# การระบุเครื่องหมายโมเลกุลที่จำเพาะต่อเพศของกุ้งกุลาดำ *Penaeus monodon* โดยการวิเคราะห์ด้วยวิธีสับแทร็กชัน

นางสาวศรีสุภาพ พูนลาภเดชา



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547

ISBN 974-53-1017-4

# IDENTIFICATION OF SEX-SPECIFIC MARKERS FROM BLACK TIGER SHRIMP *Penaeus monodon* USING SUBTRACTION ANALYSIS

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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 974-53-1017-4

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|---|---|
| Thesis Title | Identification of sex-specific markers from black tiger shrimp *Penaeus monodon* using subtraction analysis |
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| Field of study | Biotechnology |
| Thesis Advisor | Professor Piamsak Menasveta, Ph.D. |
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ศรีสุภาพ พูนลาภเดชา : การสืบค้นเครื่องหมายโมเลกุลที่จำเพาะต่อเพศในกุ้งกุลาดำ *Penaeus monodon* โดยการวิเคราะห์ด้วยวิธีสับแทร็กชัน (IDENTIFICATION OF SEX-SPECIFIC MARKERS FROM BLACK TIGER SHRIMP *Penaeus monodon* USING SUBTRACTION ANALYSIS) อ. ที่ปรึกษา : ศ. ดร. เปี่ยมศักดิ์ เมนะเศวต, อ. ที่ปรึกษาร่วม : ดร. ณรงค์ศักดิ์ พ่วงลาภ 180 หน้า. ISBN 974-53-1017-4.

กุ้งกุลาดำ (*Penaeus monodon*) เป็นสัตว์ที่มีอัตราการเติบโตแตกต่างกันระหว่างเพศโดยเพศเมียมีอัตราการเติบโตสูงกว่าเพศผู้ ดังนั้นการเลี้ยงแบบเพศเดียวจึงมีส่วนช่วยในการพัฒนาการเพาะเลี้ยงได้ อย่างไรก็ตามยังไม่มีรายงานเกี่ยวกับกลไกการกำหนดเพศและเครื่องหมายพันธุกรรมที่ใช้ในการตรวจสอบเพศในกุ้งกุลาดำ ดังนั้นจึงได้นำวิธีจีโนมิกดีเอ็นเอสับแทร็กชันซึ่งใช้แยกความแตกต่างในระดับจีโนมิกดีเอ็นเอมาใช้ในการสืบค้นเครื่องหมายโมเลกุลที่มีความจำเพาะต่อเพศของกุ้งกุลาดำเพื่อใช้ในการพัฒนาการเพาะเลี้ยงกุ้งแบบเพศเดียว โดยผลที่ได้จากสับแทร็กชันของเพศผู้แบบ PERT พบเครื่องหมายโมเลกุลที่จำเพาะต่อเพศผู้ทั้งหมด 9 โคลน และพบ 4 โคลนในแบบ RDA จากการทำสับแทร็กชันของเพศเมียพบเครื่องหมายโมเลกุลที่จำเพาะต่อเพศเมียทั้งหมด 4 โคลนในแบบ PERT และ 4 โคลนในแบบ RDA จากนั้นคัดเลือก 1 เครื่องหมายจาก PERT (PMMSH6) และ 2 เครื่องหมายจาก RDA (PMFJ200 และ PMFJ800) มาตรวจสอบความจำเพาะต่อเพศด้วยปฏิกิริยาลูกโซ่โพลีเมอเรส พบว่าไม่มีความแตกต่างกันระหว่างเพศทั้งในระดับจีโนมิกดีเอ็นเอและอาร์เอ็นเอเมื่อตรวจสอบด้วย agarose gel electrophoresis จึงนำผลิตภัณฑ์พีซีอาร์ไปตรวจสอบ single nucleotide polymorphism (SNP) ด้วยวิธี SSCP โดยให้ผลเป็นแบบ polymorphic แต่ไม่เกี่ยวข้องกับความแตกต่างระหว่างเพศเช่นเดียวกัน ในการตรวจสอบด้วยวิธีจีโนมวอล์กกิ้ง ซึ่งได้ตัดจีโนมิกดีเอ็นเอด้วยเอนไซม์ 5 ชนิด พบว่าใน PMMSH6 และ PMFJ800 ที่ตัดด้วย *Dra*I ให้ผลเฉพาะที่จำเพาะในเพศเมีย แต่เมื่อเพิ่มตัวอย่างพบว่าความแตกต่างที่ได้เป็นลักษณะเฉพาะของกุ้งแต่ละตัวที่ไม่เกี่ยวกับเพศ ในการตรวจสอบเครื่องหมายที่ได้ด้วยวิธี Southern hybridization พบว่าเครื่องหมายจากสับแทร็กชันของเพศผู้ 2 โคลน และเพศเมีย 2 โคลน แบบ PERT (PMMSH3, PMMSH8, PMFSH26 และ PMFSH32) และ 2 โคลนในเพศผู้ (PMMJ200 และ PMMN200) และ 1 โคลนในเพศเมีย (PMFJ300) ในแบบ RDA น่าจะเป็น single copy number เพราะไม่สามารถตรวจสอบได้ทั้งสองเพศ ส่วนเครื่องหมายจากสับแทร็กชันของเพศผู้ 2 โคลน (PMMJ450 และ PMMN450) และเพศเมีย 3 โคลน (PMFJ200, PMFJ800 และ PMFN300) ในแบบ RDA พบว่าสามารถตรวจพบได้จากดีเอนเอของทั้งสองเพศซึ่งแสดงว่าไม่มีความจำเพาะต่อเพศในกุ้งกุลาดำ นอกจากนี้ได้นำ degenerated primers ของ estrogen receptor gene (ER) มาสังเคราะห์ด้วยปฏิกิริยาลูกโซ่โพลีเมอเรสในจีโนมิกดีเอ็นเอและซีดีเอ็นเอของกุ้งทั้งสองเพศ พบว่าให้ผลิตภัณฑ์ดีเอ็นเอจำนวน 5 โคลน เฉพาะในรังไข่ทั้งในกุ้งกุลาดำอายุ 3 เดือนและตัวเต็มวัย แต่เมื่อตรวจสอบด้วยวิธี Southern hybridization พบว่าไม่มีความแตกต่างระหว่างเพศ ส่วนการสร้างห้องสมุดจีโนมิกดีเอ็นเอของกุ้งกุลาดำเพศผู้และเพศเมีย ได้ค่า titer เท่ากับ $7.3 \times 10^3$ และ $1.3 \times 10^4$ pfu/ml ตามลำดับ โดยพบว่าขนาดเฉลี่ยของโคลนในห้องสมุดจีโนมิกดีเอ็นเอที่ได้ของทั้งสองเพศมีความยาวประมาณ 20 kb นอกจากนี้ได้นำโคลนจากการทำปฏิกิริยาลูกโซ่โพลีเมอเรสของ ER มาโพรบกับห้องสมุดจีโนมิกดีเอ็นเอของกุ้งทั้งสองเพศด้วยวิธี plaque hybridization พบว่าสามารถโพรบติดในห้องสมุดจีโนมิกดีเอ็นเอของทั้งเพศผู้และเพศเมีย

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

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

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

# # 4572508023 : MAJOR BIOTECHNOLOGY

KEY WORD: *Penaeus monodon* / BLACK TIGER SHRIMP / SEX–SPECIFIC MARKERS / GENOMIC DNA SUBTRACTION

SRISUPAPH POONLAPHDECHA: IDENTIFICATION OF SEX-SPECIFIC MARKERS FROM BLACK TIGER SHRIMP *Penaeus monodon* USING SUBTRACTION ANALYSIS. THESIS ADVISOR: PROF. PIAMSAK MENASVETA, Ph.D., THESIS CO-ADVISOR: NARONGSAK PUANGLARP, Ph.D., 180 pp. ISBN 974-53-1017-4.

Generally, female *P. monodon* attains a relatively larger size than males reflecting the difference on growth rate between male and female *P. monodon*. However, studies of sex determining mechanisms in crustaceans are not well advanced. Sex chromosomes in *P. monodon* are not yet cytological identified. Therefore, sex specific markers of *P. monodon* will be identified by molecular biological techniques. Once sex determining system in the tiger prawn is understood, this basic knowledge will allow the possibility to develop a monosex culture of *P. monodon* to increase the efficiency of shrimp culture successfully. Genomic DNA subtraction, a powerful technique for isolating the differences of nucleic acid composition from two cell samples was carried out. Nine and four clones were obtained from PERT and RDA male subtractions, respectively while 4 and 4 clones were obtained from PERT and RDA female subtractions, respectively. Of these, 1 clone of PERT subtracted male (PMMSH6) and 2 clones of RDA subtracted female (PMFJ200 and PMFJ800) were sex-specifically verified by PCR. The results showed no difference in PCR product pattern between genders. Further analysis by SSCP revealed the polymorphic but no sex specific properties of these clones. For genome walking analysis, sex-specific characters of some candidates were initially observed. Ultimately, they were proven to be the artifacts caused by individual variation. For Southern blot analysis, 4 PERT-subtracted clones, 2 males (PMMSH3 and PMMSH8) and 2 females (PMFSH26 and PMFSH32), 4 RDA-subtracted clones 2 males (PMMJ200 and PMMN200) and 1 females (PMFJ300) were undetectable from both sexes, assuming that these fragments were parts of single copy genes. Five RDA-subtracted clones, 2 males ($PMMJ_{450}$ and $PMMN_{450}$) and 3 females (PMFJ200, PMFJ800 and PMFN300) were hybridized to genomic DNAs from both sexes, indicating no sex specificities of these candidates for sex identification in *P. monodon*. Male and female genomic DNA libraries of *P. monodon* were constructed. Titer efficiencies of male and female libraries were 7.3 x $10^3$ and 1.3 x $10^4$ pfu/ml, respectively. The average size of the clones was detected as 20 kb. Furthermore, amplification of estrogen receptor gene (ER) using 4 combinations of degenerated primers was performed, resulting 5 clones from the PCR of ovary cDNA reaction. One clone was used as probe for Southern blot analysis on *Dra*I-digested genomic DNA and plague screening from male and female genomic libraries, the results revealed positive bands and plaques from both male and female samples.

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

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

Co-advisor's signature……...

# ACKNOWLEDGMENTS

I would like to express my deepest sense of gratitude to my advisor, Professor Piamsak Menasveta, Ph.D. and my co-advisor Narongsak Puanglarp, Ph.D. for their guidances, encouragement, valuable suggestion and supports throughout my study.

I would also like to thank Assistant Professor Charoen Nitithamyong, Ph.D., Assistant Professor Thaithaworn Lirdwitayaprasit, Ph.D., Sirawut Klinbunga, Ph.D. my thesis committee who provided their valuable time to read, discuss and advice me for the improvement of my work.

I wish to acknowledge University Development Committee (UDC) Scholarship, Center of Excellence for Marine Biotechnology, and National Center for Genetic Engineering and Biotechnology (BIOTEC) for my financial support.

Many thanks are also excessively to all of my friends in the laboratory for their help, suggestion and kindness friendship that give a happy time during a study periods.

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

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| bp | Base pair |
| °C | Degree Celcius |
| cDNA | Complementary deoxyribonucleic acid |
| CIP | Calf intestinal phosphatase |
| CTAB | Cetyl trimethyl ammonium bromide |
| dATP | Deoxyadenosine triphosphate |
| dCTP | Deoxycytosine triphosphate |
| dGTP | Deoxyguanosine triphosphate |
| dNTP | Deoxyribonucleotide triphosphate |
| dTTP | Deoxythymidine triphosphate |
| DEPC | Diethylpyrocarbonate |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetate |
| g | Gram |
| *g* | Gravity (multiples of, as in centrifugal field) |
| HCl | Hydrochloric acid |
| Hepps | N-(2-hydroxyethyl) piperazine-N'-(3-propanesulfonic acid) |
| IPTG | Isopropyl-thiogalactoside |
| kb | Kilobase |
| LB | Luria-Bertani |
| Ln | Logarithm (natural) |
| M | Molar (mole/ liter) |
| $MgCl_2$ | Magnesium chloride |
| mg | Milligram |
| ml | Millilitre |
| mM | Millimolar |
| ng | Nanogram |
| OD | Optical density |
| PCR | Polymerase chain reaction |
| PERT | Phenol emulsion reassociation technique |
| pfu | Plaque forming unit |

# LIST OF ABBREVIATIONS (Cont.)

| | |
|---|---|
| RDA | Representational difference analysis |
| RNase A | Ribonuclease A |
| rpm | Revolution per minute |
| SDS | Sodium dodecyl sulfate |
| SSCP | Single strand conformation polymorphism |
| Tm | Temperature, melting |
| Tris | Tris (hydroxyl methyl) aminomethane |
| U | Unit |
| μg | Microgram |
| μl | Microliter |
| μM | Micromolar |
| UV | Ultraviolet |
| v/v | Volume/ volume (concentration) |
| w/v | Weight/ volume (concentration) |
| λ | Lambda |

# CHAPTER I

# INTRODUCTION

## 1.1 General introduction

Penaeid shrimp culture is one of the most important aquacultural industries in many countries. In Southeast Asia, black tiger shrimp, *Penaeus monodon*, is on the top list of world shrimp production. Thailand has been the leader of farmed *P. monodon* export since 1993. About 95% of all shrimp farms in Thailand today are operated by intensive systems and 5% of that are inland shrimp farms or farms operated in agricultural areas. Presently, shrimp farming has confronted several problems, such as loss of the cultured production due to bacterial and viral infections and size differences of cultivated shrimps in the same cultured pond. Other drawbacks, especially for *P. monodon*, are the shortages of wild brood stock, difficulty in captive breeding, and low survival rate of seeding (20-30%). *P. monodon* farming has relied entirely on wild broodstock, which resulted in heavy exploitation of wild shrimps. Thus, the success of breeding in the farm will be the first step to solve the critical massive requirement of broodstock for supplying postlarval shrimps for the aquaculture industry, which could partly resolve the over-exploitation problem of the wild *P. monodon* females.

Many reports have proclaimed the success of captive breeding in domesticated shrimp broodstock. However, the quality of larval production from domesticated broodstock, particularly from *P. monodon*, is still below that of wild spawners. For this reason, major improvements on the reproductive performances of domesticated *P. monodon* are needed. One of the enhancement approaches for the improvement of shrimp domestication is sex control or monosex culture. It has been proven to make more profitable in the production of many aquatic animals. Various strategies utilizing sex reversal and breeding, progeny testing, gynogenesis and androgenesis can lead to the development of predominantly, or completely, male or female populations. Furthermore, interspecific hybridization has been used to increase growth rate, manipulate sex ratios, produce sterile animals, improve flesh quality, increase disease resistance, improve tolerance of environmental extremes, and improve a variety of other traits that make

aquatic animal production more profitable. In order to do all that mentioned above, an understanding of shrimp reproductive biology and sex determination systems are primarily required. Extensive research has been initiated to determine the factors, organs, hormones and gene sequence for regulatory mechanisms of sex differentiation.

The existence of two sexes is nearly universal in the animal kingdom. Also, gonadal morphogenesis is remarkably similar across vertebrates. However, the sex-determining mechanism varies considerably. In most mammals, there is an XX/XY system where the Y carries male factors. XO mammals are sterile females. Unlike mammals, the heterogametic sex in birds is female (ZW sex chromosomes), whereas males are homogametic (ZZ sex chromosomes). In contrast to mammals and birds, reptiles do not have dimorphic sex chromosomes. Instead, sex is determined by temperature of the environment. Hormones are also critical for sex determination in birds and alligators. Many fishes change sex during their lives, as do some molluscs. Several species are hermaphrodites, bearing both male and female gonads. In ants, bees, wasps and termites there is a haplodiploid system; females are diploid, males are haploid. There are no sex chromosomes, or alternatively, it can be said that every chromosome is a sex chromosome.

Metazoan sexual differentiation is controlled by complex regulatory pathways that govern the developmentally coordinated expression of large groups of male- and female-specific gene products. Different sex determination mechanisms are even used within the same species, and in some cases individuals can belong sequentially first to one sex and then to the other. In contrast to the situation observed in vertebrates such as mammals, birds, or fish and some insects such as fruit fly, the identification of sex-determining gene in penaeid shrimps, so far, has never been investigated.

This study focuses on identifying sex-specific markers in the genome of *P monodon.* In case where sex chromosomes have not been identified, isolation of subtracted genomic DNAs between genomes of male and female shrimps is required. In addition, part of the investigation involves the constructions of male and female genomic DNA libraries which support not only the study of sex determination genes but also become useful for gene structural analyses and other important genome researches in *P. monodon*.

## 1.2 Biology of *Penaeus monodon*

### 1.2.1 General information

*P. monodon* (Black tiger prawn or Giant tiger shrimp) is named for its huge size and banded tail. The exterior of the shrimp is distinguished by a cephalothorax with a characteristic hard rostrum, and by a segmented abdomen (Figure 1.1).

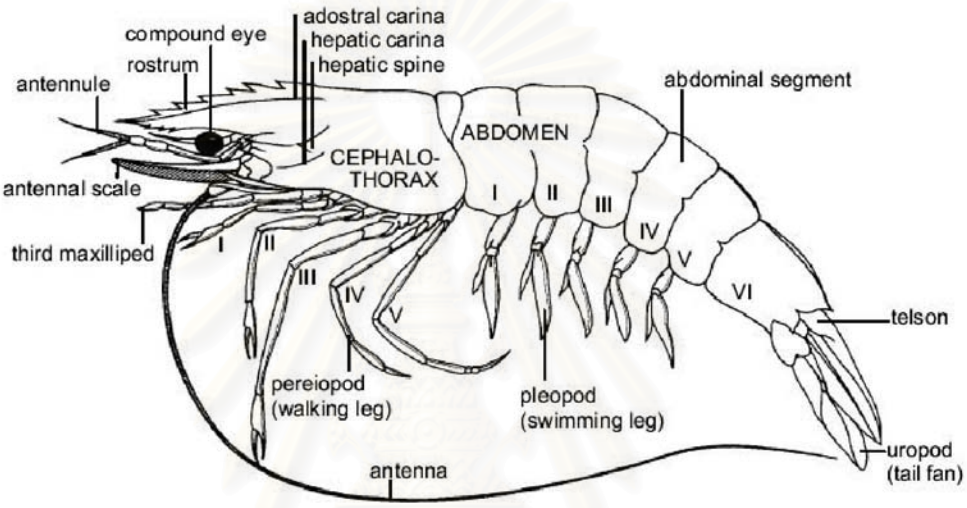



**Figure 1.1** Lateral view of the external morphology of *Penaeus monodon* (Primavera, 1990).

The penaeid shrimps are generally found from near shore shallow waters to as far as 100 km off shore at depths greater than 100 m (ASCC News, 1996). It is the largest and fastest growing farm-raised shrimp. It can reach a size of 360 mm and weight over 150 g, if allowed to grow to full size (Dore, 1994). Even at high stocking densities, *P. monodon* can reach marketable sizes of 25-30 g within 3-4 months after postlarvae stocking in cultured ponds (Muir and Roberts, 1982). The shrimp can tolerate a wide range of salinities, but is highly susceptible to two of the most lethal shrimp viruses: Yellow Head Virus (YHV) and White Spot Syndrome Virus (WSSV). Life cycle of *P. monodon* includes several distinct stages that are found in a variety of habitats (Figure 1.2).

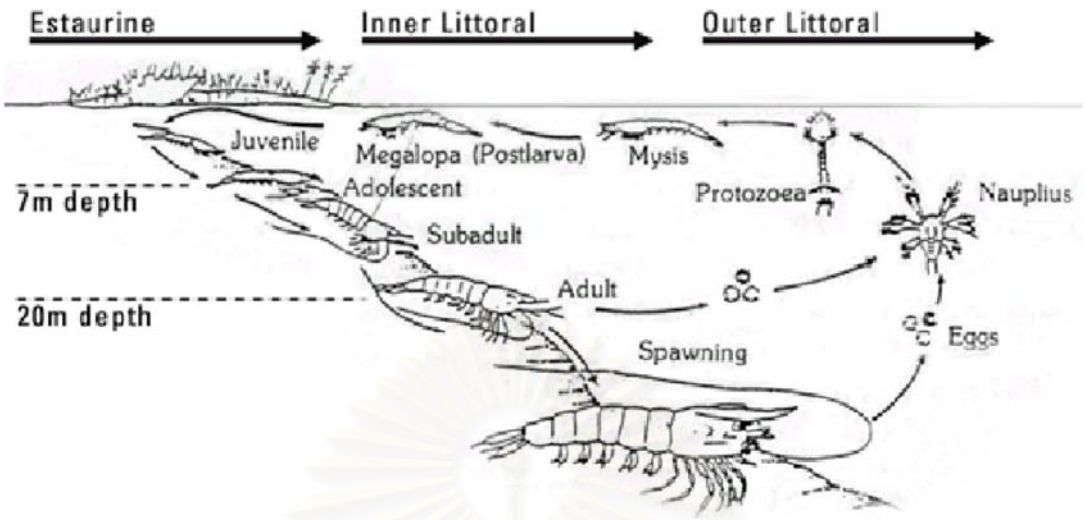



**Figure 1.2** The life cycle of *P. monodon* (ASCC, 1996).

### 1.2.2 External morphology of reproductive system

The *P. monodon* is heterosexual. The female attains a relatively larger size than the male. Live adult female and male black tiger shrimp have the following characteristic coloration: carapace and abdomen are transversely banded with red and white, the antennae are grayish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow brackish waters or when cultured in ponds, the color changes to dark and, often, to blackish brown (Solis, 1988). (Figure 1.3)

The sexually mature shrimp can be distinguished by the presence of the external genital organs: joined petasma, a pair of appendix masculina on the exopods of the second pleopods, and a genital opening on the coxa of the fifth of pereopod for the male. In females, the thelycum is situated between the fourth and fifth pair of pleopods, consists of an anterior of lateral plates (Figure 1.4).

**Figure 1.3** The black tiger shrimp, *Penaeus monodon*, female (top) and male (bottom) (Longyant, 1999).

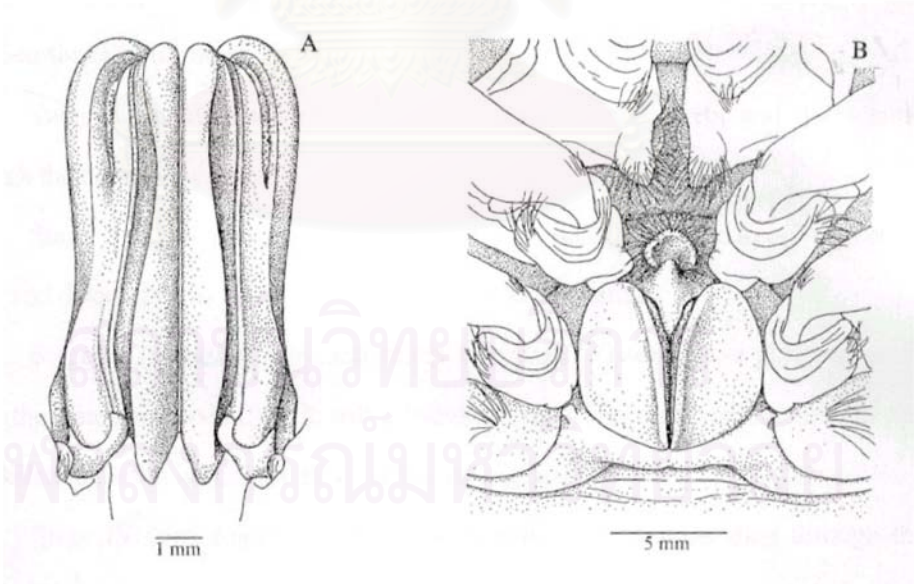



**Figure 1.4** External genitalia of male, petasma (A) and female, thelycum (B) of *Penaeus monodon* (Farfante and Kensley, 1997).

Female receives the spermatophores during mating. In *P. monodon*, the thelycum is classified as closed type. The genital opening is on the coxa of the third pereopod (Farfante and Kensley, 1997; Solis, 1988).

### 1.2.3 Domestication and sex manipulation on shrimp farming

Major improvements have already been achieved through enhanced management, nutrition, disease diagnostics and therapeutics, water quality maintenance and genetic improvement of production traits. Genetics has been used to meet many production challenges, such as disease resistance, tolerance of handling, enhanced feed conversion and spawning manipulation. Domestication effects can be observed in some fish within as few as one to two generations after removal from the natural environment (Dunham, 1996). Domestication of farmed shrimp (*penaeids*) has been relatively slow compared to that of finfish. Use of established, high-performance domestic strains is the first step in applying genetic principles to improved aquaculture management. Currently, shrimp larval production relies entirely on wild broodstocks. Therefore, the opportunity to enhance production traits such as disease resistance, tolerance of handling, enhanced feed conversion, and genetic improvement through selective breeding is limit. A number of attempts to domesticate *P. monodon* broodstock have been resulted in successful captive breeding. However, domesticated broodstock are still less cost-effective when compared to wild broodstock and the qualities of postlarvae cannot match those of wild broodstocks. For this reason, major improvements on the reproductive performances of domesticated *P. monodon* are needed.

Sex control, which has been proven to make more profitable in the production of many aquatic animals, is one of the approaches for the improvement of shrimp domestication. Sex manipulation utilizing sex reversal, progeny testing, gynogenesis and androgenesis can lead to the development of predominantly or completely male or female populations. Monosex culture has been successfully established in a large number of aquatic animals including salmonids, channel catfishs, carps and tilapias. Populations of super males (more than one Y chromosome) have been established for Nile tilapia, salmonids and marginally, for channel catfish (Dunham, 1996). All-male progeny are beneficial for catfish culture, since they grow 10-30 percent faster than females

(Benchakan, 1979; Dunham and Smitherman, 1984, 1987; Smitherman and Dunham, 1985). YY males are also viable in salmonids, Nile tilapia, goldfish and channel catfish (Donaldson and Hunter, 1982). A combination of sex-reversal and breeding to produce all-female XX rainbow trout is now the basis for stocking most of the culture industry in the United Kingdom (Bye and Lincoln, 1986), as is the case for the chinook salmon industry in Canada. Monosex chinook (*O. tshchawystcha*), and coho crossed with chinook have also been produced (Hunter et al., 1983).

The YY male technology provides a robust and reliable solution to culture problems with early sexual maturation, unwanted reproduction and overpopulation (Mair et al., 1995; Tuan et al., 1998, 1999; Abucay et al., 1999). Although the development process is time-consuming and labour-intensive, once developed, the production of monosex males can be maintained through occasional feminization of YY genotypes and existing hatchery systems without any special facilities or labour requirements. Additional costs for application of this technology at the hatchery level would be minimal. Based on impending availability of further improved GMT, along with increasing resistance to use of hormones in aquaculture, this technique is likely to impact aquaculture on a global scale. Gynogenesis and sex-reversal have also successfully induced in *Morone* spp. to produce monosex populations to avoid limitations on introductions to areas where this species is exotic (Gomelsky et al., 1998, 1999).

Among crustacean species, freshwater prawn (*Macrobrachium rosenbergii*) has, so far, become the most successful domesticated species. Differential growth and dominance of larger individuals characterize *Macrobrachium* populations. Males grow faster than the females aggravating the differential growth pattern within and between sexes. All male culture has become the new trend adopted in commercial farming of *M. rosenbergii*, in order to ensure maximum yield.

Similar to *M. rosenbergii*, male yabbies (*Cherax albidus*) in isolation grow much faster than females or mixed sex stock. A low cost method for overcoming breeding and high densities in farm pond is monosex culture. When males and females are separated, male yabbies grow about 15% faster than males in mixed sex ponds, and female yabbies grow about 30% faster than those in mixed sex farm ponds. Male yabbies grow about 70% faster than female yabbies. It also provides a 70% increase in gross return to the farmer. To make this easier and more accurate, a hybrid has been discovered that only

produces male progeny (by crossing male WA yabbies *Cherax albidus*, female *Cherax rotundus* yabbies from NSW). In research ponds, hybrids grew twice as fast as a mixed sex population.

In *P. monodon*, females exhibit greater growth rate than do males at all stages of development. To ensure maximum yield and weight/unit, all-female populations are desirable for commercial farming. Therefore, more attention has been paid to monosex culture which has been successfully established in a large number of aquatic animals. Unfortunately, the knowledge on sex determination system in crustaceans, especially decapoda, are scarce. 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.

## 1.3 Sex determination systems

Although most metazoan species have two sexes, the sex determining mechanisms appear to be very different. In most animals, sex of an individual is determined by the sex chromosomes at the time of fertilization, a process referred as genetic sex determination. However, this genetic determination is not final and irrevocable; many external and internal environmental factors may come in to operation during the developmental process which could modify or completely reverse the phenotypic expression of the genetic constitution of the individual. In mammals and birds, the genetic constitution established at the time of fertilization determines the type of gonad that develops. In many species, the situation is more complex than the two simple model systems described above. In some cases, autosomal genes can have a role in sex determination (Wachtel et al, 1991). In contrast, the sex of some poikilothermic vertebrate groups is determined solely by environmental temperature, whilst in others, there appears to be an interaction between environmental temperature and genetic system (Pieau et al., 1994).

### 1.3.1 Genetic sex determination

The term genetic sex determination (GSD) signifies that the sex of a zygote is determined entirely by its genotype; the sex of an individual is fixed at fertilization. The

most common type of GSD involves sex chromosomes. In animals exhibiting GSD, a sexual maturation control system is established which directs and controls all of the late ontogenetic processes involved in male-female differentiation of the genitalia. In polygenic sex determination, which is less common, sex is determined by a number of genes, each with minor effect, distributed throughout the chromosome complement. Wilkins (1995) and Wolf (1999) have predicted that some homologous sex determining genes should be found, but they would not be the higher-ups on the hierarchy. Rather, there would be the genes that were actually regulating the genes whose products were specific for male and female gonads. Recent studies have shown that one of these down-in-the-hierarchy genes appears to be conserved throughout much of the animal kingdom (Raymond et al., 1998). The two major systems of genotypic sex determination that exist may be referred to as the Mammalian and the Avian systems, also denoted as the XX/XY and ZZ/ZW systems, respectively. In animals exhibiting the XY/XX system, the male is the heterogametic sex (XY) while the female is homogametic (XX), half of the spermatozoa produced containing and X-chromosome and remainder the Y-chromosome. For the ZZ/ZW system, the female is the heterogametic sex. The small chromosome (equivalent to the Y-chromosome) is designated by the letter W, and the X-chromosome equivalent by the letter Z. Thus, the homozygous (ZZ) condition produces male while the heterozygous (ZW) produces female and half the ova will carry a W-chromosome and the other half a Z-chromosome. All the sperm carry a Z-chromosome (reviewed in Turner and Bagnara, 1971; Pieau et al., 1994; Fry, 1995). Although the triggers for initiating sex determination may differ among vertebrates, there is considerable conservation in the genetic cascades that underline the sex determination process. Not only are the gene sequences coding for transcription factors and proteins important to sex determination similar across vertebrates, but also the patterns of expression of these genes appear to reflect phylogenetic relationships, with turtles being more similar to mammals and crocodilians more similar to birds (Figure 1.5).

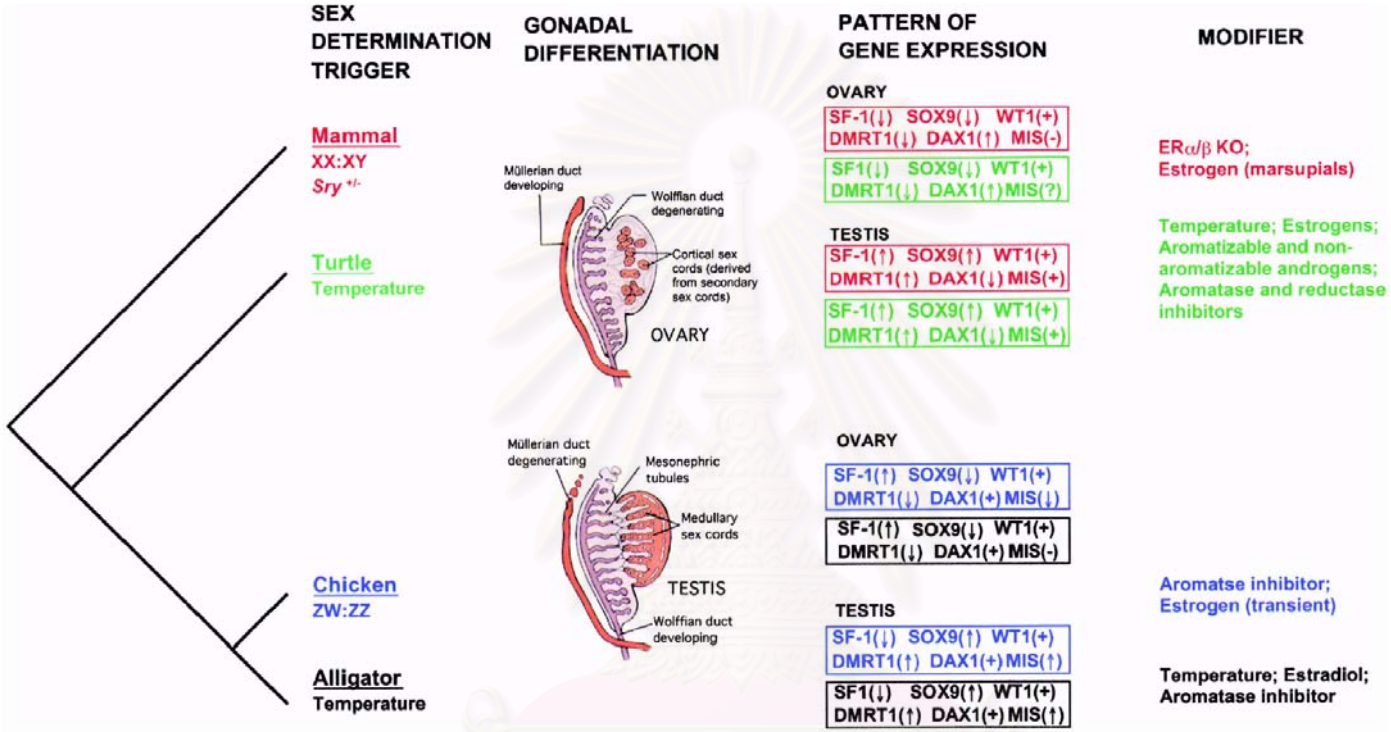



**Figure 1.5** Genes involved in sex determination in amniote vertebrates based on evidence from fossils, gene sequences, proteins, and morphology (Crews, 2003).

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. Therefore, identification of sexes in mammals at the DNA level is well-established (O'Neill and O'Neill, 1999). For non-mammalian species, no widely conserved sex-specific genes have yet been described. Therefore, a sex-specific marker isolated from one species may not even exist outside the genus (Griffiths and Tiwari, 1993). 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 still not clear. Some species of fish do not have sex chromosome. As a result, sex of these species is determined by male or female genes located on the autosomal chromosomes.

#### 1.3.1.1 Genetic control of sex determination

Numerous genes are involved in controlling determination of gonad type. *SRY* is the principal initiator of the cascade of gene interactions that determine the development of a testis from the indifferent gonad. *SOX9* plays a crucial role in this pathway where it is up-regulated by *SRY*. That *SOX9* lies downstream of *SRY* in a cascade of testis development. In XY male development, the repressor function upstream of *SOX9* is normally repressed or inhibited by *SRY*, thereby allowing *SOX9* to induce testis formation. In normal female development, *SOX9* is repressed and no testis forms. Other genes required for testis determination in humans remain to be identified. It is possible that this gene may be implicated in some forms of XY gonadal dysgenesis.

#### 1.3.1.2 Conserved genes for sex determination

Two genes encoded proteins with a DNA-binding motif were identified. They were conserved and homologous with *doublesex* (*dsx*) and *mab3* genes involved in sex development in Drosophila and Caenorhabditis, respectively. These similar regions were called the DM domain (after *Dsx* and *mab-3*). Therefore, these two genes were designated as DMRT1 and DMRT2 (*doublesex* and *mab*-3 related transcription factors). The expression of these proteins was seen only in the testis. *DMRT1*-related sequences have also been found in the chick, alligator, and mouse (Raymond et al., 1999; Smith et al., 1999). In birds, which have ZZ/ZW sex determination, *Dmrt1* is found on the Z chromosome (Nanda et al. 1999), again suggesting that two doses of *Dmrt1* (ZZ=male) might be necessary for testis development. In all vertebrates examined, *Dmrt1* is expressed in the differentiating male genital ridges and adult testis, including mammals, birds, and reptiles with temperature-dependent sex determination (Raymond et al. 1999; Smith et al. 1999; De Grandi et al. 2000; Kettlewell et al. 2000; Moniot et al. 2000). In mouse, *Dmrt1* mRNA is expressed in the genital ridges of both sexes and then becomes testis specific at the end of the sex-determining period. The number of sex-linked genes or markers found in fishes is low in comparison to more recent vertebrate groups, and most markers do not seem to be applicable to more than one fish species or even strain (Iturra *et al.*, 1997). Sex-linkage of genes or markers have been identified by: (a) deduction from mapping data (May *et al.*, 1989; Allendorf *et al.*, 1994); (b) systematic searches through the male and female genome (Devlin *et al.*, 1991; Iturra *et al.*, 1997; Kovacs *et al.*, 2001); (c) other methods (Du *et al.*, 1993; Pro¨dohl *et al.*, 1994; Moran *et al.*, 1996; Matsuda *et al.*, 1997; Coughlan *et al.*, 1999).

### 1.3.2 Environmental sex determination

The term environmental sex determination (ESD) signifies that the sex of an individual is determined irreversibly by the environment experienced during early development. Where the decisive environmental factor is temperature, a process referred as temperature-dependent sex determination (TSD); all crocodilians, many turtles, and several lizards it is the temperature experienced during the mid-trimester of embryogenesis that determines the gonadal sex of the individual. Sex determination may also be influenced by pH and by social conditions or relative juvenile size (Hardy, 2002).

In mammals and birds, embryonic development at the time of sex determination occurs under controlled temperature conditions. However, in poikilothermic species, embryonic development proceeds in full exposure to the external physical environment where relatively large temperature alterations can occur.

Temperature effects on sex have been now observed in at least eight families of jawed fishes, as well as one Agnathan species. Sex determination is controlled by the actions of a variety of biochemical pathways involving many different proteins (e.g. transcription factors, steroidogenic enzymes, receptors and second messenger systems, etc.). Since it is well known that temperature can dramatically influence the structure and function of proteins and other macromolecules, temperature fluctuations as are encountered by fish in different habitats could alter sex-determination pathways and influence the probability that development would be male or female. Temperature-dependent sex determination has been extensively studied in reptiles, where exposure to elevated temperature results in female development in some species (Bull and Vogt, 1979; Vogt and Bull, 1982).

In a study on the turtle, *Emys orbicularis*, all individuals from eggs incubated at 25-26°C became males, and all individuals from eggs incubated at 30-30.5°C became female (Zaborski et al, 1982). These temperature-dependent effects appear to be mediated in part by influencing aromatase activity and estradiol synthesis in females, and by steroid receptors in both sexes (Crews and Bergeron, 1994; Crews, 1996). In Nile tilapia (*O. niloticus*) and Japanese flounder (*P. olivaceus*), elevated temperatures (which cause masculinization) are associated with reduced aromatase mRNA levels and lower estradiol levels (Kitano et al., 1999; D'Cotta et al., 2001), and treatment with an aromatase inhibitor is able to counter the masculinizing effects of high temperature (Kwon et al., 2000). Low temperature is also capable of biasing sex differentiation toward females in two other atherinids, *O. bonariensis* and *Patagonina hatcheri*. The two species show distinctive responses to temperature: *O. bonariensis* sex ratio is influenced without threshold over a broad range of temperatures, whereas *P. hatcheri* sex is only influenced at temperature extremes and otherwise has genetically determined sexes (Struˉssmann et al., 1996a, b).

Although sex determination in tilapia species is known to be controlled polygenically by major and minor factors on the sex chromosomes and autosomes,

temperature influences on sex ratio have also been detected (Baroiller and D'Cotta, 2000). In *Oreochromis mosambicus*, genetically female groups (derived from crosses between sex-reversed XX males and regular females) exposed to low temperature (19 °C) incubation during early development resulted in 89% males (Mair et al., 1989). In other fish, indications of temperature-dependent sex determination have been suggested. In channel catfish *Ictalurus punctatus*, sex is normally determined genetically by an XY system, but high temperature extremes applied during the critical period for sex determination result in female-skewed sex ratios, which indicate influence by environmental factors as well (Patino et al., 1996). Some Perciforme hermaphroditic fishes can change sex depending on their social interaction with conspecifics (Fishelson, 1970; Robertson, 1972). The process of sex inversion can involve a complete reorganization of the reproductive system, with replacement of gonadal cell types, duct systems, hormone profiles, and sex-specific behaviours. Changes can begin immediately upon a shift in social status of an individual, and, depending on conditions, can be completed as quickly as a few weeks (e.g. *A. melanopus*, Godwin, 1994) or as long as several years (e.g. *A. frenatus*, Hattori, 1991).

### 1.3.3 Sex determination/ differentiation in invertebrates

Invertebrates exhibit both asexual and sexual reproduction, with 99% of species reproducing sexually at some point in their life cycle (Barnes et al., 1993). Some invertebrate taxa always have separate sexes assigned to separate individuals (gonochorism), whilst in others, individuals have both male and female organs at the same time (simultaneous hermaphroditism) or may lose the characteristics of one sex and gain those of the other, or may even alternate their sex during their life time (sequential hermaphroditism). Genetic sex determination is found in species of insects, arachnids, crustaceans, nematodes and the polychaete *D. gyrociliatus* (Barnes et al., 1993).

The principles of genetic sex determination basically follow those of genetic sex determination in vertebrates. Environmental conditions are also known to determine the sexual development of the embryo or larva in some invertebrate species. Photoperiod is known to influence sex determination in some crustaceans, for example in the amphipod *Gammarus duebeni* (Watt, 1994). An understanding of sexual biology of any sexual-

reproducing species is also important for designing breeding programs in that species. However, studies of sex determining mechanisms in insects and crustaceans are not well advanced. Previous researches 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).

In insects, sex determination system have been well studied in *Drosophila melanogaster* because several spontaneous mutants affecting sex determination were found. The ratio of X-chromosomes (X) to autosomes (A) is the primary signal for sex determination in this species. The gene *Sex-lethal* (*Sxl*) controls sex determination, dosage compensation, and oogenesis in *D. melanogaster* and can be activated only when the X/A was 1 or more and a female develops. Alternatively, a ratio of 0.5 (X: AA) leaves *Sxl* inactivate and male development occurs. *Sxl* is an RNA-binding protein that controls splicing of the *transformer* (*tra*) pre-mRNA, its downstream target in sex determination, by binding to any thereby blocking an optimal splicing acceptor site (Sosnowski et al., 1989; Inoue et al., 1990). This allows female *D. melanogaster* to produce the functional *tra* protein through female-specific splicing. The female *tra* protein induces female-specific splicing of the *doublesex* (*dsx*) pre-mRNA in coopreation with the *tra-2* gene product. The *dsx* gene is known as the final gene of the sex-determining cascade in *D. melanogaster*. Female- or male-specific proteins of *dsx* regulate the expression of sex-specific differentiation gene such as yolk protein gene (Burtis et al., 1991; Jursnich and Burtis, 1993; An and Wensink, 1995a, b). The *dsx* protein binds to a specific site of an enhancer sequence in the 5’ flanking region of the yolk protein genes and regulates their transcription (Garabedian et al., 1986; Coschigano and Wensink, 1993). The *dsx* proteins have a zinc finger-like domain called DM domain (Erdman and Burtis, 1993; Erdman et al., 1996; Raymond et al., 1998). The *dsx* homologues have been identified in many other species including *mab-3* from *Caenorhabditis elegans* (Shen et al., 1998), DMRT1 from human (Raymond et al., 1998; Moniot et al., 2000) and *Dmrt*1 from mouse and chicken (Raymond et al., 1999; De Grandi et al., 2000). These *dsx* homologues were all contain DM domain and considered to regulate sexual differentiation and have been evolutionarily conserved as sex-determining genes. DMRT1 has been suggested as the first conserved gene involved in sex differentiation found from invertebrates to human. Recently, a DMRT1 homologus (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 and Page, 1994), porcine (Schmoll and Schellander, 1996), cattle, sheep and goats (Aasen and Medrano, 1990), *D. melanogaster* (Pauli et al., 1995) and *C. elegans* (Raymond et al., 2000).

### 1.3.4 Sex determination in crustaceans

A number of studies reported the existence of sex hormones in crustaceans. At least malacostracan crustaceans, also arthropods, had a sex hormone. It was suggested by the observation that sacculinized Carcinus with completely modified secondary sex characteristics often retain normal testes, indicating that external characteristics were controlled by a male hormone produced by an organ independent of the testes. This hypothesis was intensively explored and the suspected organ was discovered in the amphipod *Orchestia gammarella* and named the androgenic gland (review of Martin et al, 1999). This gland induces not only the secondary sex characteristics but also male gonad differentiation. These mechanisms are unique among the animal kingdom as they are hormonally induced and triggered by the androgenic gland producing androgenic hormone. Indeed, spermatogenesis of young males is totally under the control of the androgenic gland. Most malacostracan Crustacea are gonochoristic, males and females being indistinguishable on hatching. Sexual differentiation in malacostracans is controlled by the presence or absence of the androgenic gland. In the woodlouse *Armadillidium vulgare*, ablation of the androgenic glands causes complete feminization of young males

(Suzuki and Yamasaki, 1991). By grafting androgenic gland from adult males into young females, the complete and fully functional reversal of sex has been accomplished (Katakura, 1961; Katakura and Hasegawa, 1983; and Hasegawa and Katakura, 1983) recently the same result has been obtained with a decapod, *Macrobrachium rosenbergii* (Malecha et al., 1992). In both cases, sex-reversed individuals have been used in mating experiments to determine the genetic basis of sex.

Surgical experiments (ablation or grafting of the androgenic glands) induced reversal of sex in prawns and woodlice. This permitted the determination of an animal-size or age threshold for the expression of sex determining genes (Nagamine et al., 1980). Beyond this threshold, complete reversal of reproductive function is no longer possible. Andrectomy demonstrated that in the absence of the androgenic glands, female characteristics appear and oogenesis occurs in genetic males. Androgenic gland implantations proved that genetic females are able to differentiate functional androgenic glands (Juchault and Legrand, 1964) and testes. These experimental results clearly showed that each individual, male or female, possesses the genes which induce differentiation of androgenic glands. These genes are tissue specific (Legrand et al, 1987). In females, these genes are silent and therefore no androgenic hormone is synthesized. Furthermore in the pandalid shrimp *Pandalus borealis*, which is a protandric hermaphrodite, the androgenic gland disappears at a certain stage of the shrimp's life and the animal is transformed into a functional female (Hoffman, 1968). Numerous studies have stressed the importance of the androgenic gland for sexual differentiation. The androgenic hormone of the woodlouse, a terrestrial isopod, has been shown by different methods to be a protein (Hasegawa, et al., 1987; Martin, et al., 1990; and Okuno et al., 1997) with a molecular mass of about 17 kDa, but its sequence has not been determined.

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).

Genetic basis for sex determination had been studied in only a few species, none of which were decapods. The strong influence of environmental factors (including temperature, food supply and social environment) on sex determination in some crustacean groups has been reviewed. So far, no sex chromosome has been observed in penaeids, nor any environmental sex determination reported (Malecha, 1983; Korpelainen, 1990). Karyological studies in the giant freshwater prawn (*M. rosenbergii*) revealed the chromosome number from the antennal gland in males as a diploid number (2n) of 118. This was further confirmed by the haploid chromosome number of n = 59 for 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 shrimps 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.

There are no published reports on experimental manipulations of this type for penaeids, and their sex determination mechanism remains unknown. Little data on the developmental processes of penaeids has been published, but the differentiation of the genital organs and the androgenic gland in *P. japonicus* has been described (Nakamura et al., 1992). Sex chromosomes in *P. monodon* are not yet cytological identifiable. Information on the shrimp's genetics, its genes, and its genome have good potential for breeding and improvement programs of the shrimp to make them having better growth and survival rate under an intensive farming condition.

## 1.4 Genomic DNA subtraction

Subtractive hybridization is a powerful technique that allows isolation of the differences in the nucleic acid composition of two cell samples. Differences can be at the level of RNA species represented within each sample or within the complement of genomic DNAs. Such differences include genes whose differential expression distinguishes one cell type from another, one growth phase from another, or a normal state from a diseased state. A related procedure, termed positive selection, has been used to isolate differences in cDNA and in genomic DNAs among various genotypes.

### 1.4.1 The basic concept of DNA subtraction

Subtractive hybridization uses a process called driver excess hybridization (see Figure 1.6). Nucleic acid sequences to be isolated differentially (the tester) is hybridized to complementary nucleic acid that is believed to lack sequences of interest (the driver). Driver nucleic acid is present at much higher concentration (at least 10-fold) than is tester, and it dictates the speed of the reannealing reaction. The driver and tester nucleic acid populations are allowed to hybridize, and only sequences common to the two populations can form hybrids. After hybridization, driver-tester hybrids and unhybridized driver are removed. This is the subtraction step. The tester that remains behind is enriched for sequences specific to the tester and depleted for sequences common to tester and driver. Usually, the process must be performed reiteratively in order to remove all the sequences common to both the driver and the tester. After subtraction, remaining nucleic acid can be used to prepare a library enriched in tester-specific clones or to make a probe that can be used to screen a library for tester-specific clones. The subtraction analysis consists of 4 types (Sagerström et al., 1997).

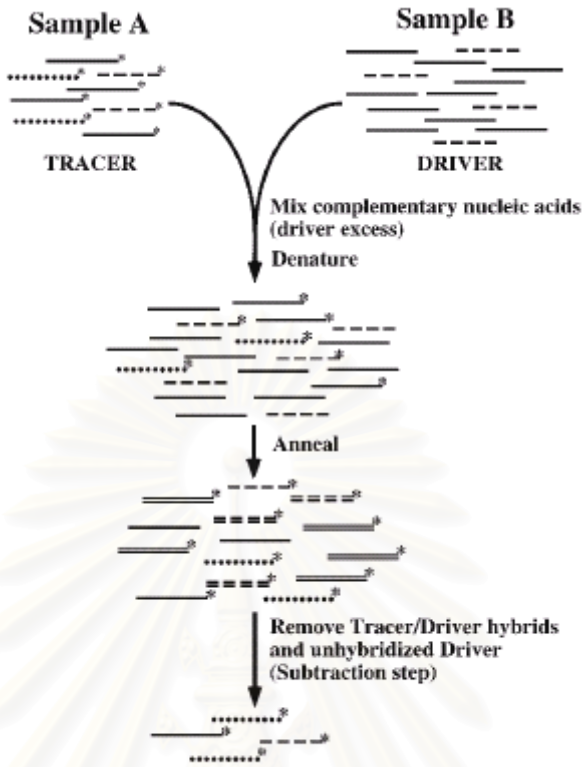



**Figure 1.6** General outline of subtractive hybridization. Complementary nucleic acids from two samples are mixed together (driver sequences are present in excess), denatured, and allowed to anneal. Duplexes formed between driver and tester (asterisks indicate tester) are then removed, as is unhybridized driver, leaving a population enriched for sequences present in the tester but absent in the driver. Different sequences are indicated by solid, dashed, and dotted lines; dotted sequences are unique to the tester (Sagerström et al., 1997).

#### 1.4.1.1 cDNA subtraction analysis

This method used first strand cDNA as the tester, poly A+ as the driver, and Hydroxyapatite (HAP) to remove hybrids. The remaining RNA can be used directly as subtracted probe. This method is still useful if single cell type is used. It is able to obtain a large quantity of starting materials. However, when materials are limiting, the use of RNA as the tester presents a problem because it is labile and may be degraded significantly during the hybridization reaction. Another disadvantage is that the subtracted products cannot be cloned directly.

#### 1.4.1.2 Library-library subtraction analysis

Specific digestion of driver-tester hybrids by restriction enzymes has been used. In a single reported use of this method, both tester and driver are single stranded. Tester is prepared from a library as single-stranded phagemids, and driver is first-strand cDNA (Rivolta et al., 1995). After hybridization, the driver-containing hybrids are digested with restriction endonuclease, and the remaining DNA is introduced into bacteria. The phagemid tester is capable of transforming bacteria, but digested driver-tester hybrids and excess driver are not. Because this method relies on the ability of phagemid DNA to transform bacteria, it cannot be adapted easily to another form of tester.

In another method, RNaseH is used to remove hybrids (Kuze et al., 1989). Labeled RNA tester is hybridized with single stranded DNA driver, and the reaction mix is treated first with RNaseH to remove hybridized tester and subsequently with DNaseI to remove excess driver. The remaining RNA can be used directly as subtracted probe. This method is simple, but the use of RNA as the tester presents a problem because it is labile and may be degraded significantly during the hybridization reaction. Another disadvantage is that the subtracted products cannot be cloned directly.

#### 1.4.1.3 PCR-based subtraction analysis

This positive selection technique uses selective amplification of tester-tester hybrids by PCR. In a variation of the compatible restriction site method, specific adaptors are selectively ligated to tester-tester ends, followed by PCR (Straus et al., 1990). Only tester-tester hybrids have adaptors at both ends of the duplex DNA and are amplified exponentially by PCR. Tester-driver hybrids are amplified with linear kinetics, whereas

driver-driver duplex and single stranded tester are not amplified at all. In another method (Lisitsyn et al., 1993), tester DNA fragments are ligated to adaptors that have both strands dephosphorylated and, therefore, become covalently attached only to the two 50-ends of double-stranded tester molecules. After hybridization, the ends of DNA molecules are filled in and subjected to PCR, and the adaptor again serves as primer. Again, only tester-tester hybrids are amplified exponentially.

#### 1.4.1.4 Positive selective based on cohesive restriction sites analysis

This method selects for the ability of tester-tester hybrids to be cloned because both ends of the hybrids contain a particular restriction site (see Figure 1.7). Driver-tester and driver-driver hybrids do not have the correct ends to be cloned. Tester is prepared by restriction endonuclease digestion that generates sticky ends, and driver is prepared by sonication, which leaves ragged ends. After hybridization the entire nucleic acid mix is combined with DNA ligase and vector with ends compatible to those of the tester-tester hybrids, which will be the only molecules cloned efficiently (Sagerström et al., 1997).

### 1.4.2 Choosing a subtraction analysis

The decision to use a particular method should be based on the amount of starting materials that can be obtained, the complexity of those materials, and the goal of cloning (for example, a subtracted probe or a subtracted library, full-length clones or partial fragments) Figure 1.7 illustrates some useful cloning schemes and summarizes distinct features of each. The basic scheme (see Figure 1.7A) is perhaps the most straightforward when similar tissues are compared and it is easy to obtain large amounts of such tissues. When the starting tissue is difficult to obtain or when complex tissue is to be compared, a library-library or PCR-based scheme must be adopted. Library-library subtraction (Figure 1.7B) is the easiest way to isolate full-length clones, but it is difficult to perform this method reiteratively. PCR-based subtraction (Figure 1.7C) is the scheme that most easily allows multiple rounds of subtraction, but representation of tester population may be biased because of multiple rounds of PCR required and because the average size of tester nucleic acids is small. The scheme shown for positive selection (Figure 1.7D) is based on sticky restriction sites. As discussed previously, positive selection is so sensitive that some clones of interest are likely to be isolated but unlikely to obtain a full spectrum of clones.

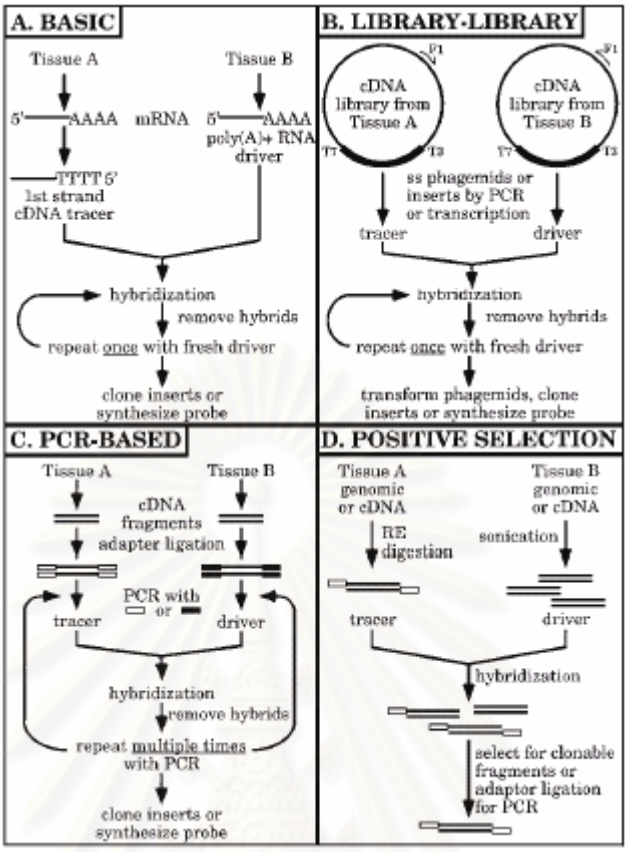



**Figure 1.7** Comparison of subtractive enrichment and positive selection. (A) Basic scheme: First strand cDNA serves as tester and mRNA as driver. A large amount of tissue is necessary for this scheme, and subtractive hybridization can be performed only twice. (B) Library-library subtraction: Solid bars represent cDNA inserts. In this scheme, the final subtracted products represent full-length cDNA. (C) PCR-based subtraction: Open and solid boxes represent different adapters. This scheme allows multiple rounds of subtraction to be performed easily. (D) Positive selection based on cohesive restriction sites method: Open boxes represent regenerated cohesive ends, which can be ligated to vector or adapter (Sagerström et al., 1997).

**1.5 Phenol emulsion reassociation technique subtraction analysis**

A phenol aqueous emulsion allows the reassociation of DNA at room temperature. Phenol emulsion reassociation technique (PERT), therefore, promotes the very rapid reassociation of DNA. The rate of DNA reassociation observed with the emulsion technique is at least dependent on: (a) the presence of an emulsion; (b) the type and concentration of ion present; (c) an appropriate temperature of incubation; (d) the proper pH; (e) the rate and manner of agitating the emulsion; (f) the amount of phenol present; (g) the fragment size of the DNA; (h) the complexity of the DNA; and (i) the concentration of DNA. The presence of salt is necessary for the emulsion reassociation. As a general rule the more chaotropic the anion or cation, the faster the DNA reassociation in the emulsion system. DNA will reassociate over a wide range of pH (5–9) and phenol concentration (5–95%). Optimum emulsion reassociation rates are obtained by vigorous shaking of the emulsion. At high DNA concentrations, the rate depends on the violence of shaking. The fragment size of the DNA used in the emulsion reaction does not greatly influence the rate of reassociation. However, the greatest rate increase observed as yet is only 50-100 times the reference aqueous rate (Kohne et al., 1977).

The following terms are the key role of the technique. The term target is designated the DNA sequence(s) to be isolated while tester is DNA (genomic DNA) containing the target. Driver is closely related DNA but distinct from tester in target sequences. Ideally, but not always, the target sequences are present in tracer and absent from driver.

Initially, driver DNA is sheared by sonication and tester DNA is completely digested with *Mbo*I. A 100-fold excess of sheared driver DNA is mixed with *Mbo*I digested tester DNA and the mixture is denatured and annealed to a $C_ot$ = 1320 (Lamar and Palmer, 1984).

PERT subtraction can be used to extract targets of different classes. There are at least three of them: absolute target, present in tester but absent from driver; amplified target, present both in tester and driver but different copy numbers and non-identical target, present both in tester and driver but in not fully identical forms. The basic idea of PERT subtraction is that tester DNA will primarily reassociate with excess driver DNA while target sequences having (ideally) no counterparts in driver will inevitably

reassociate with each other, or (when the tester is in a single-stranded form) remain single-stranded. The reassociated fragments common for driver and tester are discarded, and the remaining DNA enriched in target sequences is cloned and analysed (Figure 1.8) (Sverdlov and Ermolaeva, 1994).

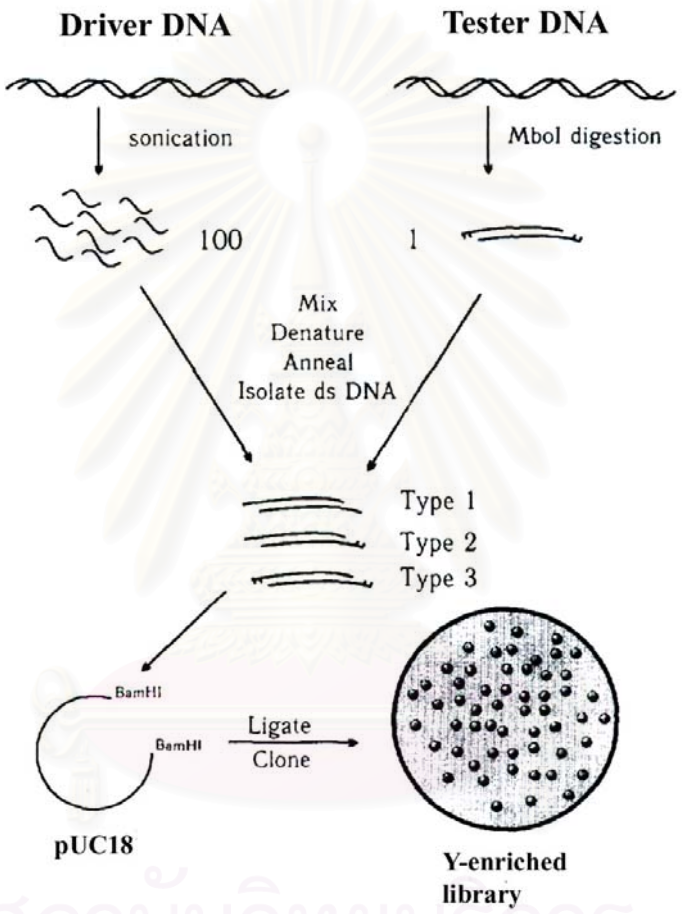



**Figure 1.8** PERT subtraction scheme (Lamar and Palmer, 1984).

**1.6 Representational difference analysis**

Representational difference analysis (RDA) is a recently described modification of the subtractive hybridization process (Lisitsyn et al., 1993). Representations of the genomes to be compared are constructed by restriction enzyme digestion and PCR amplification of the resulting restriction fragments. The representation or amplicon containing the target sequences of interest is designated the tester and the representation used to remove common sequences is designated the driver. Tester is then hybridized to excess driver. In previously described subtraction procedures driver/tester hybrids are removed by physical means; by contrast, RDA utilizes kinetic enrichment by PCR in which only tester sequences can be amplified. This kinetic enrichment is achieved by ligating a unique set of adaptors to the tester amplicon so that only self-annealing tester sequences are rendered amplifiable successive rounds of the subtraction and kinetic components of RDA result in progressive enrichment of target tester sequences to the degree where they are visible in ethidium-stained agarose gels (Figure 1.9) (Lisitsyn et al., 1994).

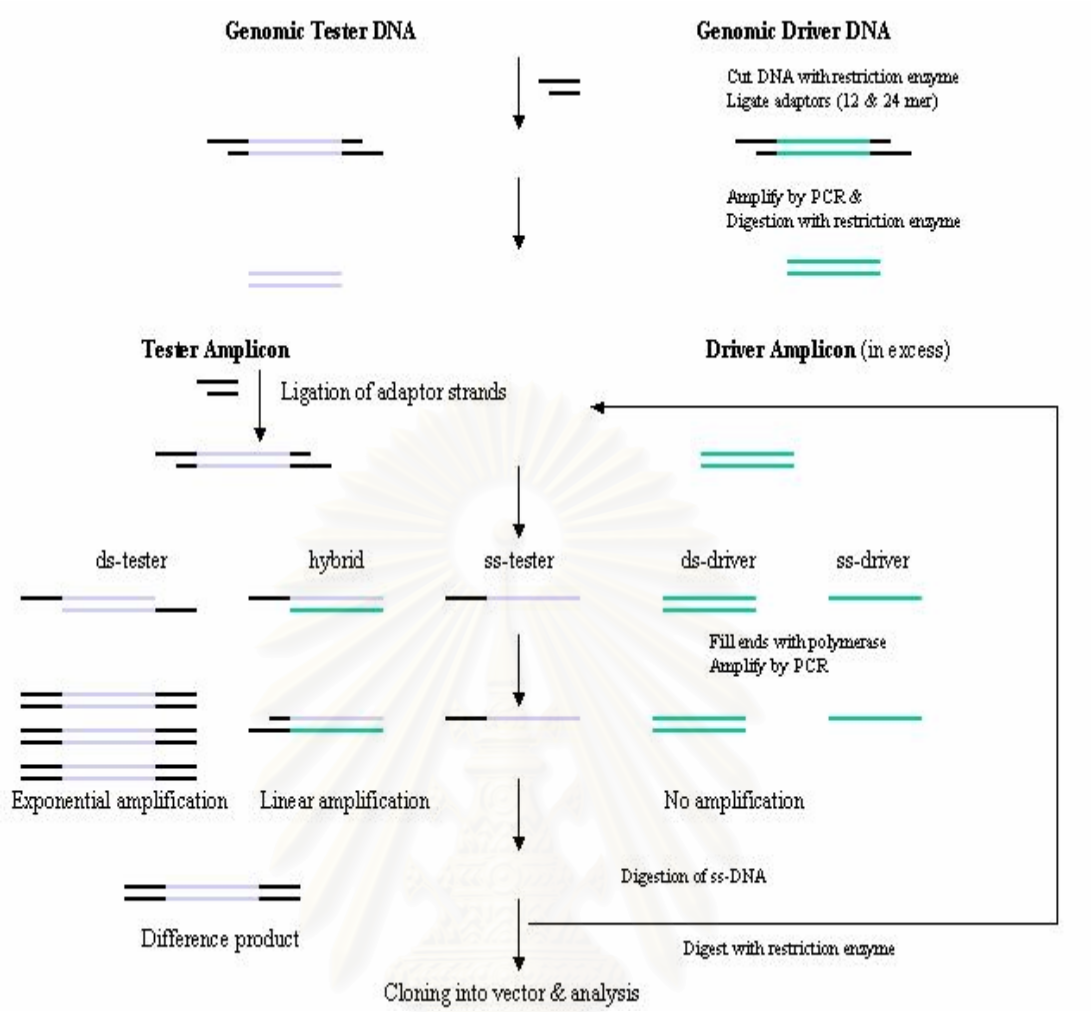



**Figure 1.9** Steps involved in the genomic DNA subtraction procedure of representational difference analysis (Lisitsyn et al., 1993).

**1.7 Genomic DNA subtraction for the isolation of sex-specific markers**

Sex of many species can usually be deduced from external morphology. However, this becomes a problem 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. Another general approach involves subtraction of sequences between one genome and another to allow selective isolation of sex-specific sequences. The basis of these approaches is that the homogametic sex contains all sequences that the heterogametic sex does, except for those in the vicinity of the sex-determination locus. Thus, in XY systems, female DNA can be subtracted from the male genome, to leave only male specific DNA sequences. Such subtraction methods include hybridization approaches (e.g. the Phenol-Enhanced Reassociation Technique (PERT); Kohne et al., 1977; Devlin et al., 1991) or PCR amplification (e.g. Representational Difference Analysis, RDA; Lisitsyn et al., 1993), and can improve the efficiency of identifying unique sequences many thousand fold, making feasible experiments searching for sex-limited DNA sequences. In cases where the mode of sex determination is known, subtraction procedures can be performed in one direction only (i.e. in XY systems, subtraction of male against female genomic DNA), but where this information is lacking subtraction must be performed bidirectionally. Once putative sex-specific DNA sequences have been isolated, they should be analyzed in defined families to ensure cosegregation with the sex-determination locus. In most cases, it will also be desirable to characterize the clones in detail to allow development of simple PCR diagnostics, to improve testing procedures (improve speed, and allow determination of genetic sex from minute, nonlethal samples of tissue or blood) and to assist in the detection of exceptional offspring (Du et al., 1993; Devlin et al., 1994; Matsuda et al., 1997; Iturra et al., 1998; Coughlan et al., 1999). Several genomic DNA subtraction techniques have been established successfully in various numbers of aquatic organisms.

Devlin et al. (1991) used subtractive hybridization methodologies to selectively clone a DNA fragment from the Y chromosome of chinook salmon (*Oncorhynchus tshawytscha*). This clone was used as probe and a single of 8-kb *Bam*HI restriction

fragment was specifically detected in Southern blots of male genomic DNAs but absent in females. This sex-specific pattern was observed in over 50 individuals from five separate chinook stocks, suggesting that the organization of this sequence is conserved in this species. The strength of the hybridization signal and the pattern observed in multiple enzyme digests indicated that this sequence was repeated in the male chinook genome. With long autoradiographic exposures, fainter additional sequences were also detected both in males and females, suggesting that other similar sequences not organized in a sex-specific fashion were found on the X chromosome and/or autosomes. Examination of the segregation of this DNA sequence in families showed that the male-specific pattern segregated from father to son, providing strong evidence that the DNA probe was from the Y chromosome. The application of Y-chromosomal probes to the commercial culture of this species would simplify the synthesis of new monosex strains and allow verification of existing genotypic female, phenotypic male stocks.

Drew and Brindley (1995) used representational difference analysis (RDA), which was a PCR-based modification of the subtractive hybridization process. It was employed to isolate female-specific sequences of *Schistosoma mansoni*. Using *Hin*dIII-derived amplicons, an excess of male DNA was employed to remove sequences common to both male and female adult genomes from female genomic DNA. Following three rounds of RDA, the enriched sequences including two female-specific repetitive elements were obtained. One of these exhibited 76% homology to the SMα family of retroposons and represents a W chromosome-specific variant of that family. The other sequence represented a novel, female-specific repetitive sequence. These sequences have been designated SMα fem-1 and W2, respectively, and both were apparently arrayed as tandem repeats on the W chromosome of *S. mansoni*. The isolation and characterization of the two female-specific sequences by RDA indicated that this procedure should also find utility in the definition of traits and sequences that differ among other groups of schistosomes.

Fujisawa et al. (2001) used RDA to isolate DNA markers for sex chromosomes of liverwort. The liverwort *Marchantia polymorpha* has X- and Y- chromosomes in the respective female and male haploids. This study reported the successful exploitation of representational difference analyses to isolate DNA markers for the sex chromosomes.

Two female-specific and six male-specific DNA fragments were genetically confirmed to originated from the X- and Y- chromosomes, respectively.

**1.8 Genome walking analysis**

DNA walking is a simple method for identifying unknown genomic DNA sequences adjacent to known sequences such as cDNA or characterized markers.

Initially, genomic DNA of interest species are separately digested with different rare or frequent blunt-end generating restriction endonucleases (usually, *Alu*I, *Dra*I, *Eco*RI, and *Hae*III). Following digestion, restricted products of each digestion are ligated with the GenomeWalker adaptor (Cato et al., 2001). The first amplification is carried out using the outer adaptor primer (AP1) and an outer gene-specific primer (GSP1) using ligated products as the template.

The primary PCR products are diluted and served as the template for the secondary PCR using the nested adaptor primer (AP2) and a nested gene specific primer (GSP2). The resulting products are electrophoretically analyzed by agarose gel electrophoresis (Figure 1.10). The major PCR product begins with known sequences at the 5’ end of the GSP2 and extends into the unknown adjacent genomic DNA can be cloned and analyzed. A new primer pairs can be designed from characterized sequence. The PCR product of different individuals can be firstly analyzed by single strand conformational polymorphism (SSCP) and subsequently by DNA sequencing.

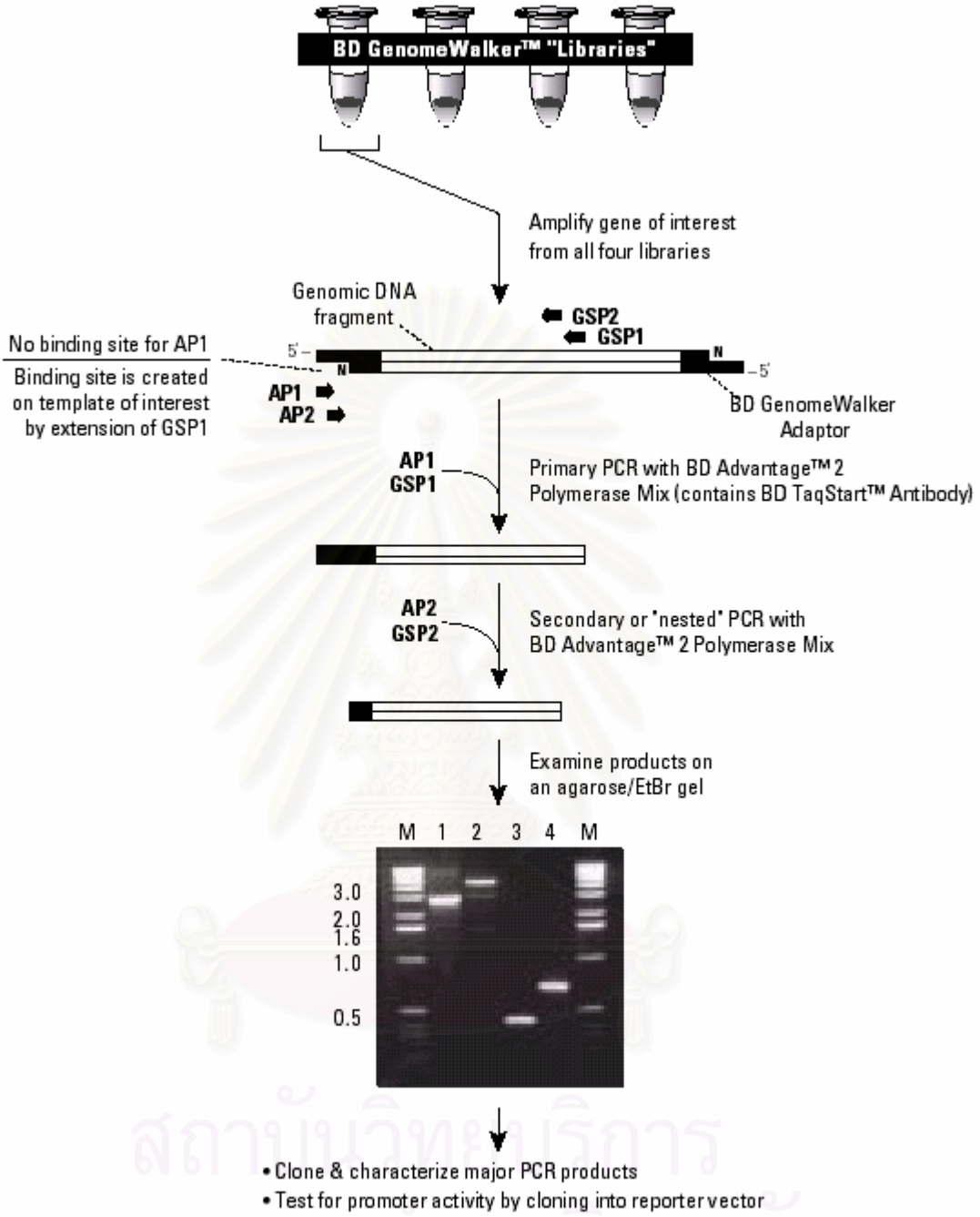



**Figure 1.10** Overview of the Genome Walking procedure. N: amino group that blocks extension of the 3' end of the adaptor-ligated genomic fragments. AP1: Adaptor primers. GSP: Gene-specific primers (Universal GenomeWalker$^{TM}$ Kit; CLONTECH, USA).

**1.9 Construction of genomic DNA library**

Gene cloning usually requires the initial preparation of multiple clones, each composed of a different DNA fragment, from the entire genome of an organism. A random collection of clones from a specific genome is called a genomic DNA library and may represent the total DNA of the organism. The basic steps involved in the preparation of a genomic DNA library were the digestion of the organism's DNA with a restriction endonuclease and the ligation of the fragment into a cloning vector. These recombinant DNA molecules are then transformed into an appropriate host cell. More details are shown in Figure 1.11. A genomic library may contain a desired gene, but identifying this clone among the thousands of clones from the library can sometimes be difficult. In recent years, some very innovative methods have been developed to simplify gene identification in genomic libraries.

In constructing a gene library, a large number of different clones must be generated to ensure that all DNA fragments are represented. This number is dependent upon the size of the cloned fragments, the size of the genome, and the probability that an individual clone will be in the library. The number of clones necessary to represent an entire genome can be calculated from the equation

$$N = \frac{In(1-P)}{In(1-f)}$$

When $N$ is the number of required recombinant clones, In is the natural log, $P$ is the desired probability of recovering a given fragment, and $f$ represents the fraction (in base pairs) of the genome present in each clone. This equation takes into consideration the need for overlapping clones to represent the library (Atherly et al, 1999).

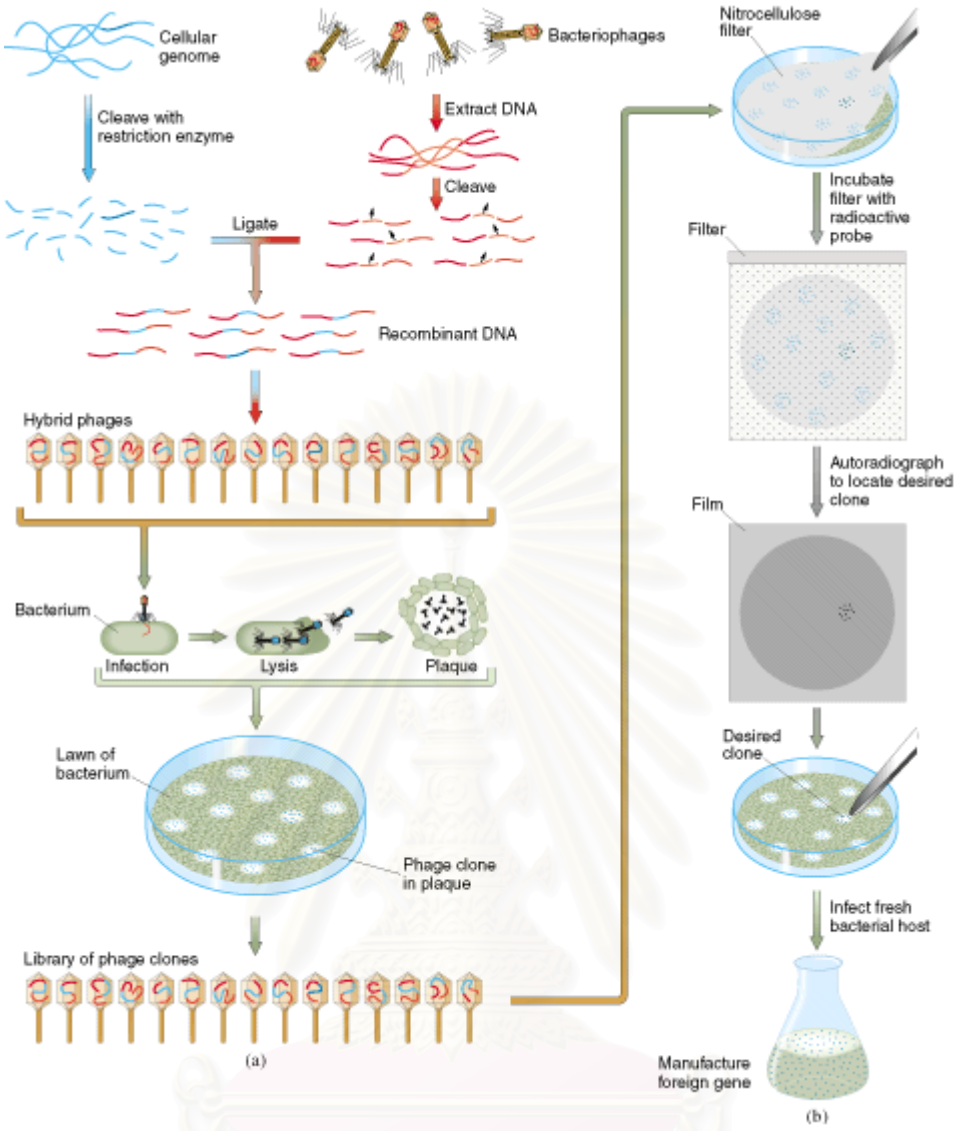



**Figure 1.11** Schematic of genomic DNA constructed. Preparing of recombinant clones, a. Recombinant clones detected with plaques hybridization analysis.

**1.10 The Objectives of the thesis**

The aims of this thesis are:

-To establish the genomic DNA subtraction between male and female of *P. monodon*,

-To identify and characterize candidate sex-specific markers obtained from genomic DNA subtraction analysis, and

-To construct male and female *P. monodon* genomic DNA libraries

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Equipments

- Autoclave : model HICLAVE, HVE – 50, HIRAYAMA, Japan
- Automatic micropipettes P2, P10, P20, P200 and P1000 (Gilson Medical Electrical S. A., France)
- Camera Pentax K1000 (Asahi Opt. Co., LTD., Japan)
- Centrifuge SORVALL® ULTRA Pro 80 (Goffin Meyvis, Netherland)
- Contherm Thermotec 2000 (Sanyo, Japan)
- -20°C Freezer (Whirlpool, USA)
- -30°C Freezer (Sanyo, Japan)
- -80°C Freezer (Sanyo, Japan)
- Gelmate 2000 (Toyobo, Japan)
- Horizontal Gel Electrophoresis Systems (GibcoBRL and BioRad, USA)
- Hybridization Oven : model Midi Dual 14 (Hybaid, England)
- Incubator (Sanyo, Japan)
- Laminar flow Cabinet : Nuaire Class II model NU-440-300E (USA)
- Microcentrifuge tube (Sorenson, USA)
- Microwave Power Boost 900 (Hitashi, Japan)
- PCR Thermal cycler
  - : PCR sprint (Hybaid, England)
  - : Omnigen (Hybaid, England)
  - : Perkin Elmer 9700 (USA)
- Pipette tips 10, 200 and 1000 μl
- Power supply (Bio-Rad Laboratories, USA)
  - : Power PAC300
  - : Power PAC Junior
  - : model 200/2.0

- Refrigerated centrifuge 3K18 (Sigma Osterode and Harz, Germany)
- Shaking incubator 1083 (GFL, Germany)
- Shaking 3015 (GFL, Germany)
- Spectrophotometer Spectronic Genesys 5, Milton Roy, Germany)
- UV Transilluminator M – 26 (UVP, USA)
- Vacuum Blotter Model 785 (Bio-Rad Laboratories, USA)
- Vacuum Pump (Bio-Rad Laboratories, USA)
- Water bath SBS30 (Stuart Scientific, UK)

### 2.1.2 Chemicals

- Absolute ethanol (BOH, England)
- Acetic Acid (Merck, Germany)
- Acrylamide (Sigma Chemical Co., USA)
- Agarose gel (FMC Bioproducts, USA)
- Bacto-agar (Oxoid, England)
- Bacto-tryptone (Oxoid, England)
- Bacto-yeast extract (Oxoid, England)
- Bis-Acrylamide (Promega Co., USA)
- Boric acid (Sigma Chemical Co., USA)
- Cetyl trimethyl ammonium bromide (CTAB) (Sigma Chemical Co., USA)
- Chloroform (Merck, Germany)
- 100 mM dATP, dCTP, dGTP and dTTP (SibEnzyme, Russia)
- Diethyl pyrocarbonate (DEPC) (Sigma Chemical Co., USA)
- DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany)
- Di-sodium hydrogen phosphate (Merck, Germany)
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid (EDTA) (Fluka Chemika, Switzerland)
- Formaldehyde (Labscan Asia Co., Thailand)
- Glucose (Sigma Chemical Co., USA)
- Improm-II$^{TM}$ Reverse Transcription System (Promega, USA)
- Isoamyl alcohol (Sigma Chemical Co., USA)
- Isopropanol (LabScan Asia Co., Ireland)

- KODAK GBX Developer and Replenisher (Kodak, USA)
- KODAK GBX Fixer and Replenisher (Kodak, USA)
- KODAK Medical X-ray Film General Purpose Blue (Kodak, USA)
- N-(2-hydroxyethyl) piperazine-N'-(3-propanesulfonic acid) (Sigma Chemical Co., USA)
- Phenol, redistilled (Aldrich Chemical Co., USA)
- QIAquick® Gel Extraction Kit (QIAGEN, Germany)
- Silver nitrate (Sigma Chemical Co., USA)
- Sodium acetate (Merck, Germany)
- Sodium hydroxide (Merck, Germany)
- Sodium carbonate (Sigma Chemical Co., USA)
- Sodium chloride (Sigma Chemical Co., USA)
- Sodium dodecyl sulfate (SDS) (Sigma Chemical Co., USA)
- Sucrose (Sigma Chemical Co., USA)
- Tri Reagent® (Molecular Research Center, Inc, USA)
- Tris-(hydroxyl methyl)-aminomethane (USB, Amersham Life Science, England)

### 2.1.3 Enzymes

- Calf intestinal phosphatase (Promega Corporation Medison, Wiscosin, USA)
- DyNAzyme™ DNA Polymerase (Finnzyme, Finland)
- Klenow fill-in (Stratagene, USA)
- Mung Bean nuclease (New England Biolabs, England)
- Proteinase K (Sigma Chemical Co., USA)
- Restriction endonucleases; *Alu*I, *Dra*I, *Eco*RI, *Hae*III, *Hind*III, *Mbo*I, *Rsa*I and *Xho*I (Promega Corporation Medison, Wiscosin, USA)
- RNase A (Sigma Chemical Co., USA)
- T4 DNA ligase (New England Biolabs, England)
- T4 DNA ligase (Promega Corporation Medison, Wiscosin, USA)
- T4 DNA ligase (Stratagene, USA)

### 2.1.4 DNA markers

- 100 base pair DNA ladder (SibEnzyme, Russia)

- Lambda DNA (Promega Corporation Medison, Wiscosin, USA)

### 2.1.5 Bacterial strains

- *Escherichia coli* strain JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi 8* (*lac-pro AB*) *F'* (*traD 36 proAB*$^{+}$ *lac*$^{q}$ *lacZ 8 M15*)
- *Escherichia coli* strain VCS257
- *Escherichia coli* strain XL1-Blue MRA [Δ(*mcrA*)*183 Δ*(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 gyrA96 relA1 lac* ($P_2$ lysogen)]

### 2.1.6 Cloning vectors

- Gigapack® III XL Packaging Extract Kit (Stratagene, USA)
- Lambda FIX II/ *Xho*I Partial Fill- In Vector Kit (Stratagene, USA)
- pGEM® -T easy vector (Promega Co., USA)

## 2.2 Samples

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



## 2.3 Nucleic acid extraction

### 2.3.1 DNA extraction

Genomic DNA was extracted from the tissue of each shrimp by the method of Winnepenninckx (1993) with modification. Detail of the method was as follow. Muscle (10 g) was dissected and immediately frozen in the mortar containing liquid nitrogen. The muscle was ground into fine powder using pestle. Muscle powder was added into 10 ml

of preheated (55°C) CTAB buffer (2% (w/v) CTAB, 0.1 M Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 0.2% (v/v) β-mercaptoethanol). The mixture was incubated for 15 minutes at 55°C with occasional gentle mixing to dissolve all nucleic acids. Proteinase K (1.5 mg) was then added and mixed by inversion. The mixture was incubated for 1-3 hours at 55°C (if tissue is not solubilized, added 150 μl of proteinase K and leave overnight at 55°C). The solution was gently extracted 2 times with chloroform:isoamyl alcohol (24:1). The aqueous phase of extracted solution was retained and isopropyl alcohol (2/3 volume) was added. The mixture was left for DNA to precipitate for a few minutes at room temperature. DNA was spooled and washed in 70% and 100% ethanol, respectively. DNA pellet was air-dried and resuspended in 200 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). RNase A was added to a final concentration of 100 μg/ml. The resulting mixture was then incubated at 37°C for 1 hour. DNA was extracted by adding an equal volume of Tris-Cl (pH 8.0) equilibrated phenol and mixed gently for 15 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a new sterile microcentrifuge tube and further extracted once with phenol and once with chloroform:isoamyl alcohol (24:1). One-tenth volume of 3 M sodium acetate (pH 5.2) was added. DNA was precipitated by adding two volume of ice-cold absolute ethanol and incubated at -80°C for 30 minutes. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 15 minutes at room temperature and briefly washed twice with 70% ethanol. DNA pellet was air-dried and resuspended in 100 μl of TE buffer. The DNA solution was incubated at 37°C for 1-2 hours for complete dissolution and kept at 4°C until further required.

**2.3.2 RNA extraction**

Total RNA was extracted from ovary and testis of each TRI REAGENT®. Tissue was dissectant immediately placed in mortar containing liquid nitrogen and ground the fine powder. The tissue powder was transferred to a microcentrifue tube containing 500 μl of TRI REAGENT® (1 ml/ 50-100 mg tissue) and homogenized. Additional 500 μl of TRI REAGENT® were added. The homogenate was left at room temperature for 5 minutes before adding 0.2 ml of chloroform. The homogenate was vortexed for 15 seconds, left at room temperature for 2–15 minutes, and centrifuged at 12000x *g* for 15 minutes at 4°C. The mixture was separated into the lower red, phenol-chloroform phase, the interphase, and the colorless upper aqueous phase. The aqueous phase inclusively

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

Total RNA was also extracted from other tissues including hemocytes and hepatopancreases of *P. monodon* using the same extraction procedure.

## 2.4 Determination of DNA and RNA concentration using spectrophotometry and gel electrophoresis

### 2.4.1 Spectrophotometry

DNA and RNA extracted from tissue samples was quantified by measuring the optical density of the solution at 260 nanometer ($OD_{260}$). An $OD_{260}$ of 1.0 was correspondent to a concentration of 50 μg/ml for double stranded DNA, 40 μg/ml for single strand RNA and 33 μg/ml for single strand DNA (Sambrook et al., 2001). Therefore, the concentration of DNA samples (μg/ml) were estimated in μg/ml by using the following equation,

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

The purity of DNA samples can be evaluated from a ratio of $OD_{260}/OD_{280}$. The acceptable ratios of approximately purified DNA and RNA were 1.8 and 2.0, respectively. The ratio lower than 1.8 indicated the contamination of residual proteins or organic solvents whereas the ratio greater than this value indicated the contamination of RNA in the DNA solution (Sambrook et al., 2001).

### 2.4.2 Electrophoresis

The quality of DNA and RNA were observed by analyzing the sample in agarose gel electrophoresis. The size and the amount of DNA were evaluated by comparing with lambda DNA.

## 2.5 Agarose gel electrophoresis

A 0.28 g of agarose gel (0.8% w/v) was weighed out and mixed with 35 ml of 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.3). The gel slurry was boiled in a microwave oven to complete solubilization, and allowed to approximately 60°C before pouring into the gel mold. A comb was inserted and the gel was left to solidify. When needed, the comb was carefully removed. The agarose gel was submerged in a chamber containing enough amount of 1x TBE buffer to cover the gel for approximately 0.5 cm. Appropriate volume from each DNA sample was mixed with the one-fifth volume of the 10x loading dye (0.25% bromophenol blue and 25% Ficoll in water) and loaded into each well. A 100 bp DNA ladder was used as the standard DNA marker. Electrophoresis was carried out at 5-6 volts/cm until bromophenol blue moved to approximately one-half of the gel. The electrophoresed gel was stained with an ethidium bromide solution (0.5 µg/ml) for 5-15 minutes and destained in running tap water to remove unbound ethidium bromide from the gel. DNA fragments were visualized under a UV transilluminator and photographed through a red filter using Fomapan Classic 100 film. The exposure time was 10-20 seconds.



## 2.6 Genomic DNA subtraction

In this study, genomic DNA subtraction was performed to identify genomic differences between male and female P. *monodon*. Genomic DNA subtraction consists of 2 types were phenol emulsion reassociation technique (PERT), based on cohesive restriction sites method, and representational difference analysis (RDA), PCR-based subtraction.

### 2.6.1 Phenol emulsion reassociation technique (PERT)

The method used in this study was adopted from the method of Devlin et al. (1991) with slight modification.

#### 2.6.1.1 Amplicon construction

Genomic DNA was extracted from male and female *P. monodon* (approximately 10 g body weight). A position selection of subtraction approach was carried out using genomic DNA of male as the tester and that of female as the driver and vice versa. Driver genomic DNA (250 μg) was randomly cut by sonication for 60 minutes and vortex for 15 minutes. Tester genomic DNA (1 μg) was completely digested with *Mbo*I in a 20 μl reaction volume containing 2 μl of 10x restriction buffer C (10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT), 0.2 μl of Acetylated BSA (10 μg/μl), 1 μg of tester genomic DNA and 0.5 μl of *Mbo*I (10 U/μl). The reaction mixture was incubated at 37°C for 1 hour.

#### 2.6.1.2 Hybridization

Denatured driver and tester DNA were hybridized in 2.5 ml of solution containing 1.25 M $NaClO_4$, 120 mM sodium phosphate, pH 6.8 and 12% phenol, pH 8.0 at room temperature for six days. After annealing, the hybridized mixture was extracted once with chloroform:isoamyl alcohol (50:1). The aqueous phase dialyzed against TE buffer, pH 8.0 and precipitated twice with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. The precipitate was washed in 70% ethanol, dried, and redissolved in TE buffer. This process yielded a population of double-stranded DNA molecules.

#### 2.6.1.3 Cloning of subtracted genomic DNA into pUC18

The fragments with sticky ends (non-hybridized DNA fragments) were selectively cloned into a pUC18 vector digested with restriction endonuclease *Bam*HI and treated with calf intestinal alkaline phosphatase.

**2.6.1.3.1 Preparation of pUC18**

A single colony of *E. coli* JM 109 containing pUC18 plasmid was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl, pH 7.0) with vigorous shaking at 37°C for 16 hours. After plasmid DNA was extracted.

**2.6.1.3.2 Extraction of plasmid**

Plasmid DNA was isolated by alkaline lysis method (Li et al., 1997) with modification. Transformed cell containing recombinant plasmid was inoculated in LB broth (3 ml) supplemented with amplicilin (150 μg) and incubated at 37°C for 16 hours with vigorous shaking. The culture was then poured into a microfuge tube and centrifuged at 12000 rpm for 30 seconds at 4°C. After centrifugation was complete, the medium was removed by aspiration, leaving the bacterial pellet as dry as possible. Bacterial pellet was resuspended in 200 μl of ice-cold GTE buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0) by vigorous vortexing. Freshly prepared alkaline lysis solution (0.2 N NaOH, 1% (w/v) SDS) (400 μl) was added to the bacterial suspension. The tube was closed tightly and the content was mixed by inverting the tube rapidly 5 times before placing on ice for 3-5 minutes. An ice-cold 3 M potassium acetate, pH 4.8 (300 μl) was added to the tube and alkaline lysis solution was dispersed through the viscous bacterial lysate by inverting the tube several times. The tube was kept on ice for 3-5 minutes prior to the centrifugation of the bacterial lysate at 12000 rpm for 5 minutes at 4°C. The supernatant was transfered to a fresh tube. Plasmid was extracted by mixing with an equal volume of Tris-Cl (pH 8.0) equilibrated phenol for 15 minutes. The mixture was centrifuged at 12000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a new sterile microcentrifuge tube and further extracted once with phenol and once with chloroform:isoamyl alcohol (24:1). One-tenth volume of 3 M sodium acetate (pH 5.2) was added. Plasmid was precipitated by an addition of two volume of ice-cold absolute ethanol and incubated at -80°C for 30 minutes. The precipitated Plasmid was recovered by centrifugation at 12000 rpm for 15 minutes at room temperature and briefly washed twice with 70% ethanol. The pellet was air-dried and resuspended in 50 μl of TE buffer. RNase A was added to a final concentration of 100 μg/ml to digest contaminating RNA. Plasmid DNA was incubated at 37°C for 1 hour and store at -20°C until further required.

**2.6.1.3.3 Digestion and dephosphorylation**

Closed circular pUC18 was digested with *Bam*HI in a 60 μl reaction volume containing 6 μl of 10x restriction buffer E (6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT), 0.6 μl of acetylated BSA (10 μg/μl), 5 μl of *Bam*HI (10 U/μl) and 10 μg of plasmid pUC18. The reaction mixture was incubated overnight at 37°C for 1 hour. An aliquot (0.1 μg) was removed and the extent to digestion was analyzed by electrophoresis through a 1.0% agarose gel containing ethidium bromide, using undigested plasmid DNA as a marker. If digestion was not complete, more restriction enzyme was added and the incubation was continued.

After digestion was complete, sample was extracted once with phenol/chloroform and the plasmid DNA was recovered by precipitation with one-tenth volume of 3 M sodium acetate (pH 5.2) was added. Plasmid DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and incubated at -80°C for 30 minutes. Plasmid DNA was recovered by centrifugation at 13000 rpm for 10 minutes at 4°C in a microfuge, and dissolved in 110 μl of 10 mM Tris-HCl (pH 8.3).

Removal of terminal 5'-phosphate groups was used to suppress self-ligation and circularization of plasmid DNA. To the remaining 90 μl of the linearized plasmid DNA, 10 μl of 10x calf intestinal phosphatase buffer (0.5 M Tris-HCl, pH 9.3, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine) and 6 μl of calf intestinal phosphatase (CIP) (1 U/μl) were added and the solution was incubated for 60 minutes at 37°C. The phosphatase activity was inactivated by heating to 65°C for 30 minutes (or 75°C for 10 minutes) in the presence of 5 mM EDTA (pH 8.0). The reaction mixture was cooled to room temperature, and then extracted once with phenol and once with phenol/chloroform. Plasmid DNA was recovered by precipitation with one-tenth volume of 3 M sodium acetate (pH 5.2) was added. Plasmid DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and incubated at -80°C for 30 minutes. Plasmid DNA was recovered by centrifugation at 13000 rpm for 10 minutes at 4°C in a microfuge. The pellet was washed with 70% ethanol at 4°C and then centrifuged. The supernatant was carefully removed and the open tube was left on the bench to allow the ethanol to evaporate. Precipitated plasmid DNA was dissolved in TE buffer (pH 8.0) at a concentration of 100 μg/ml. Plasmid DNA was stored in aliquots at -20°C.

**2.6.1.3.4 Ligation of DNA fragment into pUC18**

Subtracted DNA was ligated into the *BamH*I pre-cut pUC18 in a 50 µl reaction volume containing 25 µl of T4 DNA Ligase Buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 µg/ml BSA), 4 µl of T4 DNA ligase (400 weiss unit/µl), 300 ng of pre-cut pUC18 vector and 500 ng of DNA insert. The reaction mixture was incubated overnight at 4°C and then transformed into *E. coli* JM 109.

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

**2.6.1.3.5.1 Preparation of competent cells**

A single colony of *E. coli* JM 109 was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl, pH 7.0) with vigorous shaking at 37°C for 16 hours. The starting culture was then inoculated into 50 ml of LB broth and continued culture at 37°C with shaking to an $OD_{600}$ of 0.5 to 0.8. The cells were briefly chilled on ice for 10 minutes, and recovered by centrifugation at 2700x *g* for 10 minutes at 4°C. The pellets were resuspended in 30 ml of ice-cold $MgCl_2/CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged as above. After the cells were resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$, the concentrated cell suspension was devided to 200 µl aliquots. These competent cells were either used immediately or stored at -80°C for subsequently used.

**2.6.1.3.5.2 Transformation**

This method was adopted from the method of Sambrook et al., 2001. Competent cells were thawed on ice for 5 minutes. Two to four microlitres of the ligation mixture were added and gently mixed by pipetting and left on ice for 30 minutes. The transformation reaction was heat-shocked in a 42°C water bath for exactly 1 minute without shocking. The reaction tube was immediately placed in ice for 2-3 minutes. The mixture were removed from the tubes and added to a new tube containing 1 ml of prewarmed SOC (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). The cell suspension was incubated with shaking at 37°C for 90 minutes. The mixture was centrifuged for 20 seconds at room temperature, gently resuspended in 100 µl of SOC medium and spreaded

onto a selective LB agar plates containing 50 μg/ml of ampicillin, 25 μg/ml of IPTG and 20 μg/ml of X-gal and further incubated at 37°C for 16 hours. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

**2.6.1.4 Detection of recombinant clone by colony PCR and restriction endonuclease digestion**

An interesting colony was picked by a pipette tip and served as the template in PCR reaction. The reaction was performed in a 25 μl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 100 μM of each dNTP, 2 mM $MgCl_2$, 0.1 μM each of pUC1 (5'-CCG GCT CGT ATG TTG TGT GGA-3') and pUC2 (5'-GTG GTG CAA GGC GAT TAA GTT GG-3'), 0.5 unit of Dynazyme$^{TM}$ II DNA Polymerase (2 U/μl). PCR was carried out in a thermocycler consisting of predenaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute and extension at 72°C for 1.5 minutes. The colony PCR products were electrophoresed though 1.2% agarose gel and visualized under a UV transilluminator after ethidium bromide staining.

Colony PCR products containing an insert were separately digested with *Eco*RI and *Hin*dIII in a 15 μl reaction volume containing 1.5 μl of 10x restriction buffer E (6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT), 0.15 μl of Acetylated BSA (10 μg/μl), 0.3 μl each of *Eco*RI (12 U/μl) and *Hin*dIII (10 U/μl), 4 μl of the colony PCR product. The reaction mixture was incubated at 37°C for 16 hours. The reactions were electrophoresed though 1.2% agarose gel and visualized under a UV transilluminator after ethidium bromide staining.

**2.6.1.5 Isolation of recombinant plasmid**

Plasmid DNA was isolated by alkaline lysis method (Li et al., 1997) with modification as procedure described above in Extraction of plasmid.

**2.6.1.6 Elution of DNA fragments from agarose gel**

The required DNA fragment was fractionated through agarose gels in duplication. One was run side-by-side with a 100 bp DNA marker 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 stained with ethidium bromide (0.5 μg/ml) for 5 minutes. Position of the DNA marker and the ethidium bromide stained fragment were used to align the position of the non-stained target DNA fragment.

The desired DNA fragment was excised from the agarose 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 volumes of the QG buffer was added and mixed by inversion of the tube. The mixture was incubated at 50-55°C for 10 minutes or until the gel slice was completely dissolved. The mixture was transferred into a QIAquick column inserted in a 2 ml collection tube and centrifuged at 12000 rpm for 1 minute. The flow-though solution was discarded. Another 0.5 ml of the QG buffer was added to the QIAquick column and recentrifuged for 1 minute. After this step, 0.75 ml of the PE buffer was added to the QIAquick column and centrifuged as above. The flow-though solution was discarded. The column was recentrifuged to remove the trace amount of the washing solution. The QIAquick column was then placed into a sterile 1.5 ml microcentrifuge tube. DNA was eluted out by an addition of 15 μl of EB buffer (10 mM Tris-HCl, pH 8.5) or $H_2O$ to the center of the QIAquick membrane and left for 1 minute, before centrifuged at 12000 rpm for 1 minute. The eluted sample was stored at -20°C until further reqired.

**2.6.1.7 DNA sequencing**

The recombinant plasmid was unidirectional sequenced for undirectional sequence 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 BioSciences).

**2.6.1.8 Data analysis**

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

### 2.6.2 Representational difference analysis (RDA)

The method used in this study was adopted from the method of Drew and Brindley (1995) with modification.

#### 2.6.2.1 Amplicon construction

RDA of genomic DNA from male and female *P. monodon* was carried out using genomic DNA of male as the tester, that of female as the driver, and vice versa.

##### 2.6.2.1.1 Restriction digestion of genomic DNA

Genomic DNA from male and female *P. monodon* was digested separately with *Hin*dIII. Each digestion reaction was conducted in a 20 µl volume containing 2 µl of 10x restriction buffer E (6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT), 0.2 µl of Acetylated BSA (10 µg/µl), 1 µl of *Hin*dIII (10 U/µl) and 1 µg of genomic DNA. The reaction mixture was incubated at 37°C for 16 hours. An aliquot (2 µl) was removed and analyzed the extent to digestion by electrophoresis through a 1.0% agarose gel containing ethidium bromide. After the digestion was complete, the enzyme was heat inactivated at 65°C for 25 minutes.

##### 2.6.2.1.2 Ligation of adaptors

A 24-bp (RHind24, 5'-AGC ACT CTC CAG CCT CTC ACC GCA-3') and a 12-bp oligonucleotides (RHind12, 5'-AGC TTG CGG TGA-3') (0.5 nmol each) were added and annealed in a 30 µl reaction volume containing 3 µl of T4 DNA ligase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 µg/ml BSA) and 1 µg of genomic DNA digestion. The reaction was cooled down from 50°C to 10°C over a period of 1 hour. After these oligonucleotides from a *Hin*dIII overhang were annealed, T4 DNA ligase (20 U) was added and the solution was incubated at 16°C for 16 hours.

#### 2.6.2.2 PCR amplification

Oligomer RHind24 was used as a PCR primer. The amplification reaction was performed in a 50 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% TritonX-100, 400 µM of each dNTP, 1.5 mM $MgCl_2$, 1 µM of RHind24 and 50 ng of ligation mixture. The PCR reaction was heated at 72°C for 3 minutes prior to the

addition of 1 unit of Dynazyme$^{TM}$ DNA Polymerase and then a further 5 minutes at 72°C before 20 cycles of 95°C for 1 minute and 72°C for 3 minutes. Finally, the reaction was incubated at 72°C for 10 minutes. As differences were to be isolated from the tester amplification product, this was designated the tester amplicon according to the convention of Lisitsyn et al. (1993). The driver amplification product which was to be used to remove common sequences was designated the driver amplicon. Ten 200 μl volumes of driver DNA were amplified as above to yield approximate 250 μg of driver. The driver was digested with 200 U of *Hin*dIII at 37°C for 6 hours. After that, the enzyme was heat inactivated at 65°C for 25 minutes, extracted with phenol/chloroform, ethanol precipitated, and dissolved in TE buffer. One 200 μl volume of tester DNA was amplified as above. The tester DNA was then extracted with phenol/chloroform, ethanol precipitated, and dissolved in TE buffer. One microgram of tester was digested with *Hin*dIII (10 U) for 2 hours at 37°C and the enzyme was heat inactivated. Subsequently, the digested tester was ligated to 0.5 nmol JHind24 (5'-ACC GAC GTC GAC TAT CCA TGA ACA-3') and JHind12 (5'-AGC TTG TTC ATG-3') oligonucleotides as outlined above.

**2.6.2.3 Subtractive hybridization**

Tester DNA (500 ng) ligated to JHind oligonucleotides was ethanol precipitated along with 40 μg of driver DNA. The precipitate was then dissolved in 4 μl of 3x HE buffer (1x HE; 10 mM Hepps (N-[2-hydroxyethyl]piperazine-N'-[3-propanesulfonic acid]), 1 mM EDTA, pH 8.0). The mixture was denatured at 95°C for 5 minutes, followed by the addition of 1 μl of 5 M NaCl, and the mixture was incubated at 67°C for 20 minutes. The hybridization mixture was diluted to 30 μl with 3x HE/ 5 M NaCl (4:1). Six microliter of the diluted hybridization solution was employed as the template for PCR in a 400 μl PCR reaction inwhich dNTP, $MgCl_2$, Dynazyme$^{TM}$ II DNA Polymerase and 10x buffer were identical to those used for the manufacture of the driver and tester. The mixture was heated to 72°C for 5 minutes and the 24 bp primer JHind24 was added. The content of the tube was subjected to PCR amplification for 10 cycles of 95°C for 1 minute, 70°C for 3 minutes and the last step at 72°C for 10 minutes.

**2.6.2.4 Removal of single-stranded PCR products**

PCR reaction was digested with mung bean nuclease in a 50 μl reaction volume containing 5 μl of 10x mung bean nuclease buffer (1x buffer; 50 mM sodium acetate, pH 5.0, 30 mM NaCl and 1 mM $ZnSO_4$), 40 μl of PCR product and 20 U of mung bean nuclease (10 U/μl). The reaction mixture was incubated at 37°C for 30 minutes to remove single-stranded PCR products, diluted to 200 μl with 50 mM Tris-HCl (pH 8.0), and heated at 95°C for 5 minutes to inactivate the enzyme. The mung bean nuclease-digested products (40 μl) were subjected to 20 cycles of PCR, as above.

The products of this subtractive PCR process, expected to represent genomic differences between the male and female *P. monodon*, were extracted with phenol/chloroform, ethanol precipitated and dissolved in TE buffer.

**2.6.2.5 Change of adaptors on tester amplicon**

Change of adaptors was necessary to enable only self-annealed tester amplicon molecules to be amplified after hybridization. Subtracted DNA (1 μg) was digested with *Hin*dIII, the enzyme heat inactivated and the DNA ligated to NHind24 (5'-AGG CAG CTG TGG TAT CGA GGG AGA-3') and NHind12 (5'-AGC TTC TCC CTC-3') oligonucleotides (begin to the second round) as for RHind and JHind oligonucleotides. 100 ng of DNA from this ligation was hybridized to 40 μg of driver, and amplified by PCR using NHind24 as primer as for the previous round of subtraction with JHind oligonucleotides.

**2.6.2.6 Ligation of PCR products to the pGEM®-T easy vector**

DNA fragments were ligated to the pGEM®-T easy vector in a 10 μl reaction volume containing 5 μl of 2x Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% polyethylene glycol; MW 8000), 3 weiss units of T4 DNA ligase, 25 ng of pGEM®-T easy vector and 50 ng of DNA insert. The reaction mixture was incubated at 4°C for 16 hours before transforming to *E. coli* JM109. Recombinant clones were detected by isolation and digestion of recombinant plasmid DNA. Alternatively, Insert DNA from JM109 containing recombinant plasmid was determined by colony PCR.

#### 2.6.2.7 Digestion of recombinant plasmid DNA

The insert size of each recombinant plasmid was examined by digesting the plasmid with *Eco*RI. The digestion was carried out in a 15 µl reaction volume containing 1x restriction buffer H (90 mM Tris-HCl, pH 7.5, 10 mM NaCl and 50 mM $MgCl_4$), 1 µg of recombinant plasmid and 2-3 unit of *Eco*RI (12 U/µl) and incubated at 37°C for 3 hours. The digestion was analyzed by agarose gel electrophoresis.

## 2.7 First strand cDNA synthesis

First strand cDNA was synthesized from 1 µg of total RNA extracted from ovaries and testes using an ImProm-II$^{TM}$ Reverse Transcription System Kit. Total RNA was combined with 0.5 µg of oligo ($dT_{18}$) and appropriate DEPC-treated $H_2O$ in a final volume of 5 µl. The reaction was incubated at 70°C for 5 minutes and immediately placed on ice for at least 5 minutes. Then 5x reaction buffer, $MgCl_2$, dNTP Mix, and RNasin was added to the final concentrations of 1x, 2.25 mM, 0.5 mM and 20 units, respectively. Finally, 1 µl of ImProm-II$^{TM}$ reverse transcriptase was added and gently mixed by pipetting. The reaction mixture was incubated at 25°C for 5 minutes and 42°C for 90 minutes. The reaction was terminated by incubated at 70°C for 15 minutes to terminate the reverse transcriptase activity. The concentration of first stranded cDNA was spectrophotometrically examined at $OD_{260}$. The quality of cDNA was determined by the ratio of $OD_{260}$ and $OD_{280}$, and electrophoretically analyzed in 1.0% agarose gel. The first stranded cDNA was diluted to 500 ng/µl and kept at 20°C until required.



## 2.8 Primer design

PCR primers were designed from the sequences of candidate sex markers obtained from genomic DNA subtraction using Primer Premier 5.0 program with the following criteria: the length of the primers were between 18-25 bases, the melting temperatures were between 55-70°C and less than 5°C differences of the melting temperature of a primer pair (Table 2.1).

**Table 2.1** Sequences and melting temperatures of primers designed from candidate markers obtained from genomic DNA subtraction of *P. monodon*

| Primer | Sequences | Length (bp) | Tm (°C) |
|---|---|---|---|
| PMMSH6-F | 5'-CAA AGA AAC GGT CCA GTC AG-3' | 20 | 55.4 |
| PMMSH6-R | 5'-CTC TTG GGT GGG GGT TGG-3' | 18 | 60.4 |
| PMFFJ200-F | 5'-AGG AGG CTA TCA AAA CCA AGA-3' | 21 | 56.1 |
| PMFFJ200-R | 5'-TTG TAC CTT TCA CCT GCG GT-3' | 20 | 58.0 |
| PMFFJ800-F | 5'-GGA GGA GGA GCA GAT TTA GG-3' | 20 | 55.6 |
| PMFFJ800-R | 5'-CTG TCT GTG GTC GTC CGT T-3' | 19 | 55.8 |

**2.9 Polymerase chain reaction**

PCR reaction was performed in a 25 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% TritonX-100, 100 µM of each dNTP, 2 mM MgCl2, 0.1 µM of each primer, 1 unit of Dynazyme$^{TM}$ DNA Polymerase and 25 ng of genomic DNA of male and female *P. monodon* (or 500 ng of the first strand cDNA). PCR was carried out in a thermocycler consisting of predenaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1.5 minutes. The final extension was carried out at 72°C for 7 minutes.

The amplification products were electrophoretically analyzed through 1.2% agarose gels and visualized under a UV transilluminator after ethidium staining. Successful amplification products were further characterized follwing standard single strand conformational polymorphism (SSCP) to examine whether fixed single nucleotide polymorphisms (SNPs) was existent for each gender.

An estrogen receptor was amplified by ER-F/R (4 primer recombinations). PCR reaction was performed in a 25 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8,

50 mM KCl, 0.1% TritonX-100, 100 µM of each dNTP, 2 mM $MgCl_2$, 0.1 µM of each primer, 1 unit of Dynazyme$^{TM}$ DNA Polymerase and 25 ng of male and female P. monodon (or 500 ng of the first strand cDNA). PCR was carried out in a thermocycler consisting of predenaturation at 94°C for 3 minutes followed by 5 cycles of denaturation at 94°C for 1 minute, annealing at 45°C for 1 minute and extension at 72°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes. The final extension was carried out at 72°C for 7 minutes.

**Table 2.2** Sequences and melting temperatures of primers designed from sequence alignment of estrogen receptors from a number of fish species.

| Primer | Sequence | Length (bp) | Tm (°C) |
|---|---|---|---|
| ER-F1 | 5'-GCN WSN GGN TAY CAY TAY GG-3' | 20 | 62 |
| ER-F2 | 5'-CAN AAY CAR TGY CAN ATH GA-3' | 20 | 56 |
| ER-Rα | 5'-TCR AAD ATY TCN GCC ATN CC-3' | 20 | 59 |
| ER-Rβ | 5'-ACY TCN ARC CAR CAR CAY TC-3' | 20 | 60 |



2.10 Genome walking analysis

2.10.1 Digestion of genomic DNA

Construction of genome walk DNA libraries were performed with very clean, high-molecular weight genomic DNA. For each library construction, a total of five reactions were set up. For experimental genomic DNA, four blunt-end digestions were set up; one for each blunt-end restriction enzyme provided. Additionally, one *Pvu*II digestion of human genomic DNA was set up as a positive control.

Five 1.5-ml tubes were labelled: *Alu*I, *Dra*I, *Hae*III, *Rsa*I and positive control. For each reaction, the following were combined in a separate 1.5-ml tube to total volume of 100 μl: 10 μl of restriction enzyme buffer, 2.5 μg of genomic DNA and 80 units of restriction enzyme. The reaction was mixed gently by inverting tube and incubated at 37°C for 3 hours. The reaction was vortexed at slow speed for 5-10 seconds then returned to 37°C overnight. From each reaction tube, 2 μl was removed and run on a 0.8% agarose gel to determine whether digestion was complete.

**2.10.2 Purification of genomic DNA**

An equal volume of phenol was added to each reaction tube, vortexed at slow speed for 5-10 seconds, and centrifuged briefly to separate the aqueous and organic phases. Using a pipette, the upper (aqueous) layer was transferred into a fresh 1.5-ml tube and the lower (organic) layer was properly discarded into the chlorinated hazardous waste. To each tube, an equal volume of chloroform was added, vortexed at slow speed for 5-10 seconds, and centrifuged briefly to separate the aqueous and organic phases. Using a pipette, the upper (aqueous) layer was transferred into a fresh 1.5-ml tube and the lower (organic) layer was properly discarded into the chlorinated hazardous waste. To each tube, 2.5 volumes of ice cold 95% ethanol, 1/10 volume of 3 M sodium acetate (pH 4.5), and 20 μg of glycogen were added. The mixture was vortexed at slow speed for 5-10 seconds and centrifuged at 15000 rpm for 10 minutes. Supernatant was discarded before the pellet was washed in 100 μl of ice cold 80% ethanol and centrifuged at 15000 rpm for 5 minutes. Supernatant was decanted and the pellet was air-dried. The pellet was dissolved in 10 μl of TE buffer and vortexed at slow speed for 5-10 seconds. From each reaction tube, 1 μl was removed and run on a 0.8% agarose gel to determine the approximate quantity of DNA after purification.

**2.10.3 Ligation of genomic DNA to genome walker adaptors**

For each library construction, a total of five ligation reactions were set up. From each tube, 0.5 μg of digested, purified DNA was transferred to a fresh 0.5-ml tube and the following to total volume of 10 μl: 9 μM of genome walking adaptor, 5 μl of ligation buffer and 3 units of T4 DNA ligase were added. The reaction was incubated at 16°C for 16 hours. The mixture was incubated at 70°C for 5 minutes to stop the reactions, added with 30 μl of TE buffer, and vortexed at slow speed for 10-15 seconds.

**2.10.4 Procedure for PCR-based DNA walking in genome walk libraries**

Primary PCR master mix was prepared for all nine reactions plus one additional tube. The followimg reagents were combined in an 0.2-ml tube to total volume of 50 µl: 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% TritonX-100, 200 µM of each dNTP, 1.1 mM $MgCl_2$, 0.2 µM of AP1 primer, 0.2 µM of Forward (or Reverse) primer (primer designed from genomic DNA subtraction) and 1 unit of Dynazyme™ DNA Polymerase. The reaction tube was mixed well by vortexing (without introducing bubbles) and briefly centrifuged. The primary PCR master mix (49.5 µl) was transferred to the appropriately labeled tubes and 0.5 µl of each DNA library was also added to the appropriately labeled tubes whereas 0.5 µl of $dH_2O$ was added to the nagative control. The tubes were briefly centrifuged before the PCR reaction was carried out in a thermocycler using the following two-step cycle parameters: 7 cycles at 94°C for 25 seconds and 72°C for 3 minutes followed by 32 cycles at 94°C for 25 seconds and 67°C for 3 minutes. The final extension was carried out at the same temperature for 7 minutes. The primary PCR products (5 µl) were analyzed on a 1.5% agarose gel, along with a 100 bp DNA ladder.

### 2.11 Standard single strand conformational polymorphism (SSCP) analysis

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).

#### 2.11.1 Non-denaturating polyacrylamide gel electrophoresis

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

#### 2.11.2 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 (4 µl of Bind silane, 995 µl of 95% ethanol and 5 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.

### 2.11.3 Preparation of SSCP gel

The glass plates (PROTEIN II xi Cell) were cleaned and prepared as described previously. Different concentration of low crosslink non-denaturing polyacrylamide gel (37.5:1 or 75:1 of acrylamide and bis-acrylamide) were prepared by dilution of a 40% stock solution to required gel concentration. The acrylamide gel solution (30-40 ml) were mixed with glycerol (5% 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.

2.11.4 Electrophoresis

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

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

### 2.11.5 Silver staining

The gel plates were carefully separated using a plastic wedge. The long glass plate with the gel was placed in a plastic tray containing 1.5 litres of the fix/stop solution and agitates well for 25-30 minutes. The gel was soaked with shaking 3 times for 2 minutes with deionized water. The gel was lifted out from the tray between each wash and allowed the washed water draining out of the gel for 4 seconds. The gel was transferred to 0.1% silver nitrate (1.5 litres) and incubated with agitation at room temperature for 30 minutes. The gel was soaked in 1.5 litres of deionized water with shaking (10 forward and 10 backward agitations) and immediately placed in the tray containing 1.5 litres of the chilled developing solution. This step was 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 band was visible (usually 1.5-2 minutes). The gel was then transferred to another tray containing 1.5 litres of chilled developer and shaked until bands from every lane were observed (usually 2–3 minutes). One litre of the fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was left at room temperature.

**2.12 Southern blot analysis**

The purpose of Southern blotting and hybridization was to detect the presence or absence of specific gene fragments in a complex mixture. The length of DNA restriction fragments harboring specific genes was determined.

**2.12.1 Genomic DNA digestion**

Male and female genomic DNA (2.5 μg) was completely digested with *Dra*I in a 100 μl reaction volume containing 10 μl of 10x restriction buffer (10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT), 1 μl of Acetylated BSA (10 μg/μl), 2.5 μg of genomic DNA and 5 μl of *Dra*I (10 U/μl). The reaction mixture was incubated at 37°C for 16 hours.

**2.12.2 DNA labeling**

DNA fragments (1 μg) in 16 μl of $dH_2O$ was added into a sterile 1.5 ml microcentrifuge tube and heated in boiling water for 10 minutes before it was immediately placed on ice for 5 minutes. DIG-High Prime (4 μl) was added, mixed well, and incubated at 37°C for 20 hours. The reaction was inactivated by adding 2 μl of 0.2 M EDTA (pH 8.0) and heating to 65°C for 10 minutes. The labeled DNA was stored at -20°C until further required.

**2.12.3 Transfer of target DNA to membrane**

Target DNA was transferred to membrane by the 3-stage procedure; gel pretreatment, vacuum transfer, and post transfer.

**2.12.3.1 Gel pretreatment**

After target DNA was run on the agarose gel electrophoresis, the gel was submerged in a tray containing 0.25 N HCl with gentle shaking for 10 minutes. After the 0.25 N HCl solution was discarded, the gel was rinsed twice with deionized distilled water. The gel was then denatured in 0.5 N NaOH/ 1.5 M NaCl with gentle shaking for 15 minutes (2 times). After the 0.5 N NaOH/ 1.5 M NaCl solution was discarded, the gel was rinsed twice with deionized distilled water. If the pH of the gel is > 9 during the transfer, the gel was then neutralized in 0.5 M Tris-HCl (pH 7.5)/ 1.5 M NaCl with gentle shaking for 15 minutes (2 times).

**2.12.3.2 Vacuum transfer**

Precut nylon membrane was wet in double distilled water by slowly lowering the membrane at a 45 degree angle to the water. Then, the membrane and the filter paper were wet in 10x SSC. The wetted filter paper was placed on the Porous Vacuum Plate and the filter paper was laid in the area where the cut window of the Gasket was. The wetted membrane was then placed on top of the filter paper. The bubbles were removed by rolling a 10 ml glass pipette over the membrane. The Reservoir Seal O-ring was wet with water before the Window Gasket was placed on top of the membrane/filter paper. The gel was gently placed with well side up on top of the Window Gasket, leaving the gel edges to overlap the Window Gasket by at least 5 mm. The Sealing Frame was then placed on top of the Vacuum Stage and locked by the four latch posts. Before applying the vacuum to start blotting, the Bio-Rad Vacuum Pump was prewarmed for 10 minutes and the

Vacuum Regulator bleeder valve was unscrewed counter-clockwise several turns to prevent strong initial vacuum. The vacuum pump was then started and the bleeder valve was slowly turned clockwise until the gauge reads at 5 inches of Hg. Gentle pressure was applied with a finger on top of the gel along the window border to form a tight vacuum seal between the gel and the Window Gasket. One and a half liter of 10x SSC was gently poured into the upper reservoir without displacing the gel. Finally, the lid was placed on top of the Vacuum Blotter. During 90 minutes of the gel transferring, the buffer level was occasionally checked to make sure that it was higher than the gel and the vacuum pressure was adjusted to 5 inches of Hg as needed.

**2.12.3.3 Post transfer**

After 90 minutes, the vacuum source was turned off and the Sealing Frame spring handle was squeezed inward to release it from the latch posts. The Sealing Frame was removed to let the upper buffer drain into the Base Unit. The transferred gel was removed and stained with 1.0 μg/ml of Ethidium Bromide to check for any remaining DNA. After Window Gasket was displaced, nylon membrane was removed, soaked in 2x SSC for 5 minutes, air-dried between two sheets of filter paper, and dried in a vacuum oven at 80°C for 2 hours. At this stage, the blotted membrane was ready for hybridization.

**2.12.4 DNA hybridization**

The procedure consisted of 3 stages including prehybridization, hybridization, and stringency washing. For prehybridization, the blotted membrane was incubated in 10 μl of DIG Easy Hyb (10 ml/100 $cm^2$ filter) to hybridization temperature (42°C) for 30 minutes with gentle agitation in a roller bottle. DIG-labeled DNA probe (about 25 ng/ml DIG Easy Hyb) was denatured by heating to 100°C for 5 minutes and immediately chilled on ice. Add denatured DIG-labeled DNA probe to pre-heated DIG Easy Hyb (3.5 ml/100 $cm^2$ membrane) and mix but avoid foaming (bubbles may lead to background).

For hybridization step, prehybridization solution was poured off and probe-hybridization solution mixture was added. The membrane was hybridized with gentle agitation at 42°C for at least 16 hours.

After the hybridization was terminated, membrane was subjected to stringency washing by rinsing two times (5 minutes each) with 2x SSC, 0.1% SDS at room temperature and two times (15 minutes each) with 0.5x SSC, 0.1% SDS at 65°C.

**2.12.5 Immunological detection**

After stringency washes, membrane was rinsed briefly (1-5 minutes) in Maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl) containing 0.3% Tween 20 and incubated in blocking solution 100 ml with shaking for 30 minutes at room temperature. Membrane was then incubated for 30 minutes in antibody solution (anti-DIG-AP conjugate in blocking solution with the dilution of 1:10,000) with gentle shaking at room temperature. The membrane was washed two times for 15 minutes each with Maleic acid buffer containing 0.3% Tween 20. Membrane was equilibrated for 2-5 minutes in Detection buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl). Place membrane with DNA slide facing up on a development folder (or plastic bag) and apply 1 ml CSPD ready-to-use. Immediately cover the membrane with the second sheet of the folder to spread the substrate evenly and without airbubbles over the membrane. Incubate for 5 minutes at 15-25°C. Squeeze out excess liquid and seal the edges of the development folder. Incubate the damp membrane for 10 minutes at 37°C to enhance the luminescent reaction. Expose to an appropriate imager for 5-20 minutes or to X-ray film for 15-25 minutes at 15-25°C.

**2.12.6 Reprobe**

Membrane was rinsed thoroughly in double distilled water and washed twice for 15 minutes at 37°C in 0.2 M NaOH containing 0.1% SDS to remove the DIG-labeled probe. Membrane was rinsed again thoroughly for 5 minutes in 2x SSC. Prehybridization and hybridization with a second probe wre then carried out.



**2.13 Construction of genomic DNA library**

**2.13.1 Partial digestion of genomic DNA**

The method used in this study was adopted from the method of Sambrook et al. (2001) with slight modification.

**2.13.1.1 Pilot reactions**

Pilot reactions using the same batch of genomic DNA used to prepare fragments for cloning was set up. High-molecular-weight DNA (30 μg) was diluted by adding 900 μl of 10 mM Tris-HCl (pH 8.0) and 100 μl of the appropriate 10x restriction enzyme buffer. A sealed glass capillary was used to mix the solution gently. This mixing ensured that the high-molecular-weight DNA was distributed evenly throughout the restriction enzyme buffer. After mixing, the diluted DNA was stored for 1 hour at room temperature to allow any residual clumps of DNA to disperse. A series of microfuge was labelled tubes 1 through 10. A wide-bore glass capillary or disposable plastic pipette tip was used to transfer 60 μl of the DNA solution to a microfuge tube (Tube 1). DNA solution (30 μl) was transferred to each of nine additional labeled microfuge tubes. The tubes were incubated on ice. After adding 2 units of *Mbo*I to Tube 1, 30 μl of the reaction was transferred to the next tube in the series, mixed as before, and continued transferring to successive tubes. Nothing was added to the tenth tube (the no enzyme control), but 30 μl from the ninth tube was discarded. The reactions were incubated for 1 hour at 37°C followed by the inactivation of the restriction enzyme by heating the reactions to 70°C for 15 minutes. The reactions were cooled to room temperature and the appropriate amount of sucrose gel-loading buffer was added. Wide-bore plastic pipette tip or a disposable wide-bore glass capillary was used to transfer the solution to the wells of a 0.6% agarose gel to the lanes of an agarose gel for pulsed-field electrophoresis and electrophoresis was performed.

**2.13.1.2 Preparative reactions**

**2.13.1.2.1 Preparation of the gradient**

Sucrose gradients (10 ml of 10 and 40% w/v) were prepared in clear ultracentrifuge tubes. The gradients were stored for 1-2 hours at 4°C. A series of digestion was set up, each containing 100 μg of high-molecular-weight DNA. Three different concentrations of restriction enzyme that straddle the optimal concentration determined in the pilot experiments were used. The restrictions were incubated at 37°C for 1 hour with

*Mbo*I. An aliquot of the partial digested DNA was analyzed by gel electrophoresis to ensure that the digestion has worked according to prediction. Until the results were available, the remainder of the sample was stored at 4°C. The digested DNA was gently extracted twice with phenol/chloroform. The DNA was recovered by standard precipitation with ethanol and dissolved in 200 μl of TE (pH 8.0).

**2.13.1.2.2 Size fractionation of DNA through the gradient**

DNA sample (100 μg) was heated for 10 minutes at 68°C, cooled to 20°C, and gently layered on the top of the gradient before centrifuged at 25500 rpm for 22 hours at 20°C. A 21 gauge needle or a gradient fractionation device was used to puncture the bottom of the tube and 500 μl fractions were collected.

**2.13.1.2.3 Removal of sucrose**

The method used in this study was adopted from the method of Boulnois (1988) with slight modification.

Two volumes of distilled water, 0.1 times the new volume of 3 M Sodium acetate, pH 5.6 and two times the new volume of ethanol were added to the required fraction to precipitate the DNA. The solution was mixed thoroughly, incubated at -80°C for 30 minutes, and centrifuged at 17000 rpm, 4°C for 30 minutes to pellet the DNA. After pouring off the supernatant, draining thoroughly, and blotting the rim of the tube with tissue, DNA pellet was washed by adding 70% (v/v) ethanol to the tube and tipping back and forth a few times before centrifuging for 10 minutes at 17000 rpm, 4°C. Ethanol was discarded before the pellet was dried under vacuum and resuspend in TE buffer.

**2.13.2 Preparing of host strains**

The stored cells were revived by scraping off splinters of solid ice with a sterile wire loop and streaked onto a LB agar plate before incubating overnight at 37°C. The plate was stored at 4°C for up to 1 week. The cells were restreaked onto a fresh plate every week.

**2.13.3 Preparing a -80°C bacterial glycerol stock**

In a sterile 50 ml conical tube, 10 ml of LB medium was inoculated with one colony from the plate and allowed the cells to grow to late log phase before adding 4.5 ml of sterile glycerol-liquid medium solution (5 ml of glycerol/ 5 ml of LB medium) to the bacterial culture. The culture was mixed well and aliquoted into sterile centrifuge tubes (1 ml/tube). This preparation was stored at -20°C for 1-2 years or at -80°C for more than 2 years.

**2.13.4 Preparing the insert**

**2.13.4.1 Standard partial fill-in reaction**

Insert DNA (50 μg) was digested with *Mbo*I. Control reaction [1-1.5 μg control DNA, 2.5 μl 10x fill-in buffer (60 mM Tris-HCl, pH 7.5, 60 mM NaCl, 60 mM $MgCl_2$, 0.5% gelatin, 10 mM dithiothreitol), 1 μl dATP (10 mM), 1 μl dGTP (10 mM), 1 μl Klenow polymerase (5 U/μl), $dH_2O$ to a total volume of 25 μl] and sample reaction [50 μg digested insert DNA, 30 μl 10x fill-in buffer, 1 μl dATP (10 mM), 1 μl dGTP (10 mM), 3 μl Klenow polymerase (5 U/μl), $dH_2O$ to a total volume of 300 μl] were separately added to two sterile 1.5 ml tubes. The components of both tubes were mixed by gently pipetting the reactions up and down with a pipette tip and left at room temperature (22°C) for 15 minutes. To the total volume of 500 μl, 150 μl of 1x STE buffer (10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, pH 8.0) and 50 μl of 10x STE buffer (100 mM Tris-HCl, pH 8.0, 1 M NaCl, 10 mM EDTA, pH 8.0) were added to the sample reaction whereas 475 μl of 1x STE buffer was added to the control. An equal volume (500 μl) of phenol/chloroform was added to each reaction. Both reactions were vortexed, microcentrifuged for 2 minutes at room temperature at 13000 rpm, and the upper aqueous layer of each reaction was transferred to a fresh tube. This step was repeated if there is an interface. An equal volume (500 μl) of chloroform was added to each reaction in order to extract any residual phenol from the DNA. Each reaction was vortexed, microcentrifuged for 2 minutes at room temperature at 13000 rpm and the upper aqueous layer of each reaction was transferred to a fresh tube. Two volumes of 100% (v/v) ethanol (1ml) was added to each reaction and mixed well. The DNA was precipitated for 30 minutes at -20°C. Both reactions were microcentrifuged at 13000 rpm

for 10 minutes, 4°C. The supernatants were discarded before the pellets were washed with ice-cold 70% (v/v) ethanol and lyophilized until dry. The DNA of the sample reaction was resuspended in 25 μl of TE buffer and the DNA of the control reaction was resuspended in 3.5 μl of TE buffer.

**2.13.4.2 Analysis of partial fill-in control reactions**

The following components were added to 1 μg (1μl) of control DNA that has been partially filled in: 0.5 μl of 10x fill-in buffer, 0.5 μl of 10 mM rATP (pH7.5), 0.5 μl of T4 DNA ligase (4 weiss U/μl), 2.5 μl of $dH_2O$ for a final volume of 5 μl. In addition, the following components were added to a separate sterile 1.5 ml tube: 1.0 μl of pUC19/*Bam*HI-digested control DNA (1 μg), 0.5 μl of 10x fill-in buffer, 0.5 μl of 10 mM rATP (pH7.5), 0.5 μl of T4 DNA ligase (4 weiss U/μl), 2.5 μl of $dH_2O$ for a final volume of 5 μl. The reaction components were mixed by gently pipetting the reactions up and down with a pipette tip and incubated overnight at 4°C. The control reactions that have been ligated overnight were analyzed on a 1.0% (w/v) agarose gel. In addition, 1 μg of pUC19/*Bam* HI-digested control DNA was loaded.

**2.13.5 Ligating the insert**

The following reaction mixture were prepared in a microcentrifuge tube: 1.0 μl of the Lambda FIX II predigested with *Xho* I (1 μg), 0.8 μl of pMF/*Bam*HI insert (0.4 μg), 0.5 μl of 10x ligase buffer (500 mM Tris-HCl, pH 7.5, 70 mM $MgSO_4$, 10 mM dithiothreitol), 0.5 μl of 10 mM rATP (pH 7.5), 2 U of T4 DNA ligase and water up to a final volume of 5 μl. The reaction was incubated at 4°C overnight.

**2.13.6 Preparing the host bacteria, *E. coli* strain XL1-blue MRA (P2)**

Bacterial glycerol stock was streaked onto the appropriate agar plates and incubated the plates overnight at 37°C. LB medium, supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose was inoculated with a single colony and the culture was incubated at 37°C for 4-6 hours with shaking. Bacteria were not allowed to grow past an $OD_{600}$ of 1.0. Alternatively, the culture was grown overnight at 30°C and bacteria was pelleted at 500x *g* for 10 minutes before resuspending gently in half the original volume with sterile 10 mM $MgSO_4$ and diluted to an $OD_{600}$ of 0.5 with sterile 10 mM $MgSO_4$.

**2.13.7 Packaging protocol**

Twenty-five μl of packaging extract vials from a -80°C freezer was removed and placed on dry ice. The packaging extract was quickly thawed by holding the tube between your fingers until the contents of the tube just began to thaw. The experimental DNA was added immediately (1-4 μl containing 0.1-1.0 μg of ligated DNA) to the packaging extract. The tube was stired with a pipette tip to mix well. Gentle pipetting was allowable provided that air bubbles were not introduced. The tube was centrifuged quickly (for 3-5 seconds), if desired, to ensure that all contents were at the bottom of the tube. The tube was incubated at room temperature (22°C) for 2 hours. SM buffer (500 μl) and chloroform (20 μl) was added to the tube. The contents of the tube was mixed gently and centrifuged briefly to sediment the debris. The supernatant containing the phage was subjected to titering. The supernatant were stored at 4°C for up to 1 month.

**2.13.8 Titering the packaging reaction**

The final packaged reaction (1 μl) was added to 200 μl of host cells diluted in 10 mM $MgSO_4$ to an $OD_{600}$ of 0.5. 1 μl of a 1:10 dilution of the packaged reaction in SM buffer was added to 200 μl of host cells. The mixture of phage and the bacteria was incubated at 37°C for 15 minutes to allow the phage to attach to the cells. LB top agar (3 ml, 48°C) was added and plated immediately on pre-warmed LB agar plates. The plaques were counted and the titer was determined in plaque-forming units per mililiter (pfu/ml).

**2.13.9 Amplifying the libraries**

Final packaged solution containing library phages was aliquoted into 150 μl and each aliquot was mixed with 300 μl of freshly prepared XL1-Blue MRA(P2) host cells. The mixture was incubated for 15 minutes at 37°C. The mixture was added to 3 ml of 48°C-melted top agar and immediately poured and spreaded evenly onto a freshly poured bottom agar plate. The plates were incubated at 37°C for 6-8 hours. Then, the plates were overlaid with 8-10 ml of SM buffer and kept at 4°C overnight to allow the phages to diffuse into the SM buffer. The bacteriophage suspension was recovered from each plate and pooled into a sterile container. The plates were rinsed with an additional of 2 ml SM buffer and pooled. Chloroform was added to a 5% (v/v) final concentration, mixed well, and incubated for 15 minutes at room temperature. The cell debris was removed by

centrifugation for 10 minutes at 500x*g*. The supernatant was recovered and transferred to a sterile container. Chloroform was added to a 0.3% (v/v) final concentration and kept at 4°C. The amplified libraries were aliquoted and stored in 7% (v/v) DMSO at -80°C. The titer of the amplified libraries was checked using host cells and serial dilutions of the libraries.

**2.14 Screening library**

Phages containing genomic DNA of interest were screened by plague lift technique. The method was carried out by plating the phages at 800 pfu/plate. After the plagues with the diameter of 1 mm were present, the plate was chilled for 2 hours at 4°C to prevent the top agar from sticking to the membrane. Nylon membrane was then placed on the top of the plague and maintained for 2 minutes to let the plaques transfer onto a membrane. A needle was used to prick through the agar for orientation. The membrane was denatured after lifting by submerging in a 1.5 M NaCl and 0.5 M NaOH denaturation solution for 2 minutes, followed by neutralizing the membrane for 5 minutes by submerging the membrane in a 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) neutralization solution. The membrane was then rinsed for no more than 30 seconds by submerging the membrane in a 0.2 M Tris-HCl (pH 7.5) and 2x SSC buffer solution and blotted briefly on Whatman 3MM paper. After baking in the oven at 80°C for 2 hours, the membrane was carried on to hybridization process. The stock agar plate of the transfers was stored at 4°C to use after screening.

**2.15 Purification of lambda DNA from library**

Lambda DNAs were purified from the plate lysates using QIAGEN lambda kit. The method was conducted according to the instruction of manufactural protocol.

Lambda phages were propagated on agarose plates as described in 2.13.9. The plate (90-mm) was then overlaid with 5 ml SM buffer and incubated with shaking at room temperature for 4 hours. The liquid was transferred to a clean tube and the plate was added with additional 1 ml SM and the washing was added to the same tube. Chloroform was added (2% v/v), vortexed, and centrifuged at > 10,000x*g* for 10 minutes to remove

residual agarose. After the supernatant (~6 ml) was collected, buffer L1 (18 µl of buffer L1 per 6 ml of supernatant) was added and the mixture was incubated at 37°C for 30 minutes. A 500 µl aliquot of the sample (fraction 1) was removed and kept for an analytical gel. To the mixture, ice-cold buffer L2 (1.2 ml) was added, mixed gently, and incubated on ice for 60 minutes. After centrifugation at >10,000x*g* for 10 minutes, the supernatant was discarded while the pellet was resuspended in 0.6 ml of buffer L3 by pipetting up and down, followed by the addition of buffer L4 (0.6 ml), mixed gently, and incubated at 70°C for 10 minutes before cooling down on ice. Buffer L5 (0.6 ml) was added and mixed immediately but gently by inverting the tube 4-6 times prior to centrifugation at 4°C for 30 minutes at >15,000x*g*. The supernatant was transferred promptly to a fresh tube and centrifuged again at 4°C for 10 minutes at >15,000x*g* to obtain a particle-free cleared lysate (non-turbid). A 150 µl.aliqout of the cleared lysate (fraction 2) was removed and kept for an analytical gel. A QIAGEN-tip was equilibrated by applying 1 ml of buffer QBT, and the column was allowed to empty by gravity flow before the application of the supernatant to the QIAGEN-tip. The supernatant was allowed to enter the resin by gravity flow. At this stage, a 150 µl aliquot of the flow-through (fraction 3) was removed and kept for an analytical gel. The QIAGEN-tip was washed with buffer QC (2 ml). A 100 µl aliquot of the wash flow-through (fraction 4) was collected and kept for an analytical gel. DNA was eluted from the column with 1.5 ml of buffer QF into a clean collection tube. Again, a 75 µl aliquot of the eluate (fraction 5) was removed and kept for an analytical gel. The DNA was precipitated by adding 1 ml of room-temperature isopropanol to the eluted DNA before mixing and centrifuging at >15,000x*g* for 30 minutes at 4°C. The supernatant was arefully decanted. The pellet was air-dried for 5-10 minutes before and redissolving in a suitable volume (~10-20 µl) of TE buffer at pH 8.0.

**2.15.1 Analysis of yield and quality of lambda DNA by agarose gel electrophoresis**

Poor yields and quality could be caused by a number of different factors. To determine at what stage of the procedure any problem occurred, a number of fractions removed from different steps of lambda DNA purification procedure were analyzed using agarose gel electrophoresis.

An aliquot removed following the addition of buffer L1 (fraction 1) was extracted with phenol/chloroform to lyse the phage and free the lambda DNA. The lambda DNA was then precipitated with 1.25 M sodium acetate and 0.8 volumes of isopropanol, and resuspended in 10 μl TE, pH 8.0. An aliquot of the cleared lysate after PEG precipitation (fraction 2) was precipitated with 1 volume of isopropanol and resuspended in 10 μl TE, pH 8.0. An aliquots of the flow-through, wash, and elute (fraction 3, 4, and 5) was precipitated each with 1 volume of isopropanol, and resuspend in 10 μl TE, pH 8.0. Sample from each fraction (2 μl each) was run on a 0.8% agarose gel alongside a known amount of DNA (20-200 ng) to evaluate the amount of DNA in each fraction.

# CHAPTER III

# RESULTS

## 3.1 DNA extraction

Genomic DNA was extracted from tissue of each *P. monodon* and the quality and quantity of extracted DNA were determined by electrophoresis on a 0.8% agarose gel (w/v) comparing to an undigested lambda DNA. High molecular weight genomic DNA was located at approximately 23.1 kb, indicating good quality of extracted genomic DNA (Figure 3.1). The concentration of genomic DNA was spectrophotometrically determined at 260 nm. The $OD_{260}/OD_{280}$ ratio of extracted DNA ranged from 1.8–2.0 indicating that the purity of DNA samples was acceptable.


**Figure 3.1** A 0.8% ethidium bromide stained agarose gel showing the quality of genomic DNA extracted from the tissue of *P. monodon*. Genomic DNA was individually extracted from tissue of male and female *P. monodon* (lane 1-3 and 4-6, respectively). Lane M is 100 ng of undigested lambda DNA marker.

### 3.1.1 Purification of genomic DNA

Genomic DNA from both male and female *P. monodon* was digested with a number of restriction enzymes. This included *Eco*RI, *Hin*dIII, *Bam*HI, *Mbo*I, and *Dra*I. The results were shown in Figure 3.2. Complete digestion was obtained from genomic DNA digested by *Mbo*I and *Dra*I.

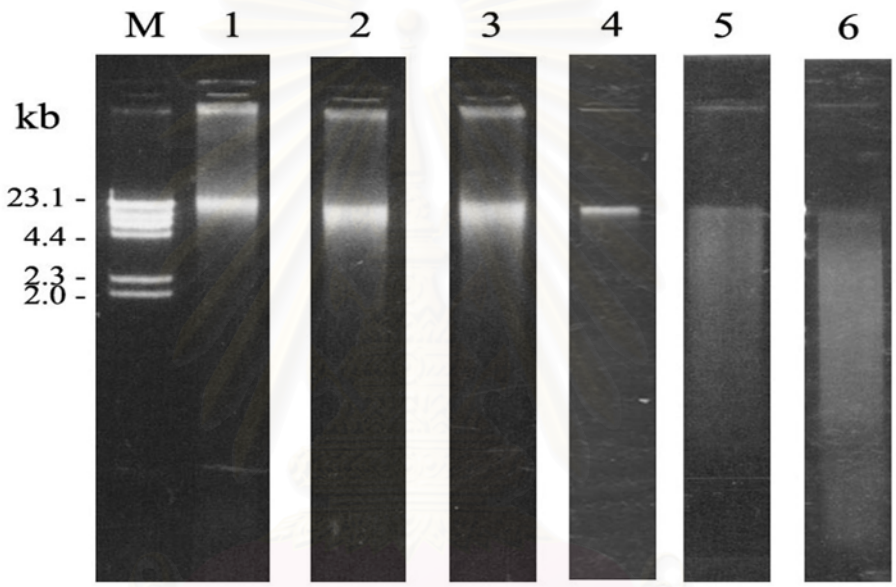



**Figure 3.2** Restriction digestion of *P. monodon* genomic DNA. DNA markers (lane M), undigested genomic DNA (lane 1), and genomic DNAs digested by *Eco*RI (lane2), *Hin*dIII (lane 3), *Bam*HI (lane 4), *Dra*I (lane 5), and *Mbo*I (lane 6) were analyzed by agarose gel electrophoresis.

### 3.2 Phenol emulsion reassociation technique (PERT) genomic DNA subtraction analysis

PERT genomic DNA subtraction procedures were used to select the sex-specific sequences in *P. monodon*. A position selection of subtraction approach was carried out using genomic DNA of male as the tester and that of female as the driver and *vice versa*.

#### 3.2.1 Male genomic DNA subtraction

The preparation of tester and driver DNAs were carried out prior to subtraction. Tester was prepared by complete restriction digestion of male genomic DNA using *Mbo*I. The result was shown in Figure 3.3. For driver preparation, female genomic DNA was randomly sheared by the sonication and vortex. As shown in 1.0% agarose gel electrophoresis (Figure 3.4), the complete shearing of genomic DNA was obtained by sonicating for 60 minutes and vortexing for 15 minutes, respectively.

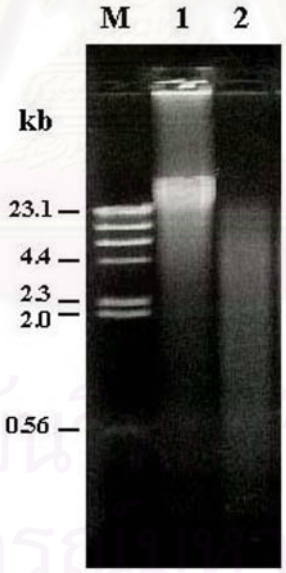



**Figure 3.3** A 1.0% agarose gel electrophoresis of genomic DNA from male *P. monodon* (tester) completely digested with *Mbo*I at 37°C for 16 hours (lane 2). Lane M and 1 are λ-*Hin*dIII DNA marker and undigested male genomic DNA, respectively.

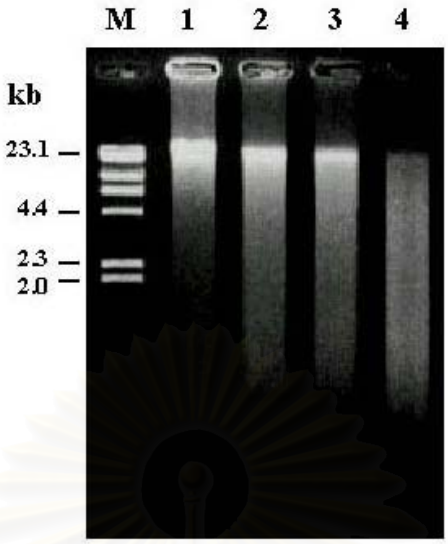



**Figure 3.4** A 1.0% agarose gel electrophoresis showing mechanical shearing of genomic DNA of female *P. monodon* (driver DNA) by sonication and vortexing. Genomic DNA was vortexed for 5 minutes followed by sonication for 20 minutes (lane 2), 10 minutes vortex and 30 minutes sonication (lane 3), and 15 minutes vortex and 60 minutes sonication (lane 4), respectively. Lane M and 1 were λ-*Hin*dIII DNA marker and extracted female genomic DNA (control), respectively.

After DNA subtraction was conducted, 51 recombinant clones from male genomic DNA subtraction were obtained. Colony PCR were carried out in each clones to specify the sizes of subtracted DNA fragment. The results (Figure 3.5, 3.6 and 3.7) indicated 9 recombinant clones containing inserted DNA larger than 100 bp. These clones were then subjected to sequence analysis. The results were shown in Figure 3.8.


**Figure 3.5** A 1.0% agarose gel electrophoresis showing colony PCR of PERT subtraction of genomic DNA of male (tester) and female (driver). Clones containing inserted DNA are found in lane 2, 3, 5 and 11. A 100 bp ladder was used as the DNA markers (lane M).

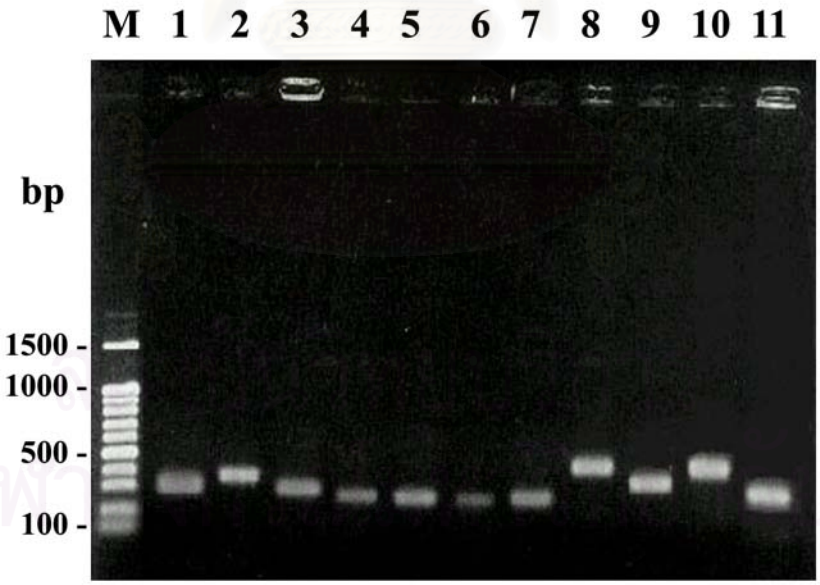



**Figure 3.6** A 1.0% agarose gel electrophoresis showing colony PCR of PERT subtraction of genomic DNA of male (tester) and female (driver). A PCR product of 203 bp in size indicates clones without DNA inserts. Larger PCR products (lane 1, 2, 3, 8, 9, 10 and 11) contain small inserts. A 100 bp ladder was used as the DNA markers (lane M).


**Figure 3.7** A 1.0% agarose gel electrophoresis showing colony PCR of PERT subtraction of genomic DNA of male (tester) and female (driver). Clones containing inserted DNA are found in lane 5 and 9. A 100 bp ladder was used as the DNA markers (lane M).

**>pPMMSH3 (119 bp)**

GAGAGGAAACTGCACAAGTTATTAGAATGTGTTTGCAATTTTTCTACACGATGTTGCTGTTGTTC
ATATTAGTGACTTTTTAATAATACCCATTGAATCAATGCATTTCTGTGGGCGGT

**>pPMMSH4 (117 bp)**

AAATATANNNCGTNACATTATATTGCTGATTCCTGCGCCTCGCCATTCCATTCTGTGAGTNCCCT
AGAGCAATATTAACAGACAATCCAGTTTCAAGGCAGGTAAACACAAACAGCGAATTGCGCATGTC
AAAACTCGCCCTTATGACCAGCGCTCTTAAGGTCCAAAATACCTCTC

**>pPMMSH5 (102 bp)**

AACAACAAAAACTGTCTAGCACACAAAAAAGAGTTGCTTCAACTGCACATGGTATGATATTTGCC
ATCTGCCACATGTACCTGTTTTTTCATAACAACAAAA

**Figure 3.8** Nucleotide sequences of positive clones resulted from PERT subtraction of male (tester) and female (driver) genomic DNA of *P. monodon* (continue).

**>pPMMSH6 (294 bp)**

AAAGACGACAACGGAGAAAAATGAAAGAAAGAGAGAAATGCAAAGAAACGGTCCAGTCAG
ATTTCGGGCTAAAGGGAATACGGTTAAAATCCCGTGACCTGCCCCGTGGGAAGACAGACC
CGTGCGTGGGAAAATATTTAGAGTGCGGGTGGCCGTCTAACGAGTGCGGCGGCGCGCGTG
GGCCGCGCGGCGTCGTCGTCGTCGTCGTCGGGAGTCTCTCCCCCCTCTTGGGTGGGGGTT
GGCGAACGACGACGACGGCACCGCCGCCGGCGCCGGCGCCGCCGCGGTTACGAC

**>pPMMSH8 (234 bp)**

AATGTACTGTCCAAGCTAATAAACGGTTATATTATCAACGACCAAGTGTCAAAGACACAT
GAAAAAGATATTTCGCTATAACGTAGAAGGCGGTGATTAACATGCCGCAGGTTTACACTA
TCAGCGAGAGAAAAAAAAGAAAAATATCACACAGGTGACAGTAAATATTTTTATATACCA
TATCCATTTATGTTCTAGTTGTCTCGATGGCATTTACAAAAAAATAAACATGGA

**>pPMMSH9 (139 bp)**

TTCATGTTTTGTCTGTAAGAGTGGTAGAAAATTTCAGTAATTTTTTTCTTTCTTTTTTCTTTCTT
CCTTAGATTAAACCCATGATATTTTTGCAGATTTAGTCACAGAAAGAATGACAGCATAGCAAGTA
CTTCTAGTG

**>pPMMSH10 (235 bp)**

AATGTACTCGTTCAACGCTAATAAACGGTTATATTATCAACACCAAGTGTCAAAGACACATGAAA
AAGATATTTCGCTATAACGTAGAAGGCGGTGATTAACATGCCGCAGGTTTACACTATCAGCGAGA
GAAAAAAAAGAAAAATATCACACAGGTGACAGTAAATATTTTTATATACCATATCCATTTATGTT
CTAGTTGTCTCGATGGCATTTACAAAAAAATAAACATGGA

**>pPMMSH12 (223 bp)**

AATGTACTGTTCAAGCTAATAAACGGTTATATTATCAACACCAAGTGTCAAAGACACATGAAAAA
GATATTTCGCTATAACGTAGAAGGCGGTGATTAACATGCCGCAGGTTTACACTATCAGCGAGAGA
AAAAAAAGAAAAATATCACACAGGTGACAGTAAATATTTTTATATACCATATCCATTTATGTTCT
AGTTGTCTCGATGGCATTTACAAAAAAATAAACATGGA

**>pPMMSH13 (87 bp)**

TCTTTTGACACACACATTAATTATAGAAAAGGAGAAAAATTACGAACGTTTGCAGCATGTGCATG
TGCAAAAGTCGACCCAAACCGT

**Figure 3.8** Nucleotide sequences of positive clones resulted from PERT subtraction of male (tester) and female (driver) genomic DNA of *P. monodon*.

The subtracted DNA fragments from these clones were further identified by sequence comparison with the DNA sequences reported in the GenBank using BLAST protocol. The result revealed that 7 clones (pPMMSH3, pPMMSH4, pPMMSH5, pPMMSH8, pPMMSH10, pPMMSH12, pPMMSH13) were identified as unknown genes. Of these, pPMMSH8, pPMMSH10 and pPMMSH12 sheared nucleotide sequences (Figure 3.9). Two clones (pPMMSH6 and pPMMSH9) were identified as DNA fragment partly similar to 18s ribosomal RNA of *Penaeus vannamei* and BAC clone RP23-389K5 from chromosome19 of *Mus musculus*, respectively (Table 3.1).

```
pPMMSH10        AATGTACTCGTTCAACGCTAATAAACGGTTATATTATCAAC-ACCAAGTGTCAAAGACAC
pPMMSH12        AATGTACT-GTTCAA-GCTAATAAACGGTTATATTATCAAC-ACCAAGTGTCAAAGACAC
pPMMSH8         AATGTACT-GTCCAA-GCTAATAAACGGTTATATTATCAACGACCAAGTGTCAAAGACAC
                ******** ** *** ************************ ******************

pPMMSH10        ATGAAAAAGATATTTCGCTATAACGTAGAAGGCGGTGATTAACATGCCGCAGGTTTACAC
pPMMSH12        ATGAAAAAGATATTTCGCTATAACGTAGAAGGCGGTGATTAACATGCCGCAGGTTTACAC
pPMMSH8         ATGAAAAAGATATTTCGCTATAACGTAGAAGGCGGTGATTAACATGCCGCAGGTTTACAC
                ************************************************************

pPMMSH10        TATCAGCGAGAGAAAAAAAAGAAAAATATCACACAGGTGACAGTAAATATTTTTATATAC
pPMMSH12        TATCAGCGAGAGAAAAAAAAGAAAAATATCACACAGGTGACAGTAAATATTTTTATATAC
pPMMSH8         TATCAGCGAGAGAAAAAAAAGAAAAATATCACACAGGTGACAGTAAATATTTTTATATAC
                ************************************************************

pPMMSH10        CATATCCATTTATGTTCTAGTTGTCTCGATGGCATTTACAAAAAAATAAACATGGA
pPMMSH12        CATATCCATTTATGTTCTAGTTGTCTCGATGGCATTTACAAAAAAATAAACATGGA
pPMMSH8         CATATCCATTTATGTTCTAGTTGTCTCGATGGCATTTACAAAAAAATAAACATGGA
                ********************************************************
```

**Figure 3.9** Multiple sequence alignments of pPMMSH8, pPMMSH10 and pPMMSH12. The alignment shows high similarities between their sequences.

**Table 3.1** BLAST analysis of nucleotide sequences of clones obtained from PERT subtraction of male (tester) and female (driver) genomic DNA of *P. monodon*.

| Clone | Insert size (bp) | Blast*N* | E-value | Blast*X* | E-value |
|---|---|---|---|---|---|
| PMMSH3 | 119 | Unknown | 0.015 | No significant | - |
| PMMSH4 | 117 | Unknown | 0.38 | No significant | - |
| PMMSH5 | 102 | Unknown | 0.2 | No significant | - |
| PMMSH6 | 294 | *Penaeus vannamei* 18s ribosomal RNA | 3E-34 | Unknown | 0.049 |
| PMMSH8 | 234 | Unknown | 0.008 | No significant | - |
| PMMSH9 | 139 | *Mus musculus* BAC clone RP23-389K5 from chromosome19 | 2E-05 | No significant | - |
| PMMSH10 | 235 | Unknown | 0.008 | Unknown | 5.8 |
| PMMSH12 | 223 | Unknown | 0.008 | Unknown | 5.8 |
| PMMSH13 | 87 | Unknown | 0.041 | No significant | - |

### 3.2.2 Female genomic DNA subtraction

Similar to male genomic DNA subtraction, tester and driver DNAs were prepared prior to subtraction. The difference was that female genomic DNA was used as tester and male genomic DNA was used as driver. The result of female DNA digestion by *Mbo*I and the DNA mechanical shearing of male DNA were shown in Figure 3.10 and 3.11, respectively. The conditions used to complete the digestion and shearing of genomic DNA from both sexes were identical to the ones earlier used.

Seventeen clones containing inserted fragments from male genomic DNA subtraction were obtained. After colony PCR from each clones was conducted, the result revealed 4 recombinant clones containing inserted DNA larger than 100 bp (Figure 3.12 and 3.13). All 4 clones were detected and subjected to sequence analysis. The results of DNA sequences were shown in Figure 3.14.

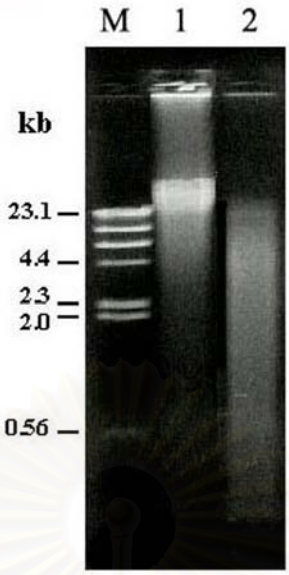



**Figure 3.10** A 1.0% agarose gel electrophoresis showing genomic DNA of female *P. monodon* (tester DNA) completely digested with *Mbo*I at 37°C for 16 hours (lane 2). Lane M and 1 are λ-*Hin*dIII DNA marker and undigested female genomic DNA, respectively.


**Figure 3.11** A 1.0% agarose gel electrophoresis showing mechanical shearing of genomic DNA of male *P. monodon* (driver DNA) by sonication and vortexing. Genomic DNA was vortexed for 5 minutes followed by sonication for 20 minutes (lane 2), 10 minutes vortex and 30 minutes sonication (lane 3), and 15 minutes vortex and 60 minutes sonication (lane 4), respectively. Lane M and 1 were λ-*Hin*dIII DNA marker and extracted male genomic DNA (control), respectively.

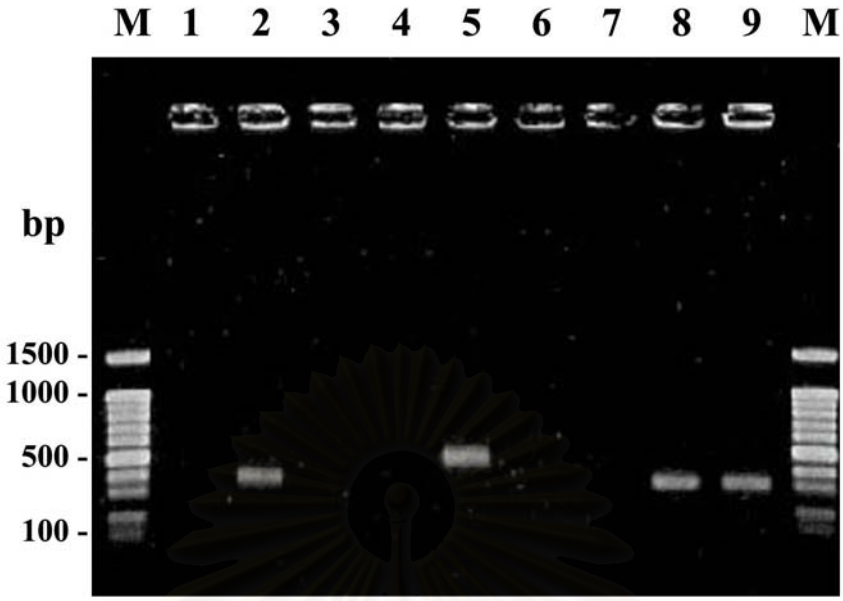



**Figure 3.12** A 1.0% agarose gel electrophoresis showing colony PCR of PERT subtraction of genomic DNA of female (tester) and male (driver). Clones containing inserted DNA are found in lane 2 and 5. A 100 bp ladder was used as the DNA markers (lane M).

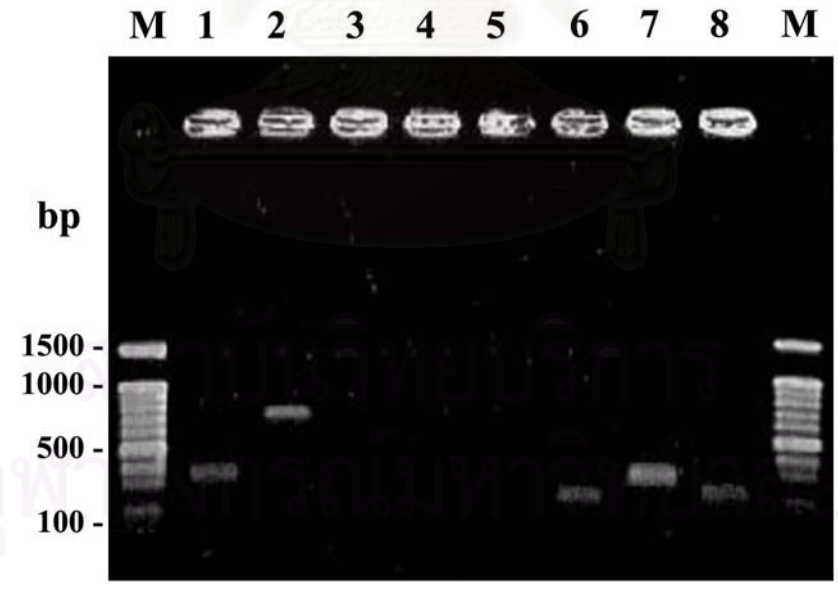



**Figure 3.13** A 1.0% agarose gel electrophoresis showing colony PCR of PERT subtraction of genomic DNA of female (tester) and male (driver). Clones containing inserted DNA are found in lane 1 and 2. A 100 bp ladder was used as the DNA markers (lane M).

**>pPMFSH17 (253 bp)**

GAGCGTCTTGCAGGTGGAGTCAGGAGTACTCCCCTCCGACTCAGACGCGGCCAGTTAGCCTTGAG
CTATGCCGCAAAGGTGGCTCGAGTCCCGAGCCACCCTAATGGCAATAGTGGCATTAGGCCCTTTG
CCACGAGACTCCACGACCTCGTCGAGAAAGTCGGTATAGATCTAGGTATTTTTTCATTACATTTA
GCAGGGGTCTCATCAATCTTAGGAGCTGTAAACTTTATAACGACCGTTATCAATATAC

**>pPMFSH26 (123 bp)**

CAGCTCTTGNGTACCGTTATTTTTACATGATTAGTTTCGAGATATTGATTTCTACTTTTTCCCCC
TTTTTTCATTATATTTTTTCCTTTTTTCTTTTACAATTTGATGATGTATGACGTTCGA

**>pPMFSH27 (433 bp)**

GGCACGAGGGCAGTGTACAGAACCTGAACTCAACAATGCGTTTATTAATTTTCCTGAGTCTCATC
TTCGTCGCCTCTGGCCTACTCCGCCCACTGGGAGATCTTGCAGAAATTGTGTGTCATCAGCCGAA
GGTGGTAGGGCCTTGCAAAGCCCGTTTCCATAGGTTCTACTATAGCTTCGATAACGAATGAATGC
ATACCGTTTATTTACGGCNGGTTGTGGAAAGAATGACAACAACTTTGAAACAAAAGAAGATTGCT
GGTTTCTCTGCGGCCGATGAAGATCCGGGAATGCACCGAACATGGAAAGGATCCACATGACCTTG
ACGAAATGATCTCAGACGCCTCTCTACAGCTTCAGAGTTTCAGCTGAAGATGCTTTCATTCTTAA
AAATTCAACTNATTGAAATNATATTCTCAGGCAAAGTGTATNN

**>pPMFSH32 (135 bp)**

ATTAATTTCCNTGAACATGCACATTTCCTTATCCTACACTACCTGAAATTTCATGTTTTTTTGTT
TTTCTATCTTCGCAAATAGATAATACCTCGAATACAATTATAAATATGTACATTTACACATACAC
ATAAT

**Figure 3.14** Nucleotide sequences of positive clones resulted from PERT subtraction of female (tester) and male (driver) genomic DNA of *P. monodon*.



These clones were further identified by sequence comparison with the data reported in the GenBank. The result indicated that 2 isolated clones (pPMFSH26, and pPMFSH26) were identified as unknown genes whereas pPMFSH17 and pPMFSH27 were identified as genes slightly similar to Cytochrome oxidase subunitI from *Macrobrachium australiense* and Protease inhibitor from *Marsupenaeus japonicus*, respectively (Table 3.2).

**Table 3.2** BLAST analysis of nucleotide sequences of clones obtained from PERT subtraction of female (tester) and male (driver) genomic DNA of *P. monodon*.

| Clone | Insert size (bp) | Blast*N* | E-value | Blast*X* | E-value |
|---|---|---|---|---|---|
| PMFSH17 | 253 | *Penaeus monodon*, complete mitochondrial genome | 9E-40 | Cytochrome oxidase subunitI (*Macrobrachium australiense*) | 2E-07 |
| PMFSH26 | 123 | Unknown | 0.001 | Unknown | 9.9 |
| PMFSH27 | 433 | Unknown | 0.064 | Protease inhibitor (*Marsupenaeus japonicus*) | 1E-13 |
| PMFSH32 | 135 | Unknown | 0.004 | No significant | - |

### 3.2.3 Detection of sex-specific markers by PCR

A candidate fragment, pPMMSH6, from PERT male subtraction with the sizes larger than 200 bp was selected and subjected to PCR amplification. Sequences and positions of the forward and reverse primers were shown in Figure 3.15. PCR reaction was performed in genomic DNA and gonad-extracted cDNA from both male and female of *P. monodon*.

**>pPMMSH6 (294 bp)**

AAAGACGACAACGGAGAAAAATGAAAGAAAGAGAGAAATG**CAAAGAAACGGTCCAGTCAG**ATTTC
GGGCTAAAGGGAATACGGTTAAAATCCCGTGACCTGCCCCGTGGGAAGACAGACCCGTGCGTGGG
AAAATATTTAGAGTGCGGGTGGCCGTCTAACGAGTGCGGCGGCGCGCGTGGGCCGCGCGGCGTCG
TCGTCGTCGTCGTCGGGAGTCTCTCCCCC**CTCTTGGGTGGGGGTTGG**CGAACGACGACGACGGCA
CCGCCGCCGGCGCCGGCGCCGCCGCGGTTACGAC

**Figure 3.15** Nucleotide sequences of pPMMSH6. Sequences and positions of the forward primer and the complementary to the reverse primer are underlined and boldfaced.

Expected PCR product at the size of 202 bp were amplified at an annealing temperature of 60°C in genomic DNA from both male and female *P. monodon* (Figure 3.16). This indicated that PMMSH6 was in the genome of both sexes and it was not sex-specific. Similar result was obtained when PCR amplification was performed with cDNAs extracted from testes and ovaries (Figure 3.17), indicating no different level in the expression of PMMSH6 between sexes.

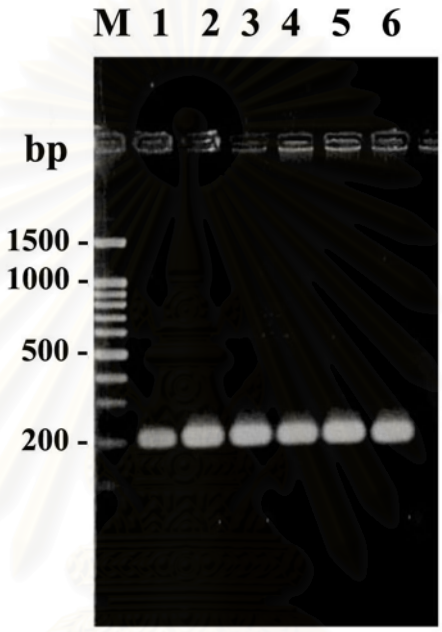



**Figure 3.16** A 1.2% agarose gel electrophoresis showing result of amplification when genomic DNA was used as template (annealing temperature at 60°C). Lane 1-6 =PMMSH6 primer (lane 1-3 = male genomic DNA and lane 4-6 = female genomic DNA) A 100 bp ladder was used as the DNA markers (lane M).

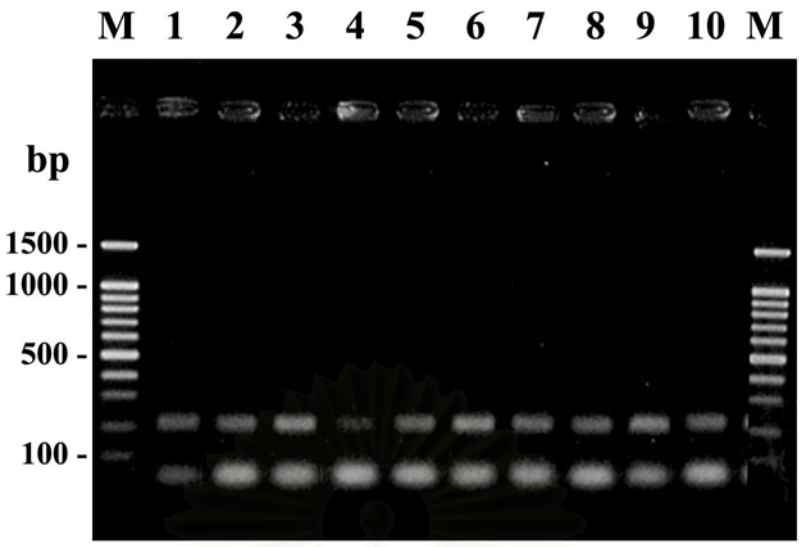



**Figure 3.17** A 1.2% agarose gel electrophoresis showing result of amplification when the first stand cDNA (testis and ovary) was used as template (annealing temperature at 60°C) with PMMSH6 primer (lane 1-5 = testis and lane 6-10 = ovary). A 100 bp ladder was used as the DNA markers (lane M).

### 3.2.4 Genome walking

In order to find the unknown genomic DNA sequences adjacent to the known sequences of PMMSH6, genome walking was carried out. Male and female genomic DNAs of *P. monodon* digested with *Alu*I, *Hae*III, *Rsa*I, and *Dra*I and ligated to adaptors were used as templates for PCR amplification. The result of PMMSH6 5' and 3' extension revealed similar patterns of amplified products in both male and female genomic DNA digested with *Hae*III, *Rsa*I, and *Dra*I whereas the 5' extention (reverse primer+AP1) of female genomic DNA digested with *Alu*I yielded 700 bp product and 3'extension (forward+AP1) yielded 600 and 1000 bp (Figure 3.18). These products were then amplified using forward and reverse primers (Figure 3.19) and digested with *Alu*I. The result revealed no digestion between male and female (Figure 3.20). When genome walking was performed in other 3 individual samples for each sex, the result revealed no difference in PCR product pattern from both sexes (Figure 3.21). This indicated that the different PCR product pattern detected from the first sample caused by nucleotide polymorphism from shrimp individual.

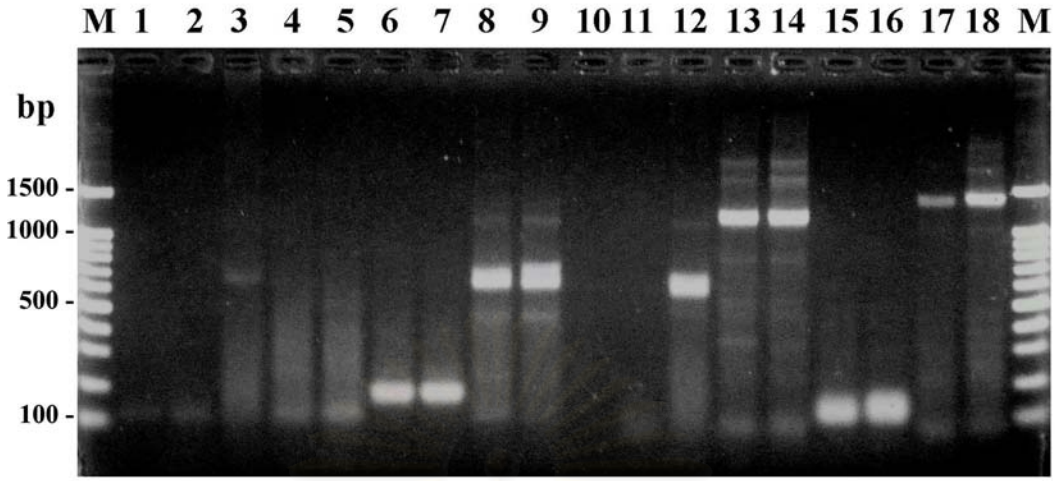



**Figure 3.18** A 1.5% agarose gel electrophoresis showing result of genome walk when using PMMSH6 primer, lane 1-9 = forward primer + AP1 and lane 10-18 = reverse primer + AP1 (lane 2-3, 11-12 = genomic DNA digested with *Alu*I, lane 4-5, 13-14 = *Dra*I, lane 6-7, 15-16 = *Hae*III and lane 8-9, 17-18 = *Rsa*I) lane 2, 4, 6, 8, 11, 13, 15, 17 = male genomic DNA and lane 3, 5, 7, 9, 12, 14, 16, 18 = female genomic DNA). A 100 bp ladder was used as the DNA markers (lane M). Negative controls of forward and reverse primer are lane 1 and 10, respectively).

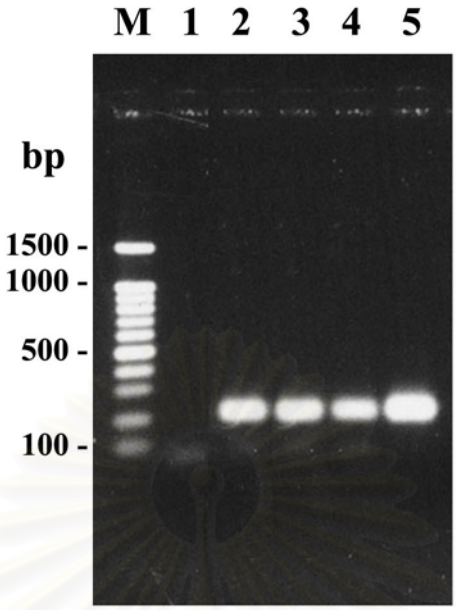



**Figure 3.19** A 1.2% agarose gel electrophoresis showing result of amplification when using the genome walk products as template with PMMSH6 primer. A 100 bp ladder was used as the DNA markers (lane M) and lane 1 = negative control of PMMSH6.

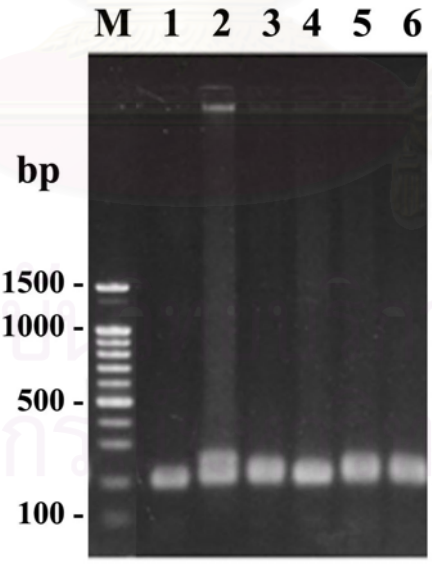



**Figure 3.20** A 1.5% agarose gel electrophoresis showing result of genome walk product from PMMSH6 primer digested with *Alu*I. Lane 1-3 = male products and lane 4-6 = female products. A 100 bp ladder was used as the DNA markers (lane M).

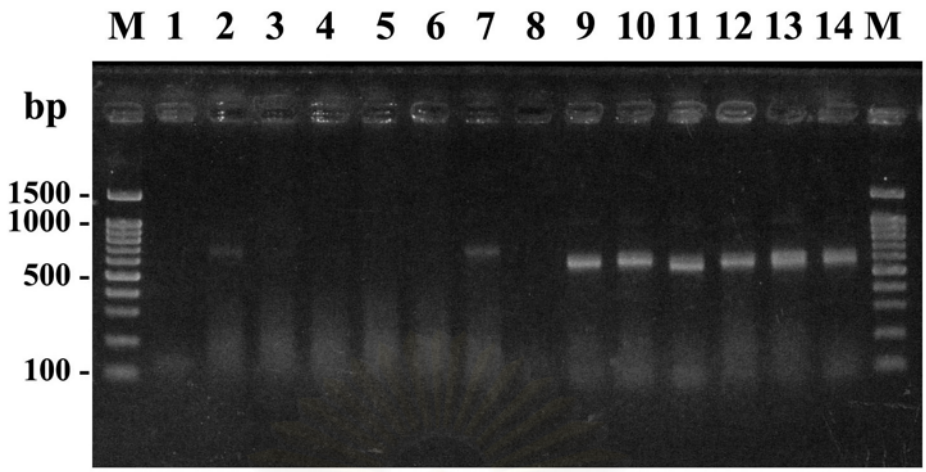



**Figure 3.21** A 1.5% agarose gel electrophoresis showing result of genome walk (repeated genomic DNA digested with *Alu*I only) when using PMMSH6 primer, lane 1-7 = forward primer + AP1 and lane 8-14 = reverse primer + AP1 (lane 2-4, 9-11 = male genomic DNA and lane 5-7, 12-14 = female genomic DNA). A 100 bp ladder was used as the DNA markers (lane M). Negative controls of forward and reverse primer are lane 1 and 8, respectively).

### 3.2.5 Identification of nucleotide polymorphism using SSCP analysis

A marker PMMSH6, not exhibiting sex-specificity when analyzed by agarose gel electrophoresis, was then conducted to SSCP analysis to investigate whether the amplified fragments would show nucleotide polymorphism between male and female *P. monodon*. The result of SSCP showed difference between fragments amplified from both male and female DNAs caused by nucleotide polymorphism from shrimp individual. This indicated that both subtraction markers did not show sex-specific nature. PMMSH6 was polymorphic across investigated individuals (Figure 3.22 and 3.23).

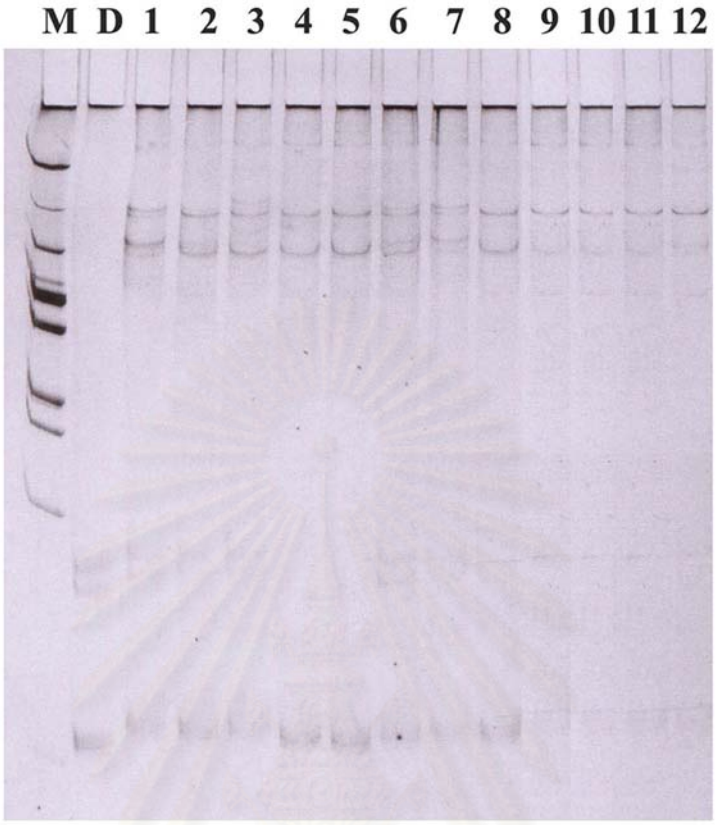



**Figure 3.22** SSCP patterns of a male genomic subtraction marker derived from PMMSH6 of male (lane 1-4) and female (lane 5-8) genomic DNA and testis (lane 9-10) and ovary (lane 11-12) *P. monodon* resulted from size-fractionation through 17.5% PAGE + 5% glycerol. Lane M = 100 bp, lane D = ds PCR product control.

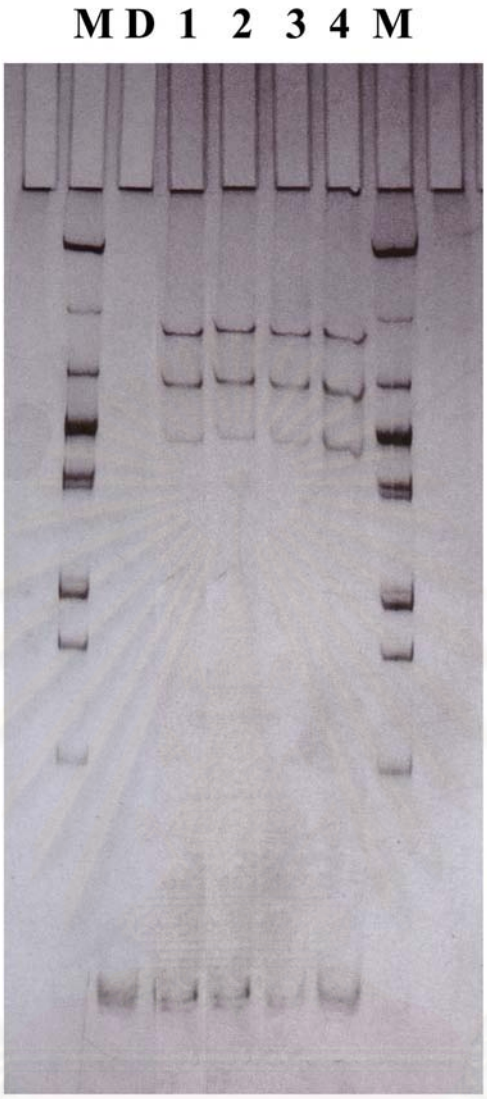



**Figure 3.23** SSCP patterns of a genome walk products of male genomic subtraction marker derived from PMMSH6 of male (lane 1 and 2) and female (lane 3-4) genomic DNA *P. monodon* resulted from size-fractionation through 17.5% PAGE + 5% glycerol. Lane 1 and 3 = forward primer + AP1 and lane 2 and 4 = reverse primer + AP1. Lane M = 100 bp, lane D = ds PCR product control.

### 3.2.6 Southern blot analysis

Clones containing insert fragments from male and female genomic DNA subtraction were chosen for further analysis on sex specificity using Southern blotting and hybridization methods. This included 2 clones from male subtraction (PMMSH3 and PMMSH8) and 2 clones for female subtraction (PMFSH26 and PMFSH32). As the results shown in Figure 3.24, 3.25, 3.26 and 3.27, no bands corresponding to all 4 clones were detected from both male and female *P. monodon*.

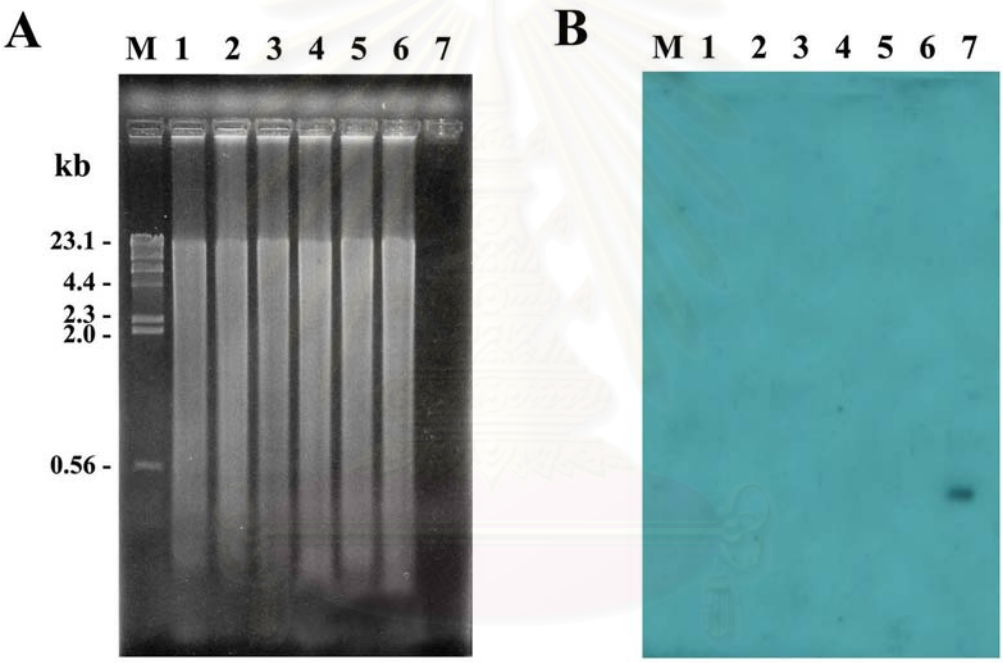



**Figure 3.24** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMMSH3 (B). An inserted DNA was non-radioactively labelled by DIG and hybridized with positive control, (an amplified product of PMMSH3 using pUC1 and pUC2 primers) (lane 7), male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I.

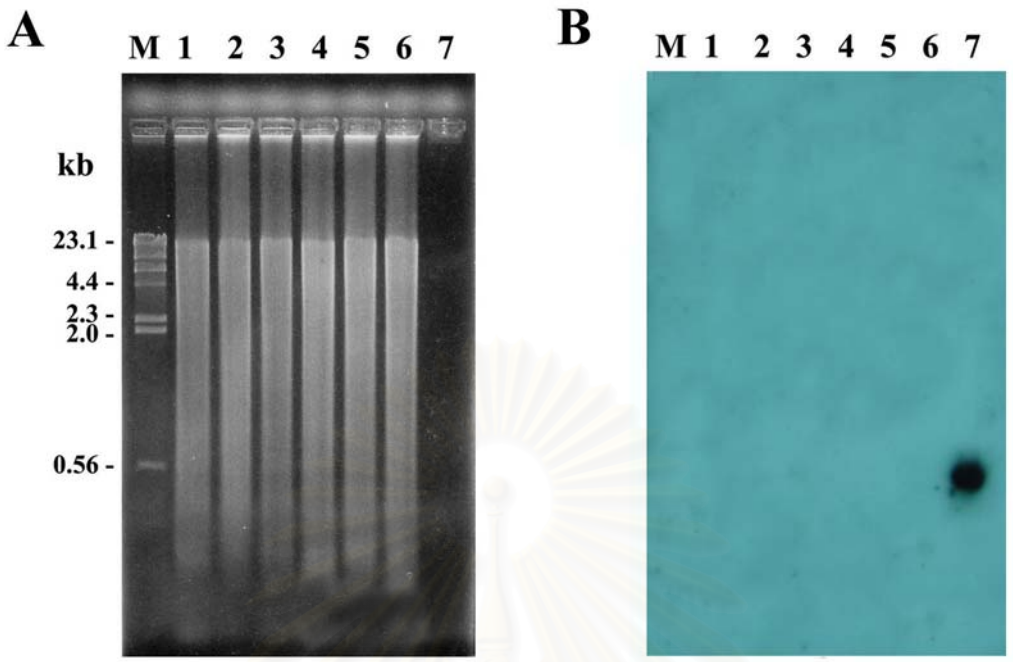



**Figure 3.25** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMMSH8 (B). An inserted DNA was non-radioactively labelled by DIG and hybridized with positive control, (an amplified product of PMMSH8 using pUC1 and pUC2 primers) (lane 7), male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I.

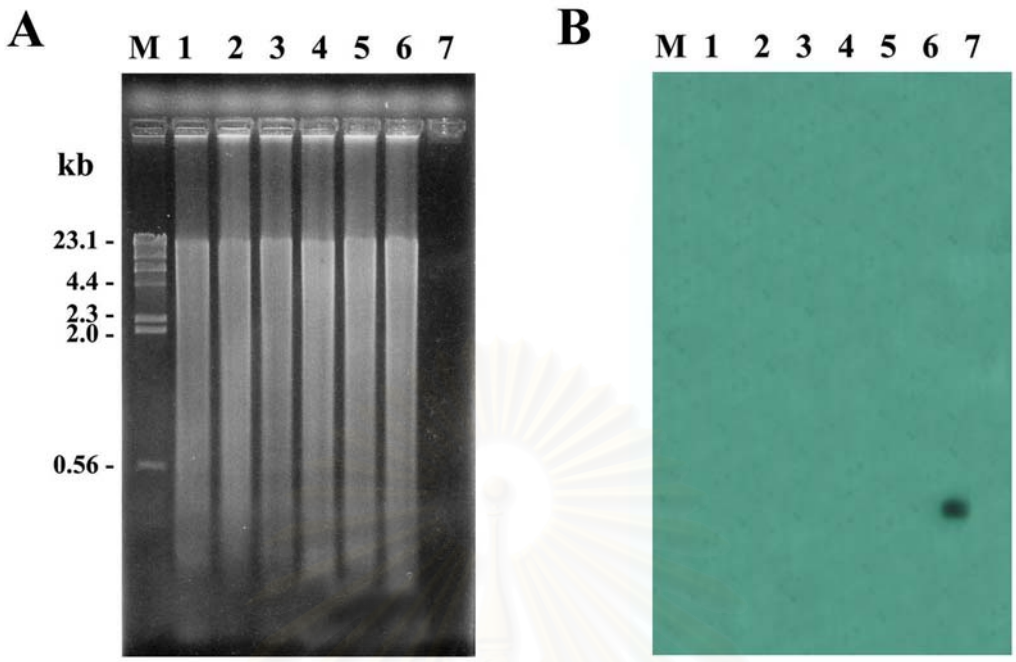



**Figure 3.26** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMFSH26 (B). An inserted DNA was non-radioactively labelled by DIG and hybridized with positive control, (an amplified product of PMMSH26 using pUC1 and pUC2 primers) (lane 7), male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I.

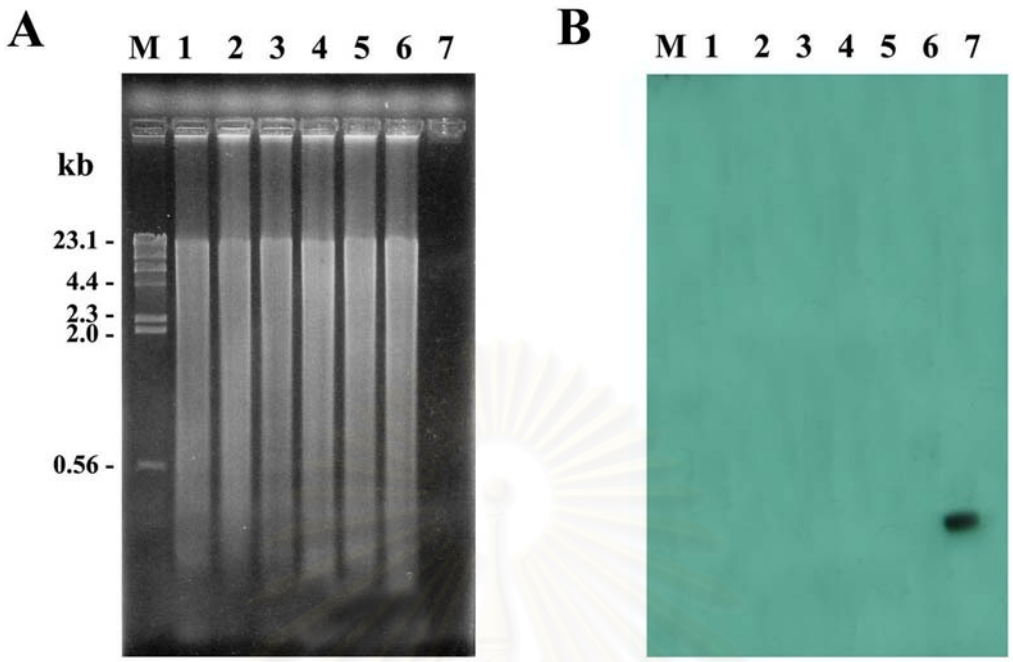



**Figure 3.27** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMFSH32 (B). An inserted DNA was non-radioactively labelled by DIG and hybridized with positive control (an amplified product of PMMSH32 using pUC1 and pUC2 primers) (lane 7), male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I.

### 3.3 Representative difference analysis genomic DNA subtraction analysis

RDA genomic DNA subtraction procedures utilize kinetic enrichment by PCR in which only tester sequences can be amplified. A PCR-based of subtraction approach was carried out using genomic DNA of male as the tester and that of female as the driver and *vice versa*. The results of both male and female genomic DNA of *P. monodon* digested with *Hin*dIII were shown in Figure 3.28. Digested DNAs from male and female were ligated with RHind12 and RHind24 single-stranded oligonucleotides by the function of ligase enzyme. The amplicons were then amplified by PCR using RHind24 as primer. This promoted a smear of amplicon between <700 and 1500 bp in size. Two major bands at the size of 900 and 1100 bp were observed in male amplicons. Two major bands at the size of 950 and 1200 bp were observed in female amplicons. (Figure 3.29).

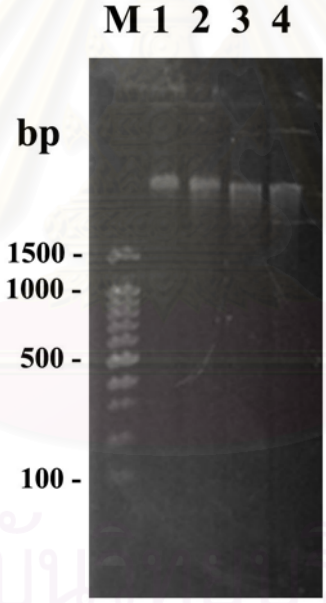



**Figure 3.28** A 1.0% agarose gel electrophoresis showing male and female *Hin*dIII-digested genomic DNA (lane 3 and 4, respectively). λ-*Hin*dIII DNA was used as the DNA markers (lane M). Undigested male and female genomic DNAs are shown in lane 1 and 2, respectively.

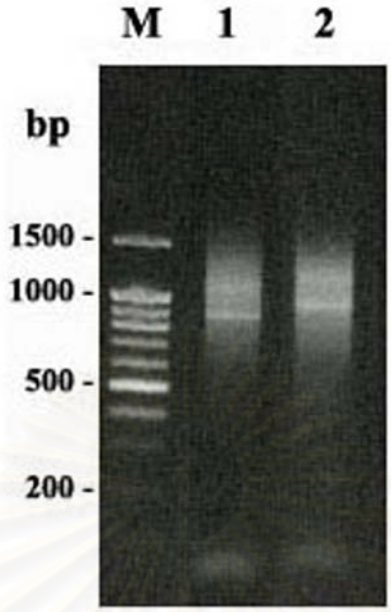



**Figure 3.29** A 1.0% agarose gel electrophoresis showing amplification of male (lane 1) and female (lane 2) *Hin*dIII-digested genomic DNA ligated to RHind12 and RHind24 oligonucleotides. A 100 bp ladder was used as the DNA markers (lane M).

### 3.3.1 Male genomic DNA subtraction

After the first round of RDA using female driver and male tester, the complexity of the subtracted male amplicon was decreased from a smear to two major bands, approximately 200 and 450 bp in size, and smear ranging in size from 200 to 450 bp (Figure 3.30, lane 1). The second round of RDA also yielded products of a similar pattern to the first round (Figure 3.30, lane 2). Four subtracted DNA fragments from the first and second rounds of RDA products were cloned and sequenced. Plasmids were digested with *Eco*RI to check the insert size before sequencing (Figure 3.31). The resulted sequences were shown in Figure 3.32.

Sequences from each clone were further analyzed by sequence comparison with data reported in the GenBank (Figure 3.32). The result indicated that all isolated clones were unknown DNA fragments (Table 3.3).

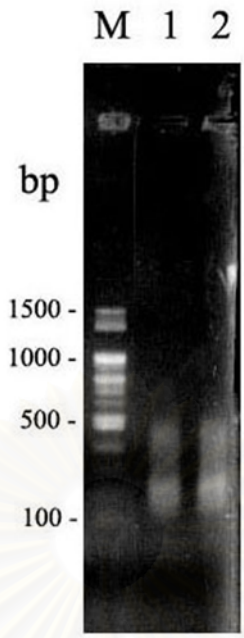



**Figure 3.30** A 1.5% agarose gel electrophoresis showing residual amplicons after one (lane 1) and two (lane 2) rounds of RDA male subtraction. A 100 bp ladder was used as the DNA markers (lane M).

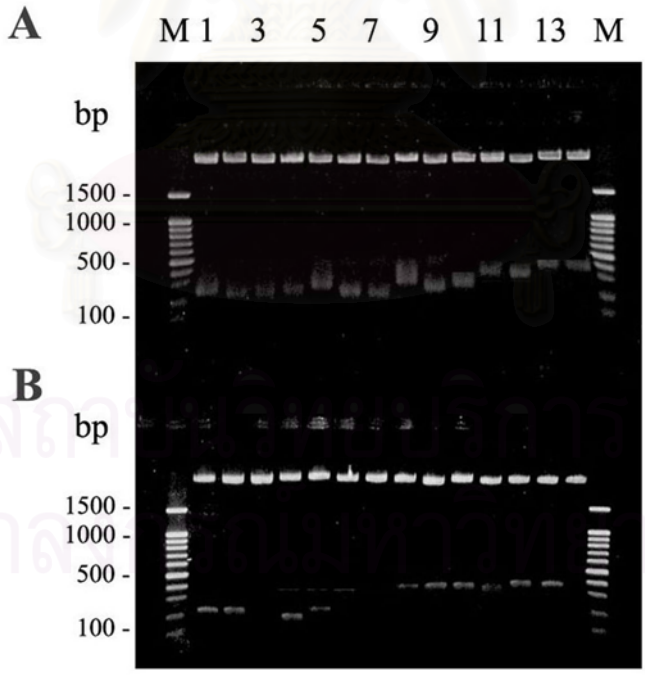



**Figure 3.31** A 1.0% agarose gel electrophoresis showing plasmid digestion of RDA subtraction of genomic DNA of male (tester) and female (driver), and the first round (panel A) and the second round (panel B) of subtractions. A 100 bp ladder was used as the DNA markers (lane M).

**>pPMMJ200 (170 bp)**

ACCGACGTCGACTATCCATGAACAGCACTCTCCAGCCTCTCACCGCAAGCTAGCACTCTCCAGCC
TCTCACCGCAAGCTAGCACTCTCCAGCCTCTCACCGCAAGCTAGCACTCTCCAGCCTCTCACCGC
AAGCTCATGAACAAGCTGTTCATGGATAGTCGACGTCGGT

**>pPMMJ450 (468 bp)**

AGGCAGCTGTGGTATCGAGGGAGAACCGACGTCGACTATCCATGAACAGAGCACTCTCCAGCCTC
TCACCGCACACCCACTCGTTCCGCTAGCTCACGGTCTGTTCTCCCTATCAAACATGCCATATCTT
GTTGGCGAGGCCTCACCCTTCTCCTAAATCTGCTCCTCCTCCCCTCGACTAAGCGCAATGCTGTA
TTTACCGCGAGGATGTAAAAAATGGTGATTATAATCATAATTGGAGCTATAACATAACTTCACAC
AACACTAAATATGTATTGACATTGATTGACTTANAATTGGTGATGCTTATAGCCATATCAAGCTT
CTCCCTCGATACCACAGNTGNCTAATCNAATNCCCGNNGNCGNCATGGNGGNCGGGANCATGCNA
NNTCNGGNCCNNNNCNNCCTATNNNGAGTCNNATNNCNNNNCNNNGGNCGNNNNNNNNNNNNNN
NNGANTGGGANNN

**>pPMMN200 (198 bp)**

AGGCAGCTGTGGTATCGAGGGAGAGAGGGAGAAGCTANCACTCTCCANCCTCTCACCGCAAGCTA
NCACTCTCCAGCCTCTCACCGNAAGCTANCACTCTCCANNCTCTCACCGNNAGCTANCACTCTCC
ANNCTCTCACCGNNNGCNCNNNCTNNTNNCTCNNNNNCNNNNNNNNCNNNNNNNNNNNCNNNNN
CNN

**>pPMMN450 (465 bp)**

AGGCAGCTGTGGTATCGAGGGAGAACCGACGTCGACTATCCATGAACACACTCTCCAGCCTCTCA
CCGCACTCGTTCCGCTAGCTCACGGCGGTGTTCTCCCTATCAAACATGCCATATCTTGTTGGCGA
GGCTTTACCCTTCTCCTAAATCTGCTCCTCCTCCCCTCTCCCCTCGACTAANCGCGATGCTGTAT
TTACCGCGAGGATGTAAAAAATGGTGATTATAATCATAATNGGGGNTATAACATAACTTCACACA
ACACTAAATATGTATTGACATNGACTGACTTANAATTGGTGATGCTTATANCCATATNAANCTTC
TCCCTCNATACCACAGNTGNNTANTCNNNTNCCCGNNNGNCGNCNNGNNGGNCGNNANCNTNNNA
NNTNNGNNCCNNNNNNNCCNNTNNNNANNCNNNTNNNNNNNNNNNGNNCNNNNNNNNNNNNNNN
NNNNNGNNAN

**Figure 3.32** Nucleotide sequences of positive clones resulted from RDA subtraction of male (tester) and female (driver) genomic DNA of *P. monodon*.

**Table 3.3** BLAST analysis of nucleotide sequences of clones obtained from RDA subtraction of male (tester) and female (driver) genomic DNA of *P. monodon*.

| Clone | Insert size (bp) | BLAST*N* | E-value | BLAST*X* | E-value |
|---|---|---|---|---|---|
| PMMJ200 | 170 | Unknown | 1E-03 | Not significant | - |
| PMMJ450 | 468 | Unknown | - | Unknown | - |
| PMMN200 | 198 | Unknown | - | Unknown | - |
| PMMN450 | 465 | Unknown | - | Unknown | - |

### 3.3.2 Female genomic DNA subtraction

RDA was conducted using male driver and female tester. The result from the first round revealed that the complexity of the female amplicon was decreased from a smear to three major bands, approximately 200, 320 and 800 bp in size, and smear ranging from 180 bp to 1 kb (Figure 3.33, lane 1). The second round of RDA yielded products at the size of approximately 300 bp, and smear ranging in size from 180 bp to 1 kb (Figure 3.33, lane 2).

Three separate RDA female subtraction products from the first round of RDA were cloned and sequenced. The sequences were shown in Figure 3.35. The size of each fragment was found to be 185, 270 and 785 bp, respectively. One separate RDA product from the second round of RDA was cloned. The insert size from the plasmid was checked before sequencing by the digestion of plasmid (Figure 3.34). When sequenced, it was found to be 158 bp in size. The result of sequencing analysis was shown in Figure 3.35.

The sequences of subtracted DNA fragment were identified by sequence comparison of clones from subtraction and those reported in the GenBank (Figure 3.35). It was revealed that all isolated clones were unknown DNA segments (Table 3.4).

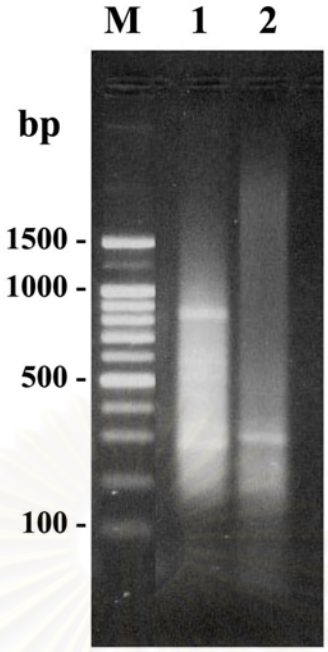



**Figure 3.33** A 1.5% agarose gel electrophoresis showing residual amplicons after one (lane 1) and two (lane 2) rounds of RDA female subtraction. A 100 bp ladder was used as the DNA markers (lane M).

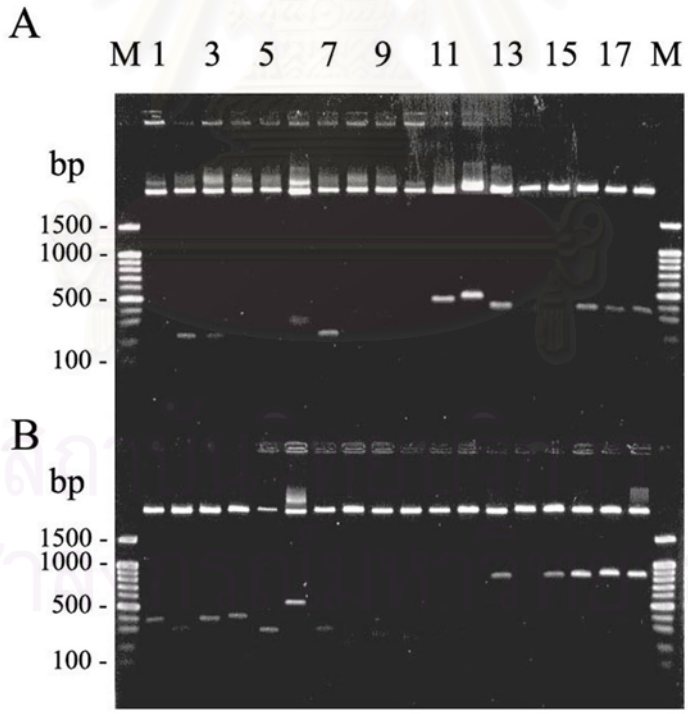



**Figure 3.34** A 1.0% agarose gel electrophoresis showing plasmid digestion of RDA subtraction of genomic DNA of female (tester) and male (driver), and the first round (panel A) and the second round (panel B) of subtractions. A 100 bp ladder was used as the DNA markers (lane M).

**>pPMFJ200 (235 bp)**

ACCGACGTCGACTATCCATGAACAAGCTTACTGCACGTCACTGCAGCAGAAATTGCAGGAGGCTA
TCAAAACCAAGAGGCACAGCATGGGGTCCCTCCTAGAAGGCAATGCCCCTGNTTCACTCTCACAT
TTCCCAGATGGAAGTGCTGTCTTGATGGCTACGGCCTTCCTCTTGTACCTTTCACCTGCGGTGAG
AGGCTGGAGAGTGCTCTGTTCATGGATAGTCGACGTCGGT

**>pPMFJ300 (318 bp)**

ACCGACGTCGACTATCCATGAACAAGCACTCTCCAGCCTCTCACCGCAGCTTGCTCACAACACTT
CCCCGGGCCTGTCGCGGCAGCCACTTTACCAATGTTATAATAATGTTGCCAAAAGTTCGACTCCA
CCGTCATTTCCTGGAATGTTACTCGAGTTCACAAATGTTATTTTATTCTTGAATTGTATCAATTG
ATTAATTACTGATTTACTGATATTACGTTAGGAAATAAGAATAAATACGAGCTGTTATTTATAAA
CATCTTAAAAGTACAAATCAAATGAGGGAAAGCTTGTTCATGGATAGTCGACGTCGGT

**>pPMFJ800 (809 bp)**

ACCGACGTCGACTATCCATGAACACATGAACAAGCTTGATATGACTATAAGCATCACCAATTCTA
AGTCAATGTCAATACATATTTAGTGTTGTGTGAAGTTATGTTATAGCTCCAATTATGATTATAAT
CACCATTTTTTACATCCTCGCGGTAAATACAACATCGCGCTTAGTCGAGGGGAGAGGGGAGGAGG
AGCAGATTTAGGAGAAGGGTGAGGTCTCGCCAACAAGATATGGCATGTTTGATAGGGAGAACAGA
CCGTGAGCTGGCGGAACGAGTGGGTGGGAAGAACAATGTGCTACAAAGATCAACAACCGAGGCGA
GCATTTCTAAAACTTTCTAGCTATCCAGAACATGGGGTTTACCTTGGGTCAAATGATTGAAGGGG
TGCGATTTCCATGGTTAGATATTCATTAACTAGGCAGTTCTTCACGAAAGGAGACGAGAAAAGTA
CTTCCATGGTGTCGCTCTATTGTTTGCCTCTTCCTCATTTCGCCAGTCCATTAATGGTCTTAGTA
TGTCGGTGAAGTCATTATCTCGTGACANGCAGGGGTATTCCCTGNATACCTGTCTGTGGTCGTCC
GTTATCTGGATATTGCCTGGAACATTGTCAGAGTTAGGAAGGCATCTTTANATAATANGGCTTAN
AACAACTGACTTCTGTTAAGATGATACATGCTATACATTTAACACCCGTATNTTTCCAGTCTGAA
AAACTGNTCAGTCATTACTTTACTCACCNCCTGNNTTGAAAAAACNGGCTTNGCTTCNTANTTTA
AANAACTGTCNGGATNTCACNCGTACAAT

**>pPMFN300 (205 bp)**

AGGCAGCTGTGGTATCGAGGGAGACCGACGTCGACTATCCATGAACACGTCAGTCACTGAGGAGA
ACGGACGCTTGCTGCACCTTTACAGATAACCAGGTCTCATAACCTAAGGAATGAACTATCTGAAC
CACACCCCTAAGCACTACAAACTTGTAAGTATCTCANNCCTAGCTAAGCTTCTCCCTCGATACCA
CAGNCTGCCT

**Figure 3.35** Nucleotide sequences of positive clones resulted from RDA subtraction of female (tester) and male (driver) genomic DNA of *P. monodon*.

**Table 3.4** BLAST analysis of nucleotide sequences of clones obtained from RDA subtraction of female (tester) and male (driver) genomic DNA of *P. monodon*.

| Clone | Insert size (bp) | BLAST*N* | E-value | BLAST*X* | E-value |
|---|---|---|---|---|---|
| PMFJ200 | 235 | Unknown | 0.006 | Unknown | 4.6 |
| PMFJ300 | 318 | Unknown | 0.002 | No significant | - |
| PMFJ800 | 809 | Unknown | 0.47 | Unknown | 0.38 |
| PMFN300 | 205 | Unknown | 5.2 | No significant | - |

### 3.3.3 Detection of sex-specific markers by PCR

Some candidate markers obtained from RDA genomic DNA subtraction of *P. monodon* for sex specificity were detected by PCR amplification. Candidate markers with the sizes larger than 200 bp were selected and subjected to primer designation. This included two fragments, PMFJ200 and PMFJ800, from RDA female subtraction. Sequences and positions of the forward and reverse primers were shown in Figure 3.36. PCR reaction was performed in genomic DNA and gonad-extracted cDNA from both male and female of *P. monodon*.

**>pPMFJ200 (235 bp)**

ACCGACGTCGACTATCCATGAACAAGCTTACTGCACGTCACTGCAGCAGAAATTGC**AGGAGGCTA**
**TCAAAACCAAGA**GGCACAGCATGGGGTCCCTCCTAGAAGGCAATGCCCCTGNTTCACTCTCACAT
TTCCCAGATGGAAGTGCTGTCTTGATGGCTACGGCCTTCCTC**TTGTACCTTTCACCTGCGGT**GAG
AGGCTGGAGAGTGCTCTGTTCATGGATAGTCGACGTCGGT

**Figure 3.36** Nucleotide sequences of pPMFJ200 and pPMFJ800. Sequences and positions of the forward primer and the complementary to the reverse primer are underlined and boldfaced (continue).

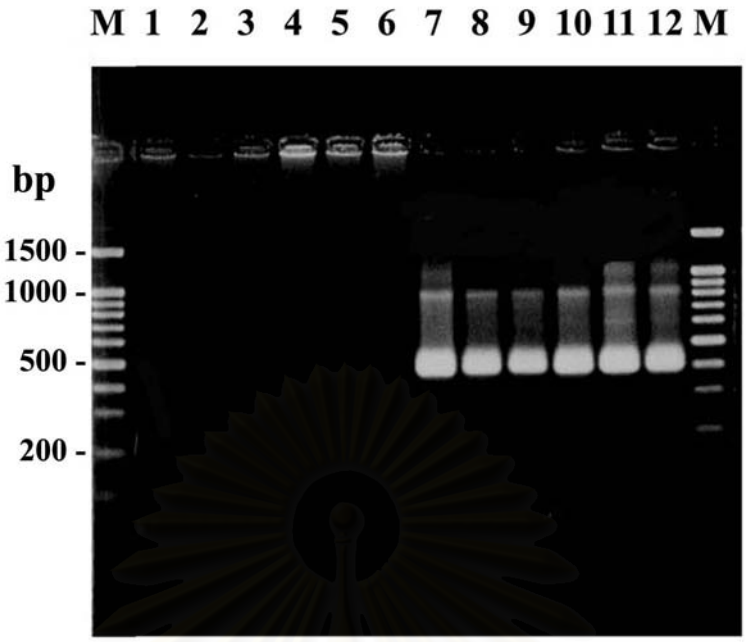
**>pPMFJ800 (809 bp)**

ACCGACGTCGACTATCCATGAACACATGAACAAGCTTGATATGACTATAAGCATCACCAATTCTA
AGTCAATGTCAATACATATTTAGTGTTGTGTGAAGTTATGTTATAGCTCCAATTATGATTATAAT
CACCATTTTTTACATCCTCGCGGTAAATACAACATCGCGCTTAGTCGAGGGGAGAGG**GGAGGAGG**
**AGCAGATTTAGG**AGAAGGGTGAGGTCTCGCCAACAAGATATGGCATGTTTGATAGGGAGAACAGA
CCGTGAGCTGGCGGAACGAGTGGGTGGGAAGAACAATGTGCTACAAAGATCAACAACCGAGGCGA
GCATTTCTAAAACTTTCTAGCTATCCAGAACATGGGGTTTACCTTGGGTCAAATGATTGAAGGGG
TGCGATTTCCATGGTTAGATATTCATTAACTAGGCAGTTCTTCACGAAAGGAGACGAGAAAAGTA
CTTCCATGGTGTCGCTCTATTGTTTGCCTCTTCCTCATTTCGCCAGTCCATTAATGGTCTTAGTA
TGTCGGTGAAGTCATTATCTCGTGACANGCAGGGGTATTCCCTGNATAC**CTGTCTGTGGTCGTCC**
**GTT**ATCTGGATATTGCCTGGAACATTGTCAGAGTTAGGAAGGCATCTTTANATAATANGGCTTAN
AACAACTGACTTCTGTTAAGATGATACATGCTATACATTTAACACCCGTATNTTTCCAGTCTGAA
AAACTGNTCAGTCATTACTTTACTCACCNCCTGNNTTGAAAAAACNGGCTTNGCTTCNTANTTTA
AANAACTGTCNGGATNTCACNCGTACAAT

**Figure 3.36** Nucleotide sequences of pPMFJ200 and pPMFJ800. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced.

For PMFJ800 amplification, product at the size of 401 bp was observed when amplified with an annealing temperature of 60°C. No different pattern of PCR product amplified from genomic DNA of male and female shrimps (Figure 3.37). Similar result was obtained with the amplification of testis and ovary cDNA (Figure 3.38). Different PCR cycles (20, 25, 30, and 35 cycles) were performed. PCR products were observed from cycle 25 to 35, however, no difference between products were detected (Figure 3.39). These indicated that PMFJ800 was not sex-specific and it was not differentially expressed between male and female *P. monodon*.

For the detection of PMFJ200, PCR was performed with 4 different annealing temperatures (45, 55, 50 and 48°C). However, no expected PCR product was amplified in genomic DNA and gonad-extracted cDNA from both sexes. Therefore, sex specificity of PMFJ200 was still non-conclusive.




**igure 3.37** A 1.2% agarose gel electrophoresis showing result of amplification when genomic DNAs were used as templates (annealing temperature at 60°C). Lane 1-6 = PMFJ200 primer and lane 7-12 = PMFJ800 primer (lane 1-3 and 7-9 = male genomic DNA and lane 4-6 and 10-12 = female genomic DNA) A 100 bp ladder was used as the DNA markers (lane M).

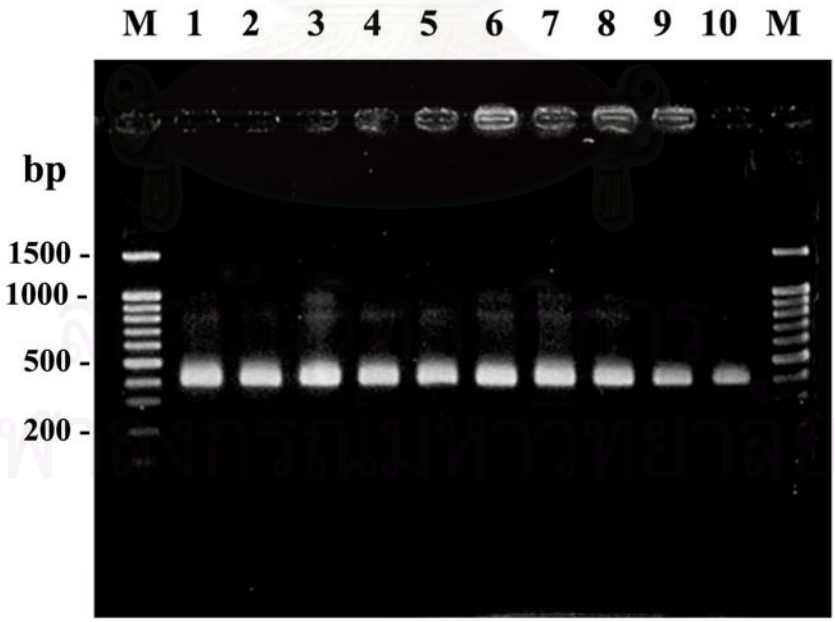



**Figure 3.38** A 1.2% agarose gel electrophoresis showing result of amplification when the first stand cDNAs (testis and ovary) were used as templates (annealing temperature at 60°C) with PMFJ800 primer (lane 1-5 = testis and lane 6-10 = ovary). A 100 bp ladder was used as the DNA markers (lane M).

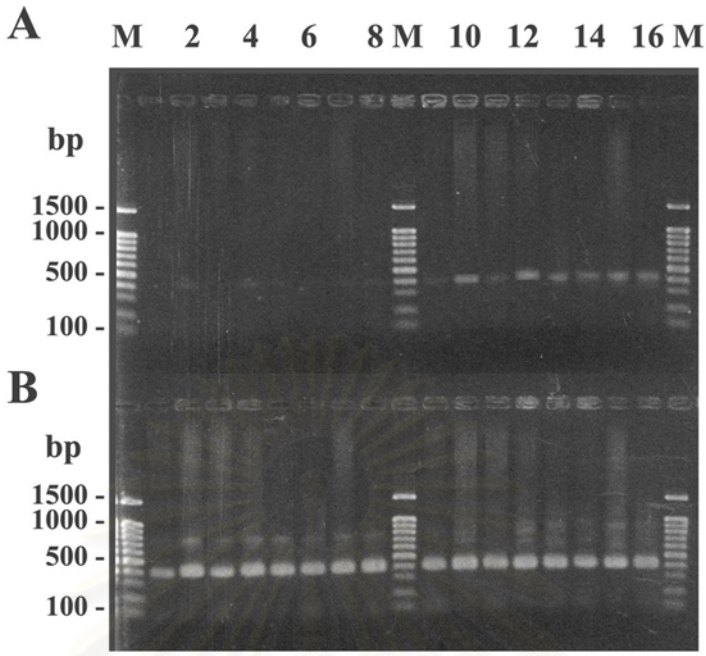



**Figure 3.39** A 1.2% agarose gel electrophoresis showing result of varying cycles of amplification when the first stand cDNAs (testis and ovary) as template (annealing temperature at 60°C) with PMFJ800 primer (lane 1-2 and 9-10 = testis of *P. monodon* 3-month old, lane 3-4 and 11-12 = testis of *P. monodon* adult, lane 5-6 and 13-14 = ovary of *P. monodon* 3-month old and lane 7-8 and 15-16 = ovary of *P. monodon* adult). Panel A was 20 (left) and 25 (right) cycles, respectively and panel B was 30 (left) and 35 (right) cycles, respectively. A 100 bp ladder was used as the DNA markers (lane M).

### 3.3.4 Genome walking

In order to find the unknown genomic DNA sequences adjacent to the known sequences of PMFJ200, and, PMFJ800, genome walking was carried out. Male and female genomic DNAs of *P. monodon* digested with *Alu*I, *Hae*III, *Rsa*I, and *Dra*I and ligated to adaptors were used as templates for PCR amplification. The result of PMFJ200 was amplified non-specific products (Figure 3.40). The result of PMFJ800 5' and 3' extension revealed similar patterns of amplified products in both male and female genomic DNA digested with *Hae*III, *Rsa*I, and *Dra*I whereas the 5' extention (reverse primer+AP1) of female genomic DNA digested with *Alu*I yielded 300, 400 and 600 bp product and 3'extension (forward+AP1) yielded 150 bp (Figure 3.41). These products

were then amplified using forward and reverse primers (Figure 3.42) and digested with *Alu*I. The result revealed no digestion between male and female (Figure 3.43). When genome walking was performed in other 3 individual samples for each sex, the result revealed no difference in PCR product pattern from both sexes (Figure 3.44). This indicated that the different PCR product pattern detected from the first sample caused by nucleotide polymorphism of each individual.

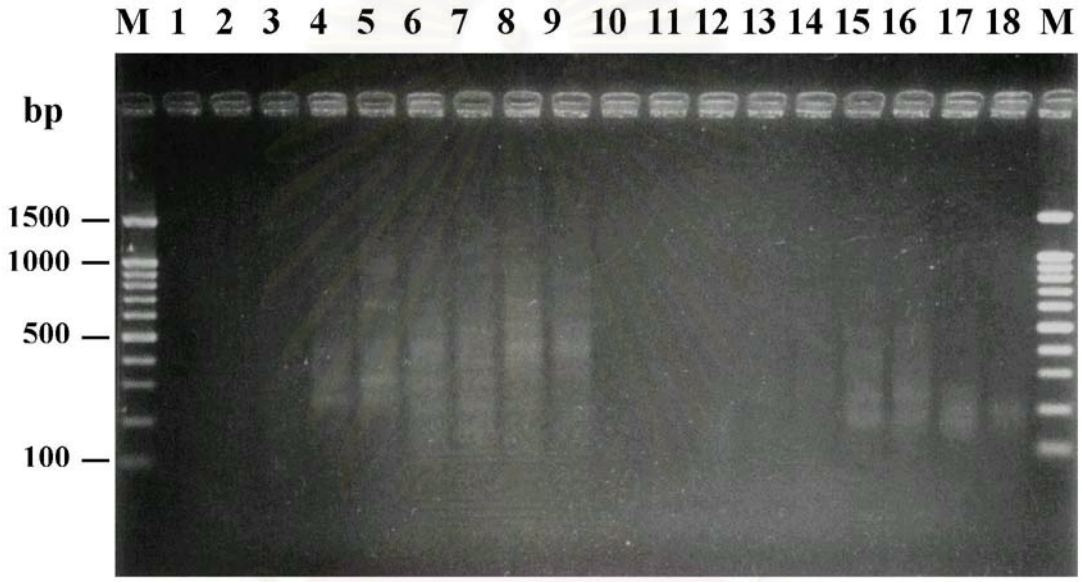



**Figure 3.40** A 1.5% agarose gel electrophoresis showing result of genome walk when PMFJ200 primer was used, lane 1-9 = forward primer + AP1 and lane 10-18 = reverse primer + AP1 (lane 2-3, 11-12 = genomic DNA digested with *Alu*I, lane 4-5, 13-14 = *Dra*I, lane 6-7, 15-16 = *Hae*III and lane 8-9, 17-18 = *Rsa*I) lane 2, 4, 6, 8, 11, 13, 15, 17 = male genomic DNA and lane 3, 5, 7, 9, 12, 14, 16, 18 = female genomic DNA). A 100 bp ladder was used as the DNA markers (lane M). Negative controls of forward and reverse primer are lane 1 and 10, respectively).

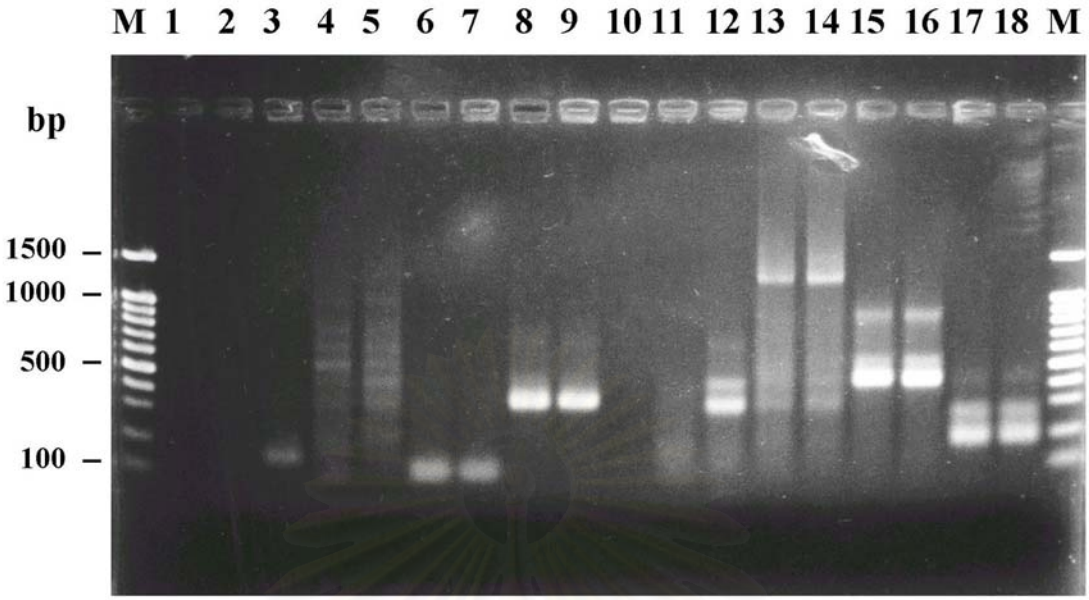



**Figure 3.41** A 1.5% agarose gel electrophoresis showing result of genome walk when PMFJ800 primer was used as probe, lane 1-9 = forward primer + AP1 and lane 10-18 = reverse primer + AP1 (lane 2-3, 11-12 = genomic DNA digested with *Alu*I, lane 4-5, 13-14 = *Dra*I, lane 6-7, 15-16 = *Hae*III and lane 8-9, 17-18 = *Rsa*I) lane 2, 4, 6, 8, 11, 13, 15, 17 = male genomic DNA and lane 3, 5, 7, 9, 12, 14, 16, 18 = female genomic DNA). A 100 bp ladder was used as the DNA markers (lane M). Negative controls of forward and reverse primer are lane 1 and 10, respectively).

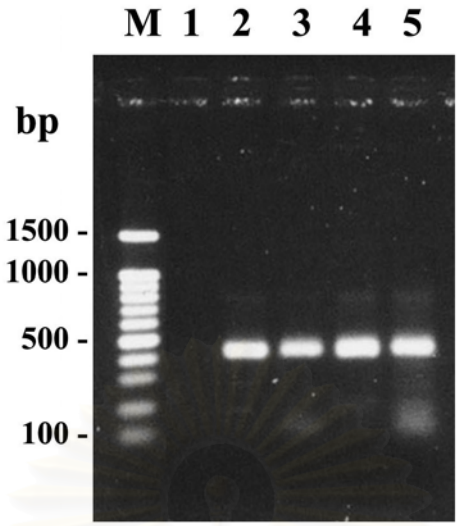



**Figure 3.42** A 1.2% agarose gel electrophoresis showing result of amplification when the genome walk products were used as template with PMFJ800 primer. A 100 bp ladder was used as the DNA markers (lane M) and lanes 1 = negative control of PMFJ800.

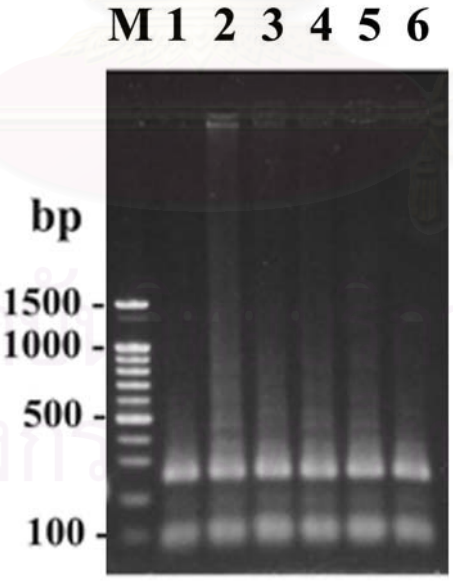



**Figure 3.43** A 1.5% agarose gel electrophoresis showing result of genome walk product from PMFJ800 primer digested with *Alu*I. Lane 1-3 = male products and lane 4-6 = female products. A 100 bp ladder was used as the DNA markers (lane M).

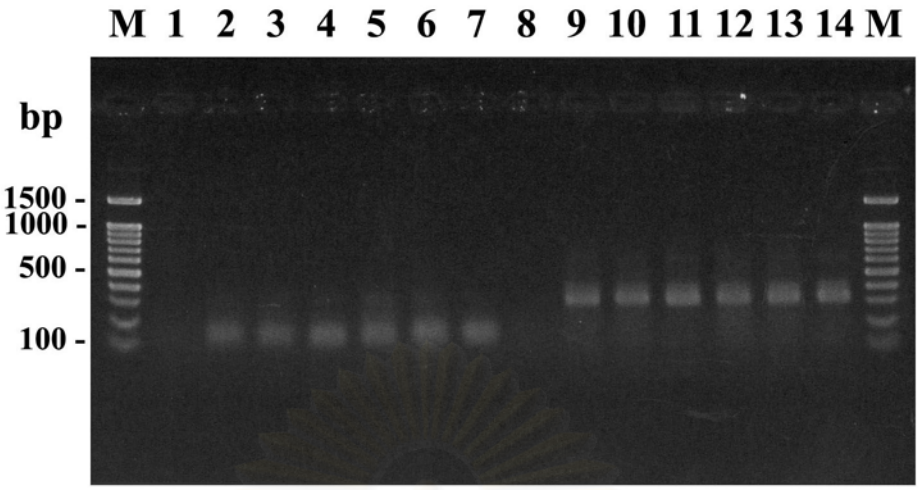



**Figure 3.44** A 1.5% agarose gel electrophoresis showing result of genome walk (repeated genomic DNA digested with *Alu*I only) when used PMFJ800 primer, lane 1-7 = forward primer + AP1 and lane 8-14 = reverse primer + AP1 (lane 2-4, 9-11 = male genomic DNA and lane 5-7, 12-14 = female genomic DNA). A 100 bp ladder was used as the DNA markers (lane M). Negative controls of forward and reverse primer are in lane 1 and 8, respectively.

### 3.3.5 Identification of nucleotide polymorphism using SSCP analysis

PMFJ800, one of candidate markers from female genomic DNA subtraction (RDA), revealed no sex-specificity when analyzed by agarose gel electrophoresis. Therefore, further study on nucleotide polymorphism of PMFJ800 was carried out using SSCP. The result products of PCR amplification and genome walking from both sexes analyzed. The results showed no sex-specific nature of this candidate. Previous result in genome walking analysis showed sex specificity of PMFJ800 was possibly caused by polymorphic across investigated individuals (Figure 3.45, 3.46 and 3.47).

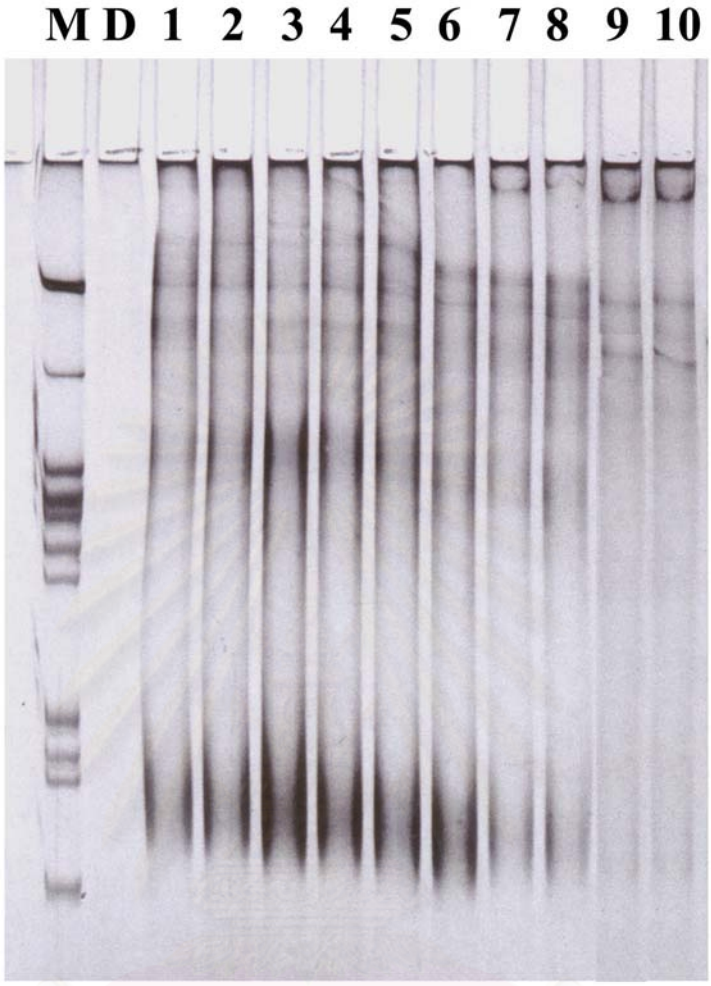



**Figure 3.45** SSCP patterns of a female genomic subtraction fragments, PMFJ800, analyzed in male (lane 1-3) and female (lane 4-6) genomic DNA and cDNAs from testis (lane 7-8) and ovary (lane 9-10) of *P. monodon*. Result was derived from size-fractionation through 12.5% PAGE + 5% glycerol. Lane M = 100 bp, lane D = ds PCR product control.

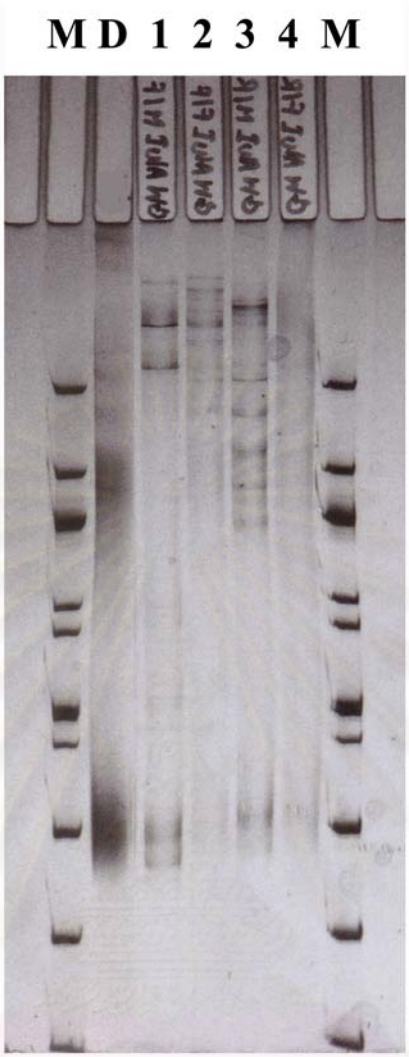



**Figure 3.46** SSCP patterns of a genome walk products using primers from PMFJ800 and AP1. The analysis was conducted in male (lane 1 and 2) and female (lane 3 and 4) genomic DNA of *P. monodon*. Result was derived from size-fractionation through 12.5% PAGE + 5% glycerol. Lane 1 and 3 = forward primer + AP1 and lane 2 and 4 = reverse primer + AP1. Lane M. Lane M = 100 bp, lane D = ds PCR product control.

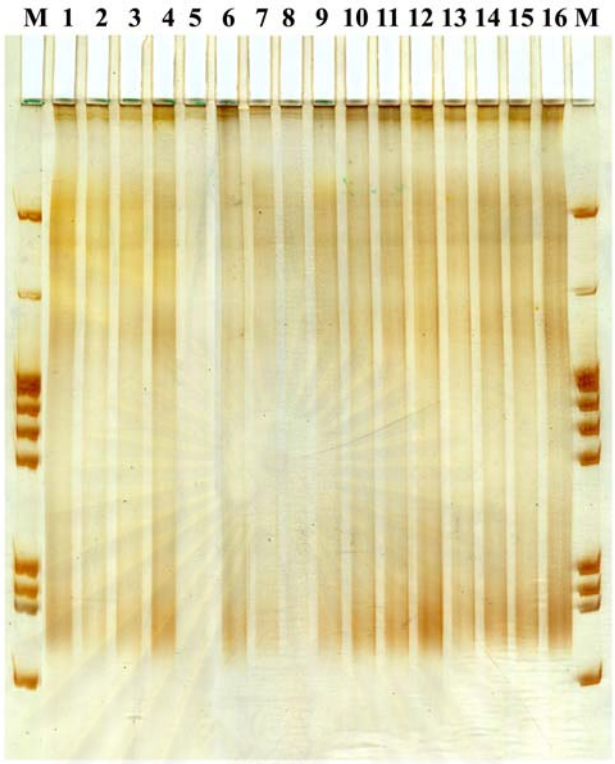



**Figure 3.47** SSCP patterns of a female genomic subtraction fragments, PMFJ800, analyzed in testes (lane 1-5), ovaries (lane 6-10), male haemocytes (lane 11-13) and female haemocytes (lane 14-16) of *P. monodon*. Result was derived from size-fractionation through 12.5% PAGE + 5% glycerol. Lane M = 100 bp.

### 3.3.6 Southern blot analysis

A number of clones containing insert fragments from male and female RDA were chosen and made as probes for further analysis on sex specificity using Southern blotting and hybridization methods. This included 4 clones from male subtraction (PMMJ200, PMMJ450, PMMN200, and PMMN450), and 4 clones from female subtraction (PMFJ200, PMFJ300, PMFJ800, and PMFN300). The results were shown in Figure 3.48, 3.49, 3.50, 3.51 and 3.52, 3.53, 3.54, 3.55, and 3.56, respectively.

For the result of hybridization with the clones from male subtraction, corresponding bands of genomic DNA from both male and female were detected after probing with PMMJ500 and PMMN500, indicating that these 2 clones were not sex specific while no positive band was detected from both male and female genomic DNA after probing with PMMJ200 and PMMN200. Similar results were obtained from the hybridization of the clones from female subtraction. Positive bands of genomic DNA from both male and female were detected after probing with PMFJ200, PMFN300, indicating no sex specificity obtained from these 2 clones. For the result of PMFJ800 probing, the major bands observed in male genomic DNA appeared slightly bigger than those in female. Thus, the hybridization pattern of PMFJ800 to the genomic DNAs digested with different enzyme (*Mbo*I) was further conducted. As shown in Figure 3.55, identical bands of both male and female genomic DNAs were detected when hybridized with PMFJ800, indicating that PMFJ800 was not sex specific. No positive band was detected from both male and female genomic DNA after probing with PMFJ300.

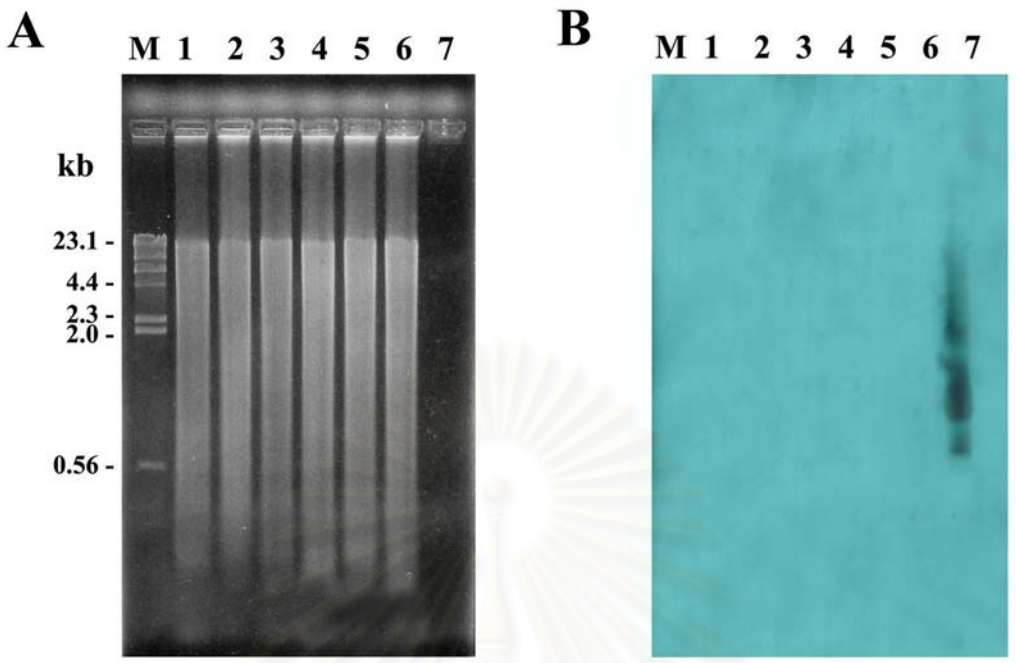



**Figure 3.48** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMMJ200 (B). The probe was non-radioactively labelled by DIG and hybridized with male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I. Positive control (an amplified product of PMMJ200 using pUC1 and pUC2 primers) was shown in lane 7.

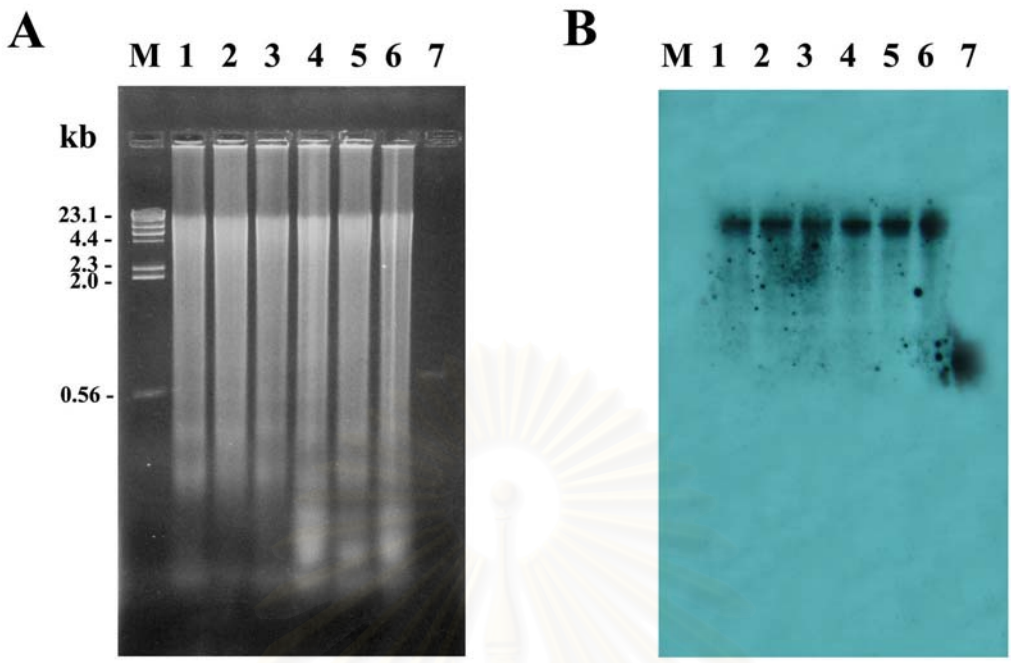



**Figure 3.49** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMMJ450 (B). The probe was non-radioactively labelled by DIG and hybridized with male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I. Positive control (an amplified product of PMMJ450 using pUC1 and pUC2 primers) was shown in lane 7.

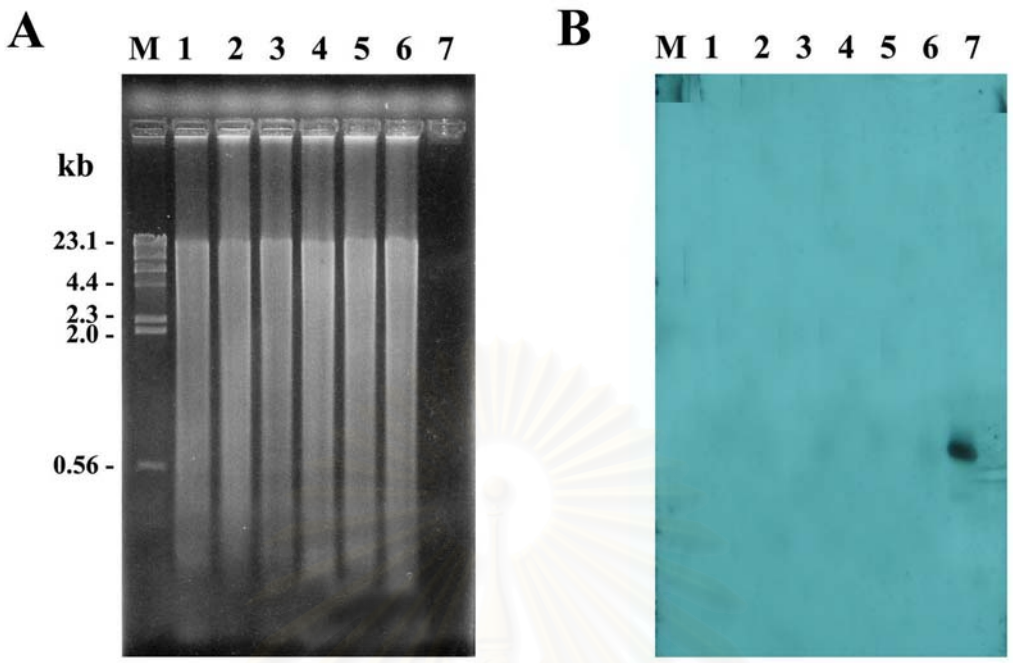



**Figure 3.50** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMMN200 (B). The probe was non-radioactively labelled by DIG and hybridized with male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I. Positive control (an amplified product of PMMN200 using pUC1 and pUC2 primers) was shown in lane 7.

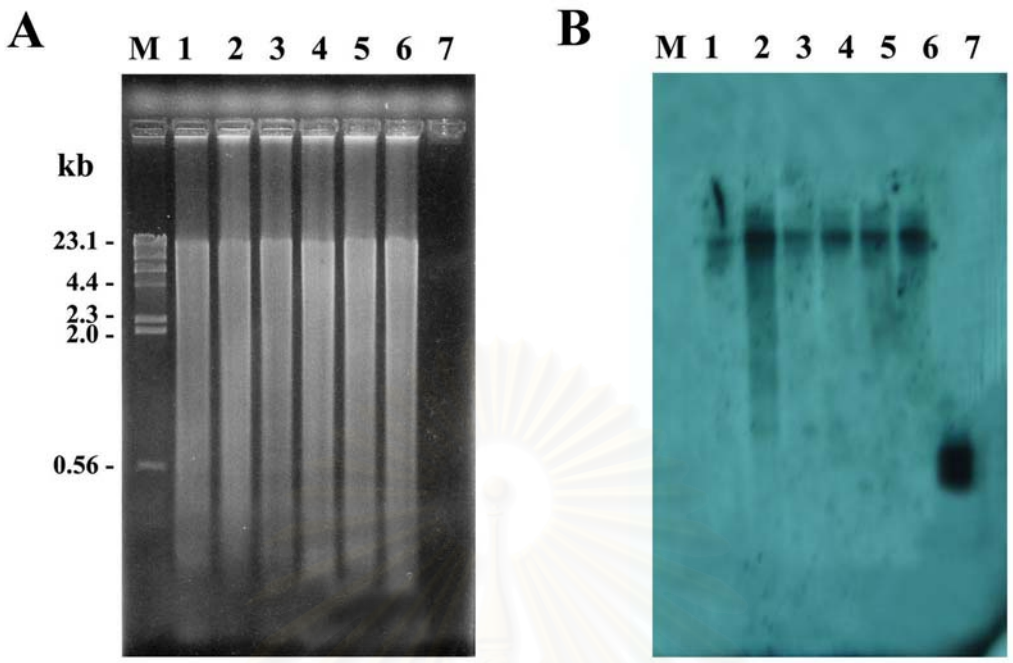



**Figure 3.51** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMMN450 (B). The probe was non-radioactively labelled by DIG and hybridized with male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I. Positive control (an amplified product of PMMN450 using pUC1 and pUC2 primers) was shown in lane 7.

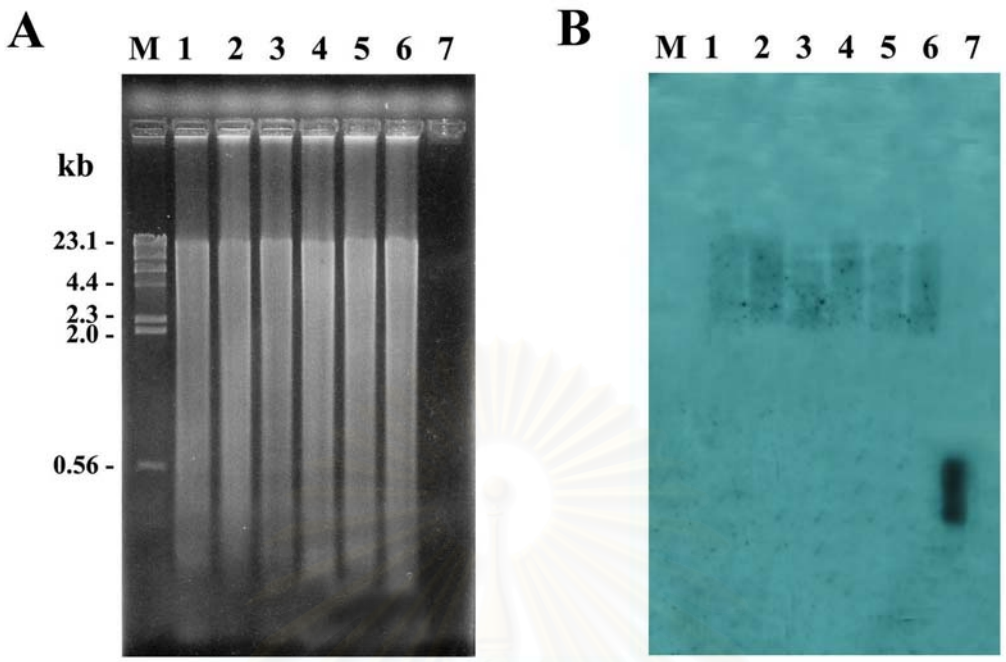



**Figure 3.52** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMFJ200 (B). The probe was non-radioactively labelled by DIG and hybridized with male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I. Positive control (an amplified product of PMFJ200 using pUC1 and pUC2 primers) was shown in lane 7.

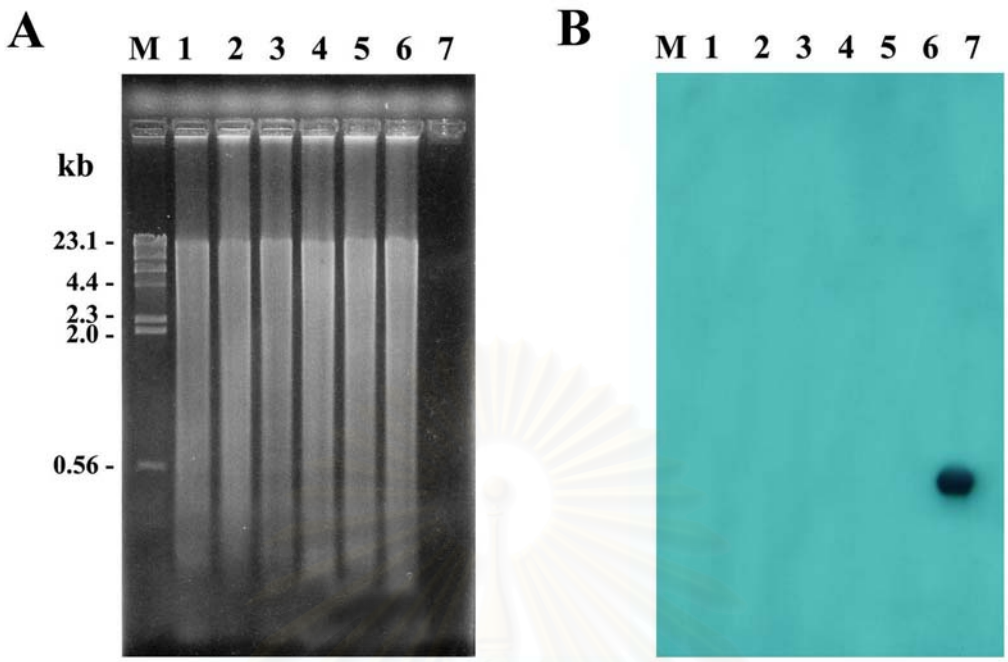



**Figure 3.53** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of pPMFJ300 (B). The probe was non-radioactively labelled by DIG and hybridized with male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I. Positive control (an amplified product of PMFJ300 using pUC1 and pUC2 primers) was shown in lane 7.

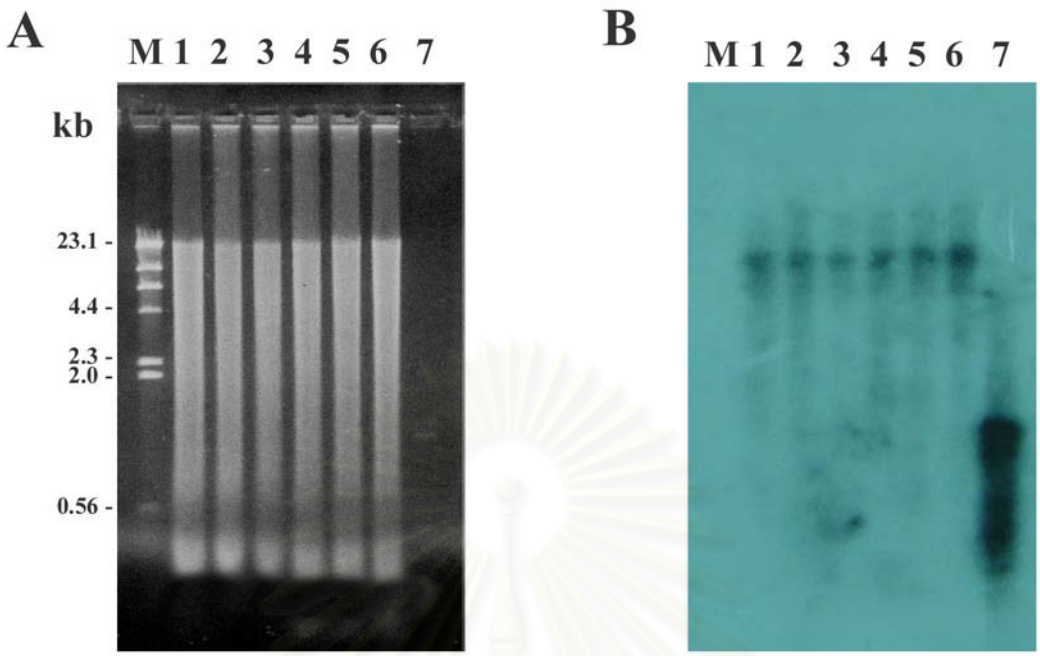



**Figure 3.54** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMFJ800 (B). The probe was non-radioactively labelled by DIG and hybridized with male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I. Positive control (an amplified product of PMFJ800 using pUC1 and pUC2 primers) was shown in lane 7.

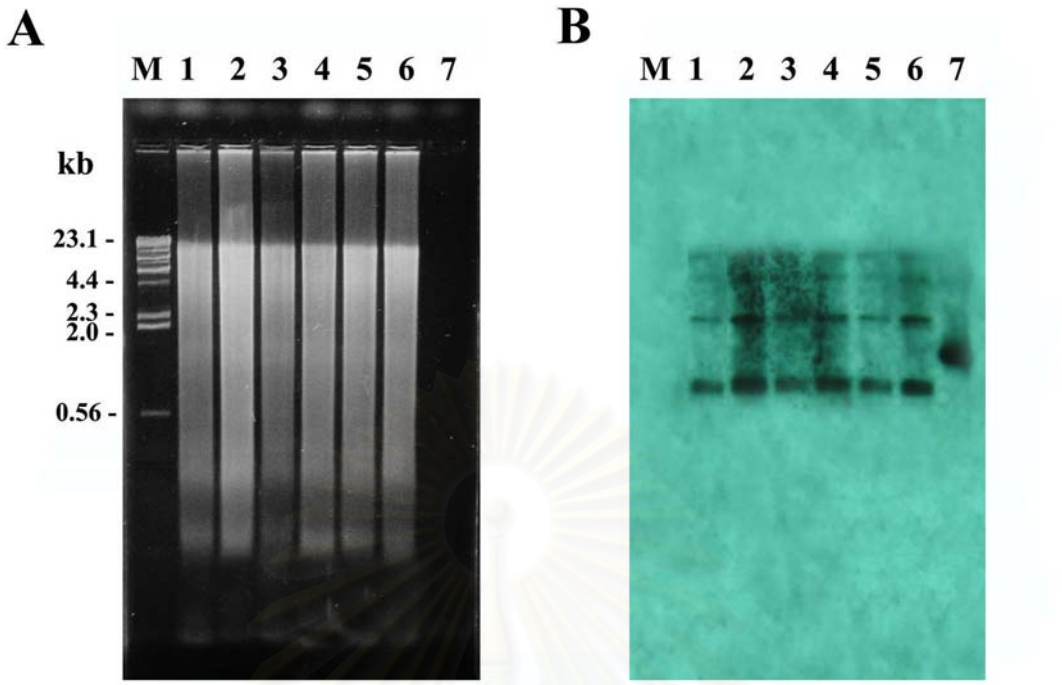



**Figure 3.55** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMFJ800 (B). The probe was non-radioactively labelled by DIG and hybridized with male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Mbo*I. Positive control (an amplified product of PMFJ800 using pUC1 and pUC2 primers) was shown in lane 7.

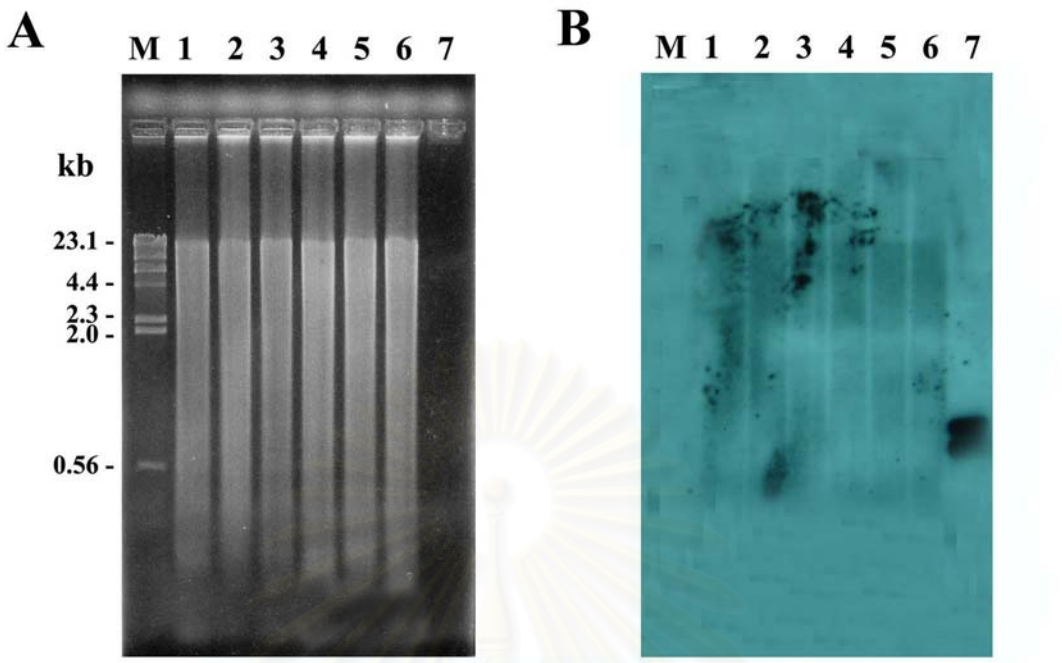



**Figure 3.56** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of PMFN300 (B). The probe was non-radioactively labelled by DIG and hybridized with male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I. Positive control (an amplified product of PMFN300 using pUC1 and pUC2 primers) was shown in lane 7.

### 3.4 Amplification of estrogen receptor homolog gene

Estrogen receptor (ER) gene was amplified in genomic DNA and cDNA of *P. monodon* using 4 combinations of primers. Forward (F1 and F2) and reverse (Rα and Rβ) degenerated primers were designed from the conserved regions of ER genes from a number of fish species (data not shown). As shown by the result in Figure 3.57, no PCR product were obtained from the amplification of 4 combination primers in genomic DNA from both sexes. No product was also obtained from the amplification with testis cDNA. However, a number of PCR products were only observed from the amplification with ovary cDNA. The amplification of F1Rα yielded the product at the size of 1400 bp while F1Rβ yielded the products of 1000 and 1400 bp, and F2Rβ yielded the products of 490, 850, and 1400 bp (Figure 3.57). After a number of samples was increased both from males (2 testes) and females (2 ovaries from 3 month old and adult shrimps), the patterns of PCR products remained the same (Figure 3.58). Some of the products were cloned and sequenced. The results were shown in Figure 3.59. These clones were further identified by sequence comparison with the data reported in the GenBank (Table 3.5).

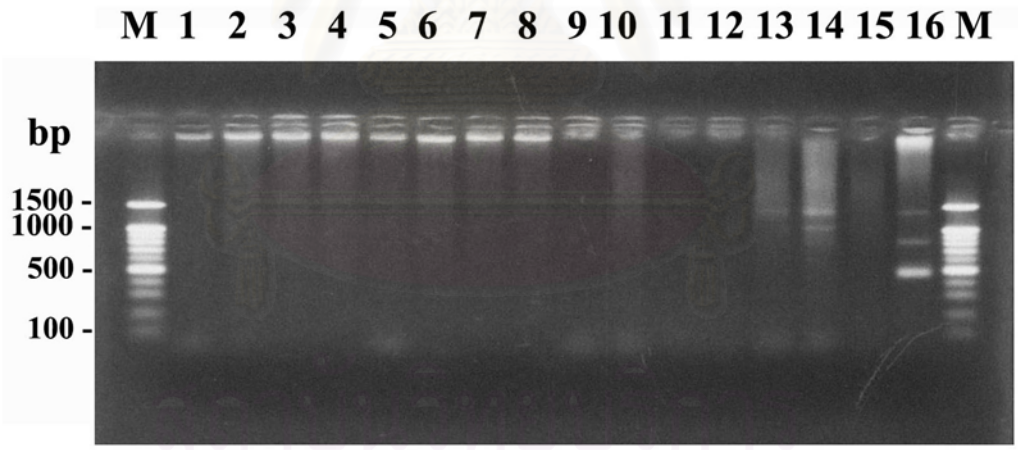



**Figure 3.57** A 1.5% agarose gel electrophoresis showing amplification of male and female genomic DNA (lane 1-4 and 5-8, respectively) and the first strand cDNA from testes and ovaries (lane 9-11 and 12-16, respectively) using different primer recombination; ER_F1/Rα (lane 1, 5, 9 and 13), ER_F1/Rβ (lane 2, 6, 10 and 14), ER_F2/Rα (lane 3, 7, 11 and 15) and ER_F2/Rβ (lane 4, 8, 12 and 16). A 100 bp ladder was used as the DNA markers (lane M).

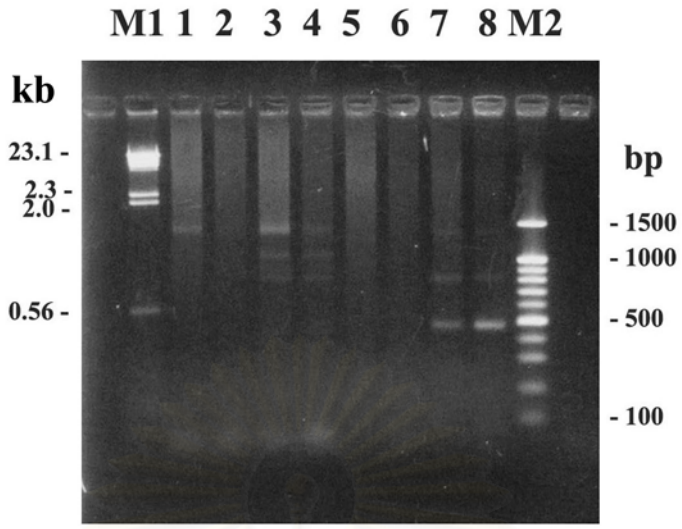



**Figure 3.58** A 1.5% agarose gel electrophoresis showing amplification of the first strand cDNA from ovaries 3 month old and adult (lane 1, 3, 5, 7 and 2, 4, 6, 8, respectively) using different primer recombination; ER_F1/Rα (lane 1 and 2), ER_F1/Rβ (lane 3 and 4), ER_F2/Rα (lane 5 and 6) and ER_F2/Rβ (lane 7 and 8). A 100 bp ladder was used as the DNA markers (lane M).

**>ER_F2β5/5 (468 bp)**

ACCTCAAACCAACAACATTCCCCAGTCAAGAGAAGCACTACCTTCCAAGGAAAGCTTTGTACTTG
CCCCATGGGCATGCTCTGCTTGTACGTTCCTAAATGACAGGGACCGCAACGTTTGTGACATGTGT
GGAAAGTCTCGCATGCCTGGTCCAGAAGTGAAGCCTCTCGTTTCTGGTGGGCGACAATGTCCACA
GTGCACTTTCATAAATGAGAGAGATGCTCAGGATTGTTCTGTATGTGGAGAACACCTGGAGGGAT
CTTCCACATACATCTAAGAAGATTAAAGTGACAGAGAAAGTGAGCCAAAGTATTCATTGTTAGTT
TTCCAGCATTGTTCAAATCTGGATTTGCCAAATGACATAAGTATTGACAAGAGATTTGTCACCTT
TTTTTCCTTTTTTATGTTTCATATGTAACAAAGAAATTACCCTTATCTGCATATTCACTCTATTG
TGCATTGGTTTGT

**Figure 3.59** Nucleotide sequences of products from F2Rβ of *P. monodon* (continue).

**>ER_F2β5/16 (571 bp)**

CAACACTCCTTGCCTCCGCGCTGCGACTTGCGGTCCACACCACCATCCCTGCTACAATCTTGTAC
TTTGAGGTGACGTCCACACACCTTTGCTCTTAGAGCAAAGTTAAAAGTCACAGCTCACAGTGTGG
GATTTTGATCCTCCACTGACACTCATTCTGCCCGCCCTTTTAATCAGTCTTACAATGGCTGAGTG
AGGCGGTGAGGTGAACACCACATGTTATGACCCACCACTCTCTTCCAGTTCACACTTGTACTTGT
TGTAGGGTGCAAATGGGGGTCGGGAAGGATCGTCAATGCGCACTACTCTCGTGCAATTTGGCCGC
AACCCATCTGTGTGCCCCAGGAACCAGTCAGTAGCAGCTCGTTGATCTGCTTCTGATACATGTCT
TGTGCACCAATCACTGTGCCAGGAAACAAGGCCACTACGCATCCTTCGACTAACATGTTCATGAC
TCTGTTTTGCTCTCTTGAGTGCTTGCTTCTTGTACATGTAGGAATCACTGTTCACTACAAATACC
ATTTTGAAGGAGGAGAGGTTGAACTTGCACTGAGTGTTGTTGGCTTGAGGT

**>ER_F2β8/14 (798 bp)**

ACCTCCAACCAACAGCACTCAACCGCTCCAACTCCTTCCTTGCTGAGACCAGAGTGAGCCTGCAC
GAGGTTAAAACCCGTCTGACCCACAACCTGCGTAGGCTAGAAAATCAAGGCCTGGCTAGTCGCGC
AGACAATTACCAGACCCTCTTGAATGCGATTGCCAGTGATATTGTCAATCAGCGGCAACACAGAA
TATCTCGAAGAATGGAGTTGCAGAGACTGCTAATGACAGCTAAACAGTTAGAGGAGAAAGAAAAG
TTCTACCGTGATCAGCTGGATTCATACCACACCTACCTCAGTACCTGCCTTGCCAATATTGCAGC
CAGAAATAAGAATGTGCATCAGGCCTCGAGCAAGCAAAATGGCGAGAAGACACGTTCAAAACAAA
CCATCAAGTACCCAGCAACCAAACTCATGGAGAAGGGTGTACTGCTAGAGGTGGAAGACCTGCCT
GAGACACAGAGGAAGAATGTGCTGTTTGAAATCACTCCTCTGGCTACCACTGGTATTTTCCATGT
TCAGGTCAAGTTCATGGGTGTGCCAATGGAGTTTGTTGAAATTGATATTCAGGAGTTACTACAGC
TTCAGTATGAAGGTGTTACAGTCAAGAAGATGTTCTCCAAAGTCAAAGTTAATGTGAACCTCCTA
CTCCACCTTCTCAATACCAAGTTCTACTCAAAGAAGAAGTGAATATACTTGAAGACAGGTCCCAC
AGTCACTGGGAAAGTATGAGTCGTAAGATATTGTACATAGGAATGAAAGTGTTGGTAAATATGAG
TTAGAAGAGTGTTGCTGGN

**>ER_ F2β8/27 (541 bp)**

ACCTCAAACCAACAACACTCATGTTTCATCCACAAGTCCTTGCATGTGAATGGTGCCAGACAAGT
GACATTGATACACTGGTTTTGAGAAATGCAGTTCTTGACCAAGTTACGGAACATTGTGGCTTGAC
CCCACTGGGTGCCATTGAGGCCTGGAGGAAGGGGAAGATGCTTGCCCTCAAGTCTGATGTCATCC
AAGCAACCCAATTTGTAATCATTATATACTTCAAAGGTCCTGATGCCTGTGTACTCTGCCTTTCC
TCCTGCATATACACCCTCTTGTTTGTCAACATCCATCAACATATGGCCTGAGAATGCCATTGTTT
CATTGTAGCGTCGCCCTTCACCACCATCCATCCTCAGAGTTGAAGTAGAGCCGTGTCTCTCCACT
GTTACAGAATGCCAGATGCCATCATTAACAGCCACTGCAGACAGCCATAAATCATGTTCTTCTGT
CCGTAAACTGTTTAGGTTGTAGCGGAACCGCAACCTTCCATCTTTGATCTCCAAGATGCCGTATT
CTCTATTGTACATTGGTTTGT

**Figure 3.59** Nucleotide sequences of products from F2Rβ of *P. monodon* (continue).

**>ER_F2β8/49 (800 bp)**

ACCTCGAACCAACAACACTCCCCAGTCAAGAGAAGCACTACCTTCCAAGGAAAGCTTTGTACTTG
CCCCATGGGCATGCTCTGCTTGTACGTTCCTAAATGACAGGGACCGCAACGTTTGTGACATGTGT
GGAAAGTCTCGCATGCCTGGTCCAGAAGTGAAGCCTCTCGTTTCTGGTGGGCGACAATGTCCACA
GTGCACTTTCATAAATGAGAGAGATGCTCAGGATTGTTCTGTATGTGGAGAACACCTGGAGGGAT
CTTCCACATACATCTAAGAAGATTAAAGTGACAGAGAAAGTGAGCCAAAGTATTCATTGTTAGTT
TTCCAGCATTGTTCAAATCTGGATTTGCCAAATGACATAAGTATTGACAAGAGATTTGTCGCCTT
TTTTTCCTTTTTTATGTTTCATATGTAACAAAGAAATTACCCTTATCTGCATATTCACTCTATTG
TGCATTGGTTTGTGCCTCCAACCAACAACACTCCCTAGTCAAGAGAAGCACTACCTTCCAAGGAA
AGCTTTGTACTTGCCCCATGGGCATGCTCTGCTTGTACGTTCCTAAATGACAGGGACCGCAACGT
TTGTGACATGTGTGGAAAGTCTCGCATGCCTGGTCCAGAAGTGAAGCCTCTCGTTTCCGGTGGGC
GACAATGTCCACAGTGCACTTTCATAAATGAGAGAGATGCTCAGGATTGTTCTGTATGTGGAGAA
CACCTGGAGGGATCTTCCACATACATCTAAGGAGATTAAAGTGACAGAGAAAGTGAGCCCAAGTA
TTCATTGTTAGTTTTCCAGC

**Figure 3.59** Nucleotide sequences of products from F2Rβ of *P. monodon*.

**Table 3.5** BLAST analysis of nucleotide sequences of clones obtained from F2Rβ of *P. monodon*.

| Clone | Insert size (bp) | BLAST*N* | E-value | BLAST*X* | E-value |
|---|---|---|---|---|---|
| ER_F2β5/5 | 468 | Unknown | 0.082 | similar to CG4057-PB (*Apis mellifera*) | 2E-17 |
| ER_F2β5/16 | 571 | Unknown | 0.040 | transcription co-repressor Sin3 (*Xenopus laevis*) | 1E-13 |
| ER_F2β8/14 | 798 | Unknown | 0.56 | testis specific IQ motif containing GTPase activating protein 2 (*Homo sapiens*) | 1E-48 |
| ER_F2β8/27 | 541 | *Caridina japonica* Cjcad mRNA for Cj-cadherin, complete cds | 1E-44 | Cj-cadherin (*Caridina japonica*) | 1E-95 |
| ER_F2β8/49 | 800 | Unknown | 0.14 | similar to CG4057-PB (*Apis mellifera*) | 6E-17 |

As results shown in Table 3.5, none of these amplified DNA fragments were identified as estrogen receptor. Surprisingly, one particular clone, ER_F2β8/14, which was amplified from ovary cDNA, was identified as a homolog of IQ motif containing GTPase activating protein 2 specifically found in testis of human. This clone was further analyzed for sex specificity using Southern blot analysis on genomic DNA of male and female *P. monodon.* The result from Southern blot analysis on genomic DNA revealed no positive band from both sexes (Figure 3.60).

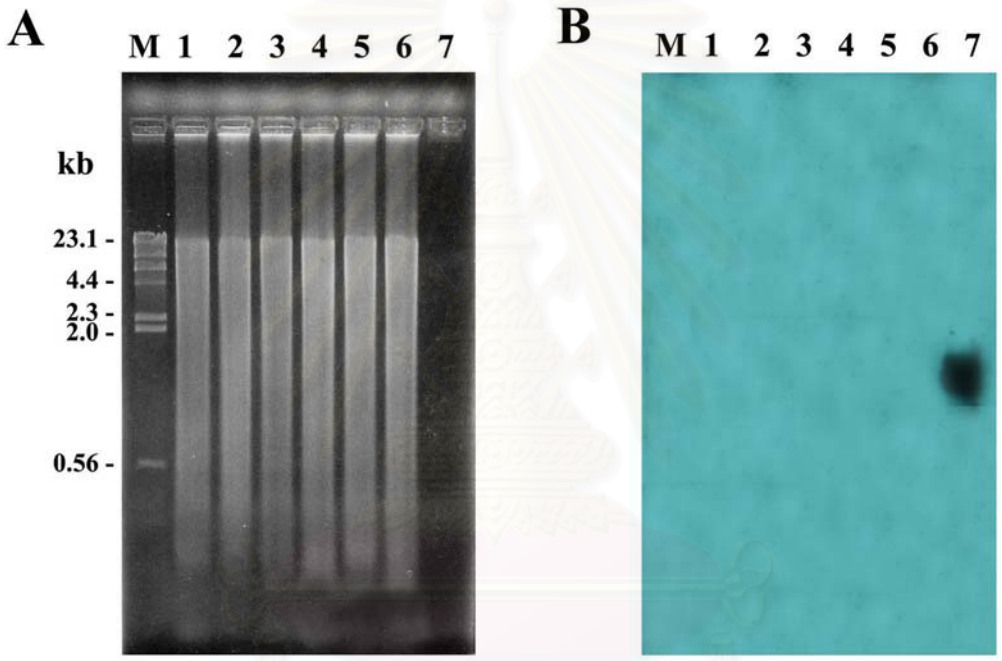



**Figure 3.60** Agarose gel electrophoresis (A) and Southern blot hybridization to examine specificity of pER_F2β8/14 (B). Probe was non-radioactively labelled by DIG and hybridized male (lane 1-3) and female (lane 4-6) genomic DNAs of *P. monodon* digested with *Dra*I. Positive control (an amplified product of pER_F2β8/14 using pUC1 and pUC2 primers) was shown in lane 7.

### 3.5 Construction of the male and female *P. monodon* genomic DNA libraries

Male and female genomic libraries was constructed by partially digesting *P. monodon* genomic DNA with *Mbo*I. Figure 3.61 and 3.62 showed the result of partial digestion on pilot scale of male and female genomic DNA, respectively. The dilutions yielded smeary pattern with the range of 4-23 kb scaled up (Figure 3.63 and 3.64), size fractionated (Figure 3.65, 3.66, 3.67, 3.68, 3.69), and ligated to Lambda FIX® II vector. The efficiencies of male and female libraries were 7.3 x $10^3$ and 1.3 x $10^4$ pfu/ml, respectively.

Approximately 1155 and 1097 clones from each of male and female libraries were subjected to phage DNA purification. The result indicated that purified DNA of male library revealed the size about 20 kb (Figure 3.70) while that of female was about 20 kb (Figure 3.71).

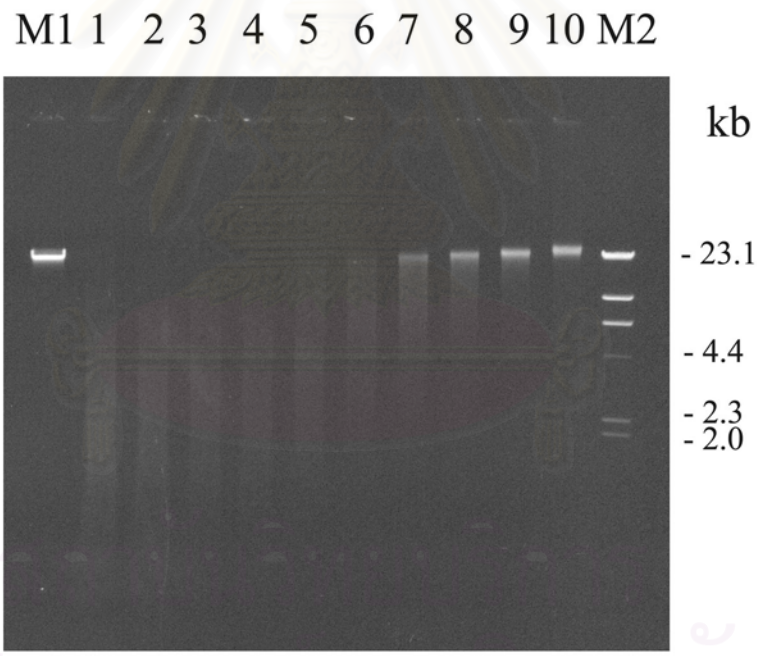



**Figure 3.61** Pilot scale of partial cleavage of high molecular weight genomic DNA of male *P. monodon* with *Mbo*I. The markers are intact lambda DNA (lane M1) and lambda DNA cleaved to completion with *Hin*dIII (lane M2).

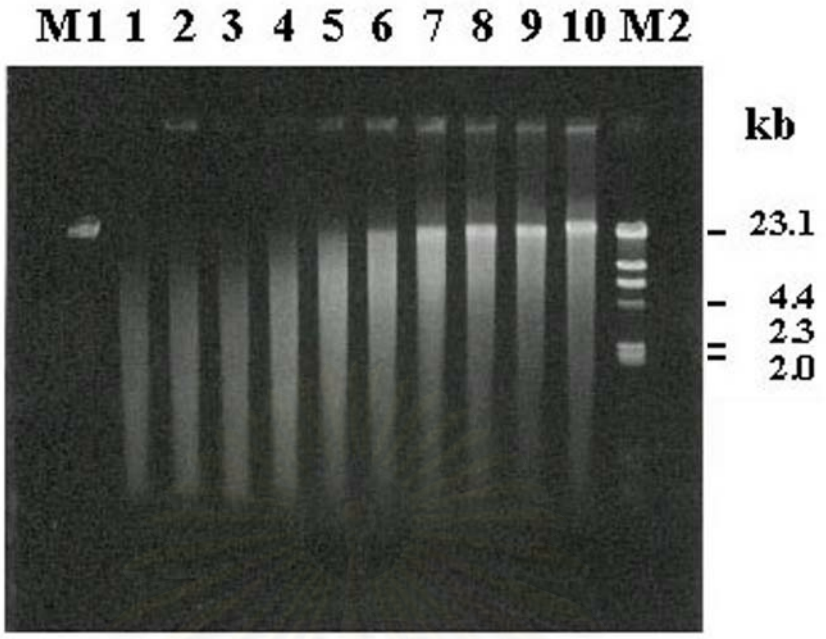



**Figure 3.62** Pilot scale of partial cleavage of high molecular weight genomic DNA of female *P. monodon* with *Mbo*I. The markers are intact lambda DNA (lane M1) and lambda DNA cleaved to completion with *Hin*dIII (lane M2).

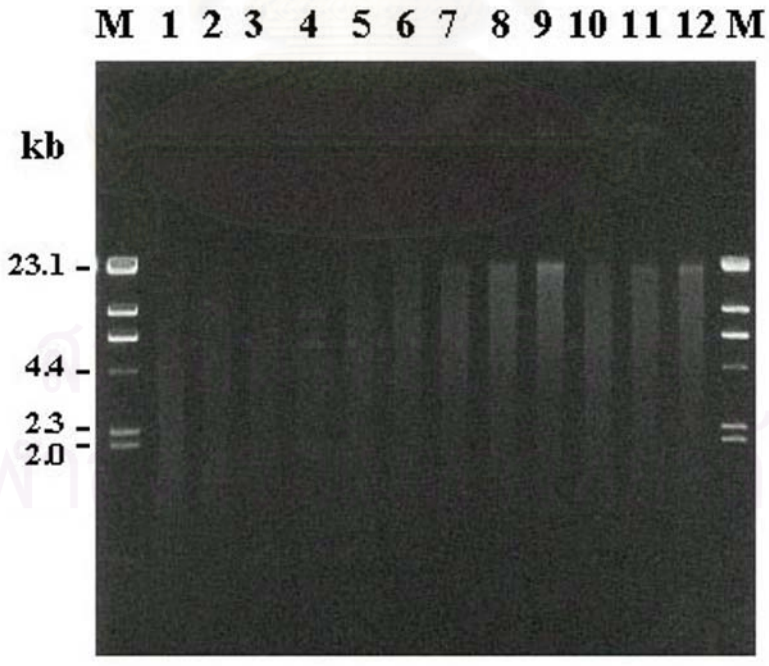



**Figure 3.63** Large scale of partial cleavage of high molecular weight genomic DNA of male *P. monodon* with *Mbo*I. The markers are intact lambda DNA (lane M1) and lambda DNA cleaved to completion with *Hin*dIII (lane M2).

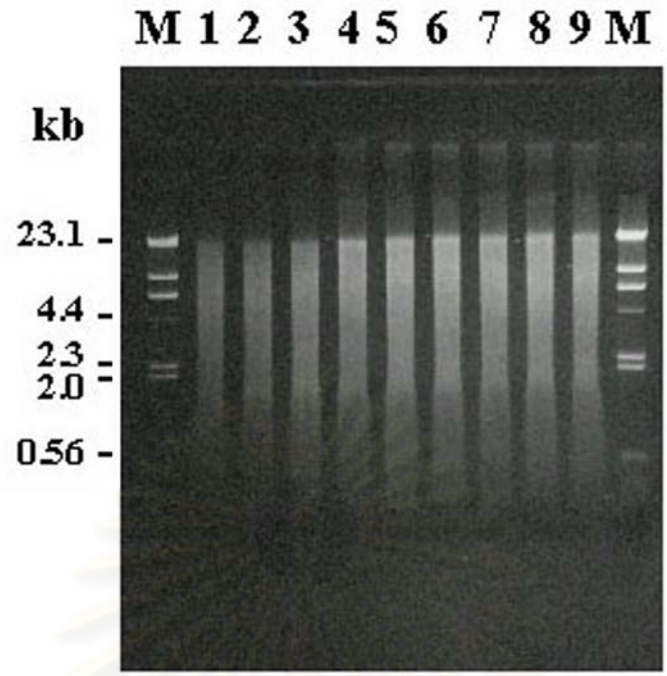



**Figure 3.64** Large scale of partial cleavage of high molecular weight genomic DNA of female *P. monodon* with *Mbo*I. The markers are intact lambda DNA (lane M1) and lambda DNA cleaved to completion with *Hin*dIII (lane M2).

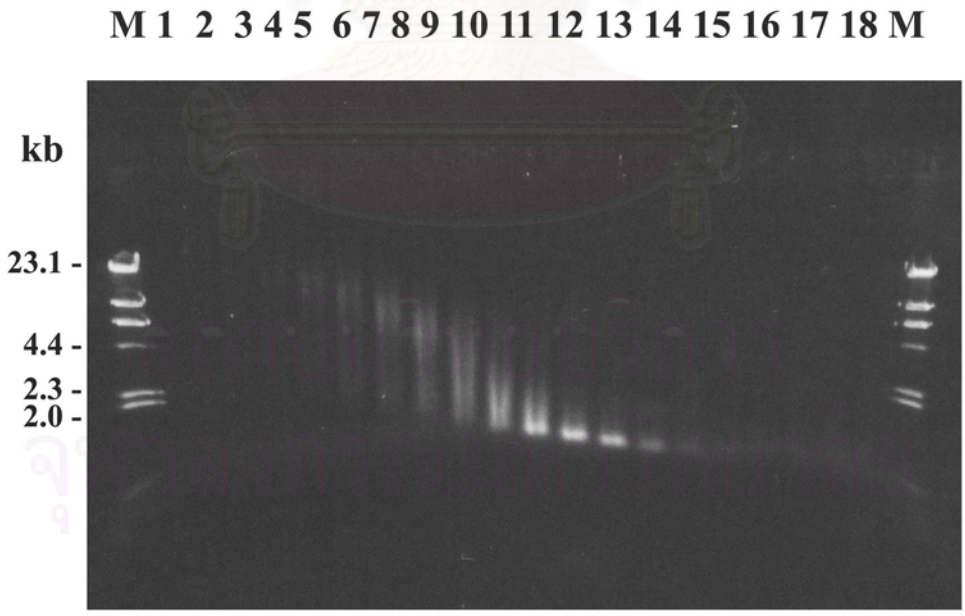



**Figure 3.65** Analysis of sucrose gradient fractions of the fifth dilution of male genomic DNA digestion by 0.6% agarose gel electrophoresis. The two end tracks contain lambda DNA cleaved to completion with *Hin*dIII to serve as size markers.

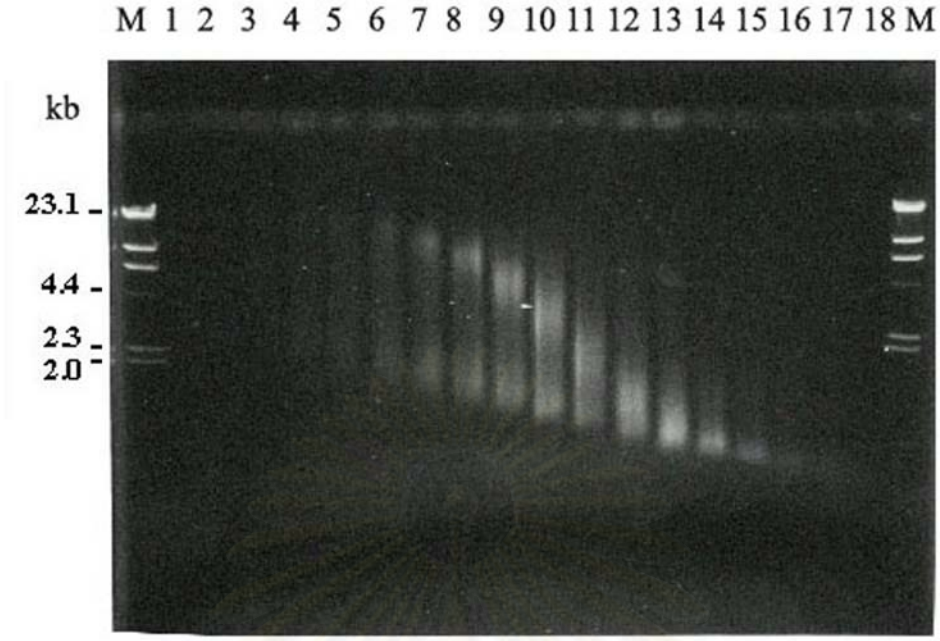



**Figure 3.66** Analysis of sucrose gradient fractions of the sixth dilution of male genomic DNA digestion by 0.6% agarose gel electrophoresis. The two end tracks contain lambda DNA cleaved to completion with *Hin*dIII to serve as size markers.

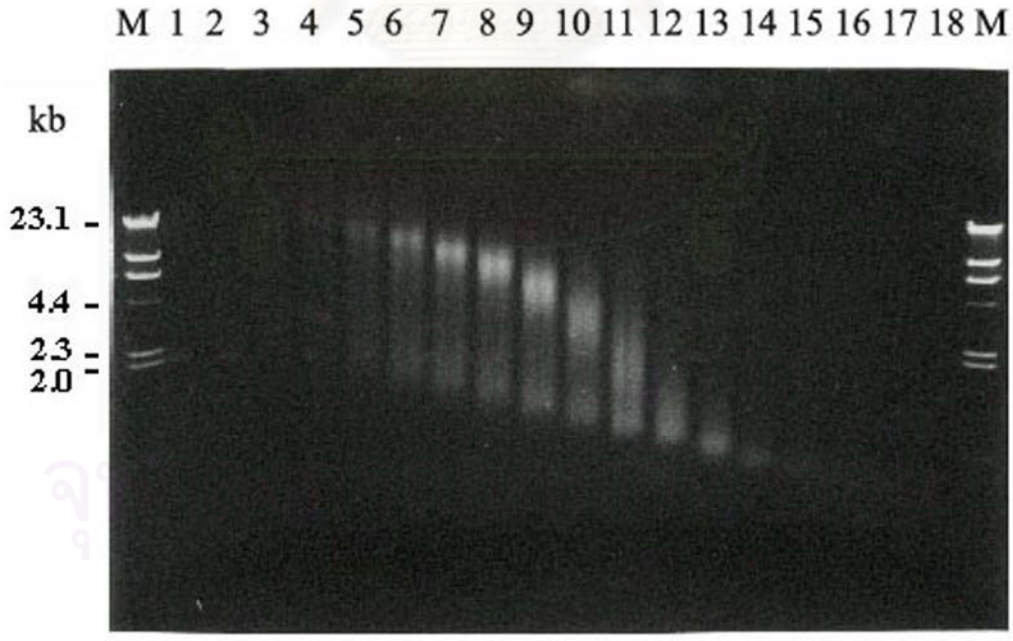



**Figure 3.67** Analysis of sucrose gradient fractions of the seventh dilution of male genomic DNA digestion by 0.6% agarose gel electrophoresis. The two end tracks contain lambda DNA cleaved to completion with *Hin*dIII to serve as size markers.

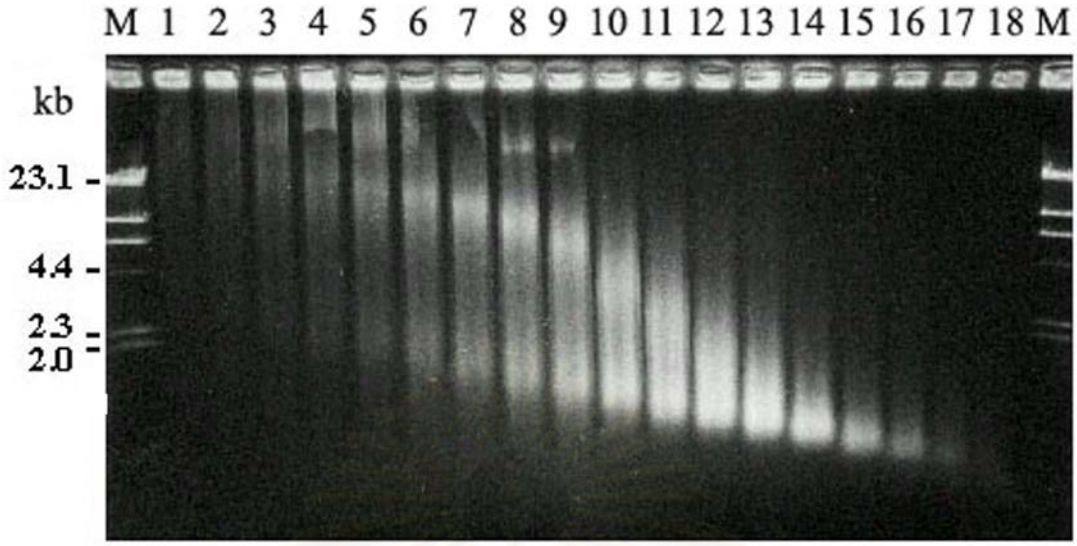



**Figure 3.68** Analysis of sucrose gradient fractions of the sixth dilution of female genomic DNA digestion by 0.6% agarose gel electrophoresis. The two end tracks contain lambda DNA cleaved to completion with *Hin*dIII to serve as size markers.

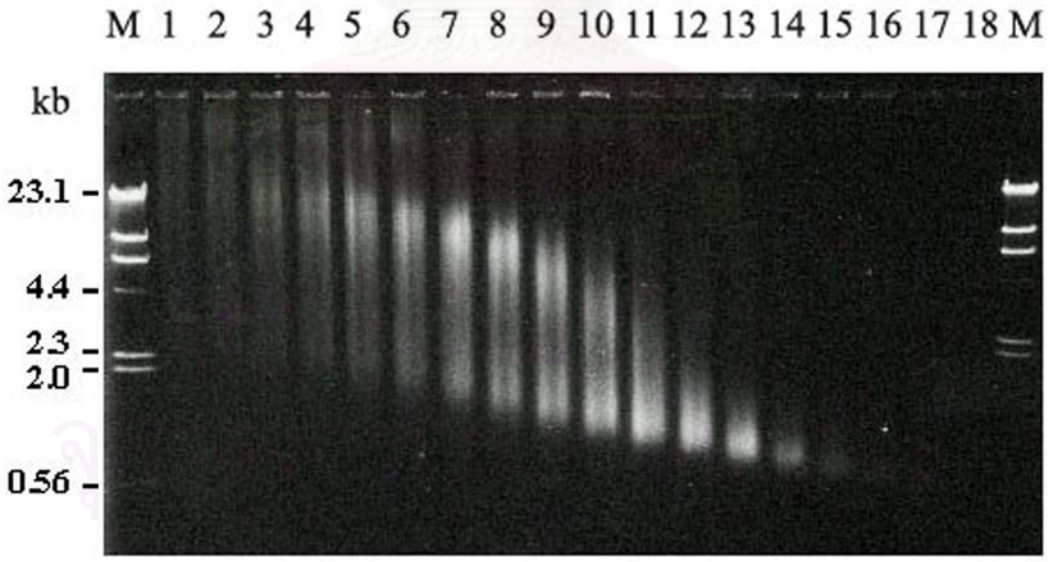



**Figure 3.69** Analysis of sucrose gradient fractions of the seventh dilution of female genomic DNA digestion by 0.6% agarose gel electrophoresis. The two end tracks contain lambda DNA cleaved to completion with *Hin*dIII to serve as size markers.

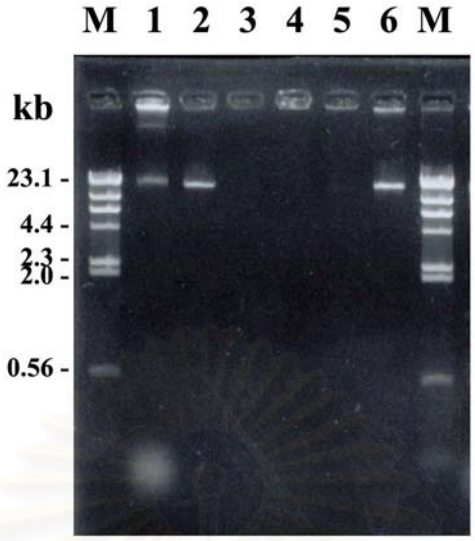



**Figure 3.70** A 0.8% ethidium bromide stained agarose gel showing the purified male genomic DNA library. Lane 1, 2, 3, 4, 5 and 6 were products from fraction 1, 2, 3, 4, 5 and 5, respectively. The two end tracks contain lambda DNA cleaved to completion with *Hin*dIII to serve as size markers.

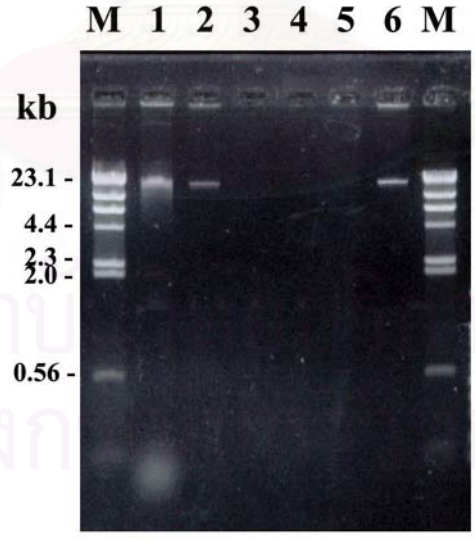



**Figure 3.71** A 0.8% ethidium bromide stained agarose gel showing the purified female genomic DNA library. Lane 1, 2, 3, 4, 5 and 6 were products from fraction 1, 2, 3, 4, 5 and 5, respectively. The two end tracks contain lambda DNA cleaved to completion with *Hin*dIII to serve as size markers.

### 3.5.1 Detection of ER_F2β8/14 in male and female *P. monodon* genomic DNA libraries

Approximately 941, and 856 clones from male and female genomic DNA libraries were detected by hybridizing with DIG-labeled ER_F2β8/14 probe. The results of male and female genomic DNA libraries were shown in Figure 3.72 and 3.73.

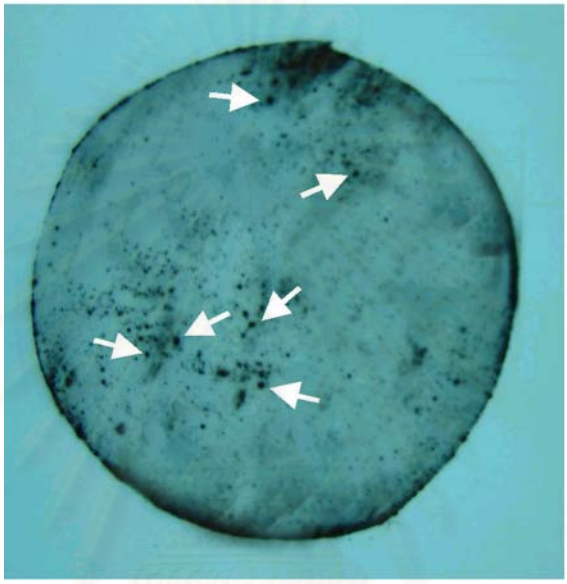


**Figure 3.72** Result of male genomic DNA libraries detected by hybridizing with DIG-labeled ER_F2β8/14 probe.

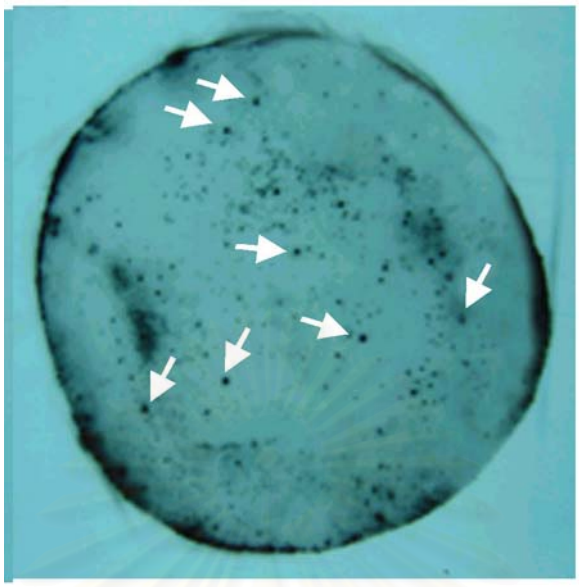


**Figure 3.73** Result of female genomic DNA libraries detected by hybridizing with DIG-labeled ER_F2β8/14 probe.

# CHAPTER IV

# DISCUSSION

Bigger female sizes in penaeid shrimps have been documented in a number of studies (Parrack, 1979; Devi, 1986; Dredge, 1990; Chow and Sandifer, 1991; Buckworth, 1992). Differential weight gains of 5.5 g/month for males and 10 g/month for females in *P. monodon* were reported (Makinouchi and Hirata, 1995). Sex-based size dimorphism with larger sizes and faster growth rates in females when compared to males may be due to a greater weight increase per molt cycle leading to a faster growth rate (Hansford and Hewitt, 1994). However, this reflects the importance of different mechanisms between genders. For this reason, culture efficiency of *P. monodon* can be increased by the development of monosex culture through sex manipulation (gynogenesis, androgenesis or crossing between neomales and normal female). Identification and characterization of sex-specific markers is an important step for understanding molecular mechanisms of sex determination/differentiation in *P. monodon*. Sex identification is problematic and time consuming in researches of many species. Sex-specific markers should be developed from fixed polymorphism in genomic DNA of male and female by the application of DNA-based technology.

Sex-chromosomal DNAs have been isolated from a variety of species from several phyla and are composed of both single-copy and repetitive sequences. For example, a variety of repetitive sex-specific or sex-associated DNAs have been described in Drosophila (Goldstein et al., 1982; Lifschytz and Hareven, 1982; Steinemann, 1982; Livak, 1984; Waring and Pollack, 1987; Pardue et al., 1987). Homologous sequences are present in both sexes, but the organization of those on the Y chromosome is distinct from those found elsewhere in the genome. Remarkably, another sequence, Bkm, is detected as repetitive and highly variable repeats and hybridizes to the sex chromosomes of both invertebrates and vertebrates (Singh et al., 1984).

In humans, repetitive sex chromosomal sequences have also been isolated (Cook 1976; Kunkel et al., 1976; Cook et al., 1982; Wolfe et al., 1984; Muller et al., 1986) and single-copy sequences (ZFY and SRY) with putative sex-determining function have been

described (Page et al., 1987; Sinclair et al., 1990). Similar studies have revealed Y-chromosome-specific sequences in mice (Larmar and Palmer, 1984; Mitchell et al., 1989; Gubbay et al., 1990). Some of these sequences have been tested for their presence in salmonids (Ferreiro et al., 1989; Lloyd et al., 1989) and although single-copy and repetitive signals were detected, no evidence for sex-specific organization was observed.

Genetically monosex female populations of shrimp are synthesized either by gynogenesis (Refstie et al., 1982) or by crossing masculinized genotypic females (androgen treated) with regular females (Hunter et al., 1983). In the latter case, musculinized females (phenotypic males) must be distinguished from regular (XX) sibling males. Although phenotypic sex in submature shrimps can be determined by morphometric analysis (see Beacham and Murray, 1986) determination of genetic sex has usually been accomplished by a test cross to determine the sex of resulting offspring. For many aquaculture operations, this process is excessively expensive and time consuming to perform.

The study of the sex-determining mechanisms in penaeid shrimps is not highly advanced. Almost nothing is known about the molecular basis of sex determination. So far, no sex-determining gene has been identified. The chromosome numbers of penaeid shrimps have been reported in *P. esculentus*, *P. monodon*, *Farfantepenaeus aztecus*, *Fenneropenaeus chinensis*, *Fenneropenaeus merguiensis*, *Fenneropenaeus penicillatus* and *Marsupenaeus japonicus* (2N = 88); *P. semisulcatus* and *Litopenaeus setiferus* (2N = 90); and *Fenneropenaeus californiensis* and *Litopenaeus occidentalis* (2N = 92) (Benzie, 1998). Neither sex chromosomes nor environmental sex determination has been reported in penaeid shrimps.



**Genomic DNA subtraction of *P. monodon***

Subtractive hybridization was first used in 1966 to identify sequences from a small deletion in the bacteriophage T4 genome (Bautz and Reilly, 1966). Subtraction approaches usually involve repeated rounds of hybridization of excess DNA from the source lacking a desired sequence with DNA from the source containing the desired sequence, followed by removal of the undesired sequence at each round. In this study, 2

genomic DNA subtractions (PERT and RDA) were applied for the selection of sex-specific sequences in *P. monodon*.

Preparation of high quality and quantity DNA is essential for genomic DNA subtraction and hybridization. High molecular weight used in this study was initially prepared by the method of Winnepenninckx (1993). This method was suitable for extracting genomic DNA from muscle in a large quantity.

The choices of restriction enzymes were also critical. A number of restriction enzymes were preliminarily used to digest genomic DNA from muscle of male and female *P. monodon*. This included *Eco*RI, *Hin*dIII, *Bam*HI, *Mbo*I, and *Dra*I. Surprisingly, only *Mbo*I, and *Dra*I were able to digest genomic DNAs from *P. monodon* completely. *Eco*RI, *Hin*dIII, and *Bam*HI, commonly used for complete digestion of DNAs from a large number of organisms, failed to give a complete digestion of *P. monodon* genomic DNA. Only small amount of digested DNA was detected when genomic DNAs were digested by *Eco*RI and *Hin*dIII. Moreover, genomic DNA appeared to be intact after digesting with *Bam*HI. This indicates a unique characteristic of *P. monodon* genomic DNA.

In PERT male subtraction, only 9 out of 51 recombinant clones containing inserted DNA larger than 100 bp were obtained from male genomic DNA subtraction. Much smaller number of clones (17 clones) containing inserted fragments was obtained in female subtraction and only 4 clones containing subtracted DNA larger than 100 bp were found. Seven clones were identified as unknown genes and 3 clones shared similar nucleotide sequences. Only 2 clones were identified as known genes and they were not sex-linked genes or genes relating to any sex determination mechanisms. Although, BLAST results indicated that these 2 clones shared similar nucleotide sequences to the known genes (Cytochrome oxidase subunitI from *Macrobrachium australiense* and Protease inhibitor from *Marsupenaeus japonicus*, respectively), the similarities were considerably low, therefore, the identities of these clones were still unclear. None of these clones hybridized specifically with either male or female genomic DNA of *P. monodon*.

The majority of clones from PERT subtraction was found to contain small inserted fragments (< 100 bp), only a small number of clones contained fragments at the sizes ranging from 200 to 800 bp. This result was in consistent with the report of Kunkel et al.

(1985). PERT technique was used to isolate and clone specific fragments of the X-chromosome. The average size of the cloned fragments was relatively small (<200 bp) and therefore was not sensitive to the loss of DNA fragment length.

Kohne et al (1977) described the improved technique of PERT. Nucleic acid reassociation has been proved to be a powerful tool for analyzing the genetic material of a wide variety of organisms from virus to man. While nucleic acid reassociation has been used to answer many important questions, a major limitation on the feasibility of using this technique in many significant biological experiments is the basic rate of reassociation seen for the standard one-phase aqueous systems. The two-phase system, water and phenol, is the basis for a new, rapid nucleic acid reassociation technique. When phenol and water are mixed in the right proportions and shaken, an emulsion form. When the shaking stops, the emulsion breaks and the two phases separate. Addition of single stranded DNA and salt to the water phase, and then shaking the mixture at room temperature results in the extremely rapid formation of double-stranded DNA. The presence of phenol also lowers the Tm of the DNA considerably (Leng et al., 1974). The rapid reassociation in PERT is apparently dependent on the presence of an emulsion. Thus, the effect of fragment size on reassociation rate is small when using the PERT (Kohne et al., 1977). For the above reasons, the small sizes of subtracted fragments obtained from PERT in this study appeared to be normal. However, the most critical part might be the shaking part to constantly emulsify the mixture at the appropriate condition for a long period of time.

Devlin et al. (1991) used genomic DNA subtraction (PERT) methodologies to selectively clone a DNA fragment, OtY1, from the Y chromosome of Chinook salmon (*Oncorhynchus tshawytscha*). This clone could specifically detect a single 8 kb *Bam*HI restriction fragment in Southern blots of male genomic DNA that was absent in females.

Another variation on the subtractive hybridization technique is representational difference analysis (RDA), which was developed by Lisitsyn et al. (1993). It is a powerful differential hybridization method to identify unique DNA sequences out of two complex and highly genomes. It detects genomic losses rearrangements and amplifications of the cell genomic as well as pathogenic organisms (Lisitsyn and Wigler, 1995).

In this study, the different patterns of the amplicons from male and female *P. monodon* were obviously seen before subtraction was started. Two major bands at the size of 800 and 1100 bp were observed in male amplicon and 2 major bands at the size of 900 and 1200 bp were observed in female amplicon. After the first round of subtraction, smear ranging in size from 450 to 200 bp and 2 subtracted bands (200 and 450 bp) were clearly observed. After the second round of subtraction, two major bands with the same sizes obtained from the first round were still observed. The difference of 2 major bands previously seen in amplicons of both sexes was no longer observed after 2 rounds of subtraction. These results were not in agreement with the result reported in some of previous studies which suggested that initial male and female amplicons were visually indistinguishable from each other. For example, DNA fragments unique to male or female plants, *M. polymorpha*, could be observed in the third-round products of *Bam*HI, the fourth-round products of *Hin*dIII, and the third-round products of *Bgl*II (Fujisawa et al., 2001). However, after the result of sequence analysis was obtained, the clones from the first round and the second round were totally different from each others. All these clones were identified as unknown DNA fragments.

On the other hand, the results of the first and the second rounds of female RDA revealed obvious difference of subtracted DNA products. First round of subtraction yielded 3 major bands (200, 320 and 800 bp) while second round yielded a single band (300 bp). Similar to the results of markers obtained from male subtraction, all isolated clones were unknown DNA fragments. All markers obtained from both male and female subtractions could hybridize to male and female genomic DNAs, indicating the non-existence of unique sex-specific sequences on the chromosomes of male and female *P. monodon*.

Several modifications of the RDA protocol have been published. Optimization of the procedure was reported by using alternative methods for PCR purification and depletion of single stranded PCR fragments together with alternative primer design (Pastorian et al., 2000). Magnetic bead technology has been reported to yield a more robust protocol with fewer false positives and requires very low amounts of starting RNA (Odeberg et al., 2000). A methylation sensitive RDA protocol was developed for scanning of differences in methylation status in mammalian genomes (Ushijima et al., 1997 and Muller et al., 2001). Ligation mediated subtraction (LIMES) is a modified RDA

protocol for detection of genomic differences, improved by an additional amplification step to avoid preferential amplification of repetitive sequences (Hansen-Hagge et al., 2001). Successful RDA has also been reported for microbial genomes (Bowler et al., 1999).

The downsides of RDA were also documented. Spinella et al. (1999) reported smaller fragments hybridize more efficiently and were amplified more efficiently by PCR. The amplification efficiencies of PCR products were also affected by the nucleotide sequence of the DNA fragment. Inevitably, PCR was reported to amplify abundant common species of DNA (Zeng et al., 1994).

Sugai et al. (1998) reported one problem of the RDA method is biased amplification of unexpected sequences by PCR. Such non-specific amplification appears to be inevitable as long as PCR amplification is involved. A number of studies reported on the success of using RDA to identify sex-specific markers in many organisms.

Drew and Brindley (1995) employed RDA to isolate female-specific sequences of *Schistosoma mansoni*. Using *Hin*dIII-derived amplicons, an excess of male schistosome DNA was employed to remove sequences common to both male and female adult *S. mansoni* genomes from female genomic DNA. Following three rounds of RDA, the enriched sequences included two female-specific repetitive elements. One of these exhibited 76% homology to the SMα family of retroposons and represents a W chromosome-specific variant of that family. The other sequence represented a novel, female-specific repetitive sequence. These sequences were designated SMαfem-1 and W2, respectively, and both were apparently arrayed as tandem repeats on the W chromosome of *S. mansoni*. The isolation and characterization of the two female-specific sequences by RDA indicated that this procedure should also find utility in the definition of traits and sequences that differ among other groups of schistosomes.

Fujisawa et al. (2001) reported the successful exploitation of RDA to isolate DNA markers for the sex chromosomes of *Marchantia polymorpha*. A total of 10 male and 10 female individuals from the $F_1$ family were used for linkage analysis. The primer sets for six male RDA-derived clones showed amplification only in the 10 male progeny, not in the females. On the other hand, the primer sets for two female RDA-derived clones showed amplification only in the 10 females. These results are statistically highly

significant evidence for linkage using the genomic DNAs from wild *M. polymorpha* plants as templates.

**Verifying the sex specificity of candidate markers from DNA subtraction**

The first technique applying for this study was PCR. It is a sensitive technique for detecting the existence of DNA sequences from different sources. It performed in both genomic DNA and gonad-extracted cDNA from both male and female of *P. monodon*. The disadvantage of PCR is that the contamination causing false positive result can easily occur. Also, when the amplification is performed on genomic DNA, PCR products can be interfered by introns of the target genes.

The sizes of most candidate markers obtained from PERT and RDA in this study were rather small. Only 3 clones with the sizes larger than 200 bp and sequences available for primer designation were obtained. Two clones were amplified from genomic DNAs from both sexes, indicating no sex specificity of these clones. Although the identities of these 2 DNA fragments were not clarified, the positive result of PCR in the same levels from both testis- and ovary-extracted cDNAs also indicated that the expression of these 2 DNA fragments were in the same level, assuming no correlation between these 2 genes and sex determination of *P. monodon*.

It should be noted that PMFJ200 were not amplified in any sources of DNA. This could simply cause by using inappropriate primers or adopting incorrect sequence of PMFJ200 for primer designation. However, it is also possible that the homology of PMFJ200 sequence is low among investigated individuals. This assumption was supported by the result of Southern blot analysis which revealed smear of hybridization between PMFJ200 and *Dra*I-digested genomic DNAs from both sexes. This result confirms the existence of this fragment in *P. monodon* genome.

Genome walking and SSCP analysis were also applied to the present study. The result of genome walking from 2 clones, PMMSH6 (from PERT) and PMFJ800 (from RDA), exhibited different patterns of PCR products between male and female, however, when the analysis was performed in increased number of individual samples, the difference of PCR pattern between males and females were no longer detected. No sex-

specific properties of these markers were also confirmed by the result of SSCP. Both PMMSH6 and PMFJ800 showed polymorphic nature across investigated individuals. Nevertheless, the polymorphisms of these genes will be valuable for the study of linkage mapping in *P. monodon*.

Southern blot analysis was used as major technique for verifying candidate markers. According to the results of male and female genomic DNA with 12 candidate markers from both PERT (4 probes) and RDA (8 probes), markers could be separated into 2 groups. One was the markers that could hybridize to DNAs from both sexes and the other was the markers that were not detectable in both male and female genomes. The first group can be clearly identified as non-specific markers for sex determination. The conclusion for sex specificity of the latter group, however, was still ambiguous. The negative results do not necessarily mean the complementary sequences of the probes do not exist in the tested genome.

It has been mentioned in many studied that the detection of single copy genes by Southern blot analysis tend to give very faint bands or no positive result at all if the signal of probe is not highly intensified and the amount of tested DNA is low. The confirmation of single copy genes can be obtained by conducting dot blot hybridization using male and female genomic DNA as probes and candidate markers as targets. In addition, the undetectable results of some markers in this study may cause by the less efficiency in probe labeling since the sizes of all markers obtained for Southern hybridization were close to 100 bp.

A sex-related gene, such as Estrogen receptor (ER), an important protein necessary for maturation in many organisms, was also under investigation. The existence of ER in *P. monodon* has never been reported. Amplification of ER genes was attempted in this study. Six DNA fragments were amplified by the degenerated primers designed from ER in ovary cDNA of *P. monodon*. Five of these DNA fragments were cloned, sequenced, and identified. None of their sequences were similar to those of ER sequences reported in GenBank. Two clones were similar to un-characterized gene of *Apis mellifera* and 3 clones were identified as genes similar to transcription co-repressor Sin3 in *Xenopus laevis,* testis specific IQ motif containing GTPase activating protein 2 in *Homo sapiens,* and Cj-cadherin in *Caridina japonica,* respectively.

It was interesting to note that one fragment (ER_F2β8/14) was identified as genes similar to testis specific IQ motif containing GTPase activating protein 2 which was quite unusual. However, after this tsIQGAP2-like fragment was used as probe for hybridization to *Dra*I-digested DNA and screening male and female genomic DNA libraries, it was indicated that ER_F2β8/14 existed in the genomes of both sexes.

Testis specific IQ motif containing GTPase-activating protein 2 (tsIQGAP2) is a testis specifc gene exhibiting 6.4-fold difference at expression level between adult and embryo human testes. The full cDNA length of tsIQGAP2 is 3388 bp and contains a 3215 bp open reading frame, encoding a 122.85 kDa protein of 1071 amino acids. Sequence analysis showed that tsIQGAP2 harbors all the domains (one WW domain, three IQ motifs, and one RasGAP homology domain) in HsIQGAP2 (IQ motif containing GTPase-activating protein 2) except for the N-terminal calponin homology domain. It was suggested the potential role of tsIQGAP2 during spermatogenesis (Wang et al., 2004).

Transcriptional corepressor SIN3 gene was reported to involve in the regulation of centromeres which play a vital role in maintaining the genomic stability of eukaryotes by coordinating the equal distribution of chromosomes to daughter cells during mitosis and meiosis (Silverstein et al., 2003).

Cadherins are long proteins composed of several modules linked together like beads on a string. They extend from the surface of a cell and, with the help of calcium ions, adhere to cadherins on neighboring cells. This interaction is specific, allowing nerve cells to connect to other nerve cells and stomach cells to connect with other stomach cells. Many different types of cadherins are made by our cells, each with a different adhesive function used in different parts of the body. When cadherins fail in their function, cells lose their ability to hold onto one another. In the case of cancer, this allows individual cells to separate from a solid tumor. They are then free to wander through the body and form metastases (Goodsell, 2002).

Attempts to investigate on sex-linked genes in *P. monodon* have been previously conducted. Leelatanawit (2003) amplified a 450 bp fragment from genomic DNA of *P. monodon* using primers designed from Zinc finger genes. Male and female shrimps from different populations were subjected to PCR amplification. A number of amplified DNA

fragments were identified as unknown genes and sex-specific markers still could not identify.

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 ratio in *P. chinensis*. The female to male ratio in triploids was approximately 4:1 (Li et al., 2003).

Based on studies in mammalian species, sex-linked genes such as X-linked zinc finger protein gene (Zfx) and Y-linked zinc finger protein gene (Zfy) usually showed fixed single nucleotide polymorphism (SNP) between males and females of a particular species. This was not found in any SSCP-tested markers in this study.

**Construction and evaluation of the male and female *P. monodon* genomic DNA libraries**

In constructing a genomic library, a large number of different clones must be generated to ensure that all DNA fragments are represented. The average sizes of male and female libraries detected in this study were approximately 20 kb. Genome of *P. monodon* was reported to contain 2 x $10^9$ bp. Therefore, the number of clones necessary to represent an entire genome can be calculated from the equation,

$$N = \frac{In(1-P)}{In(1-f)}$$

To cover the whole genome, the library was expected to be 4.6 x $10^5$ pfu/ml. However, the efficiencies of male and female *P. monodon* genomic libraries were 7.3 x $10^3$ and 1.3 x $10^4$ pfu/ml, respectively. This indicated that the libraries constructed in this study were not covered the whole genome of *P. monodon.*

It has been in agreement of the researchers that construction of a genomic DNA library, especially BAC library, with the high efficiency was extremely difficult. Attempt to construct *P. monodon* BAC libraries by a number of laboratories has yet been reportedly successful. To avoid this problem, a lambda system appears to be an appropriate choice for constructing genomic DNA libraries in *P. monodon*. Although

each of these libraries does not cover the whole *P. monodon* genome, the average sizes of their clones are considerably large enough for gene structure investigation. So far, there has been no report on the construction of genomic DNA library in *P. monodon*. The libraries in this study are the first male and female genomic DNA libraries constructed in *P. monodon*. These libraries will be useful for the extension study of EST and also provide the alternative resources for further investigation on genomic level of *P. monodon*.

# CHAPTER IV

# DISCUSSION

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The downsides of RDA were also documented. Spinella et al. (1999) reported smaller fragments hybridize more efficiently and were amplified more efficiently by PCR. The amplification efficiencies of PCR products were also affected by the nucleotide sequence of the DNA fragment. Inevitably, PCR was reported to amplify abundant common species of DNA (Zeng et al., 1994).

Sugai et al. (1998) reported one problem of the RDA method is biased amplification of unexpected sequences by PCR. Such non-specific amplification appears to be inevitable as long as PCR amplification is involved. A number of studies reported on the success of using RDA to identify sex-specific markers in many organisms.

Drew and Brindley (1995) employed RDA to isolate female-specific sequences of *Schistosoma mansoni*. Using *Hin*dIII-derived amplicons, an excess of male schistosome DNA was employed to remove sequences common to both male and female adult *S. mansoni* genomes from female genomic DNA. Following three rounds of RDA, the enriched sequences included two female-specific repetitive elements. One of these exhibited 76% homology to the SMα family of retroposons and represents a W chromosome-specific variant of that family. The other sequence represented a novel, female-specific repetitive sequence. These sequences were designated SMαfem-1 and W2, respectively, and both were apparently arrayed as tandem repeats on the W chromosome of *S. mansoni*. The isolation and characterization of the two female-specific sequences by RDA indicated that this procedure should also find utility in the definition of traits and sequences that differ among other groups of schistosomes.

Fujisawa et al. (2001) reported the successful exploitation of RDA to isolate DNA markers for the sex chromosomes of *Marchantia polymorpha*. A total of 10 male and 10 female individuals from the $F_1$ family were used for linkage analysis. The primer sets for six male RDA-derived clones showed amplification only in the 10 male progeny, not in the females. On the other hand, the primer sets for two female RDA-derived clones showed amplification only in the 10 females. These results are statistically highly

significant evidence for linkage using the genomic DNAs from wild *M. polymorpha* plants as templates.

**Verifying the sex specificity of candidate markers from DNA subtraction**

The first technique applying for this study was PCR. It is a sensitive technique for detecting the existence of DNA sequences from different sources. It performed in both genomic DNA and gonad-extracted cDNA from both male and female of *P. monodon*. The disadvantage of PCR is that the contamination causing false positive result can easily occur. Also, when the amplification is performed on genomic DNA, PCR products can be interfered by introns of the target genes.

The sizes of most candidate markers obtained from PERT and RDA in this study were rather small. Only 3 clones with the sizes larger than 200 bp and sequences available for primer designation were obtained. Two clones were amplified from genomic DNAs from both sexes, indicating no sex specificity of these clones. Although the identities of these 2 DNA fragments were not clarified, the positive result of PCR in the same levels from both testis- and ovary-extracted cDNAs also indicated that the expression of these 2 DNA fragments were in the same level, assuming no correlation between these 2 genes and sex determination of *P. monodon*.

It should be noted that PMFJ200 were not amplified in any sources of DNA. This could simply cause by using inappropriate primers or adopting incorrect sequence of PMFJ200 for primer designation. However, it is also possible that the homology of PMFJ200 sequence is low among investigated individuals. This assumption was supported by the result of Southern blot analysis which revealed smear of hybridization between PMFJ200 and *Dra*I-digested genomic DNAs from both sexes. This result confirms the existence of this fragment in *P. monodon* genome.

Genome walking and SSCP analysis were also applied to the present study. The result of genome walking from 2 clones, PMMSH6 (from PERT) and PMFJ800 (from RDA), exhibited different patterns of PCR products between male and female, however, when the analysis was performed in increased number of individual samples, the difference of PCR pattern between males and females were no longer detected. No sex-

specific properties of these markers were also confirmed by the result of SSCP. Both PMMSH6 and PMFJ800 showed polymorphic nature across investigated individuals. Nevertheless, the polymorphisms of these genes will be valuable for the study of linkage mapping in *P. monodon*.

Southern blot analysis was used as major technique for verifying candidate markers. According to the results of male and female genomic DNA with 12 candidate markers from both PERT (4 probes) and RDA (8 probes), markers could be separated into 2 groups. One was the markers that could hybridize to DNAs from both sexes and the other was the markers that were not detectable in both male and female genomes. The first group can be clearly identified as non-specific markers for sex determination. The conclusion for sex specificity of the latter group, however, was still ambiguous. The negative results do not necessarily mean the complementary sequences of the probes do not exist in the tested genome.

It has been mentioned in many studied that the detection of single copy genes by Southern blot analysis tend to give very faint bands or no positive result at all if the signal of probe is not highly intensified and the amount of tested DNA is low. The confirmation of single copy genes can be obtained by conducting dot blot hybridization using male and female genomic DNA as probes and candidate markers as targets. In addition, the undetectable results of some markers in this study may cause by the less efficiency in probe labeling since the sizes of all markers obtained for Southern hybridization were close to 100 bp.

A sex-related gene, such as Estrogen receptor (ER), an important protein necessary for maturation in many organisms, was also under investigation. The existence of ER in *P. monodon* has never been reported. Amplification of ER genes was attempted in this study. Six DNA fragments were amplified by the degenerated primers designed from ER in ovary cDNA of *P. monodon*. Five of these DNA fragments were cloned, sequenced, and identified. None of their sequences were similar to those of ER sequences reported in GenBank. Two clones were similar to un-characterized gene of *Apis mellifera* and 3 clones were identified as genes similar to transcription co-repressor Sin3 in *Xenopus laevis,* testis specific IQ motif containing GTPase activating protein 2 in *Homo sapiens,* and Cj-cadherin in *Caridina japonica,* respectively.

It was interesting to note that one fragment (ER_F2β8/14) was identified as genes similar to testis specific IQ motif containing GTPase activating protein 2 which was quite unusual. However, after this tsIQGAP2-like fragment was used as probe for hybridization to *Dra*I-digested DNA and screening male and female genomic DNA libraries, it was indicated that ER_F2β8/14 existed in the genomes of both sexes.

Testis specific IQ motif containing GTPase-activating protein 2 (tsIQGAP2) is a testis specifc gene exhibiting 6.4-fold difference at expression level between adult and embryo human testes. The full cDNA length of tsIQGAP2 is 3388 bp and contains a 3215 bp open reading frame, encoding a 122.85 kDa protein of 1071 amino acids. Sequence analysis showed that tsIQGAP2 harbors all the domains (one WW domain, three IQ motifs, and one RasGAP homology domain) in HsIQGAP2 (IQ motif containing GTPase-activating protein 2) except for the N-terminal calponin homology domain. It was suggested the potential role of tsIQGAP2 during spermatogenesis (Wang et al., 2004).

Transcriptional corepressor SIN3 gene was reported to involve in the regulation of centromeres which play a vital role in maintaining the genomic stability of eukaryotes by coordinating the equal distribution of chromosomes to daughter cells during mitosis and meiosis (Silverstein et al., 2003).

Cadherins are long proteins composed of several modules linked together like beads on a string. They extend from the surface of a cell and, with the help of calcium ions, adhere to cadherins on neighboring cells. This interaction is specific, allowing nerve cells to connect to other nerve cells and stomach cells to connect with other stomach cells. Many different types of cadherins are made by our cells, each with a different adhesive function used in different parts of the body. When cadherins fail in their function, cells lose their ability to hold onto one another. In the case of cancer, this allows individual cells to separate from a solid tumor. They are then free to wander through the body and form metastases (Goodsell, 2002).

Attempts to investigate on sex-linked genes in *P. monodon* have been previously conducted. Leelatanawit (2003) amplified a 450 bp fragment from genomic DNA of *P. monodon* using primers designed from Zinc finger genes. Male and female shrimps from different populations were subjected to PCR amplification. A number of amplified DNA

fragments were identified as unknown genes and sex-specific markers still could not identify.

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 ratio in *P. chinensis*. The female to male ratio in triploids was approximately 4:1 (Li et al., 2003).

Based on studies in mammalian species, sex-linked genes such as X-linked zinc finger protein gene (Zfx) and Y-linked zinc finger protein gene (Zfy) usually showed fixed single nucleotide polymorphism (SNP) between males and females of a particular species. This was not found in any SSCP-tested markers in this study.

**Construction and evaluation of the male and female *P. monodon* genomic DNA libraries**

In constructing a genomic library, a large number of different clones must be generated to ensure that all DNA fragments are represented. The average sizes of male and female libraries detected in this study were approximately 20 kb. Genome of *P. monodon* was reported to contain 2 x $10^9$ bp. Therefore, the number of clones necessary to represent an entire genome can be calculated from the equation,

$$N = \frac{In(1-P)}{In(1-f)}$$

To cover the whole genome, the library was expected to be 4.6 x $10^5$ pfu/ml. However, the efficiencies of male and female *P. monodon* genomic libraries were 7.3 x $10^3$ and 1.3 x $10^4$ pfu/ml, respectively. This indicated that the libraries constructed in this study were not covered the whole genome of *P. monodon.*

It has been in agreement of the researchers that construction of a genomic DNA library, especially BAC library, with the high efficiency was extremely difficult. Attempt to construct *P. monodon* BAC libraries by a number of laboratories has yet been reportedly successful. To avoid this problem, a lambda system appears to be an appropriate choice for constructing genomic DNA libraries in *P. monodon*. Although

each of these libraries does not cover the whole *P. monodon* genome, the average sizes of their clones are considerably large enough for gene structure investigation. So far, there has been no report on the construction of genomic DNA library in *P. monodon*. The libraries in this study are the first male and female genomic DNA libraries constructed in *P. monodon*. These libraries will be useful for the extension study of EST and also provide the alternative resources for further investigation on genomic level of *P. monodon*.

# CHAPTER IV

# CONCLUSION

1. In PERT subtraction, 51 and 17 DNA fragments from male and female subtractions, respectively, were obtained. Of these, 9 and 4 potential markers for male and female specificity, respectively were selected and subjected to sequence analysis. Most markers were identified as unknown sequences. Two markers contained sequences similar to known genes (Cytochrome oxidase subunitI from *Macrobrachium australiense* and Protease inhibitor from *Marsupenaeus japonicus*, respectively). These genes are not involved to any sex-linked mechanisms.

2. In RDA subtraction, the different patterns of the amplicons from male and female *P. monodon* were obviously seen before subtraction. The first round of subtraction in male subtraction yielded a smear ranging in size from 450 to 200 bp. Two subtracted bands (200 and 450 bp) were also observed clearly. After the second round of subtraction, two major bands with the same sizes as the first round still present. However, the result of sequence analysis revealed that markers from both rounds were different. All these clones were identified as unknown DNA fragments. The difference between products from the first and the second rounds of female RDA was also observed. The first round of subtraction yielded 3 major bands (200, 320 and 800 bp) while second round yielded a single band (300 bp). All identified markers were unknown DNA fragments.

3. Candidate markers obtained from PERT and RDA were small in size. Only 3 clones with the sizes larger than 200 bp were selected and subjected to PCR analysis in both genomic DNA and gonad-extracted cDNA from both male and female of *P. monodon*. Two clones were amplified from genomic DNAs from both sexes, indicating no sex specificity of these clones. One cloned was not amplified with DNA from both sexes. Since the existence of this close in *P. monodon* genome was confirmed by the result of Southern blot analysis, it was assumed that it was caused by defect primers.

4. The results of genome walking and SSCP analysis revealed no sex specific properties in candidate markers which are in agreement with the other verifying methods on sex specificity of investigated markers.

5. Southern blot analysis of male and female genomic DNA with 12 candidate markers from both PERT (4 probes) and RDA (8 probes) were conducted. According to the result, 5 markers hybridized to both sexes while 7 markers were undetectable by Southern blot analysis. This could cause by the low signal of the labeled probe hybridizing to the target gene with low copy number. Therefore, sex specificity of these candidate markers remained unconclusive.

6. Attempt to amplify Estrogen receptor gene from *P. monodon* was initiated. Six DNA fragments were amplified with ovary cDNA by the degenerated primers designed from ERs of many species. Five of these DNA fragments were cloned, sequenced, and identified. None of their sequences were similar to those of ER reported in GenBank. Two clones were similar to un-characterized gene of *Apis mellifera* and 3 clones were identified as genes similar to transcription co-repressor Sin3 in *Xenopus laevis*, testis specific IQ motif containing GTPase activating protein 2 in *Homo sapiens*, and Cj-cadherin in *Caridina japonica*, respectively.

7. Male and female *P. monodon* genomic libraries were constructed using lambda system. The efficiencies of male and female libraries are $7.3 \times 10^3$ and $1.3 \times 10^4$ pfu/ml, respectively. The average sizes of the clones are 20 kb in both libraries. After screening with the probe of tsIQGAP2-like fragment, positive plaques are detected from both libraries.

8. After the sex specificities of all potential markers have been verified by PCR and Southern blot analyses, it reveals the high possibility that there are no sex chromosomes and no gender-specific sequences on male and female genomes of *P. monodon*. Presumably, sex determination and differentiation of *P. monodon* are controlled by sex-linked genes located on autosomes.

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# APPENDICES

# Appendix A

**1. Ammonium Acetate (10 M)**

To prepare a 1 liter solution, dissolve 770 g of ammonium acetate in 800 ml of $H_2O$. Adjust volume to 1 liter with $H_2O$. Sterilize by filtration. Alternatively, to prepare a 100 ml solution, dissolve 77 g of ammonium acetate in 70 ml of $H_2O$. Sterilize the solution by passing it through a 0.22 μM filter. Store the solution in tightly sealed bottles at 4 °C or at room temperature. Ammonium acetate decomposes in hot $H_2O$ and solutions containing it should not be autoclaved.

**2. Ampicillin**

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

**3. Chloroform : Isoamyl Alcohol (50:1)**

A mixture consisting of equal parts of equilibrated phenol and chloroform:isoamyl alcohol (50:1) is frequency used to remove proteins from preparation of nucleic acids. The chloroform denatures protein and facilitates the separation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction. Neither chloroform nor isoamyl alcohol requires treatment before use. The Chloroform:isoamyl alcohol mixture may be stored under 100 mM Tris-Cl (pH 8.0) in a light-tight bottle at 4 °C for period of up to 1 month.

**4. CTAB Extraction Buffer**

2% (w/v) CTAB

100 mM Tris-HCl (pH 8.0)

20 mM EDTA (pH 8.0)

1.4 M NaCl

0.2% (v/v) β-mercaptoethanol (optional)

**5. Detection Buffer**

0.1 M Tris-HCl (pH 9.5)

0.1 M NaCl

**6. Developer and Replenisher** (3.8 liters)

| | | |
|---|---|---|
| water | 3.04 | L |
| Developer and Replenisher | 760 | ml |

**7. Developing Solution** (Prepare for 3 liters per packet)

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

**8. Dimethylsulfoxide (DMSO)**

Purchase a high grade of DMSO (HPLC grade or better). Divide the contents of a fresh bottle into 1 ml aliquots in sterile tubes. Close the tubes tightly and store at –20°C. Use each aliquot only once and then discard.

**9. EDTA (0.5 M, pH 8.0)**

Add 186.1 g of disodium ethylene diamine tetraacetate·$2H_2O$ to 800 ml of $H_2O$. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The sodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.

**10. Ethidium Bromide (10 mg/ml)**

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

**11. Fix/Stop Solution** (10% glacial acetic acid), (Prepare for 2 liters)

| | | |
|---|---|---|
| Glacial acetic acid | 200 | ml |
| ultrapure or double-distilled water | 1800 | ml |

**12. Fixer and Replenisher** (3.8 liters)

| | | |
|---|---|---|
| Fixer and Replenisher | 757 | ml |
| Water | 3.043 | L |

**13. Gelatin (2% w/v)**

Add 2 g of gelatin to a total volume of 100 ml of $H_2O$ and autoclave the solution for 15 minutes at 15 psi (1.05 $kg/cm^2$) on liquid cycle.

**14. Gel-Loading Buffer Type II**

10x buffer

0.25% bromophenol blue

0.25% xylene cyanol

25% Ficoll (type 400) in $H_2O$

Store at room temperature.

**15. Glycerol (10% v/v)**

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

**16. GTE Buffer**

50 mM glucose

25 mM Tris-HCl (pH 8.0)

10 mM EDTA (pH 8.0)

Add deionized $H_2O$ and autoclave.

**17. HCl (2.5 N)**

Add 25 ml of concentrated HCl <!> (11.6 N) to 91 ml of sterile $H_2O$. Store the diluted solution at room temperature.

**18. HE buffer (1x)**

10 mM Hepps (N-[2-hydroxyethyl]piperazine-N'-[3-propanesulfonic acid])

1 mM EDTA (pH 8.0)

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

IPTG is isopropylthio-B-D-galactoside. Make a 20% solution of IPTG by dissolving 2 g of IPTG in 8 ml of distilled $H_2O$. Adjust the volume of the solution to 10 ml with $H_2O$ and sterilize by passing it through a 0.22 μM disposable filter. Dispense the solution into 1 ml aliquots and store them at 4°C.

**20. KCl (1 M)**

Dissolve 74.56 g of KCl in 800 ml of $H_2O$. Adjust volume to 1 liter. Dispense into aliquots and sterilize by autoclaving.

**21. Luria-Bertani (LB) Broth**

10 g of NaCl

10 g of tryptone

5 g of yeast extract

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

**22. LB Agar**

10 g of NaCl

10 g of tryptone

5 g of yeast extract

20 g of agar

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

**23. LB-Ampicillin Agar**

Prepare 1 liter of LB agar. Autoclaved and cooled to 55°C

Add 50 ml of filter-sterilized ampicillin

Pour into Petri dishes (25 ml/100 mm plate)

**24. LB top agar**

10 g of NaCl

10 g of tryptone

5 g of yeast extract

Add 0.7% (w/v) agar

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

**25. Lysis Buffer**

0.2 N NaOH

1% (w/v) SDS

**26. Maleic Acid Buffer**

0.1 M Maleic acid

0.15 M NaCl

Add deionized $H_2O$ to a final volume of 1 liter. Adjust to pH 7.5 with NaOH (pellet) and autoclave.

**27. Maltose (20% w/v)**

Dissolve 20 g of maltose in a final volume of 100 ml of $H_2O$ and sterilize by passing through a 0.22 μM filter. Store the sterile solution at room temperature.

**28. $MgCl_2$ (1 M)**

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

**29. $MgSO_4$ (1 M)**

Dissolve 12 g of $MgSO_4$ in a final volume of 100 ml of $H_2O$. Sterilize by autoclaving or filter sterilization. Store at room temperature.

**30. NaCl (5 M)**

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

**31. NaOH (10 N)**

The preparation of 10 N NaOH <!> involves a highly exothermic reaction, which can cause breakage of glass containers. Prepare this solution with extreme care in plastic beakers. To 800 ml of $H_2O$, slowly add 400 g of NaOH pellets <!>, stirring continuously. As an added precaution, place the beaker on ice. When the pellets have dissolved completely, adjust the volume to 1 liter with $H_2O$. Store the solution in a plastic container at room temperature. Sterilization is not necessary.

**32. Neutralization Buffer II** (for Transfer of DNA to Nylon Membranes)

0.5 M Tris-HCl (pH 7.5)

1.5 M NaCl

Add deionized $H_2O$ to a final volume of 1 liter. Adjust to pH 7.5 with HCl and autoclave.

**33. Equilibration of Phenol**

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

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

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

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

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

**34. Phenol : Chloroform : Isoamyl Alcohol (25:24:1)**

A mixture consisting of equal parts of equilibrated phenol and chloroform:isoamyl alcohol (24:1) is frequency used to remove proteins from preparation of nucleic acids. The chloroform denatures protein and facilitates the separation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction. Neither chloroform nor isoamyl alcohol requires treatment before use. The phenol:Chloroform:isoamyl alcohol mixture may be stored under 100 mM Tris-Cl (pH 8.0) in a light-tight bottle at 4 °C for period of up to 1 month.

**35. Polyacrylamide Gel (40%, crosslink = 75:1)**

An enough amount of ingredients for a 200 ml. Composed of:

| | |
|---|---|
| Acrylamide | 78.94 g |
| Bis-acrylamide | 1.052 g |

**36. Potassium Acetate (5 M)**

| | |
|---|---|
| 5 M potassium acetate | 60 ml |
| glacial acetic acid | 11.5 ml |
| $H_2O$ | 28.5 ml |

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the buffer at room temperature.

**37. Prehybridization Solution**

Add carefully 64 ml sterile double distilled water in two portions to the DIG Easy Hyb Granules, dissolve by stirring immediately for 5 min at 37°C.

**38. Proteinase K (20 mg/ml)**

Purchase as a lyophilized powder and dissolve at a concentration of 20 mg/ml in sterile 50 mM Tris (pH 8.0), 1.5 mM calcium acetate. Divide the stock solution into small aliquots and store at –20°C. Each aliquot can be thawed and refrozen several times but should then be discarded. Unlike much cruder preparations of protease (e.g., pronase), proteinase K need not be self-digested before use.

**39. SM Buffer**

This buffer is used for phage storage and dilution.

| | | |
|---|---|---|
| NaCl | 5.8 | g |

| | | |
|---|---|---|
| $MgSO_4 \cdot 7H_2O$ | 2 | g |
| 1 M Tris-HCl (pH 7.5) | 50 | ml |
| 2% (w/v) gelatin | 5 | ml |

Add deionized $H_2O$ to a final volume of 1 liter and autoclaving. After the solution has cooled, dispense 50 ml aliquots into sterile containers. SM may be stored indefinitely at room temperature. Discard each aliquot after use to minimize the chance of contamination.

**40. SOB Medium**

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

**41. Sodium Acetate (3 M, pH 5.2)**

Dissolve 408.3 g of sodium acetate $\cdot 3H_2O$ in 800 ml of $H_2O$. Adjust pH to 5.2 with glacial acetic acid. Adjust volume to 1 liter. Dispense into aliquots and sterilize by autoclaving.

**42. Sodium Dodecyl Sulfate (SDS,** also called sodium lauryl sulfate) **10%**

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

**43. SSC (20x)**

175.3 g of NaCl

88.2 g of sodium citrate

Adjust to pH 7.0 with a few drops of concentrated HCl. Adjust to 1,000 ml. with water.

**44. Staining Solution** (Prepare for 1.5 liters)

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

**45. 10x STE Buffer**

100 mM Tris-HCl (pH 8.0)

1 M NaCl

10 mM EDTA (pH 8.0)

Sterilize by autoclaving and store the sterile solution at 4°C.

**46. 10x Tris-Borate (TBE)** (per Liter)

| | | |
|---|---|---|
| Tris-borate | 108 | g |
| Boric acid | 55 | g |
| 0.5 M EDTA (pH 8.0) | 100 | ml |

**47. TE (pH 7.5)**

10 mM Tris-HCl (pH 7.5)

1 mM EDTA (pH 8.0)

**48. TE (pH 8.0)**

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

**49. TEN Buffer (10x)**

0.1 M Tris-Cl (pH 8.0)

0.01 M EDTA (pH 8.0)

0.1 M NaCl

**50. Washing Buffer**

0.1 M Maleic acid

0.15 M NaCl

Adjust with NaOH (solid) to pH 7.5 and add 0.3% (v/v) Tween 20.

**51. X-gal Solution (2% w/v)**

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

# Appendix B

Restriction mapping of plasmid pUC18 (Figure A.1), pGEM®-T easy vector (Figure A.2) and Lambda FIX®II replacement vector (Figure A.3)

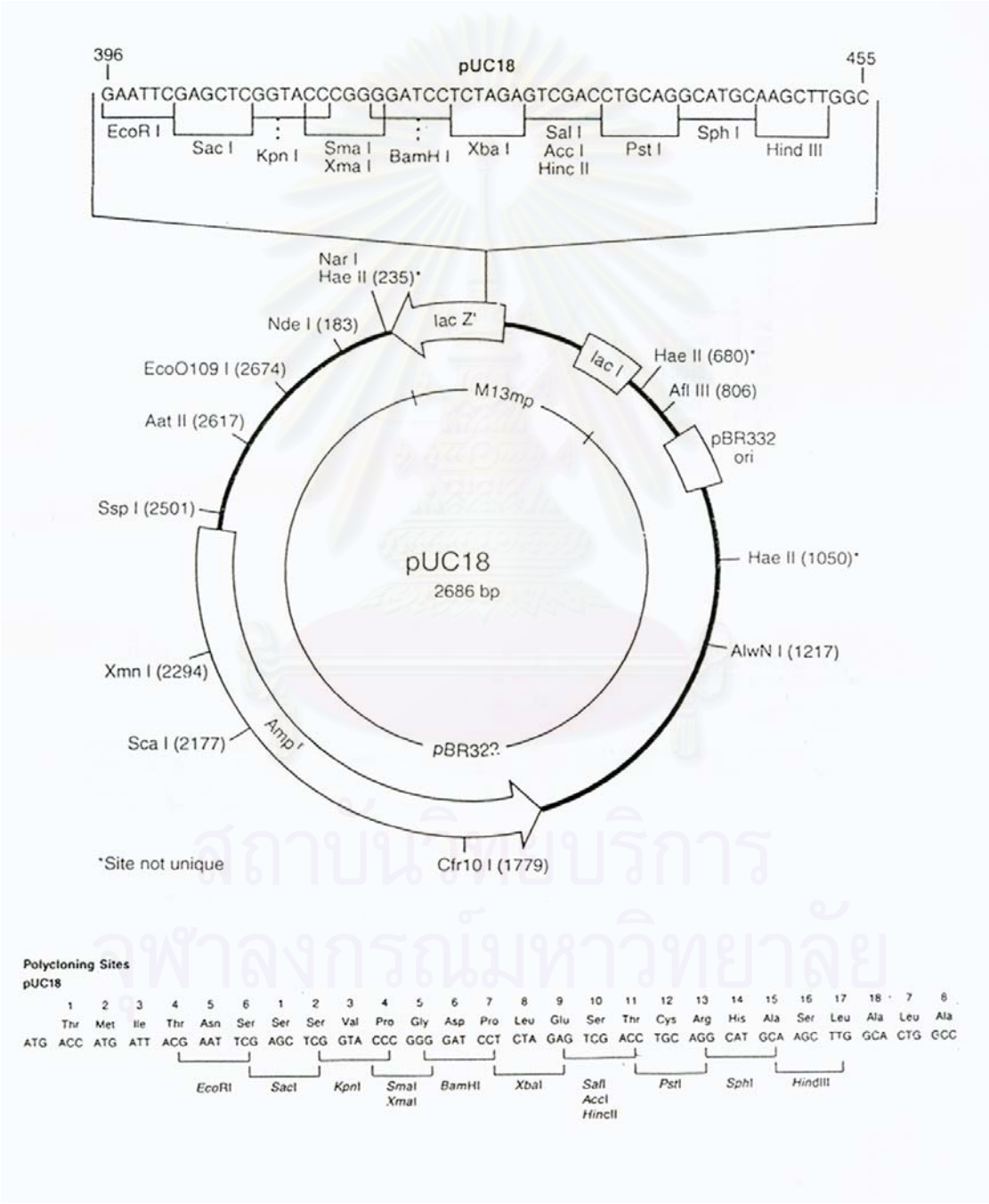



**Figure A.1** Map of the pUC18 and multiple cloning site sequence of the pUC18

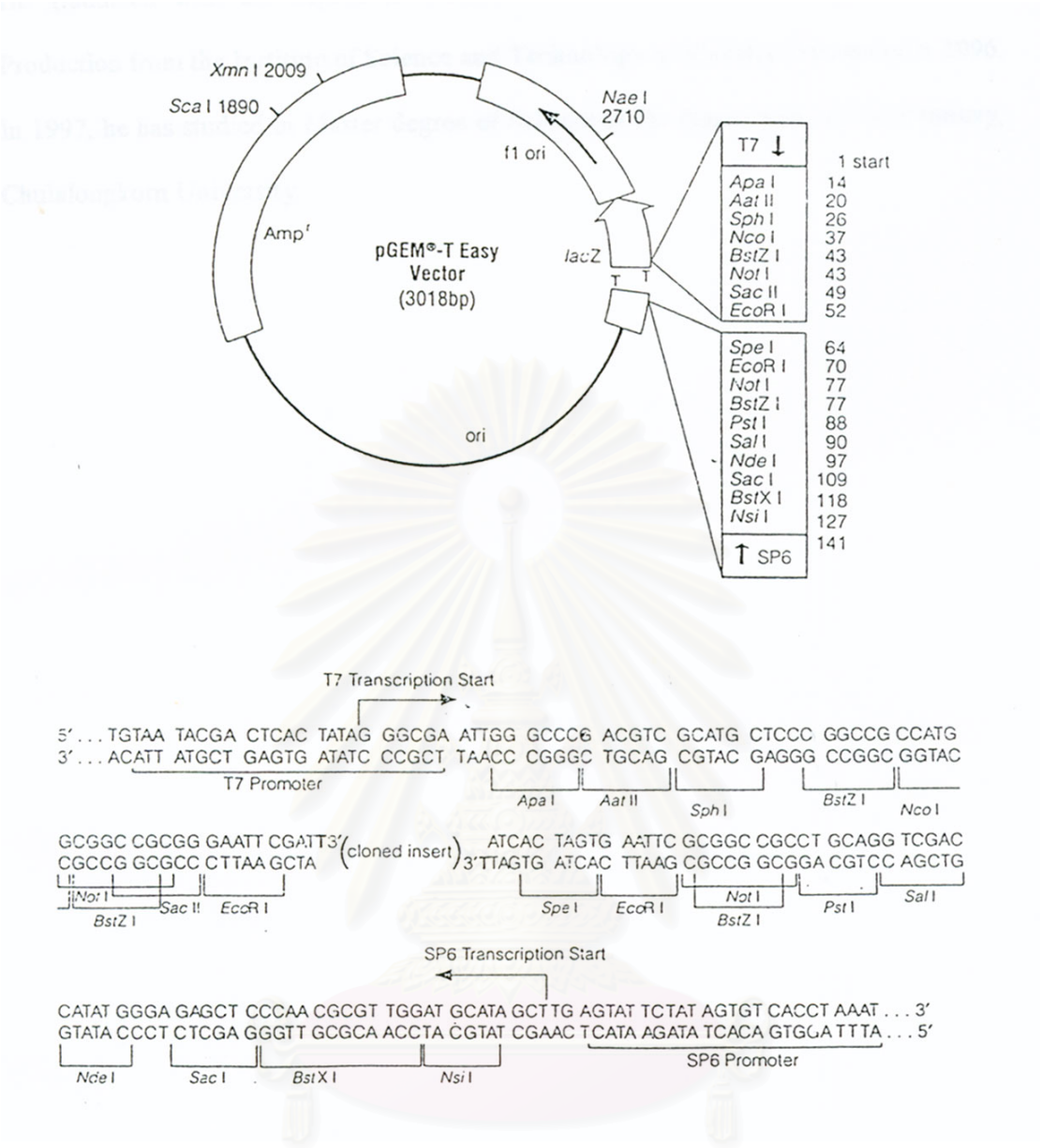
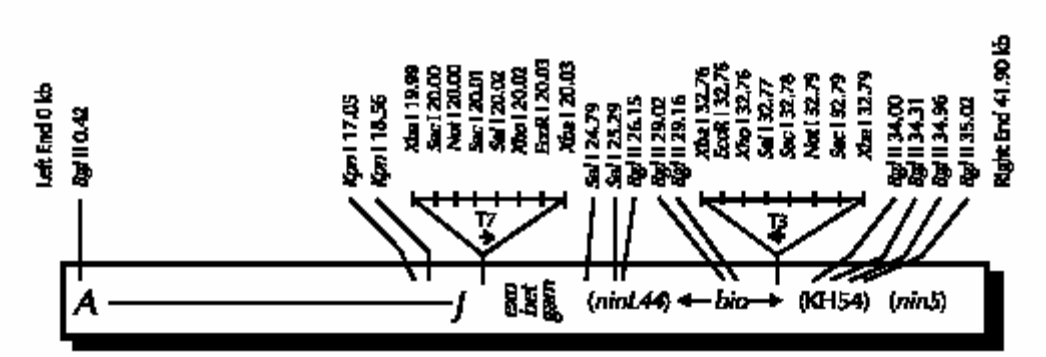



**Figure A.2** Map of the pGEM®-T easy vector and multiple cloning site sequence of the pGEM®-T easy vector




*Xba* I *Sac* I *Not* I *Sac* I T7 *Sal* I *Xho* I *EcoR* I *Xba* I
5′-TCTAGAGAGCTCGCGGCCGCGAGCTCTAATACGACTCACTATAGGGCGTCGACTCGAGAAGGAATTCTCTAGA-3′

*Xba* I *EcoR* I *Xho* I *Sal* I T3 *Sac* I *Not* I *Sac* I *Xba* I
5′-TCTAGAGAATTCGGCTCGAGTCGACTCCCTTTAGTGAGGGTTAATTGAGCTCGCGGCCGCGAGCTCTCTAGA-3′

**Figure A.3** Map of the Lambda FIX®II replacement vector and multiple cloning site sequence of the Lambda FIX®II replacement vector

# Appendix C

**Publication from this thesis**

1. Srisupaph Poonlaphdecha, Narongsak Puanglarp, Sirawut Klinbunga and Piamsak Menasveta (2004). Identification of sex-specific markers from black tiger shrimp *Penaeus monodon* using genomic DNA subtraction. 30th Congress on Science and technology of Thailand (Oral presentation).

# BIOGRAPHY

Miss Srisupaph Poonlaphdecha was born on October 29, 1980 in Ratchaburi, Thailand. She graduated with the degree of Bachelor of Science in Fisheries Science from faculty of Agricultural technology at King Mongkut's Institute of Technology Ladkrabang in 2001. In 2002, she has studied Master degree of Science at the Program of Biotechnology, Chulalongkorn University.