# การสืบค้นเครื่องหมายทางพันธุกรรมที่จำเพาะต่อเพศในกุ้งกุลาดำ *Penaeus monodon* โดยการวิเคราะห์ด้วยเอเอฟแอลพี

นางสาวสุภาพร ธรรมรุ่งธนกิจ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547
ISBN 974-17-6097-3
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

IDENTIFICATION OF MOLECULAR GENETIC MARKERS INVOLVED
IN SEX DETERMINATION IN THE
GIANT TIGER SHRIMP *Penaeus monodon*
USING AFLP ANALYSIS

Miss Supaporn Thumrungtanakit

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2004

ISBN 974-17-6097-3

| | |
|---|---|
| Thesis Title | Identification of molecular genetic markers involved in sex determination in giant tiger shrimp *Penaeus monodon* using AFLP analysis |
| By | Miss Supaporn Thumrungtanakit |
| Field of study | Biotechnology |
| Thesis Advisor | Associate Professor Padermsak Jarayabhand, Ph.D. |
| Thesis Co-advisor | Sirawut Klinbunga, Ph.D. |

---

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

………………………………………… Dean of the Faculty of Science

(Professor Piamsak Menasveta, Ph.D.)

THESIS COMMITTEE

………………………………………… Chairman

(Assistant Professor Charoen Nitithamyong, Ph.D.)

………………………………………… Thesis Advisor

(Associate Professor Padermsak Jarayabhand, Ph.D.)

………………………………………… Thesis Co-advisor

(Sirawut Klinbunga, Ph.D.)

………………………………………… Member

(Assistant Professor Thaithaworn Lirdwitayaprasit, Ph.D.)

………………………………………… Member

(Narongsak Puanglarp, Ph.D.)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

สุภาพร ธรรมรุ่งธนกิจ : การสืบค้นเครื่องหมายทางพันธุกรรมที่จำเพาะต่อเพศในกุ้งกุลาดำ *Penaeus monodon* โดยการวิเคราะห์ด้วยเอเอฟแอลพี (IDENTIFICATION OF MOLECULAR GENETIC MARKERS INVOLVED IN SEX DETERMINATION IN THE GIANT TIGER SHRIMP *Penaeus monodon* USING AFLP ANALYSIS) อ. ที่ปรึกษา : รศ.ดร. เผดิมศักดิ์ จารยะพันธุ์, อ. ที่ปรึกษาร่วม : ดร. ศิราวุธ กลิ่นบุหงา 149 หน้า. ISBN 974-17-6097-3.

ค้นหาเครื่องหมายทางพันธุกรรมที่จำเพาะต่อเพศในกุ้งกุลาดำด้วยเทคนิคเอเอฟแอลพี จากการวิเคราะห์ไพรเมอร์จำนวน 256 คู่ผสมกับตัวอย่างดีเอ็นเอ 6-10 กลุ่ม พบเครื่องหมายเอเอฟแอลพีที่จำเพาะต่อเพศเมีย จำนวน 5 เครื่องหมาย (FE10M9520, FE10M10725, FE14M16340, FE15M14400 และ FE16M8350) และเพศผู้ จำนวน 1 เครื่องหมาย (ME10M8420) ตามลำดับ ทำการโคลนและออกแบบไพรเมอร์จากเครื่องหมายที่จำเพาะกับเพศเมียทั้งหมด พบว่าเครื่องหมาย SCAR ที่พัฒนาจาก FE10M9520, FE10M10725.1, FE10M10725.2 และ FE14M16340 ให้ผลิตภัณฑ์จากปฏิกิริยาลูกโซ่โพลีเมอเรสในทั้งสองเพศซึ่งไม่แสดงความจำเพาะต่อเพศของกุ้งกุลาดำ เมื่อนำเทคนิค SSCP มาใช้ในการค้นหาความแตกต่างของนิวคลีโอไทด์ ในเครื่องหมาย SCAR ที่เพิ่มจำนวนจากดีเอ็นเอของเพศผู้และเพศเมีย พบความหลากหลายของนิวคลีโอไทด์ในเครื่องหมาย FE10M10725.2 และ FE14M16340 แต่ไม่แสดงความจำเพาะต่อเพศของกุ้งกุลาดำ นอกจากนี้ยังได้นำไพรเมอร์จำนวน 34 คู่ ที่พัฒนาจากเครื่องหมายเอเอฟแอลพี และจากยีนในกุ้งกุลาดำ กุ้งก้ามกราม และหอยเป๋าฮื้อ ที่เกี่ยวข้องกับระบบเพศของสิ่งมีชีวิตดังกล่าวมาทำการศึกษา พบว่า ยีน$TSP_{462F+288R}$, XNP-1, peritrophin, DSI, ZFP, PMO920 และ PMT1700 ในกุ้งกุลาดำ และ VCP2 ในหอยเป๋าฮื้อ ให้ความหลากหลายของรูปแบบผลิตภัณฑ์ที่ได้เมื่อวิเคราะห์ด้วยเทคนิค SSCP แต่ยังคงไม่จำเพาะต่อเพศของกุ้งกุลาดำ จึงนำเครื่องหมาย PMO920, PMT1700 , ZFP และ DSI มาศึกษาความหลากหลายทางพันธุกรรมของกุ้งกุลาดำจากธรรมชาติ จำนวน 5 แหล่ง (สตูล ตรัง พังงา ชุมพร และ ตราด) พบว่าเครื่องหมายเหล่านี้ให้ความหลากหลายทางพันธุกรรมสูงในกุ้งกุลาดำที่ทำการศึกษา โดยมีจำนวนอัลลีลต่อตำแหน่งคือ 6, 5, 12 และ 19 อัลลีล ตามลำดับและมีค่า observed heterozygosity ของ PMO920, PMT1700 และ ZFP เท่ากับ 0.3043 – 0.5128, 0.3462 – 0.4643 และ 0.5000 – 0.8108 ตามลำดับ จากการวิเคราะห์ Linkage disequilibrium แสดงให้เห็นว่าจีโนไทป์ของยีนดังกล่าวมีการแยกตัวแบบสุ่ม ($P > 0.05$) ผลการวิเคราะห์จาก PMO920 และ ZFP พบว่าประชากรในจังหวัดตรังและตราด ( $P = 0.0002$ และ $P = 0.0005$; homozygote excess) และประชากรในจังหวัดสตูลและพังงา ($P = 0.0047$ และ $P = 0.0175$ ; heterozygote excess) มีค่าเบี่ยงเบนจากสมดุล Hardy-Weinberg อย่างมีนัยสำคัญทางสถิติ ตามลำดับ นอกจากนี้ยังพบความแตกต่างทางพันธุกรรมในระดับต่ำระหว่างกลุ่มประชากรกุ้งกุลาดำในประเทศไทย ($P = 0.0077 - 0.0178$) เมื่อสร้างแผนภูมิ neighbor-joining tree จากค่าเฉลี่ยความแตกต่างทางพันธุกรรมของ PMO920, PMT1700, ZFP และ DSI พบว่าสามารถแบ่งกุ้งกุลาดำที่ทำการศึกษาออกเป็น 2 กลุ่ม กลุ่มที่ 1 ประกอบด้วย สตูล ตรัง และพังงา และกลุ่มที่ 2 ประกอบด้วย ชุมพร และ ตราด จากการทดสอบทางสถิติพบความแตกต่างทางพันธุกรรมอย่างมีนัยสำคัญทางสถิติระหว่างกลุ่มตัวอย่าง สตูล-ตราด และ สตูล-พังงา ในยีน ZFP ($P < 0.05$) นอกจากนี้ยังพบความถี่อัลลีลจำนวน 7 อัลลีลของยีน DSI ที่มีรูปแบบการกระจายตัวที่แสดงความแตกต่างทางพันธุกรรมในกลุ่มตัวอย่างที่ศึกษาทั้งหมดอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) แสดงให้เห็นถึงการแบ่งแยกประชากรทางพันธุกรรมของกุ้งกุลาดำในประเทศไทย

ลายมือชื่อนิสิต……………………………..

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

ปีการศึกษา……2547……………………..ลายมือชื่ออาจารย์ที่ปรึกษาร่วม………………………….

# # 4472465523 : MAJOR BIOTECHNOLOGY

KEY WORD : *Penaeus monodon* / GIANT TIGER SHRIMP / AFLP / SSCP

SUPAPORN THUMRUNGTANAKIT : IDENTIFICATION OF MOLECULAR GENETIC MARKERS INVOLVED IN SEX DETERMINATION IN THE GIANT TIGER SHRIMP *Penaeus monodon* USING AFLP ANALYSIS THESIS ADVISOR : ASSOC. PROF. PADERMSAK JARAYABHAND , CO-ADVISOR : Dr. SIRAWUT KLINBUNGA,

149 pp. ISBN 974-17-6097-3.

AFLP analysis was used to isolate sex-specific markers in *P. monodon*. A total of 256 primer combinations were tested against 6-10 bulked genomic DNA of *P. monodon*. Five (FE10M9520, FE10M10725, FE14M16340, FE15M14400 and FE16M8350) and one (ME10M8420) candidate female and male-specific AFLP markers were identified. The former markers were cloned and further characterized. SCAR markers derived from FE10M9520, FE10M10725.1, FE10M10725.2, FE14M16340 did not retain the original sex specificity. SSCP analysis was applied to identify whether fixed SNP was existent in SCAR markers amplified from male and female *P. monodon*. Polymorphic but not sex-linked pattern were found from FE10M10725.1 and FE14M16340-derived SCAR markers. Additionally, thirty-four primers pairs derived from sex-related AFLP and cDNA fragments of *P. monodon*, *M. rosenbergii* and *H. asinina* from previous studies were tested. Polymorphic SSCP markers that are not linked to sexes of *P. monodon* ($TSP_{462F+288R}$, XNP-1, peritrophin, DSI, ZFP, PMO920 and PMT1700 from *P. monodon* and VCP2 from *H. asinina*) were found. Four polymorphic markers (PMO920, PMT1700, ZFP and DSI) were selected for population genetic studies of natural *P. monodon* in Thai waters. Relative high genetic diversity was found at these loci. The number of allele per locus was 6, 5, 12 and 19 respectively. The observed heterozygosity was 0.3043 – 0.5128, 0.3462 – 0.4643 and 0.5000 – 0.8108 for PMO920, PMT1700 and ZFP, respectively. Linkage disequilibrium analysis indicated that genotypes of these loci segregated randomly ($P > 0.05$). Significant deviation from Hardy-Weinberg expectation was observed in Trang and Trad at PMO920 ($P = 0.0002$ and $P = 0.0005$ ; homozygote excess) and Satun and Phangnga ($P = 0.0047$ and $P = 0.0175$ ; heterozygote excess). Low genetic distance was found between pairs of geographic samples ($P = 0.0077 – 0.0178$). The neighbor-joining tree constructed from the average genetic distance of overall loci allocated Satun, Trang and Phangnga into one cluster and Chumphon and Trad into the other cluster. Population differentiation between Satun – Trad and Satun – Phangnga was found at ZFP ($P < 0.05$). In addition, seven polymorphic alleles of DSI revealed significant distribution patterns across overall samples ($P < 0.05$) implying low but significant genetic heterogeneity of *P. monodon* in Thailand.

Student's signature……………………..

Field of study…Biotechnology….. Advisor'signature…………………………………………….

Academic year…….2004…………Co-advisor's signature………………………………………

# ACKNOWLEDGMENTS

I would like to express my deepest sense of gratitude to my advisor, Associate Professor Dr. Padermsak Jarayabhand and my co-advisor, Dr. Sirawut Klinbunga for their guidances, encouragement, valuable suggestion and supports throughout my study.

The special thanks are also extended to Professor Dr. Piamsak Menasveta for supporting the laboratory at Marine Biotechnology Reserch Unit (MBRU) and Dr. Sirawut Klinbunga for his help in data computerized analysis suggestions.

My gratitude is also extended to Assistant Professor Dr. Charoen Nitithamyong, Assistant Professor Dr. Thaithaworn Lirdwitayaprasit and Dr. Narongsak Puanglarp for serving as thesis committee, for their recommendations and also useful suggestion.

I wish to acknowledge to Marine Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC) for my financial support.

Thanks are also express to Mr. Piti Amparyup for his training in the laboratory and all my friends of the Biotechnology for their helps in the laboratory and friendships.

Finally, I would like to express my deepest gratitude to my parents and my brothers for their love, care, understanding and encouragement extended throughout my study.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

# CONTENTS


| | Page |
|---|---|
| THAI ABSTRACT………………………………………………………...……... | iv |
| ENGLISH ABSTRACT……………………………………………………….... | v |
| ACKNOWLEDGMENTS..……………………………………………..…..... | vi |
| CONTENTS……………………………………………………………….…. | vii |
| LIST OF TABLES……………………………………………………….... | x |
| LIST OF FIGURES……………………………………………………..…. | xii |
| LIST OF ABBREVIATIONS…………………………………………..…. | xx |
| | |
| CHAPTER I INTRODUCTION | |
| 1.1 General introduction……………………………………...….…. | 1 |
| 1.2 Taxonomy of *P. monodon*……………………………………….…. | 3 |
| 1.3 Molecular technique commonly used for isolation of genetic markers at the DNA level ……………………..........…………..... | 5 |
| 1.4 Studies of sex-determination/differentiation marker in animals …... | 18 |
| 1.5 Population genetic studies of *P. monodon*…………………………. | 23 |
| 1.6 Objectives of the thesis…………………………………………….. | 33 |
| | |
| CHAPTER II MATERIALS AND METHODS | |
| 2.1 Experimental animals ……………………………………………… | 34 |
| 2.2 DNA extraction ……………………………………………. | 34 |
| 2.3 Measuring DNA concentrations using spectrophotometer and electrophoresis ………………………………………………….. | 35 |
| 2.4 Bulked segregant analysis (BSA) of *P. monodon* genomic DNA … | 36 |
| 2.5 Amplified fragment length polymorphism (AFLP) analysis………. | 37 |
| 2.5.1 Restriction enzyme digestion and adapter ligation……... | 37 |
| 2.5.2 Preamplification……………………………………………… | 37 |
| 2.5.3 Selective amplification………………………………….. | 37 |
| 2.6 Agarose gel electrophoresis ……………………………………. | 39 |
| 2.7 Denaturing Polyacrylamide Gel Electrophoresis…………………... | 39 |
| 2.7.1 Preparation of glass plate………………………………. | 39 |

**CONTENTS (cont.)**


**Page**

2.7.2 Preparation of denaturing polyacrylamide gel lectrophoresis 40

2.7.3 Electrophoresis………………………………………………….. 40

2.8 Silver staining…………………………………………………………….. 41

2.9 Cloning of AFLP fragments ……………………………………...….. 41

2.9.1 Elution of DNA from polyacrylamide gels……………….. 41

2.9.2 Elution of DNA from agarose gels ……………………….. 42

2.9.3 Ligation of PCR product to vector………………………… 42

2.9.4 Preparation of competent cells………………………….. 43

2.9.5 Transformation of the ligation product to *E. coli* host cells 43

2.9.6 Detection of recombinant clone by colony PCR………….. 44

2.9.7 Isolation and digestion of recombinant plasmid DNA……. 44

2.9.8 Digestion of the amplified DNA insert……………………. 45

2.9.9 DNA sequencing……………………………………………... 45

2.10 PCR amplification of derived candidate sex-specific AFLP markers in *P. monodon* and sex related genes in *P. monodon* and other related species……………………………………………… 45

2.11 Preparation of SSCP gel ………………………………………… 55

2.12 Preparation of denaturing polyacrylamide gels…………………. 55

2.13 Nuclear DNA polymorphism analyzed by SSCP and denaturing PAGE analysis…………………………………………………… 55

2.14 Data analysis…………………………………………………….. 56

CHAPTER III RESULTS

3.1 DNA extraction………………………………………………… 58

3.2 AFLP analysis………………………………………............… 58

3.3 Cloning and characterization of candidate sex-specific AFLP markers……………………………………………………… 79

3.4 Development of sex-specific SCAR marker in *P. monodon*.......... 90

3.5 Identification and characterization of sex-related genes in *P. monodon* using PCR and SSCP analysis……………………… 90

3.6 Identification of single nucleotide polymorphism (SNP) using SSCP analysis…………………………………………………… 103

**CONTENTS (cont.)** page


3.7 Genetic diversity and population differentiation of *P. monodon* using nuclear DNA polymorphism…………………………….. 111

CHAPTER IV DISCUSSION………………………………….………...... 121

CHAPTER V CONCLUSION……………………………………………… 131

REFERENCES…………………………….………………………………. 133

APPENDICES…………………………………………………………...…. 141

APPENDIX A……………………………………………………………… 142

APPENDIX B……………………………………………………………….. 143

Biography………………………………………………………………….. 149

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

## LIST OF TABLES

| | | Page |
|---|---|---|
| **Table 1.1** | Thai Frozen Shrimp Export in 2001……...................……… | 2 |
| **Table 2.1** | Specimens used for construction of male and female bulked DNA for isolation of sex-specific AFLP markers of *P. monodon* ……………………………………………………. | 36 |
| **Table 2.2** | AFLP primers and their sequences used for identification of sex-specific markers in *P. monodon*…………………………… | 38 |
| **Table 2.3** | Primers and primer sequences and melting temperature of primers designed from AFLP markers and cDNAs of *P. monodon*)…………………………………………………… | 47 |
| **Table 2.4** | Primers and primer sequences and melting temperature of heterologous primers designed from sequences of candidate sex-specific AFLP markers in *M. rosenbergii* and cDNAs of *H. asinina*………………………………………………………. | 49 |
| **Table 2.5** | PCR conditions used for amplification of candidate sex-specific/differential expression markers of *P. monodon* using primers in Table 2.3……………………………………………. | 51 |
| **Table 2.6** | PCR conditions used for amplification of candidate sex-specific/differential expression markers of *P. monodon* using heterospecific primers in Table 2.4………………………….. | 53 |
| **Table 3.1** | Candidate sex-specific markers of *P .monodon* generated by AFLP using primers $E_{+3}$-1 - 8 +$M_{+3}$ -1 - 8…………………… | 67 |
| **Table 3.2** | Candidate sex-specific markers of *P. monodon* generated by AFLP using primer combination $E_{+3}$-1 - 8/$M_{+3}$-1 - 8………… | 70 |
| **Table 3.3** | Candidate sex-specific markers of *P. monodon* generated by AFLP using primer combination $E_{+3}$-1 - 8/$M_{+3}$-9 - 16……….. | 71 |
| **Table 3.4** | Candidate sex-specific markers of *P. monodon* generated by AFLP using primer combination $E_{+3}$-9 - 16/$M_{+3}$-1 - 8……….. | 78 |
| **Table 3.5** | Candidate sex-specific markers of *P. monodon* generated by AFLP using primer combination $E_{+3}$-9 - 16/$M_{+3}$-9 - 16……… | 77 |
| **Table 3.6** | Conclusion of name of primer combination and size of marker of sex-specific AFLP marker generated by AFLP analysis…… | 79 |

**LIST OF TABLES (cont.)**


| | | Page |
|---|---|---|
| **Table 3.7** | Different restriction patterns of colony PCR found from a single insert……………………………………………………. | 82 |
| **Table 3.8** | Characterization of female-specific AFLP marker of *Penaeus monodon*……………………………………………………. | 89 |
| **Table 3.9** | Characterization of sex-related genes in *P. monodon* using PCR and SSCP analysis………………………………………. | 101 |
| **Table 3.10** | Characterization of sex-related gene homologues in *P. monodon* using heterospecific primers from the tropical abalone (*H. asinina*)…………………………………………. | 102 |
| **Table 3.11** | Allele frequencies, observed and unbiased expected heterozygosities and Hardy-Weinberg disequilibrium at PMO920, PMT1700 and zinc finger protein loci of *P. monodon* from different geographic locations of Thailand……. | 113 |
| **Table 3.12** | Allele frequencies resulted from analysis of DSI of *P. monodon* from different geographic locations of Thailand……. | 116 |
| **Table 3.13** | Genotypic disequilibrium between pairs of loci in each geographic sample of *P. monodon* in Thailand……………….. | 117 |
| **Table 3.14** | Genotypic disequilibrium between pairs of loci across overall samples of *P. monodon* in Thailand…………………………… | 118 |
| **Table 3.15** | Pairwise genetic distance between *P. monodon* originating from different geographic locations in Thailand analyzed by PMO920, PMT1700, ZFP and DSI……………………………. | 118 |
| **Table 3.16** | Pairwise comparison of genetic differentiation between geographic samples of *P. monodon* at 3 loci (PMO920, PMT1700 and zinc finger protein) using geographic heterogeneity (exact test) and $F_{ST}$ analyses…………………… | 120 |
| **Table 3.17** | Genetic differentiation of overall investigated sample of *P. monodon* at the DSI locus using geographic heterogeneity (exact test) and $F_{ST}$ analyses…………………………………… | 120 |

# LIST OF FIGURES


| | | Page |
|---|---|---|
| **Figure 1.1** | Illustration showing external anatomy of *P. monodon* ...………….. | 4 |
| **Figure 1.2** | Illustration showing petasma and thelycum used for identification of male and female *P. monodon* ……………………..…..…..… | 4 |
| **Figure 1.3** | General illustration of the polymerase chain reaction for amplifying DNA (after Oste, 1988)………………………………. | 6 |
| **Figure 1.4** | A schematic diagram illustrating principles of PCR-RFLP analysis | 8 |
| **Figure 1.5** | Schematic presentation of the RAPD procedure. Genomic DNA (indicated by long strings of lines) is used for PCR using an arbitrary short primers of identical sequences …………………… | 10 |
| **Figure 1.6** | Molecular basis of RAPD polymorphism. (A) Base substitutions in the primer binding sites, especially at the 3′ end of the primer binding sites may lead to decrease (as shown) or increase of the number of RAPD bands. (B) Insertion or deletion between two primers may lead to increase or decrease of fragment sizes………. | 10 |
| **Figure 1.7** | A schematic diagram illustrating principles of AFLP analysis……. | 12 |
| **Figure 1.8** | Automated DNA sequencing………………………………….. | 14 |
| **Figure 1.9** | A flow chart illustrating the GenomeWalker protocol……………. | 15 |
| **Figure 1.10** | A schematic diagram of SSCP analysis…………………………… | 17 |
| **Figure 1.11** | Geographic distribution of mtDNA phylogenetic clusters A and B among fourteen geographic samples of *P. monodon* analyzed by 10 polymorphic restriction enzyme…………………………… | 26 |
| **Figure 1.12** | Geographic distribution of mtDNA phylogenetic clusters A and B among fourteen geographic samples of *P. monodon* analyzed by 5 polymorphic restriction enzyme…………………………… | 27 |
| **Figure 1.13** | A UPGMA dendrogram showing relationships among 37 composite haplotypes found in Thai *P. monodon* based on sequence divergence between pairs of composite haplotypes…….. | 28 |
| **Figure 3.1** | A 0.8% ethidium bromide stained-agarose gel showing the quality of genomic DNA extracted from the pleopod of *P. monodon* …….. | 59 |
| **Figure 3.2** | A 0.8% ethidium bromide stained-agarose gel showing the quality | |

## LIST OF FIGURES (cont.)

**Page**

of genomic DNA extracted from pooled pleopods of *P. monodon* 59

**Figure 3.3** A 1.8% agarose gel electrophoresis showing preamplification of bulked female DNA (lanes 2 – 5) and bulked male DNA (lanes 7 – 10). Lane M = a 100 bp DNA marker…………………………….. 60

**Figure 3.4** A 1.8% agarose gel electrophoresis showing selective amplification products of bulked PMF1 (odd lanes) and bulked PMM1 (even lanes) using various primer combinations………….. 60

**Figure 3.5** 1.5% agarose gel electrophoresis showing selective amplification products of bulked PMF1 (lanes 1, 5, and 9), PMF2 (lanes 2, 6 and 10), PMM1 (lanes 3, 7 and 11) and PMM2 (lanes 4, 8 and 12) using primers $E_{+3}$-3/$M_{+3}$-6………………………………………… 61

**Figure 3.6** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products from 2 bulked DNA (PMF1 and PMM1) using $E_{+3}$-8/$M_{+3}$-3 (lanes 1 – 2)……………………………………… 62

**Figure 3.7** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of bulked PMF1 (lanes 1, 5, 9, 13 and 17), PMF2 (lanes 2, 6, 10, 14 and 18), PMM1 (3, 7, 11, 15 and 19) and PMM2 (lanes 4, 8, 12, 16 and 20) using primers $E_{+3}$-3/$M_{+3}$-6 (lanes 1 – 4) 63

**Figure 3.8** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP product of 4 bulked DNA; PMF1 (lanes 1, 5, 9, 13 and 17), PMF2 (lanes 2, 6, 10, 14 and 18), PMM1 (3, 7, 11, 15 and 19) and PMM2 (lanes 4, 8, 12, 16 and 20) using primers $E_{+3}$-8/$M_{+3}$-5 (lanes 1 – 4)……………………………………………………….. 64

**Figure 3.9** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP product of 4 bulked DNA; PMF1 (lanes 1, 5, 9, 13 and 17), PMF2 (lanes 2, 6, 10, 14 and 18), PMM1 (3, 7, 11, 15 and 19) and PMM2 (lanes 4, 8, 12, 16 and 20) using primers $E_{+3}$-3/$M_{+3}$-3 (lanes 1 – 4)……………………………………………………… 65

**Figure 3.10** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP product of 4 bulked DNA; PMF1 (lanes 1, 5, 9, 13 and 17), PMF2 (lanes 2, 6, 10, 14 and 18), M1 (3, 7, 11, 15 and 19) and M2

**LIST OF FIGURES (cont.)**

Page

(lanes 4, 8, 12, 16 and 20) using primers $E_{+3}$-8/$M_{+3}$-3 (lanes 1 – 4) 66

**Figure 3.11** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 6 bulked DNA; PMF1 (lanes 9 and 15), PMF2 (lanes 10 and 16), PMF3 (lanes 11 and 17), PMM1 (lanes 12 and 18), PMM2 (lanes 13 and 19), PMM3 (lanes 14 and 20) using primers $E_{+3}$-10/$M_{+3}$-4 (lanes 9 – 14)…………………………… 72

**Figure 3.12** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 10 bulked DNA; PMF1 – 5 (lanes 1 – 5), PMM1 – 5(lanes 6 – 10) using primers $E_{+3}$10/$M_{+3}$-9…………….. 73

**Figure 3.13** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 10 bulked DNA; PMF1-BF5 (lanes1 – 5), PMM1-PMM5 (lanes 6 – 10) using primers $E_{+3}$-10/$M_{+3}$-10……… 74

**Figure 3.14** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 10 bulked DNA; PMF1 – PMF5 (lanes1 – 5), PMM1–PMM5 (lanes 6 – 10) using primers $E_{+3}$-14/$M_{+3}$-15……… 75

**Figure 3.15** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 6 bulked DNA; PMF1 (lanes 1, 7 and 13), PMF2 (lanes 2, 8 and 14), PMF3 (lanes 3, 9 and 15), PMM1 (lanes 4, 10 and 16), PMM2 ( lanes 5, 11 and 17), PMM3 ( lanes 6, 12 and 18) using primers $E_{+3}$-15/$M_{+3}$-14…………………………… 76

**Figure 3.16** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 6 bulked DNA; PMF1 (lanes 1, 7 and 13), PMF2 (lanes 2, 8 and 14), PMF3 (lanes 3, 9 and 15), PMM1 (lanes 4, 10 and 16), PMM2 ( lanes 5, 11 and 17), PMM3 ( lanes 6, 12 and 18) using primers $E_{+3}$-10/$M_{+3}$-8……………………… 77

**Figure 3.17** Reamplification of candidate female-specific AFLP fragments (FE10M9520, lane 1; FE10M10725, lane 2, FE14M16340, lane 3 and ME10M8420, lane 5).Lane M = 100 bp DNA marker………. 80

**Figure 3.18** Colony PCR product of recombinant clones containing a FE10M9520 fragment (lanes 1 – 12). Lane M = A 100 bp DNA ladder………………………………………………………….. 80

## LIST OF FIGURES (cont.)

**Page**

**Figure 3.19** A 1.0% agarose gel electrophoresis showing restriction patterns of colony PCR product of the FE10M9520 insert digested with *Hind*III (lanes 1 – 6, panel A and B) and *Rsa* I (lanes 7 – 12, A and B). Lane M = a 100 bp DNA ladder………………………………… 81

**Figure 3.20** A 1.0% agarose gel electrophoresis showing restriction patterns of *EcoR*I-digested plasmid DNA from FE10M9520 (lanes 4 – 5, A) 81

**Figure 3.21** Nucleotide sequences of FE10M9520**.** Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced ……………………………………… 83

**Figure 3.22** Nucleotide sequences of FE10M10725. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced ……………………………… 84

**Figure 3.23** Nucleotide sequences of FE14M16340. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced ……………………….……… 85

**Figure 3.24** Nucleotide sequences of FE15M14400. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced ……………………….……… 86

**Figure 3.25** Nucleotide sequences of FE16M8350. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced ……………………………………… 87

**Figure 3.26** Nucleotide sequences of MF10M8420. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced ………………………………….. 88

**Figure 3.27** A 1.6% agarose gel electrophoresis showing the PCR product of a SCAR marker derived from FE10M9520 against female (lanes 1 – 4) and male (lanes 5 – 8) genomic DNA of *P. monodon*. A 100 bp ladder was used as the DNA markers………………………….. 91

**Figure 3.28** A 1.6% agarose gel electrophoresis showing the PCR product of SCAR markers derived from FE10M10725 (A) and FE14M16340 (B) against female (lanes 1 – 4) and male (lanes 5 – 8) genomic

**LIST OF FIGURES (cont.)**

**Page**

DNA of *P. monodon*…………………………………………………. 91

**Figure 3.29** A 1.6% agarose gel electrophoresis showing the PCR products of SCAR markers derived from FE10M10725.2, FE15M14400 and FE16M8350……………………………………………………. 92

**Figure 3.30** A 1.6% agarose gel electrophoresis showing the PCR product amplified from genomic DNA of female (lanes 1 – 6) and male (lanes 8 – 13) of *P. monodon* using primers $TSP_{462F+288R}$. Lane M is a 100 bp DNA marker………………………………………… 94

**Figure 3.31** A 1.6% agarose gel electrophoresis showing restriction patterns of *Ssp* I-digested PCR product of female (lanes 1 – 2) and male (lanes 3 – 4) *P. monodon* amplified by primers $TSP_{462F+288R}$. Lanes M is a 100 bp DNA marker…………………………………………… 94

**Figure 3.32** A 1.6% agarose gel electrophoresis showing XNP-1 amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon*. A 100 bp ladder was used as the DNA markers…….. 95

**Figure 3.33** A 1.6% agarose gel electrophoresis showing peritrophin1 amplified from genomic DNA of female (lanes 1 – 2) and male (lanes 3 – 4) *P. monodon*. A 100 bp ladder was used as the DNA markers…………………………………………………………. 95

**Figure 3.34** A 1.6% agarose gel electrophoresis showing Peritrophin2 amplified from genomic DNA of female (lanes 2 – 3) and male (lanes 6 – 7) *P. monodon*. Lanes M and 8 are a 100 bp DNA ladder and the negative control, respectively…………………………….. 96

**Figure 3.35** A 1.8% agarose gel electrophoresis showing PMX amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon*. A 100 bp ladder was used as the DNA markers lane M).. 96

**Figure 3.36** A 1.6% agarose gel electrophoresis showing DSI amplified from genomic DNA of female (lanes 1 – 18, panel A) and male (lanes 1 – 18, B) *P. monodon.* A 100 bp ladder was used as the DNA markers (lanes M)……………………………………………….. 97

## LIST OF FIGURES (cont.)

**Page**

**Figure 3.37** A 1.6% agarose gel electrophoresis showing a zinc finger protein gene amplified from genomic DNA of female (lanes 1 – 18, panel A) and male (lanes 1 – 18, B) *P. monodon*. A 100 bp ladder was used as the DNA markers (lanes M)………………………………… 97

**Figure 3.38** A 1.6% agarose gel electrophoresis showing PMT1700 amplified from genomic DNA of female (lanes 1 – 18, panel A) and male (lanes 1 – 18, B) *P. monodon.* A 100 bp ladder was used as the DNA markers (lanes M)………………………………………… 98

**Figure 3.39** A 1.6% agarose gel electrophoresis showing PMO920 amplified from genomic DNA of female (lanes 1 – 18, panel A) and male (lanes 1 – 18, B) *P. monodon.* A 100 bp ladder was used as the DNA markers (lanes M)…………………………………………… 98

**Figure 3.40** A 1.6% agarose gel electrophoresis showing EGF-response factor 1 amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon*……………………………………… 99

**Figure 3.41** A 1.6% agarose gel electrophoresis of the PCR product resulted from amplification of female (lanes 1 – 4) and male (lanes 5 – 8) genomic DNA of *P. monodon* with heterospecific primers VCP2. 99

**Figure 3.42** A 1.6% agarose gel electrophoresis showing the PCR product of female (lanes 5 and 6, panels A and B) and male (lanes 7 and 8; panels A and B) *P. monodon* amplified by SARIP-F/R (A) and FP-F/R (B). Lanes M is a 100 bp DNA marker……………………….. 100

**Figure 3.43** SSCP patterns of a SCAR marker derived from FE10M9520 of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* resulted from size-fractionation through 15%PAGE + 5%glycerol……….. 104

**Figure 3.44** SSCP patterns of a SCAR marker derived from FE10M10725.1 of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* resulted from size-fractionation through 15%PAGE + 5%glycerol……….. 104

**LIST OF FIGURES (cont.)**


| | | Page |
|---|---|---|
| **Figure 3.45** | SSCP patterns of a SCAR marker derived from FE14M16340 of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* resulted from size-fractionation through 17.5%PAGE…………………….. | 105 |
| **Figure 3.46** | SSCP patterns of a SCAR marker derived from FE10M10725.2 of female (lanes 2 – 8) and male (lanes 9 – 15) *P. monodon* resulted from size-fractionation through was size-fractionated through 12.5%PAGE……………………………………………………. | 105 |
| **Figure 3.47** | SSCP analysis of the PCR product amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) of *P. monodon* using primers $TSP_{462F+288R}$ The PCR product was denatured and size-fractionated through a 12.5% native polyacrylamide gel (37.5:1)…………………………………………………………… | 106 |
| **Figure 3.48** | SSCP analysis of XNP-1 amplified from genomic DNA of female (lanes 1 – 5) and male (lanes 6 – 10) *P. monodon*. The amplified XNP-1 was denatured and size-fractionated through 15% native polyacrylamide gel (37.5:1)…………………………………….. | 106 |
| **Figure 3.49** | SSCP analysis of Peritrophin-1 amplified from genomic DNA of female (lanes 1 – 5) and male (lanes 6 – 10) *P. monodon*. The amplified Peritrophin-1 was denatured and size-fractionated through 12.5% native polyacrylamide gel + 5% glycerol (37.5:1) | 107 |
| **Figure 3.50** | SSCP analysis of the PCR product amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* using VCP2 primers. The PCR product was denatured and size-fractionated through a 12.5% native polyacrylamide gel (37.5:1)… | 107 |
| **Figure 3.51** | SSCP analysis of the PCR product amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* using primers SARIP-F/R. The PCR product was denatured and size-fractionated through a 15% native polyacrylamide gel (37.5:1)…. | 108 |
| **Figure 3.52** | SSCP analysis of the PCR product amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* using primers FP-F/R. The PCR product was denatured and size- | |

**LIST OF FIGURES (cont.)**

Page

fractionated through a 20% native polyacrylamide gel (37.5:1)…... 108

**Figure 3.53** SSCP patterns of the amplified DSI of *P. monodon* individuals (lanes 1 – 17) size-fractionated through 17.5% non-denaturing PAGE (acrylamide: bisacrylamide = 75:1)………………………... 109

**Figure 3.54** SSCP patterns of the amplified Zinc finger protein gene of *P. monodon* individuals (lanes 1 - 17) size-fractionated through 15% non-denaturing PAGE (acrylamide:bisacrylamide = 75:1)………... 109

**Figure 3.55** SSCP patterns of the amplified PMT1700 of *P. monodon* individuals (lanes 1 – 17) size-fractionated through 15% non-denaturing PAGE (acrylamide:bisacrylamide = 75:1)…………… 110

**Figure 3.56** Patterns of the amplified PMO920 of *P. monodon* individuals (lanes 1 – 17) size-fractionated through 6% denaturing PAGE (19:1)……………………………………………………………… 110

**Figure 3.57** Diagram showing SSCP pattern of ZFP…………………………… 112

**Figure 3.58** A neighbor-joining tree indicating genetic relationship between each geographic sample of *P. monodon* analyzed by polymorphism of PMO920, PMT1700, zinc finger protein gene and DSI……….. 119

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

## LIST OF ABBREVIATIONS

| | |
|---|---|
| bp | base pair |
| °C | degree Celcius |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytosine triphosphate |
| dGTP | deoxyguanosine triphosphate |
| dTTP | deoxythymidine triphosphate |
| DNA | deoxyribonucleic acid |
| HCl | hydrochloric acid |
| IPTG | isopropyl-thiogalactoside |
| Kb | kilobase |
| M | Molar |
| $MgCl_2$ | magnesium chloride |
| mg | Milligram |
| ml | Millilitre |
| mM | Millimolar |
| ng | Nanogram |
| OD | optical density |
| PCR | polymerase chain reaction |
| RNA | Ribonucleic acid |
| RNase A | ribonuclease A |
| rpm | revolution per minute |
| SDS | sodium dodecyl sulfate |
| Tris | tris (hydroxyl methyl) aminomethane |
| µg | Microgram |
| µl | Microlitre |
| µM | Micromolar |
| UV | ultraviolet |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

# CHAPTER I

# INTRODUCTION

## 1.1 General introduction

The giant tiger shrimp (*Penaeus monodon*) is one of the most economically important penaeid species in Thailand and South East Asian countries. The world cultured shrimp production in 2000 were accounts for 56% by *P. monodon*, 17% by *P. merguiensis*, 16% by *P. vannamei* and 11% by other species (Rosenberry, 2001). Farming of *P. monodon* has achieved a considerable economic and social importance in the region, constituting a significant source of income and employment.

In Thailand, *P. monodon* have been intensively cultured for more than two decades. Approximately 60% of the total harvest shrimp comes from cultivation. Shrimp farms and hatcheries are scattered along the coastal areas of Thailand. Southern provinces (Nakorn Sri Thammarat and Surat Thani) account for the majority while those in the East (Chanthaburi) and Central regions (Samut Sakhon and Samut Songkhran) comprise the minority in terms of number. The intensive farming system (85%) has been used for *P. monodon* farming activity resulting in the consistent increase in the outcome production (Department of fisheries, 1999).

The success of tiger shrimp industry in Thailand has resulted in the steadily increased income for the nation. This has also elevated the quality of life for Thai farmers. The reasons for this are supported by several factors including the appropriate farming areas without the serious disturbing from typhoons or cyclone, small variable of seawater during seasons, and ideal soils for pond construction.

Thailand has been regarded as the leader for *P. monodon* production for over a decade. Thailand had a severe outbreak of diseases during 1995-1997 causing the decrease in the shrimp production at that period. Nevertheless, Thailand is still the largest *P. monodon* producer. Although the shrimp production from Thailand in 2001 was estimated to be approximately 280,000 metric tons, prawn farming presently faces several problems such as reduction of high quality wild broodstock, loss of the

cultured production due to bacterial and viral infections and size differences of cultivated *P. monodon* in the same cultured pond.

The United States of America and Japan are the major shrimp importers of shrimps (Table 1.1). Approximately 68% of *P. monodon* from Thailand are imported to these countries, worthing 38,859 million baht. The remaining important markets are Europe, Asian countries, Australia and others.

**Table 1.1** Thai Frozen Shrimp Export in 2001

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

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

## 1.2 Taxonomy of *P. monodon*

The taxonomic definition of the giant tiger shrimp, *P. monodon* is as follows (Bailey-Brook and Moss, 1992):

**Phylum** Arthropoda

**Subphylum** Crustacea

**Class** Malacostraca

**Subclass** Eumalacostraca

**Order** Decapoda

**Suborder** Natantia

**Infraorder** Penaeidea

**Superfamily** Penaeoidea

**Family** Penaeidae Rafinesque, 1985

**Genus** Penaeus Fabricius, 1798

**Subgenus** Penaeus

**Species** monodon

**Scientific name**: *Penaeus monodon* (Fabricius), 1798 (Figure 1.1)

**Common name**: giant tiger prawn or black tiger shrimp

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

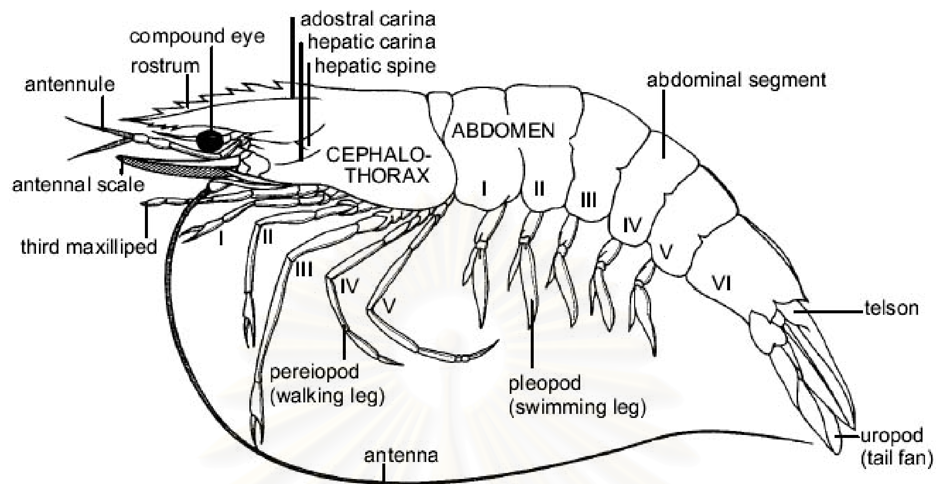



**Figure 1.1** Illustration showing external anatomy of *P. monodon*

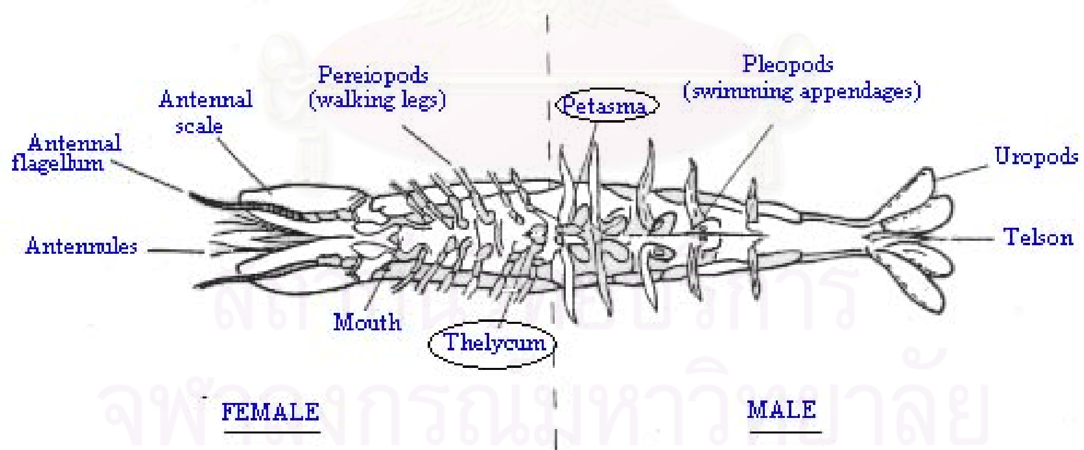



**Figure 1.2.** Illustration showing petasma and thelycum used for identification of male and female *P. monodon*

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

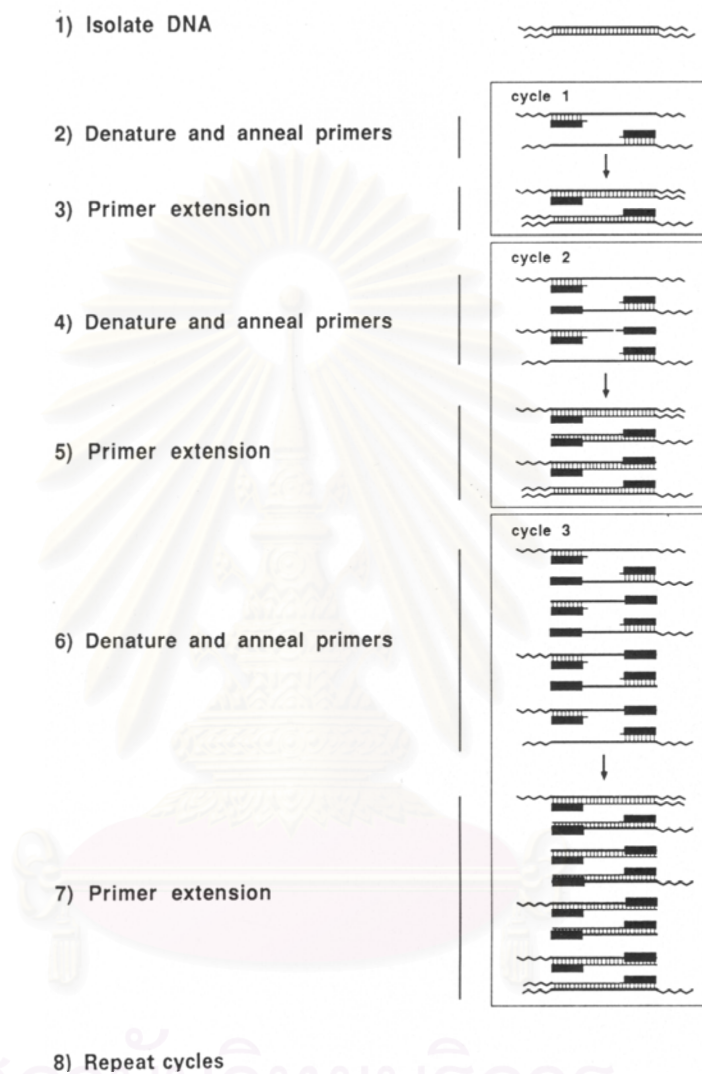
**1.3 Molecular technique commonly used for isolation of genetic markers at the DNA level**

The introduction of the polymerase chain reaction (PCR) by Mullis et al. (1987) has opened a new approach for molecular genetic studies. PCR is a method for an *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. Million copies of the target DNA sequence can be synthesized from a low amount of starting DNA template within a few hours. The PCR reaction components are constituting of DNA template, a pair of primers for the target sequence, dNTPs (dATP, dCTP, dGTP and dTTP), buffer and heat-stable DNA polymerase (usually *Taq* polymerase). The amplification reaction consists of three steps; denaturation of double stranded DNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal temperature, and extension of the annealed primers by heat-stable DNA polymerase. The cycle is repeated for 30-40 times (Figure1.3). The amplification product is determined by electrophoresis.

Molecular markers are useful for genetic studies of various organisms. Several molecular techniques can be used for identification of genetic markers at the genomic DNA and the cDNA level for different applications. The former level includes restriction fragment length polymorphism-PCR (RFLP-PCR), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single strand conformational polymorphism (SSCP), DNA sequencing and genome walking analysis. The latter level includes reverse transcription (RT)-PCR, differential display (DD-PCR), RNA arbitrary primed PCR (RAP-PCR) and Expressed Sequence Tags (ESTs).

**1.3.1 PCR-restriction fragment length polymorphism (PCR-RFLP)**

Restriction fragment length polymorphism (RFLP) analysis is indirectly used to determine genetic variation at the DNA level by comparison of shared restriction fragments or sites. Basically, variation in restriction enzyme cleavage sites (usually detected by Southern blot hybridization) generates size differences of the resulting fragments.



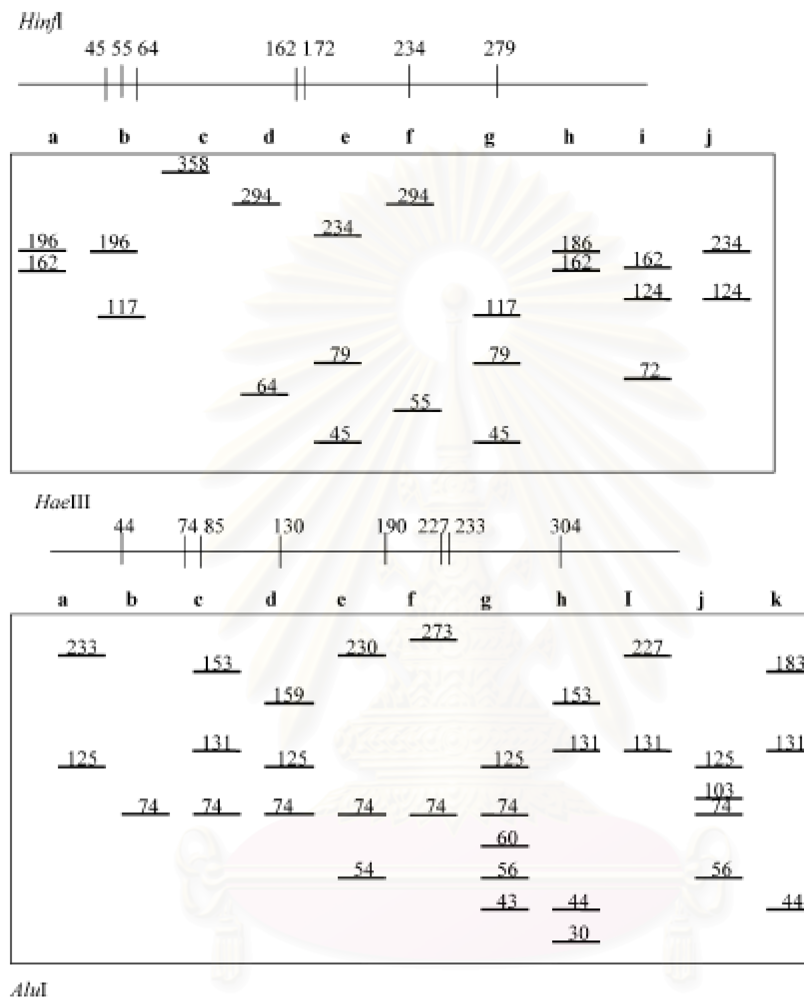

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Fig. 1.3** General illustration of the polymerase chain reaction for amplifying DNA.

Conventional RFLP analysis is carried out by digestion of genomic DNA with an appropriate restriction endonuclease followed by electrophoresis of digested DNA through the agarose gel. The fractionated DNA is then transferred to a supporting (nylon or nitrocellulose) membrane before hybridized with the specific labeled DNA probe. The results are visualized by autoradiography.

The PCR-amplified fragment can be included into the analysis by amplification of the target DNA fragment followed by digestion with restriction endonuclease (restriction site or fragment length polymorphism). The restricted fragments are fractionated in the agarose gel (or polyacrylamide gel), stained with ethidium bromide (or silver) and visualized by a UV transilluminator. The incorporation of PCR to RFLP (or site) analysis reduces time consuming and risks from using radiolabelled probe from hybridization-based RFLP analysis. Accordingly, PCR-RFLP has increasingly utilized and has replaced the use of the conventional RFLP method at present.

PCR-RFLP analysis is much simpler than the conventional RFLP approach. Therefore, it is one of the initial techniques widely used to indirectly detect genetic variation at the DNA level (Figure 1.4). It examines size variation of specific DNA fragments due to substitutions (transitions or transversions) at the recognizing sites of a particular restriction endonuclease. Different restriction patterns are created and can be used for evaluation of genetic diversity in various organisms. This technique has successfully been used for determination of genetic diversity and population differentiation of several marine species in Thai waters including; *P. monodon* (Klinbunga et al., 2001), cupped oyster of the genera *Crassostrea* and *Saccostrea* (Klinbunga et al., 2003) and abalone, *Haliotis asinina*, *H. ovina* and *H. varia* (Klinbunga et al., 2003).




สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Figure 1.4** A schematic diagram illustrating principles of PCR-RFLP analysis.

### 1.3.2 Randomly amplified polymorphic DNA (RAPD)-PCR analysis

RAPD was first independently developed by Welsh and McClelland 1990 and William et al., 1990. It is a PCR-based method but a short oligonucleotide primer (typically 10 bp long) which have random sequence and G+C content grater than 50% rather than a pair of specific primers is used in the reaction under low stringency conditions (Figure 1.5). The amplified fragments are those regions of the genome that are flanked by “inward-oriented” sequences complementary to the primer and a number of PCR products are generated from random locations within the genome.

Allelic variation of RAPD-PCR depends on the presence or absence of the particular amplification products, which can be separated on agarose gels stained with ethidium bromide. Polymorphic alleles may result from mutations of a primer recognition site which prevent its amplification or from insertion that change the size of DNA segment (William et al., 1990).

RAPD method is quick, simple, relative inexpensive and numerous markers can be developed easily by changing sequences or number of nucleotide in the primer. However, RAPD markers are mostly inherited in a dominant fashion. As a result, information on the parental origin of alleles may be inaccessible (Lewis and Snow, 1992). Owing to short length of primer and low stringency of PCR conditions, RAPD markers may produce some artifact of amplification products therefore careful control of DNA quality and amplification conditions is necessary to ensure reproducible banding patterns (Carson et al. 1991, Riedy et al., 1992, Scott et al., 1993).

The advantages of RAPD PCR are; first, RAPD analysis is a simple, rapid and inexpensive method for detecting DNA polymorphism at different taxonomic levels, second, RAPD does not require knowledge of the genome under investigation, third, RAPD is a PCR-based method and, therefore, requires only small quantity of DNA template per reaction. Forth, RAPD-PCR does not require the use of radiolabelled probes for hybridization. Finally, unlimited numbers of RAPD primers can be screened for suitable molecular markers of various applications within a short period of time.

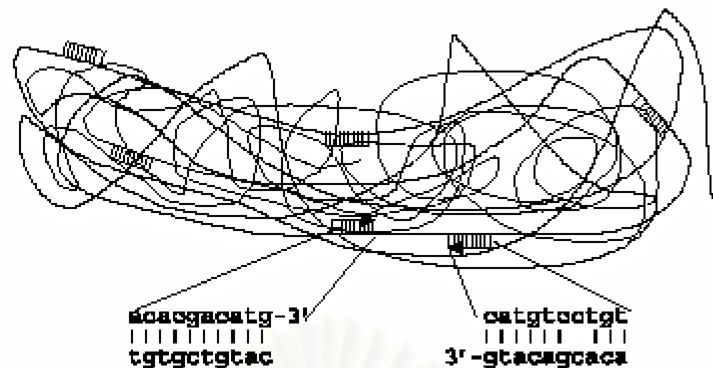


**Figure 1.5** Schematic presentation of the RAPD procedure. Genomic DNA (indicated by long strings of lines) is used for PCR using an arbitrary short primers of identical sequences (indicated by short segments annealing to their complementary sites in the genome either perfectly or non-perfectly) under low annealing temperatures. When the two primers bind to sites close enough (often less than 2000 base pairs) on opposite strands of DNA (indicated by arrowed segments with base pairing), a PCR product results.

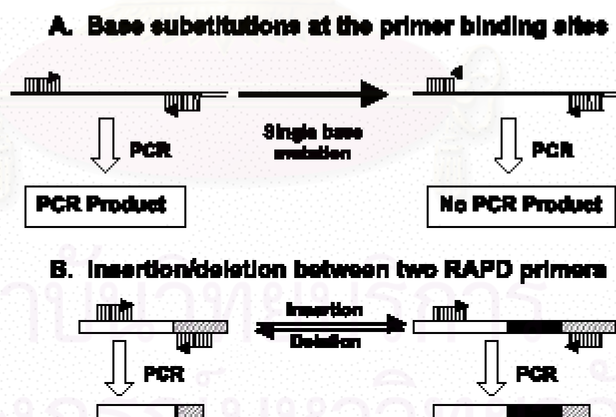



**Fig. 1.6** Molecular basis of RAPD polymorphism. (A) Base substitutions in the primer binding sites, especially at the 3′ end of the primer binding sites may lead to decrease (as shown) or increase of the number of RAPD bands. (B) Insertion or deletion between two primers may lead to increase or decrease of fragment sizes.

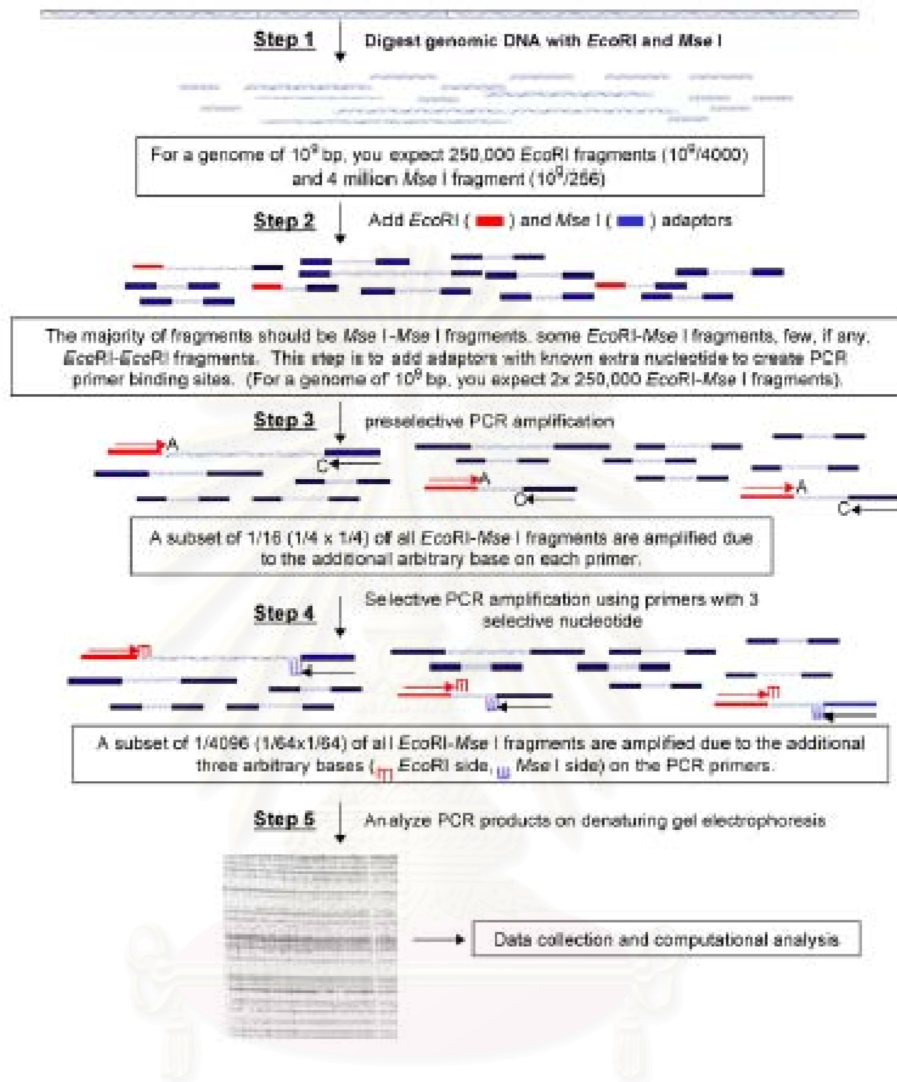
The disadvantages of RAPD PCR is that more than 90% of polymorphisms segregate as dominant alleles. Moreover, reproducibility of amplification results is quite low. Accordingly, RAPD markers found from the experiments are usually converted to sequence-characterized amplified region (SCAR) markers through cloning and sequencing of the original marker. A pair of primer is designed and used for specific amplification of the target fragment.

### 1.3.3 Amplified fragment length polymorphism (AFLP) analysis

Amplified fragment length polymorphism (AFLP) was developed by Vos *et al*. (1995) to increase the potential of RFLP and increase reproducibility of 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 number of amplified fragments are significantly reduced and can be simply analyzed by polyacrylamide gel electrophoresis (Figure 1.6).

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.




สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Figure 1.7** A schematic diagram illustrating principles of AFLP analysis.

Similar to other dominant markers, heterozygosity can not be deduced from AFLP data. This reduces its potential for examination population genetic parameters of artificially propagated stocks in the hatcheries.

**1.3.4 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 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.

DNA sequencing is the most optimal method for population genetic applications particularly phylogenetic studies of organisms. This technique provides high resolution and facilitating interpretation. However, sequencing of a large number of individuals using conventional method is extremely tedious and prohibitively possible. The sequencing method has been facilitated by the direct and indirect use of DNA fragments generated through PCR. At present, automatic DNA sequencing has been introduced and commonly used (Fig. 1.8). This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

**1.3.5 Genome walking**

DNA walking is a method for identifying unknown genomic DNA sequences adjacent to known sequences such as cDNA or characterized markers (e.g. AFLP and RAPD). Genomic DNA is separately digested with different blunt-end generating restriction endonucleases (usually, *Eco* RI, *Dra* I, *Pvu* II and *Ssp* I). The digested genomic DNA in each tube was then ligated to the adaptor to generate the template for PCR amplification. PCR was carried out with the primer complementaty to the adaptor (AP1) and the interesting gene (gene specific primer 1; GSP1). The resulting product is amplified with nested primers (AP2 and GSP2). The amplified

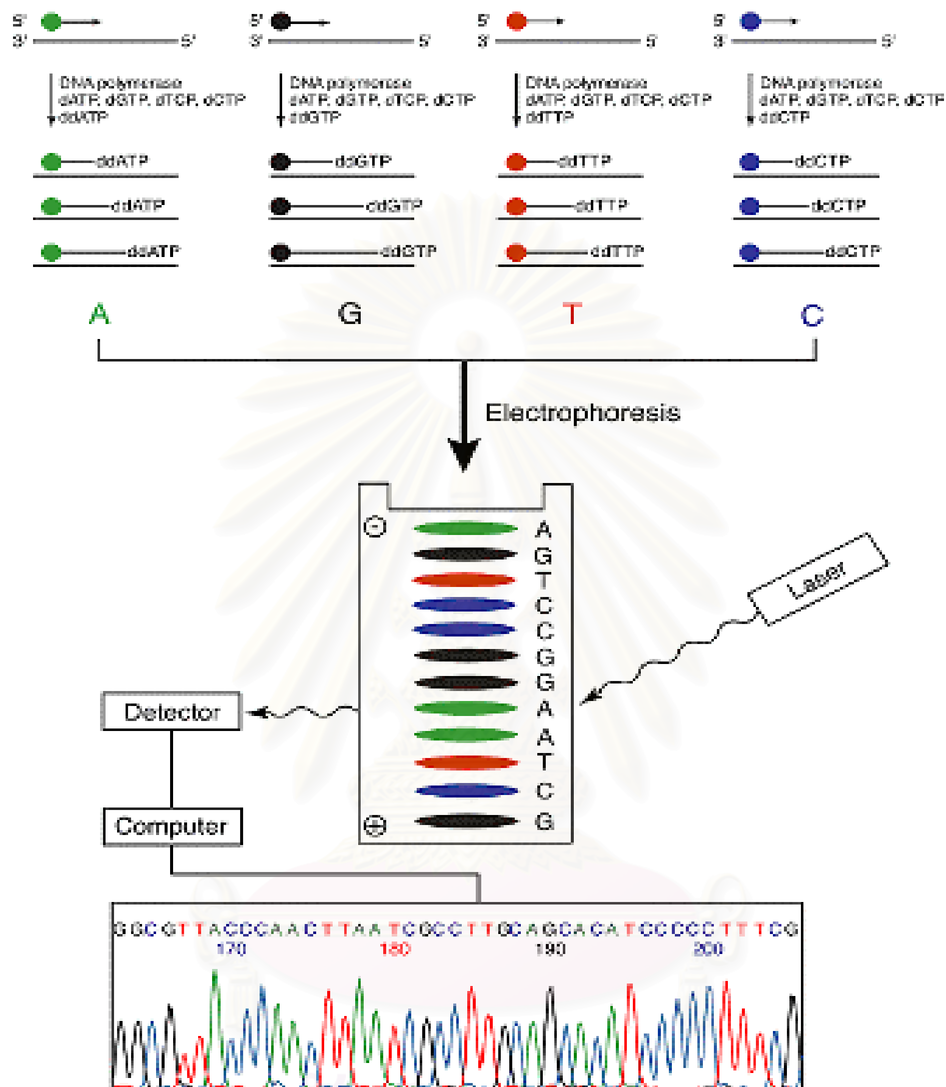



**Fig. 1.8** Automated DNA sequencing.

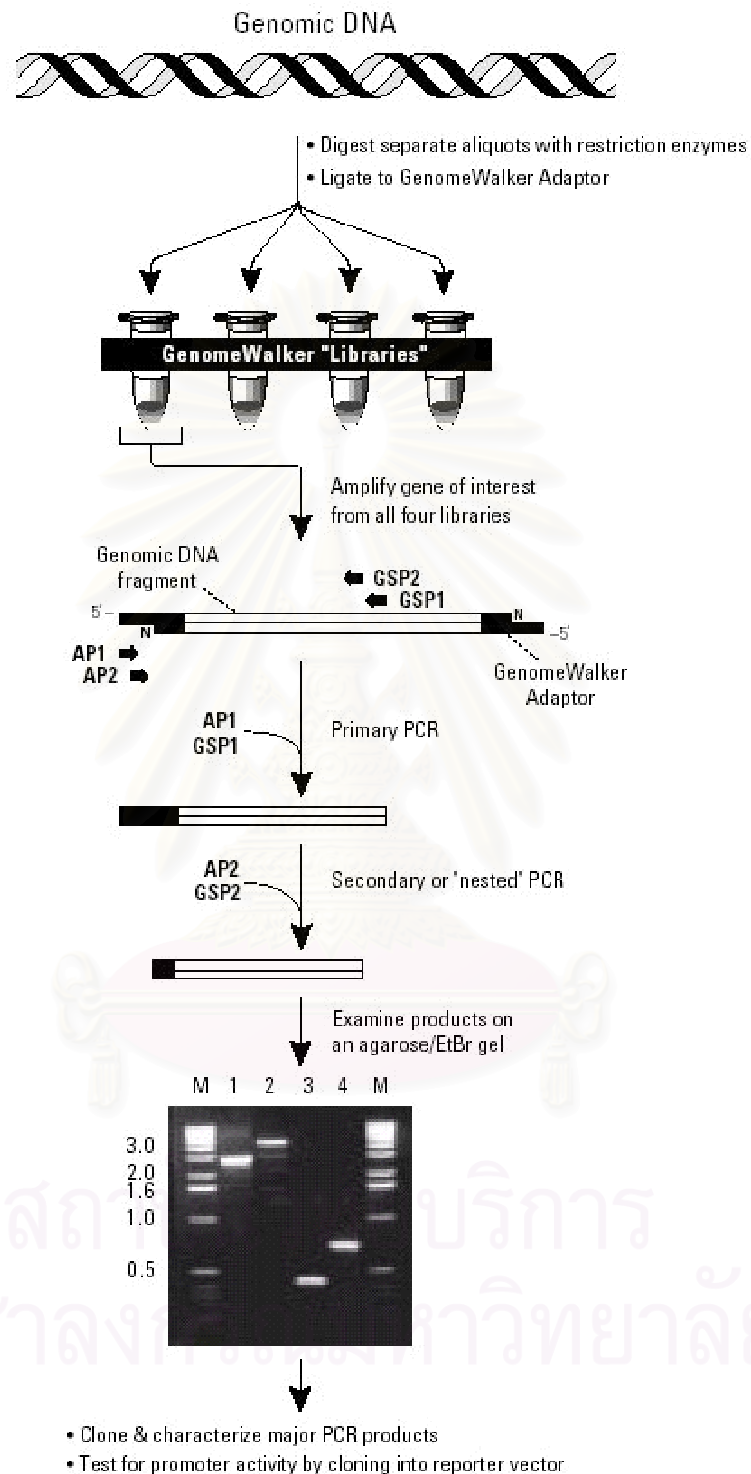



**Figure 1.9** A flow chart illustrating the GenomeWalker protocol.

adjacent fragment can be cloned and characterized (Figure 1.8). This technique allows isolation of the promoter region of interesting genes and 3' and 5' adjacent regions of AFLP markers that required further characterization.

**1.3.6 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 (Figure 1.9).

The major advantage of SSCP is that many individual PCR products may be screened for variation simultaneously. Heteroduplexes can occasionally 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 cause the SSCP patterns complicate for estimation of allele frequencies precisely.

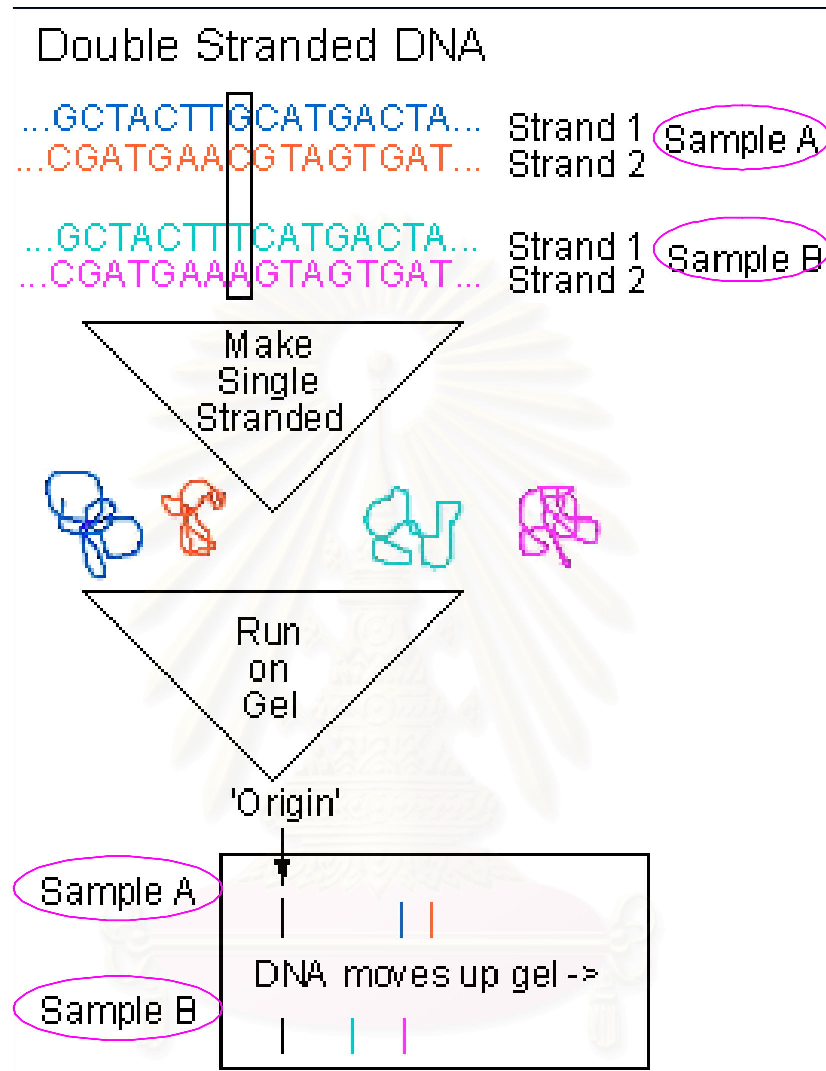



**Figure 1.10** A schematic diagram of SSCP analysis.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

### 1.4 Studies of sex-determination/differentiation marker in animals

The genetic sex of many species cannot be deduced from external morphology. This problem is usually arisen when dealing with embryonic or juvenile forms of interesting species. One effective solution is to exploit DNA markers to diagnose sex of each individual. Such markers are present in the genome whenever sex determination is genetically controlled. In many organisms sexual differentiation is governed by chromosomal sex determination, where the sex determination genes are carried on a specialized pair of sex chromosomes. The two main forms are male heterogamy, where the male has X and Y chromosomes and the female is XX, and female heterogamy, where the female is WZ and the male is ZZ. The Y or W chromosomes are, thus, unique to one sex, so their presence or absence in a sample of genomic DNA is indicative of sex.

For mammals, this problem has largely been solved by the discovery of *SRY* gene (*Sex*-determining *Region-Y* chromosome) which is structurally conserved and Y-linked across the class.

In mammals, sex determining region-Y chromosome (SRY) has been discovered for more than a decade. The gene is structurally conserved and Y-linked across the class. Therefore, identification of sexes in mammals at the DNA level is well established (O'Neill and O'Neill, 1999). Nevertheless, the homologue of mammalian SRY has not been identified in non-mammalian species. Although sex-specific markers have also been described in several non-mammalian species, they are not usually conserved. as a result, sex specific-marker isolated from one species may not exist outside the genus (Griffiths and Tiwari, 1993).

Griffiths and Tiwari (1993) used 16 primers of 21 – 29 mers and 20 of 10 mers to isolate candidate sex-specific RAPD marker in birds. Of these, ss2 (5′-CGG TCG GGA GGT TTC AAG GAA TG-3′) amplified female-specific 724 bp fragment of the great tit (*Parus major*) whereas 2 decanucleotide primers; AB09 (GGGCGACTAC) and AB18 (CTGGCGTGTC) yielded a candidate female-specific fragments in the jackdaw (*Corvus monedula*; 1100 bp) and the zebra finch (*Taenopygia guttata*; 900 bp), respectively. Each of these amplified bands was used as the probe against

genomic DNA of males and females of each species by Southern Blot analysis. Results confirmed their W-linked and also indicated the occurrence of related sequences elsewhere in the genome.

Griffiths and Orr (1999) demonstrated the use of AFLP for isolation of sex-specific markers in the ostrich (*Struthio camelus*) possessing ZW heterogamy for females. The shag (*Phalacrocorax aristotelis*) was also included into the analysis. Screening of 3 males and 3 females ostriches with a single E-primer ($E_{AGG}$) in combinations of each of the eight of M primers provided 2 W-linked markers from $E_{AGG}/M_{CAG}$ and $E_{AGG}/M_{CAA}$ primer combinations, respectively. These two primer combination were tested against 8 individuals of ostriches and female individuals can be identified correctly. Additionally, 2 W-linked markers in the shag were also found from $E_{AAG}/M_{CAA}$ and $E_{ACT}/M_{CAC}$, respectively. Primers ScW1F/R and ScW2F/R designed from cloned AFLP markers of the ostriches exhibited female specificity when further tested with 12 individuals of ostriches.

Studies of sex determining and chromosome systems in fish are well advanced compared to other aquatic species. Nine sex determination systems have been reported in fish. Sex is controlled by sex chromosome in eight: XX/XY, ZW/ZZ, XX/XO, ZO/ZZ, X1X1X2X2/X1X2Y, ZW1W2/ZZ, XX/XY1Y2 and the WXY (where XY and YY fish are males while XX, WX and WY are females) of these systems.

The sex chromosome of some fish species are heteromorphic (morphological distinct) and can be directly identified whereas those of several fish species are homomorphic (not distinct morphologically) and inferred from chromosome manipulation, hybridization experiment, and sex reversal studies (Tave, 1993). Surprisingly, the medaka (*Oryzias latipes*) has been used as a model fish in several biochemical, physiological and genetic studies, sex determination system is not clear. Sex determination markers in this species are being investigated (Musuda et al., 2000).

Griffiths et al. (2000) isolated sex-specific markers of the three-spined stickleback (*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 various 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).

Kovacs et al. (2001) identified sex-specific DNA markers in the African catfish, *Clarias gariepinus* (Burchell, 1822) by random amplified polymorphic DNA (RAPD) analysis against pooled genomic DNA of males and females. Two sex-linked RAPD markers (2.6 kb for CgaY1 and 458 bp for CgaY2) were identified from the pooled DNA of male *C. gariepinus* and specificity also confirmed on individual samples. Both RAPD markers were cloned and sequenced. Southern blot analysis using the CgaY1 probe under stringency conditions showed strong hybridized fragments only in males but not in females indicating the presence of multiple copies of CgaY1 in the male genome. CgaY2 produced similar hybridization pattern in both sexes of *C. gariepinus.* Specific primers were designed to the sequences and the markers were amplified in multiplex PCR reactions together with a control band common to all individuals. Three band (male) versus one band (female) patterns found in *C. gariepinus* allowed for rapid molecular sexing of this catfish species.

Sex determination may not be controlled by sex chromosomes but is controlled autosomally. Some species of fish do not have sex chromosome as a result sex in these species is determined by male or female genes located on the autosomal chromosomes.

An understanding of sexual biology of any sexual-reproducing species is important for designing breeding programmes in that species. However, studies of sex determining mechanisms in insects and crustaceans are not well advanced. Previous researches has shown that most of the isopod species display chromosomal sex

determination in both XX/XY and ZW/ZZ systems but heteromorphism of the sex chromosomes were observed in very few cases (Barzotti et al., 2000).

DMRT1 is a gene encoding a protein with a DNA binding motif called DM domain. DMRT1 has been suggested as the first conserved gene involved in sex differentiation found from invertebrates to human. Recently, a DMRT1 homologous (rtDMRT1) was cloned in the rainbow trout (*Oncorhynchus mykiss*). Expression of this gene was found during testicular differentiation but not during ovarian differentiation (Marchand et al., 2000). The DMRT1 has been fully characterized in human and shows similarity with sex determination gene in invertebrates; double sex (*dsx*) in *Drosophila melanogaster* and *mab*-3 gene in *Caenorhabditis elegans*. These genes share a number of properties and contain the DM domain, a zinc finger-like DNA binding motif (Raymond et al., 2000). In addition, repetitive sex-specific DNA has been described on the Y chromosome of *D. melanogaster* (Goldstein et. al., 1982).

The genes encoding for zinc finger proteins composed of the Y-linked zinc finger gene (Zfy) located on the Y-chromosome, the closely related X-linked gene (Zfx) located on the X chromosome and the autosomal zinc finger gene (Zfa). The zinc finger gene has been used for sex determination of several vertebrate and invertebrate species for instance, human, (Stacks and Witte.,1996), mouse (Luoh, S,-W, 1994), porcine (Schmoll and Schellander., 1996), cattle, sheep and goats (Aasen and Medrano, 1990), *D. melanogaster* (Pauli et al., 1995) and *C. elegans* (Raymond and Rousset., 2000).

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

An isopod crustacean, *Asellus aquaticus*, consists of 8 homomorphic chromosomes in both sexes but a heteromorphic sex chromosome is present in one-

quarter of the males in natural populations. The sex chromosomes in this species cannot be differentiated by conventional staining techniques (G- or R-banding). Genomic *in situ* hybridization cannot reveal any sex chromosome differentiation between homomorphic males and females whereas males exhibiting heteromorphic chromosomes showed differentially labeled regions with male-derived DNA probe (Barzotti et al., 2000).

Karyological studies in the giant freshwater prawn (*Macrobrachium rosenbergii*) were conducted. The chromosome number of the antennal gland in males reveal a diploid number of 2n = 118. This was further confirmed by the haploid chromosome number (n = 59) from testes. While most of investigated females exhibit identical number of chromosome, a certain number of cells having 117 and 111 chromosomes were also observed. Therefore, it was not possible to conclude the chromosome number of female *M. rosenbergii* unambiguously. Moreover, sex chromosomes in *M. rosenbergii* could not be identified cytologically (Justo et al., 1991).

Maleacha et al. (1992) examined sex-ratio and sex determination in progeny of crosses betweeen masculinised genotypic females and normal females of the giant freshwater prawn (*M. rosenbergii*). Cumulative sex-ratios in the progeny support a hypothesis that sex of prawns is differentiated with female heterogamous (ZW) and male homogamous (ZZ). Nevertheless, variation of sex-ratios among different crosses implied that sex determination in *M. rosenbergii* is more complex than the simple ZW/ZZ system.

In *P. monodon*, females exhibit greater growth rate than do males at all stages of development. Nevertheless, a lack of obvious heteromorphic sex chromosomes in this species causing limited knowledge on sex chromosome (XY or ZW) and their segregation patterns. This prohibits the possibility to elevate culture efficiency of *P. monodon* through monosex farming. Accordingly, sex-specific markers in *P. monodon* need to be developed.

Sex determination in the crustacean has been reviewed by Legrand et al. (1987) who note that the genetic basis for sex determination had been studied in only a few species and none of which were decapods. Korpelainen (1990) has reviewed the strong influence of environmental factors (including temperature, food supply and social environment) on sex determination in some crustacean groups. Neither sex chromosome in penaeids, nor any environmental sex determination has been observed (Korpelainen, 1990).

Limit knowledge on sex reproductive markers in non-mammalian species prohibits understanding of reproductive biology in several important species. In this research, sex determination markers of the giant tiger shrimp (*P. monodon*) will be identified by molecular techniques. Specificity of the markers will be extensively tested in *P. monodon.*

### 1.5 Population genetic studies of *P. monodon*

One of the most important goals for fishery management is to primarily obtain detailed information of genetically different stocks and understand interactions among stocks. On the basis of allozyme analysis, it has been reported that the level of genetic variability in crustaceans is relatively low (mean heterozygosity was 0.073 for 97 species) (Ward and Grewe., 1994). Genetic variation and population differentiation of *Penaeus monodon* in Thailand at the DNA level were examined by several molecular approaches.

#### 1.5.1 Genetic diversity of *P. monodon* in based on mitochondrial DNA polymorphism

Genetic variation based on mtDNA polymorphism was applied for stock structure analysis in *P. monodon* because of its more rapid evolutionary rate compared to single copy nuclear (scn) DNA and allozymes. Due to its haploid and maternally inherited nature, the estimated effective population size for mtDNA is smaller than that of allozymes and scnDNA increasing its susceptibility to genetic drift, inbreeding and bottleneck events.

Intraspecific genetic diversity of *P. monodon* was examined using mtDNA-RFLP analysis. A total of 212 wild *P. monodon* individuals collected from ten sites (Lamu in Kenya, Medan, North and South Java in Indonesia, Satun, Surat and Trad in Thailand and Lingayen in the Philippines) were analysed with eleven restriction endonucleases (*Ava* II, *Bam* HI, *Cla* I, *Dra* I, *Eco* RV, *Hind* III, *Pvu* II, *Sac* I, *Sca* I and *Xba* I).

Sixty-three mtDNA composite haplotypes were identified and 28 of these were found in Thai samples. These could be placed into one or other of two clonal lineages, A and B. The most easterly sites (Lingayen, Philippines) were fixed for cluster A haplotypes and the most westerly site (Lamu, Kenya) was fixed for a B haplotype. At the other sites, both clusters were present with the A haplotypes generally more common in the South China and Java Sea than from samples in the Andaman Sea (Figure 1.11).

Haplotype diversity of *P. monodon* mtDNA was 0.7689 ± 0.00186. The average nucleotide diversity within and between populations was 1.7276 ± 0.0007% and 2.7230 ± 0.0003% respectively. Significant population differentiation was observed with a major discontinuity between the Andaman Sea, Java and South China Sea and west African samples of *P. monodon*.

Duad (1995) determined genetic diversity and population differentiation in 6 geographic samples from Malaysia (Kedah and Kampung Pulau Sayak from the west coast, Dungun Kemaman and Sabah from the east coast and Pengerang from the further south of the Malaysian peninsula) using restriction analysis of the entire mtDNA with *Bam* HI, *Sac* I, *Eco* RV, *Pvu* II and *Hind* III. Combining that with the above data (on 5 restriction enzymes) provided comparable results when 10 geographic samples of *P. monodon* was analyzed by 11 enzymes (Figure 1.10). Based on genetic diversity of 13 geographic samples in South-East Asia and additional samples from western Africa, a pattern of increasing genetic diversity in South-East Asia samples moving west from the Philippines towards Pengerang was observed.

The Philippine sample had the lowest haplotype diversity whereas the highest value was in Pengerang, the site which lies on the boundary between the Andaman Sea and the South China Sea and Java Sea groups. The diversity levels were similar in

the Andaman Sea sites to those of the east. The Kenyan sample was fixed for a single haplotype so the genetic diversity was effectively zero. However the high diversity seen in the populations on either side of the Malaysian peninsula appears to have been resulted from two previously isolated populations mixing. Pengerang exhibited the highest genetic diversity is the probable interface between these samples.

Benzie et al., (2002) surveyed mtDNA variation in 5 geographic samples of southeast African, 5 of Australian and 3 of southeast Asian *P. monodon* using RFLP and provided clear information that the Indo-West Pacific region is the site of accumulation of genetic diversity rather than the site of origin of genetic diversity. The dominant haplotype was different in the Australian and southeast Asian samples. Genetic diversity was greastest in Indonesia, less in the Philippines and Australia and markedly less in the southeast African and west Australia. The high diversity in the Southeast Asian samples resulted from the occurrence of a set of haplotypes found only in the southeast Asian samples derived from the southeast African haplotypes. These genetic variants were evolved in the Indian Ocean and subsequently migrated into the Indo-West Pacific region. Low genetic variation in geographically marginal samples in southeast African and west Australia is possibly resulted from the consequence of bottleneck effects but mismatch haplotype distributions suggest that large population sizes have been maintained in Indonesian samples for long periods.

Genetic diversity of *P. monodon* in Thailand based on PCR-RFLP of 16S rDNA and cytochrome subunit I-II (COI-II) was analyzed using specimens collected in 1997 from Satun ($N$ = 30), Trang ($N$ = 30), and Phangnga ($N$ = 31) in the Andaman Sea; and Chumphon ($N$ = 39) and Trad ($N$ = 24) in the Gulf ofThailand. Digestion of *P. monodon* 16S rDNA (560 bp in length) with *Mbo*I and COI-COII (1700 bp) with *Alu*I, *Mbo*I, *Taq*I, *Hin*fI and *Dde*I produced 48 restriction fragments with an average of 8.0 fragments per enzyme. Linkage disequilibrium analysis indicated that haplotypes from the 16S rDNA and an intergenic COI-COII were associated nonrandomly ($P$ < .0001). In total, 37 composite haplotypes were identified among the 5 samples of Thai *P. monodon* (Fig. 1.13). Of these, 22 composite haplotypes were carried by single individuals. Only 2 composite haplotypes (I, ABBBBA; and VII, BAAAAB) were commonly found, in 16.23% and 28.57% of overall specimens, respectively. These haplotypes were not population-

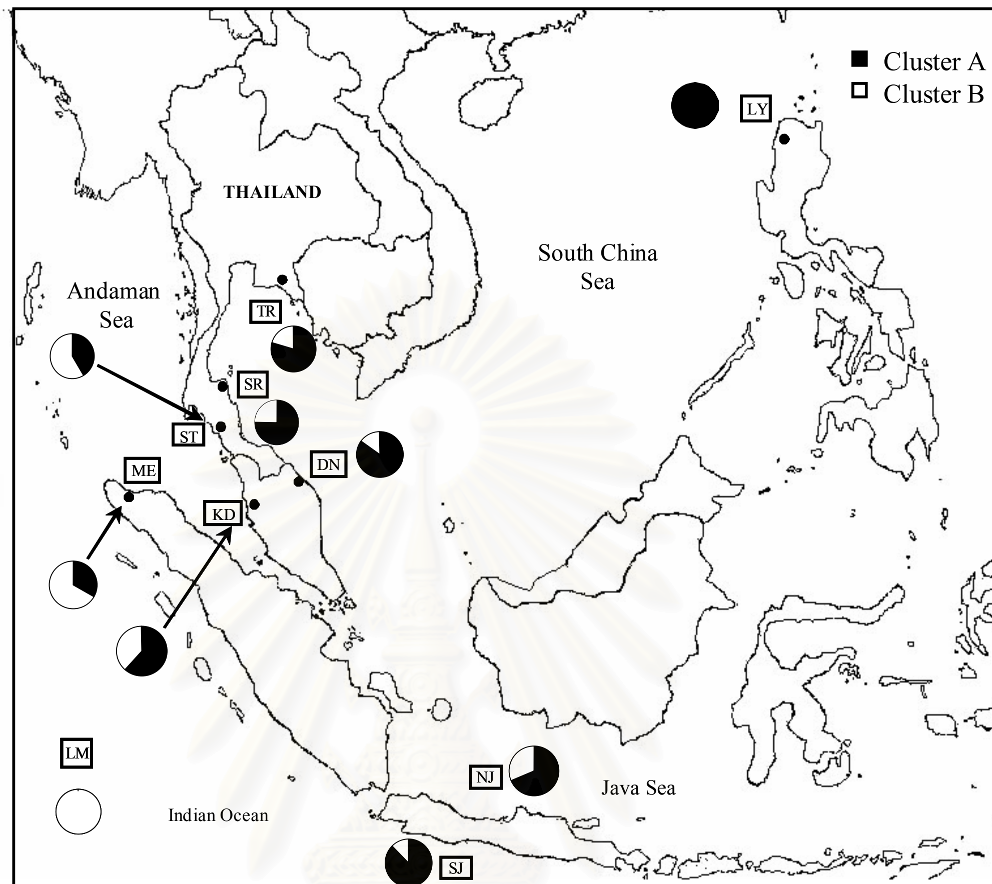



**Fig. 1.11** Geographic distribution of mtDNA phylogenetic clusters A and B among fourteen geographic samples of *P. monodon* analyzed by 10 polymorphic restriction enzymes.

LY = Lingayen, Philippines; DN = Dungun, KD = Kedah, Malaysia; SR = Surat, ST = Satun, TR = Trad, Thailand; ME = Medan, NJ = North Java, SJ = South Java, Indonesia; LM = Lamu, Kenya. Noted that Kenya is located in Africa.

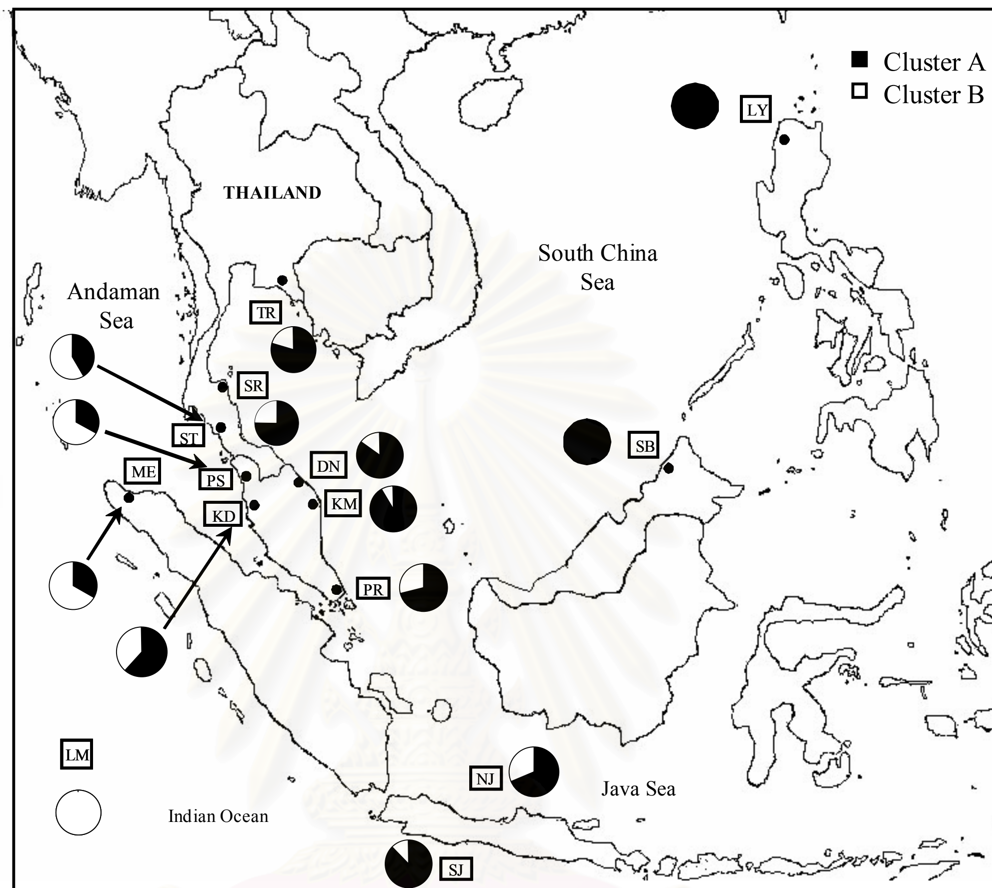



**Fig. 1.12** Geographic distribution of mtDNA phylogenetic clusters A and B among fourteen geographic samples of *P. monodon* analyzed by 5 polymorphic restriction enzymes.

LY = Lingayen, Philippines; DN = Dungun, KD = Kedah, KM = Kemaman, PR = Pengarang, PS = Kampung Pulau Sayak, SB = Sabah, Malaysia; SR = Surat, ST = Satun, TR = Trad, Thailand; ME = Medan, NJ = North Java, SJ = South Java, Indonesia; LM = Lamu, Kenya. Noted that Kenya is located in west Africa.

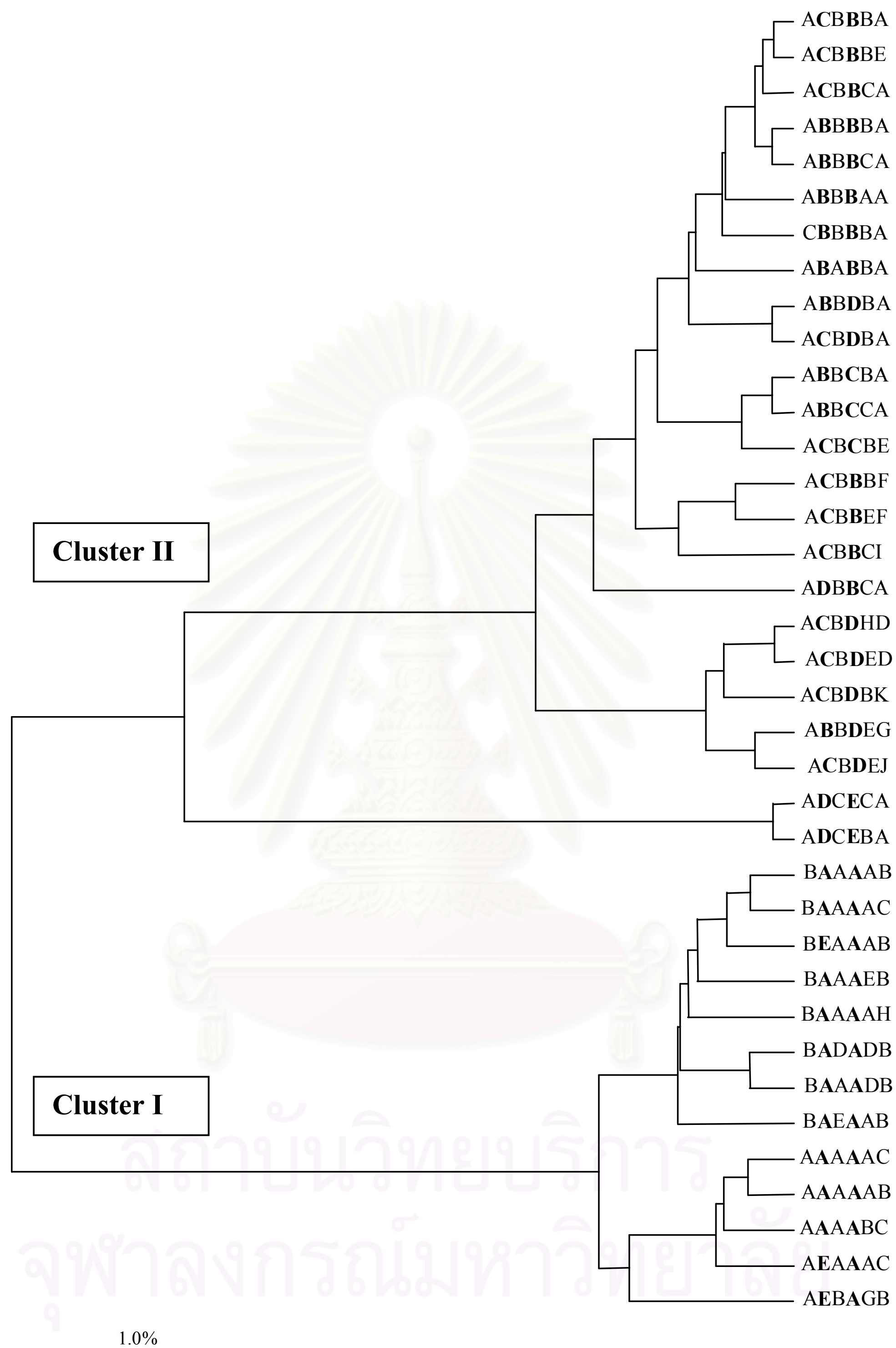



**Fig. 1.13** A UPGMA dendrogram showing relationships among 37 composite haplotypes found in Thai *P. monodon* based on sequence divergence between pairs of composite haplotypes (arranged from 16S rDNA digested with *Mbo*I and an intergenic COI-CO II digested with *Alu*I, *Mbo*I, *Taq*I, *Hin*fI, and *Dde*I, respectively). Boldface letters refer to restriction patterns of *Alu*I and *Mbo*I-digested COI-COII that precisely represent the frequencies of phylogenetic clusters I and II (equivalent to clusters B and A in Klinbunga et al., 1999, respectively) of Thai *P. monodon.*

specific but found in all geographic samples. The UPGMA cluster analysis based on sequence divergence estimated among composite haplotypes indicated 2 major phylogenetic clusters of mtDNA haplotypes (clusters I and II) joined in the dendrogram with a genetic distance of 3.028% (Fig. 1.13). The former cluster contained 13 composite haplotypes, whereas the latter consisted of 24 remaining composite haplotypes. Each phylogenetic cluster corresponds to 50% of overall *P. monodon* individuals.

The distributions of these 2 clusters overlapped in all geographic samples with different proportions. The highest frequency of cluster I (identical to cluster B in Klinbunga et al., 1999) was observed in Satun (0.600) followed by Chumphon (0.564), Trang (0.500), Phangnga (0.433), and Trad (0.375). The distribution of cluster II genotypes in a particular sample was reversed. Considering digestion patterns obtained from restriction enzymes in their study, patterns from digestion of an intergenic COI-COII with *Alu*I and *Taq*I could represent the frequency of each phylogenetic cluster accurately (Fig. 1.12). This simplification opens the possibility of genetic-based stock enhancement (restocking) programs of *P. monodon* in Thailand without significant disturbance of its local gene pools.

**1.5.2 Genetic diversity of *P. monodon* in based on Nuclear DNA polymorphism**

Although mtDNA polymorphism clearly provided information on genetic stock structure of *P. monodon* in Thailand, other genetic markers which are useful for breeding programs of *P. monodon* are still required. As a result, rDNA, randomly amplified polymorphic DNA (RAPD) and microsatellite analysis were further examined to evaluate intraspecific population differentiation and identify additional genetic markers in *P. monodon*.

A study of nuclear rDNA polymorphism of *P. monodon* showed individual-specific genotypes when genomic DNA of *P. monodon* was restricted with *Sac* I and *Bam* HI. This indicated rather high genetic diversity of wild *P. monodon*. Nevertheless, rDNA polymorphism of *P. monodon* showed both inter- and intraindividual polymorphism (length heteroplasmy) resulted in difficulties on interpretation and identification of useful rDNA markers in this species.

RAPD analysis was used to examine genetic variation in wild black tiger shrimp, *P. monodon*. Specimens were collected from five geographically separated locations (Satun-Trang, Phangnga, and Medan in the Andaman Sea and Chumphon and Trad in the Gulf of Thailand). A total of 100 *P. monodon* individuals were investigated using seven arbitrarily selected primers (Tassanakajon et al., 1998a). Fifty-eight (72.5%) of eighty reproducible RAPD fragments ranging in size from 200 to 2200 bp were polymorphic. The percentages of polymorphic bands of the 5 geographic samples investigated varied from 51.5 to 57.7%. The genetic distance between populations and UPGMA dendrograms indicated that the Medan population was genetically different from Thai *P. monodon* ($d_{\mathrm{RAPD}}$ = 14.976%). Within Thailand, the Satun-Trang *P. monodon* was separated from the remaining geographic populations with a genetic distance of 2.632%.

RAPD analysis in their study yielded a total of 252 genotypes. A Monte Carlo analysis illustrated geographic heterogeneity in genotype frequencies within this species, suggesting that genetic population structure does exist in this taxon ($P$ < 0.001 for all primers). Significant differences in genotype frequencies between Thai and Indonesian (Medan) *P. monodon* were observed ($P$ < 0.0001). Within Thailand, the Andaman Sea *P. monodon* was significantly different from that of the Gulf of Thailand ($P$ values between 0.0000 and 0.0387), indicating population differentiation between *P. monodon* from these two main fishery regions of Thailand. This was concordant with an extremely high $F_{\mathrm{ST}}$ estimate (0.4810 for overall populations and 0.2104 for Thai *P. monodon*).

Surprisingly, the Phangnga sample located in the Andaman Sea was clustered as a sister taxon to Chumphon’s from the Gulf of Thailand in the UPGMA dendrogram. An inability to discriminate their genotype heterogeneity was also observed when using primers 174 ($P$ = 0.1825), 268 ($P$ = 1.0000), 456 ($P$ = 0.4409), and 457 ($P$ = 0.0526). A possible explanation could be that the population being analyzed was disturbed and might not be typical for the area. Moreover, Phangnga is one of the major shrimp farming areas on the west coast. The local population may have been disturbed by escapees or recruits from enhancement programs because Phangnga is a bay where the water is quite shallow and mostly enclosed by land.

RAPD analysis of *P. monodon* originating from the same geographic locations (Chumphon and Trad from the Gulf of Thailand and Satun, Trang and Phangnga from the Andaman Sea) was additional analyzed by 3 additional primers; UBC268, UBC273 and UBC299 to determine whether that anomalous population differentiation was consistent (Klinbunga et al., 2001). Results also indicated the existence of differentiation between *P. monodon* from the Andaman Sea and Gulf of Thailand ($P < 0.0001$).

Surprisingly, paired comparisons of distances between Chumphon and each of the Andaman Sea samples ($d_{RAPD}$ = -0.002 to 0.003) indicated closer genetic relationships than between Chumphon and Trad ($d_{RAPD}$ = 0.034). Genetic distances between samples within the Andaman Sea samples were -0.001 to 0.005. Overall comparisons of RAPD genotype frequencies revealed significant geographic heterogeneity among Thai *P. monodon* ($P < .0001$), and between coastal regions ($P < .0001$). Not all RAPD primers used in Klinbunga et al. (2001) indicated geographic heterogeneity for all possible comparisons between Chumphon and each of the Andaman Sea samples ($P > 0.05$), but all primers revealed highly significant heterogeneity between Chumphon and Trad ($P < .0001$) (Table 2). This indicated that the Chumphon *P. monodon* showed genetically closer relationship to each of the Andaman populations than Trad located in the same coastal area.

Domestication and closed-life-cycle culture of *P. monodon* should be established using wild stock with high genetic diversity, including desirable culture traits. Our RAPD-PCR results indicated high genetic diversity with Thai *P. monodon.* We observed a large number of RAPD genotypes and high genetic differences between Trad and the remaining samples (Klinbunga et al., 2001). Tassanakajon et al. (1998a) found that the primer UBC428 yielded a population-specific RAPD marker (950 bp) with Satun *P. monodon*. The UBC428 marker coupled with that of the UBC268 can be used to verify growth and survival among different *P. monodon* stocks in commercial culture settings.

Microsatellites in *P. monodon* were characterized and first reported by Tassanakajon et al., 1998b. Using these markers, high levels of genetic diversity were observed in five different geographic samples of *P. monodon* in Thailand (Chumphon

and Trad from the Gulf of Thailand and Satun, Trang and Phangnga from the Andaman Sea).

The average observed heterozygosity of 0.78 in *P. monodon* was still lower than that of wild *P. vannamei* collected from Salinas (Ecuador) when analyzed with the *M1* locus (*H*o = 1.0). Conversely, the average effective number of alleles of *P. monodon* in their study was slightly higher than that of the Ecuadorian *P. vannamei,* implying similarly high levels of diversity of allele frequencies in these two species.

A number of microsatellite alleles found in the Andaman sample were not available in Trad, but some of those existed in Chumphon. The failure to detect significant allele distribution frequencies between Chumphon and each of the west coast samples might have resulted from mixing of the gene pools of different *P. monodon* stocks as a consequence of extensive transplantation of *P. monodon* in Thailand. Collection of more *P. monodon* samples from farther south of the Thai-Malaysian Peninsula and increasing the microsatellite loci may clarify the differentiation patterns in these areas.

Although high genetic diversity was observed in each geographic samples of *P. monodon*, low degrees of population differentiation were observed in this species. A high level of gene flow during planktonic larval stages of *P. monodon* may be responsible for this limited differentiation. Notably, the present population differentiation of *P. monodon* may be underestimated as it results from both unintentional transplantation and restocking programs of this species in Thailand.

Five *P. monodon* samples could be allocated to three different populations : A (the Andaman Sea), B (Chumphon), and C (Trad). More importantly, further screening of wild *P. monodon* covering a larger geographic scale (e.g., Southeast Asia) based on microsatellite analysis should be undertaken to elucidate the patterns of intraspecific genetic structure in this species at macrogeographic scales. Although a limited level of subdivision has been observed, three isolated *P. monodon* populations should, from the management point of view, be treated as separately exploited stocks.

**1.6 Objectives of the thesis**

The aim of this thesis is to identify and characterize candidate sex-specific marker using AFLP analysis in *P. monodon.* In addition, polymorphic markers (Disulfide isomerase, Zinc finger protein gene, PMT1700 and PMO920) were used for population genetic studies of natural *P. monodon* in Thai waters by SSCP analysis.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Experimental animals

Broodstock of *P. monodon* was collected from Chumphon (*N* = 28) and Trad (*N* = 29) located in the Gulf of Thailand and Satun (*N* = 39), Trang (*N* = 23) and Phangnga (*N* = 29) located in the Andaman Sea. These specimens were used for population genetic studies of *P. monodon* in Thai waters using nuclear DNA polymorphism. For isolation of candidate sex-diagnostic AFLP markers in *P. monodon*, some wild *P. monodon* were included in the experiment. In addition, cultured juvenile *P. monodon* (approximately 20 g body weight) showing well characterized petasma (male) and thelycum (female) purchased from local farms were also included in the analysis. Pleopods of each shrimp were collected and kept at -80°C until required.

## 2.2 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) solutions were added to a final concentration of 1.0 % (w/v) and 100 $\mu g.ml^{-1}$, respectively of The resulting mixture was then incubated at 37°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 300 $\mu g.ml^{-1}$ and further incubated at 55 °C for 3 – 4 hours. An equal volume of buffer-equilibrated phenol: chloroform: isoamylalcohol (25:24:1) was added and gently mix for 10 minutes. The solution was centrifuged at 10,000 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 once 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 -80$^{o}$C for 30 minutes. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature and washed twice with 1 ml of 70% ethanol (5 minutes and 2 – 3 minutes, respectively). After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 50 – 80 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.

**2.3 Measuring DNA concentrations using spectrophotometer and electrophoresis**

The concentration of extracted DNA is estimated by measuring the optical density at 260 nanometre ($OD_{260}$). An $OD_{260}$ of 1.0 corresponds to a concentration of 50 μg.ml$^{-1}$ double stranded DNA. Therefore, the concentration of DNA samples (μg/ ml) is estimated by multiplying an $OD_{260}$ value with a dilution factor and 50. The purity of DNA samples can be guided by a ratio of $OD_{260}$ /$OD_{280}$. The ratio lower than 1.8 indicated contamination of residual proteins or organic solvents whereas the ratio greater than this value indicate contamination of RNA in the DNA solution (Kirby, 1992).

On the basis of the direct relationship between the amount of DNA and the level of fluorescence after ethidium bromide staining, agarose gel electrophoresis can be carried out for rough estimation of high molecular weight DNA quantitvely. Genomic DNA was run in a 0.8 – 1.0% agarose gel prepared in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.0 mM EDTA, pH 8.3) at 4 V.cm$^{-1}$. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg.ml$^{-1}$). DNA concentration was estimated from the intensity of the fluorescent band by comparing with that of undigested λ - DNA.

**Part I : Isolation and characterization of sex-specific markers in *P. monodon***

### 2.4 Bulked segregant analysis (BSA) of *P. monodon* genomic DNA

Five bulked DNA of female (PMF1 – 5) and male (PMM1 – 5) were generate by pooling 30 – 50 ng of genomic DNA from each broodstock-sized (PMF1 – 2 and PMM1 – 2) and that from pooled pleopod tissue of juvenile shrimps (PMF3 – 5 and PMM3 – 5) (Table 2.1).

**Table 2.1** Specimens used for construction of male and female bulked DNA for isolation of sex-specific AFLP markers of *P. monodon*

| No. of bulked DNA | **Individual** |
|---|---|
| Female (PMF) 1 | Chumphon (CHU)2, CHU9, Satun (SAT)1, SAT8, SAT9, SAT20, SAT29 and Trang (TNG)16 |
| Female (PMF) 2 | CHU10, CHU20, CHU21, SAT10, SAT25, TNG19, TNG32 and TNG41 |
| Female (PMF) 3 | 5 juvenile shrimps purchased from the commercial farm in Pathumthani showing well developed thelycum |
| Female (PMF) 4 | 5 juvenile shrimps purchased from the commercial farm in Pathumthani showing well developed thelycum |
| Female (PMF) 5 | 5 juvenile shrimps purchased from the commercial farm in Pathumthani showing well developed thelycum |
| Male (PMM) I | CHU1, CHU3, CHU14, CHU15, SAT11, SAT23, TNG13,and TNG35 |
| Male (PMM) 2 | CHU3, CHU4, CHU6, SAT4, SAT11, SAT16, TNG9 and TNG14 |
| Male (PMM) 3 | 5 juvenile shrimps purchased from the commercial farm in Pathumthani showing well developed petasma |
| Male (PMM) 4 | 5 juvenile shrimps purchased from the commercial farm in Pathumthani showing well developed petasma |
| Male (PMM) 5 | 5 juvenile shrimps purchased from the commercial farm in Pathumthani showing well developed petasma |

## 2.5 Amplified fragment length polymorphism (AFLP) analysis

### 2.5.1 Restriction enzyme digestion and adaptor 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 $CH_3COOK$ 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 2.2) 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.

### 2.5.2 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 µM of each dNTP,1.5 mM $MgCl_2$, 30 ng of $E_{+A}$ (5$^{/}$-GAC TGC GTA CCA ATT C**A**-3$^{/}$) $M_{+C}$ ( 5$^{/}$-GAT GAG TCC TGA GTA A**C**-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 minutes. The preamplification product was then diluted for 12.5 fold.

### 2.5.3 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 2.2), 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

**Table 2.2** AFLP primers and their sequences used for identification of sex-specific markers in *P. monodon*

| Primer | Sequences |
|---|---|
| *Adaptor 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 |
| $E_{+3}$-9 | $E_{+A}$GT |
| $E_{+3}$-10 | $E_{+A}$GA |
| $E_{+3}$-11 | $E_{+A}$TG |
| $E_{+3}$-12 | $E_{+A}$TC |
| $E_{+3}$-13 | $E_{+A}$TA |
| $E_{+3}$-14 | $E_{+A}$TT |
| $E_{+3}$-15 | $E_{+A}$AA |
| $E_{+3}$-16 | $E_{+A}$AT |
| $M_{+3}$-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 |
| $M_{+3}$-9 | $M_{+C}$GA |
| $M_{+3}$-10 | $M_{+C}$GT |
| $M_{+3}$-11 | $M_{+C}$GC |
| $M_{+3}$-12 | $M_{+C}$GG |
| $M_{+3}$-13 | $M_{+C}$CA |
| $M_{+3}$-14 | $M_{+C}$CT |
| $M_{+3}$-15 | $M_{+C}$CG |
| $M_{+3}$-16 | $M_{+C}$CC |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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.

**2.6 Agarose gel electrophoresis (Sambrook et al., 1989)**

Appropriate amount of agarose was weighed out and mixed with 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.0 mM EDTA, pH 8.3). The gel slurry was heated until complete solubilization in the microwave. The gel solution was cooled down to approximately 55°C before poured into a gel mould. The comb was inserted. The gel was allow to solidify at room temperature for approximately 45 minutes. When needed, the gel mould was placed in the gel chamber and sufficient 1x TBE buffer was added to cover the gel for approximately 0.5-cm. The comb was carefully withdrawn. To carry out agarose gel electrophoresis, one-fourth volume of the gel-loading dye (0.25% bromphenol blue and 25% ficoll) was added to each sample, mixed and loaded into the well. A 100-bp DNA ladder or λ-*Hind* III was used as the standard DNA markers. Electrophoresis was carried out at 4 - 5 $V.cm^{-1}$ until the tracking dye migrated about three-quartered of the gel. After electrophoresis, the gel was stained with ethidium bromide (0.5 $\mu g.ml^{-1}$) for 5 minutes and destained to remove unbound EtBr by submerged in $H_2O$ for 15 minutes. The DNA fragments were visualized under the UV light using a UV transilluminator.

**2.7 Denaturing Polyacrylamide Gel Electrophoresis**

**2.7.1 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.

### 2.7.2 Preparation of denaturing polyacrylamide gel electrophoresis

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 tape were carefully removed. The top of the gel was rinsed with 1x TBE. The sharkstooth comb was rinsed with water.

### 2.7.3 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 ul 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).

**2.8 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 agitate well for 40 minutes (25 – 30 minutes for SSCP gels). The gel was soaked with shaking 3 times for 3 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 of 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 lanes 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).

**2.9 Cloning of AFLP fragments**

**2.9.1 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.

### 2.9.2 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 staınined 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 13,000 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 13,000 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 13,000 rpm for 1 minute. The eluted sample was stored at -20°C until further required.

**2.9.3 Ligation of PCR product to vector**

The ligation reaction was set up in the total volume of 10 μl containing of 3 ul 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.

**2.9.4 Preparation of competent cells**

A single colony of *E. coli* JM109 was innoculated 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 3,000 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.

**2.9.5 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 μl. 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 $MgSO_4$ 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 12,000 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 $\mu g.ml^{-1}$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.

**2.9.6 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 µM 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 0.5 unit of DyNAzyme™ 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.

**2.9.7 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 $\mu g ml^{-1}$ 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 12,000xg 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 12,000 g for 10 minutes. The supernatant was transferred into the QIAprep column and centrifuged at 12,000 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 µl of the buffer EB (10 mM Tris-

Cl, pH 8.5 ) was added to eluted the extracted plasmid DNA. The column was left at room temperature for 1 minute and centrifuge at 12,000 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.

### 2.9.8 Digestion of the amplified DNA insert

Clones showing corresponded DNA insert size were separately digested with *Hin*d III and *Rsa* I to verify whether a single insert contained only one type of sequence. Typically, the digestion reaction was set up in the total volume of 15 µl containing appropriate restriction enzyme buffer (buffer E; 6 mM Tris-HCl; pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT for *Hin*d III and buffer C; 10 mM Tris-HCl; pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT for *Rsa* I), 5 µl of the amplified product and 2 units of either *Hin*d III or *Rsa* I. The reaction mixture was at incubated at 37°C for 3 – 4 hours. Digestion patterns were analyzed by agarose gel electrophoresis.

### 2.9.9 DNA sequencing

The recombinant plasmid was unidirectional sequenced for undirectional sequenced using a Thermo Sequence Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Biosciences, Sweden) with the M13 reverse or M13 forward primers on an automated DNA sequencer (MegaBace1000, Amersham BioScience).

## 2.10 PCR amplification of derived candidate sex-specific AFLP markers in *P. monodon* and sex related genes in *P. monodon* and other related species

PCR was carried out using primers designed from cDNA sequences of sex-linked gene homologues and sequences of candidate sex-specific AFLP markers in *P. monodon* (Table 2.3) and those of the giant freshwater prawn, *Macrobrachium rosenbergii* and the tropical abalone, *Haliotis asinina* (Table 2.4).

The amplification reaction was performed in a 50 µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 0.2 - 0.4 µM of a primer, 25 ng of genomic DNA of male and female *P. monodon* and 1.0 unit of DyNAzyme$^{TM}$ II DNA Polymerase (Finnzymes). The amplification profiles were composed of predenaturation at 94°C for 3 minutes followed denaturation at 94°C for 1 minute, an appropriate annealing temperature for 1 minute and extension at 72°C for 1.5 minutes for 35 cycles. The final extension was carried out at the same temperature for 7 minutes. The amplification products were electrophoretically analysed through 1.8% agarose gels and visualised under a UV transilluminator after ethidium bromide staining. Successful amplification products were further characterized following standard single strand conformational polymorphism (SSCP) to examine whether fixed single nucleotide polymorphisms (SNPs) was existent for each gender.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 2.3** Primers and primer sequences and melting temperature of primers designed from AFLP markers and cDNAs of *P. monodon*

| Gene/Primer | Sequence | Length | Tm (°C) |
|---|---|---|---|
| **Candidate sex-specific AFLP markers in *P. monodon*** | | | |
| $FE_{10}$ $M_{9}$520-F | 5′-AAC CAT AGC GAT GTT GAC GG-3′ | 20 | 60 |
| $FE_{10}M_{9}$520-R | 5′-TTT GCT TTG CCA TTT CGT AT-3′ | 20 | 54 |
| $FE_{10}$ $M_{10}$725-F | 5′-CAC GGG ATG TTT TGA CTA ATG ATG T-3′ | 25 | 70 |
| $FE_{10}$ $M_{10}$725-R | 5′-GGA TAA AGA TAA AGG AGG TGG CTG T-3′ | 25 | 72 |
| $FE_{10}$ $M_{10}$7252-F | 5′-ATC TAA CAC CAA CAA AAG TCA TCC T-3′ | 25 | 59 |
| $FE_{10}$ $M_{10}$7252-R | 5′-GTA GCC AGT CAT AAA GCG TAG TCA G-3′ | 25 | 60 |
| $FE_{14}$ $M_{16}$340-F | 5′-GCC GTC ACT TCA AAC ACA AAC CCT G-3′ | 25 | 76 |
| $FE_{14}$ $M_{16}$340-R | 5′-CAA ATC TTC CGC TGA GCC CCA ACT A-3′ | 25 | 76 |
| $FE_{15}$ $M_{14}$400-F | 5′-CCG ACT GGA TAG GCT GGA C-3′ | 19 | 57 |
| $FE_{15}$ $M_{14}$400-R | 5′-GAA ATG GGT GAG GGT GGC-3′ | 18 | 57 |
| $FE_{16}$ $M_{8}$350-F | 5′-AAG GTT TCG GGA GTG ATC TGC-3′ | 21 | 60 |
| $FE_{16}$ $M_{8}$350-R | 5′-TCC AAT GTC TCT GCC TCG TCT A-3′ | 22 | 60 |
| **Disulfide isomerase (DSI)** | | | |
| DSI-F | 5′-GCC GTT GCC AAT AAG GAC GA -3′ | 20 | 58 |
| DSI-R | 5′-TCA CCC GCC TTG AGA TTG GT -3′ | 20 | 58 |
| **Thrombospondin (TSP)** | | | |
| TSP288-F | 5′-AAC CCT CTC TCG GGA AAT CGA AC-3′ | 23 | 60 |
| TSP288-R | 5′-GTC GCG GCC GAG GTA CTA ATC T-3′ | 22 | 62 |
| TSP462-F | 5′-ATG GCT AAT CCG GGG CAG TTA T-3′ | 22 | 59 |
| TSP462-R | 5′-CGG GCA GGT ACA AAC TCC TAC G-3′ | 22 | 62 |
| TSP288-1 | 5′-ATT AGT ACC TCG GCC GCG ACC ACC G-3′ | 25 | 67 |
| TSP371-1 | 5′-TCT CCT TCA GGC TCG TTC TGT GCT AGA-3′ | 27 | 64 |
| TSP462-2 | 5′-TTT TCT AAT TCA GGT ACC TCG GCC GC-3′ | 26 | 62 |
| **Sex lethal protein (Sxl)** | | | |
| Sxl-F | 5′-GCG AAC AAA CCT TAT CAT CAA CTA C-3′ | 25 | 70 |
| Sxl-R | 5′-CTT CAT TTT GCC GTG CTC CT-3′ | 20 | 60 |
| **Sex-linked nuclear protein 1 (X-linked in *C. elegans*)** | | | |
| XNP1-F | 5′-TTC CAC CCT TTT GCT TGC TA-3′ | 20 | 58 |
| XNP1-R | 5′-GGA TTT GCC TGA GAC ACC TAA-3′ | 21 | 62 |
| **Hypothetical protein ENSANGP00000010123 (X-linked in *Drosophila melanogater*)** | | | |
| ENS40 – F | 5′-TCA TGT CGG AGG TCG TGA GTA A-3′ | 22 | 66 |
| ENS40 – R | 5′-AGA ACA AGG AAG AGG AGG GAT T-3′ | 22 | 64 |
| ENS44 – F | 5′-GCG TCA TCA AGT TGT CCG AGT C-3′ | 22 | 68 |
| ENS44 – R | 5′-TGA GGG GCA GTG AAG ATA GGT G-3′ | 22 | 68 |

**Table 2.3 (cont.)**

| Gene/Primer | Sequence | Length | Tm (°C) |
|---|---|---|---|
| **Redimentary protein** | | | |
| Rudimentary-F | 5′-CCA GGT GCG ACT CAC AAG GAA G-3′ | 22 | 70 |
| Rudimentary-R | 5′-AGC AGC GGA ACG AGG GGC-3′ | 18 | 62 |
| **Ubiquitin specific protease 9-X linked** | | | |
| USP9X-F | 5′-GGA AAT GGA CCT GGG CGG-3′ | 18 | 60 |
| USP9X-R | 5′ TCT TCT GGA ACT GCT ACC TCT GC-3′ | 23 | 70 |
| **EGF-response factor (Zinc finger protein, X chromosome in *Homo sapiens*)** | | | |
| EGF-F | 5′-GAA TCG GTG TGT TCT CTG GGC-3′ | 21 | 66 |
| EGF-R | 5′-GGC GGC AAG GCT CGG TCT-3′ | 18 | 62 |
| **Zinc finger protein** | | | |
| Zincfinger-F | 5′-TCG AAA CCC TTG CCG CAT AT-3′ | 20 | 60 |
| Finger-R | 5′-TGC TTG AAT CTC CCT CAT CCC-3′ | 21 | 64 |
| **Candidate sex-specific cDNA in *P. monodon*** | | | |
| PMT1700-F | 5′-CGT AAC CAG TAA GAG ATC GGG AG-3′ | 23 | 70 |
| PMT1700-R | 5′-GCT TTT TGG CAG TTT AAG AGA GTC-3′ | 24 | 68 |
| PMO920-F | 5′-TTG AAC CAG TGT TTC TGC AAG T-3′ | 22 | 62 |
| PMO920-R | 5′-TCG AAT AGC ACC AAG TCA TCA A-3′ | 22 | 62 |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 2.4** Primers and primer sequences and melting temperature of heterologous primers designed from sequences of candidate sex-specific AFLP markers in *M. rosenbergii* and cDNAs of *H. asinina*

| Gene/Primer | Sequence | Length | Tm (°C) |
|---|---|---|---|
| **Candidate sex-specific AFLP markers in *M. rosenbergii*** | | | |
| ME$_4$M$_8$517-F | 5′-TGT TAT CCT TCG TTC CCT CC-3′ | 20 | 60 |
| ME$_4$M$_8$517-R | 5′-AAA GTG ACA GTC CTG GCA AA-3′ | 20 | 58 |
| ME$_8$M$_1$310-F | 5′ TTG TCA GAT GGC TAA TAG TGT C 3′ | 22 | 62 |
| ME$_8$M$_1$310-R | 5′ CAA ATG AGA AAT GAA GTG GAA G 3′ | 22 | 60 |
| **Vitelline coat protein of *H. asinina*** | | | |
| VCPg1-F | 5′ GGC TGC ACC AGA CCG ATG AAC GAT ACA C 3′ | 28 | 68 |
| VCPg1-R | 5′ TGC TTC AAC ACC ATA CCG TCT CCA CAA C 3″ | 28 | 65 |
| VCPg2-F | 5′ ACT GGG CTT TCT ACC ATC AAC GTC CTG T 3′ | 28 | 64 |
| VCPg2-R | 5′ CGA CGA CCC CTT GTT CTG GAT AAT CTC A 3′ | 28 | 65 |
| VCPg3-F | 5′ ATA CAC AAT CTC GTG CTC GTT CGG TTC A 3′ | 28 | 64 |
| VCPg3-R | 5′ CAC TAT TCC GTC TCC GCA ACC TGC TCT T 3′ | 28 | 66 |
| VCPg7-F | 5′ TGC TCA GTG CTG GTG GTG CTC TGC TGG G 3′ | 28 | 71 |
| VCPg7-R | 5′ TGG CTG GGC GGT GCC TTG TTA CCT TCT A 3′ | 28 | 70 |
| VCPHA49-F | 5′ ACC CCA CAG GAG GAA CAA ACC 3′ | 21 | 66 |
| VCPHA49-R | 5′ CCG CAG CCA GCT CTA AGG ATA 3′ | 21 | 66 |
| VCPHA75-F | 5′ AGG ATG TGG TGA CGG TAT TGT G 3′ | 22 | 66 |
| VCPHA75-R | 5′ CTT TAT AGT GCG AGC GTT TGG T 3′ | 22 | 64 |
| **Axonemal p66.0 of *H. asinina*** | | | |
| Axonemal-F | 5′ GCA CGA CGA AAC TTC GCC CTG 3′ | 21 | 68 |
| Axonemal-R | 5′ AAC GCC CGC TTC GCT CCC CA 3′ | 20 | 68 |
| **Tektin A1 of *H. asinina*** | | | |
| Tektin-F | 5′ TGT TGA CAG GGA TGG TGC GG 3′ | 20 | 64 |
| Tektin-R | 5′ CTC CTT TGC CTA CAG TTG AGA TTG 3′ | 24 | 70 |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 2.4 (cont.)**

| Gene/Primer | Sequence | Length | Tm (°C) |
|---|---|---|---|
| **Vitellogenin-1 of *H. asinina*** | | | |
| VTG1-F | 5′ ACA TCA GAA CCG ACG GCA AC 3′ | 20 | 62 |
| VTG1-R | 5′ TGA GGC AAG GTA GGC GAG G 3′ | 19 | 62 |
| **Small androgenreceptor-interacting protein (SARIP) of *H. asinina*** | | | |
| SARIP-F | 5′ GGC TTA GTG ACT GAA CGC CTC TA 3′ | 23 | 70 |
| SARIP-R | 5′ GCT GCT CTA CTA CGC ACA ACA C 3′ | 22 | 68 |
| **Gonadotropin inducible ovarian transcription factor 1 (GIOT1) of *H. asinina*** | | | |
| GIOT1-F | 5′ GAC CAC CCA CGC ACA GGA C 3′ | 19 | 64 |
| GIOT1-R | 5′ TAG CAG CAC TAA TAA AGC CCC G 3′ | 22 | 66 |
| **Hydroxysteroid dehydrogenase-like protein (HSD) of *H. asinina*** | | | |
| HaHSD-F | 5′ GCC GTG GAA GAA GCA GTT GGA 3′ | 21 | 68 |
| HaHSD-R | 5′ CAG GGG GTT GAG GTT GAG TGG 3′ | 21 | 68 |
| **Fertilization protein (FP) of *H. asinina*** | | | |
| HaFP – F | 5′ CGA CCC ATA GCG GCG TAG TT 3′ | 20 | 64 |
| HaFP – R | 5′ AAG GTC CCA AAG AAA AGC CAG TA 3′ | 23 | 66 |
| **Sperm lysin of *H. asinina*** | | | |
| Halysin–F | 5′ CGA GAA GGC AGA CAG CCA GAC 3′ | 21 | 68 |
| Halysin–R | 5′ ATC CAG TGC TTG ATG CTT GAC G 3′ | 22 | 66 |
| **DMRT1 of *H. asinina*** | | | |
| DMRT1-F | 5′ GGA AAT GGA CCT GGG CGG 3′ | 18 | 60 |
| DMRT1-R | 5′ TCT TCT GGA ACT GCT ACC TCT GC 3′ | 23 | 70 |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 2.5** PCR conditions used for amplification of candidate sex-specific/differential expression markers of *P. monodon* using primers in Table 2.3

| Primer | dNTPs (μM) | $MgCl_2$ (mM) | Primer (μM) | PCR conditions |
|---|---|---|---|---|
| 1. $FE_{10}$ $M_9$520 | 200 | 1.5 | 0.5 | 94°C for 3 min;1 cycle followed by<br>94°C for 30 s<br>55°C for45 s<br>72°C for 45 s for 35 cycles and<br>72°C for 7 min; 1 cycle |
| 2. $FE_{10}$ $M_{10}$725.1 | 200 | 1.5 | 0.5 | As described in1. |
| 3. $FE_{10}$ $M_{10}$725.2 | 200 | 1.5 | 0.5 | 94°C for 3 min;1 cycle followed by<br>94°C for 45 s<br>45°C for 60 s<br>72°C for 60 for 5 cycles and<br>94°C for 45 s<br>53°C for 60 s<br>72°C for 60 s for additional 35 cycles and<br>72°C for 7 min;1 cycle |
| 4. $FE_{14}$ $M_{16}$340 | 200 | 1.5 | 0.5 | As described in1. |
| 5. $FE_{15}$ $M_{14}$400 | 200 | 1.5 | 0.5 | As described in 3. |
| 6. $FE_{16}$ $M_8$350 | 200 | 1.5 | 0.5 | As described in 3. |
| 7. Disulfide isomerase (DSI) | 200 | 1.5 | 0.2 | 94°C for 3 min; 1 cycle followed by<br>94°C for 45 s<br>55°C for 60 s<br>72°C for 60 s for 35 cycles and<br>72°C for 7 min ;1 cycle |
| 8. Thrombospondin $(TSP)_{288\text{-}1+371\text{-}1}$ | 200 | 2 | 0.4 | 94°C for 3 min; 1 cycle followed by<br>94°C for 45 s<br>58°C for 60 s<br>72°C for 2 min; for 35 cycles and<br>72°C for 7 min ; 1 cycle |
| 9. Thrombospondin $(TSP)_{462\text{-}F+288\text{-}R}$ | 200 | 2 | 0.4 | As described in 8. |
| 10. Sex lethal protein (Sxl) | 100 | 2 | 0.5 | 94°C for 3 min ; 1 cycle followed by<br>94°C for 30 s<br>55°C for 60 s<br>72°C for 60 s; for 35 cycles and<br>72°C, 7 min ;1 cycle |
| 11. XNP-1 | 100 | 2 | 0.2 | 94°C for 3 min; 1 cycle followed by<br>94°C for 30 s<br>50°C for 60 s<br>72°C for 60 s ; for 5 cycles and<br>94°C for 30 s<br>55°C for 60 s<br>72°C for 60 s ; for additional 35 cycles and<br>72°C for7 min; 1 cycle |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Tavle 2.5 (cont.)**

| Primer | dNTPs (µM) | $MgCl_2$ (mM) | Primer (µM) | PCR conditions |
|---|---|---|---|---|
| 12. Rudimentary protein | 200 | 2 | 0.4 | 94°C, 3 min; 1 cycle followed by<br>94°C for 30 s<br>45°C for 60 s<br>72°C for 90 s ; for 5 cycles and<br>94 for 30 s<br>55°C for 60 s<br>72°C for 90 s; for additional 35 cycles and<br>72°C for 7 min ; 1 cycle |
| 13. Ubiquitin P9 | 200 | 2 | 0.4 | As described in 12. |
| 14. EGF | 200 | 2 | 0.3 | 94°C, 3 min ; 1 cycle followed by<br>94°C for 30 s<br>58°C for 45 s<br>72°C for 45 s ; for 35 cycles and<br>72°C for 7 min ; 1 cycle |
| 15. Zinc finger protein (Zincfing-F/ Finger-R) | 200 | 1.5 | 0.2 | As described in 7. |
| 16. PMT1700 | 200 | 1.5 | 0.2 | As described in 7. |
| 17. PMO920 | 200 | 1.5 | 0.2 | As described in 7. |
| 18. Peritrophin1 | 200 | 1.5 | 0.2 | 94°C for 3 min; 1 cycle followed by<br>94°C for 45 s<br>53°C for 60 s<br>72°C for 60 s; for 35 cycles<br>72°C for 7 min; for 1 cycle |
| 19. Peritrophin2 | 200 | 1.5 | 0.2 | 94°C for 3 min; 1 cycle followed by<br>94°C for 30 s<br>55°C for 60 s<br>72°C for 60 s; for 35 cycles and<br>72°C for 7 minute; 1 cycle |
| 20. AFLP 517 | 200 | 1.5 | 0.2 | 94°C for 3 min; 1 cycle followed by<br>94°C for 45 s<br>58°C for 60 s<br>72°C for 60 s; for 35 cycles and<br>72°C for 7 min;1 cycle |
| 21. TSP1300 | 200 | 1.5 | 0.2 | As described in 7 |
| 22. HEP44 | 200 | 2 | 0.4 | 94°C for 3 min ; 1 cycle followed by<br>94°C for 30 s<br>55°C for 45 s<br>72°C for 90 s; for 35 cycles<br>72°C for 7 min;1 cycle |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 2.6** PCR conditions used for amplification of candidate sex-specific/differential expression markers of *P. monodon* using heterspecific primers in Table 2.4

| Primer | dNTPs (μM) | $MgCl_2$ (mM) | Primer (μM) | PCR conditions |
|---|---|---|---|---|
| 1. $ME_4M_8517$ | 200 | 1.5 | 0.5 | 94°C for 3 min;1 cycle followed by<br>94°C for 30 s<br>55°C for 45 s<br>72°C for 45 s; for 35 cycles<br>72°C for 7 min; 1 cycle |
| 2. $ME_8M_1310$ | 200 | 1.5 | 0.5 | 94°C for 3 min; 1 cycle followed by<br>94°C for 30 s<br>42°C for 45 s<br>72°C for 30 s; for 5 cycles and<br>94°C for 30 s<br>53°C for 45 s<br>72°C for 30 s; for additional 35 cycles and<br>72°C for 7 min; for 1 cycle |
| 3. VCPg1 | 100 | 1.5 | 0.5 | 94°C for 3 min; 1 cycle<br>94°C for 30 s<br>42°C for 60 s<br>72°C for 90 s; for 5 cycles and<br>94°C for 30 s<br>55°C for 60 s<br>72°C for 90 s; for additional 35 cycles and<br>72°C for 7 min; 1 cycle |
| 4. VCPg2 | 200 | 1.5 | 0.5 | 94°C for 3 min; 1 cycle followed by<br>94°C for 30 s<br>45°C for 60 s<br>72°C for 90 s; 5 cycles and<br>94°C for 30 s<br>55°C for 60 s<br>72°C for 90 s; for additional 35 cycles and<br>72°C for 7 min; 1 cycle |
| 5. VCPg3 | 100 | 1.5 | 0.5 | As described in 3 |
| 6. VCPg7 | 100 | 1.5 | 0.5 | As described in 3 |
| 7. VCPHA49 | 100 | 2 | 0.5 | 94°C for 3 min;1 cycle followed by<br>94°C for 30 s<br>55°C for 60 s<br>72°C for 90 s; for 35 cycles and<br>72°C for 7 min; 1 cycle |
| 8. VCPHA75 | 100 | 2 | 0.5 | As described in 7. |

**Table 2.6 (cont.)**

| Primer | dNTPs (µM) | $MgCl_2$ (mM) | Primer (µM) | PCR conditions |
|---|---|---|---|---|
| 9. Axonemal | 200 | 1.5 | 0.3 | 94°C for 3 min; 1 cycle followed by<br>94°C for 45 s<br>45°C for 45 s<br>72°C for 45 s; for 5 cycles and<br>94°C for 45 s<br>58°C for 45 s<br>72°C for 42 s; for additional 35 cycles and<br>72°C for 7 min; 1 cycle |
| 10. Tektin A1 | 200 | 1.5 | 0.3 | As described in 9. |
| 11. VTG1 | 200 | 2 | 0.4 | 94°C for 3 min; 1 cycle followed by<br>94°C for 60 s<br>42 for 60 s<br>72 for 2 min; for 5 cycles and<br>94°C for 60 s<br>55°C for 60 s<br>72°C for 2 min; for additional 35 cycles and<br>72°C for 7 min; 1 cycle |
| 12. SARIP | 200 | 2 | 0.4 | As described in 11. |
| 13. GIOT1 | 200 | 2 | 0.4 | As described in 11. |
| 14. HaHSD | 200 | 2 | 0.4 | As described in 11. |
| 15. Halysin | 200 | 2 | 0.4 | As described in 11. |
| 16. DMRT1 | 200 | 2 | 0.4 | 94°C for 3 min; 1 cycle followed by<br>94 for 30 s<br>45°C for 45 s<br>72°C for 45 s; for 5 cycles and<br>94°C for 30 s<br>55°C for 45 s<br>72°C for 45 s; for 35 cycles<br>72°C for 7 min; 1 cycle |
| 17. HaFP | 100 | 2 | 0.4 | 94°C for 3 min; 1 cycle followed by<br>94°C for 60 s<br>42°C for 60 s<br>72°C for 2 min; for 5 cycles and<br>94°C for 60 s<br>55°C for 60 s<br>72°C for 2 min; for additional 35 cycles and<br>72°C, 7 min ; 1 cycle |
| 18. Adam | 100 | 2 | 0.5 | 94°C for 3 min;1 cycle followed by<br>94°C,for 30 s<br>55°C for 60 s<br>72°C for 60 s; for additional 35 cycles and<br>72°C, 7 min; 1 cycle |

**Part II : Genetic diversity of *P. monodon* using nuclear DNA polymorphism**

**2.11 Preparation of SSCP gel**

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.

**2.12 Preparation of denaturing polyacylamide gels**

Denaturing polyacrylamide gels were prepared in essentially identical to that described for AFLP analysis with the exception that the gel format (PROTEAN II xi Cell) and thickness (0.5 mM) was different from those of the sequencing gel (SA32 adjustable sequencing gel apparatus).

**2.13 Nuclear DNA polymorphism analyzed by SSCP and denaturing PAGE analysis**

Four primer pairs for amplification of disulfide isomerase (DSI), PMT1700, zinc finger protein gene and PMO920 were used for determination of genetic diversity and population differentiation of *P. monodon* in Thai waters. Originally, DSI and zinc finger protein gene were isolated from cDNA from ovaries of *P. monodon* whereas PMO920 and PMT1700 were fragments of unknown transcripts showing specific expression patterns in ovaries and testes of *P. monodon*, respectively. The amplification product of these DNA segment exhibited polymorphism when analyzed by SSCP analysis. As a result, the experiment was extended for larger sample sizes.

In a total, 141 individuals of *P. monodon* originating from Satun ($N$ = 38), Trang ($N$ = 22) and Phangnga ($N$ = 26) located in the Andaman Sea and Chumphon ($N$ = 28) and Tard ($N$ = 27) located in the Gulf of Thailand were genotyped at 4 nucleare loci. Conditions for amplification of these nuclear DNA markers were described in Table 2.3.

For SSCP analysis, 6 μl of the amplified DSI, zinc finger protein gene and PMT1700 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 products of DSI (12.5% + 5% glycerol; 37.5:1 crosslink), zinc finger protein gene and PMT1700 (15% without glycerol supplementation; 75 : 1 crosslink) were electrophoretically analyzed in native polyacrylamide gels at 250 – 300 volts for 16 – 24 hours at 4°C. Fractionated bands were visualized by silver staining

For genotyping using denaturing polyacrylamide gel electrophoresis, 6 μl of the amplified PMO920 were mixed with 3 μl of the acrylamide gel loading dye (98% formamide, 200 μl EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol), denatured in a boiling bath for 5 minutes and immediately cooled on ice for 3 minutes. The denatured product of PMO920 was size-fractionated through 6% denaturing polyacrylamide gel at 35 W for 2.5 hours. The fractionated bands were visualized by silver staining.

**2.14 Data analysis**

Nucleotide sequences of AFLP fragments were compared with those previously deposited in the GenBank using BLAST*N* and BLAST*X* (Altschul et al., 1990 available at http://www.ncbi/nlm/nih.gov). Significant probabilities of matched nucleotides/proteins were considered when the E-value was < $10^{-4}$.

For PMO920, zinc finger protein gene and PMT1700, observed and expected heterozygosity were calculated (Nei, 1987). The effective number of alleles at each locus was examined (Crow and Kimura, 1965). Hardy-Weinberg expectations and genotypic disequilibrium were analysed using the exact test. Geographic heterogeneity in allele distribution frequencies among compared samples was carried out with the Markov chain approach for $\chi^2$ analysis (Guo and Thompson, 1992). $F_{ST}$ between pairs of samples (Weir and Cockerham, 1984) was determined if it was significantly different from zero. All tests were routine in GENEPOP (Raymond and Rousset, 1995).

For DSI, each RAPD fragment is treated as an independent character. Sizes of the bands were estimated by comparing with a 100 bp ladder and recorded in a binary matrix to represent the presence (1) or the absence (2) of a particular band.

The average genetic distance between geographic samples were calculate. Genetic distance between paired samples was then calculated and used to construct a neighbor-joining tree (Saitou and Nei, 1987) using Neighbor in PHYLIP (Felsenstein, 1993). Geographic heterogeneity and $F_{ST}$ statistics (θ) between pairs of samples were analyzed using TFPGA (available at http://www.public.asu.edu/~mmille8/tfpga.htm).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

# CHAPTER III

# RESULTS

## 3.1 DNA extraction

Genomic DNA was extracted from a piece of pleopod of each *P. monodon* using a phenol-chloroform-proteinase K method (Klinbunga et al., 1999). The quality of extracted genomic DNA was electrophoretically determined using a 0.8 % agarose gel (w/v). High molecular weight DNA at 23.1 kb along with sheared DNA was obtained from pleopods of broodstock-sized shrimp kept at –30 °C since 1997 (Figure 3.1) whereas the quality of genomic DNA extracted from farmed shrimps clearly showed higher quality than the first sample set (Figure 3.2). The ratio of $OD_{260}/OD_{280}$ of extracted DNA ranged from 1.8 – 2.0 indicating that DNA samples were relatively pure. Some DNA samples contained RNA contamination as visualized by the smear at the bottom of gel.

## 3.2 AFLP analysis

Smear preamplification products with the molecular weight greater than 300 bp was observed (Figure 3.3). This indicated successful restriction/ligation of genomic DNA of bulked female and male *P. monodon*. The preamplification products were then subjected to selective amplification and the resulting products analyzed by agarose gel electrophoresis revealed different band patterns between the same template but different selective primers combinations (Figure 3.4 and 3.5) implying the successful development of genomic DNA template for further screen of candidate sex-specific markers in *P. monodon* by AFLP analysis.

The two-step amplification strategy, firstly with a pair of preamplification primers (one selective nucleotide) and secondly with a pair of selective primers (three selective nucleotides) allow the possibility to screen a total of 256 primer combinations (16 primers of $E_{+3}$ and $M_{+3}$). These primers were divided to 4 separate set ($E_{+3}$-1-8 + $M_{+3}$-1-8, $E_{+3}$-1-8 + $M_{+3}$-9-16, $E_{+3}$-9-16 + $M_{+3}$-1-8 and $E_{+3}$-9-16 + $M_{+3}$ - 9-16) and screened against bulked genomic DNA of male and female *P. monodon*.

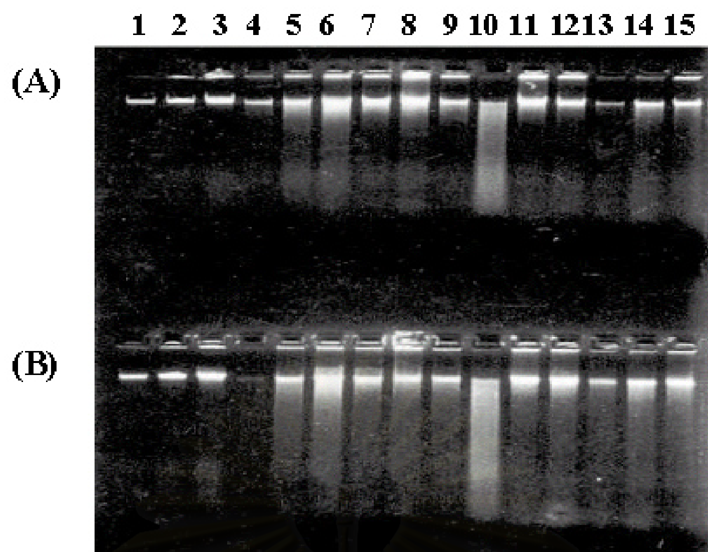



**Figure 3.1** A 0.8% ethidium bromide stained-agarose gel showing the quality of genomic DNA extracted from the pleopod of *P. monodon*
Lanes 1 – 3 = 50, 100 and 200 ng of undigested lambda DNA, respectively.
Lanes 4 – 15 (panels A and B) = Genomic DNA individually extracted from pleopod of *P. monodon*

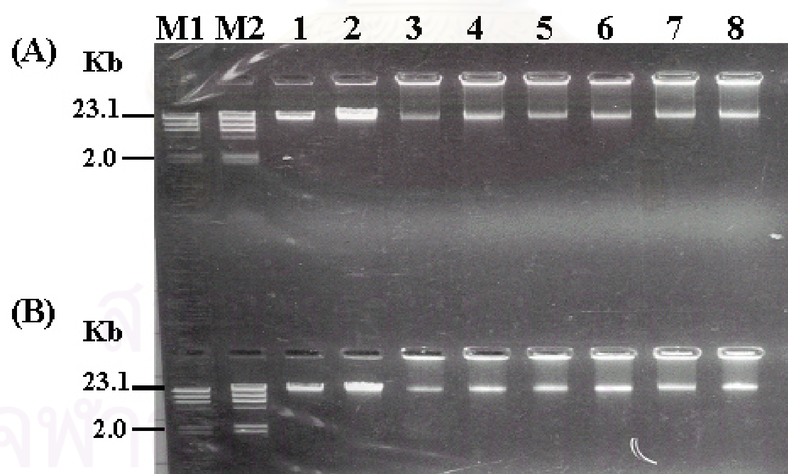



**Figure 3.2** A 0.8% ethidium bromide stained-agarose gel showing the quality of genomic DNA extracted from pooled pleopods of *P. monodon*
Lanes M1 and M2 = 100 and 200 ng of λ-*Hin*d III
Lanes 1 and 2 = 100 and 200 ng of undigested λDNA
Lanes 3 – 8 = Genomic DNA extracted from pooled pleopods of *P. monodon* ($N = 5$)

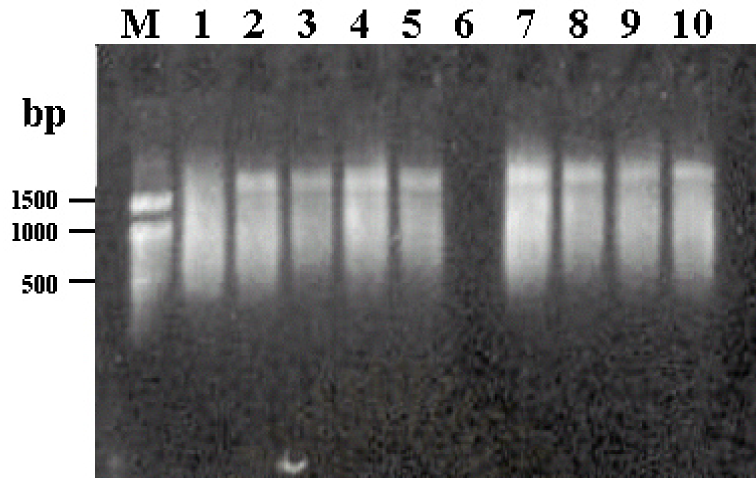
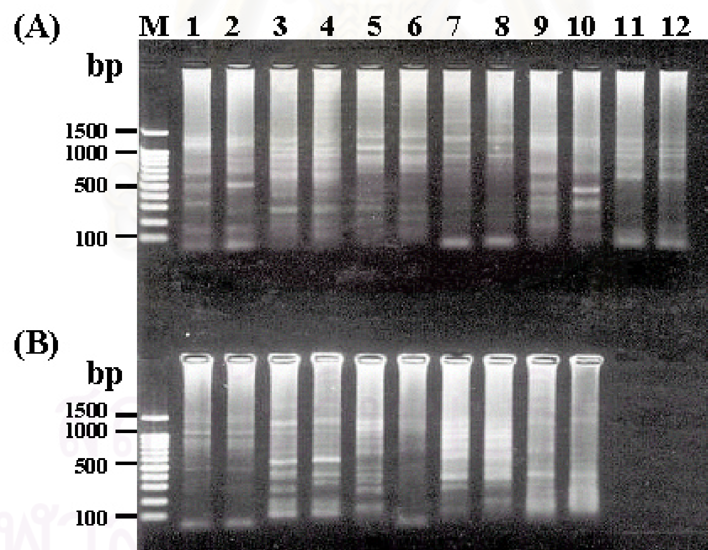



**Figure 3.3** A 1.8% agarose gel electrophoresis showing preamplification of bulked female DNA (lanes 2 – 5) and bulked male DNA (lanes 7 – 10). Lane M = a 100 bp DNA marker.




**ure 3.4** A 1.8% agarose gel electrophoresis showing selective amplification products of bulked PMF1 (odd lanes) and bulked PMM1 (even lanes) using various primer combinations. Lane M; a 100 bp DNA marker, Panel A, lanes 1 – 2; $E_{+3}$-3/$M_{+3}$-3, lanes 3 – 4; $E_{+3}$-3/$M_{+3}$-4, lanes 5 – 6; $E_{+3}$-4/$M_{+3}$-1, lanes 7 – 8; $E_{+3}$-4/$M_{+3}$-2, lanes 9 – 10; $E_{+3}$-4/$M_{+3}$-3, lanes 11 – 12; $E_{+3}$-4/$M_{+3}$-4. Panel B, lanes1 – 2; $E_{+3}$-5/$M_{+3}$-1, lanes 3 - 4; $E_{+3}$-5/$M_{+3}$-2, lanes 5 – 6; $E_{+3}$-5/$M_{+3}$-3, lanes 7 – 8; $E_{+3}$-5/$M_{+3}$-4, lanes 9 – 10; $E_{+3}$-6/$M_{+3}$-1.

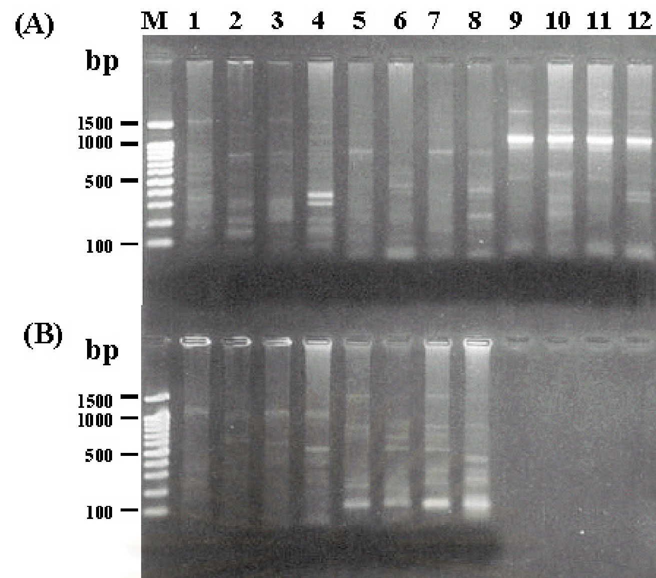
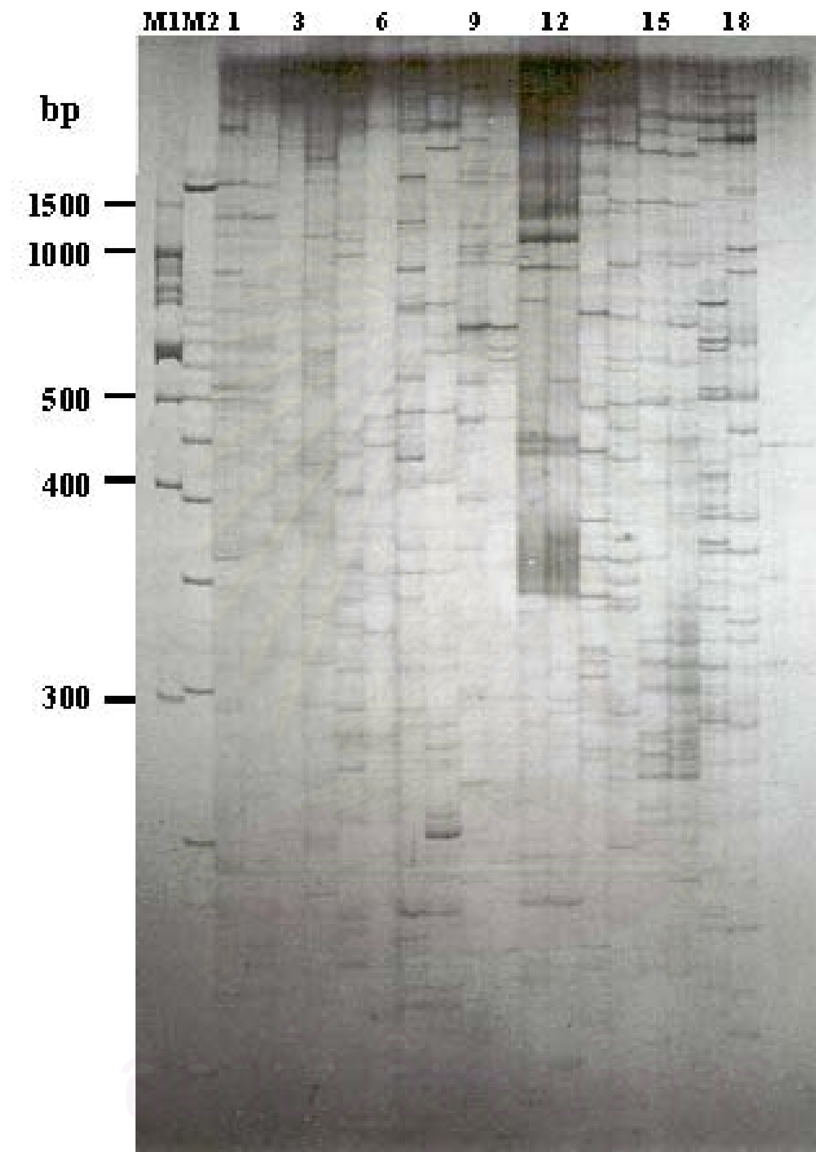



**Figure 3.5** A 1.5% agarose gel electrophoresis showing selective amplification products of bulked PMF1 (lanes 1, 5, and 9), PMF2 (lanes 2, 6 and 10), PMM1 (lanes 3, 7 and 11) and PMM2 (lanes 4, 8 and 12) using primers $E_{+3}$-3/$M_{+3}$-6 (lanes 1 - 4, panel A) $E_{+3}$-3/$M_{+3}$-7 (lanes 5 – 8, A), $E_{+3}$-3/$M_{+3}$-8 (lanes 9 – 12, A), $E_{+3}$-4/$M_{+3}$-5 (lanes 1 – 4, B) and $E_{+3}$-4/$M_{+3}$-6 (lanes 5 – 8, B). Lanes M was a 100 bp DNA marker.

The selective amplification products were size-fractionated through 4.5 % denaturing polyacylamide gels (19 : 1 crosslink, Figure 3.6). Initially, 64 primer combinations ($E_{+3}$-1 - 8 +$M_{+3}$-1 - 8) were screened against 2-bulked DNA (PMF1 and PMM1) and a total of 168 and 108 candidate female- and male-specific AFLP markers with the molecular length greater than 300 bp were observed.

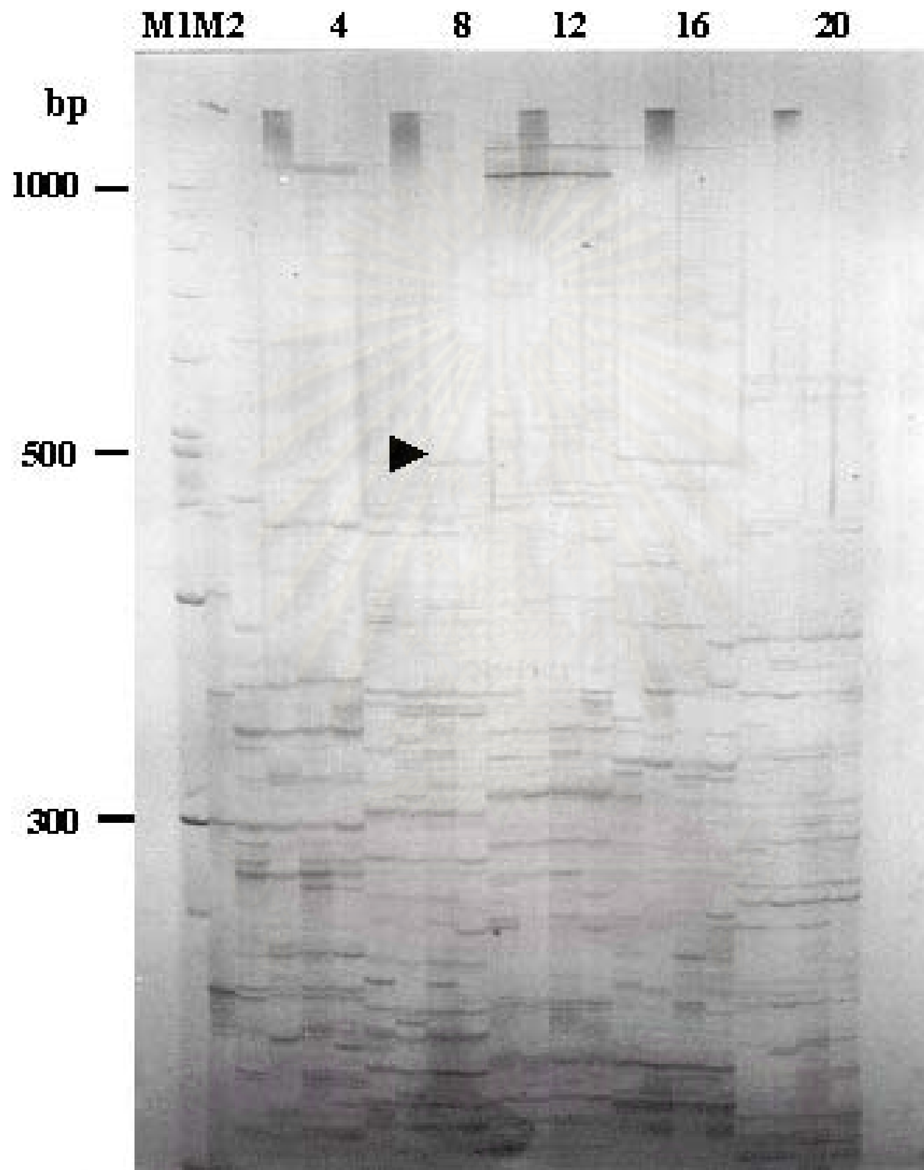
To reduce the false positive amplification bands, 46 informative primer combinations were re-screened with 4 bulked DNA (PMF1, PMF2, PMM1 and PMM2) and the number of promising markers were decreased to only 8 fragments in females and 15 markers in males of *P. monodon* (Table 3.1 and Figs.3.7-3.10).

The third screening was then carried out against bulked PMF3 and PMM3 established from genomic DNA of juvenile *P. monodon* showing well characterized thelycum (female) and petasma (male). No sex-specific AFLP markers were found after three round screening with the first set of primers (Table 3.2).



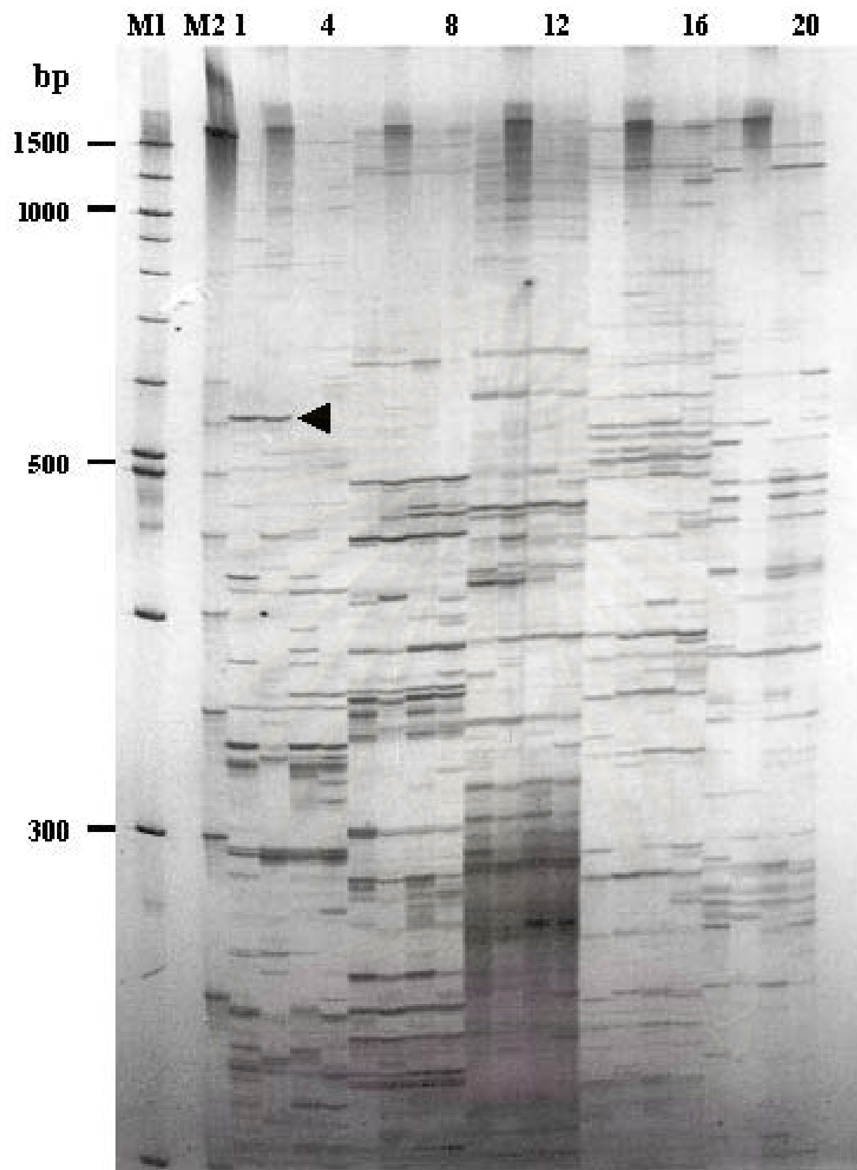

จุฬาลงกรณ์มหาวิทยาลัย

**Figure 3.6** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products from 2 bulked DNA (PMF1 and PMM1) using $E_{+3}$-8/$M_{+3}$-3 (lanes 1 – 2), $E_{+3}$-8/$M_{+3}$-4 (lanes 3 – 4), $E_{+3}$-1/$M_{+3}$-5 (lanes 5 – 6), $E_{+3}$-1/$M_{+3}$-6 (lanes 7 – 8), $E_{+3}$-1/$M_{+3}$-7 (lanes 9 – 10), $E_{+3}$-1/$M_{+3}$-8 (lanes 11 – 12), $E_{+3}$-2/$M_{+3}$-5 (lanes 13 – 14), $E_{+3}$-2/$M_{+3}$-6 (lanes 15 – 16) and $E_{+3}$-2/$M_{+3}$-7 (lanes 17 – 18).
Lane M1 and M2 were 100 bp and 50 bp DNA markers, respectively.




จุฬาลงกรณ์มหาวิทยาลัย

**Figure 3.7** A 4.5% denaturing polyacrylamide gel elctrophoresis showing AFLP products of 4 bulked DNA PMF1; (lanes 1, 5, 9, 13 and 17), PMF2 (lanes 2, 6, 10, 14 and 18), PMM1 (3, 7, 11, 15 and 19) and PMM2 (lanes 4, 8, 12, 16 and 20) using primers $E_{+3}$-3/$M_{+3}$-6 (lanes 1 – 4), $E_{+3}$-3/$M_{+3}$-7 (lanes 5 – 8): $E_{+3}$-3/$M_{+3}$-8 (lanes 9 – 12) $E_{+3}$-4/$M_{+3}$-6 (lanes 13 – 16), $E_{+3}$-4/$M_{+3}$-7 (lanes 17 – 20). An arrowhead indicates a candidate sex-specific AFLP marker from $E_{+3}$-3/$M_{+3}$-7.Lanes M1 and M2 were 100 bp and 50 bp DNA markers, respectively.




สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Figure 3.8** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP product of 4 bulked DNA; PMF1 (lanes 1, 5, 9, 13 and 17), PMF2 (lanes 2, 6, 10, 14 and 18), PMM1 (3, 7, 11, 15 and 19) and PMM2 (lanes 4, 8, 12, 16 and 20) using primers $E_{+3}$-8/$M_{+3}$-5 (lanes 1 – 4), $E_{+3}$-2/$M_{+3}$-2 (lanes 5 – 8), $E_{+3}$-6/$M_{+3}$-4 (lanes 9 – 12), $E_{+3}$-7/$M_{+3}$-2 (lanes 13 – 16) and $E_{+3}$-1/$M_{+3}$-7 (lanes 17 – 20). An arrowhead indicates a candidate sex-specific AFLP marker from $E_{+3}$-8/$M_{+3}$-5. Lanes M1 and M2 were 100 bp and 50 bp DNA markers, respectively.

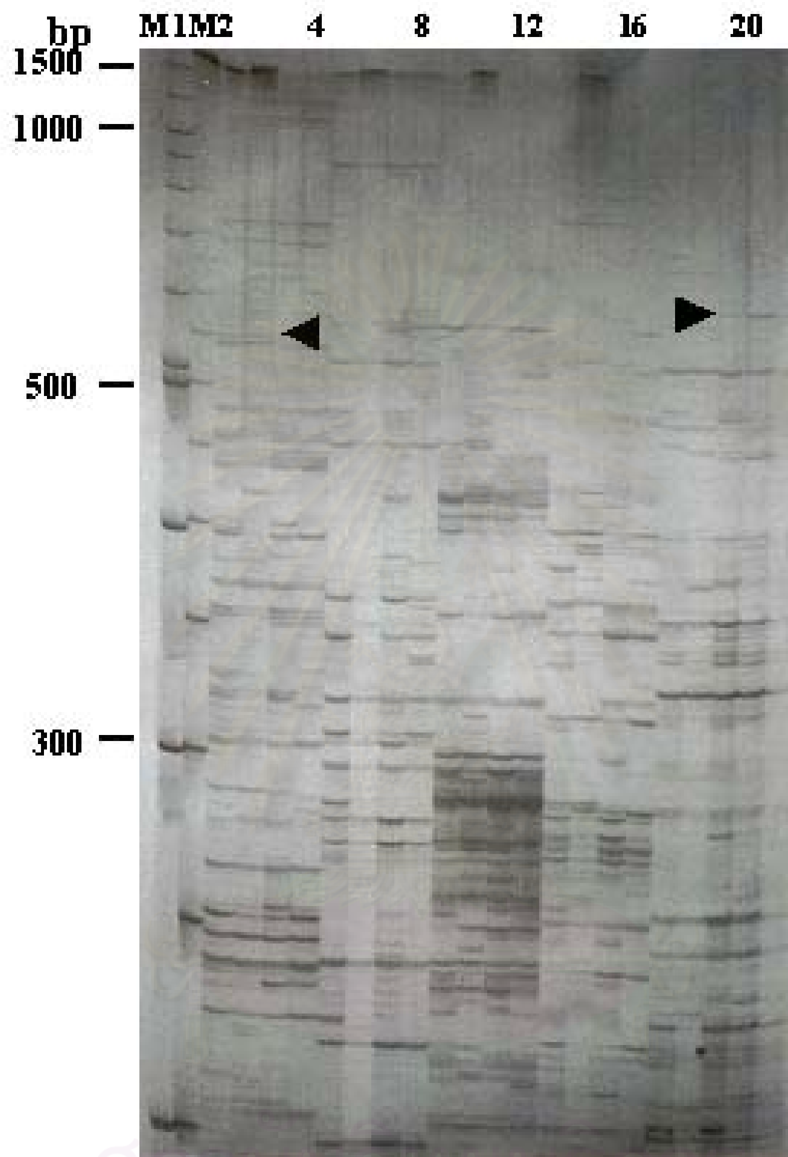
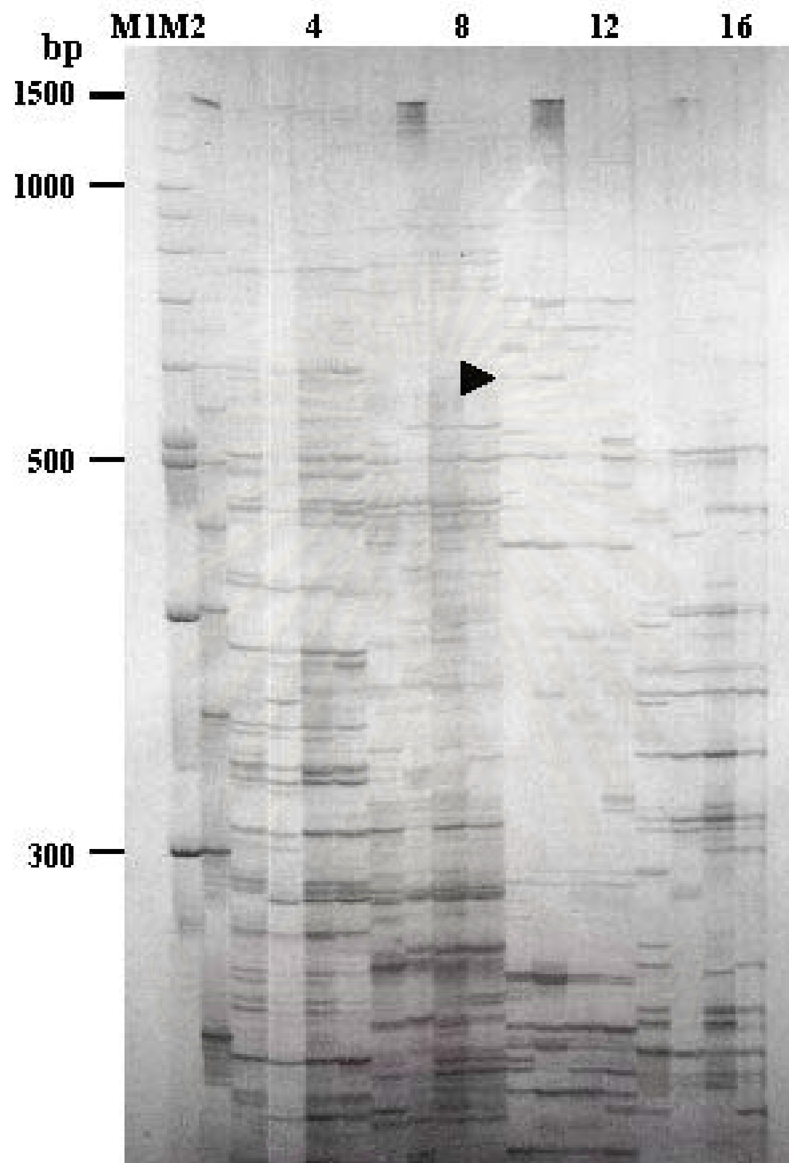



**Figure 3.9** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP product of 4 bulked DNA; PMF1 (lanes 1, 5, 9, 13 and 17), PMF2 (lanes 2, 6, 10, 14 and 18), PMM1 (3, 7, 11, 15 and 19) and PMM2 (lanes 4, 8, 12, 16 and 20) using primers $E_{+3}$-3/$M_{+3}$-3 (lanes 1 – 4), $E_{+3}$-4/$M_{+3}$-2 (lanes 5 – 8), $E_{+3}$-4/$M_{+3}$-3 (lanes 9 – 12), $E_{+3}$-4/$M_{+3}$-4 (lanes 13 – 16) and $E_{+3}$-6/$M_{+3}$-2 (lanes 17 – 20). Arrowheads indicate candidate sex-specific AFLP markers. Lanes M1 and M2 were 100 bp and 50 bp DNA markers, respectively.




สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Figure 3.10** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP product of 4 bulked PMF1 (lanes 1, 5, 9 and 13), PMF2 (lanes 2, 6, 10 and 14), M1 (3, 7, 11, and 15) and M2 (lanes 4, 8, 12, and 16) using primers $E_{+3}$-8/$M_{+3}$-3 (lanes 1 – 4), $E_{+3}$-8/$M_{+3}$-4 (lanes 5 – 8), $E_{+3}$-1/$M_{+3}$-5 (lanes 9 – 12) and $E_{+3}$-1/$M_{+3}$-6 (lanes 13 – 16). Arrowheads indicate candidate sex-specific AFLP markers. Lanes M1 and M2 were 100 bp and 50 bp DNA markers, respectively.

**Table 3.1** Candidate sex-specific markers of *P. monodon* generated by AFLP using primers $E_{+3}$-1 - 8 +$M_{+3}$-1 - 8

| Primer combinations | 2 Bulked DNA (bp) | | 4 Bulked DNA (bp) | |
|---|---|---|---|---|
| | PMF1 | PMM1 | PMF1–PMF2 | PMM1–PMM2 |
| $E_{+3}$-1/$M_{+3}$-5 | 175,190,210,1000, 1100 | 230,350,400 | 600*** 850*** | - |
| $E_{+3}$-1/$M_{+3}$-6 | 190,330,830,1200, 1600 | 150,180 | - | - |
| $E_{+3}$-1/$M_{+3}$-7 | 170,260,1100,1500 | - | - | - |
| $E_{+3}$-1/$M_{+3}$-8 | 600 | 200,400 | - | |
| $E_{+3}$-2/$M_{+3}$-2 | - | 350 | - | 400*** |
| $E_{+3}$-2/$M_{+3}$-4 | - | 470 | - | - |
| $E_{+3}$-2/$M_{+3}$-5 | 110,200,340,390 | 140,145 | - | - |
| $E_{+3}$-2/$M_{+3}$-6 | 155,170,260,390 | 140,190,240,270,555 | - | 300*** |
| $E_{+3}$-2/$M_{+3}$-7 | 130,140,190,210,250, 260,280,320,400,500, 520,600 | 230,350,800,1000, 1500 | - | - |
| $E_{+3}$-2/$M_{+3}$-8 | 210,240 | 300 | - | - |
| $E_{+3}$-3/$M_{+3}$-3 | 150,180,190,235, 265,310,1080 | - | 550*** | |
| $E_{+3}$-3/$M_{+3}$-6 | 140,180,190,200, 1000 | 135,160,195,210,240, 285,1500, | - | 360*** |
| $E_{+3}$-3/$M_{+3}$-7 | - | 145,250,350,800, 1500 | | |
| $E_{+3}$-3/$M_{+3}$-8 | 250,350,700 | - | - | 460** |
| $E_{+3}$-4/$M_{+3}$-2 | 180,320,500 | 190,240 | - | - |
| $E_{+3}$-4/$M_{+3}$-3 | 160,230,240,245, 275,320 | 150,165 | - | - |
| $E_{+3}$-4/$M_{+3}$-4 | 240,1000 | 230 | - | 325*** |
| $E_{+3}$-4/$M_{+3}$-5 | 150,160,250,350,400, 700,900,1000,1400, 1500 | 1200,500 | - | - |
| $E_{+3}$-4/$M_{+3}$-6 | 150,165,170,200,225 | 155,230,1500 | 420*** | |
| $E_{+3}$-4/$M_{+3}$-7 | 155,230,260,400, 450 | 350 | - | - |
| $E_{+3}$-4/$M_{+3}$-8 | - | 165,170,250 | - | - |
| $E_{+3}$-5/$M_{+3}$-1 | 400 | 1050 | - | - |
| $E_{+3}$-5/$M_{+3}$-2 | 390 | 280,300,400 | - | - |
| $E_{+3}$-5/$M_{+3}$-3 | 230,250,260,600, 1500 | 275,300 | | 600** |
| $E_{+3}$-5/$M_{+3}$-4 | 210 | 150,320 | 350*** | - |
| $E_{+3}$-5/$M_{+3}$-5 | 125,170,210,600,700 | 330,650,800 | - | - |
| $E_{+3}$-5/$M_{+3}$-6 | 215,230,1300 | 160,240 | - | - |
| $E_{+3}$-5/$M_{+3}$-7 | 155,165,425 | 140,500 | - | - |
| $E_{+3}$-5/$M_{+3}$-8 | 155,160,175,220,260, 300,425 | - | - | - |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 3.1 (continued)**

| Primer combinations | 2 Bulked DNA (bp) | | 4 Bulked DNA (bp) | |
|---|---|---|---|---|
| | **PMF1** | **PMM1** | **PMF1–PMF2** | **PMM1–PMM2** |
| $E_{+3}$-6/$M_{+3}$-2 | 240,245,300,360 | 290,450 | - | 450** 575** |
| $E_{+3}$-6/$M_{+3}$-3 | 240,300,330,360, 700,1500 | 210,220,245,400, 1000 | - | - |
| $E_{+3}$-6/$M_{+3}$-4 | 235,550,700,1500 | 240,1400 | - | 625*** |
| $E_{+3}$-6/$M_{+3}$-6 | 170,180,220,375,400 | - | 780*** | - |
| $E_{+3}$-6/$M_{+3}$-7 | 160,190,200,250,350 | - | - | - |
| $E_{+3}$-6/$M_{+3}$-8 | 220,230,240,300,500 900 | 250 | | 1300*** |
| $E_{+3}$-7/$M_{+3}$-1 | 240,250,290,340 800 | 270,450,600,1400 | - | 400** |
| $E_{+3}$-7/$M_{+3}$-2 | 220,300,370 | 270,450,1000 | - | 360*** 460*** |
| $E_{+3}$-7/$M_{+3}$-3 | 285,300,330,450,800 | 245,260,340,1000 | 800*** | - |
| $E_{+3}$-7/$M_{+3}$-4 | 290 | 330,390 | - | - |
| $E_{+3}$-7/$M_{+3}$-5 | 170,200,310,800 | 295,850,1500 | - | - |
| $E_{+3}$-7/$M_{+3}$-7 | 180,210,390,700, 1000 | 170,220 | - | |
| $E_{+3}$-8/$M_{+3}$-1 | 370 | 240,270,390,700 | - | - |
| $E_{+3}$-8/$M_{+3}$-2 | - | 250,265,270,275, 300,450 | - | - |
| $E_{+3}$-8/$M_{+3}$-3 | 260,800,1500 | - | - | 640*** |
| $E_{+3}$-8/$M_{+3}$-4 | - | 220,240,430,700, 1500 | - | 400*** |
| $E_{+3}$-8/$M_{+3}$-5 | 170,185 | 235,1000 | 550*** | - |
| **Total** | **168** | **108** | **8** | **15** |

*** = indicate fragments found in one sex but absent from the opposite sex of *P. monodon* where the intensity of bands was identical.

** = indicate fragments found in one sex but absent from the opposite sex of *P. monodon* where intensity of bands was not identical

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Results from the first set of primers suggested that the use of only 2 bulked DNA (one from females and the other from males) provided a large number of false positive AFLP bands.

Screening of the second set of primers ($E_{+3}$-1-8 +$M_{+3}$-9-16; 64 primer combinations) were then carried out against 4 bulked genomic DNA (PMF1 - 2 and PMM1 – 2). A total of 4 and 22 promising AFLP bands exhibited female and male specificity, respectively. Further screening of the informative primers with 6 bulked DNA (PMF1, PMF2, PMF3, PMM1, PMM2 and PMM3) did not yield consistent AFLP markers across screened samples (Table 3.3). For the third set of 64 primer combinations ($E_{+3}$-9-16 + $M_{+3}$-1-8), six DNA bulks (PMF1 - 3 and PMM1 - 3) were simultaneously screened. Two candidate female-specific ($Eco_{+3}$-16/$Mse_{+3}$-8) and male-specific ($E_{+3}$-10/$M_{+3}$-8) AFLP markers were identified (Table 3.4).

The final set of 64 primer combinations ($E_{+3}$-9-16 + $M_{+3}$-9-16) were screened against 4 bulked DNA (PMF1 – 2 and PMM1 – 2) and only 6 candidate female-specific and 14 candidate male-specific AFLP markers were found. The informative primers were tested against 10 bulked genomic (PMF1 – 5 and PMM1 – 5). Only 4 candidate female-specific AFLP fragments were found (Table 3.5).

In a total, 5 candidate female-specific and 1 male-specific AFLP markers were observed from screening of various DNA bulks of male and female *P. monodon* with 256 primer combinations. Name of informative primers, sizes of AFLP markers and the original specificity are illustrated by Table 3.6.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 3.2** Candidate sex-specific markers of *P. monodon* generated by AFLP using primer combination $E_{+3}$-1-8/$M_{+3}$-1-8

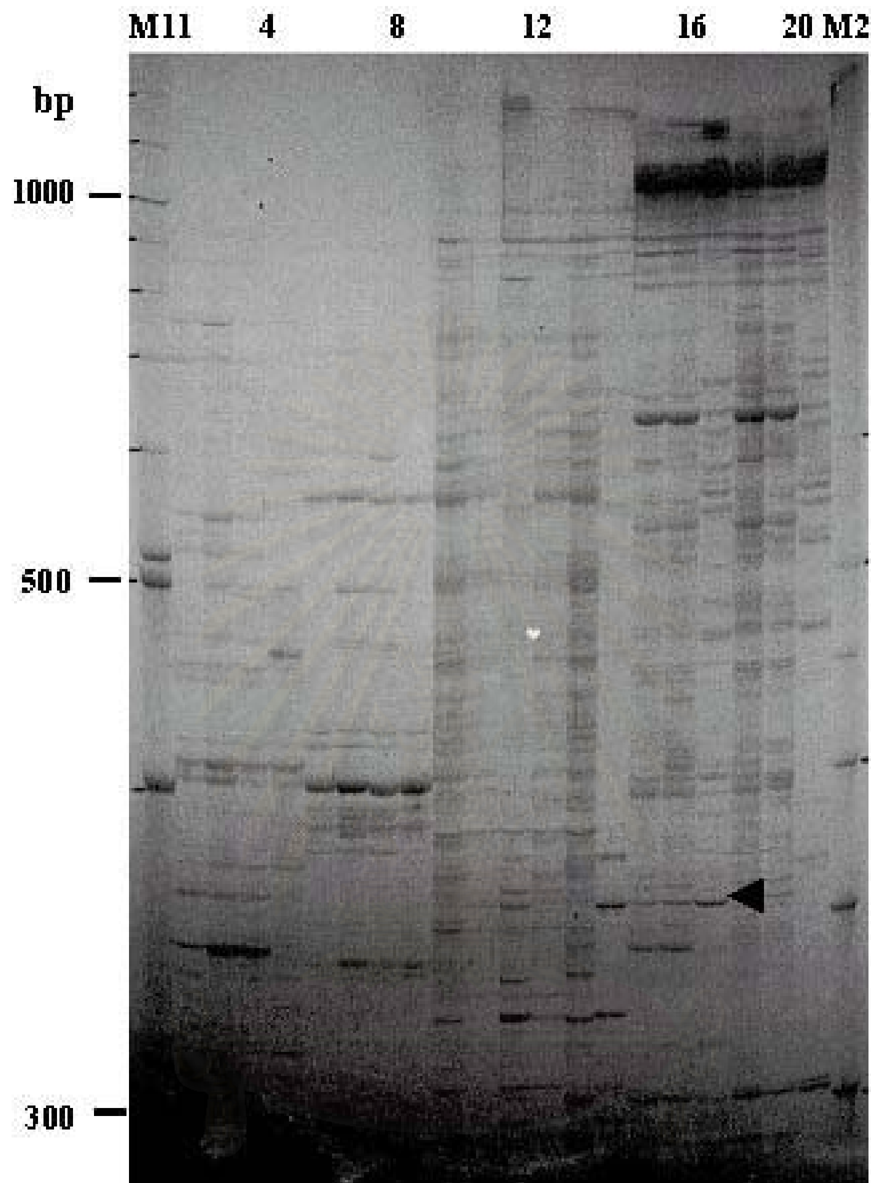
| Primer combinations | AFLP bands from 4 DNA bulks (bp) | | AFLP bands from 6 DNA bulks (bp) | |
|---|---|---|---|---|
| | Female (PMF1–PMF2) | Male (PMM1–PMM2) | Female (PMF1–PMF3) | Male (PMM1–PMM3) |
| $E_{+3}$-1/$M_{+3}$-5 | 600*** | - | - | - |
| | 850*** | - | - | - |
| $E_{+3}$-2/$M_{+3}$-4 | - | 400*** | - | - |
| $E_{+3}$-2/$M_{+3}$-6 | - | 300*** | - | - |
| $E_{+3}$-3/$M_{+3}$-3 | 550*** | | - | - |
| $E_{+3}$-3/$M_{+3}$-6 | - | 360*** | - | - |
| $E_{+3}$-3/$M_{+3}$-8 | - | 460** | - | - |
| $E_{+3}$-4/$M_{+3}$-4 | - | 325*** | - | - |
| $E_{+3}$-4/$M_{+3}$-6 | 420*** | | - | - |
| $E_{+3}$-5/$M_{+3}$-3 | - | 600** | - | - |
| $E_{+3}$-5/$M_{+3}$-4 | 350*** | | - | - |
| $E_{+3}$-6/$M_{+3}$-2 | - | 450** | - | - |
| | | 575** | - | - |
| $E_{+3}$-6/$M_{+3}$-4 | - | 625*** | - | - |
| $E_{+3}$-6/$M_{+3}$-6 | 780*** | | - | - |
| $E_{+3}$-6/$M_{+3}$-8 | - | 1300*** | - | - |
| $E_{+3}$-7/$M_{+3}$-1 | - | 400*** | - | - |
| $E_{+3}$-7/$M_{+3}$-2 | - | 360*** | - | - |
| | | 460*** | - | - |
| $E_{+3}$-7/$M_{+3}$-3 | 800*** | | - | - |
| $E_{+3}$-8/$M_{+3}$-3 | - | 640*** | - | - |
| $E_{+3}$-8/$M_{+3}$-4 | - | 400*** | - | - |
| $E_{+3}$-8/$M_{+3}$-5 | 550*** | | - | - |
| **Total** | **8** | **15** | **0** | **0** |

*** = indicate fragments found in one sex but absent from the opposite sex of *P. monodon* where the intensity of bands was identical.
** = indicate fragments found in one sex but absent from the opposite sex of *P. monodon* where intensity of bands was not identical
* = indicate fragments found in both males and females but the intensity of bands was not identical

**Table 3.3** Candidate sex-specific markers of *P. monodon* generated by AFLP using primer combinations $E_{+3}$-1-8/$M_{+3}$-9-16

| Primer combinations | AFLP bands from 4 DNA bulks (bp) | | AFLP bands from 6 DNA bulks (bp) | |
|---|---|---|---|---|
| | Female (PMF1–PMF2) | Male (PMM1–PMM2) | Female (PMF1–PMF3) | Male (PMM1–PMM3) |
| $E_{+3}$-1/$M_{+3}$-11 | - | 520*** | - | - |
| $E_{+3}$-1/$M_{+3}$-12 | 320** | - | - | - |
| $E_{+3}$-1/$M_{+3}$-14 | 450** | 500*** | - | - |
| $E_{+3}$-1/$M_{+3}$-15 | - | 370** | - | - |
| | - | 500*** | | |
| | - | 600** | | |
| $E_{+3}$-2/$M_{+3}$-9 | - | 625*** | - | - |
| $E_{+3}$-2/$M_{+3}$-12 | - | 310*** | - | - |
| $E_{+3}$-2/$M_{+3}$-13 | - | 550** | - | - |
| $E_{+3}$-2/$M_{+3}$-14 | - | 440** | - | - |
| $E_{+3}$-3/$M_{+3}$-9 | 450*** | - | - | - |
| $E_{+3}$-3/$M_{+3}$-11 | - | 370*** | - | - |
| $E_{+3}$-4/$M_{+3}$-9 | 510** | 500** | - | - |
| $E_{+3}$-4/$M_{+3}$-10 | - | 320*** | - | - |
| | - | 700** | - | - |
| $E_{+3}$-4/$M_{+3}$-13 | - | 420*** | - | - |
| | | 500*** | | |
| | | 780*** | | |
| $E_{+3}$-4/$M_{+3}$-14 | - | 550*** | - | - |
| $E_{+3}$-5/$M_{+3}$-12 | - | 900*** | - | - |
| $E_{+3}$-5/$M_{+3}$-14 | - | 410** | - | - |
| $E_{+3}$-6/$M_{+3}$-11 | - | 420*** | - | - |
| $E_{+3}$-6/$M_{+3}$-13 | - | 325*** | - | - |
| $E_{+3}$-7/$M_{+3}$-14 | - | 450*** | - | - |
| **Total** | **4** | **22** | **0** | **0** |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย




สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Figure 3.11** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 6 bulked DNA; PMF1 (lanes 9 and 15), PMF2 (lanes 10 and 16), PMF3 (lanes 11 and 17), PMM1 (lanes 12 and 18), PMM2 (lanes 13 and 19), PMM3 (lanes 14 and 20) using primers $E_{+3}$-10/$M_{+3}$-4 (lanes 9 – 14) and $E_{+3}$-16/$M_{+3}$-8 (lanes 15 – 20). An arrowhead indicates a candidate sex-specific AFLP marker. Lanes M1 and M2 are 100 bp and 50 bp DNA markers, respectively.

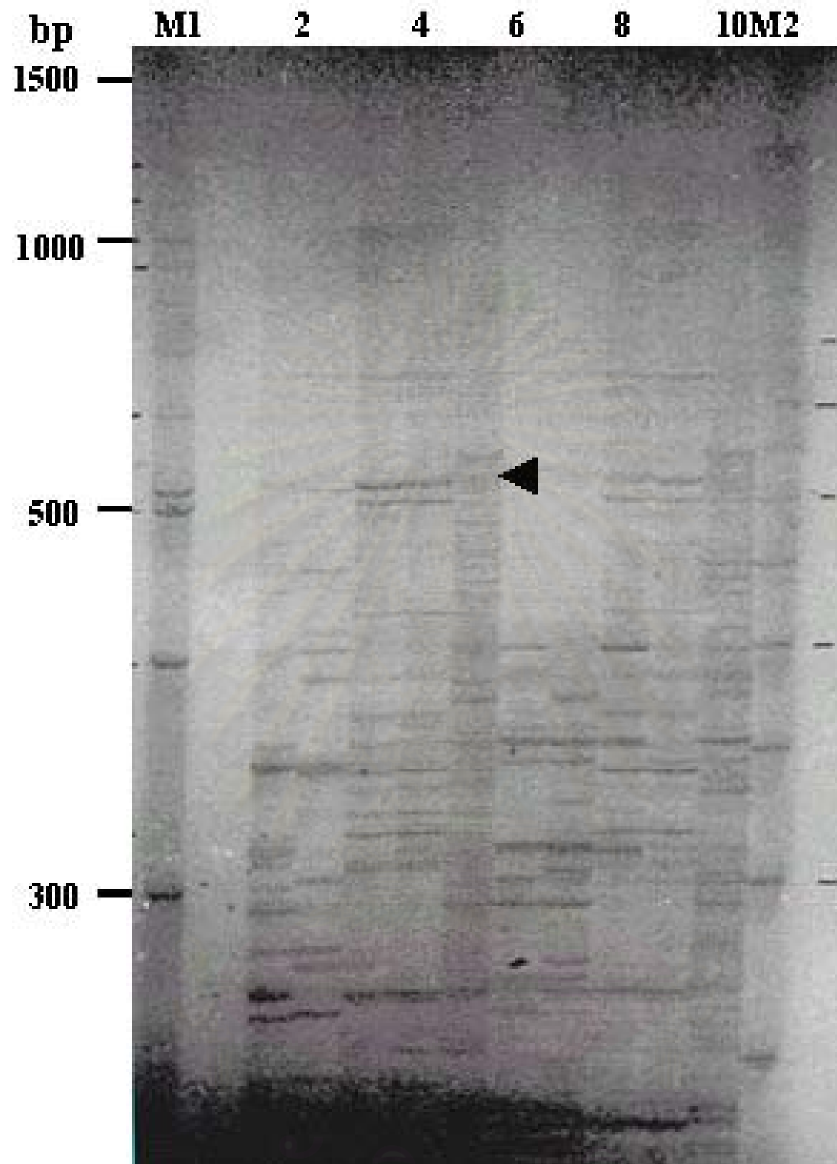
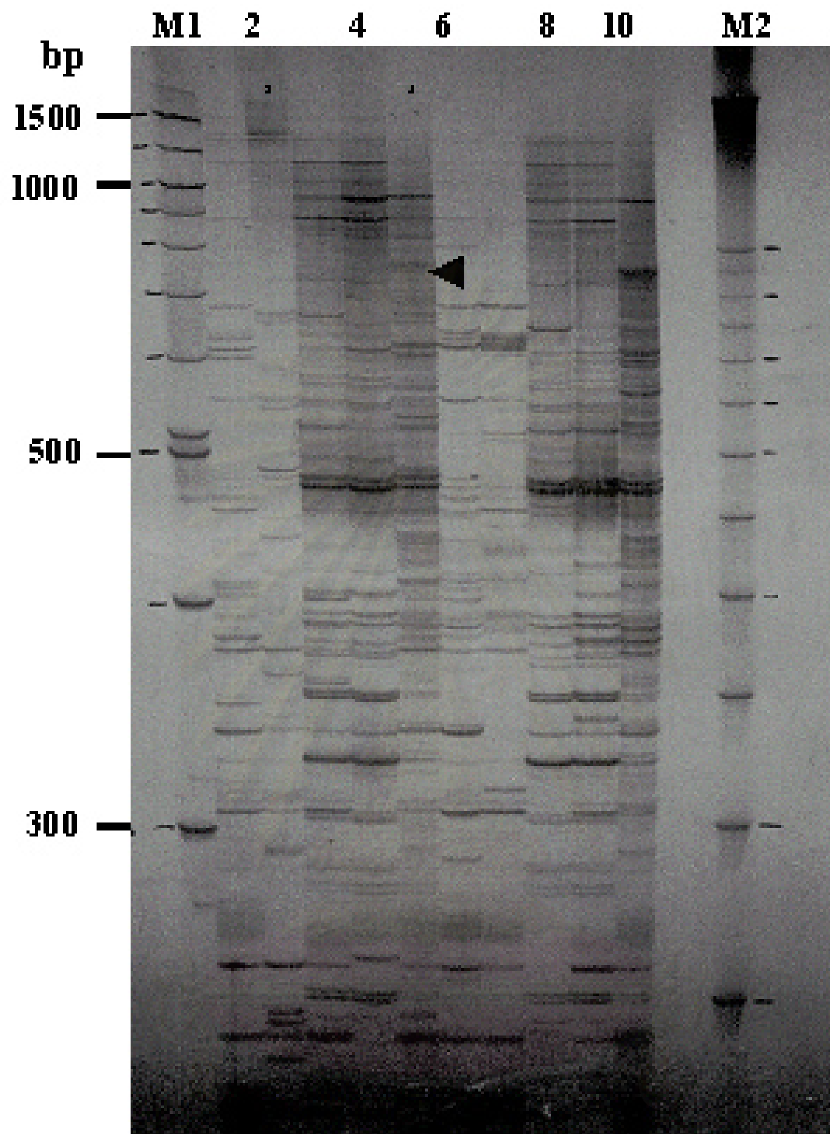
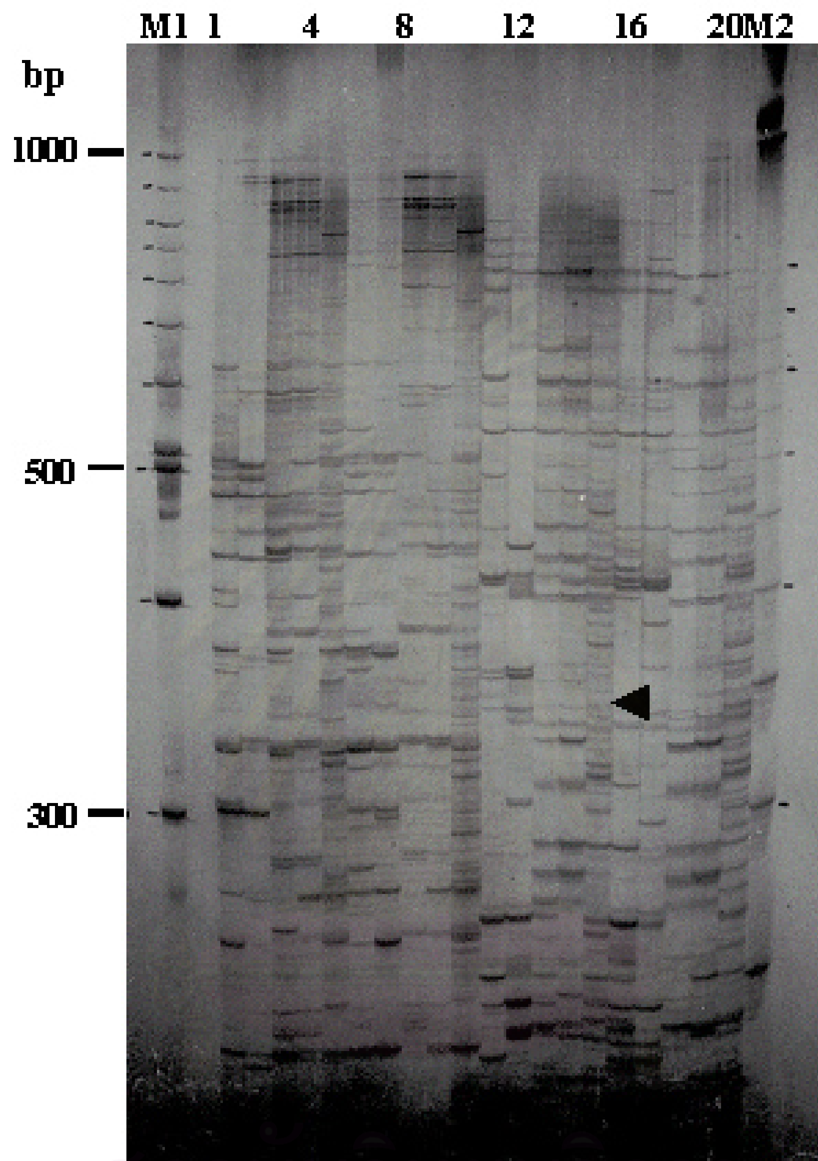



**Figure 3.12** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 10 bulked DNA; PMF1 – 5 (lanes 1 – 5), PMM1 - 5(lanes 6 – 10) using primers $E_{+3}$-10/$M_{+3}$-9 An arrowhead indicates a candidate sex-specific AFLP marker. Lanes M1 and M2 are 100 bp and 50 bp DNA markers, respectively.



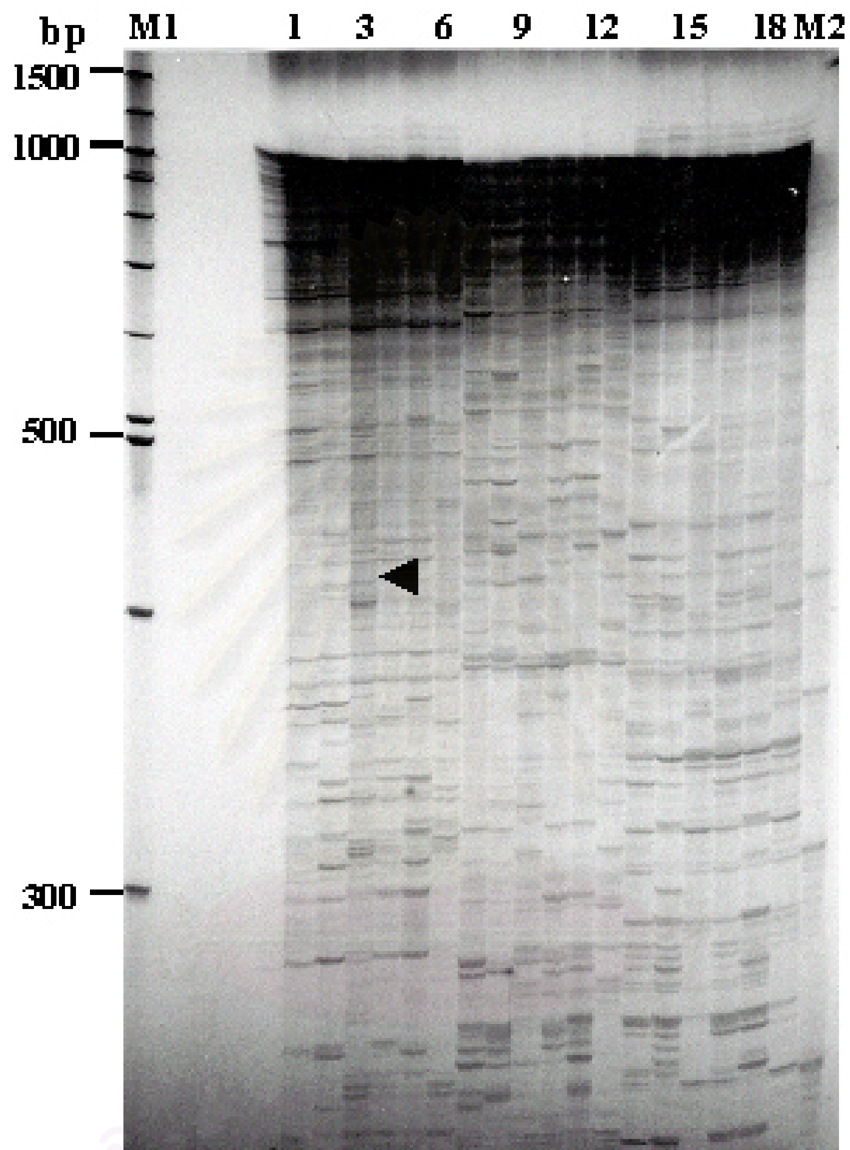

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Figure 3.13** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 10 bulked DNA; PMF1 – 5 (lanes 1 – 5), PMM1 – 5 (lanes 6 – 10) using primers $E_{+3}$-10/$M_{+3}$-10 An arrowhead indicates a candidate sex-specific AFLP marker. Lanes M1 and M2 are 100 bp and 50 bp DNA markers, respectively.




สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Figure 3.14** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 10 bulked DNA; PMF1 – 5 (lanes 1 – 5), PMM1 – 5 (lanes 6 – 10) using primers $E_{+3}$-14/$M_{+3}$-15; PMF1 – PMF5 (lanes 11 – 15), PMM1 – PMM5 (lanes 16 – 20) using primers $E_{+3}$-14/$M_{+3}$-16; An arrowhead indicates a candidate sex-specific AFLP marker. Lanes M1 and M2 are 100 bp and 50 bp DNA markers, respectively.




สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Figure 3.15** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 6 bulked DNA; PMF1 (lanes 1, 7 and 13), PMF2 (lanes 2, 8 and 14), PMF3 (lanes 3, 9 and 15), PMM1 (lanes 4, 10 and 16), PMM2 (lanes 5, 11 and 17), PMM3 (lanes 6, 12 and 18) using primers $E_{+3}$-15/$M_{+3}$-14 (lanes1 – 6), $E_{+3}$-16/$M_{+3}$-13 (lanes 7 – 12), $E_{+3}$-16/$M_{+3}$-14 (lanes 13 – 18). An arrowhead indicates a candidate sex-specific AFLP marker. Lanes M1 and M2 were 100 bp and 50 bp DNA markers, respectively.

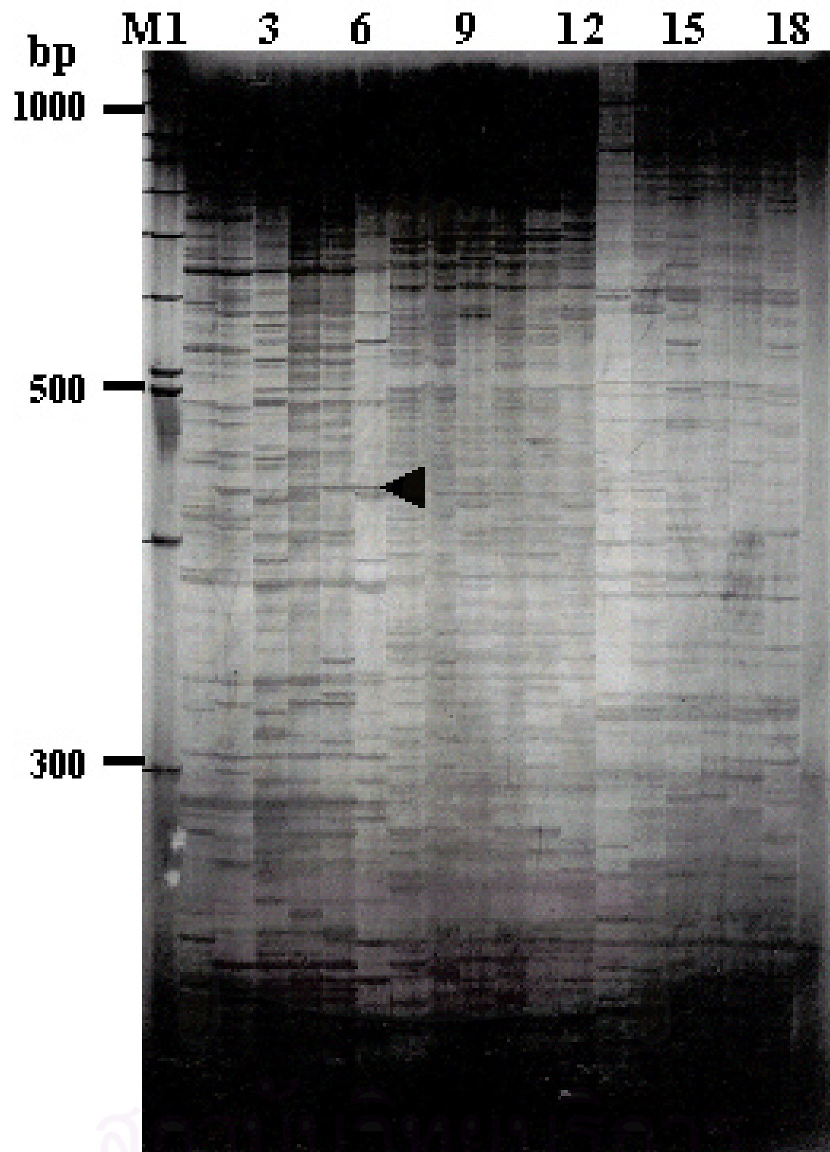



**Figure 3.16** A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP products of 6 bulked DNA; PMF1 (lanes 1, 7 and 13), PMF2 (lanes 2, 8 and 14), PMF3 (lanes 3, 9 and 15), PMM1 (lanes 4, 10 and 16), PMM2 ( lanes 5, 11 and 17), PMM3 ( lanes 6, 12 and 18) using primers $E_{+3}$-10/$M_{+3}$-8 (lanes 1 – 6), $E_{+3}$-11/$M_{+3}$-1 (lanes 7 – 12), $E_{+3}$-11/$M_{+3}$-2 (lanes 13 – 18). An arrowhead indicates a candidate sex-specific AFLP marker. Lanes M1 and M2 were 100 bp and 50 bp DNA markers, respectively

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 3.4** Candidate sex-specific markers of *P. monodon* generated by AFLP using primer combination $E_{+3}$-9-16/$M_{+3}$-1-8

| Primer combinations | AFLP bands from 6 DNA bulks (bp) | |
|---|---|---|
| | Female (PMF1–PMF3) | Male (PMM1–PMM3) |
| $E_{+3}$-10/$M_{+3}$-8 | - | 420** (Fig 3.16) |
| $E_{+3}$-16/$M_{+3}$-8 | 350** (Fig. 3.11) | - |
| **Total** | **1** | **1** |

**Table 3.5** Candidate sex-specific markers of *P. monodon* generated by AFLP using primer combination $E_{+3}$-9-16/$M_{+3}$-9-16

| Primer combinations | AFLP bands from 4 DNA bulks (bp) | | AFLP bands from 10 DNA bulks (bp) | |
|---|---|---|---|---|
| | Female (PMF1–PMF2) | Male (PMM1–PMM2) | Female (PMF1–PMF5) | Male (PMM1–PMM5) |
| $E_{+3}$-9/$M_{+3}$-16 | - | 350*** | - | - |
| $E_{+3}$-10/$M_{+3}$-9 | 520*** | 360*** | 520 (Fig. 3.12) | - |
| | | 700*** | | |
| $E_{+3}$-10/$M_{+3}$-10 | 725*** | 380*** | 725 (Fig. 3.13) | - |
| $E_{+3}$-10/$M_{+3}$-11 | - | 460*** | - | - |
| $E_{+3}$-10/$M_{+3}$-12 | 390*** | - | | |
| $E_{+3}$-10/$M_{+3}$-14 | 520*** | - | | |
| $E_{+3}$-11/$M_{+3}$-14 | 950***- | 490*** | - | - |
| | | 320*** | | |
| $E_{+3}$-11/$M_{+3}$-15 | - | 850*** | - | - |
| $E_{+3}$-12/$M_{+3}$-16 | - | 300*** | - | - |
| | | 4008** | | |
| $E_{+3}$-13/$M_{+3}$-9 | - | 430*** | - | - |
| $E_{+3}$-14/$M_{+3}$-10 | - | 520*** | - | - |
| $E_{+3}$-14/$M_{+3}$-13 | - | 525*** | - | - |
| $E_{+3}$-14/$M_{+3}$-16 | 350*** | 590*** | 350 (Fig. 3.14) | - |
| $E_{+3}$-15/$M_{+3}$-14 | 400*** | - | 400(Fig. 3.15) | - |
| **Total** | **6** | **14** | **4** | **0** |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 3.6** Conclusion of name of primer combination and size of marker of sex-specific AFLP marker generated by AFLP analysis

| Primer combination | Size of AFLP marker (bp) | Sex-specificity |
|---|---|---|
| 1. $E_{+3}$ -16/$M_{+3}$-8 | 350 | Female |
| 2. $E_{+3}$ -10/$M_{+3}$-9 | 520 | Female |
| 3. $E_{+3}$ -10/$M_{+3}$-10 | 725 | Female |
| 4. $E_{+3}$ -14/$M_{+3}$-16 | 340 | Female |
| 5. $E_{+3}$ -15/$M_{+3}$-14 | 400 | Female |
| 6. $E_{+3}$ -10/$M_{+3}$-8 | 420 | Male |

### 3.3 Cloning and characterization of candidate sex-specific AFLP markers

Six candidate sex-specific AFLP markers constituting of a 520 bp fragment from $E_{+3}$-10/$M_{+3}$-9 (called FE10M9520), a 725 bp fragment from $E_{+3}$-10/$M_{+3}$-10 (FE10M10725), a 340 bp fragment from $E_{+3}$-14/$M_{+3}$-16 (FE14M16340), a 400 bp fragment from $E_{+3}$-15/$M_{+3}$-14 (FE15M14400), a 350 bp from $E_{+3}$-16/$M_{+3}$-8 (FE16M8350) and a 420 bp fragment from $E_{+3}$-10/$M_{+3}$-8 (ME10M8420) were successfully reamplified (Figure 3.17). The gel-eluted PCR product was cloned. Colony PCR was performed to evaluation of the insert sizes (Figure 3.18). The colony PCR products (Figure 3.19) or alternatively the recombinant plasmid (Figure 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) revealed that more than one type of sequences were found from a single insert (Figure 3.19 and 3.20 and Table 3.7).

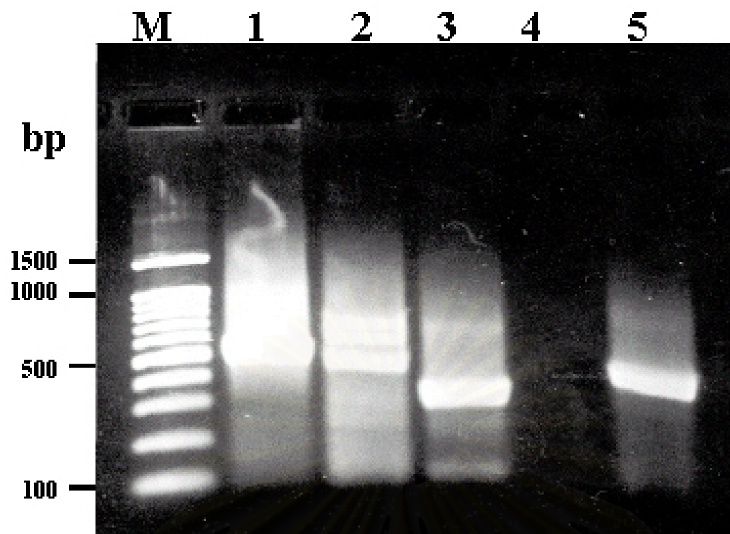



**Figure 3.17** Reamplification of candidate female-specific AFLP fragments (FE10M9520, lane 1, FE10M10725, lane 2, FE14M16340, lane 3 and ME10M8420, lane 5).Lane M = 100 bp DNA marker

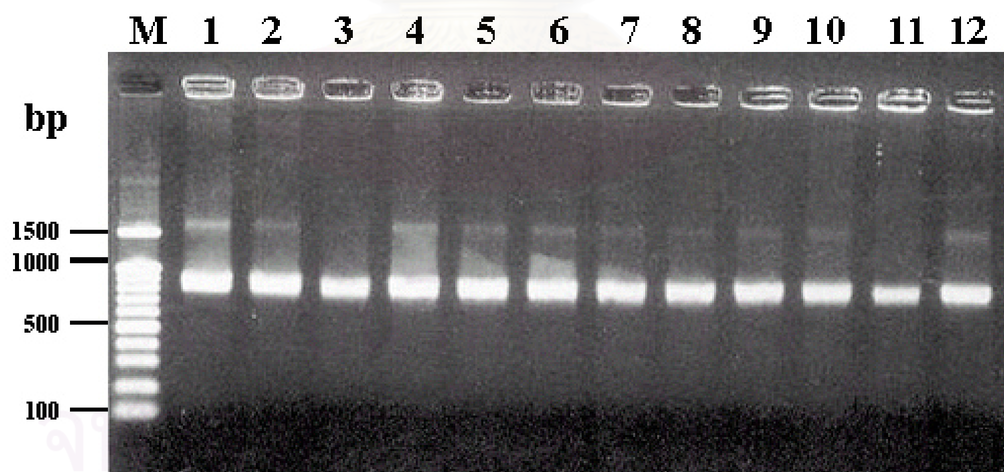



**Figure 3.18** Colony PCR product of recombinant clones containing a FE10M9520 fragment (lanes 1 – 12). Lane M = A 100 bp DNA ladder.

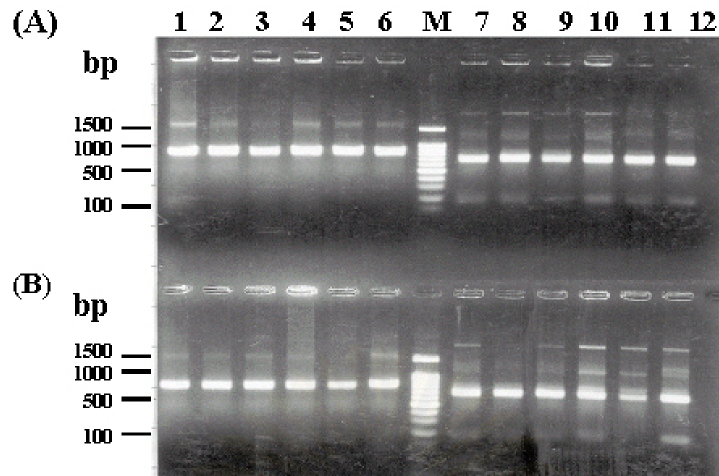



**Figure 3.19** A 1.0% agarose gel electrophoresis showing restriction patterns of colony PCR product of the FE10M9520 insert digested with *Hind*III (lanes 1 – 6, panel A and B) and *Rsa* I (lanes 7 – 12, A and B). Lane M = a 100 bp DNA ladder.

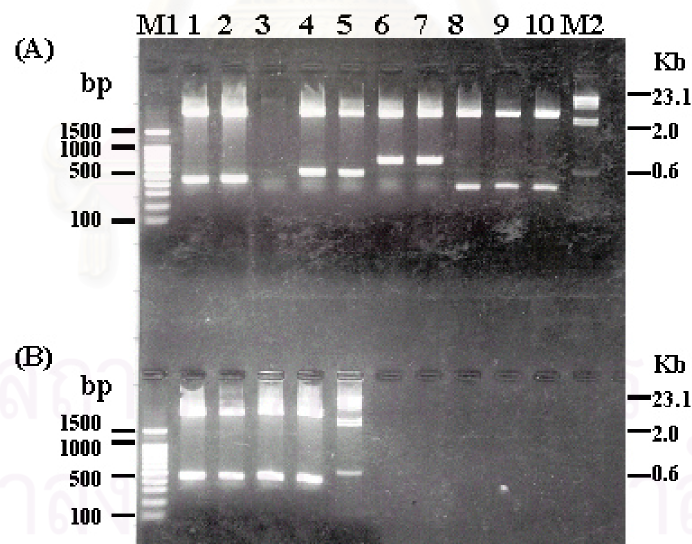



**Figure 3.20** A 1.0% agarose gel electrophoresis showing restriction patterns of *Eco*RI-digested plasmid DNA from FE10M9520 (lanes 4 – 5, A), FE10M10725 (lanes 6 – 7, A), FE14M16340 (lanes 8 – 10, A) and ME10M8 (lane 11 – 14, B) inserts.Lane M1 and M2 are a 100 bp DNA ladder and λ-*Hin*d III, respectively.

At least 4 positive colonies were digested by *Hind* III and *Rsa* I. The restriction pattern of each enzyme was combined (= composite restriction patterns, Table 3.7). Plasmid DNA was extracted from a representative of each clone type. Seventeen recombinant plasmid DNA were subjected to DNA sequencing (Figure 3.21 – 3.26). Nucleotide sequences of these clones were aligned and further confirmed that different nucleotide sequences were obtained from a single insert

Homology search using BLAST*N* and BLAST*X* did not show significant matching between nucleotide sequences of these clones and those previously deposited in the GenBank. Therefore, they were regarded as unknown sequences (Table 3.8).

**Table 3.7 Different restriction patterns of colony PCR found from a single insert**

| Insert | Composite digestion pattern | No. of clone showing identical pattern | Primer design |
|---|---|---|---|
| FE10M9520 | AA | 12 | **Yes** |
| FE10M10725 | AB | 2 | Yes |
| | BA | 2 | Yes |
| FE14M16340 | AA | 3 | Yes |
| | AB | 2 | No |
| | AC | 1 | No |
| FE15M14400 | AA | 7 | Yes |
| | AB | 3 | No |
| | AC | 1 | No |
| FE16M8350 | AA | 6 | Yes |
| | AB | 2 | No |
| | AC | 1 | No |
| ME10M8420 | AA | 2 | No |
| | AB | 2 | No |
| | AC | 1 | No |
| | AD | 1 | No |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**FE10M9520.1**

GATGAGTCCTGAGTAACGACGAAGATTTCCTTAAAAATTCTCTAAAAAAAAGTAAAACAG
CCACTCTCATGAACGAGAGTCTTGCCAAAAAGTTATCCAACGGCGTAGTGAATATCACAG
GACGATTTGGGAAAGTGTTGATATACGGAGGCATAGCTTATGG**AACCATAGCGATGTTGA**
**CGG**CATCAACCATTCACTCCTCTAAAGGTGCACACTACAACGTTATCTCTCGATACTGTC
CGAGCGGTGTCAAATCTTACAAAATCCTGAAATACTCTTGCGTGGACAAGAGCCTAGGGA
ACGGCGAAAGCATGGAACACCCGTTTGAGACTCACATCTCGCAATATATTGCGAACAATA
TCAACGATCTGAAACAGGGGGCATACGTCAAAAACCCTA**ATACGAAATGGCAAAGCAAAA**
CAAAGGGGTATGCGCCGATCTGTTGTGAAGACGACGTGAATTTCCACGGACCCTGCGGGG
GATGGGCTGTTTTTTCCGACACGTCTGAATTGGTACGCAGTCA

**FE10M9520.2**

GATGAGTCCTGAGTAACGACGAAGATTTCCTCAAAAATTCTCTAAAAGAAAGTAGAACAG
CCACTCTCATGAACGAGAGTCTTGCCAAAAAGTTATCCAACGGCGTAGTGAATATCACAG
GACGATTTGGGAAAGTGTTGATATACGGAGGCATAGCTTATGGAACCATAGCGATGTTGA
CGGCATCAACCATTCACTCCTCTAAAGGTGCACACTACAACGTTATCTCTCGATACTGTC
CGAGCGGTGTCAAATCTTACAAAATCCTGAAATACTCTTGCGTGGACAAGAGCCTAGGGA
ACGGCGAAAGCATGGAACACCCGTTTGAGACTCACATCTCGCAATATATTGCGAACAATA
TCAGCGATCTGAAACAGGGGGCATACGTCAAAAACCCTAATACGAAATGGCAAAGCAAAA
CAAAGGGGTATGCGCCGATCTGTTGTGAAGACGACGTGAATTTCCACGGACCCTGCGGGG
GATGGGCTGTTTTTTCCGACACGTCTGAATTGGTACGCAGTCA

**Figure 3.21** Nucleotide sequences of FE10M9520**.** Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**FE10M10725.1**

GATGAGTCCTGAGTAACGTTTGGCATGGATGTACAAGATTTCAGCAAGCTTCCAACACCT
ACAAGCAGACTTGCCTCACCATCGCCACCCCAGGGTCCCCTCCACTCGATCTCGGATTCT
GTGCAAGTGACTGCACTACATGATGCCACTGTTGCACTCAAAACTCGATTTGCATCACTT
ACAGAGCATTTTGATGCAATCGAAGCTCGCCTCGATGGTTTGGTTAGCAAACAAGCTATA
TTCGAGATCACACTAAACTCAATTGTTGAGTCACAAAAGGTTGAGATTTGTACAATTACT
GTGCTCACTGAGAAGTTTCGAGAGTACATGTTTCGAGAACATAACCCCAATCCCCCCCAA
GGAGCCTTTTC**CACGGGATGTTTTGACTAATGATGT**CTGCAGTACACCTGCTACTCATAC
TTCCACAGCTGCTTGTATCACCAACACCGCCCATGTCAACATCGCCACTCCTGCCACTAC
TACTTCATCAGTCGGTCATCGTCGCCACAAAGGAAAAGTTCAGTAACCAGATGAAGCTAC
ATGTCATCTCATGGAATGCCTGCGGTATTACAAACTGAGCGAAACTTACAGGGATATGTA
CACA**GCCACCTCCTTTATCTTTATCC**AAGAAGCATTCATTAGACTAGCCCTACCCAGAGG
GGAAGTACCCTCACTCAGTGGCTAGGTGTCCTACATGCATCATGTCTGAATTGGTACGCA
GTCA

**FE10M10725.2**

GATGAGTCCTGAGTAACGTTAGCGTAATATATCAG**ATCTAACACCAACAAAAGTCATCCT**
TTAGAAGTCACCGAAACGGAAAATGTTCTTGGCTATAGCAATGAAAATTCCTATACTTTT
ACGATTTCGGCCAAGAGGTAGCACATAATACTGCGACCAAGAATAGACTTCAGAAAGATA
TACTTGAAAAAAGTGTACATGGCAGGCTTACTGA**CTACGCTTTATGACTGGCTAC**TGGAT
CTCGAATATCCAAGCAATCTAAATGTCCTAGCGTAAGACAGTGGTAAGACAACACATAAG
GAACATCACAATTTCCGCGCCCACTCCTTCACGGTTCTCAGCCAGCCCGTGTTGACTGCG
CCCTCGGAAATAGTTGCAAGCCCTTGCTTCGCATTCATTACAGCAACATTTCTACCATTT
ACAGTGGGGGTATTGCCGTCACCACTCTCAGCCGGAAACAGATTTTGCTTCTTCTCGGAC

**Figure 3.22** Nucleotide sequences of FE10M10725. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**FE14M16340.1**

GACTGCGTACCAATTCATTTTTTTTCCCTCTAATCTTGCGCTTTATGTGGAGTTCCGCGA
TGACTTACTGAAGGGATGTTTATG**GCCGTCACTTCAAACACAAACCCTG**CTTCTCCCTCA
AGTGTTCACAGTTTACTCTCCAGTGTCTTCGGTGGCAACAAGACACGTCTAACCAGCCAG
GCGCCCCGTTCTCTCCAAAGACATGAGGGCGACAGGCGGCGTTTGGGGGGGGGCGAACT
CCCTCCCCTTGTGAGGAAGGAGGGTGATGCTTTGCTTC**TAGTTGGGGCTCAGCGGAAGAT**
**TTG**TGGAGGGAAGGGTGAAGGGTTACTCAGGACTCATCA

**FE14M16340.2**

GACTGCGTACCAATTCATTTTTTTATGAGAATTTAAATAATATTAACAAAAATAGATTTT
GGTAAAATAAAAATGGGTCTGGCATAACCTTTAATTAACCCTTTGCTGCCAGGTGGCATG
TATGTACATGCCATGGAATGCCTGGACTATATTCCGGGTGGCATGTGCATACATGCCATG
GTGCACCAGTCCTGTCTTCTGGGGGCATGTTTGGTAATGTCGTGCATGCTATGATGCCAA
CACGATCCCCCGGCAGTGGAACCTGGTTCACCATAAATACAGCCTGGGAGAGAGAGCTTT
TGTATCAGAATCCAGCCAGCTGCATTGGGTTACTCAGGACTCATCA

**FE14M16340.3**

CTACTCAGGACTCATTGGGCCTAGACAACCCGGTGCGGATATCGGTATTATTGTTTGTTT
GAGGAGTATGGTCCATGTCGATATCCGTTGTGTTGCGTGTTCTCGTACAACTCACCTTTC
AAGGTGATTTACATCGTCTGAGGGTCCGGTTTGCCGTCCGGCCACCGTCCTCAGTGGTTT
CAGTCACTGTGGTTCTCTAAAGACTATCAGTAACTCCCCAACTTGTAACATACTATTCTT
GACACTCCCGTTGATTTGTTAGAGAGAGGTCATACACATACTTACACATTAGTTTTATAA
AGGATATCCATTTTATTACTTAACCATGCGTCAGT

**Figure 3.23** Nucleotide sequences of FE14M16340. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**FE15M14400.1**

GATGAGTCCTGAGTAACCTGAGATTTTATGTATCTATTCATCTGTTTGTCTATTTTTTTA
TCTATCCATTTACCTATACATCTCTCTTTTTATTCATCTATTTATCTATACATTTATCTT
TTTATCTATCTGTTTATCTATACTTCTATCTGTCCATCTATTGATTTATCTATTTATCTA
TGTAAAATATAGATAAGATTTGATGTATGCTGGACCGCACTTCCTTATAGAAGTTGCATG
TCTTTTTTATTCTGATTTCAATATTACTTTGTGTTGTGTCCCTTTGTTTTAGTTTCTCTC
TCTCTCTCTCTCTCTTGCTTTCTTGTTGTTTTCTTTTCTTTTCTCTCTGTTTTGTTTTCT
GACTTATCATTAGCATCGTCATTTTGAATTGGTACGCAGTC

**FE15M14400.2**

GACTGCGTACCAATTCAAAAAATTGATACCTAGAGCCACAAGAACTGGGTAGAAACTGAT
CCAGATCCAATTAT**CCGACTGGATAGGCTGGAC**AACACCTGCGAACCCACAGCCCCCATC
TCGTGTGAGGAAGTAAAAAAAAAGAAAAAGAGGTTACCTCGTAAGGCTCAGGCTCCTCAC
AGATCGGACGCGACGCTCTTATTCTGAGATTTGGCCGGCCGCCCTCCCACACAGCCTCAT
GTGGTGGAGGGGAAGCCCCACGTGCCCGAGGACACCCACACCGAACACGAAAGATTCAAG
ATGGCGCTGT**GCCACCCTCACCCATTTC**CTCCTTTCAATGAACCCACTACAGTTATTACC
TCAAACTTTACCCTGCATTCTAGGTTACTCAGGACTCATC

**FE15M14400.3**

GATGAGTCCTGAGTAACCTGAGAATTTTATGTATCTATTCATCTGTTTGTCTATTTTGTT
TATCTATCCATTTACCTATACATCTTTCTTTTTATTCATCTATTTATCTATACATTTATC
TTTTTATCTATCTGTTTATCTATACTTCTATCTGTTCATCTATTGATTTATCTATTTATC
TATGTAAAATATAGATAAGATTTGATGTATGCTGGACCGCACTTCCTTATAGAAGTTGCA
TGTCTTTTTTTATTCTGATTTCAATATTACTTTGTGTTGTGTCCCTTTGTTTTAGTTTCTC
TCTCTCTCTCTCTCTCTTGCTTTCTTGTTGTTTTCTTTTCTTTTCTCTCTGTTTTGTTTT
CTGACTTGTCATTAGCATCGTCATTTTGAATTGGTTCGCAGTC

**Figure 3.24** Nucleotide sequences of FE15M14400. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**FE16M8350.1**

GACTGCGTACCAATTCAATGTGGTTGGAATAATTGGAGA**AAGGTTTCGGGAGTGATCTGC**
GACAAGAGAGTGCCCATAAAGTTGAAGGGAATGGTACATAAGTCTGTGGTCAGACCAGAG
AAGAAACGAAGATCATGTGGGAAGACAGGCAATGGAAATGGAAATAGAGGGAAACAGACC
AAGAGGAAGGCCTAAGACCAGATGGAAGGATGTTGTTCAAAAAGATATGAGGGAGAAACA
TC**TAGACGAGGCAGAGACATTGGA**GAGAACTCTATGGCGAAGTCTCATTAGAAATGGCGA
CCCCGGGTACGGAAACGCTAAGACAAGGAACAAGAAGAAGTTACTCAGGACTCATCA

**FE16M8350.2**

GATGAGTCCTGAGTAACTTCTTCTTGTTCCTTGTCTTAGCGTTTCCGTACCCGGGGTCGC
CATTTCTAATGAGACTTCGCCATAGAGTTCTCTCCAATGTCTCTGCCTCGTCTAGATGTT
TCTCCCTCATATCTTTTTGAACAACATCCTTCCATCTGGTCTTAGGCCTTCCTCTTGGTC
TGTTTCCCTCTATTTCCATTTCCATTGCCTGTCTTCCCACATGATCTTCGTTTCTTCTCT
GGTCTGACCACAGACTTATGTACCATTCCCTTCAACTTTATGGGCACTCTCTTGTCGCAG
ATCACTCCCGAAACCTTTCTCCAATTATTCCAACCACATTGAATTGGTACGCAGTC

**FE16M8350.3**

ATAGATTGAGTCCTTGTGTGAAATTGTTATCCGCTACCTCATGCTCCAGAAACTGCCTTC
ATGTTGTCTTCTCTCTCTCTCTCCCATCTCCCNTCATCCCCCTCCCATCTCCCTCTTT
CTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCGTCTTTCCTCTTTCTCTTTTCTCATTTCT
CTTTCCTCTTTCGTCTCCTCGTTTCTCANTATTTCGNTCATTTAGTCGNTCCNTTTATCC
TTCCTTTAGTCGTCCNTNTATCGTCCTTTTATCATCATTTTTCGTCCTTATTTCCATCCG
TTATATCCTTCCTATTTATCGCTCCATTTANCGATCGATCGATCNAGTAC

**Figure 3.25** Nucleotide sequences of FE16M8350. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**ME10M8420.1**

ACAGGTTNGCNAGCTATTTAGGTGACACGTATAAATACTCAAGCGATGCATCCAACGGCG
TTGGGGAGCTCTCCCATATGGTCGACCTGCGAGGCGGCCGCGAATTGNACTAGTGATTGA
TGAGTCCTGAGTAACTTGGACGCAGGGTAGTGTCAAGATTCCTTTGCCAGGACCCACCCA
TTAGTGAGGATCCACACTCCCTGACTGAAGTCAGGAAGGGCACTCTCCAAGCTGAATAGT
AGTAAAGCAGCAGGTATCTGCAGCATCCCAGCTGATCTGCTGAAAGCTGGTGGAGAACCT
ATGGCTAGGGGTTTGCATGCTGTCCTGTCTGCAATTTGGCAAGACTGGTACCATTCCCCC
TGAGCTGCTGAGGGGTATGGCCATCCTTCTCTGGAAGGGGTGAGGGGATCAGAGGGACTG
CAGCAATCACTGAGGCATTACTCTACTTAGTATATCGGGCAGGGTTCTCGCTCACATCCT
TCTGAGACGTATCAGAGACCACCTGCTGAGTCACCAGAGACCAGAGCAATCTGAATTGGT
ACGCAGTCA

**ME10M8420.2**

GATGAGTCCTGAGTAACTTTCTTATCCTCTTCCTGGTCTTCATCCTTTCCTTCCTCCTGT
GCATGTAGTGCAAAACTCAATCCAAGCAATACCAACATTACTTGAATGCGGAACATAGTT
TTCATATAGAAAATATTATATTTTGCTGCAATGTATTCCAACTGCCACAACAATACAATA
TCTAATGGTTTTATTTTTTCATGCCAGCTATTACCAATGAGAGGTAGTCCTCACGACGCC
CACCTGTGCGGCGGCAGATGTCGTTGTATTCGCGGACAATTTTTTCCAGATCGCGGTATT
TATAACCGTCCTGACCAATTTTGTTGCCCATGGCGGGCTGTCCCAGCATGAGTTTTTGCC
GCATTTTTTTTCTGAAATTCAACTTACCGACGCGGAAGGCATTATCTCCTCTGAATTGGT
ACGCAGTCA

**ME10M8420.3**

GACTGCGTACCAATTCAGATGAAGATATGACATCGCTAAACCTAAAGAAAACAGGCGTAA
ACACCGTACGTGAACACACACGGAAGCACTGACACAAGAGAAATTGAAAGAAAGCCTTTT
TTTTCTTTCTTTCATTTGAGGTAGCTAATTAGCCTTAGTATGTTATTCACTCTTATTTGT
GCCTTTCAGGCCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
GAAGAAAGAAAGACAGGGACAGAGACACATAGGTAAGCCACCCTATTTTACAATGCAAAA
ATCCGTTCGCAACATTAGAAAAAAATATAAACCTGTAGCCACTTCACACCATGTTTCCTG
CAATCAGATGAATATGCATTGGATCTAAAGTCAATTTATACAGTTCCCTAGTAAGTTACT
CAGGACTCATCA

**ME10M8420.4**

GACTGCGTACCAATTCAGATGTAACTTTTACAAGGCATGAATGAAACTTAGCATGAAACT
TAGCCTACTTGTTTTTGGAGACAATATTGGAAGAGGTGGATATTGTCTGAGTAAAGGACT
GTTGGAGGTATCACAAAGAACTGATCCTGCTTGGCTGGGCTAAAAAGTACTCAAAATGTT
TGTAGTATGTAAGTAGTAGACGAACGAGGACAGAAGTAAGAAATAATGGTGTGGAGTGAG
TAAGTACTATGGGGAAAACAATAACGATGAAGAGTAGTAATATGAGGAGAAGGGGTCGAA
AATGAAGAAGATTGTACAGGAGGAGGGGAGTAGACTTTAGGAAAGGAAGGGGTGTATTCT
GAAGGTTGGGTAATGAATTAGGTGGATGATTTGGAACTGGATCAAGTTACTCAGGACTCA
TCA

**Figure 3.26** Nucleotide sequences of MF10M8420. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced

**Table 3.8** Characterization of female-specific AFLP marker of *Penaeus monodon*

| AFLP primer | Sex specificity | Size of AFLP marker | No. of clone | Clone no. | Length of sequence | Name of primer | Expected product size (bp) | BLASTN | BLASTX |
|---|---|---|---|---|---|---|---|---|---|
| $E_{+3}$-10/$M_{+3}$-9 | Female | 520 | 2 | 520 10/9 F1 | 523 | FE10M9520 | 256 | Unknown | Unknown |
| | | | | 520 10/9 F2 | 523 | | | Unknown | Unknown |
| $E_{+3}$-10/$M_{+3}$-10 | Female | 725 | 2 | 725 10/10 F1 | 724 | FE10M10725 | 255 | Unknown | Unknown |
| | | | | 725 10/10 F2 | 480 | | 200 | Unknown | Unknown |
| $E_{+3}$-14/$M_{+3}$-16 | Female | 340 | 3 | 340 14/16 F1 | 339 | FE14M16340 | 220 | Unknown | Unknown |
| | | | | 340 14/16 F2 | 346 | | | Unknown | Unknown |
| | | | | 340 14/16 F3 | 335 | | | Unknown | Unknown |
| $E_{+3}$-15/$M_{+3}$-14 | Female | 400 | 3 | 400 15/14 F1 | 401 | FE15M14400 | 254 | Unknown | Unknown |
| | | | | 400 15/14 F2 | 400 | | | Unknown | Unknown |
| | | | | 400 15/14 F3 | 403 | | | Unknown | Unknown |
| $E_{+3}$-16/$M_{+3}$-8 | Female | 350 | 3 | 350 16/8 F1 | 357 | FE16M8350 | 225 | Unknown | Unknown |
| | | | | 350 16/8 F2 | 356 | | | Unknown | Unknown |
| | | | | 350 16/8 F3 | 350 | | | Unknown | Unknown |
| $E_{+3}$-10/$M_{+3}$-8 | Male | 420 | 4 | 420 10/8 M1 | 549 | - | - | Unknown | Unknown |
| | | | | 420 10/8 M2 | 429 | | | Unknown | Unknown |
| | | | | 420 10/8 M3 | 432 | | | Unknown | Unknown |
| | | | | 420 10/8 M4 | 423 | | | Unknown | Unknown |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

### 3.4 Development of sex-specific SCAR marker in *P. monodon*

Six SCAR markers were derived from sequences of candidate female-specific AFLP markers Seventeen recombinant clones from six specific AFLP fragments (FE10M9520, FE10M10725.1, FE10M10725.2, FE14M16340, FE15M14400, FE16M8350 and ME10M8420) were sequenced. Sex-specificity was tested against genomic DNA of male ($N$ = 4) and female ($N$ = 4) individuals of *P. monodon*. The expected size from each marker was observed. Nevertheless, SCAR markers were not specifically found in female *P. monodon*. The amplification product was, however, observed in both genders implying that conversion of sex-specific AFLP markers to sex-specific SCAR markers was unsuccessful (Figures 3.27 – 3.29).

### 3.5 Identification and characterization of sex-related genes in *P. monodon* using PCR and SSCP analysis

Thirty-four primer pairs derived from AFLP markers of *M. rosenbergii* and cDNA of *P. monodon* and *H. asinina*, Eight of which including those for vitellin coat protein (VCP) 3, VCP49, VCP75, sex lethal protein (sxl), ENSANGP00000010123 (ENSAN44), Thromospondin (TSP)-T1300, ME4M8517 and ME8M1310, did not provide the amplification product. Fourteen of overall primers including those for VCP1, VCP3, VCP7, Tektin, Axonemal protein, vitellogenin (VTG) 1, gonadotrphin inducible ovarian transcriptional factor 1 (GIOT1), hydroxysteroid dehydrogenase (HSD), sperm lysine, $TSD_{288-1+371-1}$, EGF-response factor 1, rudimentary protein and X-linked ubiquitin-specific protease 9 (USP9X), gave non-specific amplification products.

Only 12 primer pairs; VCP2, small androgen receptor interacting protein (SARIP), 18 kDa fertilization protein (FP), $TSP_{462F+288R}$, X-linked nuclear protein 1 (XNP-1), peritrophin1, peritrophin2, PMX, disulfide isomerase (DSI), PMO920, PMT1700 and zinc finger protein (ZFP), generate discrete amplification products in both female and male *P. monodon*.

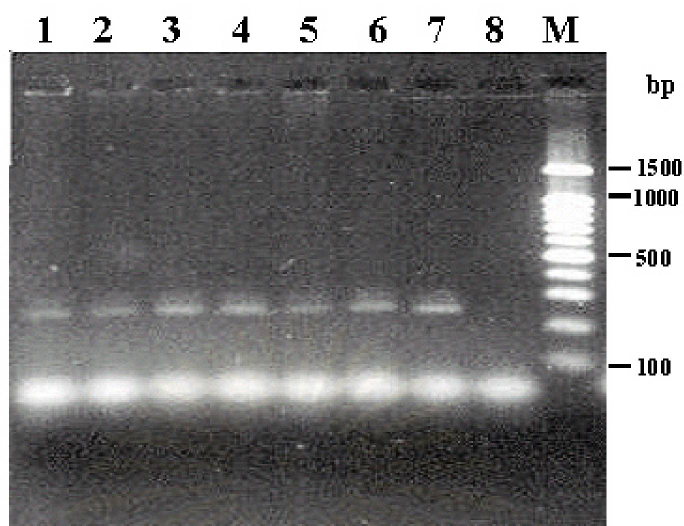



**Figure 3.27** A 1.6% agarose gel electrophoresis showing the PCR product of a SCAR marker derived from FE10M9520 against female (lanes 1 – 4) and male (lanes 5 – 8) genomic DNA of *P. monodon*. A 100 bp ladder was used as the DNA markers.

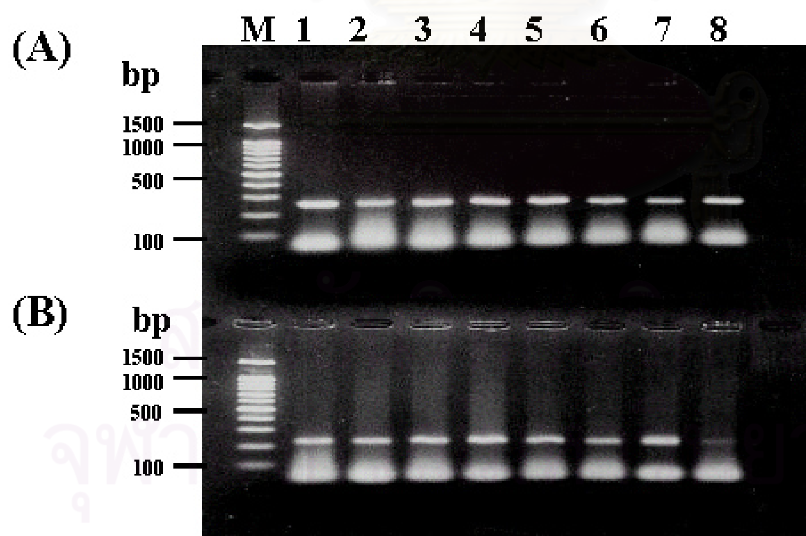



**Figure 3.28** A 1.6% agarose gel electrophoresis showing the PCR product of SCAR markers derived from FE10M10725.1 (A) and FE14M16340 (B) against female (lanes 1 – 4) and male (lanes 5 – 8) genomic DNA of *P. monodon*. A 100 bp ladder was used as the DNA marker (lanes M).

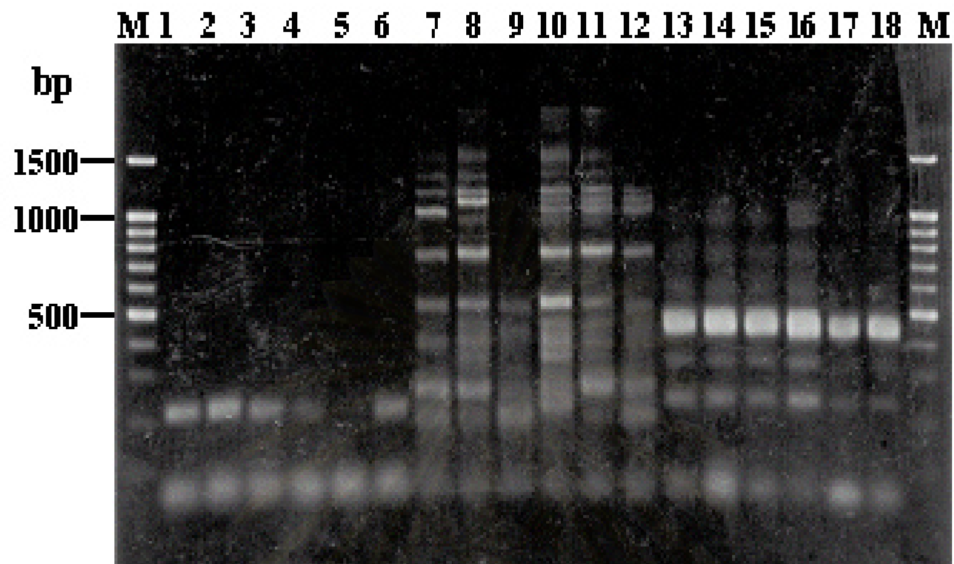



**Figure 3.29** A 1.6% agarose gel electrophoresis showing the PCR products of SCAR markers derived from FE10M10725.2, FE15M14400 and FE16M8350 against female (lanes 1 – 3, 7 – 9 and 13 – 15) and male (lane 4 – 6, 10 – 12 and 16 – 18) genomic DNA of *P. monodon*. Lanes M is a 100 bp DNA marker.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

$TSP_{462F+288R}$ provided a large amplification product (approximately 1200 bp in length) accompanying with , in some specimens, a smaller fragment of approximately 400 bp in length (Figure 3.30). Restriction analysis of the amplified TSP fragment initially provided different patterns between male and female *P. monodon* (Figure 3.31). However, this polymorphism was not fixed in each gender of *P. monodon* when the larger sample size ($N = 12$) was tested.

XNP-1 did not show presence/absence or length polymorphism between male and female *P. monodon* (Figure 3.32). Peritrophin1 also provide monomorphic band of 269 bp but 2 amplified bands (300 and 900 bp in length) were observed for peritrophin 2 implying that peritrophin may be encoded by more than a single locus (Figures 3.33 and 3.34) . Likewise, the amplified PMX, DSI, zinc finger protein and PMT1700 did not exhibit sex-specific amplification or sex-related length polymorphism (Figure 3.35 – 3.38).

The amplification patterns of PMO920 revealed both homozygotic and heterozygotic states of investigated individuals. Genotyping at this locus required further characterization using denaturing polyacrylamide gel electrophoresis.

EGF-response factor 1 may relate with growth and development of *P. monodon* and considered as an important gene in this species. However, non-specific amplification products were observed. The amplification reactions were carefully adjusted (e.g. concentration of PCR ingredients and amplification profiles) but non-specific amplification products were still sustained.

Homologues of SARIP and FP were also identified in *P. monodon* using primers originally developed in *H. asinina*. The monomorphic amplification fragment of the SARIP homologue was observed in male and female *P. monodon* whereas no amplification product was found when primers for FP were used against genomic DNA of *P. monodon*.

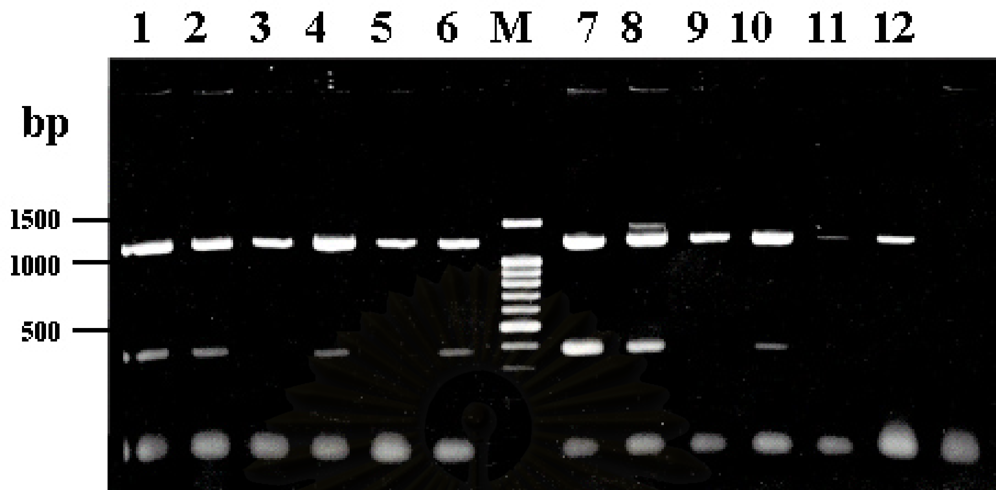



**Figure 3.30** A 1.6% agarose gel electrophoresis showing the PCR product amplified from genomic DNA of female (lanes 1 – 6) and male (lanes 7 – 12) of *P. monodon* using primers $TSP_{462F+288R}$. Lane M is a 100 bp DNA marker.

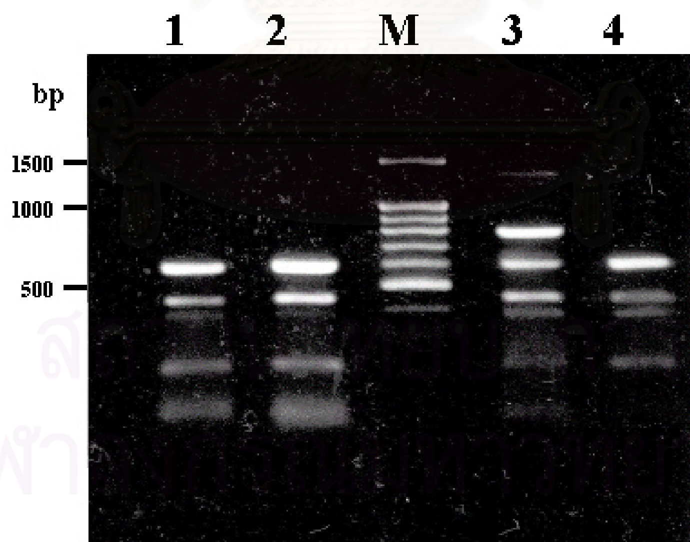



**Figure 3.31** A 1.6% agarose gel electrophoresis showing restriction patterns of *Ssp* I-digested PCR product of female (lanes 1 – 2) and male (lanes 3 – 4) *P. monodon* amplified by primers $TSP_{462F+288R}$. Lanes M is a 100 bp DNA marker.

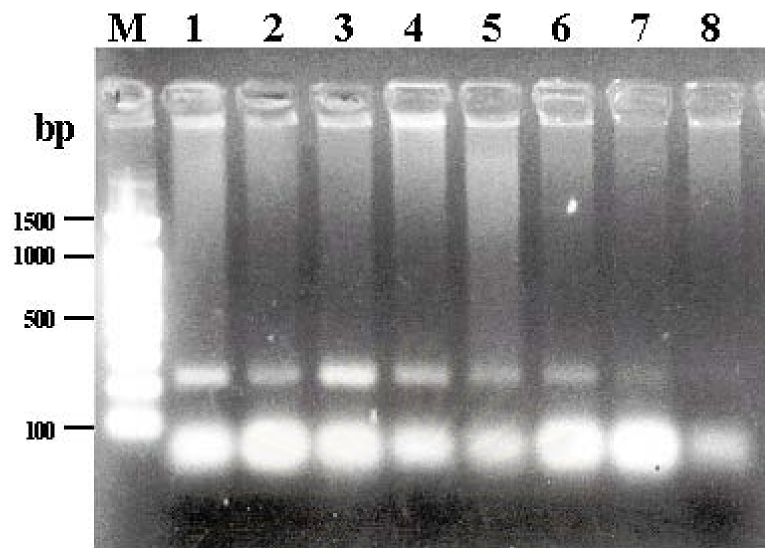



**Figure 3.32** A 1.6% agarose gel electrophoresis showing XNP-1 amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon*. A 100 bp ladder was used as the DNA markers.

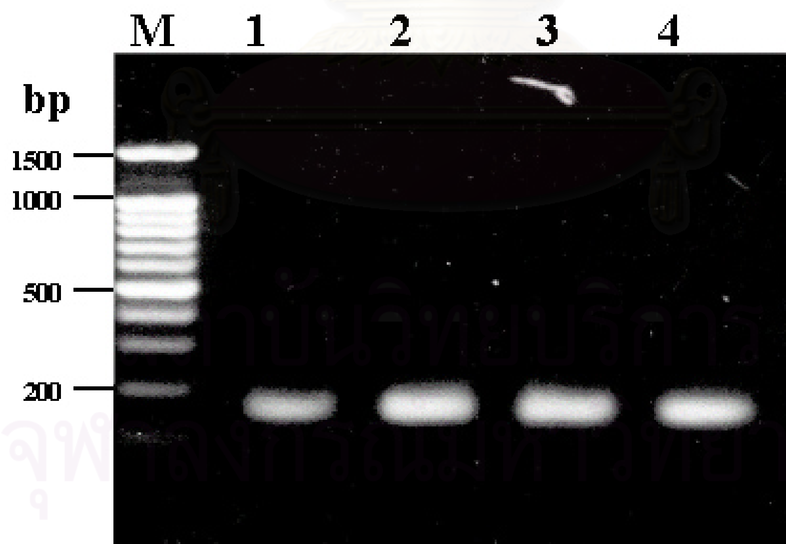



**Figure 3.33** A 1.6% agarose gel electrophoresis showing peritrophin1 amplified from genomic DNA of female (lanes 1 – 2) and male (lanes 3 – 4) *P. monodon*. A 100 bp ladder was used as the DNA markers.

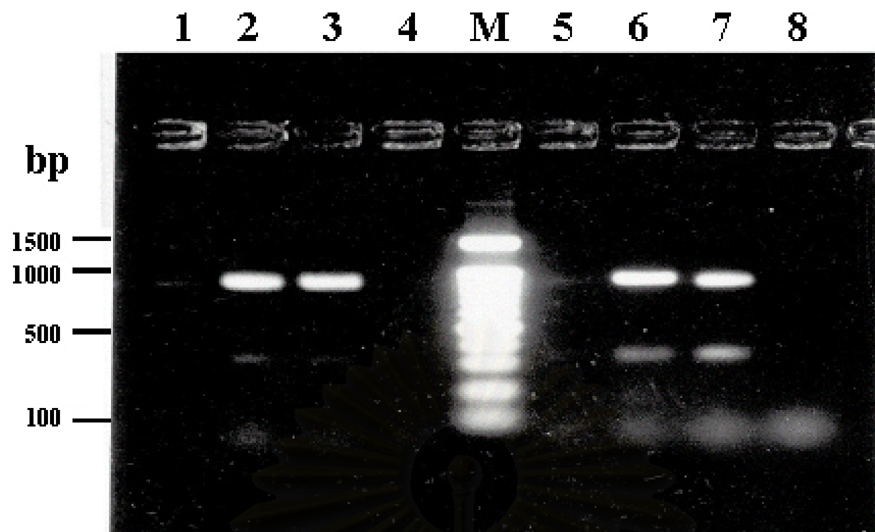



**Figure 3.34** A 1.6% agarose gel electrophoresis showing Peritrophin2 amplified from genomic DNA of female (lanes 2 – 3) and male (lanes 6 – 7) *P. monodon*. Lanes M and 8 are a 100 bp DNA ladder and the negative control, respectively.

**Figure 3.35** A 1.8% agarose gel electrophoresis showing PMX amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon*. A 100 bp ladder was used as the DNA markers (lane M).

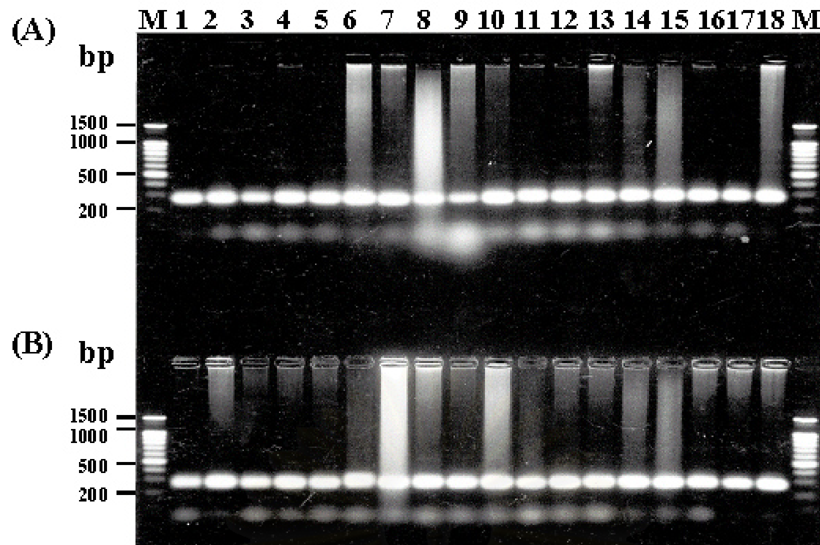



**Figure 3.36** A 1.6% agarose gel electrophoresis showing DSI amplified from genomic DNA of female (lanes 1 – 18, panel A) and male (lanes 1 – 18, B) *P. monodon*. A 100 bp ladder was used as the DNA markers (lanes M).

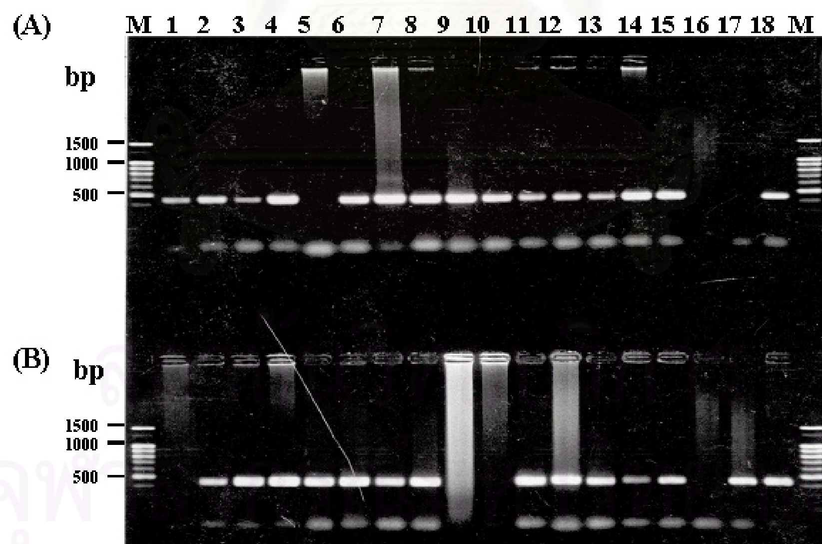



**Figure 3.37** A 1.6% agarose gel electrophoresis showing a zinc finger protein gene amplified from genomic DNA of female (lanes 1 – 18, panel A) and male (lanes 1 – 18, B) *P. monodon*. A 100 bp ladder was used as the DNA markers (lanes M).

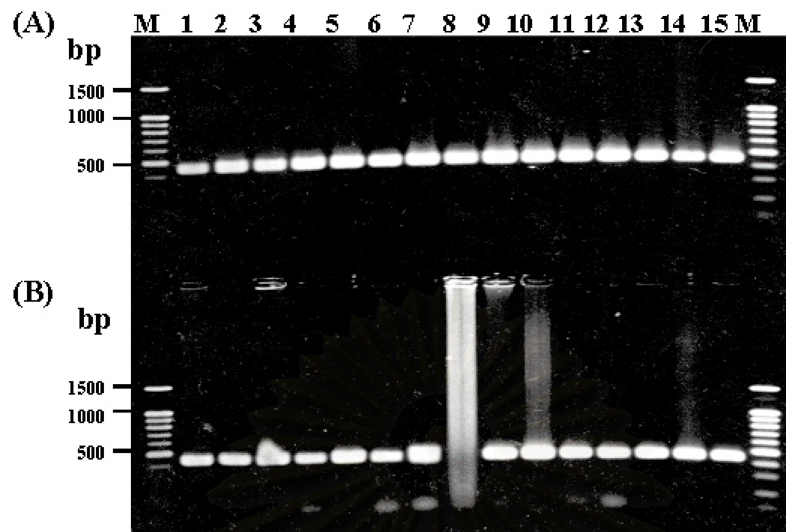



**Figure 3.38** A 1.6% agarose gel electrophoresis showing PMT1700 amplified from genomic DNA of female (lanes 1 – 18, panel A) and male (lanes 1 – 18, B) P. monodon. A 100 bp ladder was used as the DNA markers (lanes M).

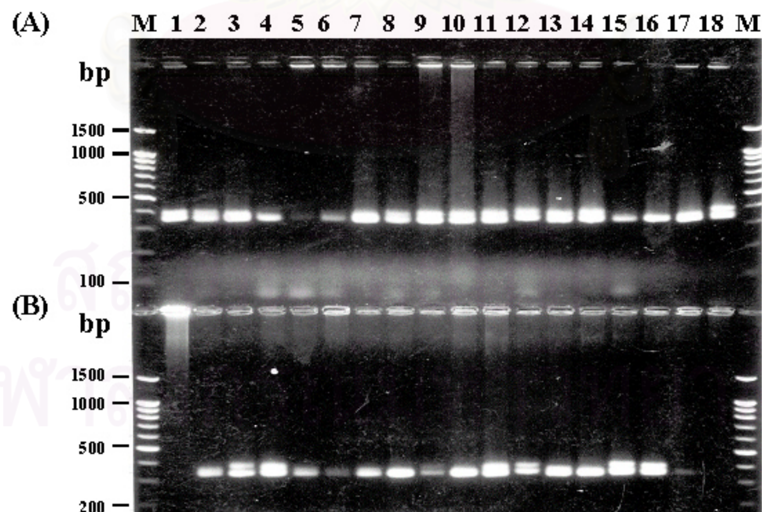



**Figure 3.39** A 1.6% agarose gel electrophoresis showing PMO920 amplified from genomic DNA of female (lanes 1 – 18, panel A) and male (lanes 1 – 18, B) P. monodon. A 100 bp ladder was used as the DNA markers (lanes M).

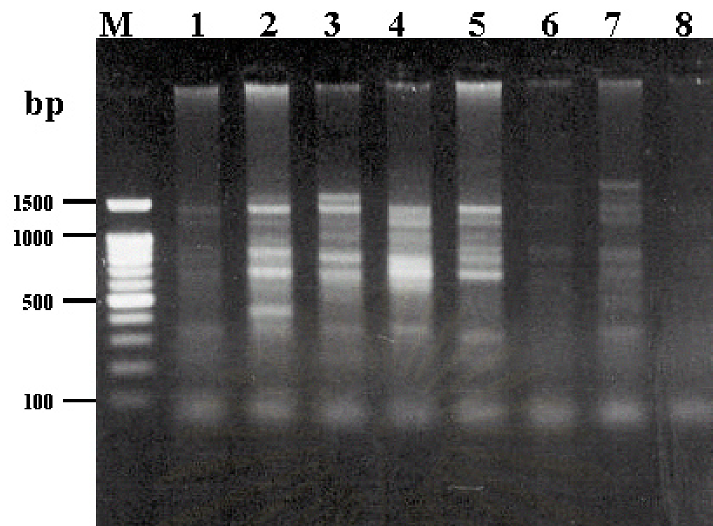



**Figure 3.40** A 1.6% agarose gel electrophoresis showing EGF-response factor 1 amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon*. A 100 bp ladder was used as the DNA markers (lane M).

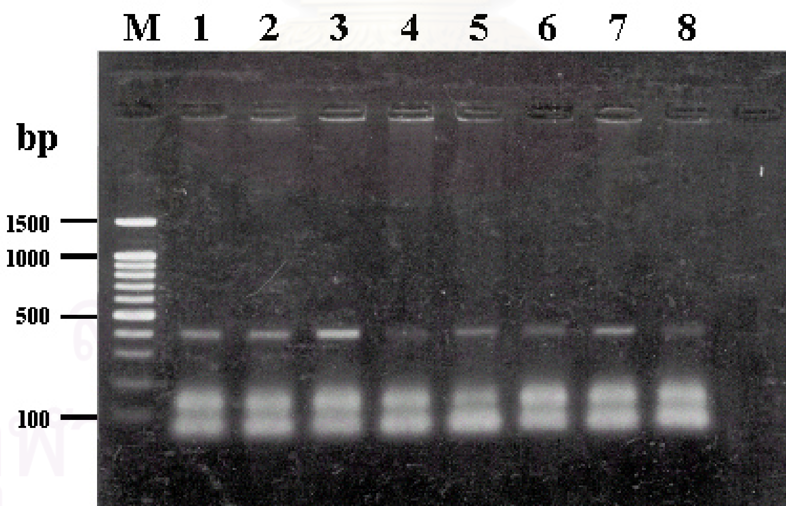



**Figure 3.41** A 1.6% agarose gel electrophoresis of the PCR product resulted from amplification of female (lanes 1 – 4) and male (lanes 5 – 8) genomic DNA of *P. monodon* with heterospecific primers VCP2. Lane M is a 100 bp DNA marker.

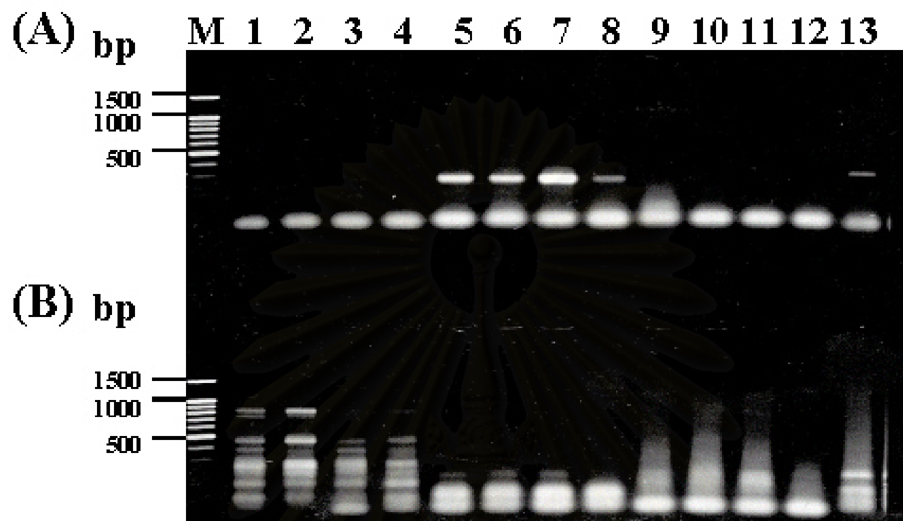



**Figure 3.42** A 1.6% agarose gel electrophoresis showing the PCR product of female (lanes 5 and 6, panels A and B) and male (lanes 7 and 8; panels A and B) *P. monodon* amplified by SARIP-F/R (A) and FP-F/R (B). Lanes M is a 100 bp DNA marker.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 3.9** Characterization of sex-related genes in *P. monodon* using PCR and SSCP analysis

| Gene | PCR | | SSCP | |
|---|---|---|---|---|
| | **Female** | **Male** | **Female** | **Male** |
| Ubiquitin X-specific protease 9 (USP9X) | NS | NS | ND | ND |
| Rudimentary protein | NS | NS | ND | ND |
| EGF-response factor1 | NS | NS | ND | ND |
| $TSP_{462F+288R}$ | 1200 and 400 bp | 1200 and 400 bp | Polymorphism | Polymorphism |
| $TSP_{288-1+371-1}$ | NS | NS | ND | ND |
| Sex-linked XNP-1 | 250 bp | 250 bp | Polymorphism | Polymorphism |
| Sex lethal protein | - | - | ND | ND |
| ENSANGP000000101123 (ENSAN44) | - | - | ND | ND |
| Peritrophin1 | 269 bp | 269 bp | Polymorphism | Polymorphism |
| Peritrophin2 | 300 and 900 bp | 300 and 900 bp | ND | ND |
| PMX | 150 bp | 150 bp | Monomorphism | Monomorphism |
| TSP T1300 | - | - | ND | ND |
| DSI | 250 bp | 250 bp | Polymorphism | Polymorphism |
| PMO920 | 350 bp | 350 bp | Polymorphism | Polymorphism |
| PMT1700 | 450 bp | 450 bp | Polymorphism | Polymorphism |
| Zinc finger protein | 450 bp | 450 bp | Polymorphism | Polymorphism |
| AFLP 517* | NS | NS | ND | ND |
| ME4M8517* | - | - | ND | ND |
| ME8M1310* | - | - | ND | ND |

* primer derived from candidate male-specific AFLP markers of *M. rosenbergii*

Abbreviations:, - = no amplification product; NS = non-specific amplification; ND = not determined.

**Table 3.10** Characterization of sex-related gene homologues in *P. monodon* using heterospecific primers from the tropical abalone (*H. asinina*)

| Gene | PCR | | SSCP | |
|---|---|---|---|---|
| | Female | Male | Female | Male |
| VCP1 | NS | NS | ND | - |
| VCP2 | 400 bp | 400 bp | Polymorphism | Polymorphism |
| VCP3 | NS | NS | ND | ND |
| VCP7 | NS | NS | ND | ND |
| VCP49 | - | - | ND | ND |
| VCP75 | - | - | ND | ND |
| Tektin A1 | NS | NS | ND | ND |
| Axonemal protein | NS | NS | ND | ND |
| Vitellogenin 1 | NS | NS | ND | ND |
| SARIP | 246 bp | 246 bp | Monomorphism | Monomorphism |
| GIOT 1 | NS | NS | ND | ND |
| HSD | NS | NS | ND | ND |
| FP | 248 bp | 248 bp | Polymorphism | Polymorphism |
| Sperm lysin | NS | NS | ND | ND |
| DMRT1 | - | - | ND | ND |

Abbreviations:, - = no amplification product; NS = non-specific amplification; ND = not determined.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

### 3.6 Identification of single nucleotide polymorphism (SNP) using SSCP analysis

Six SCAR markers converted from candidate female-specific AFLP markers (FE10M9520, FE10M10725.1, FE10M10725.2, FE14M16340, FE15M14400 and FE16M8350) did not exhibit sex-specificity when the amplified products were analyzed by agarose gel electrophoresis.

SSCP was then carried out whether the amplified fragments showed SNP between male and female *P. monodon*. Results indicated that all SCAR markers did not show sex-specific nature. FE10M9520 and FE10M10725.2 were monomorphic across investigated individuals (Figure 3.43 and 3.46) whereas FE14M16340 and FE10M10725.1 were polymorphic. Polymorphism of the latter can be used for population genetic studies and construction of genetic linkage maps in *P. monodon* (Figures 3.44 and 3.45).

In addition, polymorphism of sex-related genes of *P. monodon* and those from other related aquatic species *M. rosenbergii* and *H. asinina*, was also characterized by SSCP analysis or denaturing gel electrophoresis (PMO920) (Figures 3.47 – 3.56). Several gene products including TSP (Figure 3.47), XNP-1 (Figure 3.48), PERI-1(Figure 3.49), VCP2 (Figure 3.50), FP(Figure 3.52) DSI (Figure. 3.53), zinc finger protein (Figure 3.54), PMT1700 (Figure 3.55) and PMO920 (Figure 3.56) were polymorphic. Conversely, SARIP (Figure 3.51) were monomorphic.

Four genes (DSI, zinc finger protein, PMT1700 and PMO920) were then selected for further used for studies of genetic diversity and population differentiation of natural *P. monodon* in Thai waters for which no information was not available based on type I (coding sequences) markers.

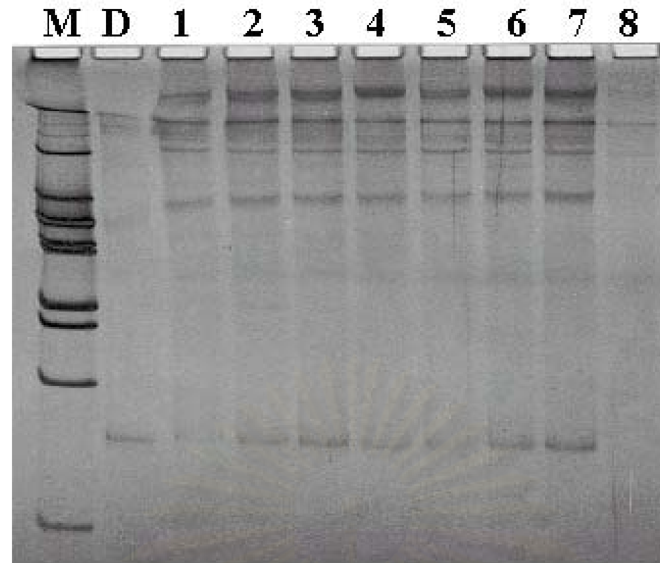



**Figure 3.43** SSCP patterns of a SCAR marker derived from FE10M9520 of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* resulted from size-fractionation through 15% PAGE + 5% glycerol. Lane M = a 100 bp DNA marker, Lane D = ds PCR
product control.

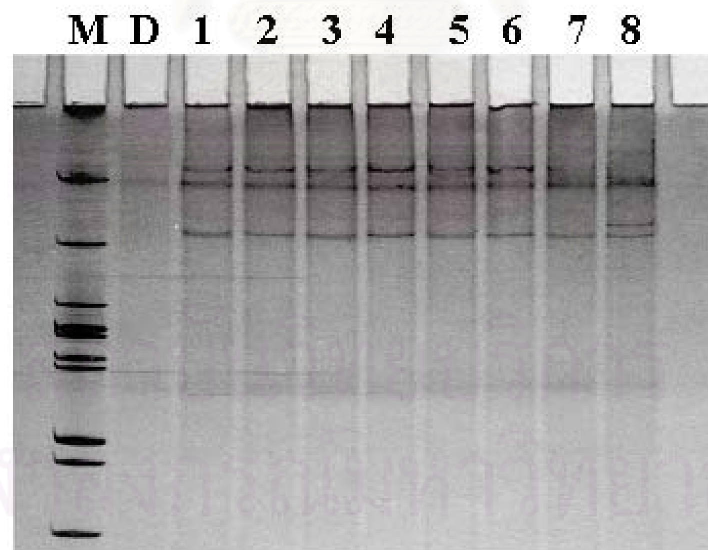



**Figure 3.44** SSCP patterns of a SCAR marker derived from FE10M10725.1 of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* resulted from size-fractionation through 15% PAGE + 5% glycerol Lane M = a 100 bp DNA marker, Lane D = ds PCR product

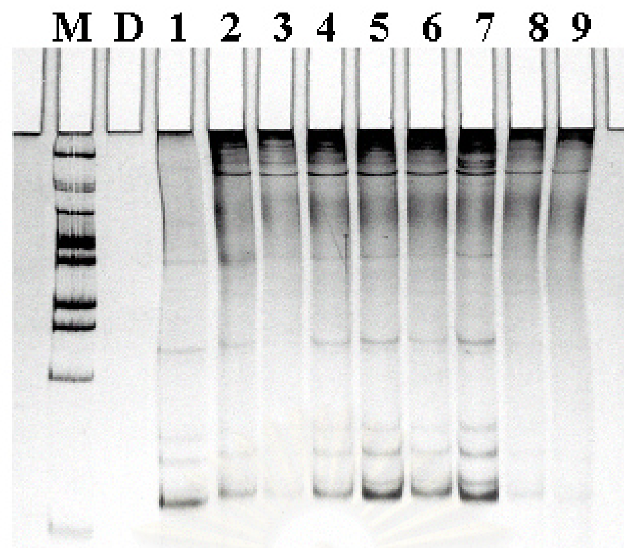



**Figure 3.45** SSCP patterns of a SCAR marker derived from FE14M16340 of female (lanes 1 – 4) and male (lanes 5 – 9) *P. monodon* resulted from size-fractionation through 17.5% PAGE. Lane M = a 100 bp DNA marker, Lane D = ds PCR control

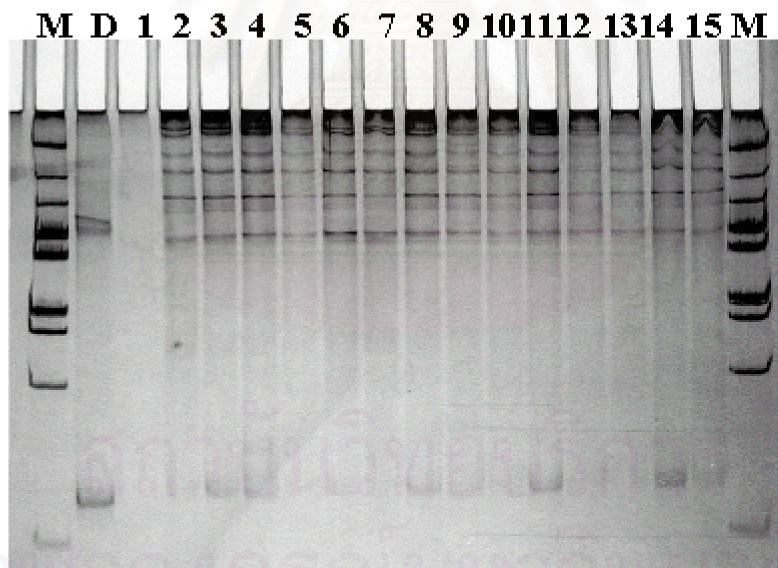



**Figure 3.46** SSCP patterns of a SCAR marker derived from FE10M10725.2of female (lanes 2 – 8) and male (lanes 9 – 15) *P. monodon* resulted from size-fractionation through was size-fractionated through 12.5%PAGE. Lane M = a 100 bp DNA marker, Lane D = ds PCR control.

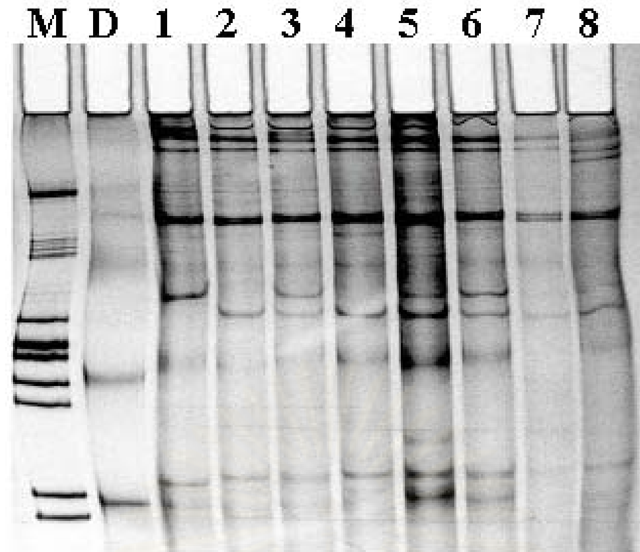



**Figure 3.47** SSCP analysis of the PCR product amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) of *P. monodon* using primers $TSP_{462F + 288R}$. The PCR product was denatured and size-fractionated through a 12.5% native polyacrylamide gel (37.5:1). Lanes M and D are a 100 bp DNA marker and ds PCR product, respectively.

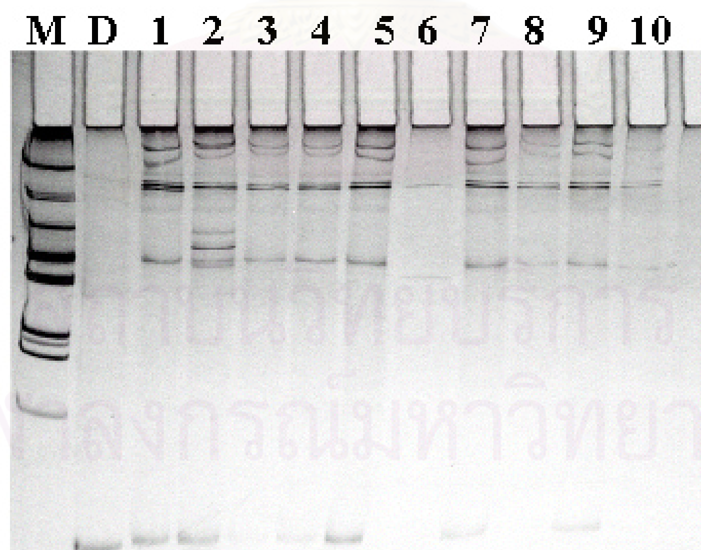



**Figure 3.48** SSCP analysis of XNP-1 amplified from genomic DNA of female (lanes 1 - 5) and male (lanes 6 – 10) *P. monodon*. The amplified XNP-1 was denatured and size-fractionated through 15% native polyacrylamide gel (37.5:1). Lanes M and D are a 100 bp DNA marker and ds PCR product, respectively.

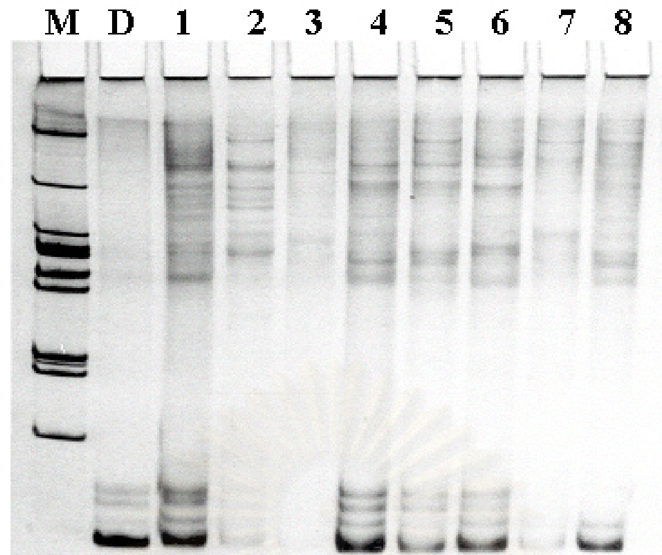



**Figure 3.49** SSCP analysis of Peritrophin-1 amplified from genomic DNA of female (lanes 1 – 5) and male (lanes 6 – 10) *P. monodon*. The amplified Peritrophin-1 was denatured and size-fractionated through 12.5% native polyacrylamide gel, 5% glycerol (37.5:1). Lanes M and D are a 100 bp DNA marker and ds PCR product, respectively

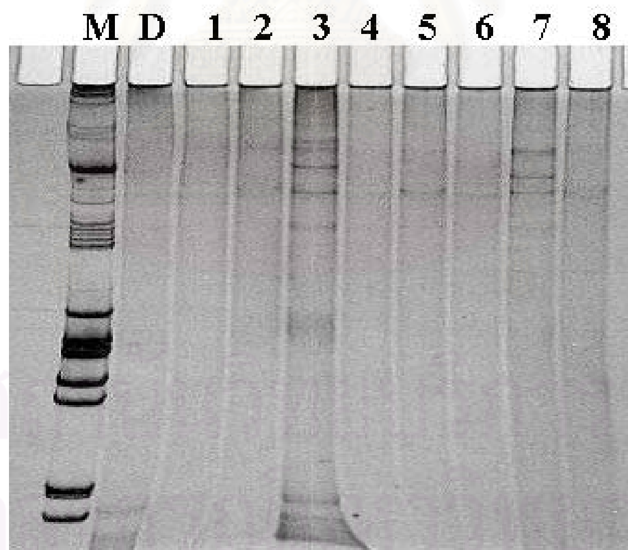



**Figure 3.50** SSCP analysis of the PCR product amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* using VCP2 primers. The PCR product was denatured and size-fractionated through a 12.5% native polyacrylamide gel (37.5:1). Lanes M and D are a 100 bp DNA marker and ds PCR product, respectively

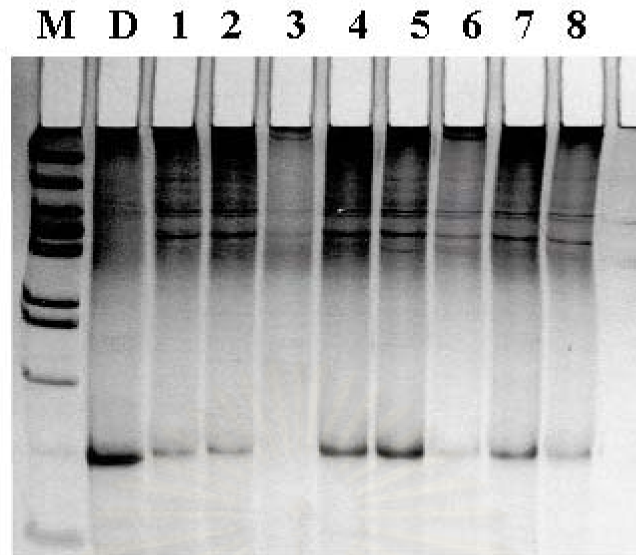



**Figure 3.51** SSCP analysis of the PCR product amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* using primers SARIP-F/R. The PCR product was denatured and size-fractionated through a 15% native polyacrylamide gel (37.5:1) and silver stained. Lanes M and D are a 100 bp DNA marker and the ds PCR product, respectively.

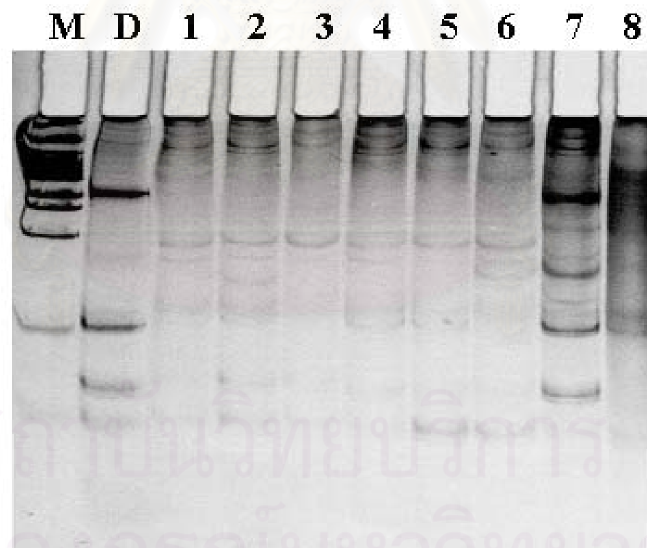



**Figure 3.52** SSCP analysis of the PCR product amplified from genomic DNA of female (lanes 1 – 4) and male (lanes 5 – 8) *P. monodon* using primers FP-F/R. The PCR product was denatured and size-fractionated through a 20% native polyacrylamide gel (37.5:1) and silver stained. Lanes M and D are a 100 bp DNA marker and the ds PCR product, respectively. Notably, polymorphism observed from the SSCP gel was resulted from patterns of primer dimers rather than the amplified FP product.

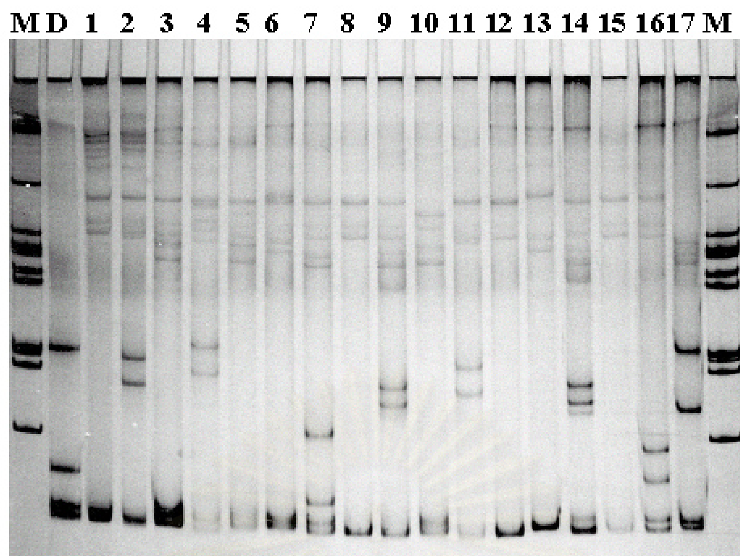



**Figure 3.53** SSCP patterns of the amplified DSI of *P. monodon* individuals (lanes 1 – 17) size-fractionated through 17.5% non-denaturing PAGE (acrylamide:bisacrylamide = 75:1). Lane M = 100 bp ladder, lane D = non – denatured PCR product (double strand control).

**Figure 3.54** SSCP patterns of the amplified ZFP of *P. monodon* individuals (lanes 1 – 17) size-fractionated through 15% non-denaturing PAGE (acrylamide:bisacrylamide = 75:1) Lane M = 100 bp ladder, lane D = non – denatured PCR product (double strand control).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

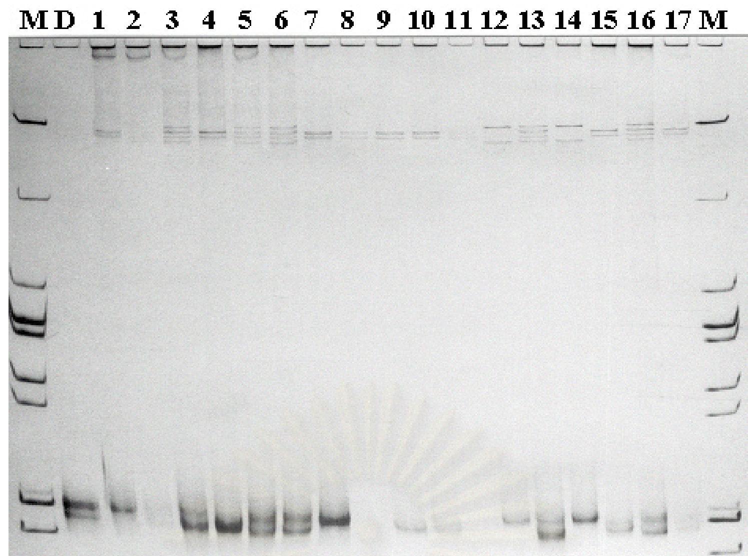



**Figure 3.55 SSCP patterns of the amplified PMT1700 of *P. monodon* individuals (lanes 1 – 17) size-fractionated through 15% non-denaturing PAGE (acrylamide : bisacrylamide = 75:1). Lane M = 100 bp ladder, lane D = non – denatured PCR product (double strand control).**

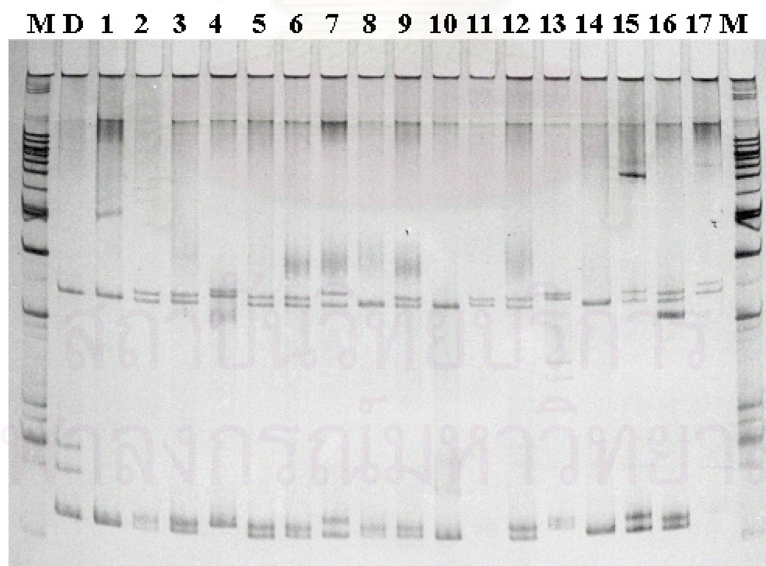


**Figure 3.56 Patterns of the amplified PMO920 of *P. monodon* individuals (lanes 1 –**

**17) size-fractionated through 6% denaturing PAGE (19:1). Lane M = 100 bp ladder, lane D = non – denatured PCR product (double strand control).**

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

### 3.7 Genetic diversity and population differentiation of *P. monodon* using nuclear DNA polymorphism

Four genes (PMO920, PMT1700, ZFP and DSI) were chosen for population genetic studies of *P. monodon* in geographically different samples (Satun, Trang and Phangnga located in the Andaman sea and Chumphon and Trad located in the Gulf of Thailand) in Thai waters.

DSI and ZFP genes were previously isolated from the EST libraries established from mature and immature ovaries of *P. monodon*, respectively. In constrast, PMO920 and PMT1700 were unknown transcripts isolated by RT-PCR. These transcripts exhibited sex differential expression in ovaries and testes of *P. monodon*, respectively.

All four markers were successfully amplified when genomic DNA was used as the template. Sizes of the amplification products were identical to those from the first strand cDNA template suggesting the absence of intron in the gene regions.

Initial screening of these genes using representative individuals of *P. monodon* followed by agarose gel electrophoresis revealed both homozygotic and heterozygotic states for PMO920 (Figure 3.56) whereas the amplification products of the remaining genes did not show length polymorphism between alleles (Figures 3.54 – 3.56). As a result, PMO920 was analyzed by denaturing polyacrylamide gel eletrophoresis to examine polymorphism between alleles whereas DSI, PMT1700 and ZFP were analyzed by SSCP analysis. Results indicated complicate band patterns of the amplified DSI. This gene was then scored as the dominant marker (homo and heterozygotes can not be distinguished) but the remaining genes were scored as the co-dominant markers.

PMO920 showed relatively high genetic diversity in *P. monodon*. Six alleles were found at this locus and allele 2 and 4 were the common alleles found at the frequencies of 0.5536 (Trad) – 0.6087 (Trang) and 0.1034 (Phangnga) – 0.2143 (Chumphon), respectively. The remaining alleles were variants found in relatively low frequencies across geographic samples (Table 3.11).

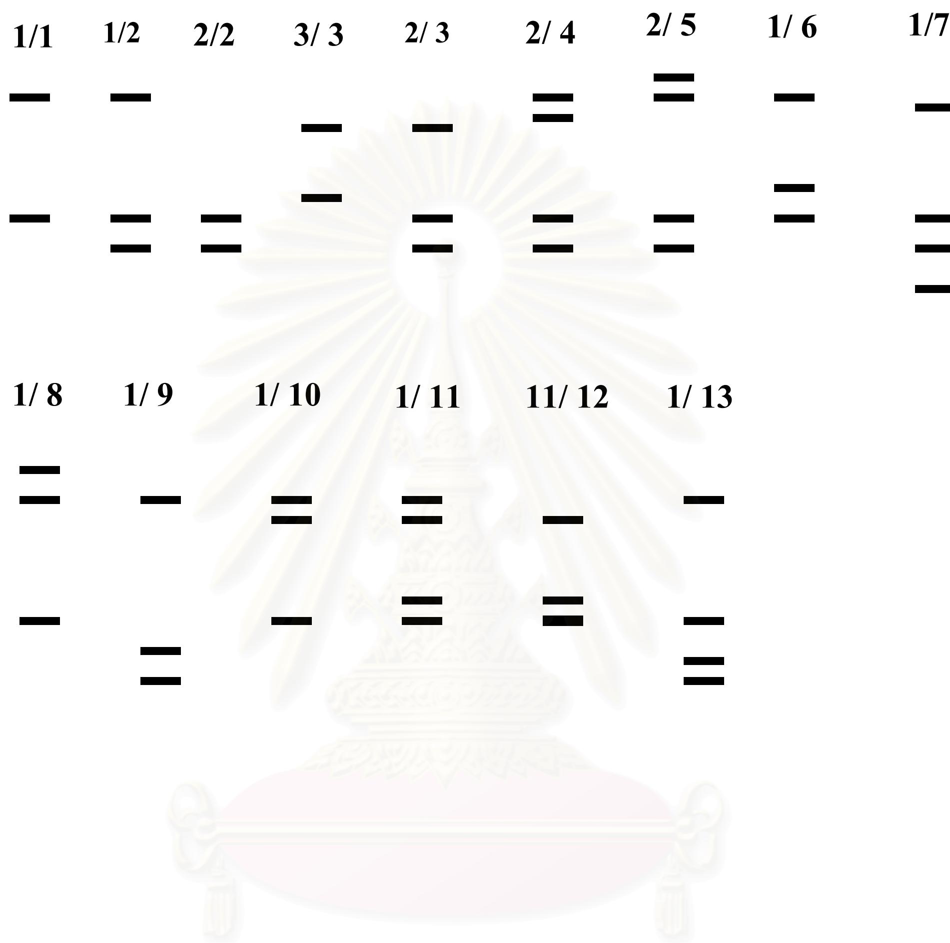



**Figure 3.57** Diagram showing SSCP pattern of ZFP

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 3.11** Alllele frequencies, observed and unbiased expected heterozygosities and Hardy-Weinberg disequilibrium at PMO920, PMT1700 and zinc finger protein loci of *P. monodon* from different geographic locations of Thailand

| Locus/allele | Allele frequency | | | | |
|---|---|---|---|---|---|
| | Satun | Trang | Phangnga | Chumphon | Trad |
| **PMO920** | | | | | |
| 1 | 0.1410 | 0.1739 | 0.1034 | 0.0714 | 0.1250 |
| 2 | 0.5897 | 0.6087 | 0.6034 | 0.6071 | 0.5536 |
| 3 | 0.0513 | 0.0435 | 0.1724 | 0.0357 | 0.0714 |
| 4 | 0.2051 | 0.1522 | 0.1034 | 0.2143 | 0.1786 |
| 5 | 0.0128 | 0.0217 | 0.0172 | 0.0357 | 0.0357 |
| 6 | 0.0000 | 0.0000 | 0.0000 | 0.0357 | 0.0357 |
| $H_0$ | 0.5128 | 0.3043 | 0.4827 | 0.5000 | 0.3571 |
| He | 0.5951 | 0.5865 | 0.5947 | 0.5870 | 0.6500 |
| H-W (P-value) | 0.1209[ns] | 0.0002* | 0.2455[ns] | 0.3276[ns] | 0.0005* |
| *N* | 39 | 23 | 29 | 28 | 28 |
| **PMT1700** | | | | | |
| 1 | 0.7436 | 0.7609 | 0.8077 | 0.6429 | 0.7321 |
| 2 | 0.2436 | 0.1957 | 0.1731 | 0.3571 | 0.2500 |
| 3 | 0.0128 | 0.0217 | 0.0000 | 0.0000 | 0.0000 |
| 4 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0179 |
| 5 | 0.0000 | 0.0217 | 0.0192 | 0.0000 | 0.0000 |
| $H_0$ | 0.3846 | 0.3478 | 0.3462 | 0.4286 | 0.4643 |
| He | 0.3926 | 0.3903 | 0.3235 | 0.4675 | 0.4084 |
| H-W (P-value) | 0.4744 | 0.1789 | 0.6562 | 0.4800 | 0.8868 |
| *N* | 39 | 23 | 28 | 28 | 28 |
| **Zinc finger protein** | | | | | |
| 1 | 0.4865 | 0.5000 | 0.6379 | 0.6429 | 0.6923 |
| 2 | 0.3919 | 0.3696 | 0.2759 | 0.2500 | 0.2500 |
| 3 | 0.0405 | 0.0000 | 0.0000 | 0.0536 | 0.0000 |
| 4 | 0.0000 | 0.0000 | 0.0172 | 0.0000 | 0.0000 |
| 5 | 0.0000 | 0.0217 | 0.0000 | 0.0179 | 0.0385 |
| 6 | 0.0135 | 0.0217 | 0.0517 | 0.0179 | 0.0000 |
| 7 | 0.0135 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 8 | 0.0000 | 0.0435 | 0.0172 | 0.0000 | 0.0192 |
| 9 | 0.0135 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 10 | 0.0270 | 0.0000 | 0.0000 | 0.0179 | 0.0000 |
| 11 | 0.0135 | 0.0217 | 0.0000 | 0.0000 | 0.0000 |
| 12 | 0.0000 | 0.0217 | 0.0000 | 0.0000 | 0.0000 |
| $H_0$ | 0.8108 | 0.6522 | 0.6897 | 0.5000 | 0.5385 |
| He | 0.6150 | 0.6232 | 0.5227 | 0.5299 | 0.4653 |
| H-W (P-value) | 0.0047[b] | 0.6531[a] | 0.0175[b] | 0.5641[a] | 0.9038[a] |
| *N* | 37 | 23 | 29 | 28 | 26 |

H-W = Hardy-Weinberg disequilibrium analysis; ns = not significant; * = significant at $P < 0.05$; [a] = heterozygote deficiency test; [b] = heterozygote excess test.

Observed and expected heterozygosities at PMO920 were between 0.3043 (Trang) – 0.5128 (Satun) and 0.5865 (Trang) – 0.6500 (Trad). Homozygote excess was clearly observed in Trang and Trad samples leading to deviations from Hardy-Weinberg equilibrium of these samples at this locus.

The genetic diversity at the locus PMT1700 was slightly lower than that of PMO920. Five alleles were found at this locus and allele 1 and 2 were the common alleles found at the frequencies of 0.6429 (Chumphon) – 0.8077 (Phangnga) and 0.1731 (Phangnga) – 0.3571 (Chumphon), respectively. The remaining alleles were variants found in relatively low frequencies across geographic samples (Table 3.11).

Observed and expected heterozygosities at the locus were 0.3462 (Phangnga) – 0.4643 (Trad) and 0.3235 (Phangnga) – 0.4675 (Chumphon). Homozygote excess was not observed in all investigated samples of *P. monodon*. All geographic samples conformed Hardy-Weinberg equilibrium at this locus.

The ZFP gene exhibited the highest genetic diversity among overall investigated loci. Twelve alleles were found at this locus and allele 1 and 2 were the common alleles found at the frequencies of 0.4865 (Satun) – 0.6923 (Trad) and 0.2500 (Chumphon and Trad) – 0.3919 (Satun), respectively. The remaining alleles were variants found in relatively low frequencies across geographic samples (Table 3.9).

Observed and expected heterozygosities at the locus were between 0.5000 (Chumphon) – 0.8108 (Trad) and 0.4653 (Trad) – 0.6232 (Trang). Interestingly, the observed heterozygosity in all samples except Chumphon was greater than the expected heterozygosity. Chumphon, Trad and Tang conformed Hardy-Weinberg equilibrium whereas Satun and Phangnga exhibited heterozygote excess at this locus.

Allele frequencies of DSI was calculated from presence and absence of the band and illustrated by Table 3.12.

Generally, all investigated loci did not show genotypic disequilibrium within each geographic samples ($P > 0.05$, Table 3.13) with the exception of PMO920 and PMT1700 in Satun ($P < 0.0457$). This was not significant after the significance value was further adjusted using the sequential Bonferroni method ($P < 0.0167$, Rice, 1989). Analysis of overall sample also showed that genotypes of these loci associated randomly ($P > 0.05$).

The average genetic distance between geographic samples was 0.0031 (Satun-Trang) – 0.0178 (Trang – Chumphon). Genetic distances between samples from the different coastal areas were 0.0079 (Phangnga – Trad) – 0.0178 (Chumphon – Trang) whereas the distance within the Gulf of Thailand and the Andaman Samples were 0.0077 and 0.0031 – 0.0135, respectively (Table 3.13).

The neighbor-joining tree constructed from the genetic distance between paired samples of *P. monodon* indicated two separate groups of investigated samples according to coastal regions (the Gulf of Thailand and the Andaman Sea) (Figure 3.58)

Geographic heterogeneity analysis using the exact test (allele frequencies) and $F_{ST}$ statistics (genotypic frequencies) indicated population differentiation was not significant in almost all of the samples at PMO920, PMT1700 and ZFP loci ($P < 0.05$) except between Satun–Phangnga ($P < 0.0307$) and Satun – Trad ($P < 0.0092$) at the ZFP locus. $F_{ST}$ statistics between Satun and Chumphon at this locus was 0.0563. As a result, larger sample size should be used for genotyping before unambiguous conclusion can be made from the data.

Likewise, 7 alleles (6, 7, 9, 10, 12, 14 and 15 called as locus in Table 3.17 ) frequencies of DSI showed significant different against overall samples ($P < 0.05$). Nevertheless, genotypic frequencies of this gene was statistically significant at the 6, 7, 9, 10, 12, 14 and 15 assumed loci (Table 3.15).

**Table 3.12** Alllele frequencies resulted from analysis of DSI of *P. monodon* from different geographic locations of Thailand

| Locus*/sample | Satun | Trang | Phangnga | Chumphon | Trad |
|---|---|---|---|---|---|
| **Locus 1** | | | | | |
| Allele 1 | 0.0132 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| Allele 2 | 0.9868 | 1.0000 | 1.0000 | 1.0000 | 1.0000 |
| **Locus 2** | | | | | |
| Allele 1 | 0.0000 | 0.0000 | 0.0351 | 0.0364 | 0.0174 |
| Allele 2 | 1.0000 | 1.0000 | 0.9649 | 0.9636 | 0.9826 |
| **Locus 3** | | | | | |
| Allele 1 | 0.0000 | 0.0445 | 0.0351 | 0.0364 | 0.0174 |
| Allele 2 | 1.0000 | 0.9555 | 0.9649 | 0.9636 | 0.9826 |
| **Locus 4** | | | | | |
| Allele 1 | 0.5133 | 0.5337 | 0.5451 | 0.5371 | 0.5451 |
| Allele 2 | 0.4867 | 0.4663 | 0.4549 | 0.4629 | 0.4549 |
| **Locus 5** | | | | | |
| Allele 1 | 0.0823 | 0.0675 | 0.0351 | 0.0364 | 0.0531 |
| Allele 2 | 0.9177 | 0.9325 | 0.9649 | 0.9636 | 0.9469 |
| **Locus 6** | | | | | |
| Allele 1 | 0.0000 | 0.0220 | 0.0351 | 0.0000 | 0.0351 |
| Allele 2 | 1.0000 | 0.9780 | 0.9649 | 1.0000 | 0.9649 |
| **Locus 7** | | | | | |
| Allele 1 | 0.0681 | 0.0220 | 0.1490 | 0.1136 | 0.0903 |
| Allele 2 | 0.9319 | 0.9780 | 0.8510 | 0.8864 | 0.9097 |
| **Locus 8** | | | | | |
| Allele 1 | 0.4380 | 0.4102 | 0.4128 | 0.4024 | 0.5087 |
| Allele 2 | 0.5620 | 0.5898 | 0.5872 | 0.5976 | 0.4913 |
| **Locus 9** | | | | | |
| Allele 1 | 0.0968 | 0.1924 | 0.1290 | 0.1762 | 0.0531 |
| Allele 2 | 0.9032 | 0.8076 | 0.8710 | 0.8238 | 0.9469 |
| **Locus 10** | | | | | |
| Allele 1 | 0.0000 | 0.0000 | 0.0174 | 0.0364 | 0.0000 |
| Allele 2 | 1.0000 | 1.0000 | 0.9826 | 0.9636 | 1.0000 |
| **Locus 11** | | | | | |
| Allele 1 | 0.1115 | 0.1153 | 0.1094 | 0.0937 | 0.1490 |
| Allele 2 | 0.8885 | 0.8847 | 0.8906 | 0.9063 | 0.8510 |
| **Locus 12** | | | | | |
| Allele 1 | 0.1571 | 0.1153 | 0.0903 | 0.0742 | 0.1906 |
| Allele 2 | 0.8429 | 0.8847 | 0.9097 | 0.9268 | 0.8094 |
| **Locus 13** | | | | | |
| Allele 1 | 0.0823 | 0.0675 | 0.0715 | 0.1136 | 0.0715 |
| Allele 2 | 0.9177 | 0.9325 | 0.9285 | 0.8864 | 0.9285 |
| **Locus 14** | | | | | |
| Allele 1 | 0.0541 | 0.0445 | 0.1290 | 0.0551 | 0.1094 |
| Allele 2 | 0.9459 | 0.9555 | 0.8710 | 0.9449 | 0.8906 |

**Table 3.12 (continued)**

| Locus*/sample | Satun | Trang | Phangnga | Chumphon | Trad |
|---|---|---|---|---|---|
| **Locus 15** | | | | | |
| Allele 1 | 0.0000 | 0.0000 | 0.0351 | 0.0000 | 0.0000 |
| Allele 2 | 1.0000 | 1.0000 | 0.9649 | 1.0000 | 1.0000 |
| **Locus 16** | | | | | |
| Allele 1 | 0.2053 | 0.1403 | 0.1094 | 0.1136 | 0.2122 |
| Allele 2 | 0.7947 | 0.8597 | 0.8906 | 0.8864 | 0.7878 |
| **Locus 17** | | | | | |
| Allele 1 | 0.0132 | 0.0220 | 0.0531 | 0.0364 | 0.0174 |
| Allele 2 | 0.9868 | 0.9780 | 0.9469 | 0.9636 | 0.9826 |
| **Locus 18** | | | | | |
| Allele 1 | 0.0132 | 0.0220 | 0.0715 | 0.0180 | 0.0351 |
| Allele 2 | 0.9868 | 0.9780 | 0.9285 | 0.9820 | 0.9649 |
| **Locus 19** | | | | | |
| Allele 1 | 0.0132 | 0.0000 | 0.0174 | 0.0180 | 0.0000 |
| Allele 2 | 0.9868 | 1.0000 | 0.9826 | 0.9820 | 1.0000 |

* DSI was regarded as the diploid/dominant marker. As a result, the presence (allele 1) and absence (allele 2) of each character (a locus) were considered.

**Table 3.13** Genotypic disequilibrium between pairs of loci in each geographic sample of *P. monodon* in Thailand.

| Geographic sample | Locus 1 | Locus 2 | P-value |
|---|---|---|---|
| Satun | PMO920 | PMT1700 | 0.0457 |
| Satun | PMO920 | ZFP | 0.9292 |
| Satun | PMT1700 | ZFP | 0.4256 |
| Trang | PMO920 | PMT1700 | 0.2630 |
| Trang | PMO920 | ZFP | 0.7852 |
| Trang | PMT1700 | ZFP | 0.0982 |
| Phangnga | PMO920 | PMT1700 | 0.7684 |
| Phangnga | PMO920 | ZFP | 0.5747 |
| Phangnga | PMT1700 | ZFP | 1.0000 |
| Chumphon | PMO920 | PMT1700 | 0.0709 |
| Chumphon | PMO920 | ZFP | 0.9268 |
| Chumphon | PMT1700 | ZFP | 0.2054 |
| Trad | PMO920 | PMT1700 | 0.4559 |
| Trad | PMO920 | ZFP | 0.85999 |
| Trad | PMT1700 | ZFP | 0.69233 |

**Table 3.14** Genotypic disequilibrium between pairs of loci across overall samples of *P. monodon* in Thailand.

| Locus pair | Chi-square | df | P-value |
|---|---|---|---|
| PMO920&PMT1700 | 16.234 | 10 | 0.0931 |
| PMO920&ZFP | 2.192 | 10 | 0.9947 |
| PMT1700&ZFP | 10.252 | 10 | 0.4187 |

**Table 3.15** Pairwise genetic distance between *P. monodon* originating from different geographic locations in Thailand analyzed by PMO920, PMT1700, ZFP and DSI

| | Satun | Trang | Phanganga | Chumphon | **Trad** |
|---|---|---|---|---|---|
| Satun | - | | | | |
| Trang | 0.0031 | - | | | |
| Phanganga | 0.0135 | 0.0102 | - | | |
| Chumphon | 0.0144 | 0.0178 | 0.0169 | - | |
| Trad | 0.0129 | 0.0129 | 0.0079 | 0.0077 | - |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

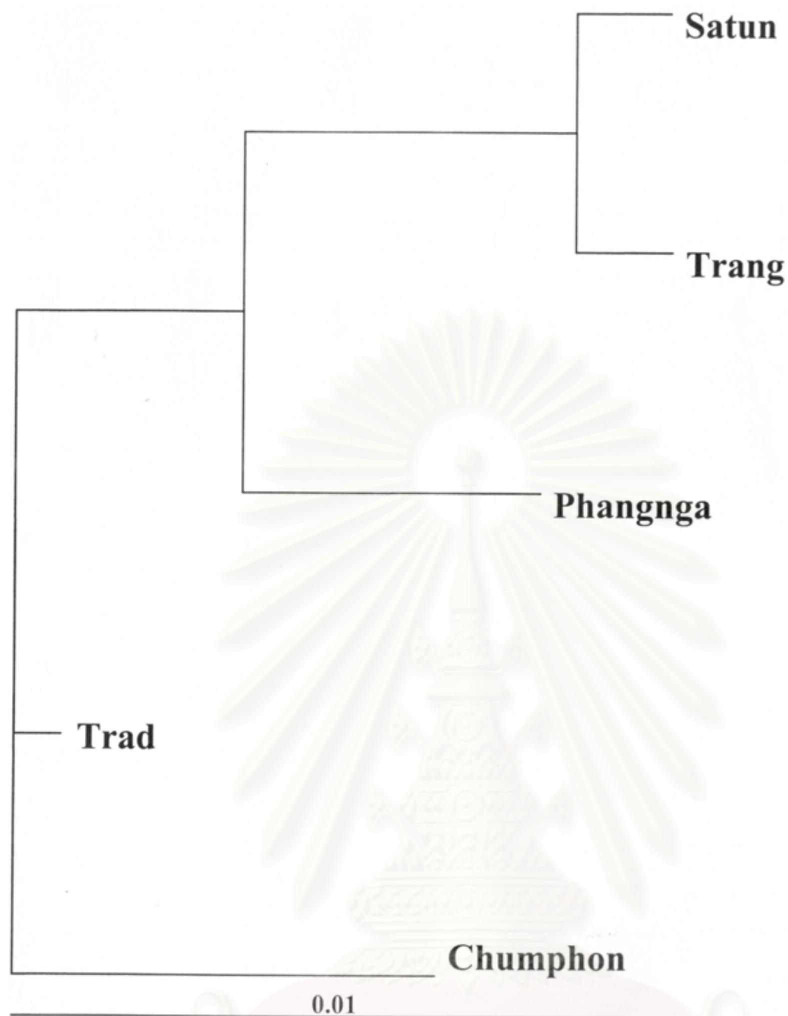



**Figure 3.58** A neighbor-joining tree indicating genetic relationship between each geographic sample of *P. monodon* analyzed by polymorphism of PMO920, PMT1700, zinc finger protein gene and DSI.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**Table 3.16** Pairwise comparison of genetic differentiation between geographic samples of *P. monodon* at 3 loci (PMO920, PMT1700 and zinc finger protein) using geographic heterogeneity (exact test) and $F_{ST}$ analyses

| Geographic sample | Geographic heterogeneity test (P-value) | | | $F_{ST}$ (P-value) | | |
|---|---|---|---|---|---|---|
| | **PMO 920** | **PMT 1700** | **ZFP** | **PMO 920** | **PMT 1700** | **ZFP** |
| **SAT – TNG** | 0.9420 | 0.5719 | 0.3575 | 0.9222 | 0.7659 | 0.8043 |
| **SAT – PHA** | 0.0963 | 0.4193 | 0.1006 | 0.2682 | 0.3823 | 0.0307* |
| **SAT – CHM** | 0.4460 | 0.2398 | 0.4757 | 0.4757 | 0.2320 | 0.0563 |
| **SAT – TRD** | 0.6091 | 0.8067 | 0.0336* | 0.9287 | 1.0000 | 0.0092* |
| **TNG – PHA** | 0.2311 | 0.8455 | 0.3738 | 0.4827 | 0.7886 | 0.1479 |
| **TNG – CHM** | 0.5171 | 0.0888 | 0.1470 | 0.6418 | 0.1281 | 0.1491 |
| **TNG – TRD** | 0.8346 | 0.4993 | 0.2840 | 0.9048 | 0.6585 | 0.0745 |
| **PHA – CHM** | 0.0565 | 0.0397 | 0.3335 | 0.2278 | 0.0515 | 0.7235 |
| **PHA – TRD** | 0.3041 | 0.3792 | 0.3352 | 0.5528 | 0.3241 | 0.5100 |
| **CHM – TRD** | 0.8916 | 0.3052 | 0.5176 | 0.8872 | 0.3042 | 0.6826 |

*significant at P < 0.05

**Table 3.17** Genetic differentiation of overall investigated sample of *P. monodon* at the DSI locus using geographic heterogeneity (exact test) and $F_{ST}$ analyses

| Allele | Geographic heterogeneity (P-value) | *F*-statistics (θ)* |
|---|---|---|
| 1 | 1.000 | -0.0048 |
| 2 | 0.3067 | 0.0018 |
| 3 | 0.3597 | -0.0025 |
| 4 | 1.0000 | -0.0166 |
| 5 | 0.7891 | -0.0087 |
| 6 | 0.2506 | 0.0023* |
| 7 | 0.2128 | 0.0081* |
| 8 | 0.8813 | -0.0098 |
| 9 | 0.1774 | 0.0110* |
| 10 | 0.2000 | 0.0080* |
| 11 | 0.9358 | -0.0134 |
| 12 | 0.3655 | 0.0037* |
| 13 | 0.9437 | -0.0129 |
| 14 | 0.4058 | 0.0025* |
| 15 | 0.1336 | 0.0186* |
| 16 | 0.3976 | 0.0020 |
| 17 | 0.7273 | -0.0069 |
| 18 | 0.4740 | 0.0016 |
| 19 | 0.9369 | -0.0098 |

*95% CI from boostrapping data 10000 replication; upper = 0.0022 and lower = -0.0100

# CHAPTER IV

# DISCUSSION

The most important step for understanding molecular mechanisms of sex differentiation in *P. monodon* is identification and characterization of sex determination markers in *P. monodon*. The molecular markers, if obtained, can be used to verify the genetic system of sex chromosomes in *P. monodon*. Increasing of the production through monosex culture is then possible in the future. Moreover, the markers can be used to evaluate the possibility to use genetic manipulation (gynogenesis, andogenesis or crossing between neomales and normal female) methods in *P. monodon.*

DNA-based mapping approaches has been developed to the point where a genetic linkage map can be developed in any species within a relatively short period of time (Lyons and Li, 2002). The performance and production traits (growth and disease resistance) are usually controlled by multiple genes and inherited as quantitative traits (referred to quantitative trait loci, QTL). QTL can be identified through the construction of genetic linkage maps in combination of breeding and assessment of markers that are tightly linked to quantitative traits of interest. This initially requires polymorphic DNA markers (microsatellites, SNP and AFLP) and the mapping families. Unfortunately, the mapping families (e.g. three generations of a few families) are not available at present. Therefore, identification of multiple gene controlling loci through the mapping approach is currently not possible.

Bulk segregrant analysis (BSA) and ampliflied fragment length polymorphism (AFLP) were combined for isolation of sex-specific DNA markers of *P. monodon*. Genomic DNA of males and females of *P. monodon* was separately pooled to product bulked male and bulked female DNA. Three different types of AFLP products are expected; 1) identical AFLP fragments found in both males and females, 2) amplified fragment from polymorphic loci among different individuals having the same sex and 3) the AFLP-amplified fragments derived from the heterogametic sex (W/Y- or X/Y-linked loci). The third type of markers should be characterized by cloning, sequencing and designing of specific primers and tested against genomic DNA of separate individuals of male and female *P. monodon* to eliminate products of a rare polymorphism.

In a total, 5 and 1 candidate female and male-specific AFLP markers were found when screened with bulked DNA (6 – 10 bulks). The probability for identifying the correct gender was P = $1.56x10^{-2}$ and P = $9.77x10^{-4}$ for markers tested against 6 and 10 bulked DNA, respectively and assumed that a bulked genomic DNA is represented by a single individuals. The number of candidate female-specific AFLP markers outnumbered that of male indirectly implying that female *P. monodon* should have represented by the heterogametic sex system, if sex chromosome is existent in this species.

Li et al. (2003) constructed a genetic linkage map in *P. japonicus* using polymorphic AFLP markers in a pseudo testcross strategy. Fifty-six progeny (top and botttom 8 % of the body weight at 6 months) of the intermediate $F_2$ cross family were genotypes by 54 primer combination. A total of 502 polymorphic AFLP fragment were found and 359 and 138 of these segregated in 1:111 and 3:1 ratios. The markers with a 1:1 segreation ratio were combined with those previously examined (Moore et al., 1999) and 217 AFLP markers were ordered into 43 linkage groups of the paternal map and 125 AFLP markers were in 31 linkage groups of the maternal map. A female-linked AFLP marker was tightly linked to the linkage group 28 of the female map. This implies that the female of *P. japonicus* exhibits the heterogametic sex. Notably, no sex chromosome has been reported in penaeid shrimps and the authors of this publication are still not able to develop a SCAR marker derived from the sex-linked AFLP markers in that species.

Sex-relate transcripts have been isolated and characterized in *P. monodon.* These included homologues of PMO920, PMT 1700, thrombospondin (TSP), ENSANGP00000010123, and Ubiquitin X-specific protease 9 (Usp9X). PMO920 and PMT1700 were differentially expressed in females and males of *P. monodon*. Expression of TSP was specifically observed in ovaries of broodstock-sized *P. monodon* but differentially expression was found in ovaries and testes of 4-month-old *P. monodon*. Coversely, Usp9x exhibited temporal female-specific expression in juvenile *P. monodon*. ENSANGP00000010123 showed female-specific expression in both juvenile (4-month-old) and broodstock *P. monodon*. Sex-specific expression markers provided important information for further studies on mechanisms (or cascades) of differentiation of sexes in *P. monodon* at the molecular level. Nevertheless, molecular markers at the genomic DNA level are still required for identification of sexes in this species to avoid sacrifice of specimens.

Leelatanawit (2003) amplified a 450 bp fragment from genomic DNA of *P. monodon* using Zincfinger-F + Finger-R. Based on previous studies in mammalian species, X-linked zingfiger proten gene (Zfx) and Y-linked zincfinger protein gene (Zfy) usually showed fixed single nucleotide polymorphism (SNP) between males and females of a particular species. Therefore, ten males (2, 2, 2, and 4 from Satun, Trang and Phangnga and Chumphon) and females (2 individuals each from Satun, Trang, Phangnga, Chumphon and Trad) broodstock of *P. monodon* was amplified, electrophoretically analyzed, gel-eluted and separately cloned into pGEM-T Easy. Positive colonies were selected using standard method. The insert size was estimated by colony PCR. Plasmid DNA was extracted from two recombinant clones representing each insert and sequenced for both directions. Nucleotide sequences of each clone was blasted against those of the GenBank. Six of which (pZFF 1/1, pZFF 1/2, pZFF10/2, pZFM 2/1, pZFM 2/2 and pZFM 8/2) were unknown genes. Comparing of a zinc finger gene homologue indicated substitutional polymorphism within male and female shrimps. Polymorphism between genders was also found but did not fix for each gender. Therefore, sex-specific markers still could not identify in *P. monodon.*

SCAR markers derived from candidate female-specific AFLP fragments were successfully amplified genomic DNA of both male and female. Sex-related transcripts exhibiting sex-specific or sex-differential expression in ovaries and testes of *P. monodon* and *H. asinina* described by Leelatanawit (2003) and Amparyup et al. (2004) were tested against genomic DNA of male and female *P. monodon*. Positive amplification was found in TSP, XNP-1, peritrophin 1 and 2, PMX, DSI, PMO920, PMT1700, zinc finger protein, VCP2, SARIP and fertilization protein but all of them did not show sex-specificity at the genomic DNA level.

Digestion of large amplified TSP fragments resulted in polymorphic PCR-RFLP paterns but the resulting patterns were not related to sex-specificity. The remaining amplified products from other genes were analyzed by SSCP. Almost all of the amplified fragment except PMX and SARIP showed polymorphism between screened *P. monodon* individuals.

AFLP is a popular approach to identify DNA markers related phynotypic characters and for genomic mapping in agricultural species particularly for which microsatellites are unavailable. For penaeid shrimp, AFLP have been used to construct the genetic linkage

maps in several comercially important marine species, including the eastern oyster (Yu and Guo,2002,), the Pacific oyster (Li and Guo,2004), the Kumura shrimp (Moore et al., 1999.Li et al,2001) and the giant tiger shrimp (Wilson et al., 2002). It is a powerful technique for identification of sex-specific markers where the species of interest possesses sex heterochromatic systems.

The first genetic linkage map of *P. monodon* was reported based on an AFLP approach (Wilson et al,2002). Three reference families of Australian *P. monodon* were genotyped by 23 primer combinations. A total of 673 polymorphic AFLP markers that conformed expected Mendelian segregation ratios were used to construct separate male and female linkage maps for each family. The linkage maps has 20 linkage groups covering a total genetic distance of 1412 cM. Sex-linked AFLP markers on the map-based approach were not found in their studies.

Li et al. (2002) collected 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*.

Isolation and characterization of sex-diagnostic markers in *M. rosenbergii* are currently carried out by two different research groups in Thailand. Mekdang (unpublished data) identified 6 AFLP primer combinations provided sex-specific bands in male and female *M. rosenbergii.* These 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.

Preechaphool (2004) analyzed pooled DNA of small orange claw (SOC, *N* = 10) and blue-claw (BC, *N* = 5) males and females (*N* = 10) using 64 AFLP primer combinations and found 90 and 42 AFLP markers in male and female *M. rosenbergii*, respectively. Additional sample set of SOC (*N* = 5), OC (*N* = 15) and BC (*N* = 10) males and female (*N* = 20) *M. rosenbergii* originating from different geographic locations with that of the first sample set were reanalyzed by 46 informative primers previously used. Morphotype-specific AFLP markers were not found in the study. In a total, 5 candidate male-specific and 4 candidate female-specific AFLP markers were finally identified. These markers were cloned and characterised. A PCR-based method for sex determination of *M. rosenbergii* was developed but sex-specificity of AFLP-derived markers was not observed.

Development of sex identification markers was also carried out in *H. asinina* using AFLP analysis. A total of 7 candidate female-specific and 7 candidate male-specific AFLP markers were found from screening 224 primer combinations with 4 bulked DNA of male and female *H. asinina*. As described previously, SCAR markers developed from candidate sex-specific AFLP fragments did not reveal their initial specific nature. Further characterization by SSCP analysis did not provide fixed polymorphism between genders (P. Amparyup, personal communication).

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. Sequeence-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).

Gonad development characteristics and sex ratio of triploid shrimp (*Penaeus chinensis*) were recently reported. The development of gonad in triploid *P. chinensis* is impaired especially in females. Interestingly, triploidy affected the sex ration in *P. chinensis*. The female to males ration in triploids was approximately 4:1 (Li et al., 2003).

In the parallel of this study, large numbers of cDNA (e.g. from RT-PCR, EST and subtraction approaches) were isolated and characterized. The coding sequences are regarded as the type I markers which are more efficient than type II (non-coding sequences) for mapping analysis. Polymorphic genes encoding interest cDNAs can be mapped to the genetic linkage map. Several cDNAs were examined against the genomic DNA of different individuals of *P. monodon*. Polymorphic markers examined by SSCP analysis indicated the possibility to identify SNP markers from functionally important genes in *P. monodon*. Correlation between expression level and SNP patterns (genotypes) can then examined and be applied to selective breeding programmes of *P. monodon* based on gene selection.

Tong et al. (2002) developed type I markers for genome mapping and other genetic studies of *P. monodon*. Primers were designed from ESTs established from the cephalothorax of *P. monodon*. Thirty-four primer pairs successfully amplified PCR products from genomic DNA of *P. monodon*. SSCP analysis indicated that approximately 30% of the EST tested were polymorphic in the tested individuals and segregation of

EST-derived markers followed Mendelian inheritance following the test against 2 mapping families. Some ESTs were also cross-species amplified from other species (*P. chinensis*, *P. japonicus* and *P. vannamei*) allowing the possibility to be used for comparative mapping between related species.

Although genetic diversity and population differentiation of *P. monodon* in Thai waters have been studies using rDNA (Klinbunga et al., 1998), mitochondrial DNA (Klinbunga et al., 1999 and 2001), RAPD (Tassanakajon et al., 1997 and 1998) and microsatellites (Supungul et al., 2000), there have been no publication concerning coding nuclear DNA markers in this species. Four polymorphic genes (PMO920, PMT1700, ZF and DSI) were then selected to determine whether their alleleic or genotypic frequencies were different among geographic samples of *P. monodon*.

Tong et al. (2002) used pirmer pairs developed from EST of *P. monodon* to amplify genomic DNA of *P. japonicus* originating from Australia and the South China Sea using a polymorphic CU89 marker. The primer pair is successfully used for amplification of *P. monodon*, *P. chinensis*, *P. japonicus* and *P. vannamei* but the amplification product of each species was different in size. This marker showed 2 distinct genotypes in investigated *P. monodon* individuals from Australian and the Gulf of Thailand and each of which predominated in each of the population.

The number of alleles and heterozygosity were relatively high and did not revealed significant reduction of genetic diversity in any geographic samples of *P. monodon*. DSI was the most polymorphic locus and could not be scored as the co-dominant marker. A band sharing approach was then applied. Among the remaining genes, ZFP exhibited greater polymorphic level than did PMO920 and PMT1700, respectively. Allele distribution patterns indicated that major alleles of each locus distributed across geographic samples implying low degree of population differentiation in this species.

Ten of possible 15 tests showed Hardy-Weinberg expectation across all loci and geographic sample ($P > 0.05$). Trang and Trad samples were significantly deviated from Hardy-Weinberg expectations at PMO920 due to homozygote excess ($P < 0.05$) whereas Satun and Phangnga samples were significantly deviated from this expectation owing to heterozygote excess at ZFP All geogrphic samples conformed Hardy-Weinberg expectations at PMT 1700.

Supungul et al (2000) examined genetic diversity of *P. monodon* in Thailand by microsatellites (*CUPmo1*, *CUPmo18*, *Di25*, *CSCUPmo1* and *CSCUPmo2*) using the same sample set as in this study. In 19 of 25 possible tests, significant deviation from the Hardy-Weinberg expectation was observed following correction for multiple tests using the sequential Bonferroni procedure (P < 0.001). All of these exhibited an excess of homozygotes. et al., 1998). However, Mendelian segregation was confirmed at all five loci using pedigree samples (P > 0.05, data not shown). The results showed the absence of nonamplifying (null) alleles at these loci.

In addition, three microsatellite loci (*Di27, CSCUPmol,* and *CSCUPmo2*) did not reveal any significant genetic heterogeneity in all possible comparisons after the probability level was adjusted by the sequential Bonferroni procedure (Rice, 1989). In contrast, *CUPmo18* and *Di25* indicated that the Andaman Sea and Trad *P. monodon* were genetically different) as were Trad and Chumphon (*P* < .001), whereas Andaman Sea and Chumphon *P. monodon* were genetically similar. Low degrees of population differentiation were observed in this species. A high level of gene flow during planktonic larval stages of *P. monodon* may be responsible for this limited differentiation. Notably, the present population differentiation of *P. monodon* may be underestimated as it results from both unintentional transplantation and restocking programs of this species in Thailand. On the basis of the present study, five *P. monodon* samples could be allocated to three different populations: A (the Andaman Sea), B (Chumphon), and C (Trad).

In the present study, only PMO920 and PMT1700 showed genotypic disequilibrium in the Satun sample (P < 0.0457). Nevertheless, this was not significant after further correction using the sequential Bonferroni method (P < 0.017). Results indicated that genotypes of PMO920, PMT1700 and ZFP did not associate non-randomly.

The average genetic distance between pairs of geographic sample was quite low suggesting the low degree of population differentiation in this species. Nevertheless, genetic relations inferred from the neighbor-joining tree showed different allocation between the Andaman and the Gulf of Thailand samples of *P. monodon.*

Genetic heterogeneity analysis between paired samples was tested using the exact test and $F_{ST}$ statistics. Almost all of the tests were not significant (P > 0.05). Only samples from Satun and Phannga and Satun and Trad at the ZFP locus were significant.

Seven fragments of DSI exhibited genetic heterogeneity across overall samples of *P. monodon*.

Klinbunga et al. (2001) examined genetic diversity of the giant tiger shrimp (*Penaeus monodon*) collected from 5 areas, Chumphon and Trad (Gulf of Thailand), and Phangnga, Satun, and Trang (Andaman Sea), was examined by randomly amplified polymorphic DNA (RAPD) and mitochondrial DNA (16S ribosomal DNA and an intergenic COI-COII) polymorphism. A total of 53 polymorphic fragments from UBC299, UBC273, and UBC268 were consistently scored across all samples. From the respective primers 26, 32, and 30 genotypes were generated. A 260-bp RAPD fragment generated by the primer UBC268 was specifically observed in 95.8% of Trad *P. monodon,* suggesting that this RAPD could be used as a marker for comparing phenotypic performance of *P. monodon* from Trad and other geographic samples. In addition, 37 mtDNA composite haplotypes were observed from restriction analysis of the same *P. monodon* samples. High haplotype diversity (0.855) and nucleotide diversity (3.328%) of Thai *P. monodon* were observed. Strong population differentiation of *P. monodon* between the Andaman Sea and Gulf of Thailand was clearly illustrated by both techniques ($P < 0.0001$).

Molecular population genetic techniques provide necessary information required for elevating culture and management efficiency of *P. monodon*. Molecular markers and the basic genetic information found in *P. monodon* are applicable for several disciplines including selection of appropriate broodstock for domestication of wild *P. monodon*, comparison on the performance of economical important traits between different stocks, genetic improvement through selective breeding programs and enhancement of natural *P. monodon* stocks. Nevertheless, general considerations for genetic management of *P. monodon* should emphasize conservation of its genetic diversity as much as possible. An overexploitation of wild *P. monodon* broodstock for the industry leads to immediate requirement on domestication and subsequently, selective breeding of local *P. monodon* stocks in Thailand.

The basic knowledge on genetic diversity and population differentiation of *P. monodon* not only yields critical information on historical and evolutionary aspects of *P. monodon* in Thailand but also the ability to construct effective breeding programs and restocking projects in this species.

Currently, genetic improvement of aquaculture species based on the genetic linkage and QTL mapping are not advanced compared to other production species (e.g. tomato, soybean, cattle and pig). Improvement of *P. monodon* broodstock has been carried out using traditional selective breeding techniques. DNA marker technology has yet to significantly affect the *P. monodon* industry but one of the possible applications of genetic markers to selective breeding programmes of *P. monodon* is to identify marker loci which are associated with loci that control economical important traits. They could be used as marker-assisted selection (MAS) leading to convertibility of polygenic variation into defined Mendelianly quantifiable entities. Various molecular approaches such as amplified fragment length polymorphism (AFLP), type I and type II microsatellite and single nucleotide polymorphism (SNP) markers can be used to construct the genetic linkage maps for identifying MAS in *P. monodon*. Upon the avalability of mapping families of Thai *P. monodon*, polymorphic DNA markers developed in this study will be applied for construction of the genetic linkage map of this economically important species.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

# CHAPTER V

# CONCLUSIONS

1. Five and one candidate female- and male-specific AFLP markers were identified from screening 256 primer combinations against 6 or 10 bulked genomic DNA of *P. monodon* (P = 0.0156 and P = 0.0010, respectively)

2. Four SCAR markers derived from FE10M9520, FE10M10725.1, FE10M10725.2 and FE14M16340) generated the expected product in both male and female *P. monodon* while FE15M14400 and FE16M8350 provided non-specific amplification results.

3. Further characterization of the successfully amplified product by SSCP analysis revealed monomorphism patterns for FE10M9520 and FE10M10725.2). However, the polymorphic markers (FE10M10725.1 and FE14M16340) were not sex-linked in *P. monodon*.

4. Thirty-four sex-related genes previously isolated from *P. monodon*, *M. rosenbergii* and *H. asinina* were tested. Polymorphic SSCP markers that are not linked to sexes of *P. monodon* ($TSP_{462F+288R}$, XNP-1, peritrophin, DSI, ZFP, PMO920 and PMT1700 from *P. monodon* and VCP2 from *H. asinina*) were found.

5. Four polymorphic markers (PMO920, PMT1700, ZFP and DSI) were further used for population genetic studies of natural *P. monodon* in Thai waters. Relatively high genetic diversity in Thai *P. monodon* were observed at these loci. The number of allele per respective locus was 6, 5, 12 and 19 respectively. The observed heterozygosity was 0.3043 – 0.5128, 0.3462 – 0.4643 and 0.5000 – 0.8108 for PMO920, PMT1700 and ZFP, respectively.

6. The average genetic distance between geographic samples was 0.0031 (Satun – Trang) – 0.0178 (Trang – Chumphon). Genetic distances between samples from the different coastal areas were 0.0079 (Phangnga – Trad) 0.0178 (Chumphon – Trang) whereas the distance within the Gulf of Thailand and the Andaman Samples were 0.0077 and 0.0031 – 0.0135, respectively.

7. The neighbor-joining tree constructed from the average genetic distance of 4 scnDNA loci allocated investigated samples to 2 groups according to the coastal regions (the Gulf of Thailand and the Andaman Sea populations).

8. Significant genetic differentiation was found between Satun – Trad ($P < 0.05$) and Satun-Phangnga ($P < 0.05$) at the ZFP locus. In addition, seven (6, 7, 9, 10, 12, 14 and 15) of nineteen alleles of DSI revealed significant allele distribution frequencies across overall investigated samples of Thai *P. monodon* ($P < 0.05$). The information implied low but significant intraspecific genetic heterogeneity of *P. monodon* in Thailand.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

# REFERENCES

Aasen, E., and Medrano, J.F. 1990. Amplification of the ZFY and ZFX genes for sex identification in humans, cattle, sheep and goats. *Biotechnol.* 8 : 1279-1281.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. *J mol Biol.* 215 : 403-410.

Amparyup, P., Klinbunga, S., Preechaphol, R., Tassanakajon, A., Hirono, I., Aoki, T. and Jarayabhand, P. 2004. Expressed sequence tag (EST) analysis of ovaries and testes from the tropical abalone (*Haliotis asinina*). *Mar Biotechnol* (in press).

Baily-Brook, J.H., and Moss, S.M. 1992. Penaeid taxonomy, biology and zoogeography. In A.W. Fast and L.J. Lester (eds.), *Marine shrimp culture Principles and practices*, Amsterdam : Elsrvier Science Publishers.

Barzotti, R., Pelliccia, F. and Rocchi, A. 2000. Sex chromosome differentiation revealed by genomic *in-situ* hybridization. *Chromosome Research* 8 : 459-464.

Beatty, R. 1964. Chromosome deviations and sex in vertebrates. *In Intersexuality in vertebrates including Man* ( Armstrong, C.N. & Marshall, A.J. eds) 17-144.

Benzie, J.A., Ballment, E., Forbes, A.T., Demetriades, N.T., Sugama, K., Haryanti, Moria, S. 2002. Mitochondrial DNA variation in Indo-Pacific populations of the giant tiger prawn, *Penaeus monodon*. *Mol Ecol*. 11 : 2553-2569.

Carlson, J.E., Tulsieram, L.K. Glaubitz, J.C., Luk, V.W.K., Kauffeldt, C., Rutledge, R., 1991. Segregation of random amplified DNA markers in F1 progeny of conifers. *Theor. Appl. Genet.* 83 : 194-200.

Crow, J. F., Kimura, M. 1965. Evolution and sexual and asexual populatios. *Am Natur.* 99 : 439-450.

Department of Fisheries. 1999. *Fisheries Statistics of Thailand 1996*. Department of Fisheries, Ministry of Agriculture and Cooperative.

Daud, S.K. 1995. Population genetics of *Penaceus monodon Fabricius* and *Penaeus merguiensis de Man* in Malaysia. Ph.D. thesis, University of Stirling, Scotland. Ph.D. thesis, University of Stirling, Scotland.

Goldstein, L.S.B., Hardy, W. and Lindsley, D.L. 1982. Structural genes on the Ychromosome of *Drosophila melanogaster. Proc .Natl. Acad. Sci.*USA. 79 : 7405-7409.

Griffiths, R. and Tiwari, B. 1993 The isolation of molecular genetic markers for the identification of sex. *Proc.Natl.Acad.Sci* USA. 90 : 8324-8326.

Griffiths, R., Orr, KJ., Adam, A. and Barber, I.2000. DNA sex identification in the three-spined stickleback. *J of Fish Biol*. 57 : 1331-1334.

Griffiths, R., Orr, K. 1999. The use of amplified fragment length polymorphism (AFLP) in the isolation of sex-specific markers. *Mol Ecol*. 8 : 671-674.

Guo, S.W. and Thompson, E.A. 1992. Performing the extract test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 48 : 361-372.

Hawthorne, D.J. 2001. AFLP-based genetic linkage map of the Colorado potato beetle Leptinotarsa decemlineata: sex chromosomes and a pyrethroid-resistance candidate gene. *Genetics*. 158 : 695-700.

Justo, C.C., Murofushi, M., Aida, K., and Hanyu, I. 1991. Karyological studies on the freshwater prawn *Macrobrachium rosenbergii*. *Aquaculture*.97 : 327-334.

Kirby, L.T. 1992. *DNA Fingerprinting : An introduction* New York : W.H. Freeman and Company.

Klinbunga, S., Penman, D.J., McAndrew, B.J., Tassanakajon, A., and Jarayabhand, P. 1998. Genetic variation, population differentiation and gene flow of the giant tiger shrimp (*Penaeus monodon*) inferred from mtDNA-RFLP data.In: A*dvances in shrimp biotechnology,* Flegel, T.W. (ed.). National Center for Genetic Engineering and Biotechnology, 50-59.

Klinbunga, S., Penman, D.J., McAndrew, B.J., and Tassanakajon, A. 1999. Mitochondrial DNA diversity in three populations of the giant tiger shrimp, *Penaeus monodon*. *Mar Biotechnol* 1 : 113-121.

Klinbunga, S., Siludjai, D., Wudthijinda, W., Tassanakajon, A., Jarayabhand, P., and Menasaveta, P. 2001. Genetic Heterogeneity of the Giant Tiger Shimp (*Penaeus monodon*) in Thailand Revealed by RAPD and Mitochondrial DNA RFLP Analyses. *Mar Biotechnol* 3 : 428-438.

Klinbunga, S., Khamnamtong, N., Tassanakajon, A., Puanglarp, N., Jarayabhand, P. and Yoosukh, W. 2003. Molecular genetic tools for three commercial cultured oysters (*Crassostrea belcheri*, *C. iredalei* and *Saccostrea cucullata*) in Thailand. *Mar. Biotechnol.* 5 : 27-36.

Klinbunga, S., Pripue, P., Khamnamtong, N., Tassanakajon, A., Jarayabhand, P.and Menasveta, P. 2003 Genetic diversity and molecular markers of the tropical abalone (*Haliotis asinina*) in Thailand. *Mar. Biotechnol.* (in press).

Korpelainen, H. 1990. Sex ratios and conditions required for environmental sex determination in animals. *Biol Rev Camb Philos Soc*. 65 : 147-184.

Klinkhardt, M. B. and Buuk, B. 1990. Karyological studies in several species of fresh-water fishes from brackish coastal waters of the south-western Baltic. 3. The 3-Spined Stickleback *(Gasterosteusa aculeatus* (Linnaeus, 1758)) and the 9-Spined Stickleback *(Pungitius pungitius* (Linnaeus,1758)). Zoologisclier Anzeiger. 225 : 341-352.

Kovacs, B., Egedi, S., Bartfai, R., Orban, L. 2000 Male-specific DNA markers from African catfish (*Clarias gariepinus*). *Genetica*. 110 : 267-276.

Leelatanawit, R., Klinbunga, S., Tassanakajon, A., Jarayabhand, P. and Menasveta, P. 2003. Cloning and characterizatuion of a sex – linked Zinc Finger X/Y homologue in the giant tiger shrimp (*Penaeus monodon*). 29$^{th}$ Congress on Science and Technoloy of Thailand.

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

Lewis, P.O. and Snow, A.A. 1992. Deterministic paternity exclusion using RAPD markers. *Mol. Ecol.* 1 : 155-160.

Li, Y., Byrne, K., Miggiano, E., Whan, V., Moore, S., Keys, S., Crocos, P., Preston, N., and Lehnert, S. 2003. Genetic mapping of the kuruma prawn *Penaeus japonicus* using AFLP markers. *Aquaculture*. 219 : 143-219.

Li, Y., Hill, J.A., Yue, G.H., Chen, F., and Orban, L. 2002. Extensive search does not identify genomic sex markers in *Tetradon nigroviridis*. *J. of Fish Biol*. 61 : 1314-1317.

Li, F., Xiang, J., Zhang, X., Zhou, L., Zhang, C., and Wu, C. 2003. Gonad development characteristics and sex ratio in triploid Chinese shrimp (*Fenneropenaeus chinensis*). *Mar Biotechnol*. 5 : 528-35.

Li, L., and Guo, X. 2004. AFLP-based genetic linkage maps of the pacific oyster *Crassostrea gigas* Thunberg. *Mar Biotechnol* (NY). 6 : 26-36.

Luoh,S-W. and Page, D.C. 1994.The Structure of the Zfx Gene on the Mouse X chromosome. *Genomics* 19 : 310-319.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Malecha, S.R., Nevin, P.A., Ha, P., Barck, L.E., La Madrid-Rose, Y., Masuno, S., and Hedgecock, D. 1992. Sex-ratios and sex determination in progeny from crosses of surgically sex-reversed freshwater prawns, *Macrobrachium rosenbergii.* Aquaculture. 105 : 201-218.

Maxam, A.M. and Gilbert, W. 1977. A new method for sequencing DNA. *Proc. Natl. Acad Sci*. 74 : 560.

Marchand, O., Govoroun, M., D'Cotta, H., McMeel, O., Lareyre, J., Bernot, A., Laudet, V., and Guiguen, Y. 2000. DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. *Biochimica et Biophysica Acta* 1493 : 180-187.

Mullis, K, B. and Faloona, F. 1987. *Meth. Enzymol*. 155 : 335.

Moore, S.S., Whan, V., Davis, G.P., Byrne, K., Hetzel, D.J.S., Preston, N., 1999. The development and application of genetic markers for the Kuruma prawn *Penaeus japonicus*. *Aquaculture* 173 : 19-32.

Nei, M., and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Nat Acad Sci USA* 76 : 5269-5273.

Nei, M. 1987. *Molecular evolutionary genetics*. Columbia University Press, New York

O'Neill, M.J., and O'Neill, R.J. 1999 Whatever happened to SRY? *Cell Mol Life Sci*. 56 : 883-893.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci U S A*. 868 : 2766-2770.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Pauli, D., Oliver, B., and Mahowald, A.P. 1995. Identification of regions interacting with ovoD mutations: potential new genes involved in germline sex determination or differentiation in *Drosophila melanogaster*. *Genetics*. 1392 : 713-732.

Preechaphol, R. 2004. Sex determination markers and gene expression in androgenic gland of different male morphotypes of the giant freshwater prawn *Macrobrachium rosenbergii.* M.sc. Thesis.Chulalongkorn university.

Raymond, C.S., Murphy, M.W., O'Sullivan, M.G., Bardwell, V.J., and Zarkower, D. 2000. Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev* 14 : 2587-2595.

Raymond, M. and Rousset, F. 1995. Genepop: population genetic software for exact test and ecumenism. *J. Hered* 86 : 248-250.

Rice, W.R. 1989. Analyzing tables of statistical tests. *Evolution*. 41 : 223-235.

Riedy, M.F., Hamilton, W.J. and Aquadro, C.F. 1992. Excess of non-parental bands in offspring from known pedigrees assayed using RAPD PCR. *Nucl. Acids Res.* 20 : 918.

Rosenberry, B. 2001. World shrimp farming 2001. Sandiego : Shrimp News International.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Mollecular Cloning : A Laboratory manual*, 2 nd ed. New York: Cold Spring Habor Laboratory Press.

Sanger, F., Nicklen, S. and Coulson, A.R. 1977. DNA sequencing with chainterminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74 : 5463-5467.

Schmoll, F. and Schellander, K. 1996 Sex determination of porcine embryos by restriction fragment lenght polymorphism analysis of ZFX/ZFY loci .*Theriogenology.* 45 : 255 Proceedings Annual Conference of the Internatinal Embryo Transfer Society.

Scott, M.P., Haymes, K.M. and Williams, S.M. 1993. Parentage analysis using RAPD PCR. *Nucl. Acids. Res.* 20 : 5493

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4 : 406-425.

Stacks, B., and Witte, M.M. 1996 Sex determination of dried blood stains using the polymerase chain reaction (PCR) with homologous X-Y primers of the zinc finger protein gene. *J Forensic Sci*. 41 : 287-290.

Supungul, P., Sootanan, P., Klinbunga, S., Kamonrat, W., Jarayabhand, P., and Tassanakajon, A. 2000. Microsatellite polymorphism and the population structure of the black tiger shrimp (*Penaeus monodon*) in Thailand. *Mar Biotechnol*

Tassanakajon, A., Pongsomboon, S., Rimphanitchayakit, V., Jarayabhand, P. and Boonsaeng, V. 1997. Random amplified polymorphic DNA (RAPD) markers for determination of genetic variation in wild populations of the black tiger prawn (*Penaeus monodon*) in Thailand. *Mol. Mari. Biol. Biotechnol.* 6 : 110-115.

Tassanakajon, A., Pongsomboon, S., Jarayabhand, P., Klinbunga, S. an Boonsaeng, V. 1998a. Genetic structure in wild populations of black tiger shrimp (*Penaeus monodon*) using randomly amplified polymorphic DNA analysis. *J. Mar. Biotechnol.* 6 : 249-254.

Tonga, J., Lehnertc, b, S. A., Byrnec, K., Kwana, H. S. and Chua, K. H. 2002. Development of polymorphic EST markers in *Penaeus monodon*: applications in penaeid genetics , *Aquaculture* .208 : 69-79.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res*. 23 : 4407-4414.

Ward R.D. and Grewe P.M. 1994. Appraisal of molecular genetic techniques in fisheries. *Rev Fish Biol Fisheries* 4 : 300-325

Weir, B.S. and Cockerham, C.C. 1984. Estimation *F*-statistics for the analysis of population structure. *Evolution* 38 : 1358-1370.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* 18 : 7213-7218.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18 : 6531-6535.

Wilson, K. et al. 2002. Genetic mapping of the black tiger shrimp *Penaeus monodon* with amplified fragment length polymorphism. *Aquaculture*. 204 : 297-309.

Yu, Z., Guo, X. 2003 Genetic linkage map of the eastern oyster *Crassostrea virginica* Gmelin. *Biol Bull*. 204 : 327-38.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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

| | | |
|---|---|---|
| Glacial acetic acid | 200 | ml |
| Ultrapure or deionized water | 1800 | ml |

**5. Staining solution, 1.5 liters**

| | | |
|---|---|---|
| $AgNO_3$ | 1.5 | g |
| 37% Formaldehyde | 2.25 | ml |

**6. Developing solution, 3 liters**

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

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

## APPENDIX B

**A summary of genotypes across 5 geographic samples of *P. monodon* at 4 nuclear DNA loci (PMO920, PMT1700,ZFP and DSI)**

| Specimens | Locus | | | |
|---|---|---|---|---|
| | PMO920[a] | PMT1700[a] | ZFP [a] | DSI [b] |
| SAT 1 | 0404 | 0102 | 0102 | 0001000100000000000 |
| SAT 2 | 0204 | 0101 | 0101 | 0001000100100101000 |
| SAT 4 | 0202 | 0102 | 0102 | 0001000110000000000 |
| SAT 3 | 0204 | 0102 | 0102 | 0000100000010101001 |
| SAT 8 | 0202 | 0101 | 0102 | 0001000110000000000 |
| SAT 6 | 0202 | 0101 | 0102 | 0001000100000000000 |
| SAT 9 | 0102 | 0102 | 0102 | 0001000100000000000 |
| SAT 10 | 0203 | 0101 | 0102 | 0001000100010101000 |
| SAT 11 | 0202 | 0102 | 0102 | 0001000100000000000 |
| SAT 19 | 0104 | 0101 | 0102 | 0001000100011001000 |
| SAT 20 | 0101 | 0101 | 0102 | 1000000010001000100 |
| SAT 14 | 0404 | 0203 | 0102 | 0001001000001001000 |
| SAT 21 | 0202 | 0101 | 0106 | 0001000100000000000 |
| SAT 22 | 0202 | 0101 | 0101 | 0001000110010000000 |
| SAT 15 | 0202 | 0102 | 0207 | 0001000110001000000 |
| SAT 23 | 0101 | 0101 | 0101 | 0001000100100001000 |
| SAT 24 | 0102 | 0202 | 0203 | 0001100100100000000 |
| SAT 17 | 0204 | 0102 | 0102 | 0001000100100001000 |
| SAT 25 | 0102 | 0101 | 0203 | 0001101000000000000 |
| SAT 27 | 0103 | 0102 | 0203 | 0000000100110000000 |
| SAT 26 | 0202 | 0101 | 0102 | 0000100010100001000 |
| SAT 28 | 0102 | 0102 | 0110 | 0001000100000000000 |
| SAT 33 | 0204 | 0102 | 0102 | 0001000100100000000 |
| SAT 31 | 0102 | 0102 | 0102 | 0001000100101000000 |
| SAT 41 | 0304 | 0102 | 0102 | 0000100000001000000 |
| SAT 5 | 0202 | 0101 | 0110 | 0001000100010001000 |
| SAT 7 | 0204 | 0101 | 0102 | 0001000100000100010 |

| Specimens | Locus | | | |
|---|---|---|---|---|
| | PMO920[a] | PMT1700[a] | ZFP [a] | DSI [b] |
| SAT 42 | 0405 | 0102 | 0102 | 00000000000010001000 |
| SAT 45 | 0202 | 0101 | 0000 | 00011000000000000000 |
| SAT 47 | 0204 | 0202 | 0101 | 00010001000000000000 |
| SAT 48 | 0202 | 0101 | 0102 | 00010011000000000000 |
| SAT 49 | 0202 | 0101 | 0000 | 00010001100000000000 |
| SAT 50 | 0202 | 0101 | 0101 | 00010011000000000000 |
| SAT 12 | 0204 | 0101 | 0109 | - |
| SAT 16 | 0204 | 0102 | 0202 | 00000000000010001000 |
| SAT 18 | 0204 | 0101 | 0111 | 00010001000000000000 |
| SAT 30 | 0202 | 0101 | 0102 | 00010000000010001000 |
| SAT 38 | 0202 | 0101 | 0202 | 00000000000010001000 |
| SAT 39 | 0203 | 0101 | 0102 | 00000010000010001000 |

[a]Genotypes at PMO920, PMT1700 and ZFP were scored as co-dominant markers. [b]Genotypes at DSI were scored as dominant markers (0 and 1 = absence and presence of a particilar band, respectively).

| Specimens | Locus | | | |
|---|---|---|---|---|
| | **PMO920[a]** | **PMT1700[a]** | **ZFP [a]** | **DSI [b]** |
| TNG 1 | 0102 | 0102 | 0108 | 0001000100000000000 |
| TNG 2 | 0202 | 0101 | 0108 | 0000000010010101000 |
| TNG 11 | 0202 | 0101 | 0102 | 0001000110000000000 |
| TNG 3 | 0202 | 0102 | 0101 | 0001000100000000000 |
| TNG 4 | 0202 | 0101 | 0102 | 0001000100000000000 |
| TNG 14 | 0202 | 0101 | 0202 | 0001000010000000000 |
| TNG 8 | 0202 | 0102 | 0101 | 0001000100010101000 |
| TNG 26 | 0205 | 0101 | 0102 | 0001000100000000000 |
| TNG 16 | 0202 | 0101 | 0202 | 0001000110000000000 |
| TNG 18 | 0202 | 0101 | 0102 | 0001000110000000000 |
| TNG 17 | 0204 | 0206 | 0102 | 0001000010001001000 |
| TNG 19 | 0204 | 0101 | 0102 | 0010100100110001000 |
| TNG 21 | 0202 | 0101 | 0106 | 0000010010001001010 |
| TNG 27 | 0204 | 0101 | 0101 | 0001000100000000000 |
| TNG 24 | 0101 | 0101 | 0102 | 0011000000110000000 |
| TNG 25 | 0101 | 0101 | 0102 | 0001000010001000100 |
| TNG 27 | 0104 | 0101 | 0101 | 0001000100000000000 |
| TNG 32 | 0303 | 0102 | 0202 | 0001000100000000000 |
| TNG 41 | 0101 | 0103 | 0205 | 0000100000100000000 |
| TNG 29 | 0202 | 0102 | 0101 | 0001000100110000000 |
| TNG 12 | 0404 | 0101 | 0102 | 0001000100000000000 |
| TNG 28 | 0204 | 0202 | 0102 | 0001000100100001000 |
| TNG 30 | 0202 | 0102 | 1112 | 0000101000000000000 |

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

| Specimens | Locus | | | |
|---|---|---|---|---|
| | **PMO920**[a] | **PMT1700**[a] | **ZFP**[a] | **DSI**[b] |
| PHA 2 | 0204 | 0101 | 0102 | 0000000010010111010 |
| PHA 3 | 0204 | 0101 | 0106 | 0000000010010101000 |
| PHA 1 | 0104 | 0000 | 0106 | 0001000100010101000 |
| PHA 4 | 0202 | 0102 | 0102 | 0001000010000000000 |
| PHA 5 | 0202 | 0102 | 0102 | 0001000100000010000 |
| PHA 14 | 0202 | 0101 | 0101 | 0000000000010101001 |
| PHA 10 | 0204 | 0102 | 0102 | 0011000100001000000 |
| PHA 11 | 0202 | 0102 | 0102 | 0000101000000000000 |
| PHA 6 | 0202 | 0101 | 0106 | 0001001100100000000 |
| PHA 12 | 0202 | 0206 | 0102 | 0100001000001000000 |
| PHA 13 | 0202 | 0101 | 0108 | 0001001001000000000 |
| PHA 7 | 0202 | 0000 | 0101 | 0001000100000001010 |
| PHA 25 | 0202 | 0102 | 0101 | 0001000100000100010 |
| PHA 24 | 0102 | 0101 | 0101 | 0001010110000000100 |
| PHA 17 | 0405 | 0101 | 0102 | 0001000110000000000 |
| PHA 26 | 0203 | 0102 | 0101 | 0001000010001000100 |
| PHA 28 | 0102 | 0101 | 0101 | 0000011000110000100 |
| PHA 29 | 0103 | 0101 | 0102 | 0001000110000000000 |
| PHA 8 | 0102 | 0101 | 0102 | 0001000100101000000 |
| PHA 16 | 0203 | 0101 | 0102 | 0001101100000000000 |
| PHA 18 | 0202 | 0101 | 0101 | 0101000100000000000 |
| PHA 33 | 0304 | 0000 | 0204 | 0001000100000001000 |
| PHA 34 | 0103 | 0101 | 0101 | 0001000100000000000 |
| PHA 30 | 0202 | 0102 | 0101 | 0011000100000000000 |
| PHA 35 | 0202 | 0101 | 0102 | 0001000100100100000 |
| PHA 15 | 0202 | 0102 | 0102 | 0001000100100100000 |
| PHA 36 | 0203 | 0101 | 0102 | 0001000100000000000 |
| PHA 37 | 0303 | 0101 | 0102 | 0001001000100000010 |
| PHA 39 | 0303 | 0101 | 0102 | 0001001100000000000 |

| Specimens | Locus | | | |
|---|---|---|---|---|
| | PMO920[a] | PMT1700[a] | ZFP [a] | DSI [b] |
| CHM 2 | 0204 | 0101 | 0203 | 0001000100000000000 |
| CHM 9 | 0405 | 0101 | 0101 | 0000100100100000000 |
| CHM 1 | 0304 | 0202 | 0102 | 0001000100001000100 |
| CHM 10 | 0304 | 0202 | 0101 | 0101000100000000000 |
| CHM 18 | 0405 | 0202 | 0101 | 0000000010010001001 |
| CHM 4 | 0202 | 0102 | 0101 | 0001000100000000000 |
| CHM 20 | 0202 | 0102 | 0106 | 0001000100000000000 |
| CHM 21 | 0202 | 0101 | 0101 | 0011001000100000000 |
| CHM 6 | 0104 | 0101 | 0101 | 0001000011001000000 |
| CHM 7 | 0206 | 0101 | 0202 | 0001000100000000000 |
| CHM 22 | 0202 | 0202 | 0203 | 0001000100001001000 |
| CHM 23 | 0202 | 0101 | 0110 | 0001001010000000000 |
| CHM 13 | 0202 | 0101 | 0101 | 0100001000001001000 |
| CHM 24 | 0202 | 0102 | 0102 | 0001000010000000000 |
| CHM 27 | 0202 | 0102 | 0205 | 0001000000110100000 |
| CHM 15 | 0204 | 0102 | 0102 | 0000000010000100010 |
| CHM 28 | 0404 | 0102 | 0101 | 0001000100000000000 |
| CHM 29 | 0202 | 0102 | 0102 | 0010000010001000000 |
| CHM 37 | 0202 | 0102 | 0102 | 0001000100001001100 |
| CHM 30 | 0102 | 0101 | 0101 | 0000101010000000000 |
| CHM 34 | 0102 | 0101 | 0101 | 0001000100000000000 |
| CHM 25 | 0202 | 0102 | 0102 | 0001000100110000000 |
| CHM 3 | 0204 | 0101 | 0203 | 0001000100000000000 |
| CHM 5 | 0202 | 0101 | 0101 | 0001000110000000000 |
| CHM 14 | 0202 | 0102 | 0101 | 0001000100010101000 |
| CHM 17 | 0104 | 0102 | 0102 | 0001001110000000000 |
| CHM 41 | 0204 | 0102 | 0101 | 0001001101000000000 |
| CHM 26 | 0206 | 0101 | 0102 | 0001000100100001000 |

| Specimens | Locus | | | |
|---|---|---|---|---|
| | PMO920[a] | PMT1700[a] | ZFP [a] | DSI [b] |
| TRAD 13 | 0202 | 0101 | 0101 | 0001000100000101000 |
| TRAD 14 | 0202 | 0202 | 0102 | 0001000100000000000 |
| TRAD 4 | 0202 | 0101 | 0102 | 0001000100000000000 |
| TRAD 15 | 0206 | 0102 | 0101 | 0001000100000000000 |
| TRAD 18 | 0204 | 0102 | 0102 | 0001000100000000000 |
| TRAD 6 | 0205 | 0102 | 0101 | 0000000100100001000 |
| TRAD 21 | 0204 | 0104 | 0101 | 0001001010100101000 |
| TRAD 22 | 0405 | 0101 | 0102 | 0001000100000000000 |
| TRAD 9 | 0202 | 0101 | 0102 | 0001000100000000000 |
| TRAD 23 | 0404 | 0102 | 0101 | 0001001000011001000 |
| TRAD 24 | 0202 | 0102 | 0101 | 0001000100000000000 |
| TRAD 11 | 0204 | 0102 | 0101 | 0001000100011001000 |
| TRAD 25 | 0101 | 0101 | 0101 | 0000000010000100010 |
| TRAD 28 | 0202 | 0101 | 0101 | 0001000100000000000 |
| TRAD 12 | 0303 | 0101 | 0205 | 0011000100000000000 |
| TRAD 30 | 0102 | 0101 | 0000 | 0001000010000000000 |
| TRAD 31 | 0102 | 0101 | 0101 | 0000000000111000010 |
| TRAD 32 | 0202 | 0101 | 0102 | 0000100100000000000 |
| TRAD 33 | 0202 | 0102 | 0102 | 0001100100000000000 |
| TRAD 34 | 0202 | 0102 | 0101 | 0100000100110000000 |
| TRAD 16 | 0303 | 0101 | 0102 | 0001000100010101000 |
| TRAD 17 | 0202 | 0101 | 0000 | 0001000100010101000 |
| TRAD 19 | 0202 | 0101 | 0205 | 0001000100010101000 |
| TRAD 1 | 0101 | 0102 | 0102 | 0001001100000000000 |
| TRAD 3 | 0102 | 0102 | 0102 | 0001101000100000000 |
| TRAD 5 | 0206 | 0102 | 0108 | 0001000100110001000 |
| TRAD 7 | 0404 | 0102 | 0102 | 0001010100110001000 |
| TRAD 10 | 0404 | 0101 | 0101 | 0001000100110001000 |
| TRAD 35 | 0202 | 0101 | 0101 | 0000011000001000100 |

# Biography

Miss Supaporn Thumrungtanakit was born on September 30, 1977 in Surin Province, Thailand. She graduated with the degree of Bachelor of Science (Biotechnology) from Ramkhamhaeng University in 1999. She has studied for the degree of Master of Science (Biotechnology) at the Program of Biotechnology, Chulalongkorn University since 2001.

**Publications during graduate study**

1. Klinbunga, S., Amparyup, P., **Thumrungtanakit, S.**, Tassanakajon, A., Hirono, I., Aoki, T., Jarayabhand, P. and Menasveta, P. (2004). Population genetics and species-specific markers of the tropical abalone (*Haliotis asinina*) in Thailand. ***Mar Biotechnol*** (in press).

2. **Thumrungtanakit, S.**, Klinbunga, S., Tassanakajon, A., Jarayabhand, P. and Menasveta, P. (2003). Development of sex-specific marker of the giant tiger shrimp (*Penaeus monodon*) using amplified fragment length polymorphism (AFLP) analysis. BioThailand 2003, 17-20 July 2003, Chonburi, Thailand (Poster presentation).

3. **Thumrungtanakit, S.**, Klinbunga, S., Tassakajon, A., Jarayabhand, P. and Menasveta, P. (2003). Development of sex specific marker of the giant tiger shrimp (*Penaues monodon*) using amplified fragment length polymorphism (AFLP) analysis. 29$^{th}$ Congress on Science and Technology of Thailand. 20 – 22 October 2003, Khon Kean, Thailand (Oral presentation).

4. **Thumrungtanakit, S.**, Leelatanawit, R., Klinbunga, S., Jarayabhand, P. and Menasveta, P. (2004). Single copy nuclear DNA markers for genetic diversity studies of the giant tiger shrimp *Penaeus monodon*. 30$^{th}$ Congress on Science and Technology of Thailand. 19 – 21 October 2004, Bangkok, Thailand (Oral presentation).

5. Klinbunga, S., Ponsomboon, S., Supungul, P., Khamnamtong, B., **Thumrungtanakit, S.**, Tassanakajon, A. and Menasveta, P. (2004). Genetic diversity and population structure of the giant tiger shrimp *Penaeus monodon* in Thailand. 7$^{th}$ Asian Fisheries Forum. 30 November – 4 December 2004, Penang, Malaysia (Oral presentation).