markers in *P. monodon*. Bulked genomic DNA from male (3 - 5 bulks) and female (3 – 5 bulks) were tested across 256 primer combinations (*Eco* RI_{1-16} – *Mse* I_{1-16}). The number of well characterized AFLP markers showing specificity to male and female *P. monodon* was 5 and 1 fragments, respectively. All candidate female-specific AFLP markers were cloned and characterized through sex-specific PCR and SSCP analysis. The developed markers were polymorphic but not related to sexes in *P. monodon*.

Candidate sex-specific AFLP fragments were usually obtained but SCAR markers converted from the AFLP fragment did not retain the original sex-specificity. The circumstances were observed in *M. rosenbergii* (this study and Mekdang, unpublished data), *P. monodon* (Leelatanawit, 2003; Thamrungtanakit, 2004) and *Haliotis asinina* (Amparyup, personal communication). On the one hand, isolated AFLP may have represented false positive markers in those species. On the other hand, specificity of sex-specific AFLP markers may be true but polymorphism of the positive AFLP markers and the orthologous fragment is existent only at one of the restriction cutting site (*Eco* RI or *Mse* I). As a result, amplification of the characterized fragment using newly designed primers from sequences of AFLP fragments would surely provide the amplification product in both genders.

To solve this possibility, PCR-based genome walk analysis should be further carried out. The primary application of 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. Initially, genomic DNA of interesting species is 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. The major PCR product begins with known sequence at the 5' (or 3') end of the GSP2 and extends into the unknown adjacent genomic DNA can be cloned and analyzed. A new primer pairs can be designed. The PCR product of different male and female individuals can be analyze by DNA sequencing.

Sex chromosomes have not been cytologically identified in *M. rosenbergii* and penaeid species. However, Li et al. (2003) constructed the genetic linkage map of the kuruma prawn (*P. 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 thee female map but not the paternal map leading to an interesting argument that female is the heterogametic sex in *P. 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 the female map of *P. japonicus* discovered by Li et al. (2004) may be resulted artifact AFLP bands.

Drew and Brindley (1995) found two female-specific sequences from *Schistosoma mansoni* by representational difference analysis (RDA), which is 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.

Li et al. (2002) collected the green spotted pufferfish (*Tetraodon nigroviridis*, *N* = 83) and used for identification of genomic sex markers using RAPD (600 primers and 1700 primers for the first and the second set of pooled DNA), AFLP (64 primer combinations) and representational difference analysis (RDA, 1 set of adaptors) methods. A total of 59, 126, 16 and 16 putative sex-specific markers were found after the primary screening. Nevertheless, secondary screening (re-testing of DNA from individuals for RAPD and AFLP and using the putative RDA markers as the probes for genomic Southern analysis of male and female DNA) did not demonstrate the presence of sex-specific marker in *T. nigroviridis.*

Sex-specific/differential expression markers were found in *M. rosenbergii*

Since the role of the androgenic gland in crustacean was discovered (Charniaux-Cotton, 1954), better understanding of sex-determining system in crustaceans was obtained through various experiments but the actual mechanism of sex differentiation in this group of animals has not been defined. In crustaceans, the generally accepted model for sex differentiation is that androgenic gland hormone (AGH) acts not only as a sex-determining factor but also as a sex-differentiating factor in males and female characteristics develop in the absence of this hormone (Sagi et al., 1997).

Sun et al. (2000) examined the total protein content and morphological characteristics of AG of three different male morphotypes (orange-claw, orange-blue-claw and blue-claw) of *M. rosenbergii*. Results from histological and transmission electron microscopy revealed at least 3 different cell types vary greatly among different male morphotypes. The BC morphotype has the highest cell density. Organization of the rough endoplasmic reticulum (RER) was also different between these males where RER in the BC is the most abundant, highly organized and formed into stacks in some areas implying the active capability of AG in the BC male. The highest protein content was found in the BC male (76 μg/AG) as compared to the OBC (45 μg/AG) and the OC (19 μ g/AG) morphotypes. SDS-PAGE analysis of the cell free extract of the AG from BC, OBC and OC revealed 4 major proteins (approximately 16, 18, 23 and 26 kDa) exhibiting quantitatively increase from the immature OC to the sexual mature BC morphotypes.

AGH has not been isolated and characterized in *M. rosenbergii*. Degenerate primes designed from 3 species of isopods did not provide the positive amplification product that can be further characterized by RACE-PCR (to obtain the complete cDNA of AGH in *M. rosenbergii*). Accordingly, candidate sex-specific AFLP markers of *M. rosenbergii* and sex-related transcripts previously isolated in *P. monodon* and *H. asinina* were studies by RT-PCR.

$ME_{+3}8M_{+3}1310.1$ primers generated 2 non-specific amplification fragments (500 bp and 700 bp) in AG (SOC, OC and BC males) but not in testes of all male morphotypes and oviducts and ovaries of female *M. rosenbergii*. After characterization, a 500 bp fragment was a homologue of the house keeping gene, β-actin whereas a 700 bp fragment was a homologue of sarco/endoplasmid reticulum Ca^{2+} ATPase (SERCA) initially isolated from the crayfish (*P. clarkii*). Higher expression levels were observed in AG of SOC, OC and BC male morphotype than in oviducts of female *M. rosenbergii*.

Zhang et al. (2000) characterized the muscle isoform (from axial abdominal muscle) of *P. clarkii* SERCA. The greatest expression level was found in the intermoult and the level of expression decreased around the ecdysis in response to either hormonal or mechanical stimuli.

Recently, Chen et al. (2002) cloned and functionally characterized the heart muscle isoform of SERCA from *P. clarkii*. The open reading frame (ORF) of this transcript was 3060 coding for a protein of 1020 amino acids. Expression of the cardiac muscle SERCA correlated with the molting stages for which high expression was found in the intermolt and decreased in the premolt. Nevertheless, the expression level was rapidly restored within 2 days after molting (postmolt). Therefore, correlation between the ovarian isoform of SERCA and molting and/or development of ovaries should be further examined.

RT-PCR of peritrophin revealed female-specific expression patterns in this gene homologue in ovaries but not in oviduct, AG and testes. However, the amplification product was approximately 600 bp which is larger than the expected size in the original target species (269 bp in *P. monodon*). Yamonto et al. (2004) isolated and characterized complete sequences of three closely related TSP homologue encoding proteins of 1114, 1032, and 991 amino acids (accession numbers AB121209, AB121210, and AB121211) with unknown functions in *P. japonicus* and reported that Peritrophin and TSP are possibly coded from the same locus but different proteins are generated through the alternative splicing of exons.

Peritrophin is highly expressed during oocyte development of marine shrimp. Two forms of transcripts, which have identical sizes of open reading frames (ORFs) (834 bp) encoding closely related proteins of 277 amino acids and exhibit nucleotide differences in the 3' UTR, were recently isolated from ovaries of *P. monodon* (accession numbers AF510331 and AF510332; http://www.ncbi.nlm.nih.gov). 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.

In contrast to the female-specific pattern in *M. rosenbergii*, non-differentially expression patterns of both forms of peritrophin were found in testes, ovaries, lymphoid organ and antennal ganglions of *P. monodon* (Leelatanawit et al., 2004). At present, sex-reversal of females to males is carried out in *M. rosenbergii* by eyestalk ablation and chemical treatment. The experiments require cultivation of the experimental prawn

reaching the stage that can be sex-differentiated morphologically. Therefore, expression patterns of peritrophin (expressed only in ovaries), SERCA (differentially expressed between AG and oviducts) and $ME_{+3}8M_{+3}1310.1$ (700 bp fragment in AG but not in testes, ovaries and oviducts) may be implemented to follow consequence effects of treatment for sex-reverse experiments.

Subtractive cDNA libraries of ovaries and testes of the giant tiger shrimp (*Penaeus monodon*) were constructed. A total of 218 clones (157 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% of total expressed sequence tags, ESTs), peritrophin (17 clones, 10.8%), and unknown transcripts (78 clones, 49.7%). Conversely, almost all of the ESTs in *P. monodon* testes were unknown transcripts (59 clones, 96.7%). Rapid amplification of cDNA ends–polymerase chain reaction (RACE–PCR) was carried out for further characterization of TSP. Homologues of elongation factor-2, oxidoreductase, peritrophin, transketolase, hypothetical protein FLJ23251, and sex-linked ENSANGP00000010123 and X-linked protein 1 (XNP-1) were also isolated. Gender-specific expression of candidate sex-linked gene homologues was examined by reverse transcriptase–polymerase chain reaction (RT-PCR). While XNP-1 and peritrophin were expressed in both ovaries and testes, TSP and ENSANGP00000010123 homologues revealed sex-specific expression in female *P. monodon* (Leelatanawit et al., 2004).

Normal and subtractive complementary DNA libraries were established from ovaries and testes of the tropical abalone (*H. asinina*). A total of 588 randomly selected clones were unidirectionally sequenced. Results indicated that 109 (54.5%) and 73 (61.9%) of normal cDNA libraries from *H. asinina* ovaries and testes significantly matched with known genes (E value $<10^{-4}$). Of these, vitelline coat proteins (VCPs; 40 clones, 20.0%) were predominant in the former library, but sperm lysin (9 clones, 7.6%) was the most abundant transcript in the latter library. For subtractive cDNA libraries, 71 clones (64.5%) and 56 clones (35.0%) of ovary (forward subtraction) and testis (reverse subtraction) libraries were known transcripts. Expression of sex-related genes was further examined using reverse transcriptase polymerase chain reaction. Gender-specific expression was found from homologues of VCP1, VCP3, VCP7, VCP49, and VTG1 and axonemal p66.0, tektin A1, and sperm lysin in female and male *H. asinina*, respectively.

Therefore, sex-specific markers in *H. asinina* were successfully developed at the cDNA level but not at the genomic DNA level (Amparyup et al., 2004).

Gender-specific gene expression has been reported in a mosquito-borne filarial nematode (*Brugia malayi*) isolated by differential display (DD) - PCR and in silico subtraction of EST cluster database and further confirmed by RT-PCR. Six (27%) of 12 and 7 (47%) of 15 initially identified by DD-PCR and in silico subtraction revealed gender-specific expression in that species (Michalksi 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 and 961 nucleotides of the female-specific cDNA (Ohbayashi et al., 2001).

RAP-PCR was applied for identification of sex-specific/differential expression markers in different male morphotypes and between male and female *M. rosenbergii*. The clear and attractive advantages of RAP-PCR are that very small amounts of total RNA can be used as starting materials. The second advantage is that the method is highly versatile in that a wide representation of differentially expressed gene fragments can be achieved for multiple conditions by visualization of side-by-side comparisons and that both upregulation and downregulation of gene expression can be detected.

The most disadvantage of RAP-PCR is that samples should be collected with caution because contamination of nucleic acids (both DNA and RNA) from other organisms can interfere the results by generating additional false positive of the desired fragments. Mathieu-Daude et al. (1996) reported that the candidate RAP-PCR marker is often a mixture of products of similar size that may provide false positive results. In addition, reamplification of the product may obviate differential expression of the original fragment from the original results.

Two interesting SOC- and female-specific RAP PCR fragments (340 bp and 315 bp, respectively) were found with all primer combinations of the cDNA-synthesizing primer and other 29 combined primers. Unfortunately, specific primers designed from those sequences did not provide the expected amplification products.

M122/159RAP.2 revealed obvious differentially expression patterns between male and female *M. rosenbergii*. Interestingly, expression patterns of SOC268/273RAP in AG, testes, oviducts and ovaries of *M. rosenbergii* implied the existence of 2 isoform of this

transcript possibly through alternative splicing of this gene region. Therefore, organization of this gene should be further characterized at the genomic DNA level. Patterns of BC428/273RAP expression require further characterization. It is interesting to examined this transcript against a large number of additional male and female *M. rosenbergii* to determine whether a larger amplified fragment found in ovaries and oviduct of *M. rosenbergii* is not artifact.

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 sexes, 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 juveniles 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*.

Like other previously examined aquatic species, sex-diagnostic markers was not successfully developed at the genomic DNA but markers obtained from the present study are still useful for other applications. SSCP analysis against genomic DNA of male and female individuals of *M. rosenbergii* revealed monomorphism for M122/159RAP.2 but polymorphic SSCP patterns was found for $ME_{+3}3M_{+3}7425.1$, $ME_{+3}3M_{+3}7425.2$, $ME_{+3}7M_{+3}6425$, $ME_{+3}4M_{+3}5570$, $ME_{+3}8M_{+3}1310.1$, $ME_{+3}4M_{+3}8517$, $FE_{+3}5M_{+3}1390$ and $FE_{+3}8M_{+3}6710$, SOC268/273RAP and BC428/273RAP.

The monomorphic AFLP- and RAP-PCR derived markers found in this study may be used for identification the species origin of *M. rosenbergii* and other closely related and morphologically similar *Macrobrachium* species. In contrast, the polymorphic SSCP markers can be directly applied for examination of genetic diversity and differentiation of natural stocks of *M. rosenbergii* in Thailand. Some markers showing clearly scored patterns and high polymorphism (e.g. SOC268/273RAP and BC428/273RAP can be applied for construction of the genetic linkage map in this species.

CHAPTER V

CONCLUSIONS

1. Five and four candidate male- and female-specific AFLP markers were identified from screening 64 primer combinations with 5 (SOC1, SOC2, OC1, BC1 and BC2) and 2 bulked (F1 and F2) genomic DNA of male and female *M. rosenbergii*, respectively.

2. SCAR markers developed from sex-specific AFLP fragment generated the positive amplification product in both males and females of *M. rosenbergii*. All SCAR markers except that derived from $FE_{+3}8M_{+3}270.2$ – F/R were polymorphic but their SSCP patterns did not show sex-specificity in *M. rosenbergii*.

3. A homologue of Sarco / endoplasmic reticulum Ca^2 + ATPase C (SERCA) were isolated from $ME_{+3}8M_{+3}1310.1$ primers ($E > 1 \times 10^{-103}$). An amplified SERCA (279 bp) clearly revealed differential expression patterns between AG of male and oviducts of female *M. rosenbergii*.

4. RT-PCR using primers developed from sex-related transcripts of related species (*P. monodon* and *H. asinina*) provided a female-specific expression marker (peritrophin), and a differential expression marker in the BC male (DSI). The sex-specific/differential expression markers can be used to follow the success of sex-reverse experiment in *M. rosenbergii*.

5. RAP-PCR analysis was also carried out to isolate various types of expression markers. In a total, 43 and 24 of male- and female-specific and 29 differential expression RAP-PCR markers were found. Additionally, 10, 12 and 13 RAP-PCR fragments exhibited morphotype-specific expression in BC, SOC and OC males were also isolated.

6. Two fragment generated from RAP-PCR were SOC-specific (340 bp) and female-specific (315 bp) when screened with combination of the first primers and all investigated second primers (30 primer combination) but conversion of these to SCAR markers was not successful.

7. The expression levels of M122/159RAP.2 (significantly similar to I–connectin mRNA of the crayfish, *P. clarkii*) clearly showed differential expression patterns in AG of males and oviducts of female *M. rosenbergii*

8. The amplification patterns of SOC268/273RAP and BC428/273RAP implied the possible existence of length polymorphism through alternative splicing. The former suggested differential expression between different isoforms in various tissues of *M. rosenbergii*. Nevertheless, the latter requires further characterization (e.g. sequencing of the amplified product) before considered as sex-specific length polymorphic markers in *M. rosenbergii*.

9. Polymorphic patterns were found from characterization of the SOC268/273RAP and BC428/273RAP products by SSCP analysis. SNP found in AFLP- and RAP-PCR derived markers can be used for other applications including construction of genetic linkage map and/or population genetic studies of natural populations of *M. rosenbergii*).

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APPENDICES

Appendix A

Chemicals for Preparation of Polyacrylamide Gels and Silver Staining

1. 4.5% Denaturing acrylamide solution, 500 ml

Acrylamide	21.375	g
Bis-aclylamide	1.125	g.
7 M urea	210	g.

2. 40% acrylamide solution (crosslink = 37.5:1), 500 ml

Acrylamide	194.80	g.
Bis-aclylamide	5.19	g.

3. 40% acrylamide solution (crosslink = 75:1), 200 ml

Acrylamide	78.94	g.
Bis – aclylamide	1.052	g.

4. Bind silane

95% Ethanol	995	µl
Bind silane	4	µl
Acetic acid	5	µl

5. Fix/stop solution (10% glacial acetic acid), 2 liters

Glacial acetic acid	200	ml
Ultrapure or deionized water	1800	ml

6. Staining solution, 1.5 liters

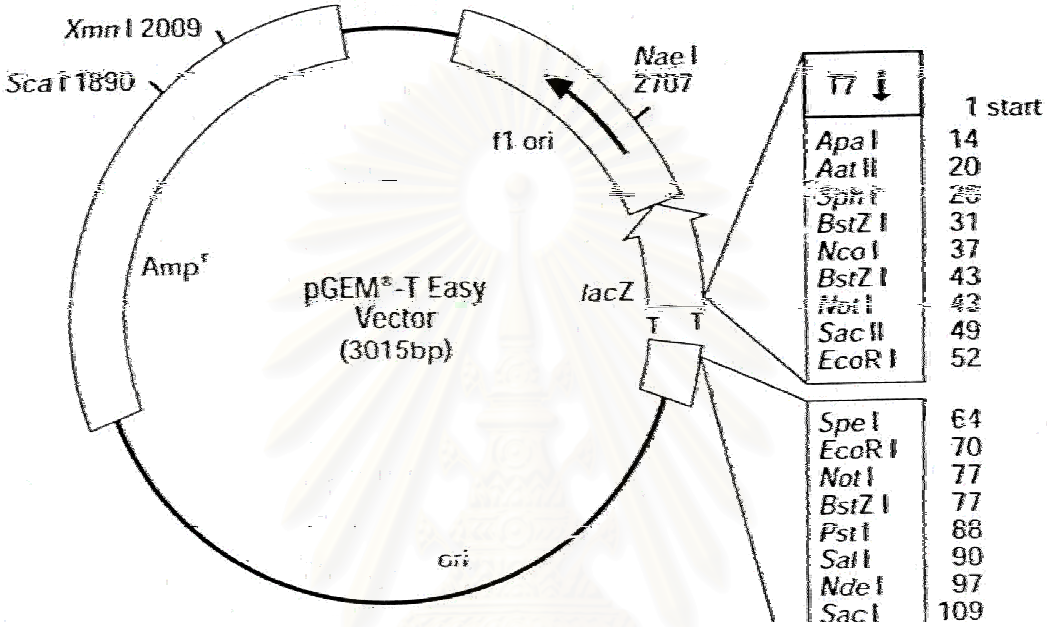
$AgNO_3$	1.5	g.
37% Formaldehyde	2.25	ml

7. Developing solution, 3 liters

Na_2CO_3	90	g.
37% Formaldehyde	5	ml
Sodium Thiosulfate (10mg/ml)	600	µl

Appendix B

Restriction mapping of pGEM® T-easy Vector



Appendix C

Data about specimens of the giant freshwater prawn (*M. rosenbergii*) used in this study

Samples	Collected Site	Total length (cm.)	Head length (cm.)	Body length (cm.)	Weight (g.)	Claw length (L,R) (cm.)	Relative claw length
AFLP analysis							
BC 001	Sam-yan Market	20.5	10.5	10.0	105.0	26.5,0	2.65
BC 002	Sam-yan Market	22.0	11.0	11.0	124.8	28.0,28.0	2.55
BC 003	Bangkla, Chachoengsao	21.5	12.5	9.0	170.3	28.5,30.0	3.33
BC 004	Bangkla, Chachoengsao	24.0	14.0	10.0	167.5	31.5,31.5	3.15
BC 005	Bangkla, Chachoengsao	24.5	12.5	12.0	191.7	35.0,35.0	191.7
BC 010	Sam-yan Market	21.0	10.5	10.5	102.5	29.0,0	2.76
BC 011	Sam-yan Market	20.0	10.0	10.0	110.1	27.0,27.0	2.70
BC 016	Sam-yan Market	18.0	8.0	10.0	101.3	24.5,24.5	2.45
BC 017	Sam-yan Market	20.0	10.5	9.5	108.5	28.5,24.5	3.00
BC 019	Sam-yan Market	20.0	10.5	9.5	112.6	28.5,27.5	2.95
BC 021	Ayuthaya	18.5	9.2	9.3	106.5	24.8,25.2	2.70
BC 022	Ayuthaya	18.9	9.5	9.4	92.2	25.1,24.6	2.64
BC 024	Ayuthaya	17.4	8.6	8.8	87.0	24.8,24.8	2.82
BC 025	Ayuthaya	18.5	9.2	9.3	94.4	26.0,25.0	2.74
BC 027	Ayuthaya	18.8	9.6	9.2	105.3	24.5,25.5	2.72
OC 001	Sam-yan Market	19.5	10.0	9.5	-	17.0,0	1.79
OC 002	Sam-yan Market	20.0	9.5	10.5	-	18.5,17.5	1.71
OC 003	Sam-yan Market	21.0	9.5	11.5	99.6	17.5,18.0	1.54
OC 004	Sam-yan Market	21.0	10.5	10.5	106.1	19.0,17.0	1.81
OC 005	Sam-yan Market	22.5	11.0	11.5	116.4	20.0,20.0	1.74
OC 006	Sam-yan Market	21.5	10.5	11.0	108.7	19.0,19.0	1.73

Samples	Collected Site	Total length (cm.)	Head length (cm.)	Body length (cm.)	Weight (g.)	Claw length (L,R) (cm.)	Relative claw length
OC 007	Bangkla, Chachoengsao	24.5	12.5	12.0	-	20.0,0	1.67
OC 008	Bangkla, Chachoengsao	22.0	12.0	10.0	102.9	18.5,20.0	2.00
OC 009	Bangkla, Chachoengsao	21.5	11.5	10.0	103.0	20.0,19.5	1.98
OC 010	Bangkla, Chachoengsao	21.0	11.5	9.5	85.4	19.5,18.5	2.00
OC 012	Sam-yan Market	20.6	10.2	10.4	101.3	20.5,20.0	1.95
OC 017	Sam-yan Market	20.0	9.5	10.5	97.5	20.5,20.5	1.95
OC 018	Sam-yan Market	18.5	9.0	9.5	83.8	15.5,15.5	1.63
OC 019	Sam-yan Market	19.5	10.0	9.5	90.7	18.0,17.0	1.84
OC 021	Sam-yan Market	20.0	10.0	10.0	85.0	15.0,17.5	1.75
OC 022	Sam-yan Market	21.5	11.0	10.5	110.0	18.0,18.0	1.71
OC 024	Sam-yan Market	20.5	10.2	10.3	89.4	18.7,18.0	1.78
OC 025	Sam-yan Market	19.0	10.0	9.0	82.1	17.0,17.5	1.92
OC 026	Sam-yan Market	18.0	9.0	9.0	71.4	15.5,15.5	1.72
OC 029	Sam-yan Market	20.5	10.8	9.7	91.4	18.0,18.0	1.86
OC 030	Sam-yan Market	19.5	9.8	9.7	84.8	16.0,15.0	1.60
OC 031	Ayuthaya	19.3	9.0	10.3	78.9	17.5,17.5	1.70
OC 032	Ayuthaya	18.5	9.2	9.3	87.3	16.9,16.9	1.82
OC 034	Ayuthaya	19.5	9.8	9.7	90.0	15.9,15.9	1.64
OC 036	Ayuthaya	19.5	9.5	10.0	88.9	16.5,16.5	1.65
SOC 016	Ayuthaya	17.5	8.2	9.3	59.0	14.0,13.5	1.48
SOC 018	Ayuthaya	17.5	8.3	9.2	62.8	13.7,13.7	1.49
SOC 019	Ayuthaya	14.6	7.5	7.1	35.1	10.0,9.9	1.40
SOC 023	Ayuthaya	16.9	8.0	8.9	48.3	11.1,12.9	1.45
SOC 024	Ayuthaya	15.1	7.5	7.6	36.1	8.3,11.4	1.50
Fe 001	Bangkla, Chachoengsao	23.0	11.0	12.0	140.9	20.5,17.5	1.71

Samples	Collected Site	Total length (cm.)	Head length (cm.)	Body length (cm.)	Weight (g.)	Claw length (L,R) (cm.)	Relative claw length
Fe 003	Bangkla, Chachoengsao	18.0	10.0	8.0	39.6	11.5,12.5	1.50
Fe 004	Bangkla, Chachoengsao	17.0	9.0	8.0	31.7	10.0,10.0	1.25
Fe 005	Bangkla, Chachoengsao	19.0	10.0	9.0	47.8	7.5,7.5	0.83
Fe 006	Bangkla, Chachoengsao	20.0	9.5	10.5	79.6	15.0,14.0	1.38
Fe 007	Bangkla, Chachoengsao	18.5	9.0	9.5	45.0	11.5,11.0	1.18
Fe 008	Bangkla, Chachoengsao	16.5	8.5	8.0	27.3	8.5,8.5	1.06
Fe 009	Bangkla, Chachoengsao	17.0	8.5	8.5	34.0	7.5,0	0.88
Fe 010	Bangkla, Chachoengsao	15.5	8.0	7.5	32.7	9.5,0	1.27
Fe 011	Bangkla, Chachoengsao	16.0	8.0	8.0	30.7	9.0,7.0	1.13
Fe 012	Chachoengsao	15.0	7.0	8.0	23.9	9.0,8.0	1.06
Fe 013	Chachoengsao	14.5	7.0	7.5	26.5	8.0,8.0	1.07
Fe 014	Chachoengsao	13.0	6.0	7.0	19.0	7.5,0	1.07
Fe 015	Chachoengsao	13.5	6.0	7.5	20.2	9.5,0	1.27
Fe 016	Chachoengsao	15.0	7.0	8.0	23.5	8.0,0	1.00
Fe 017	Chachoengsao	14.0	6.5	7.5	21.8	8.5,6.5	1.13
Fe 018	Chachoengsao	16.0	7.0	9.0	29.5	11.5,8.5	1.28
Fe 019	Chachoengsao	12.5	5.5	7.0	14.6	6.5,6.5	0.93
Fe 021	Chachoengsao	13.0	6.0	7.0	15.7	6.5,6.5	0.93
Fe 022	Chachoengsao	14.5	6.5	8.0	18.7	7.0,6.0	0.81
Fe 024	Chachoengsao	14.0	6.0	8.0	17.9	7.0,0	0.88
Fe 025	Chachoengsao	15.0	6.5	8.5	22.5	7.5,8.0	0.91
Fe 028	Chachoengsao	11.5	5.0	6.5	13.0	7.5,7.5	0.65
Fe 029	Chachoengsao	12.0	6.0	6.0	18.9	7.5,0	1.20

Samples	Collected Site	Total length (cm.)	Head length (cm.)	Body length (cm.)	Weight (g.)	Claw length (L,R) (cm.)	Relative claw length
Fe 047	Ayuthaya	13.7	6.0	7.7	32.6	10.0,0	1.30
Fe 048	Ayuthaya	15.3	7.3	8.0	34.7	10.3,8.0	1.29
Fe 049	Ayuthaya	14.0	5.7	8.3	34.2	10.1,10	1.22
Fe 050	Ayuthaya	15.0	7.2	7.8	34.0	8.9,6.8	1.14
Fe 053	Ayuthaya	14.9	6.9	8.0	33.3	8.8,9.7	1.16
Fe 054	Ayuthaya	14.4	7.0	7.4	27.5	8.4,0	1.14
RAP-PCR analysis							
BC 020	Ayuthaya	17.8	9.0	8.8	94.7	21.5,25.5	2.90
BC 021	Ayuthaya	18.5	9.2	9.3	106.5	24.8,25.2	2.70
BC 022	Ayuthaya	18.9	9.5	9.4	92.2	25.1,24.6	2.64
OC 032	Ayuthaya	18.5	9.2	9.3	87.3	16.9,16.9	1.82
OC 034	Ayuthaya	19.5	9.8	9.7	90.0	15.9,15.9	1.64
OC 036	Ayuthaya	19.5	9.5	10.0	88.9	16.5,16.5	1.65
SOC 016	Ayuthaya	17.5	8.2	9.3	59.0	14.0,13.5	1.48
SOC 018	Ayuthaya	17.5	8.3	9.2	62.8	13.7,13.7	1.49
SOC 019	Ayuthaya	14.6	7.5	7.1	35.1	10.0,9.9	1.40
Fe 047	Ayuthaya	13.7	6.0	7.7	32.6	10.0,0	1.30
Fe 048	Ayuthaya	15.3	7.3	8.0	34.7	10.3,8.0	1.29
Fe 049	Ayuthaya	14.0	5.7	8.3	34.2	10.1,0	1.22
RT-PCR analysis							
Androgenic gland tissues							
BC 020	Ayuthaya	17.8	9.0	8.8	94.7	21.5,25.5	2.90
BC 021	Ayuthaya	18.5	9.2	9.3	106.5	24.8,25.2	2.70
BC 022	Ayuthaya	18.9	9.5	9.4	92.2	25.1,24.6	2.64
BC 029	Ayuthaya	21.0	11.0	10.0	147.5	32.3,30.0	3.23
BC 031	Ayuthaya	19.5	10.5	9.0	121.9	27.3,26.2	2.97
BC 076	Ayuthaya	16.5	8.5	8.0	50.0	23.0,23.5	2.90

Samples	Collected Site	Total length (cm.)	Head length (cm.)	Body length (cm.)	Weight (g.)	Claw length (L,R) (cm.)	Relative claw length
BC 080	Ayuthaya	19.5	9.2	10.3	70.0	26.0,25.5	2.50
OC 032	Ayuthaya	18.5	9.2	9.3	87.3	16.9,16.9	1.82
OC 034	Ayuthaya	19.5	9.8	9.7	90.0	15.9,15.9	1.64
OC 036	Ayuthaya	19.5	9.5	10.0	88.9	16.5,16.5	1.65
OC 059	Ayuthaya	17.0	9.0	8.0	59.6	13.4,13.4	1.68
OC 060	Ayuthaya	17.0	8.5	8.5	61.8	16.0,15.8	1.88
OC 074	Ayuthaya	19.3	8.3	11.0	55.0	17.0,15.7	1.60
OC 078	Ayuthaya	19.0	8.7	10.3	55.0	17.5,15.8	1.70
SOC 026	Ayuthaya	12.0	6.0	6.0	17.3	8.3,8.3	1.38
SOC 027	Ayuthaya	12.0	5.5	6.5	16.0	7.5,7.5	1.15
SOC 028	Ayuthaya	14.0	7.0	7.0	29.1	10.0,11.0	1.50
SOC 033	Ayuthaya	15.5	7.0	8.5	30.0	6.8,7.0	0.81
SOC 034	Ayuthaya	14.3	6.7	7.6	25.0	8.5,7.0	1.12
Oviducts tissues							
Fe 060	Ayuthaya	15.0	7.0	8.0	29.9	9.5,9.0	1.19
Fe 061	Ayuthaya	16.0	8.0	8.0	35.9	10.5,0	1.31
Fe 065	Ayuthaya	16.0	8.0	8.0	35.0	10.5,10.5	1.31
Fe 066	Ayuthaya	15.5	8.0	7.5	35.8	11.0,9.5	1.47
Fe 069	Ayuthaya	14.0	7.0	7.0	28.6	8.5,8.5	1.21
Fe 072	Ayuthaya	14.5	7.0	7.5	28.8	10.0,9.0	1.33
Fe 078	Ayuthaya	13.0	6.5	6.5	19.2	7.5,0	1.15
Fe 080	Ayuthaya	16.0	7.5	8.5	35.5	9.0,0	1.06
Fe 082	Ayuthaya	13.0	6.0	7.0	19.4	7.5,0	1.07
Fe 124	Ayuthaya	15.4	7.2	8.2	41.8	11.5,10.5	1.40
Fe 125	Ayuthaya	16.1	8.6	7.5	40.3	11.3,0	1.51
Fe 126	Ayuthaya	15.7	7.4	8.3	34.7	10.5,10.5	1.27
Fe 136	Ayuthaya	16.0	7.0	9.0	30.0	9.2,9.2	1.02
Fe 140	Ayuthaya	14.3	6.8	7.5	20.0	7.0,8.5	1.13

Samples	Collected Site	Total length (cm.)	Head length (cm.)	Body length (cm.)	Weight (g.)	Claw length (L,R) (cm.)	Relative claw length
Testes tissues							
BC 029	Ayuthaya	21.0	11.0	10.0	147.5	32.3,30.0	3.23
BC 031	Ayuthaya	19.5	10.5	9.0	121.9	27.3,26.2	2.97
OC 059	Ayuthaya	17.0	9.0	8.0	59.6	13.4,13.4	1.68
OC 060	Ayuthaya	17.0	8.5	8.5	61.8	16.0,15.8	1.88
SOC 026	Ayuthaya	12.0	6.0	6.0	17.3	8.3,8.3	1.38
SOC 027	Ayuthaya	12.0	5.5	6.5	16.0	7.5,7.5	1.15
Ovaries tissues							
Fe 124	Ayuthaya	15.4	7.2	8.2	41.8	11.5,10.5	1.40
Fe 125	Ayuthaya	16.1	8.6	7.5	40.3	11.3,0	1.51
Fe 126	Ayuthaya	15.7	7.4	8.3	34.7	10.5,10.5	1.27

Appendix D

A summary of candidate AFLP fragments exhibited sex- and morphotype-specific markers in the giant freshwater prawn (*M. rosenbergii*) generated from different primer combinations

Primer combination	Size (bp)	First sample set			Second sample set			
		BC1	SOC1	Fe1	BC2	OC1	SOC2	Fe2
Eco RI_1 / *Mse* I_1	600	+	+	-				
	1,000	+	+	-				
Eco RI_1 / *Mse* I_2	255	+	-	-				
	285	+	-	-				
	275	-	+	-				
	295	-	-	+				
Eco RI_1 / *Mse* I_5	350	-	+	-				
	480	+	+	-	+	+	+	-
Eco RI_1 / *Mse* I_6	500	+	-	-				
	715	+	-	-	+	-	-	-
	750	-	+	-	-	-	+	-
	490	+	+	-	+	+	+	-
Eco RI_1 / *Mse* I_7	330	+	-	-				
	385	+	-	-				
	570	+	-	-				
	610	+	-	-				
	295	-	+	-				
	510	-	+	-				
	675	-	+	-				
	290*	**-**	**-**	**+**				
	325	-	-	+				
	625	-	-	+				
	375	-	-	-	-	+	-	-
	290*	**-**	**-**	**+**	**-**	**-**	**-**	**+**
	655	-	-	+	-	-	-	+
	660	-	-	+	-	-	-	+
Eco RI_1 / *Mse* I_8	280	+	-	-				
	370	+	-	-				
	455	+	-	-				
	480	+	-	-				
	750	+	-	-				
	285	-	+	-				
	370	-	+	-				
	440	-	+	-				
	475	-	+	-				
	750	-	+	-				
	300	+	+	-				
	335	+	+	-				
	340	-	-	+				
	595	-	-	+				
	324	+	-	-	+	-	-	-
	517	+	-	-	+	-	-	-
	325	-	+	-	-	-	+	-
Eco RI_2 / *Mse* I_1	270	+	-	-				

Primer combination	Size (bp)	First sample set			Second sample set			
		BC1	SOC1	Fe1	BC2	OC1	SOC2	Fe2
	470	+	-	-				
	500	+	-	-				
	275	-	+	-				
	475	+	+	-	+	+	+	-
Eco RI_2 / *Mse* I_2	345	+	-	-				
	340	-	+	-				
	350	-	+	-				
	355	-	+	-				
	335	+	-	-	+	-	-	-
Eco RI_2 / *Mse* I_4	400	+	+	-				
	600	+	+	-				
	415	-	-	-	-	+	-	-
	520	-	-	-	-	+	-	-
	700	-	-	+	-	-	-	+
	700	-	-	+	-	-	-	+
Eco RI_2 / *Mse* I_6	260	+	-	-				
Eco RI_2 / *Mse* I_7	275	+	-	-				
	410	+	-	-				
	530	+	-	-				
	310	-	+	-				
	375	-	+	-				
	600	-	+	-				
	550	+	+	-				
Eco RI_2 / *Mse* I_8	375	+	-	-				
	575	+	-	-				
	725	+	-	-				
	840	+	-	-				
	295	-	+	-				
	300	-	+	-				
	310	-	+	-				
	650	-	+	-				
	655	-	+	-				
	720	-	+	-				
	830	-	+	-				
	830	-	+	-				
	315	+	+	-				
	340	+	+	-				
	380	+	+	-				
	480	+	+	-				
	510	+	+	-				
	625	+	+	-				
	640	+	+	-				
	775	+	+	-				
	340	-	-	+				
Eco RI_3 / *Mse* I_2	650	-	+	-				
	345	+	+	-				
	340	-	-	+				
	700	-	-	+				
	1,000	-	-	+				
	365	-	-	+	-	-	-	+
Eco RI_3 / *Mse* I_3	315	+	-	-				
	395	+	-	-				
	325	+	+	-				

Primer combination	Size (bp)	First sample set			Second sample set			
		BC1	SOC1	Fe1	BC2	OC1	SOC2	Fe2
Eco RI_3 / *Mse* I_5	275	-	+	-				
	500	-	+	-				
	900	-	+	-				
	600	+	+	-				
	700	+	+	-				
	800	-	-	+				
	1,100	-	-	+				
	455	-	-	-	-	+	-	-
	492	-	-	+	-	-	-	+
Eco RI_3 / *Mse* I_6	475	-	+	-				
	500	-	+	-				
	1,500	+	+	-				
	475	-	-	+	-	-	-	+
Eco RI_3 / *Mse* I_7	285	+	-	-				
	475	+	-	-				
	600	+	-	-				
	517	-	+	-				
	425*	**+**	**+**	**-**				
	325	-	-	+				
	460	-	-	+				
	495	-	-	+				
	500	-	-	+				
	510	-	-	+				
	545	-	-	+				
	550	-	-	+				
	552	-	-	+				
	425*	**+**	**+**	**-**	**+**	**+**	**+**	**-**
Eco RI_3 / *Mse* I_8	295	+	-	-				
	310	+	-	-				
	368	+	-	-				
	500	+	-	-				
	580	+	-	-				
	645	+	-	-				
	308	-	+	-				
	370	-	+	-				
	420	-	+	-				
	421	-	+	-				
	650	-	+	-				
	320	+	+	-				
	335	+	+	-				
	470	+	+	-				
	540	+	+	-				
	640	+	+	-				
	350	-	-	+				
	520	+	+	-	+	+	+	-
Eco RI_4 / *Mse* I_1	310	+	+	-				
Eco RI_4 / *Mse* I_2	250	-	+	-				
Eco RI_4 / *Mse* I_3	400	-	+	-				
	450	-	+	-				
	600	-	+	-				
	595	+	+	-				
	605	-	-	+				
Eco RI_4 / *Mse* I_4	350	+	+	-				

Primer combination	Size (bp)	First sample set			Second sample set			
		BC1	SOC1	Fe1	BC2	OC1	SOC2	Fe2
	355	+	+	-				
	485	-	-	-	-	+	-	-
Eco RI_4 / *Mse* I_5	260	+	-	-				
	450	-	+	-				
	700	-	+	-				
	750	-	+	-				
	800	+	+	-				
	570*	**+**	**+**	**-**	**+**	**+**	**+**	**-**
Eco RI_4 / *Mse* I_6	750	+	-	-				
	260	-	+	-				
	600	-	+	-				
	700	-	+	-				
	800	+	+	-				
Eco RI_4 / *Mse* I_7	280	+	-	-				
	310	+	-	-				
	350	+	-	-				
	440	+	-	-				
	540	+	-	-				
	260	-	+	-				
	280	-	+	-				
	625	-	+	-				
	810	-	+	-				
	325	+	+	-				
	305	-	-	+				
	624	-	-	+				
	590	-	-	-	-	+	-	-
Eco RI_4 / *Mse* I_8	290	+	-	-				
	295	+	-	-				
	340	+	-	-				
	360	+	-	-				
	370	+	-	-				
	454	+	-	-				
	695	+	-	-				
	720	+	-	-				
	299	-	+	-				
	365	-	+	-				
	370	-	+	-				
	375	-	+	-				
	400	-	+	-				
	452	-	+	-				
	590	-	+	-				
	517*	**+**	**+**	**-**				
	300	-	-	+				
	353	-	-	+				
	355	-	-	+				
	415	-	-	+				
	517*	**+**	**+**	**-**	**+**	**+**	**+**	**-**
Eco RI_5 / *Mse* I_1	253	-	+	-				
	280	-	+	-				
	322	-	+	-				
	325	-	+	-				
	352	-	+	-				
	353	-	+	-				

Primer combination	Size (bp)	First sample set			Second sample set			
		BC1	SOC1	Fe1	BC2	OC1	SOC2	Fe2
	356	-	+	-				
	357	-	+	-				
	358	-	+	-				
	369	-	+	-				
	394	-	+	-				
	397	-	+	-				
	800	-	+	-				
	354	-	-	+				
	370	-	-	+				
	390*	**-**	**-**	**+**				
	403	-	-	+				
	390*	**-**	**-**	**+**	-	-	-	+
Eco RI_5 / *Mse* I_4	285	+	-	-				
	352	+	-	-				
	355	+	-	-				
	420	+	-	-				
	710	+	-	-				
	712	+	-	-				
	307	-	+	-				
	335	-	+	-				
	353	-	+	-				
	383	-	+	-				
	385	-	+	-				
	415	-	+	-				
	495	-	+	-				
	425	+	+	-				
	445	+	+	-				
	545	+	+	-				
	575	+	+	-				
	380	-	-	+				
	452	-	-	+				
	550	-	-	+				
Eco RI_5 / *Mse* I_5	850	+	-	-				
	1,250	+	-	-				
	410	+	+	-				
	550	+	+	-				
Eco RI_5 / *Mse* I_7	290	+	-	-				
	350	+	-	-				
	392	+	-	-				
	396	+	-	-				
	530	+	-	-				
	650	+	+	-	+	+	+	-
	652	+	+	-	+	+	+	-
	900	-	-	+	-	-	-	+
	902	-	-	+	-	-	-	+
Eco RI_5 / *Mse* I_8	275	+	-	-				
	325	+	-	-				
	327	+	-	-				
	340	+	-	-				
	385	+	-	-				
	550	+	-	-				
	552	+	-	-				
	274	-	+	-				

Primer combination	Size (bp)	First sample set			Second sample set			
		BC1	SOC1	Fe1	BC2	OC1	SOC2	Fe2
	288	-	+	-				
	490	+	+	-				
	492	+	+	-				
	400	-	-	+				
	640	-	-	+				
	398	+	+	-	+	+	+	-
	400	+	+	-	+	+	+	-
Eco RI_6 / *Mse* I_4	300	+	-	-				
	600	+	-	-				
Eco RI_6 / *Mse* I_5	260	+	-	-				
Eco RI_6 / *Mse* I_7	260	-	-	+				
Eco RI_6 / *Mse* I_8	270	+	-	-				
	470	+	-	-				
	472	+	-	-				
	325	-	+	-				
	338	-	+	-				
	340	-	+	-				
	360	-	+	-				
	370	-	+	-				
	602	-	+	-				
	603	-	+	-				
	309	+	+	-				
	310	+	+	-				
	348	+	+	-				
	350	+	+	-				
	640	+	+	-				
	322	-	-	+				
Eco RI_7 / *Mse* I_1	425	+	-	-				
	450	+	-	-				
	455	+	-	-				
	470	-	+	-				
	700	-	+	-				
	850	-	+	-				
	725	+	+	-				
	360	+	+	-	+	+	+	-
Eco RI_7 / *Mse* I_2	330	-	+	-				
	375	-	+	-				
	387	-	+	-				
	405	-	+	-				
	415	-	+	-				
	355	+	+	-				
	385	+	+	-				
	440	+	+	-				
	675	+	+	-				
	900	+	+	-				
	700	-	-	+				
	450	+	+	-	+	+	+	-
Eco RI_7 / *Mse* I_3	306	+	-	-				
	375	+	-	-				
	307	-	+	-				
	310	-	+	-				
	330	+	+	-				
	320	+	+	-				

Primer combination	Size (bp)	First sample set			Second sample set			
		BC1	SOC1	Fe1	BC2	OC1	SOC2	Fe2
Eco RI_7 / *Mse* I_4	680	+	-	-				
	360	-	+	-				
	650	-	+	-				
	325	+	+	-				
	510	+	+	-				
	600	+	+	-				
	330	-	-	+				
Eco RI_7 / *Mse* I_5	322	+	-	-				
	325	-	+	-				
	415	-	+	-				
	475	+	+	-				
	302	-	-	+				
Eco RI_7 / *Mse* I_6	325	-	+	-				
	315	+	+	-				
	345	+	+	-				
	365	+	+	-				
	425*	**+**	**+**	**-**				
	497	+	+	-				
	370	+	+	-	+	+	+	-
	372	+	+	-	+	+	+	-
	425*	**+**	**+**	**-**	**+**	**+**	**+**	**-**
Eco RI_7 / *Mse* I_7	287	+	-	-				
	291	+	-	-				
	310	+	-	-				
	345	+	-	-				
	470	+	-	-				
	278	-	+	-				
	286	-	+	-				
	290	-	+	-				
	445	-	+	-				
	450	-	+	-				
	500	-	+	-				
	625	-	+	-				
	422	+	+	-				
	700	+	+	-				
Eco RI_7 / *Mse* I_8	435	+	-	-				
	459	+	-	-				
	460	+	-	-				
	488	+	-	-				
	489	+	-	-				
	490	+	-	-				
	497	+	-	-				
	498	+	-	-				
	290	-	+	-				
	317	-	+	-				
	432	-	+	-				
	433	-	+	-				
	449	-	+	-				
	450	-	+	-				
	510	-	+	-				
	516	-	+	-				
	517	-	+	-				
	520	-	+	-				

Primer combination	Size (bp)	First sample set			Second sample set			
		BC1	SOC1	Fe1	BC2	OC1	SOC2	Fe2
	585	-	+	-				
	660	-	+	-				
	700	-	+	-				
	305	+	+	-				
	350	+	+	-				
	365	+	+	-				
	410	+	+	-				
	550	+	+	-				
	310	-	-	+				
Eco RI_8 / *Mse* I_1	**310***	**+**	**+**	**-**				
	445	+	+	-				
	385	+	-	-	+	-	-	-
	310*	**+**	**+**	**-**	**+**	**+**	**+**	**-**
Eco RI_8 / *Mse* I_2	305	+	-	-				
	302	-	+	-				
	415	-	+	-				
	820	-	+	-				
	824	-	+	-				
	315	+	+	-				
	475	+	+	-				
	360	+	+	-	+	+	+	-
	450	+	+	-	+	+	+	-
Eco RI_8 / *Mse* I_3	380	+	+	-				
	517	+	+	-				
	580	+	+	-				
	270*	**-**	**-**	**+**	**-**	**-**	**-**	**+**
Eco RI_8 / *Mse* I_4	330	+	-	-				
	255	-	+	-				
	350	+	+	-				
	335	-	-	+				
Eco RI_8 / *Mse* I_5	276	+	-	-				
	280	+	-	-				
	273	-	+	-				
	278	-	+	-				
	397	-	+	-				
	260	+	+	-				
	350	+	+	-				
	395	+	+	-				
Eco RI_8 / *Mse* I_6	255	+	-	-				
	245	-	+	-				
	375	+	+	-				
	300	+	+	-	+	+	+	-
	710*	**-**	**-**	**+**	**-**	**-**	**-**	**+**
Eco RI_8 / *Mse* I_7	257	+	-	-				
	267	+	-	-				
	275	+	-	-				
	280	+	-	-				
	303	+	-	-				
	305	+	-	-				
	315	+	-	-				
	323	+	-	-				
	340	+	-	-				
	349	+	-	-				

Primer combination	Size (bp)	First sample set			Second sample set			
		BC1	SOC1	Fe1	BC2	OC1	SOC2	Fe2
	350	+	-	-				
	374	+	-	-				
	375	+	-	-				
	450	+	-	-				
	490	+	-	-				
	517	+	-	-				
	650	+	-	-				
	725	+	-	-				
	255	-	+	-				
	265	-	+	-				
	272	-	+	-				
	337	-	+	-				
	342	-	+	-				
	385	-	+	-				
	390	-	+	-				
	435	-	+	-				
	440	-	+	-				
	775	-	+	-				
	800	-	+	-				
	360	+	+	-				
	460	+	+	-				
	590	+	+	-				
Eco RI_8 / *Mse* I_8	270	+	-	-				
	364	+	-	-				
	365	+	-	-				
	374	+	-	-				
	377	+	-	-				
	378	+	-	-				
	408	+	-	-				
	428	+	-	-				
	448	+	-	-				
	480	+	-	-				
	352	-	+	-				
	575	-	+	-				
	315	+	+	-				
	350	+	+	-				
	373	+	+	-				
	410	+	+	-				
	450	+	+	-				

* = The AFLP markers that were further characterized by cloning and sequencing and converted to SCAR markers

Appendix E

I. RAP-PCR fragment exhibits sex- and morphotype-specific transcripts in each primer combinations

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
UBC 268 – UBC 101					
280	+++	+	+	+	DS
300	++	++	++	-	DM
517	++	++	++	+	DS
1,000	++	+	+	-	DM
UBC 268 – UBC 119					
200	-	+	-	-	OC
520	+++	+++	+++	+	DS
685	++	++	++	-	M
UBC 268 – UBC 122					
230	+++	+++	+++	-	M
240	++	-	-	-	BC
250	-	-	-	+	F
440	++	-	-	-	BC
560	-	-	++	-	SOC
690	+	+	+++	+	DS
UBC 268 – UBC 128					
220	++	++	++	-	M
225	++	++	++	-	M
280	+++	+++	+++	-	M
685	++	++	++	-	M
UBC 268 – UBC 135					
310	+	+	+	-	M
375	++	++	++	+	DS
455	+	+	+	-	M
460	-	++	-	-	OC
UBC 268 – UBC 138					
320	+	++	-	-	DM
UBC 268 – UBC 158					
275	+	++	+	-	DM
525	+++	-	+	-	DM
690	+	+	+	-	M
690	+	+	+	-	M
UBC 268 – UBC 159					
355	+	+	+	-	M
365	+	-	-	-	BC
450	+	+	+	++	DS
695	+	+	+	-	M
700	-	-	-	+	F
UBC 268 – UBC 169					
270	++	++	+	-	DM
273	++	++	+	-	DM

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
300	-	-	+	-	SOC
325	+	+	+	-	M
460	++	++	++	-	M
690	++	++	-	-	DM
UBC 268 – UBC 174					
490	+	-	++	-	DM
UBC 268 – UBC 191				-	
600	+	+	++	-	DM
603	+	+	++	-	DM
785	+	+	+	-	M
UBC 268 – UBC 217					
220	++	++	++	-	M
420	-	-	+	-	SOC
UBC 268 – UBC 222					
270	+	-	-	-	BC
295	+	+	+	-	M
UBC 268 – UBC 228					
495	+	++	++	-	DM
UBC 268 – UBC 268					
230	+	+	+	-	M
270	++	++	++	-	M
275	++	++	++	-	M
UBC 268 – UBC 273					
250	+	+	+	-	M
500	-	-	++	-	SOC
UBC 268 – UBC 299					
290	-	-	-	+	F
425	+	++	+	+	DS
460	+	+	+	-	M
510	+	+	-	-	DM
910	++	+	+	-	DM
950	+	+	-	-	DM
1,050	+	+	+	-	M
1,500	+	+	+	-	M
UBC 268 – UBC 428					
275	+	+	+	-	M
320	+	++	+	-	DM
340	-	+	-	-	OC
600	-	+	+	-	DM
700	+	+	+	-	M
UBC 268 – UBC 456					
245	-	-	++	-	SOC
	-	-	++	-	SOC
UBC 268 – UBC 457					
233	-	-	-	+	F
235	+	+	+	-	M
315	++	-	++	-	DM

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
515	-	+++	-	-	OC
UBC 268 – OPA 02					
330	+	++	+	+	DS
375	++	++	++	+	DS
400	++	++	++	+	DS
460	+	+	+	-	M
600	++	++	+++	+	DS
650	++	++	+	-	DM
UBC 268 – OPA 09					
345	+	+	+	++	DS
393	+	+	+	-	M
395	+	-	-	-	BC
580	+	+	+	-	M
690	+	+	+	-	M
UBC 268 – OPA 16					
585	+	-	-	-	BC
595	+	+	+	-	M
690	++	++	++	+	DS
UBC 268 – OPA 17					
350	+	+	++	+	DS
UBC 268 – OPZ 09					
225	+	+	+++	+	DS
270	-	+	+	-	DM
360	-	-	+	-	SOC
UBC 268 – OPB 10					
480	-	-	++	-	SOC
UBC 428 – UBC 101					
220	+	+	+	-	M
223	+	+	+	-	M
263	+	+	+	-	M
265	+	+	+	-	M
340	-	-	+++	-	SOC
370	-	-	-	++	F
420	+	+	+	-	M
423	+	+	+	-	M
UBC 428 – UBC 119					
220	+	+	+	-	M
223	+	+	+	-	M
340	-	-	+++	-	SOC
395	-	++	-	-	OC
UBC 428 – UBC 122					
340	-	-	+++	-	SOC
425	-	++	-	-	OC
UBC 428 – UBC 128					
340	-	-	+++	-	SOC
410	-	-	+	-	SOC

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
UBC 428 – UBC 135					
340	-	-	+++	-	SOC
UBC 428 – UBC 138					
340	-	-	+	-	SOC
UBC 428 – UBC 158					
340	-	-	+++	-	SOC
645	-	-	-	+	F
UBC 428 – UBC 159					
340	-	-	+	-	SOC
UBC 428 – UBC 169					
340	-	-	+++	-	SOC
UBC 428 – UBC 174					
340	-	-	+++	-	SOC
515	+++	+++	+++	+	DS
550	+++	+++	+++	+	DS
635	++	++	++	+	DS
UBC 428 – UBC 191					
250	++	-	++	-	DM
280	-	-	-	+	F
340	-	+	+++	-	DM
UBC 428 – UBC 217					
340	-	-	++	-	SOC
UBC 428 – UBC 228					
220	+++	-	-	-	BC
275	+++	+++	+++	-	M
295	+	+	+	-	M
340	-	-	++	-	SOC
370	+	-	-	-	BC
400	+	++	++	+	DS
UBC 428 – UBC 263					
230	++	++	++	-	M
245	-	-	+	-	SOC
278	+	+	+	-	M
280	-	+	+	-	DM
335	-	-	++	-	SOC
340	-	-	++	-	SOC
355	-	+	+	-	DM
UBC 428 – UBC 268					
205	+	+	+	-	M
238	+	-	-	-	BC
240	-	-	++	-	SOC
252	++	++	-	-	DM
255	+	++	++	-	DM
340	-	-	++	-	SOC
365	+	+	+	-	M
520	-	-	-	+	F

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
UBC 428 – UBC 273					
238	+++	+++	+++	-	M
340	-	-	++	-	SOC
370	++	+	+	-	DM
580	++	+	++	-	DM
710	+++	-	-	-	BC
800	+++	+++	+++	-	M
UBC 428 – UBC 299					
340	-	-	+++	-	SOC
UBC 428 – UBC 428					
230	++	++	++	-	M
233	+	+	+	-	M
340	-	-	+	-	SOC
345	+	++	++	-	DM
355	-	+	-	-	OC
410	-	-	++	-	SOC
535	+	+	+	-	M
UBC 428 – UBC 456					
290	++	++	++	+++	DS
315	+	+	+++	-	DM
340	-	-	++	-	SOC
400	-	-	-	+++	F
625	++	++	+	-	DM
1,025	-	-	-	+	F
UBC 428 – UBC 457					
340	-	-	+++	-	SOC
UBC 428 – UBC 459					
340	+	+	+	-	M
365	-	-	-	+	F
470	+	+++	-	-	DM
UBC 428 – OPA 01					
228	-	-	-	+++	F
230	-	-	-	++	F
340	-	-	+	-	SOC
353	-	+	-	-	OC
400	-	+	-	-	OC
545	+	+	+	-	M
548	-	-	-	+	F
UBC 428 – OPA 02					
230	-	-	-	++	F
232	-	++	++	-	DM
245	-	-	+	-	SOC
280	+	+	+	+++	DS
340	+	+	+	-	M
550	+	++	++	-	DM
875	+	+	+	-	M

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
UBC 428 – OPA 07					
208	-	-	+	-	SOC
210	-	-	-	+	F
285	+	-	-	-	BC
340	+	+	+	-	M
400	++	-	-	++	DS
650	+	+	+	-	M
UBC 428 – OPA 09					
225	++	++	+	-	DM
240	-	-	-	++	F
330	-	-	-	++	F
340	-	-	+	-	SOC
350	+++	+	+	+	DS
380	+++	+++	+++	-	M
385	-	-	-	+++	F
610	-	-	-	++	F
700	+	+	+	-	M
950	-	-	-	++	F
UBC 428 – OPA 16					
220	-	-	-	++	F
230	++	++	++	-	M
340	+	+	++	-	DM
385	+	++	++	-	DM
425	-	+	-	+++	DS
UBC 428 – OPA 17					
240	+	++	++	-	DM
400	++	++	++	-	M
UBC 428 – OPB 10					
270	-	-	-	+	F
305	-	-	+	-	SOC
335	-	-	++	-	SOC
340	+	-	+	-	DM
360	-	-	-	+++	F
395	-	-	-	+++	F
410	-	-	-	+	F
900	++	++	++	-	M
UBC 122 – UBC 101					
315	-	-	-	+	F
375	-	-	-	+	F
380	+	+	+	-	M
455	-	-	-	+	F
470	+	+	+	-	M
475	+	+	+	-	M
UBC 122 – UBC 119					
315	-	-	-	+	F
345	++	-	-	-	BC

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
UBC 122 – UBC 122					
280	-	-	-	+++	F
315	+	+	+	++	DS
320	-	++	-	-	OC
615	++	++	++	-	M
700	++	++	++	-	M
UBC 122 – UBC 128					
220	-	-	-	+++	F
315	+	+	+	++	DS
UBC 122 – UBC 135					
300	-	-	-	++	F
315	-	-	-	+	F
370	++	++	++	-	M
414	+++	+++	+++	-	M
615	-	-	-	++	F
1,000	++	++	++	-	M
UBC 122 – UBC 138					
265	-	-	++	-	SOC
315	-	-	-	+	F
915	-	++	-	-	OC
UBC 122 – UBC 158					
250	-	-	-	+	F
275	-	-	-	+	F
315	+	+	-	++	DS
375	-	-	-	+	F
UBC 122 – UBC 159					
210	-	-	-	++	F
260	+	+	+	++	DS
315	+	+	+	+++	DS
425	-	+++	-	-	SOC
UBC 122 – UBC 169					
280	-	-	-	++	F
315	+	+	+	++	DS
550	+++	+++	+++	+	DS
630	+++	++	++	+	DS
UBC 122 – UBC 191					
315	-	-	-	+	F
520	++	++	++	+	DS
580	++	++	++	-	M
UBC 122 – UBC 217					
315	-	-	-	+	F
470	+	+	+	+++	DS
530	-	-	-	++	F
625	+++	+++	+++	+	DS
UBC 122 – UBC 222					
215	+++	+++	+++	+	DS
225	+++	+++	+++	+	DS

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
315	-	-	-	+	F
360	+++	+++	+++	+	DS
525	++	++	++	-	M
UBC 122 – UBC 228					
185	-	-	+++	-	SOC
210	+++	+	+	-	DM
275	++	++	++	-	M
315	-	-	-	+	F
370	++	++	++	+	DS
1,150	+	+	+	-	M
UBC 122 – UBC 263					
315	+	+	+	++	DS
UBC 122 – UBC 268					
270	-	+	-	++	DS
275	++	+	++	-	DM
280	++	++	++	-	M
315	+	+	+	++	DS
345	+++	+++	+++	-	M
UBC 122 – UBC 273					
310	+	-	-	-	BC
315	-	-	-	+	F
395	++	+	+	-	DM
UBC 122 – UBC 299					
315	+	+	+	++	DS
325	-	++	-	-	OC
390	++	++	++	+	DS
415	++	++	++	-	M
525	+	+	+	+++	DS
UBC 122 – UBC 428					
280	+	-	-	-	BC
315	-	-	-	+	F
415	-	+	-	-	OC
UBC 122 – UBC 456					
315	-	-	-	+	F
585	+++	++	++	-	DM
915	-	+	-	-	OC
1,150	+	+	+	-	M
UBC 122 – UBC 457					
215	-	-	-	+++	F
315	+	+	+	+++	DS
UBC 122 – UBC 459					
315	+	+	+	++	DS
395	+	+	+++	-	DM
UBC 122 – OPA 01					
230	++	++	++	-	M
315	+	+	+	++	DS
340	+	++	++	-	DM

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
UBC 122 – OPA 09					
230	++	++	++	-	M
415	-	-	++	-	SOC
1,100	+	+	+	-	M
UBC 122 – OPA 16					
255	-	++	-	-	OC
440	++	-	++	-	DM
500	-	-	-	++	F
UBC 122 – OPA 17					
210	++	++	++	-	M
825	++	+	+	-	DM
UBC 122 – OPZ 09					
315	-	-	-	++	F
UBC 122 – OPB 10					
235	+++	+++	+++	-	M
270	++	++	++	-	M
315	-	-	-	++	F
450	++	++	++	-	M
OPA 02 – UBC 119					
300	+++	+	-	-	DM
375	++	-	-	-	BC
725	++	-	-	+	DS
OPA 02 – UBC 135					
320	++	+	++	-	DM
630	++	++	++	-	M
1,050	-	++	+	-	DM
OPA 02 – UBC 138					
255	++	++	++	-	M
285	++	++	++	+	DS
475	+++	++	++	+	DS
530	+++	++	++	+	DS
825	++	++	++	-	M
OPA 02 – UBC 158					
375	+	++	-	+	DS
530	+++	++	++	-	DM
715	++	-	-	-	BC
825	++	++	++	-	M
OPA 02 – UBC 159					
720	++	+	+	-	DM
735	-	-	-	+	F
OPA 02 – UBC 191					
460	+	+	+	-	M
530	++	+	++	-	DM
OPA 02 – UBC 217					
530	++	++	++	-	M
OPA 02 – UBC 222					
530	++	+++	+++	+	DS

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
1,500	+++	+++	+++	-	M
OPA 02 – UBC 228					
200	+++	+++	+++	-	M
380	-	-	-	++	F
OPA 02 – UBC 268					
245	++	+	+	-	DM
350	+++	+++	+++	-	M
390	+++	+	+	-	DM
450	-	++	-	-	OC
OPA 02 – OPA 01					
530	++	+++	+++	+	DS
OPA 02 – OPA 02					
820	+	+	+	-	M
OPA 02 – OPA 17					
470	++	++	++	-	M
OPA 02 – OPB 10					
395	-	++	-	-	OC
400	++	-	-	-	
530	++	+++	+++	+	DS
OPA 16 – UBC 101					
212	-	-	-	+	F
215	+	+	+	-	M
320	+	+	+	-	M
520	+	+	-	++	DS
620	++	++	++	+	DS
OPA 16 – UBC 119					
260	+	+	+	-	M
350	-	++	+	-	DM
405	+++	+	+++	-	DM
675	+++	+	++	-	DM
OPA 16 – UBC 122					
215	-	-	++	-	SOC
OPA 16 – UBC 128					
185	-	-	-	++	F
OPA 16 – UBC 135					
240	++	++	++	-	M
253	-	-	-	+	F
255	+	+	++	-	DM
353	-	-	+++	-	SOC
455	+++	+++	+++	++	DS
OPA 16 – UBC 158					
285	-	-	-	++	F
355	-	+	++	-	DM
OPA 16 – UBC 159					
235	++	+	++	-	DM
260	-	+	-	+++	DS
317	-	-	++	-	SOC

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
320	-	++	-	-	OC
OPA 16 – UBC 191					
220	++	++	++	-	M
365	+++	+++	+++	+	DS
390	-	++	-	-	OC
392	+++	-	+++	-	DM
395	-	++	++	-	DM
397	++	-	-	-	BC
400	++	++	-	-	DM
405	++	-	-	-	BC
425	-	++	-	-	OC
510	++	++	++	-	M
625	-	-	+	-	SOC
675	+	+	+	-	M
OPA 16 – UBC 222					
195	-	-	++	-	SOC
285	++	++	++	-	M
305	+++	+++	+++	-	M
307	++	++	++	-	M
OPA 16 – UBC 228					
310	++	++	++	+	DS
315	++	++	++	-	M
1,050	++	++	++	-	M
OPA 16 – UBC 268					
250	++	++	++	+	DS
OPA 16 – UBC 273					
500	-	-	-	+	F
520	-	-	-	+++	F
875	++	+	++	-	DM
OPA 16 – UBC 457					
420	+++	+++	+++	-	M
490	+++	+++	+++	-	M
750	+++	+++	++	-	DM
OPA 16 – UBC 459					
255	+++	+++	+++	+	DS
OPA 16 – OPA 01					
275	+	++	++	-	DM
278	+	++	++	-	DM
OPA 16 – OPA 02					
460	++	++	+	-	DM
OPA 16 – OPA 07					
280	+++	+	++	-	DM
OPA 16 – OPA 09					
235	++	-	-	-	BC
500	-	-	+	-	SOC
OPA 16 – OPA 16					
225	+++	++	++	-	DM

Primer combinations / sizes (bp)	Male markers BC	OC	SOC	Female markers	Remarked
410	+++	+++	+++	-	M
450	-	+++	+	+	DS
OPA 16 – OPA 17					
260	+	+	+++	-	DM
345	+	+	++	-	DM
OPA 16 – OPZ 09					
275	-	-	-	+++	F
315	+	++	++	-	DM
470	++	++	+++	-	DM
598	++	+	+	-	DM
OPA 16 – OPB 10					
275	+	++	++	-	DM
315	+++	+++	+++	+	DS
320	+	+	+	-	M
340	+++	+++	+++	+	DS

Abbreviations

- = the absence of a particular band
- M = candidate male – specific RAP-PCR markers
- F = candidate female – specific RAP-PCR markers
- DM = RAP-PCR markers showing differential expression patterns between male morphotypes
- DS = RAP-PCR markers showing differential expression patterns between males (AG) and females (oviducts)
- BC = candidate RAP-PCR markers found only in blue-claw males
- OC = candidate RAP-PCR markers found only in orange-claw males
- SOC = candidate RAP-PCR markers found only in small orange-claw males

Intensity of the amplification products was assigned as +, ++, +++ in order.

Appendix F

Nucleotide sequence of recombinant clones obtained from candidate SOC-specific (I), female-specific (II) and male-specific and morphotype-specific (III) RAP-PCR markers. Sequence and positions of primer combinations used for RAP-PCR analysis are illustrated in boldface. Those of the forward primers and the complementary sequences of the reverse primers for development of sex-specific/differential expression markers of SOC males, females, male and male morphotypes of *M. rosenbergii* are underlined and boldface.

I. A SOC-specific RAP-PCR markers (SOCRAP340)

>SOC340-1
GTAGACGAGCAAGCAACTTGGAACGGGGAAGAAATAGCTTTCTTTCCAATCGGCATGACATTACACTCGGTG
AAAGTAGCAGCCGTTAGACCTGTTGTTCGGATGCCTTTTTTTCTCTTTTTTTTGTACAGATTTCTGCAGGCG
AGAAACTTTTCTTTTCAGAGATGACGTAGCATGGCAGAATGCTTTCCCTTTCGTTTCCACTTTCATGTATCG
TCATATCGGCCCCGTAAGGAGGTAGTGCCGTCAGTGCACCTTACGCGGAGCACTGTAGGCATTACTTGAGGT
TCTTTGCAGCGTCCCTTCGGTCCCTAGCTGGACCCCTTTTCATTTCTTTTCCTG**TACCGCAGCC**

>SOC340-2
GGCTGCGGTACTAAGCAACCCGACTCTACGGTAAAGACCCCTCCGCCCGAATTGCCTCCAACCTTTTTACAG
GCGCCGCCACACGGGCCTACCACCCTCCTCGGGGCTGCATTCAAGCAATGACTTAGGCGGTCAGCAACTCGA
GCGGTACGGATCCACCAAAACGCAACACCCCTCGACGGCCACAACAAGACCGCAAGGTTATGCGCTGGGCTC
ATCCCCTTTCAGTCGCCCTTACTCAGGGAATCCCGGTTGGTTTCTTTTCCTCCGCTTATTAATATGCTTAAA
TTTGGCGGGTAGTCTCCCCACGGACGAGGTCCTAACAGCCAAACACTCTCTCT**CTCCAGGCGC**

>SOC340-4
GTAGACGAGCAAGCAACTTGGAACGAGGAAGAAATAGCTTTCTTTCCAATCGGCATGACATTACACTCGGTG
AAAGTAGCAGCCGTTAGACCTGTTGTTCGGATGCCTTTTTTTCTCTTTTTTTTTGTTCAGATTTCTGCAGGC
GAGAAACTTTTCTTTTCAGAGATGACGTAGCATGGCAGAATGCTTTCCCTTTCGTTTCCACTTTCATGTATC
GTCATATCGGCCCCGTAAGGAGGTAGTGCCGTCAGTGCACCTTACGCGGAGCACTGTAGGCATTACTTGAGG
TTCTTTGCAGCGTCCCTTCGGTCCCTAGCTGGACCCCTTTTCATTTCTTTTCCTG**TACCGCAGCC**

>SOC340-5
ATTGGGCGATTGAGAGATGGGCCATATGCGAACACTCCTTTCCGCCACATTACGCCGTTTCCATGATATGTG
ACATAACGAGATTTCAGTGGCAAATCACGGCGGAGTACCATAGCGACGGAGTATCTTAGAGTACTTATTTTT
AACATAATTAATAGTTAGCCATTCTGTAGCATAACCTATGTTTATTAGTCTTGTACTACCGGATTCAATTCC
GGCTGGTTTTGCCTGACGACACGCATGTTTCATCACCCATAAATCTGTGTTAAGGTAGCCTATTACCGCCGG
ACCTAACTCTGGCGGGCCTTGCCTTGACGTTACTCAT**TACCGCAGCC**

>SOC340-6
ATTGGGCGATTACCTCATTACCGCAGCCAACAAAATGGAACCGAGATCATCTTCCATCATTCCATGCAGGTA
ACATTCGGGCATTGTATTCCAGAGTAGCCTGCTTTGAGCACTCTCATTTACTCAAGGTGAACTGGCCGGCCG
CGCGGGACGCTCGGTAAAGAGCACCCCGGCAGAACCGGCTTGGCTTCCACTCACGGAAGCTGTTCGGAGCCT
GACGGGGAAGCACCGGGCGGTGACCTCCCACCCCAGTCCGGAACTGACCTCCAACTACGAGCTTTTTAACCG
CAACAACTTTAATATACGCTATTGGGCGATTACCTCAT**TACCGCAGCC**

>SOC340-7
GGCTGCGGTAATGAGGTAATCGCCCAATAGCGTATATTAAAGTTGTTGCGGTTAAAAAGCTCGTAGTTGGAG
GTCAGTTCCGGACTGGGGTGGGAGGTCACCGCCCGGTGCTTCCCCGTCAGGCTCCGAACAGCTTCCGTGAGT
GGAAGCCAAGCCGGTTCTGCCGGGGTGCTCTTTACCGAGTGTCCCGCGCGGCCGGCCAGTTCACCTTGAATA
AATGAGAGTGCTCAAAGCAGGCTACTCTGGAATCTGCGATGAAAGACCTCACTCCATCATCCTTAGTCTAAC
AATTCGGGGAGAAAGGGTTTCTCTCGCGCCACCTGAC**ATCGCCCAAT**

>SOC340-15
GGCTGCGGTACTAAGCAACCCGACTCTACGGTAAAGACCCCTCCGCCCGAATTGCCTCCAACCTTTTTACAG
GCGCCGCCACACGGGCCTACCACCCTCCTCGGGGCTGCATTCAAGCAATGACTTAGGCGGTCAGCAACTCGA
GCGGTACGGATCCACCAAAACGCAACACCCCTCGACGGCCACAACAAGACCGCAAGGTTATGCGCTGGGCTC
ATCCCCTTTCAGTCGCCCTTACTCAGGGAATCCCGGTTGGTTTCTTTTCCTCCGCTTATTAATATGCTTAAA
TTTGGCGGGTAGTCTCCCCACGGACGAGGTCCTAACAGCCAAACACTCTCTCT**CTCCAGGCGC**

>SOC340-31
GGCTGCGGTAAGTACAAATCGTTGGCTCATAAATCACGAAATTAATCGCTAATCTCTTGGTAGAATTCAATT
TGCTTTGTTGGATTTGTAAGCCTTCACAATAATCTCGACTAGTGTCCTTGAGTCGACATTTGACACGATGCG
TATAGACGCTCAGTTCAACGAAACTTTTTATATTCTCTCAGTTTACCTCCAGAAGTTCTTAAATCTCTGAGT
ATCGTACTTACTGTATGCTGTCAATGTACATTATCAGTACCTTGCCTAACGCTCCTGCCAGTTTTATCACAC
CTTAACGAGAAAAGAGAAGCTTCTTTTCCGC**GCCACGGCTA**

>SOC340-38
TAGCCGTGGCAATAAATCAGTGTAGTCATCAA***GAGTTTCTTTTACCTGTGCGTGG***GTGTCTAGTACATCCTC
GGAAAAATAAACAGTTCCCAATGACGACCTTGCATCATTCATTAACTCCATAGCTTCTGCAAATTTCTCAGA
TATATCATCAACAGCCCTTTTAACTTCCTTTTCTGTCATTTCACTACCAGTGACAGACGAATACATAGAATT
TCCAATGCTCGCAACATCAGGGTACTGCATCAGTGTTAGTGCACTTGATGTGGTCTGTGGTGTGACTGGCAA
CGACATCTTCTTTTGAAGGTTATACTTCCCAATAC**TACCGCAGCC**

>SOC340-41
GGCTGCGGTAAGTA***CAAATCGTTGGTTCATAAATCAC***GAAATTAATCGCTAATCTCTTGGTAGAATTCAATT
TGCTTTGTTGGATTTGTAAGCCTTCACAATAATCTCGACTAGTGTCCTTGAGTCGACATTTGACACGATGCG
TATAGACGCTCAGTTCAACGAAACTTTTTATATTCTCTCAGTTTACCTCCAGAAGTTCTTAAATCTCTGAGT
ATCGTACTTACTGTGTGCTGTCAATGTACATTATCAGCACCT***TGCCTAACGCTCCTGCCAGTT***TTATCACAC
TTTAACGAGAAAAGAGAAGCTTCTTTTCCGC**GCCACGGCTA**

II. A female-specific RAP-PCR markers (FeRAP315)

>Fe315-5
GTAGACGAGCTTCATT***CAACGTACTACTCTTGTTCATC***ACGTGAAATCTAATATAAAACTTACTTTAAATTT
CAAAACAAAAATACATCGCCCCCCCCAGCATAATTTAAAGTACAGCTTTTTATTAAATACCAAACAAGTTTA
CACTACTGCAATTTCTGGTCACAAAAACCAAGGGGTTTTCTTTTTCTATCTCCA***TAATAGGCACTTTGGAAC
AAT***CCAGCCTTGTATTTTCAGTCATTCCATTAGCACCCTGCAGTACCTAAATTCTCAAATACGACTTAACCT
GCAATTTGATCCATCAACT**GCTCGTCTAC**

>Fe315-9
GTAGACGAGCTGGTCTGCCACAACAGTCACTCTACTAAGTACCAAGTATGGAAGGAGAGAGATGCTCCTTTC
TTCTGCCCAGGTATCTTGCTGGTAAAGATAACAATGGCTACGACTTTGTGCCTCCTAAGTTTTTCAAGCAAG
CTAACAATGTATTGTTAGCTTACTTATTCATACCACCAGCATCTCTAGCCAACTGAAATGAAACTCCCACTT
CTCTGCCAACCAGACAGTTGCTACATGTTGGTTGGCCACAACTACTCCCCAGTCTTAGGTTAATGTATCTCA
ATATGATACAAAGTCTGG**GCTCGTCTAC**

>Fe315-47
GTAGACGAGCTTGAGAAAGAGAGAGAGAGAGAGAGATTGACCATTAGTATAAAACACATGGGCATTTACAGA
GAGAGAGAGAGAGAATGACCACGAGCACACAACACGTACCCAATTACAAAGCAACAGAACTCTCCCTAAATT
AGCGCCCATTACAAGTAACTAACAAAAAAAAAATAATAATAATAAAATCACAGTAACACTCACGTAACCCCT
TCTCTCTACAACGGCACCCAGATTCGTAACTAACCTGAAACATAACACCTTTCGATCTTTCCATTACACCCA
CTCACGACACAAGTTCATAGC**GCTCGTCTAC**

>Fe315-50
CACCCCAGTCACATCAGCACACTACTCGCATTCCATGCAATCAACGTCTTTCATTTCAGTCGTATCACATCA
GTATCATGAGCCATTTTAAAAATATGATTTATGAAATATCACTTGCCATTAATTCCATATCATTATCGTCAT
TAATATCTACAATTTCACTCAAGTGATGTCACTTATTCCTTCCACAGCAGATCAGGGACATACTCACTGCTT
CATTCTCATGGTACCAATATCACAATTTCTTTACATTTCATATAATTACGACAGTTTCATTCACTTCATATT
AGTCTTGTT**GCTCGTCTAC**

>Fe315-55
CTGCTGGGACAAGCACGAGTTGAGATCAGTAATTAACTCCCACTTCAATTGCCTTCCTCA***TCCTACTACTAG CCAAGACGATT***GTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCATTCATAAATCCCACCAACACCCAC
TATCCTTTCGAATGACCGTTTTCGCTATTTCCATCTGAAATCGCCGGGTTATTTTAAGACGTTGGCAATTTT
AGCGACCGATTCGATTTCGTTTTATTTATAA***CTTGGGTCATCTGCCTGGTCG***CGGAATATGGGGCTAATCGG
TTACATGCTTCTTGA**GCTCGTCTAC**

>Fe315-69
CACCCCAGTCCAGTAAAGCATCCGAAAACACACACACACACATATACATATATACATATATATACGAATATA
AAGTTTGTATTCATTACCTTTTGAGCCACAAAGAAAGATGGAATAGAAAATGGCAGTCCAACAGTATTCCCA
AGTTTATTCCATCTTTTCACACTCGCTAACCACTAAAGCTTGCCACGTTTATTTACACACGAGCGGTCTTTA
AATCCCCCATGGAAAATACGGGGATGGTAGATAACACAGCCAGCTTTAAAAGGGTCTCGAGCTCGAGGGAAC
ATCAGCGTGGTAGTATTTGA**GCTCGTCTAC**

III. Male-specific and morphotype-specific RAP-PCR markers

>M268/128RAP
GCATATTCCGTAAAATGGGATATATTTCAATTGGCTAGTTCGTCAAAGAACAGTCCCCGGGG***GTGGGGGGAG TGCCGTCAGT***GCACCTCACTCGGTGCACTGTAGGCATTACTTAAGGTTCTTTGCAGCGTCCCTTCGGCCCCT
AGCTGCAACCCCTTTCATTCCTTTTACTGTACCTCCTTTCATATTCTTTGTTCCATCTTATTTCCCACCATC
TTCTATCAACTGCGAA***GTTTTTCTCCTGTTACACCTTTGT***AACCCTTTACTGCAATTTTCCTG**TAAGCGGCC T**

>M428/228RAP
GCTGGGCCGAAGAGATGCTGC***AAAGAACCTCAAGTAATGCCT***ACAGTGCACCCGCATATGGTGCACTGACGG
CACTAACCCCCTTGGGTTTAGGCAAAGGAGACGAAATATAGATTTAAGTGAAATATGTTTAGAGAAATTCAA
ATGAAATATACTGAACAGAGGTTTAAATACAACATATTTAAACGGGTTTAGTGCCTCAATGGCACCTTTATA
GAGCAAGTACTTAATGCGCCGAAGT***TTCTTCGGAGCAAACGAGTT***TTCTG**TACCGCAGCC**

>M122/135RAP
AAGCTGCGAGAAGGAGAGGTACGGAAGACTCGATGAGG***TCAGACTACATACCCCTTCAAT***GCCCAGATCCTA
CTACTGTTGCTGCCTTACAAGAGGTGAAGCATCTCCTCCAGTCTATGCTGAAGAAGAGCAAAGACTTGCGCC
GTGGCCTGACCAAGCAAACGAGCGAGGGCGTCGACCTCCTGAAGTTATTGCACCAGAGATTCTCTGTTCTCC
TGAGGCGCATGAACACTGAGCCTGACCACGGTCAACAGCTTGTAGCAGAGATGCAGGACTTGGGGTCCCTTG
TAGAATCCTCTTACA***CTGCTGTTTTGGTCGCCCA***GAACGCCTTCATCGACTCTTGCAAGAGGATCCAAGCCG
AGGAGCCGACTCTGGAGCACAAGATCGCCGAAAATTCTGGATAA**GCTCGTCTAC**

>MA16/222RAP
AGCCAGCGAACAGAACATGACATAAGAAAAGCCAGCAGGCAAAA***CATAAGAGCGAATGAACGAAGCA***TAGAA
AAAGCTAGCAGACAGAACATGACATAAGAAAAGCTAGCAGACAGAACATGACGTAAGAAAAGCTAGCAGACA
GAACACGACATAAGGAAAACCAGC***AGACGAAACATTACAAATAAGGA***ATGCCAGCAGACAGAACATGACATA
AGAAAAGCCAGCATACGAAACGTGACATAATTGAGGCTAGCAATCGGAACATGACATAAAGAGTATATAACG
GGA**GGGGAGGCTT**

>M122/159RAP.1
GTAGACGAGCCTTCATCCTGATCGTTATGTCTGAAGTAAGTTTGCTTCGGTCCAACCCTTAACACCCAGGTT
CCTTTATATTCCATTTCTTCCCATCAGCCAAAAACAAATTTGGTCTTCCAAGGAGATTTT***CTGCTGCTACCT TTGGCTATTA***GATTTTGTGTGTGATGGGAGTTATACACCCCTATTCTCACGTCGGTCTAGGAGTCACACATT
GTAGTCCTTATGAAGCAAGTTGAATTCACGAGCATTTAGATCACTCCATTTTATCACTGTGCAACAGCTACA
CCTAAAAAAAAAAAAATGACACTGAAATACCTACTTATCCACAGCACAGTGACAATACTGTAATTTCCCTCGG
CAAAATTCAACT***GGTGACAACTGTAACTGACGC***CAGTGACTTCACTAATTAAATTAAT**GCTCGTCTAC**

>M122/159RAP.2
GTAGACGAGCAAGCAAGTCAAGGGAAGATCCCGTCTTAACCACTTGGGCCTGCAAGGGAAGTGTGATCCGCG
GTGCCTGACGCAGTTCACATTTCTCGAGCATCTCGATGTCTT***CGACGGTAATGTATGAAGCAGT***CTCGTCGC
GGCCATATTGGTTTACTGCCACGCAGCGATAAAGACCTTTGTCATCAATGAATATTTCTCTGATTGTAAGAC
TGACCCGTCCCGTAGTTTCATCGTGCTCTTGGATGAACTCCTGGGACGGCGCCACTTCTTTATCGAGGTGAT
ACCACAGAACTCGAGGGGTCGGTTGTCCTCGCACTGTGGCTTCCAGCACCACCTTTGAGCCATGTTTGACTT
GAAGTGGCTCAAGTGGTACCACAA***ACTTTGGTGGTTCTCCTCGGG***GTTTATCTTTG**CTACGGGCTC**

>SOC268/273RAP
AATGCCGCCATACCAGAGCTGAAACAGGCACTGTAA***ATATTTGGTGCTCCATCGTA***GCTGAAAGCTGCGTAG
AGCATCAACTAACTTGAAGTATTCGCTACGGATTTTCTTCATTGTGAATGTGTCCATCATACTTTCATTGGG
TGCTGCAGCTGCCAGCAGGTGAATAAGTCTACCTCGTAGAAGAT***GCAATGGAGTGAGGACTTCA***TCTTTGCG
GCCATCACCACAGTACTGTACA**TAAGCGGCCT**

>SOC122/228RAP
GTAGACGAGCAGTG***ACGACGACGGCGACGAACG***AGCAGAGTGAAGAATTCGGAACGGCGATAAAATTTAGAA
GTCAACGCGTTGATAACCGCCTCTCGAATGGGAAGTGACGCCTTCAATCAACAATTTGGAAGGATTCCAAAT
TTTTTCTTCGCCAA***ATTCAGCAAGTATGGACTGCAA***G**TCGGCCCAGC**

>BC428/228RAP
GGCTGCGGTAAGTACTGCTAAGTAACAAAA***TACACTGCGGAAAGTAATGCT***AGGTAAAAAATACACTGAATG
TGTGCACATACATACATACATACATACATATATATATATATGTATGTATGTATGTATGAATGTATGTACC
ACAATAAGCTGTAGGTCCCGTTGCTAAGTAACCAAATGGTTCTTAGCCA***CGTAAAATAAGTCTAAGTCCT***T**C
GGCCCAGC**

>BC428/273RAP
AATGTCGCCACTTCAAAATACAACTGTTATAATTTTTTTACTTAAAATATAAATACTTTCTCACTGTATGCC
ATCTCCCTCCAAAAATATACAAGACAATGTCAGTAAGAAACCTTATATGAGCAATAACTGTCCTTATGGATT
TCCATTCTCAGTACTGCAATTTCATATTCATATTTGCTTTTA***CAAGACTGTGGGAAATCGTGT***ATCTTGTAG
GCTACCCAATCTTGCAATTTAAGCATCCGATGCTTTTCATTTTCTCCAAAACCTCGACTGTCTATACTTTTC
TACCTACAACAACGCAATAATTTTCCTCCATCATCTCCTTAATGCAATGCTAGTAATTCCTCCTTTGGCAAG
CATTTCAAACTTCCTTTCTCTCCTTTTCCCAGAATCTAAAATGANACAATA***TCTTTTCTTCAGCATCTCATT***
AATAATAATTTCATTTACTAACACCCTGACTGTAGCATCAGAATCTAAATAGCAACAATATCCTTTCTTGTA
CATCTCAATAAACATTCCATTCTCCGACACCTAGAACTGTAGCATCAATCGATACTACATCAATAAAGGAAA
GCGTTAGTGTATCGTTTACCATCCTCCCCAAGTAGAAATGCCCAAGTGAGCATATCACAGCTAATGTTAACA
TATCCACAAATATATACAAAGTACAACGGGT

Abbreviation

M = males

SOC = small orange-claw males

BC = blue-claw males

BIOGRAPHY

Miss Rachanimuk Preechaphol was born on August 20, 1980 in Chonburi. She graduated with the degree of Bachelor of Science from the Faculty of Agricultural Technology at King Mongkut's Institute of Technology Ladkrabang in 2000. She has studied for the degree of Master of Science (Biotechnology) at the Program of Biotechnology, Chulalongkorn University since 2001.

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