การระบุชิงเกิลนิวคลีโอไทด์พอลิมอร์ฟิซึมของยีนในรังไข่และอัณฑะของกุ้งกุลาดำ *Penaeus monodon*

นางสาว สิริกานต์ ประเสริฐลักษณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM OF GENES IN OVARIES AND TESTES OF THE GIANT TIGER SHRIMP *Penaeus monodon*

Miss. Sirikan Prasertlux

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM OF GENES IN OVARIES AND TESTES OF THE GIANT TIGER SHRIMP *Penaeus monodon*
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สิริกานต์ ประเสริฐลักษณ์ : การระบุซิงเกิลนิวคลีโอไทด์พอลิมอร์ฟิซึมของยีนในรังไข่และอัณฑะของกุ้งกุลาดำ *Penaeus monodon*(IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM OF GENES IN OVARIES AND TESTES OF THE GIANT TIGER SHRIMP *Penaeus monodon*)

อ. ที่ปรึกษา: ศ.ดร.เปี่ยมศักดิ์ เมนะเศวต, อ.ที่ปรึกษาร่วม: ดร.ศิราวุธ กลิ่นบุหงา 145 หน้า.

ค้นหา Single nucleotide polymorphism (SNP) ของยีนในกุ้งกุลาดำ โดยออกแบบไพรเมอร์จำนวน108 คู่จากห้องสมุดยีนของเม็ดเลือด รังไข่และอัณฑะของกุ้งกุลาดำ พบไพรเมอร์จำนวน 56 คู่ (51.85%) ที่สามารถให้ผลิตภัณฑ์พีซีอาร์โดยพบว่ามีส่วนของอินทรอนแทรกอยู่ในชิ้นยีนจำนวน 31 ยีน ทำการศึกษาการแปรผันทางพันธุกรรมชนิด SNP ของยีนจำนวน 42 ยีนกับจีโนมิกดีเอ็นเอของกุ้งกุลาดำจากธรรมชาติ (N = 15) ด้วยวิธี SSCP พบยีนจำนวน 37 ยีน (เช่น *splicing factor 3a, subunit 1*, *phosphoglucose isomerase*, *solute carrier family 3 member 2*, *rasputin*, *RNA helicase*, *heterogeneous nuclear ribonucleoprotein 87F* และ *laminin-β chain*)แสดงความแปรผันทางพันธุกรรมในกลุ่มตัวอย่างธรรมชาติ จึงทำการวิเคราะห์ลำดับนิวคลีโอไทด์ของตัวอย่างที่แสดงรูปแบบต่างๆ ของยีน *X-box protein*, *NADP-dependent leukotriene-12-hydroxydehydrogenase* (*LTB4DH*), *phosphatidylinositol 4 kinase*, *RUVB-like protein*, *phosphatidylserine receptor* และ *solute carrier family 3 member 2* พบว่าให้ผลสอดคล้องกับผลจากการศึกษาด้วย SSCP แสดงว่าวิธี SSCP ซึ่งมีค่าใช้จ่ายน้อย มีประสิทธิภาพสามารถใช้แทนการหาลำดับนิวคลีโอไทด์โดยตรงได้

จากนั้นนำยีนจำนวน 19 ยีนที่แสดงความแปรผันทางพันธุกรรมในกุ้งกุลาดำจากธรรมชาติ มาทำการตรวจสอบความแปรผันทางพันธุกรรมเบื้องต้นกับตัวอย่างกุ้งกุลาดำอายุ 132 วันที่มาจากครอบครัวเดียวกัน (G2) จำนวน 10 ตัว พบยีนจำนวน 7 ยีน (*FII1-7*, *FII-I4*, *FIII-8*, *solute carrier family 3 member 2, DDPG*, *phosphatidylinositol 4 kinase* และ *phosphatidylserine receptor short*) ที่ไม่มีการแสดงความแปรผันทางพันธุกรรมกับกลุ่มตัวอย่างนี้ และพบว่าชิ้นส่วนของยีนจำนวน 9 ชิ้นที่ได้มาจาก RAP-PCR (*457/OPA01*, *428/OPB17*, *MI-36*, *MII-51*, *FI-40*, *FIV-33*, *FV-1*, *FV-27* และ *FV-42*) แสดงความแปรผันทางพันธุกรรมในตัวอย่างที่ศึกษา แต่ชิ้นส่วน RAP-PCR เหล่านี้เป็นยีนที่ไม่ทราบหน้าที่ (unknown genes) ดังนั้นจึงเลือกยีน *X-box binding protein* (N = 76), *LTB4DH* (N = 60) และ *RUVB-like protein 2* (N =61) ซึ่งแสดงความแปรผันทางพันธุกรรม มาทำการวิเคราะห์ความสัมพันธ์ระหว่าง SNP จาก SSCP และอัตราการเจริญเติบโตของกุ้งกุลาดำ ซึ่งจากผลการทดลองไม่พบความสัมพันธ์ระหว่างน้ำหนักตัวของกุ้งกุลาดำอายุ 132 วันกับรูปแบบ SSCP ของยีน *X-box protein* (3 จีโนไทป์) และ *LTB4DH* (2จีโนไทป์) (P > 0.05) แต่พบความสัมพันธ์ระหว่าง จีโนไทป์ (รูปแบบ SSCP) ในยีน *RUVB-like protein* กับน้ำหนักตัวของกุ้งกุลาดำอายุ 132 วัน ในกลุ่มที่โตเร็ว โดยกุ้งที่มีจีโนไทป์ II (28.6875 ± 2.3564) มีน้ำหนักมากกว่าจีโนไทป์ I (26.1333 ± 4.5566) อย่างมีนัยสำคัญทางสถิติ (P < 0.05) เมื่อทำการยืนยันผลการทดลองในกุ้งกุลาดำจากฟาร์มเพาะเลี้ยง ที่มีอายุประมาณ 3 เดือน (17.39 ± 4.36 g, N = 359) พบว่าน้ำหนักตัวของกุ้งกุลาดำ ที่มี จีโนไทป์ A (19.2768 ± 3.640, N=34)และ จีโนไทป์ B (19.2929 ± 4.5477, N=78) มีความแตกต่างกับน้ำหนักตัวของกุ้งกุลาดำที่มี จีโนไทป์ C (16.5277 ± 3.8466, N=94) และ จีโนไทป์ D (16.3645 ± 4.3780, N=129) อย่างมีนัยสำคัญทางสถิติ (P < 0.05) โดยผลจากการหาลำดับเบสพบเครื่องหมาย SNP 3 ตำแหน่ง ($G \rightarrow A_{81}$, $A \rightarrow T_{196}$ และ $G \rightarrow T_{248}$) ซึ่งสามารถแบ่งแยกจีโนไทป์ต่างๆได้

ทำการค้นหาลำดับนิวคลีโอไทด์ที่สมบูรณ์ของยีน *Rasputin* (ORF ขนาด 1659 bp โดยมี 5′ และ 3′UTRs เท่ากับ 132 และ 2305 bp), *LTB4DH* (ORFขนาด 1038 bp โดยมี 5′ and 3′UTRs เท่ากับ 165 และ 1384 bp), *RUVB-like protein* (ORF ขนาด 1395 bp โดยมี 5′ and 3′UTRs เท่ากับ 436 และ 2413 bp) และ *X-box binding protein* (ORF of 858 bp with 5′ and 3′UTRs of 265 and 278 bp) ทำการทดสอบผลของ 5-HT (โดยฉีดครั้งเดียว และ ฉีดซ้ำ 2 ครั้ง ในความเข้มข้น 50 μg. g^{-1} ของน้ำหนักตัว) ต่อระดับการแสดงออกของยีนในรังไข่ของกุ้งกุลาดำโดย semiquantitative RT-PCR พบความแตกต่างของระดับการแสดงออกของยีน *LTB4DH*, *RuvB like protein* และ *X-box binding protein* ในกลุ่มทดลองเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ (P < 0.05)

สาขาวิชา หลักสูตรเทคโนโลยีชีวภาพ
ปีการศึกษา 2549

ลายมือชื่อนิสิต.....สิริกานต์ ประเสริฐลักษณ์.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD: *Penaeus monodon* / GIANT TIGER SHRIMP/SNP/SSCP
SIRIKAN PRASERTLUX: IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM OF GENES IN OVARIES AND TESTES OF THE GIANT TIGER SHRIMP *Penaeus monodon*.
THESIS ADVISOR: PROF. PIAMSAK MENASVETA, Ph.D.
THESIS COADVISOR: SIRAWUT KLINBUNGA, Ph.D. 145 pp.

Single nucleotide polymorphism (SNP) in functionally important genes of the giant tiger shrimp (*Penaeus monodon*) was examined by SSCP analysis. A total 108 pairs of primers were designed from ESTs of ovaries and testis of *P. monodon* and tested against genomic DNA of representative individuals of wild *P. monodon* (N=15). Fifty-six primer pairs (51.85%) generated the positive amplification product. Thirty-one of which gave larger product sizes than those expected from the coding sequences suggesting the existence of intron (s) in the amplified fragments. The amplification product of 42 genes using genomic DNA of wild *P. monodon* (N = 15) as the template was analyzed by SSCP. Thirty-seven gene homologues (e.g. *splicing factor 3a, subunit 1*, *phosphoglucose isomerase*, *solute carrier family 3 member 2*, *rasputin*, *RNA helicase*, *heterogeneous nuclear ribonucleoprotein 87F* and *laminin-β chain*) were polymorphic. Nucleotide sequences of an individual representing each SSCP genotype of *X-box binding protein*, *NADP-dependentleukotriene-12-hydroxy-dehydrogenase* (*LTB4DH*), *phosphatidylinositol 4 kinase*, *RUVB-like protein*, *phosphatidylserine receptor* and *solute carrier family 3 member 2* were examined by direct sequencing of the PCR product and showed compatible results with those from SSCP analysis. Therefore, SSCP analysis is an alternative cost-effective and potential for identifying polymorphism of various gene homologues.

Nineteen gene homologues initially exhibited polymorphism in wild *P. monodon* were further tested against the 132-day-old G2 family of *P. monodon* (N = 10). Seven genes (*FII1-7*, *FII-I4*, *FIII-8*, *solute carrier family 3 member 2, DDPG*, *phosphatidylinositol 4 kinase* and *phosphatidylserine receptor*) did not revealed polymorphic patterns in these specimens. Although nine RAP-PCR fragments (*457/OPA01*, *428/OPB17*, *MI-36*, *MII-51*, *FI-40*, *FIV-33*, *FV-1*, *FV-27* and *FV-42*) were polymorphic, they were unknown transcripts and were not further analyzed. Therefore, only homologues of *X-box binding protein* (N = 76), *LTB4DH* (N = 60), and *RUVB-like protein* (N = 61) were subjected to preliminary association analysis. Results indicated no correlation between the body weight of 132-day-old *P. monodon* and SSCP genotypes of *LTB4DH* (2 genotypes) and *X-box binding protein* (3 genotypes) (P >0.05). Nevertheless, a statistical significance between genotypes (SSCP patterns) of *RUVB-like protein* and the body weight of a 132-day-old shrimp was found in presumably the fast growing shrimps exhibiting the genotype II (28.6875 ± 2.3564) and the genotype I (26.1333 ± 4.5566; P < 0.05). A new sample set of *P. monodon* juveniles was collected from a commercial farm (17.39 ± 4.36 g, N = 359). Polymorphism of *RUVB-like protein* was tested and resulted indicated that the body weight of shrimp carrying the genotype A (19.2768 ± 3.640, N=34) and B (19.2929 ± 4.5477, N=78) was significantly different from that of shrimp carry the C (16.5277 ± 3.8466, N=94) and D (16.3645 ± 4.3780, N=129) genotypes. Three SNP positions ($G \rightarrow A_{81}$, $A \rightarrow T_{196}$ and $G \rightarrow T_{248}$) were found from cloned nucleotide sequences of *RUVB-like protein* in representative individuals of these specimens (P <0.05).

The full length cDNA of *raputin* (ORF of 1659 bp with 5′ and 3′UTRs of 132 and 2305 bp) *LTB4DH* (ORF of 1038 bp with 5′ and 3′UTRs of 165 and 1384 bp), *RUVB-like protein* (ORF of 1395 bp with 5′ and 3′UTRs of 436 and 2413 bp) and *X-box binding protein* (ORF of 858 bp with 5′ and 3′UTRs of 265 and 278 bp) was successfully identified and reported for the first time.

A time course effect of 5-HT (single or double injection of 50 μg. g^{-1} of the body weight of 5-HT) on expression of *LTB4DH*, *X-box binding protein* and *RUVB like protein* in ovaries of juvenile *P. monodon* were examined using semiquantitative RT-PCR. Results indicate that 5-HT potentially stimulated the expression levels of all of the investigated gene homologues (P < 0.05).

Student's signatuure.....
Field of study.....Biotechnology.....Advisor's signature.....
Academic year.....2006.....Co-advisor's signature.....

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LIST OF ABBREVIATIONS

bp	base pair
ºC	degree celcius
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
Kb	kilobase
M	Molar
$MgCl_2$	magnesium chloride
mg	milligram
ml	milliliter
mM	millimolar
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
RACE	Rapid amplification of cDNA Ends
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	revolution per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
Tris	tris(hydroxyl methyl) aminomethane
µg	microgram
µl	microlitre
µM	micromolar
UV	ultraviolet

CHAPTER 1

INTRODUCTION

1.1 General introduction

Farming of the giant tiger shrimp (*Penaeus monodon*) has been practiced for food and the livelihood of coastal people in several Asian countries including Indonesia, the Philippines, Taiwan, Vietnam and Thailand for a long period of time. Initially, *P. monodon* was originally harvested together with other shrimp species from extensive growing ponds. In Thailand, extensive and semi-intensive farms were commercially established in 1972 and 1974, respectively, after the first success in breeding of *P. monodon* at the Phuket Fisheries Station in 1972. Subsequently, the ability to develop formulated feed commercially led to the success development of the intensive culture system of this species. The global production of *P. monodon* has increased from approximately 50000 metric tines (MT) in 1985 to greater than 600000 MT annually in 2000 (Fig 1.1).

Nevertheless, the production cycle of *P. monodon* has yet to be completed. Farming of *P. monodon* presently relies almost entirely on wild-caught broodstock for the seed supply because breeding of *P. monodon* in captivity is extremely difficult. This open production cycle and reliance on wild stocks of *P. monodon* results in heavy exploitation of female broodstock from wild populations. In addition, the introduction or importation of wild broodstock is commonly practiced among the major producing countries due to insufficient of local supplies.

Thailand has been regarded as the leader for *P. monodon* production for over a decade. Thailand had a severe outbreak of diseases during 1995-1997 causing the decrease in the shrimp production at that period. Farming of *P. monodon* has consistently encountered production losses from infectious diseases, particularly from white spot syndrome virus (WSSV), yellow head virus (YHV) and *Vibrio* sp..

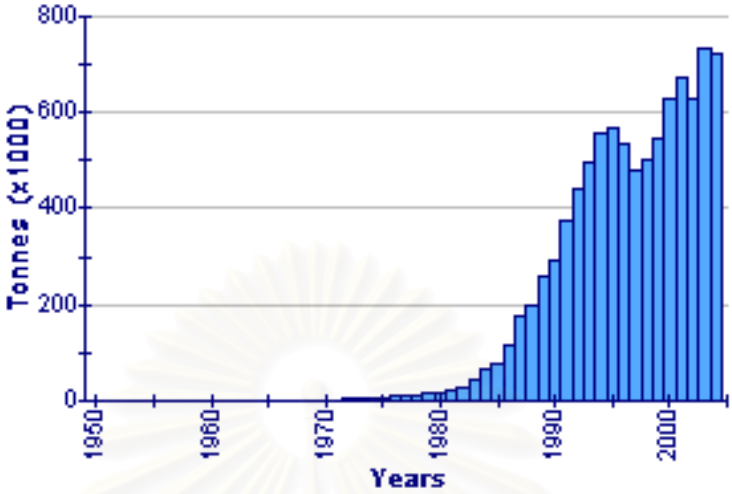

Figure 1.1 Global aquaculture production of *Penaeid shrimp.* (FAO Fishery Statistics, 2005).

Farming of *P. monodon* presently faces several problems such as the reduction of high quality wild broodstock and loss of the cultured production due to bacterial and viral infections and size differences of cultivated *P. monodon* in the same cultured pond. The annual production of *P. monodon* was significantly reduced from approximately 200,000 MT between 1992 – 2002 to 69,412 MT in 2005 (Table 1.1). and about 30,000 MT in 2006 (C.Limsuwan, personal communication) As a result, the Pacific white shrimp (*Litopenaeus vannamei*) has been introduced to Thailand as an alternative cultured species and initially contributed approximately 20000 MT of the production in 2002 and dramatically increased to 170000 and 220000 MT in 2003 and 2004, respectively (Limsuwan, 2004).

Genetic improvement and other biotechnology applications are crucial to the future development of this industry (Benzie, 1998; Brody, 1998). Progress in genetic and biotechnology researches in penaeid shrimps have been slow because a lack of knowledge on fundamental aspects of their biology (Benzie, 1998). A research concerning domestication of *P. monodon* has been carried out by production of high

quality pond-reared *P. monodon* broodstock (Withyachumnarnkul *et al.*, 1998) but was recently collapsed by the WSSV infection.

The domesticated program of *P. monodon* is start over and it is expected that selective breeding programmes of *P. monodon* will provide shrimps having commercially desired phenotypes (e.g. high growth rate and/or disease resistance) and produce *P. monodon* stocks with the ability to induce high quality egg development in domesticated females without the irreversible side-effects caused by a typical eyestalk ablation technique (Lyons and Li, 2002). Phenotypic improvement can be accomplished through conventional breeding programs and knowledge from genome studies and molecular markers linked to important traits (marker-assisted selection, MAS) can also be directly applied to improve artificial selection processes more efficiently.

Sustainable shrimp industry can be promoted through applications of knowledge from genetic and biotechnological studies including development of genetic markers to assist selective breeding programmes of *P. monodon*. The basic knowledge gained can be applied for increasing culture efficiency of *P. monodon*. Researches in both basic (molecular and cellular levels of gonad developmental processes) and applied (microsatellite/SNP genotyping to assist selective breeding of high quality pond-reared *P. monodon*) disciplines are required to elevate the culture efficiency and subsequence to sustain the industry of this economically important species.

Table 1.1 The export of the gient tiger shrimp *P.monodon* from Thailand between 2002 – 2005

Month	2002		2003		2004		2005	
	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)
Jan	11,345	3,894.80	13,360	4,671.66	11,746	3,145.25	5,625	1,510.76
Feb	10,821	3,763.43	11,453	3,916.07	12,606	3,356.08	4,193	1,098.32
Mar	12,578	4,260.60	11,594	3,890.99	4,610	1,332.69	4,537	1,248.03
Apr	12,308	4,026.19	11,230	3,895.05	5,782	1,785.85	3,603	968.76
May	14,655	4,946.44	12,594	4,120.20	7,082	1,937.04	4,313	1,194.83
Jun	15,545	5,468.02	12,446	4,089.82	8,414	2,780.45	6,623	1,749.64
Jul	14,285	5,019.06	14,055	4,563.97	9,902	3,142.45	6,857	1,885.88
Aug	17,295	5,741.69	15,731	5,036.36	8,327	2,595.77	6,678	1,870.81
Sep	19,808	7,196.31	17,988	5,611.11	10,498	3,358.83	7,339	1,946.05
Oct	21,265	8,041.29	18,057	5,358.97	10,853	3,321.91	6,864	1,857.45
Nov	18,939	6,982.56	11,857	3,454.28	9,797	2,997.04	6,618	1,817.32
Dec	12,206	4,485.87	10,832	2,974.41	8,904	2,458.51	6,162	1,605.64
Total	181,050	63,826.26	161,197	51,582.89	108,521	32,211.87	69,412	18,753.49

Source: Office of Agricultural Economics, Ministry of Agriculture and Cooperatives (2006)

1.2 Taxonomy of *P. monodon*

Penaeid shrimps are taxonomically recognized as members the largest phylum in the animal kingdom, the Arthropoda. This group of animals is characterized by the presence of paired appendages and a protective cuticle or exoskeleton that covers the whole animal.

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

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Subclass: Eumalacostraca

Order: Decapoda

Suborder: Natantia

Infraorder: Penaeidea

Superfamily: Penaeoidea

Family: Penaeidae Rafinesque, 1985

Genus: Penaeus Fabricius, 1798

Subgenus: Penaeus

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

1.3 Morphology of *P.monodon*

The external morphology of penaied shrimp is distinguished by a cephalothorax with a characteristic hard rosthum, and by a segmented abdomen (Figure 1.2). Most organs are located in cephalothorax, while the body muscles are mainly in the abdomen. The internal morphology of penaeid shrimp is outlined by Figure 1.3.

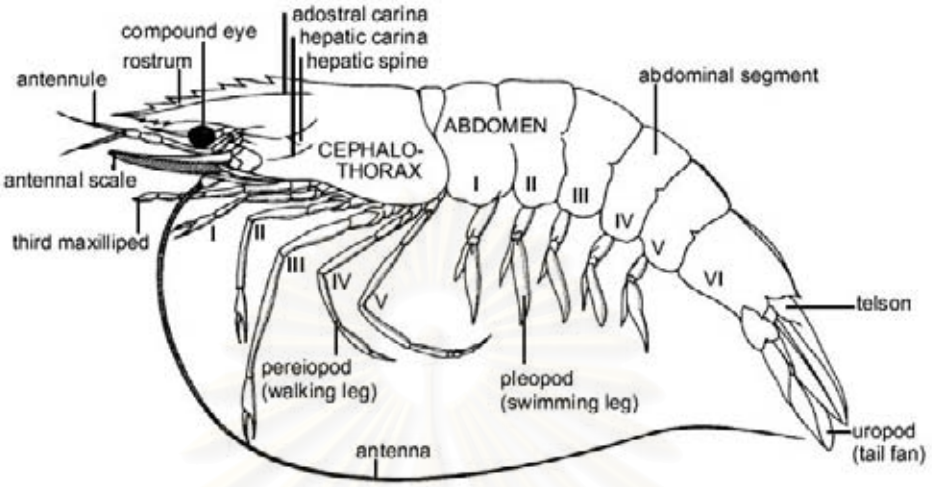

Figure 1.2 Lateral view of the external morphology of *P. monodon*. (Primavera, 1990)

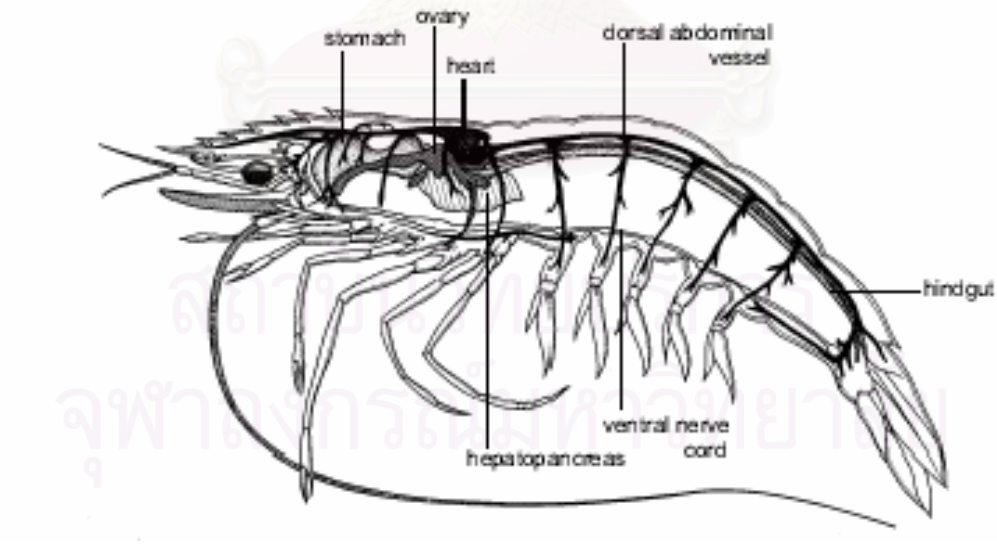

Figure1.3 Lateral view of the internal anatomy of a female *P. monodon.* (Primavera, 1990)

1.4 Domestication and selective breeding program of *P. monodon*

Domestication and subsequently selective breeding programmes (SBPs) are long-term processes used to commercially important trait in selected populations through domestication both (artificial and natural selection). Basically, short generation time and high fecundity of shrimps suggested that genetic improvement of *P. monodon* is promising and can be done in a reasonable period of time.

To carry out effective SBPs in *P. monodon*, high genetic diversity stocks should be established. Integrated knowledge on population genetics for estimation of genetic variation levels, molecular genetics for the identifying genetic markers at different levels and quantitative genetics for selection scheme and estimation of heritability for economically important phenotypes are required. At present, molecular genetic tools in *P. monodon* have been developed to the level that can be directly implemented for practical approaches.

Domestication and SBPs should use the advantage of population genetic differentiation (genetic differences among geographically different samples) between major stocks of *P. monodon*; the Andaman, the upper Gulf of Thailand (Trat) and the lower Gulf of Thailand (Chumphon and Surat) previously reported by molecular genetic markers (Supungul *et al.*, 2000; Klinbunga *et al.*, 2001).

Domestication and breeding programs to improve broodstock performance and seed production of *P. monodon* in Thailand have been conducted for a period of time (Withyachumnarnkul *et al.*, 1998). The trait which has been mainly investigated in penaeid shrimps is the growth rate but selection of shrimps based on disease resistance to harmful pathogenic bacterial and viral agents to *P. monodon* is an alternative approach to overcome the low production efficiency related to sensitivity of shrimps to bacterial and viral infection.

When desired traits showed significant heritability (additive genetic effects) selection can be carried out by individual selection, within family selection and/or combined selection. It is estimated that family and/or combined selection may increase 6 -14% production per generation in terms of the growth rate (body weight)

In contrast, hybridization can be performed when interested traits are dominantly transmitted non-additively. In this case, studies whether different strains of *P. monodon* exhibiting different performance should be firstly carried out.

Genetic diversity of the stocks should be maximized and regularly monitored using appropriate genetic markers (e.g. microsatellites). Physical tagging is also useful for selection of the growth lines through individual and family selection. The most important problem limiting effective SBPs in *P. monodon* is the low ability for spawning captive *P. monodon*. Additional researches on maturation of *P. monodon* through feed technology and hormonal systems are also important for solving the problems appropriately.

In terms of aquaculture, the success of domestication and subsequently, selective breeding programs is an important step to partly resolve problems of over-exploitation of wild *P. monodon*.

At present, several serious diseases found in commercially cultured *P. monodon* are still not well understood or controllable. Practically, a rule of thumb for the primary prevention of all diseases has stressed management of pond environments. Researchers in shrimp diseases can certainly diagnose new diseases but treatment of cultured *P. monodon* by various anti-bacterial and anti-viral agents is actually far from being achieved. Theoretically, a specific pathogen resistance (SPR) stock needs to be developed in the near future. If this is successful, the mature SPR *P. monodon* will serve as an alternative source of broodstock for commercial purposes.

In artificial propagation programs, for instance breeding of *P. monodon*, individuality and family groups of propagated progeny reared in the large communal tank need to be determined. In this case, molecular genetic markers exhibiting high polymorphic levels and segregating in a co-dominant fashion are needed and microsatellites are ideal for such purposes. Currently, a relatively large number of microsatellites have been isolated and well characterized in *P. monodon* (Pongsomboon, 2002). Microsatellite-based genotyping techniques (typical and multiplex PCR) are ready to be applied for family identification in selective breeding programs of *P. monodon* based on within family and/or family selection approaches.

The most important goal for breeding programs is to increase the biological production efficiency utilizing genetic variability of individuals within a population and that of populations (stocks) within a species. Microsatellite markers would assist *P. monodon* domestication to obtain the maximum response when SBPs are implemented.

Theoretically, a large number of local *P. monodon* stocks are needed to be tested under farming conditions for their producing ability. The basic data on genetic population differentiation of *P. monodon* in Thailand allow selection of high genetic diversity of founder populations naturally. Due to large genetic differences between *P. monodon* possessing different mitochondrial clusters, further studies to compare the performance of *P. monodon* carrying different common mitochondrial genotypes in each stock should also be carried out even though it has been generally believed that mtDNA is selective neutrally.

Another possible application of genetic markers to SBPs of *P. monodon* is to identify marker loci which are associated with loci that control economical important traits. They could be used as marker-assisted selection (MAS) leading to convertibility of polygenic variation into defined Mendelianly quantifiable entities. Various molecular approaches such as amplified fragment length polymorphism (AFLP), type I and type II microsatellite and single nucleotide polymorphism (SNP) markers can be used to construct the genetic linkage maps for identifying MAS in this economically important species.

The growth line is the priority for *P. monodon*. Broodstock exhibiting high genetic diversity should be quarantined. Good pedigree record is the key basis for successful selective breeding programs. Genetic diversity must be monitored for estimation of effective numbers of founders ($N_e > 50$) and inbreeding coefficiency (< 0.1% per generation) for each generation. Stocks should be established from different founder populations and be maintained separately. Therefore, hybridization among different lines can be subsequently carried out

Determining the relative effect that male and female broodstock quality is having on reproductive performance, particularly for key parameters such as hatching rate, will enhance the rate at which improvements reproductive performance of

domesticated stocks can be achieved. Previous studies assessing the reproductive performance of reciprocally crossed wild and pond-reared broodstock found that the wild females outperformed domesticated females in terms of maturation, spawning and total egg production in *P. monodon* (Menasveta *et al.*, 1993)

Performances of broodstock should be tested outside the nuclear breeding center (NBC). Heritability for the growth rate has been reported in *P. monodon* and other penaeid shrimps (h^2 = 0.2 – 0.8 depending on breeding plans) but heritability for disease resistance was only reported in the TSV-resistant *P. vannamei* line at approximately 0.2. Generally, family selection may be suitable for selection of disease-resistant lines whereas within family selection may by appropriate for selection of *P. monodon* exhibiting the high growth rates.

1.5 Molecular technique used in this thesis

1.5.1 PCR

The introduction of the polymerase chain reaction (PCR) by Mullis *et al.* (1987) has opened a new approach for molecular genetic studies. This method is a molecular biology technique for enzymatically replicating DNA without using a living organism, such as *E. coli* or yeast and is a method using specific DNA sequences by the two oligonucleotide primers, 17-25 nucleotides in length. Million copies of the target DNA sequence can be synthesized from the low amount of starting DNA template within a few hours.

The PCR reaction components are composed of DNA template, a pair of primers for the target sequence, dNTPs (dATP, dCTP, dGTP and dTTP), buffer and heat-stable DNA polymerase (usually *Taq* polymerase). The amplification reaction consists of three steps; denaturation of double stranded DNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal annealing temperature, and extension of the annealed primers by heat-stable DNA polymerase. The cycle is repeated for 30 - 40 times (Figure 1.4). The amplification product is determined by agarose or polyacrylamide electrophoresis.

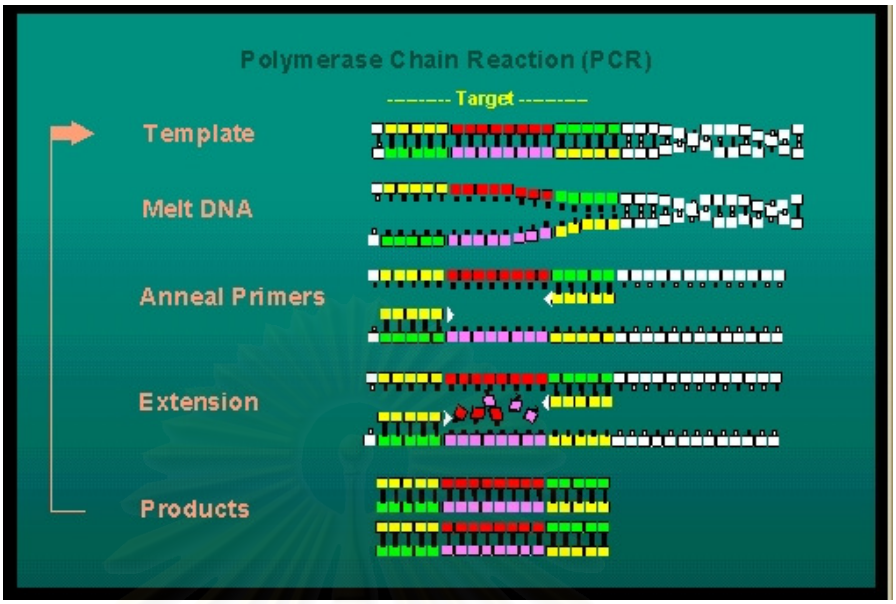

Figure 1.4 General illustration of the polymerase chain reaction (PCR) for amplifying DNA. (http://wheat.pw.usda.gov/~lazo/docs/cotton/)

1.5.2 PCR-SSCP

Single-strand conformational polymorphism (SSCP) analysis was originally described by Orita *et al.* (1989) SSCP is one of the most widely used techniques for the detection of mutations and variation of the DNA (deletions, insertions and single nucleotide polymorphism, SNP). The amplified PCR product (usually less than 400 bp in length) is denatured and loaded into low crosslink non-denaturing polyacrylamide (with or without glycerol supplementation). Base substitutions (transitions and transversions which are commonly called single nucleotide polymorphism, SNP) result in alteration of the folding affecting the migration of single stranded DNA through polyacrylamide gels. Therefore, SNP can be conveniently detected through SSCP. The general procedure of SSCP is illustrated by Figure 1.5.

The major advantage of SSCP is that polymorphism of the PCR product of several investigated individuals can be simultaneously examined. SSCP is relatively simple and does not require expensive equipment. Heteroduplex double strand DNA (located at the middle of the gel) can occasionally be resolved from homoduplex double strand DNA (located the bottom of the gel) and give additional information on the presence of variants resulted from single strand DNA (located at the top of the gel). Therefore, SSCP is regarded as one of the potential techniques that can be used to detect polymorphism in various species prior to confirmation of the results by nucleotide sequencing.

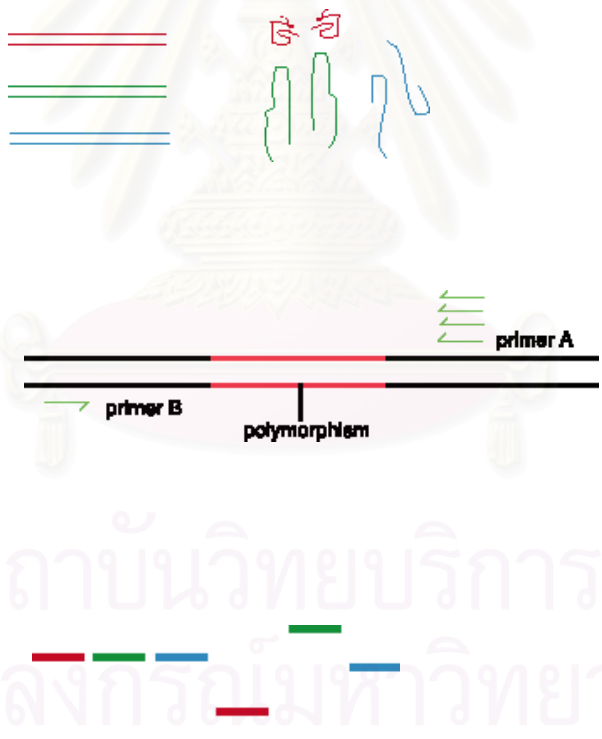

Figure 1.5 A schematic diagram of SSCP analysis for determination of polymorphism of DNA. (www.austmus.gov.au/.../tour/sscp.htm)

The other advantage of SSCP is that small PCR amplicons are required. Generally, small sizes of PCR products are relative easy to amplify. The disadvantage of SSCP is reproducibility of the technique because SSCP patterns are affected by temperature and degree of crosslinking. Additionally, multi-allelic patterns of some nuclear DNA markers may cause the SSCP patterns too complicated for estimation of allele frequencies precisely.

1.5.3 Genome walking analysis

Genome walking analysis is a method for identifying unknown genomic regions flanking a known DNA sequences. Initially, genomic DNA is separately digested with different blunt-end generating restriction endonucleases (e.g. *Hae* III, *Dra* I, *Pvu* II and *Ssp* I). The digested genomic DNA in each tube was then ligated to the adaptor. The ligated product is used as the template for PCR amplification. PCR was carried out with the primer complementary to the adaptor (AP1) and the interesting gene (gene specific primer; GSP). The resulting product is amplified with nested primers (AP2 and nested GSP). The nested PCR products were cloned and characterized (Figure 1.6). This technique allows isolation of the promoter region of interesting genes and 3′ and 5′untranslated regions (UTR) that required further characterization of SNPs at 3′ and 5′UTR.

1.5.4 DNA sequencing

DNA sequencing is the process of determining the exact order of the bases (A, T, C and G) in a piece of DNA. In essence, the DNA is used as a template to generate a set of fragments that differ in length from each other by a single base. The fragments are then separated by size, and the bases at the end are identified, recreating the original sequence of the DNA. There are two general methods for sequencing of DNA segments: the “chemical cleavage” procedure described by Maxam and Gilbert, 1977 and the “chain termination” procedure described by Sanger, 1977. Nevertheless, the latter method is more popular because chemical cleavage procedure requires the use of several hazardous substances. DNA fragments generated from PCR can be directly sequenced or alternatively, those fragments can be cloned and

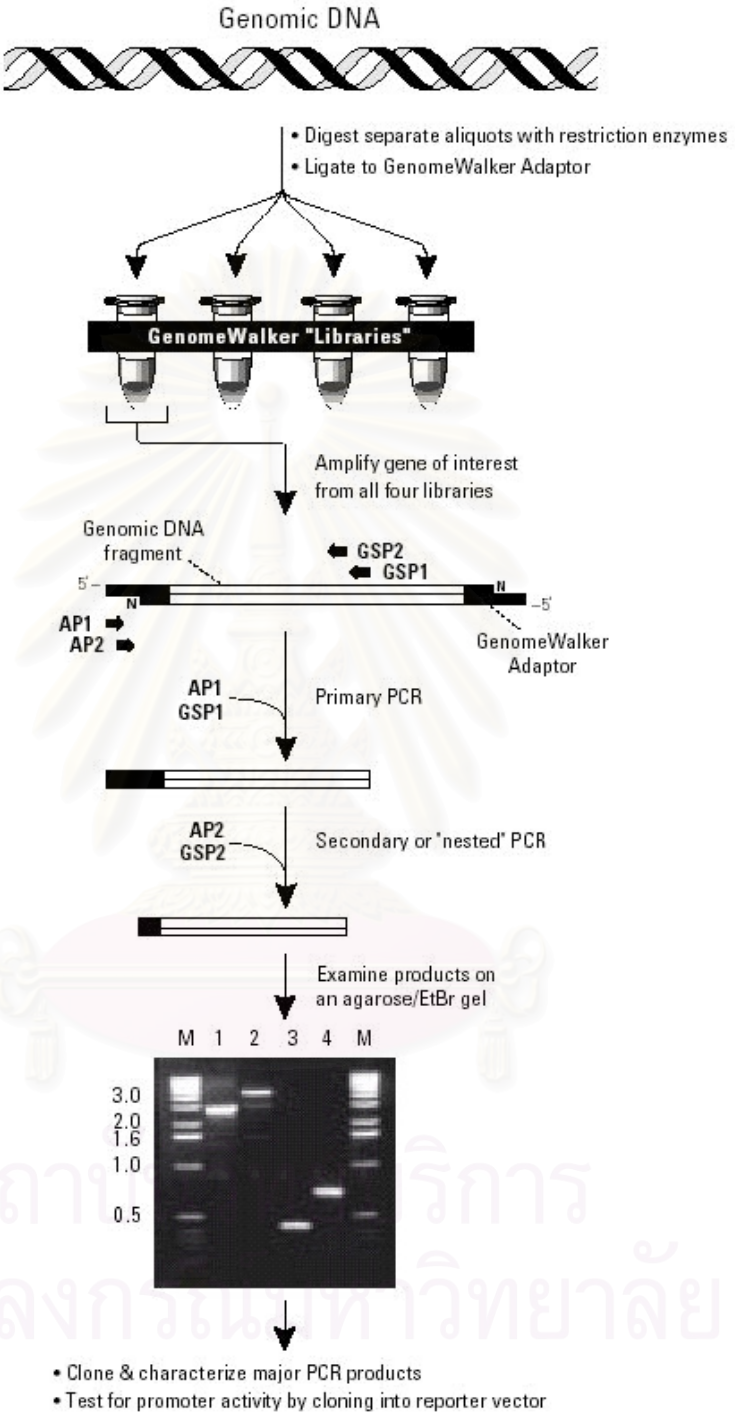

Figure 1.6 A flow chart illustrating the genome walking analysis protocol. (www.bio.mtu.edu/.../tracyneher.htm)

sequenced. This eliminates the need to establish a genome library and searching of a particular gene in the library.

DNA sequencing is the most optimal method for several genetic applications. This technique provides high resolution and facilitating interpretation. DNA fragments generated from PCR can be directly sequenced or alternatively, those fragments can be cloned and sequenced. This eliminates the need to establish a genome library and searching of a particular gene in the library. The enzymatic sequencing approach has presently been developed to automated method (Figure 1.7). DNA sequences can be detected using a fluorescence-based system.

DNA sequencing is particularly suitable for detection of single nucleotide polymorphism (SNP) because the DNA sequence of gene segments from each individual is directly compared.

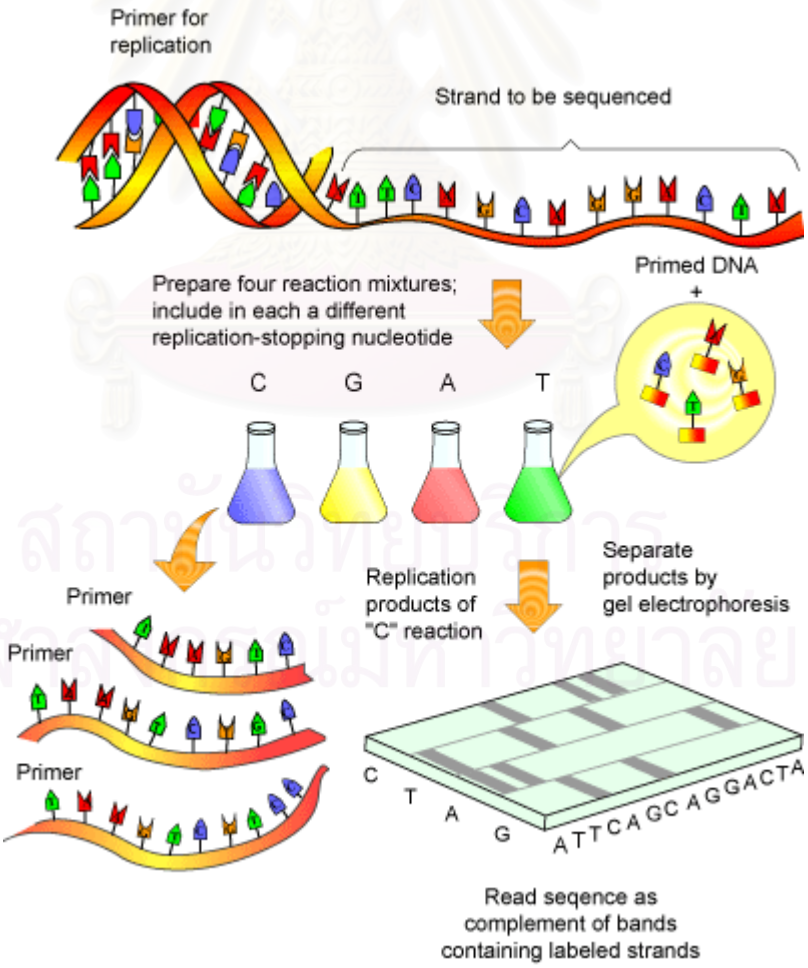

Figure 1.7 A flow chart illustrating DNA sequencing (www.scq.ubc.ca/?p=392)

1.5.5 EST

ESTs are the large-scale single-pass sequences of randomly picked clones from a cDNA library usually constructed from mRNA at a particular developmental stage and/or tissues. This method has been widely employed for discovering novel and uniquely expressed genes, and for characterizing the gene expression profiles of several tissues.

The general principles for construction of a cDNA library (Figure 1.8) begin with purification of the target mRNA that is reverse transcribed to the firststrand cDNA. This step is catalyzed by a reverse transcriptase using in oligo (dT) primer as the synthesizing primer. The secondstrand DNA is then copied from the firststrand cDNA using *E. coli* DNA polymerase I. The doublestrand cDNA is ligated to adapter and subsequently to an appropriate vector using T4 DNA ligase. The recombinant vector-cDNA molecules are packaged (λ vector) *in vitro* and transfected to the appropriate host. If a plasmid is used recombinant plasmid is transformed into *E. coli* host cells to generate a cDNA library.

ESTs can be sequenced from either $5'$ or $3'$ ends of cloned cDNA. However,The $3'$ UTR usually exhibit high polymorphism and is a promising location for SNP identification. The $3'$ end of the insert is usually marked by the poly A stretch which is often problematic for thermostable polymerase sequencing, and sequencing through poly T can reduce the length and quality of the subsequent sequence. The $5'$ ESTs have the advantage of being more likely to include some of the open reading frame (ORF) of the cDNA and thus facilitate identification of the encoded product.

EST sequences are used as the tag to homology search through the sequence data in the GenBank (Altschl *et al.*, 1997). The Blast*N* program uses nucleotide sequence to compare against the NCBI nucleotide database whereas the BLAST*X* uses the translated protein products to compare against the NCBI protein database in all possible 6 reading frames. Sequences are considered to be significantly matched when the possibility value (E-value) is less than 10^{-4} and the match length is > 100 nucleotides for Blast*N* and a match length is > 10 amino acid residues for Blast*X*, respectively (Anderson and Brass, 1998).

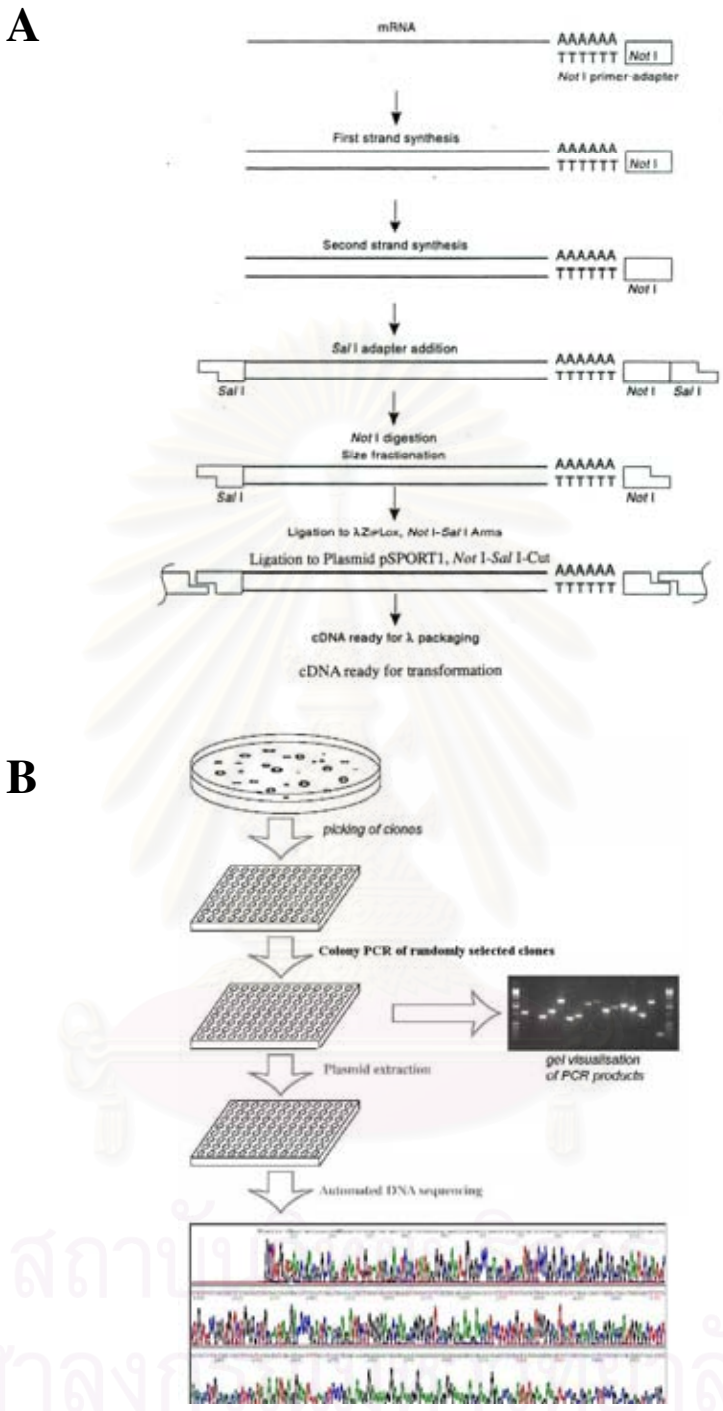

Figure 1.8 Overview for construction of cDNA inserts (A) and automated DNA sequencing (single-pass) of randomly selected cDNA clones (the entire process simply called EST analysis).

EST analysis is an important tool for several applications. They have mainly applied for rapid gene discovery of genes, comparative genomics and functional genomics in various organisms. After characterization and annotation, cDNA or designed oligonucleotides of transcripts can be further used for microarray analysis. Construction of genetic linkage maps and/or physical maps of interesting species can be carried out by development and sequencing of EST-derived markers using genomic DNA of species under investigation (Liu and Cordes, 2004).

1.5.6 Reverse transcription-polymerase chain reaction (RT-PCR) and semiquantitative RT-PCR

RT-PCR is the method that was used to amplify, isolate or identify a known sequence transcripts. This method is a comparable method of conventional PCR but the first strand cDNA rather than genomic DNA was used as the template for amplification. This method contained 2 steps, synthesis of the first strand cDNA from total RNA (or mRNA) using a reverse transcriptase and amplification of the target by PCR for which the second strand cDNA was synthesized by the first cycle of PCR.

Reverse transcription of total RNA can be performed with oligo (dT) or random primer using a reverse transcriptase. The product is then subjected to the typical PCR.

Semi-quantitative RT-PCR is an quantitative approach where the target genes and the internal control (e.g. a housekeeping gene) are separately or simultaneously amplified using the same template. The internal control (such as *β-actin*, *elongation factor EF-1α* or *G3PDH*) is used under the assumption that those coding genes are transcribed constantly and independently from the extracellular environment stimuli and that their transcripts are reverse transcribed with the same efficiency as the product of interesting transcript.

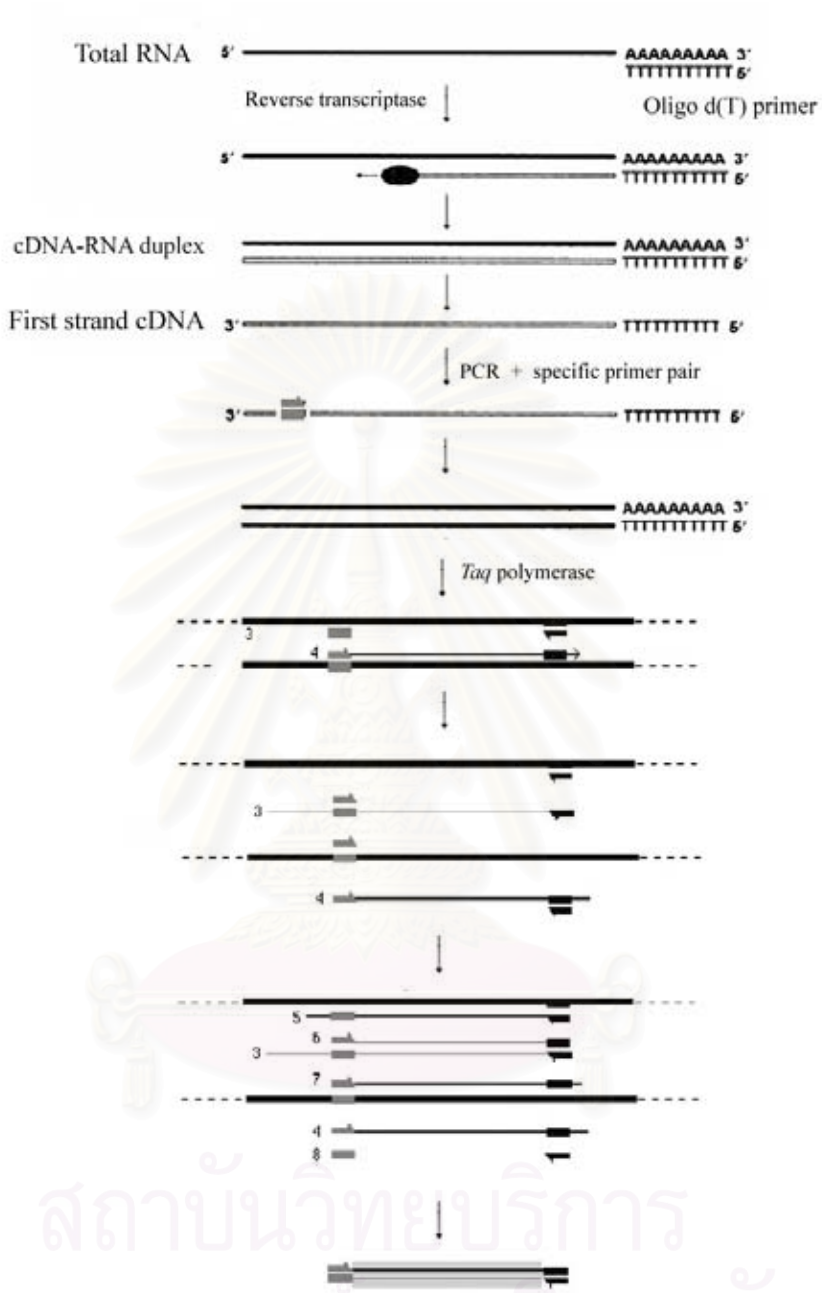

Figure 1.9 Overall concepts of the RT-PCR procedure. During the firststrand cDNA synthesis an oligo d(T) primer anneals and extends from sites present within the total RNA. The second strand cDNA synthesis primed by the 18 – 25 base specific primer proceeds during a single round of DNA synthesis catalyzed by a thermosatable DNA polymerase. These DNA fragments serve as templates for subsequent PCR amplification.

1.5.7 Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR)

RACE-PCR is an approach used for isolation of the full length of characterized cDNA. This method generates cDNA fragments by using PCR to amplify sequences between a single region in the mRNA and either the 3′- or the 5′-end of the transcript. To use RACE-PCR, it is necessary to know or to deduce a single stretch of sequence within the mRNA. From this sequence, specific primers are chosen which are oriented in the 3′ and 5′ directions, and which usually produce overlapping cDNA fragments (Primrose, 1998).

Using SMART (Switching Mechanism At 5′ end of RNA Transcript) technology, terminal transferase activity of Powerscript Reverse Transcriptase (RT) adds 3-5 nucleotides (predominantly dC) to the 3′end of the first strand cDNA. This activity is harnessed by the SMART oligonucleotides whose terminal stretch of dG can anneal to the dC-rich cDNA tail and serve as an extended template for reverse transcriptase. A complete cDNA copy of original mRNA is synthesized with the additional SMART sequence at the end (Figure 1.10).

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

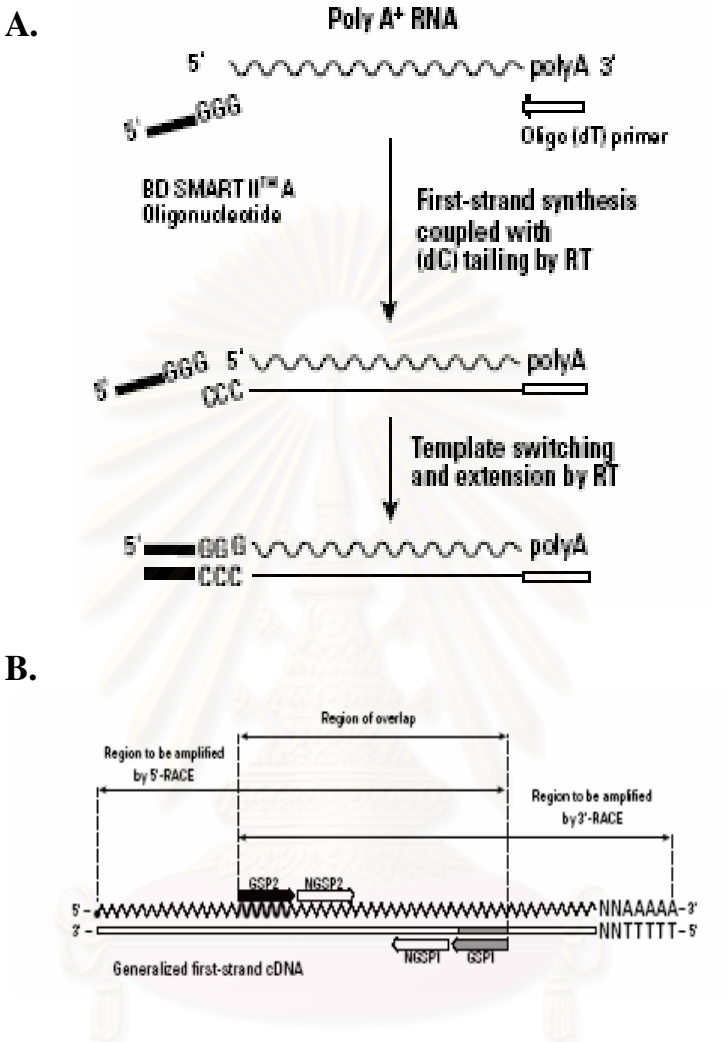

Figure 1.10 Mechanisms of a SMART™ technology cDNA synthesis. The firststrand synthesis is primed using a modified oligo (dT) primer (A). After Powerscript reverse transcriptase reaches the end of the mRNA template, it adds several dC residues. The SMART II A oligonucleotide anneals to the tail of the cDNA and serves as an extended template for PowerScript RT. The relationship of gene-specific primers to the cDNA template in 5′ and 3′ RACE-PCR is illustrated in B. (www.takarabiomed.com.cn/cplook.asp?id=231)

1.5.8 Single nucleotide polymorphism (SNP)

Single nucleotide polymorphism (SNP) is a single base change in the DNA sequence, with a usual alternative of three possible nucleotides at a given position. For such a base position with sequence alternatives in genomic DNA to be considered as an SNP, the lowest frequent allele should have a frequency of 1% or greater. Although in principle, at each position of a sequence stretch, any of the four possible nucleotide bases can be present, SNPs are usually biallelic. One of the reasons for this is that the low frequency of single nucleotide substitutions at the origin of SNPs, estimated to being between 1×10^{-9} and 5×10^{-9} per nucleotide and per year at neutral positions in mammals. Therefore, the probability of two independent base changes occurring at a single position is very low. Another reason is due to a bias in mutations, leading to the prevalence of two SNP types (transitions).

Mutation mechanisms result either in transitions: purine-purine (A ↔ G) or pyrimidine-pyrimidine (C ↔ T) exchanges, or transversions: purine-pyrimidine and *vice versa* (A↔C, A↔T, G↔C, G↔T) exchanges. Theoretically, transversions should be as twice greater than transitions if mutations are random. However, the observed data indicate a clear bias towards transitions. Results obtained from a identification of SNPs from EST sequences gave a transition to transversion ratio of 1.7 in human. Such a ration is greater in EST (2.3 - 4.0) and non-coding DNA (138 SNPs with a ratio of 2.36) in chicken (Vignal and Weigend,). One probable explanation for this bias is the high spontaneous rate of deamination of 5-methyl cytosine (5mC) to thymidine in the CpG dinucleotides, leading to the generation of higher levels of C/T SNPs (or G/A SNPs on the reverse strand). In addition, one base pair indels (insertions or deletions) is also recognized as SNPs even though they are occurred by a different mechanism.

1.5.8.1 Classification of SNP

SNP can be divided to noncoding SNP (or type II SNP) which is composed of that found in 5′ or 3′nontransribed regions, 5′ or 3′ untranslated regions, introns and intergenic spacers and coding SNP (or type I SNP) which is located in exons and

results in non-replacement (synonymous) and replacement (nonsynonymous) of amino acids in the polypeptide chains.

1.5.8.2 SNP identification and characterization

Several molecular techniques may be used to identify and subsequently to characterize SNP under investigation. DNA sequencing is the direct method for SNP identification. Nevertheless, several indirect methods are also commonly used for SNP identification (Table 1.2). This include restriction fragment length polymorphism (RFLP), SSCP and Denaturing gradient gel electrophoresis (DGGE), primer extension, oligonucleotide ligation assay (OLA), *TaqMan* exonuclease detection, PCR-allele specific amplification (PASA) and bidirectional PCR-allele specific amplification (Bi-PASA) and mass spectroscopy (MS).

Table 1.2 Comparisons of selected mutation screening methods (Shastry, 2002)

Method	Fragment length (bp)	Advantage	Disadvantage	Efficiency (%)
Single strand conformational polymorphism	~300	No expensive equipment	Small fragments. Temperature variation	80
Heteroduplex analysis	300-600	No expensive equipment,	Conditions to be determined	80
Denaturing gradient gel electrophoresis	100-1000	Simple, long and short fragments	Gradient gel required, mutation in GC region may not be detected	100 with GC clamp
Enzymatic mismatch detection	300-1000	Long and short fragments	Identifies all kinds of mutations	100
Base excision sequence scanning	50-1000	Accurate	Expensive instruments	100
RNAase cleavage	1.6 kb	Longer fragment and rapid analysis	Requires special kit	100
Chemical cleavage	1-2 kb	Large fragment	Multi-steps, labor intensive and hazardous chemicals.	100
DNA sequencing	500	Rapid and easy, no additional sequencing	Labor intensives	100

1.6 SNP studies in shrimp

SNP has been widely used in several applications including evolution and population genetic studies, gene discovery, pharmacology and construction of genetic linkage maps and linkage disequilibrium mapping for identification of quantitative trait loci (QTL). Nevertheless, research on association analysis of SNP and gene expression is rather limited in aquatic species.

Association between genotype (non-synonymous SNP) and phenotype (disorders) were found in both non-coding and coding regions. The mutated SNP influence promoter activity or DNA and pre-mRNA conformation, and play a direct or indirect role in phenotypic expression. Studies about correlation between SNP and diseases have been extensively carried out in human (Immervoll *et al.*, 2001**,** Colomb *et al.*, 2001, Tan *et al.*, 2001). Nevertheless, SNP studies in shrimp are limited and still at the initial stage.

Tong *et al*. (2002) developed type I markers for genome mapping and other applications. Primers were designed from ESTs established from the cephalothorax of *P. monodon*. Thirty-four primer pairs successfully generated PCR products from genomic DNA of *P. monodon*. SSCP analysis indicated that approximately 30% of the EST tested were polymorphic in investigated shrimps and exhibited Mendelian segregation patterns. Some ESTs were also cross-species amplified in other shrimps (*P. chinensis*, *P. japonicus* and *P. vannamei*) allowing the possibility to be used for comparative mapping between related species. SSCP analysis of CU89 primer also revealed 2 distinct genotypes in investigated *P. japonicus* individuals from Australian (100% of genotype A, $N = 5$) and the South China Sea (80% and 20% of genotypes B and A, $N = 5$).

Glenn *et al.* (2005) studied association analysis of SNP of *alpha-amylase* (*AMY2*) and *cathepsin-L* (*CTSL*) and the body weight in 2 populations of *Litopenaeus vannalei* (LV1 and LV2, $N = 75$ and 30 with the mean BW of 0.35 ± 0.06 and 2.52 ± 0.30 g, respectively) and a mapping population of *P. monodon* ($N = 41$) of investigated shrimps. SNP genotypes were carried out using PCR-RFLP of *AMY2* with *Sca I* and *CTSL* with *Pvu* II. Neither polymorphism of *AMY2* and *CTSL* were not found to be significantly associated with BW of LV1 and LV2 populations.

SNP by EST (SBE) were developed by amplification of 102 previously identified ESTs in ovaries and hemocytes of *P. monodon*. A total of 48 successfully amplified genes were further characterized by SSCP analysis and 44 of which were polymorphic. The full length of *ribophorin I* and *receptor for activated protein kinase C* (*RACK*) were successfully characterized by RACE-PCR. Semi-quantitative RT-PCR of *ribophorin I* and *RACK* were carried out. Significantly different expression levels of *ribophorin I* ($P < 0.05$) but not in *RACK* ($P > 0.05$) were observed in ovaries and testes of *P. monodon* broodstock. Although the preliminary study did not reveal significant association between SNP (SSCP patterns) and levels of *ribophorin I* and *RACK* expression in the limited sample size of *P. monodon* broodstock, this approach demonstrates the possibility to further test for association between SNP of candidate genes allied with growth and reproduction with related phenotypes (degrees of gene expression by the wild type/mutant alleles, growth rate and reproductive performance) of *P. monodon* (Buaklin, 2004).

1.7 Molecular studies on growth and reproduction of organisms

Genetic recombination plays a critical role in maintaining gene diversification through chromosomal rearrangement and also genome stability through the repair of DNA damage. The activities of many proteins are required for recombination. *RUVB-like protein*, also called a *TATA-binding protein* (*TBP*) is a central component for transcriptional regulation, forms complexes with various transcription regulators (Makino *et al.*, 1998).

Lim *et al.* (2000) isolated and characterized two highly conserved *RUVB*-like putative DNA helicases, p47/TIP49b and p50/TIP49a, in the eukaryotes. Tih2p is required for vegetative cell growth and localizes in the nucleus. Immunoprecipitation analysis revealed that Tih2p tightly interacts with Tih1p, the counterpart of mammalian p50/TIP49a, which has been shown to interact with the TATA-binding protein and the RNA polymerase II holoenzyme complex. Furthermore, a motif, which is required for nucleotide binding and hydrolysis, showed that this motif plays indispensable roles in the function of Tih2p. When a temperature-sensitive *tih2* mutant, *tih2–160*, was incubated at the nonpermissive temperature, cells were rapidly arrested in the G1 phase. Northern blot analysis revealed that Tih2p is required for transcription of G1 cyclin and of several ribosomal protein genes. The similarities

between the mutant phenotypes of *tih2–160* and those of *taf145* mutants suggest a role for *TIH2* in the regulation of RNA polymerase II-directed transcription.

The small GTPase p21Ras (or Ras) performs a central function during many cellular responses, including proliferation and differentiation. In response to signals from receptor tyrosine kinases (RTKs), Ras is activated by guanine nucleotide exchange factors (GEFs), which convert the inactive GDP-bound form to the active GTP-bound form. Activated Ras stimulates downstream effectors such as the serine/threonine kinase Raf, which is the first in a cascade of kinases, the MAPK cascade, conveying the signal to the nucleus. Ras is inactivated by GTPase activating proteins (GAPs), which stimulate conversion to the inactive GDP-bound form (McCormick, 1989). Ras is required for the differentiation of all *Drosophila* photoreceptors (Simon *et al.*, 1991) and is activated by the RTKs EGF-receptor (Egfr) and Sevenless (Sev), via the adaptor Drk and the GEF Sos. Ras then recruits and activates Raf, which initiates the MAPK cascade via Dsor1 (MEK) and the MAPK/ERK (reviewed in Zipursky and Rubin, 1994).

The *Drosophila* homologue of the RasGAP-binding protein G3BP encoded by rasputin (rin). The rin mutants which are viable and display defects in photoreceptor recruitment and ommatidial polarity in the eye were identified and characterized. Mutations in rin/G3BP genetically interact with components of the Ras signaling pathway that function at the level of Ras and above, but not with Raf/MAPK pathway components. These interactions suggest that Rin is required as an effector in Ras signaling during eye development, supporting an effector role for RasGAP (Pazman *et al.*, 2000).

The primary catabolic pathway of prostaglandins and related eicosanoids is initiated by the oxidation of 15(S)-hydroxyl group catalyzed by NAD+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) followed by the reduction of delta13 double bond catalyzed by NADPH/NADH dependent delta13-15-ketoprostaglandin reductase (13-PGR). 13-PGR was also found to exhibit NADP+-dependent leukotriene B4 12-hydroxydehydrogenase (12-LTB4DH) activity. These enzymes are considered to be the key enzyme responsible for biological inactivation of prostaglandins and related eicosanoids. Recent biochemical and molecular

biological studies have revealed interesting catalytic properties, structure, and activity relationship, and regulation of gene expression of these enzymes (Tai *et al.*, 2002).

The estrogen receptor alpha (ER-α) play an important role for reproduction of organisms. In addition, PGR has been well reported as one of the ER-α-induced genes. Recently, additional genes promoting the expression of ER-α in human breast cancer were identified by cDNA microarrays and real-time RT-PCR. This included *GATA3*, *TFF3*, *MYB*, *STC2*, *HPN/HEPSIN*, *FOXA1*, *XBP1*, *SLC39A6/LIV-1*, etc. Some of the genes identified could be useful for diagnosis or for predicting endocrine responsiveness, and could form the basis for novel therapeutic strategies (Tozlu *et al.*, 2006).

Souid *et al.*(2007) characterized *Dxbp-1*, the *Drosophila* homologue of the xpb-1 gene that encodes a "bZIP"-containing transcription factor, that plays a key role in the unfolded protein response (UPR). *Dxbp-1* is ubiquitously transcribed, and high levels are found in embryonic salivary glands and in the ovarian follicle cells committed to the synthesis of the respiratory appendages. Loss of function of *Dxbp-1* induced a recessive larval lethality, thus, revealing an essential requirement for this gene. The *Dxbp-1* transcript was submitted to an "unconventional" splicing that generated a processed *Dxbp-1* transcript encoding a DXbp-1 protein isoform, as is the case for yeast, *Caenorhabditis elegans* and vertebrate *hac1/xbp-1* transcripts after UPR activation. However, in the absence of exogenously induced ER stress, the *Dxbp-1s* transcript was also detectable not only throughout embryonic and larval development but also in adults with a high level of accumulation in the male sexual apparatus and, to a lesser extent, in the salivary glands of the third-instar larvae. Using a *Dxbp1GFP* transgene as an *in vivo* reporter for *Dxbp-1* mRNA unconventional splicing, it was confirmed that *Dxbp-1* processing took place in the salivary glands of the third-instar larvae. The *Dxbp-1* gene appears, thus, to play an essential role during the development of *Drosophila*, hypothetically by stimulating the folding capacities of the ER proteins in cells committed to intense secretory activities.

The fundamental controls of growth in penaeid shrimps are largely unstudied. Many peptides related to vertebrate growth factors have been isolated from crustaceans. Vertebrate-like growth factor peptides have been reported in *L.*

vannamei, *L. stylirostris* and *Fenneropenaeus indicus* and their levels shown to change with developmental stage (Toullec *et al.*, 1991).

It has been reported that growth retardation may be resulted from viral infection, inappropriate management and genetics of the cultured shrimp. In Thailand, the problem of unusual, widespread growth retardation in cultured *P. monodon* was first observed in 2001 and called monodon slow growth syndrome (MSGS). Disregarding effects from pathogens, good management practices on the high quality shrimp would promote the growth rate in shrimp.

Kenway *et al.* (2006) identifying genetic correlations between growth and survival rates in *P. monodon*. Shrimp were reared in captivity in tanks over three generations with full pedigree information. Weights of animals were measured at six ages between 7 and 54 weeks along with survival in each period. Females were more variable in weight than males after week 16, and variances between each sex were standardized prior to estimation of heritability and genetic correlations. The phenotypic mean ± standard deviation of weights at week 40 was 35 ± 6 g for males and 44 ± 10 g for females. Heritability with standard errors at 16, 30, 40 and 54 weeks was 0.56 ± 0.04, 0.55 ± 0.07, 0.45 ± 0.11 and 0.53 ± 0.14 respectively. Heritability for family survival, determined from mean survival within each family, was 0.51 ± 0.18, 0.36 ± 0.18 and 0.71 ± 0.17 over periods 4 to 10, 10 to 16 and 16 to 32 weeks respectively. The genetic correlations between weight and survival revealed no significant trend. The results indicated significant concurrent improvements in both growth and survival are possible through selective breeding.

Biogenic amines (e.g serotonin or 5-HT, epinephrine and dopamine) and peptide neuroregulators are known to modulate the release of neuropeptide hormones from the sinus gland. Injections of serotonin and dopamine antagonist, spiperone (25 $\mu g\ g^{-1}$ body weight + 1.5 or 5 $\mu g\ g^{-1}$ body weight) induced ovarian maturation and spawning in wild *L. stylirostris* and pond reared *L. vannamei* (Alfaro *et al.*, 2004).

Meeratana *et al.* (2006) investigated effects of serotonin on ovarian development in *M. rosenbergii*. Adult female prawns at the ovarian stage I (spent) were injected with 5-HT at 1, 5, 10, 20 and 50 $\mu g\ g^{-1}$ body weight (BW) intramuscularly on days 0, 5 and 10, and sacrificed on day 15. The low-dose,

especially at 1 μg g^{-1} body weight caused prawns to exhibit a significant increase in gonadosomatic index (5.79 ± 0.09%) as compared to the control (1.49%). The ovaries of most of these prawns could develop to stage IV (mature) and contained synchronously mature oocytes while most of the control ovaries remained at stages I and II. The medium- and high-dose treated prawns exhibited ovaries that could reach stages III and IV and contained various types of oocytes of different maturity. Pretreatment with 5-HT receptor antagonist, cyproheptadine (CYP), at 10 μg g^{-1} body weight before 5-HT injection significantly suppressed the effect of 5-HT. Intramuscular injection of the culture medium of thoracic ganglion preincubated with 1 μg ml^{-1} of 5-HT into CYP-pretreated prawns resulted in the increase of ovarian index about 5-6 times more than in the control. The ovaries of most prawn could develop up to stage IV and contained synchronously developed vitellogenic and mature oocytes.

Effects of exogenous 5-HT on the reproductive performance of *P. monodon* was examined. 5-HT solution was injected into domesticated *P. monodon* broodstock at 50 μg g^{-1} body weight and ovarian maturation and spawning were recorded. The levels of 5-HT in ovaries were measured by ELISA. The 5-HT-injected *P. monodon* developed ovarian maturation and spawning rate at the level comparable to that of unilateral eyestalk-ablated shrimp. (Wongpraset *et al.*, 2006).

Recently, Preechaphol *et al.*, (2007) isolated and characterized genes in ovaries of *P. monodon*. A total of 1051 clones were unidirectionally sequenced from the 5′ terminus. Nucleotide sequences of 758 ESTs (71.7%) significantly matched known genes previously deposited in the GenBank (E-value < 10^{-4}) whereas 308 ESTs (29.3%) were regarded as newly unidentified transcripts (E-value > 10^{-4}). A total of 559 transcripts (87 contigs and 472 singletons) were obtained. *Thrombospondin* (*TSP*) and *peritrophin* (79 and 87 clones accounting for 7.5 and 8.3% of clones sequenced, respectively) predominated among characterized transcripts. Expression patterns of 14 of 25 sex-related gene homologues in ovaries and testes of *P. monodon* broodstock were examined by RT-PCR. Several transcripts except *disulfide isomerase related P5 precursor* and *adenine nucleotide translocator 2* were higher expressed in ovaries than testes of *P. monodon* broodstock. A homologue of *ubiquitin specific preteinase 9, X chromosome* (*USP9X*) revealed a

preferential expression level in ovaries than testes of broodstock-sized *P. monodon* (*N* = 13 and 11, $P < 0.05$) but was only expressed in ovaries of 4-month-old shrimp (*N* = 5 for each sex).

Several genes encoding vertebrate-like growth factors and cell cycle regulation (*cyclin*, *cyclin dependent kinase*, *cell division cycle 2* and *epithelial growth factor 1*) were recently identified and may be used for further association analysis between genotyes (SNP) and phenotype (e.g growth and survival rates) of *P. monodon* (Preechaphol *et al.*, 2007).

1.8 Objectives of this thesis

The objective of this thesis is identification of SNP in functionally important genes of the giant tiger shrimp (*Penaeus monodon)* by SSCP and to examine correlations between genotype frequencies of SSCP patterns of functionally important genes and an age-specific growth rate of domesticated (*RUVB-like protein*, *NADP-dependent leukotriene-12-hydroxydehydrogenase* and *x-box binding protein*) and commercially cultured (*Ruvb-like protein*) *P. monodon*. In addition, effects of serotonin (5-HT) administration on expression of these genes were examined.

CHAPTER II

MATERIALS AND METHODS

2.1 Experimental animals

Broodstock-sized *P. monodon* was collected from Chumphon and Trat located in the Gulf of Thailand and Satun, Trang and Phangnga located in the Andaman Sea. (N = 3 for each geographic samples). These specimens were used for screening of genes illustrating polymorphic SSCP patterns. Specimens used for RACE-PCR were broodstock-sized *P. monodon* caught from chonburi.

Moreover, five hundred individuals of the 2nd generation of a domesticated family of *P. monodon* (132 days of age, $\overline{X}$ = 18.8609 ± 4.64 g) were kindly supplied by Prof. Dr. Boonsirm Withyachamnarnkul, Mahidol University. Ten percent of five hundred shrimp exhibiting from the top (N = 50, $\overline{X}$ = 26.86 ± 3.33 g) and the bottom (N = 50, $\overline{X}$ = 10.71 ± 2.02 g) according to the body weight were selected and used for preliminary analysis of correlation between SSCP genotypes (or SNP) of *X-box binding protein*, *RUVB-like protein* and *LTB4DH* and the growth rate of the shrimp. After the preliminary screening, polymorphism of *RUVB* was tested against a new sample set of juvenile *P. monodon* (N = 359, $\overline{X}$ = 17.39 ± 4.36 g) collected from a commercial farm in Chonburi.

Juvenile shrimp used for RT-PCR analysis were also purchased from a commercial farm. Tissues of each shrimp (pleopods, testes and ovaries) were dissected and kept at -80°C until required.

2.2 Nucleic acid extraction

2.2.1 Genomic DNA extraction

2.2.1.1 A phenol-chloroform-proteinase K method (Klinbunga et al., 1999)

A piece of pleopod tissue was dissected out and placed in a prechilled microcentrifuge tube containing 500 µl of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl; pH 8.0) and briefly homogenized with a micropestle. SDS (10%) and RNase A (10 mg/ml) solutions were added to a final concentration of 1.0 % (w/v) and 100 µg/ml, respectively. The resulting mixture was then incubated at 37^{o}C for 1 hour. At the end of the incubation period, a proteinase K solution (10 mg/ml) was added to the final concentration of 300 µg/ml and further incubated at 55 ^{o}C for 3 – 4 hours. An equal volume of buffer-equilibrated phenol was added and gently mixed for 10 min. The solution was centrifuged at 8000 *g* for 10 min at room temperature. The upper aqueous phase was transferred to a newly sterile microcentrifuge tube. This extraction process was then repeated once with phenol and phenol:chloroform:isoamyl alcohol (P:C:I, 25:24:1) and twice with chloroform:isoamyl alcohol (C:I, 24:1). The aqueous phase was transferred into a sterile microcentrifuge. One-tenth volume of 3 M sodium acetate, pH 5.2 was added. DNA was precipitated by an addition of two volume of prechilled absolute ethanol and mixed thoroughly. The mixture was incubated at -80^{o}C for 30 minutes. The precipitated DNA was recovered by centrifugation at 12,000 *g* for 10 minutes at room temperature and washed twice with 1 ml of 70% ethanol (5 min each). After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 50 – 80 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The DNA solution was incubated at 37 ^{o}C for 1 – 2 hours and kept at 4 ^{o}C until further needed.

2.2.1.2 A Chelex®-based method

A piece of the muscle tissue was homogenized in 200 µl of a 5% Chelex® (w/v). The homogenate was incubated at 60 °C for 3 hr. To stop the DNAse activity, the homogenate was placed in the boiling water for 10 min, left to room temperature and briefly centrifuged at 14,000 rpm for 2 - 3 min. The supernatant was transferred to a new tube and kept at 4 °C. This DNA solution was used as the template for PCR.

2.2.2 RNA extraction

Total RNA was extracted from various tissues (ovaries and testes) of *P. monodon* using TRI REAGENT® (Molecular Research Center). A piece of tissue was

immediately placed in a mortar containing liquid nitrogen and ground to the fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 µl of TRI REAGENT® (50 - 100 mg tissue per 1 ml) and homogenized. Additional 500 µl of TRI REAGENT® was then added. The homogenate was left for 5 minutes at room temperature before adding 0.2 ml of chloroform. The homogenate was vortexed for at least 15s and left at room temperature for 2 - 15 min and centrifuged at 12000 *g* for 15 min at 4 ^{o}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. Total RNA was precipitated by an addition of 0.5 ml pf isopropanol and mixed thoroughly. The mixture were left at room temperature for 10 - 15 min and centrifuged at 12000 *g* for 10 min at 4 ^{o}C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75 % ethanol centrifuged at 7500g for 5 min. Total RNA was dissolved in appropriate volume of DEPC-treated H_2O for immediately used. Alternatively, the total RNA pellet was kept under absolute ethanol in a -80 ^{o}C freezer for the long storage.

2.3 Measuring concentrations of nucleic acids

2.3.1 Estimation of DNA and RNA concentration by spectrophotometry

The concentration of extracted DNA or RNA was estimated by measuring the optical density at 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml double stranded DNA, 40 µg/ml single stranded RNA and 33 µg/ml oligonucleotide (Sambrook and Russell, 2001). Therefore, the concentration of DNA/RNA samples (µg/ml) were estimated by multiplying an OD_{260} value with a dilution factor and 50, 40, 33 for DNA, RNA and oligonucleotides, respectively.

The purity of DNA samples can be guided by a ratio of OD_{260} /OD_{280}. For the extracted DNA, the ratio much lower than 1.8 indicated contamination of residual proteins or organic solvents whereas the ratio greater than this value indicate contamination of RNA in the DNA solution (Kirby, 1992). The ratio of approximately 2.0 indicates the good quality of the extracted RNA.

2.3.2 Estimation of the amount of DNA by mini-gel electrophoresis

The amount of high molecular weight DNA can be roughly estimated on the basis of the direct relationship between the amount of DNA and the level of fluorescence after ethidium bromide staining using agarose gel electrophoresis. Genomic DNA was run in a 0.8 - 1.0% agarose gel prepared in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.0 mM EDTA, pH 8.3) at 4 V/cm. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml). DNA concentration was estimated from the intensity of the fluorescent band by comparing with that of undigested λDNA.

2.4 Identification of SNP in genomic DNA of *P. monodon* using SSCP analysis

2.4.1 Primer design

PCR primers were designed from EST sequences of homologues of known transcripts from hemocyte, ovarian and testes cDNA libraries of *P. monodon* (Table 2.1) using Primer Premier 5.0. The criteria for primer designing were the primer length of 18 - 25 bases, the melting temperature of 55 – 70 °C, and random base distribution of the primers to avoid polypurine and polypyrimidine tracts, less than 5 °C differences of the melting temperature of a primer pair.

2.4.2 PCR

Generally, PCR was carried out in a 25 μl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 μM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 0.1 - 0.4 μM of each primer, 25 ng of genomic DNA of *P. monodon* and 1.0 unit of DyNAzyme™ II DNA Polymerase (Finnzymes). The amplification profiles were initially carried out by predenaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 45s, annealing at 53 °C for 1 mim and extension at 72 °C for 2 min. The final extension was carried out at 72 °C for 7 min. Subsequently, more appropriate amplification conditions were further adjusted as, for example, described in Table 2.2.

2.4.3 Agarose gel electrophoresis (Sambrook and Russell, 2001)

Appropriate amount of agarose was weighed out and mixed with 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.0 mM EDTA, pH 8.3). The gel slurry was heated until complete solubilization in the microwave. The gel solution was left at room temperature to approximately 55°C before poured into a gel mould.

Table 2.1 Gene homologues, primer sequences, length and the melting temperature of primers designed from EST of *P. monodon*

Gene	Sequence	Length	Tm
1. *Y-box protein**	F: 5′-CGGAGACACAAGCCAAGCCT-3′	20	64
	R: 5′-GGTGGAACCCAACCAGCAAC-3′	20	64
2. *Hepatocarcinogenesis-related transcription factor* (*X-box binding protein*)*	F: 5′-TGATGAACTTCGGGACCTAA-3′	20	58
	R: 5′-CCTCAACGACAACTGCTGCG-3′	20	64
3. *Adipose differentiation related protein**	F:5′-TCTTGCTCTTGCCTGTGCTT-3′	20	60
	R:5′-CCGTTGGCTTGGTTATGATG -3′	20	60
4. *ESO3 protein***	F:5′-GTAACAAATGCCATGGGTGAAA-3′	22	62
	R:5′-GCTGTACTGGAGGTCCAAACTG-3′	22	68
5. *Exocyst complex component Sec6***	F:5′-GAACATCATTCACAAGACAGCC-3′	22	64
	R:5′-AAGTTAGGGTCCAACATTACGG -3′	22	64
6. *Rasputin **	F:5′-GGAGGGAGGAAGTGAAGTGG-3′	20	64
	R:5′-TTGGAGAAAGAGGGGCAGTG-3′	20	62
7. *Survival motor neuron**	F:5′ -TAACGATAAGGAACTCACCCAT-3′	22	62
	R:5′ -CATCACCTGTGTGCCACTCAAT-3′	22	66
8. *Small nuclear ribonucleoprotein D2 – like protein***	F:5′ -CCGAAATCTCAGCCAGCGTCA- 3′	21	66
	R:5′ -TGCCTTTGCTTTCTTCTGTCCC- 3′	22	66
9. *Phosphatidylinositol-4 kinase**	F:5′-CAACGCCATCAACTCCATCAC- 3′	21	64
	R:5 -CTTCCAGCACCACACAGTTTTAT- 3′	23	66
10. *TATA –binding protein(TBP) -associated factor9***	F:5′-TATGGGCATCACGGACTACGAG-3′	22	68
	R:5′-GCAGGCAGAGAGGCAATAACGA-3′	22	68
11. *Proactivator polypeptide precursor (Prosaposin)***	F:5′ -GCCATAAAGTTCTGCCCCCACC-3′	22	70
	R:5′ -GCCCTCCCAATATCTACATCCA-3′	22	66
12. *Hypothetical protein XP* (*cyclic nucleotide gated channel beta subunit 1*)*	F:5′ -TCTCCCTTCTATGCCTGTGTCC-3′	22	68
	R:5′ -GCTCGCTTCAACCAAACTGC-3′	20	62
13. *Carboxylesterase precursor***	F:5′ -GCAAGACAACATCCGTGACCTC-3′	22	68
	R:5′ -GGGACATTCCTCAAACAAGCGA-3′	22	66
14. *O-methyltransferase***	F:5′ -AGCACCGTAGAGCGGCGATGTT-3′	22	70
	R:5′ -CGAAGGCGATGACTCCACCAGA-3′	22	70
15. *Rab-protein 10*	F:5′- CTATTACAGAGGGGCAATGGGC-3′	22	68
	R:5′ -TTTTCTTTGGCAATGACACGCT-3′	22	62
16. *NADP-dependent leukotriene B4 12-hydroxydehydrogenase **	F: 5′-AGAGGGGATGCCCAAGAAGG-3′	20	64
	R: 5′-AGCCAGACGGTGAAACAACG-3′	20	62
17. *Phosphoglucose isomerase***	F: 5′-TTCTGGGACTGGGTTGGTGG-3′	20	64
	R: 5′-TGGCAATAAGGCATGGGTTT-3′	20	58

Table 2.1 (cont.)

Gene	Sequence	Length	Tm
18. *PEF- protein with a long N-terminal hydrophobic domain (peflin)***	F: 5′-CAAGGGTATGGGCATCCACAGG -3′	22	70
	R: 5′-GGTCCACAGCACGGAACCAAGAGA-3′	24	76
19. *Adenine nucleotide translocator 2**	F: 5′-GTCCGCATCCCAAAGGAACGAG -3′	22	70
	R: 5′-CGAGCGAAGTCAAGGGGGTAGA-3′	22	70
20. *TRAP-like protein precursor***	F: 5′-CCAATGGTGCCAAGGATGTT-3′	20	60
	R: 5′-TAGTAGATGAGGATAGCGAAGATG-3′	24	68
21. *Hyaluronan receptor**	F: 5′-TGTGCTCCTCCTCCAACAAAAT-3′	22	64
	R: 5′-CGCCTCCTGACACTGATGAACT-3′	22	68
22. *Keratinocyte associated protein2**	F: 5′ -AGGGAAGGGGAGGAGGACCAGT-3′	22	72
	R: 5′ -TAGGACGAGACACAGGGCGACC-3′	22	72
23. *Heterogeneous nuclear ribonucleo protein 87F***	F: 5′-TTTTCCTACAATGGAGGCAAGTGGCT-3′	26	76
	R: 5′-AGGCTACAACAACCCTGGTCCTAACA-3′	26	78
24. *Laminin beta chain **	F: 5′ -TGCTAAACCCCAGGAAATCAGG- 3′	22	66
	R: 5′ -ACAACACGCTTTGCTACACCCA- 3′	22	66
25. *Ras interacting protein RIPA**	F: 5′ -GTGAGAGTGAGGGGGAAGAGGGTAA-3′	25	78
	R: 5′ -GGGAGGTTTGGTTTTTGGGGG-3′	21	66
26. *U5 small nuclear ribonucleoprotein***	F: 5′-GCGAGAAAGTGCCAGAACAGATGACG-3′	26	80
	R: 5′-CAGGAGAGGAATTAGGAAGGCCAAAG-3′	26	78
27. *Stromal membrane associated protein***	F: 5′-CTTTCTTACCTGACGCTGGATGCTGA-3′	26	78
	R: 5′-TCGGTGTCCACAAGAGCGGAAGCAGC-3′	26	84
28. *Tissue specific transplantation antigen p35B like**	F:5′-ATGGTTTTTGGGCTCATTAGTG-3′	22	62
	R:5′-ATTGAATCGTAGGAAATCGCAG-3′	22	62
29. *RUVB-like protein 2**	F: 5′-TGGCAGGTCAGGCAGTTCTCAT-3′	22	68
	R: 5′-CCAGTGGCAGGACGGTCAATCT-3′	22	70
30. *Phosphopyruvate hydratase***	F: 5′-GGAGAACTGGACCAAGATGACC-3′	22	68
	R: 5′-CTCACCAGACCTATGGGAAACC-3′	22	68
31. *Microspherule protein1***	F: 5′-GGAGGACCAGGTTTCTAAGTGGCAAG-3′	26	80
	R: 5′-ATTATTCATTATCCATTTCCATTGTG-3′	26	66
32. *Solute carrier family 25, member 14 isoform UCP5S **	F: 5′-CCAGATTGTTGCCAAGCGATAC-3′	22	66
	R: 5′-GAACGCCCACTCTGTAACCTGA-3′	22	68
33. *CG1681-PA**	F: 5′-ATCAAAGCCATTCATTGCGAGC-3′	22	64
	R: 5′-AACCAGACAAAAATAAAACCAAAT-3′	24	60
34. *Carbonyl reductase1****	F: 5′-GCTTCGCTCCTCGTTTTCATCA-3′	22	66
	R: 5′-ATACGCCAACTTTGCTCTACCAC-3′	23	68
35. *Contractile ring component anillin****	F: 5′-TGTTTGAGGATGTTGGGGGCT-3′	21	64
	R: 5′-AACTGGAAGGTATGCTGACGGG-3′	22	68
36. *NADP-ferrihemoprotein reductase; NADPH cytochroome P450 reductase**	F: 5′-CCACCCTCAGTCTGGCAAATCT-3′	22	68
	R: 5′-GCTTTATTTAGCCTTCAGCCGAGA-3′	24	70
37. *Dynactin 4****	F: 5′-AAGTTATCGTGCTTAGTGGTGTG-3′	21	66
	R: 5′-TCTCGGTGAGGCAAGACTGTTT-3′	21	66
38. *Cystathionine gammalyase****	F: 5′-CCCAGCAGATTTTAAGGCATTTGA-3′	24	68
	R: 5′-GTGCGTGATGGTGGTTGTCG-3′	20	64

Table 2.1 (cont.)

Gene	Sequence	Length	Tm
39. *Splicing factor 3a,subunit 1***	F: 5′-AGTTGAAAGAACGAGGTGGACG-3′	22	66
	R: 5′-TACCCAATACACGCAAATAACC-3′	22	62
40. *Phosphatidylserine receptor short form **	F: 5′-CAAGTGTGGAGAAGATAATGAAGG-3′	24	68
	R: 5′-CCAGCGGTAAGGGGGGCGTC-3′	20	70
41. *BCS-2****	F: 5′-TGAAGTGTAAAGTGCTGTGGGG-3′	22	66
	R: 5′-TGGGCGGTGAACTCCGTGGT-3′	20	66
42. *Proteasome (prosome, macropain)26S subunit, ATPase2****	F: 5′-AACTCTCCAGAATGAGCAGCCA-3′	22	66
	R: 5′-TACTTATTACGATGCCACACCCAC-3′	24	70
43. *USO1****	F: 5′-GCTGACCTATTCCTGCGTCTTTG-3′	23	70
	R: 5′-TCGTGTTTCTTGGCGACCCTTTG-3′	23	70
44. *Small androgen receptor-intercting protein**	F: 5′-ATGACAGACTACAAGGAAGAACAGAA-3′	26	72
	R: 5′-CTCCAATGAGTATGAGACAAGCG-3′	23	68
45. *Cdc2 ****	F:5′-ACTTTCTTCTCGGTATTCTATTGTCG-3′	26	72
	R:5′-CCCACGTCCGTCGCCTTTGGTTTT-3′	24	76
46. *Cycilc AMP-regulated protein like protein***	F:5′-AAGGGACCCCGTCAATCCACTG-3′	22	70
	R:5′-CGTGATTTTATCCGAACGAAGCCG-3′	24	60
47. *Vitellogenin VTG**	F:5′-AGCCGAAGAAACGAAGGGA-3′	19	58
	R:5′-CACTACATCCAAAGCAACTGTCTCTG-3′	26	76
48. *SPAPC****	F:5′-AGCGTATCACCCCTCGCCATCT-3′	22	70
	R:5′-TCTCAAACACCTCCCTACTCCATCA-3′	25	74
49. *RNA helicase***	F:5′-GAAGGGAAGGCAGCCAGGAG-3′	20	66
	R:5′-CGACGGATTTCAGCCATTACATAG-3′	24	70
50. *Death associated protein 5****	F:5′-CAACCTCCTAACCAGAAGCCAACA-3′	24	60
	R:5′-CAACATTCATAAGTCCTTCACTGCGA-3′	26	74
51. *Nucleolar phosphatase**	F:5′-ACAGAACCCTCAAGGACACTCG-3′	22	68
	R:5′-ATTCAACAAATAAACAACTCCCACA-3′	25	66
52. *Probable hormone receptor **	F:5′-GGTTGGCTTGTCTTGAGTTCTTGC-3′	24	60
	R:5′-TGGATTCTTGGACTCCTCGCCG-3′	22	70
53. *Solute carrier family 3 member 2 **	F:5′-GCACGAAGCCCTACCGAGCC-3′	20	68
	R:5′-CGTCTTCCTGCGGAGCCATC-3′	20	66
54. *Mitotic checkpoint****	F:5′-CGAGTCTGAAGTCGGCAAAATG-3′	22	66
	R:5′-GCTGACCATCTAAGCCTCCACT-3′	22	68
55. *Serine palmitoyl transferase LCB2 subunit****	F:5′-ATCCAACAACTGTCTCGCAATG-3′	22	64
	R:5′-ATCCAACCCCTACGCCAGCCAC-3′	22	72
56. *Small ubiquitin-like modifier****	F:5′-GGAAGGGAACGAATACATCAAA-3′	22	62
	R:5′-GCCTGGTCTGTGCGAAAATCTC-3′	22	68
57. *Putative cold-induced protein****	F:5′-TGTGTTCTTCATTGCCTCTCCG-3′	22	66
	R:5′-TATTTGGAAGTGGGGCATCTGA-3′	22	64
58. *Multiple inositol polyphosphate****	F:5′-TATTCCAAGGACAACCCAGGCT-3′	22	66
	R:5′-TTTACATCCCCTCGTCCCGCTT-3′	22	68
59. *Low molecular weight neurofilament protein XNF-L****	F:5′-GGGAGGAAGAACACCCCAATG-3′	21	66
	R:5′-TACACGCTGAGCAACGAGAACG-3′	22	68
60. *Cell divition protein kinase 7****	F:5′-CGGAAGACAGGATGGAAGTAGAA-3′	23	68
	R:5′ATGTTGGATGGGCGTGAGGATG-3′	22	68
61. *Adapter-related protein complex 1 beta 1 subunit (beta-adeptin 1)****	F:5′-TTCCCTGATGTGGTCAACTGTATG-3′	24	70
	R:5′-TGGTCTACGCAGATAGCAGCAG-3′	22	68

Table 2.1 (cont.)

Gene	Primer sequence	Length	Tm
62. *Gene flightless-I protein**	F:5′-AGAGAAAATCGGATAAATACGGA-3′	23	62
	R:5′-TCTGGAGTTTGTTGTGGCGGAC-3′	22	68
63. *Salivary gland secretion 1****	F:5′-ATTTTTGCTTGGCGTAGCGACC-3′	22	66
	R:5′-ATGAAGGGCAGCAGCGGGAACA-3′	22	70
64. *TERA protein (chromosome 12 open reading frame 14)**	F:5′-GGACCCGTGACCTCGCTCGCCT-3′	22	76
	R:5′-CTTCATACTTCTTAGAGTCTGTAAACCG-3′	28	78
65. *Splceosome-association protein9**	F:5′-TCTACCTCTGCTGACCGCTAAGT-3′	23	70
	R:5′-AACCGTTACCCTATTATCACAATCTAT-3′	27	72
66. *Ovarian lipoprotein receptor **	F:5′-CGCCCGAGTCCCATCATTTC-3′	20	64
	R:5′-TGTCGCCCTCCATCCCAC-3′	18	60
67. *Transposase****	F:5′-CCCGTTGAAATGGTTGTTGCTG-3′	22	66
	R:5′-GCTACCTGCTTTTCTCTTTTGCG-3′	23	68
68. *Probable 26s proteasome non-ATPase regulatory subunit 3 ******	F:5′-CGCCTGGTTGAACGCAGCATTG-3′	22	70
	R:5′-TTCTCTGGTCGCCCATAGTAAGT-3′	23	68
69. *Oncoprotein nm23****	F:5′-CTGACAAGCCCTTCTACCCTGG-3′	22	70
	R:5′-AGAGCAATCTCCTTGTTGGCAG-3′	22	66
70. *Voltage-dependent anion-selective channel protein 2**	F:5′-CAAGATGTGTTTGGCAAGGGAT-3′	22	64
	R:5′-GAAGTCGGGGTTGGTGAAGC-3′	20	64
71. *Metaxin2****	F:5′-AGATACTGCTCCCTGATAATGCCCA-3′	25	74
	R:5′-GCCGTCTGTCAAGGTCACTCCC-3′	22	72
72. *Acyl-CoA oxidase (ACX3)****	F:5′-CACTGCGTATCTTTGCTGCTC-3′	21	64
	R:5′-TCATCCGTTTCCTTGTCGTAA-3′	21	60
73. *Synaptobrevin like protein****	F:5′-CTCCTCTACAGTGTCATTGCTCGG-3′	24	74
	R:5′-AAGTGCGGTGTGAACTCGGCT-3′	21	66
74. *Proteasome (prosome,macropain) subunit,alpha type3.****	F:5′-AAAGATGGTGTTGTGTTTGCTGTAG-3′	25	70
	R:5′-AGAGGGTATAGGCATGAAGGTAGG-3′	24	60
75. *Laminin beta chain****	F:5′-ATGCGATGAGTGTGCCCGAG-3′	20	64
	R:5′-GCCAAAGAAATGCGTTGTGTAGTG-3′	24	70
76. *Ubiquitin carboxyl-terminal hydrolase14****	F:5′-ATACTGCCGAACCCCCAATG-3′	20	62
	R:5′-CAGTGTTCCTCTCAGCTTCAGTCA-3′	24	60
77. *Putative U3 sno RNP protein IMP4****	F:5′-TGTCTGAGCAGTTATCCCCATCT-3′	23	68
	R:5′-GTCTTTGCCTCCATCAACATTTC-3′	23	66
78. *Cell divition protein kinase2 ****	F:5′-CGACTGCTGGATGTGGCGTA-3′	20	64
	R:5′-CCGAGGAGAATCTGTGGGGC-3′	20	66
79. *SRPK2 protein****	F:5′-CACTGTTTGGCTGTGTTGGG-3′	20	62
	R:5′-CCTGGTAATTGGAGCGGATG-3′	20	62
80. *Multiprotein bridging factor****	F:5′-TGCCACCACCTTCAACACAG-3′	20	62
	R:5′-CATCCCAATAGCCTTCTCAATC-3′	22	64
81. *Arginyl-tRNA-protein transferase1****	F:5′-GGGATGGAGACGGAGTGGAA-3′	20	64
	R:5′-TGGCATCTGGAGGGATACACC-3′	21	66
82. *Ubiquitin isopeptidase ****	F:5′-CAAGTTGGCTGCCCCTGAAG-3′	20	64
	R:5′-GTTGCCTGCTCTCGTGTGAATC-3′	22	68
83. *COP9 signalosome subunit5****	F:5′-CGGTCTGGAGGCACACTTGAG-3′	21	68
	R:5′-CATTTTCCTGGCGACCAACCT-3′	21	62
84. *Programmed cell death7****	F:5′-CCCTGACAGCCCTGCGACA-3′	19	64
	R:5′-GCACTTTCACCCATCATAACCCG-3′	23	70
85. *Inhibitor of apoptosis protein****	F:5′-CCTGAAGAGTTAGCAGCAGATGG-3′	23	70
	R:5′-TACTTGCTTTTGGAGGATTGTCAC-3′	24	68

Table 2.1 (cont.)

Gene	Sequence	Length	Tm
86. *Nudix-type motif9 isofrom A****	F:5′-CGCACTGATGATAAGACTCCTCG-3′	23	70
	R:5′-CCAGCATCCATTTTCCACCG-3′	20	62
87. *CyclophilinA****	F:5′-ATGGGCAACCCCAAAGTCTTTTTCGA-3′	26	76
	R:5′-TTACAGCTGGCCGCAGTTGGCG-3′	22	72
88. *26s proteasome regulatory particle****	F:5′-GGCATCAGTCCCACCAAGG-3′	22	62
	R:5′-CCGTTCCCGTAAGGAGTCG-3′	19	62
89. *E1B-55kDa-associated protein5 *****	F:5′-CCTTGGTGCTCTCCATTGACTG-3′	19	68
	R:5′-CTGTTGATGAGGCTGGGCTG-3′	22	64
90. *Polyadenylate binding proteinII****	F:5′-CCCTCTAGCCTCGCTCTATGTG-3′	20	70
	R:5′-GTCTAGGGCTCGTTCAGCATCA-3′	22	68
91. *SRY-box 7; SOX7 transcription factor**	F:5′-TCATCGGGTCAAAAAGGTCCAA-3′	22	64
	R:5′-TGCTGAAGTGCCTGTGCCATCT-3′	22	68
92. *Chk1 checkpoint kinase*	F:5′-TTTTATGGAAGTCGCCGATGAA-3′	22	62
	R:5′-ATTCTCGTCAAGCAGCAAGTTCT-3′	23	66
93. *457/OPA01*	F:5′-CTTCTTATGTCTGTCCTTTGATGA-3′	24	66
	R:5′-TTCTTAGGGAAACTGCTTGC-3′	20	58
94. *428/OPB17*	F:5′-CTCTGACTGGTGGAGGGAAT-3′	20	62
	R:5′-CTGGCTCGTGGGAGTGTAAT3′	20	62
95. *MI-36*	F:5′-ATGTATGTTTGTGTATGTAGGTGTG-3′	25	66
	R:5′-AGACGGCAAGGAAAGATGAG-3′	20	60
96. *MII-51*	F:5′-CCTGATGAAATCGGGTCAAAAC-3′	22	64
	R:5′-ATACTCTCCTCTGCCGCTCG-3′	20	64
97. *FI-1*	F:5′-GTATTCCATCCTCAACAACTG-3′	21	60
	R:5′-ACTGGGAGCACTACCATCTT-3′	20	60
98. *FI-40*	F:5′-AATAACCGTTCTCAGCAGCA-3′	20	58
	R:5′-ATTCAAGGCGTTCACATCC-3′	19	56
99. *FI-6*	F: 5′-AGATGGAGATGGGTTAGGA-3′	19	56
	R:5′-AGGAGTAGACGCCGTTTGT-3′	19	58
100. *FII-17*	F:5′-TGGAGGAGTAGATGCCGTT-3′	19	58
	R:5′-GTGATGGGTTAGGTGATGC-3′	19	58
101. *FIII-4*	F: 5′-GCAATCTCGCACAGCCAATACT-3′		66
	R: 5′-CGGAAAGACAGGGCAGCAAC-3′		64
102. *FIII-8*	F:5′-CCTCATAAACCAGGCACTAA-3′	20	58
	R:5′-AGAATCATCCCAGGAATCAC-3′	20	58
103. *FIII-39*	F:5′-ATCTCGCCAGGAGGAAATAA-3′	20	58
	R:5′-CCTTGTTCAGTTCTTGCCAC-3′	20	60
104. *FIV-2*	F:5′-CGTATGCCACATCCCACA-3′	18	56
	R:5′-TTCTTTTCTGAAGGAGGTCG-3′	20	58
105. *FIV-33*	F:5′-TGGGACTGTTTGTTTCTTG-3′	19	54
	R:5′-TCTCTTGGTTAGGTGTTGGT-3′	20	58
106. *FV-1*	F:5′-CGTATGCCACATCCCACAGA-3′	20	62
	R:5′-GAGCCCGTACCATTGAGAAA-3′	20	60
107. *FV-27*	F:5′-GCCCTGGCACAGCACTTAG-3′	19	62
	R:5′-GTCCCAATCTTCCTCTTTCA-3′	20	58
108. *FV-42*	F:5′-AAGTGACCTTGATATGAGTG-3′	20	56
	R:5′-ATCCCTTCGTTGTAAGTAGA-3′	20	56

*Primers designed from EST found in the hemocyte cDNA library, **Primer pairs from EST found in the ovarian cDNA libraries and ***primers designed from EST found in testes cDNA libraries. Primer pairs numbers 93-108 were from RNA-arbitrary primed (RAP)-PCR.

Table 2.2 Examples of PCR profiles and composition (dNTPs and $MgCl_2$).

Primer	dNTPs (μM)	$MgCl_2$ (mM)	PCR condition
1. *RUVB-like protein*	200	2.0	94 °C, 3 min, 1 cycle followed by 94 °C, 30 s; 63 °C, 1 min and 72 °C, 45 s for 10 cycles where the annealing temperature were lowered for 2 °C in every 2 cycles and 94 °C, 30 s; 53 °C, 1 min and 72 °C, 45 s for additional 32 cycles and 72 °C, 7 min; 1 cycle
2. *LTB4DH*	100	2.0	94 °C, 3 min; 1 cycle followed by 94 °C, 30 s; 55 °C, 1 min and 72 °C, 45s for 35 cycles and 72 °C, 7 min; 1 cycle
3. *X-box binding protein*	100	2.0	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 55 °C, 1 min and 72 °C, 1 min for additional 25 cycles and 72 °C, 7 min; 1 cycle
4. *Rasputin*	100	1.5	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 53 °C, 1min and 72 °C, 2min; for 35 cycles and 72 °C, 7 min; 1 cycle
5.*Phosphatidylinosi-tol-4 kinase*	100	1.5	As described in 4.
6. *Phosphatidylserine receptor short form*	200	2.0	As described in 2.
7. *Gene flightless-I protein*	100	2.0	As described in 4.
8. *Vitellogenin VTG*	200	2.0	94 °C, 3 min; 1 cycle followed by 94 °C, 45 s; 55 °C, 1min and 72 °C, 2min; for 35 cycles and 72 °C, 7 min; 1 cycle
9. *COP9 signalosome subunit5*	100	2.0	As described in 4.
10 *Ovarian lipoprotein receptor*	100	2.0	As described in 4.
11. *Programmed cell death7*	100	2.0	As described in 4.
12. *SRY-box 7; SOX7 transcription factor*	100	1.5	As described in 4.

The comb was inserted. The gel was allowed to solidify at room temperature for approximately 45 minutes. When needed, the gel mould was placed in the gel chamber and sufficient 1x TBE buffer was added to cover the gel for approximately 0.5 cm. The comb was carefully withdrawn. One-fourth volume of the gel-loading dye (0.25% bromophenol blue and 25% ficoll, MW 400,000 prepared in sterile deionized H_2O) was added to each sample, mixed and loaded into the well. A 100-bp DNA ladder and/or λ-*Hind* III was used as the standard DNA markers. Electrophoresis was carried out at 4 - 5 V/cm until the tracking dye migrated about three-quartered of the gel. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) for 5 minutes and destained to remove unbound EtBr by submerged in H_2O for 15 minutes. The DNA fragments were visualized using a UV transilluminator.

2.5 Single strand conformational polymorphism (SSCP) analysis

The PCR products of gene homologues that were successfully amplified and did not exhibit size polymorphism or allelic variants between individuals were further characterized using single strand conformational polymorphism (SSCP) to examine whether the amplification product of the same genes in different shrimp individuals were polymorphic due to the existence of single nucleotide polymorphism (SNP).

2.5.1 Preparation of glass plates

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

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

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

2.5.2 PCR and electrophoresis

PCR was carried out against genomic DNA of *P. monodon* using condition described in Table 2.2 Six microlitres of the amplified PCR products of 22 gene homologues were mixed with four volumes of the SSCP loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured in a boiling bath for 5 minutes and immediately cooled on ice for 3 minutes. The denatured products were electrophoretically analyzed in native polyacrylamide gels at 250 - 300 volts for 14 - 18 hours at 4°C.

2.5.3 Silver staining

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

developing solution and continued shaking for 3 minutes each. The gel was placed in the plastic bag and air-dried.

2.6 Genotyping of commercially cultivated shrimp using microsatellite

2.6.1 Sampling and DNA extraction

Three hundred and fifty-nine juvenile of commercially *P. monodon* farm in Chonburi. (*N* = 359) were collected. Pleopods were dissected from each individual and stored at-80°C until required. Genomic DNA was extracted from frozen plepods of *P. monodon* using a phenol-chloroform-proteinase K method (Klinbunga et al., 1999) .

2.6.2 Polymerase Chain Reaction and Gel Electrophoresis

Two microsatellite loci of *P. monodon* (*CUPmo02* and *CUPmo13*) were used to examine the possible number of families contributed in commercially cultured specimens (N=16, Table2.3) PCR conditions were optimized for each microsatellite primer pair. The amplification of *CUPmo02* and *CUPmo13* were as previously described (Table 2.4). The reaction mixture in 25 µl containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 2 µM of each primer, 25 ng of genomic DNA of *P. monodon* and 1.0 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes). The PCR product were electrophoretically separated on 8% denaturing polyacrylamide gels and visualized by silver staining.

Table 2.3 PCR primers, and the annealing temperatures for *P. monodon* microsatellite loci

Locus	Sequence	Tm (°C)
CUPmo02	F: 5′-CCAAGATGTCCCCAAGGC-3′	58
	R: 5′-CTGCAATAGGAAAGATCAGAC-3′	60
CUPmo13	F: 5′-GACTTCGTGGTATCAATGACTGT-3′	66
	R: 5′-TGTCAGTTCATGTAGTCTGCTC-3′	64

Table 2.4 The amplification conditions for *P. monodon* microsatellite loci

Gene homologue	Amplification condition
CUPmo02	1 cycle of 94 °C for 3 min.,
	35 cycles of 94 °C for 45 s, 53 °C for 1 min , 72 °C for 45 s, and the final extension at 72 °C for 7 min
CUPmo13	1 cycles of 94 °C for 5 min
	35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s and the final extension at 72 °C for 7 min

2.7 Correlations between SSCP genotypes and the growth rate of *P. monodon*

Genomic DNA was extracted from specimens of the G2 family of *P. monodon* showing 10% from the top and the bottom according to the body weight. The SSCP pattern of *RUVB-like protein*, *12-leukotriene B4 dehydrogenase* (*LTB4DH*) and *x-box binding protein* of each individual was analyzed. Relationships between the frequencies of identified genotypes and the body weight of shrimp showing presumably slow growth rate and high growth rate were statistically analyzed separately using one way analysis of variance (ANOVA).

Subsequently, frequencies of SSCP genotypes of *RUVB-like protein* in commercially cultivated shrimp (N = 335) was examined and statistically tested as above.

2.8 Identification of SNP by direct DNA sequencing of PCR products

2.8.1 PCR and electrophoresis

The PCR product of *RUVB-like protein*, *LTB4DH* and *X-box binding protein* was amplified from genomic DNA of representative individuals of the G2 family and electrophoretically analyzed in duplication. One was run side-by-side with a 100 bp DNA markers and the other was loaded into the distal well of the gel. After electrophoresis, lanes representing the DNA standard and its proximal DNA sample were cut and stained with ethidium bromide (0.5 μg/ml) for 5 minutes. Positions of

the DNA markers and the EtBr-stained fragment were used to align the position of the non-stained target DNA fragment.

2.8.2 Elution of the PCR product from agarose gels and direct sequencing of the PCR product of gene homologues

The PCR product of *RUVB-like protein*, *LTB4DH* and *X-box binding protein* of individuals of the G2 family was size-fractionated and excised from the agarose gel using a sterile scalpel and placed in a pre-weighed microcentrifuge tube and eluted out from the gel using a HiYield™ Gel Elution Kit (RBC).

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

The gel-eluted PCR products were sequenced on the automated DNA sequencer (MACROGEN, Korea) using the original forward or reverse primer of investigated genes as the sequencing primer. The obtained sequences were aligned by Clustal X.

2.9 Identification of SNP by cloning and sequencing of the amplified *RUVB- like protein* in commercially cultured *P. monodon*

2.9.1 Ligation of the PCR product of *RUVB- like protein* to the pGEM®-T Easy vector

The gel-eluted *RUVB- like protein* was ligated to the pGEM®-T Easy vector in a 10 µl reaction volume containing 5 µl of 2x Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DDT, 2 mM ATP and 10% PEG8000), 3 Weiss unit of T4 DNA ligase, 25 ng of the pGEM®-T Easy vector and approximately 50 ng of the DNA insert. The reaction mixture was incubated overnight at 4 - 8 °C before transformed to *E. coli* JM 109.

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

2.9.2.1 Preparation of competent cell

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

2.9.2.2 Transformation

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

and 25 µl of 25 µg/ml of IPTG for approximately 1 hr before using) and further incubated at 37 °C overnight (Sambrook and Russell, 2001). The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.9.3 Recombinant plasmid DNA extraction and sequencing

Plasmid DNA was isolated using a HiYield™ Plasmid Mini Kit (RBC). A discrete white colony was inoculated into a sterile culture tube containing 3 ml of LB broth supplemented with 50 µg/ml of amplicillin and incubated with shaking (200 rpm) at 37 °C overnight. The culture was transferred into a sterile 1.5 ml microcentrifuge tube and centrifuged at 14,000 rpm for 1 min. The supernatant was discarded. The bacterial pellet was resuspended in 200 µl of the PD 1 buffer containing RNaseA and thoroughly mixed by vortexed. The resuspended cells were lysed by the addition of 200 µl of the PD 2 buffer and mixed gently by inverting the tube for 10 times. The mixture was stand for 2 minutes at room temperature. After that, 300 µl of the PD3 buffer was added to neutralize the alkaline lysis step and mixed immediately by inverting the tube for 10 times. The mixture was then centrifuged at 14,000 rpm for 15 min.

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

The recombinant plasmids were sequenced for both directions using the automated DNA sequencer (MACROGEN, Korea). The obtained sequences were aligned by Clustal X.

2.10 Genome walking analysis

The genomic sequence of *phosphatidylserine receptor* (*PSR*), *X-box binding protein* and *phophatidylinositol-4-kinase* (*PI4K*) were further characterized by genome walking analysis.

2.10.1 Digestion of genomic DNA

Two and a half microgrammes of genomic DNA extracted from a single individual of *P. monodon* were singly digested with 40 units of a blunt end generating restriction enzyme (*Rsa* I,, *Dra* I, *Eco* RV, *Ssp* I) in a reaction volume of 100 µl containing 1X of an appropriate restriction enzyme buffer. The reaction was incubated at 37ºC for 4 hours. Five microlitres of the digest was run on a 0.8% agarose gel to determine whether the digestion was complete.

2.10.2 Purification of digested DNA

An equal volume (100 µl) of buffer-equilibrated phenol was added. The mixture was vortexed at the low speed for 5-10s and centrifuged for 5 min at room temperature to separate the aqueous and organic phases. The upper layer was transferred into a fresh 1.5 ml microcentrifuge tube. An equal volume of chloroform: isoamylalcohol (24:1) was added, vortexed and centrifuged. The upper layer was transferred into a new microcenttrifuge tube and ethanol precipitated as described previously. The DNA pellet was air-dried. DNA was dissolved in 10 µl of TE (10 mM Tris, pH 8.0, 0.1 mM EDTA).

2.10.3 Ligation of digested genomic DNA to Genome Walker Adaptors

The ligation reaction was set up in a 10 µl reaction volume containing 4 µl of digested DNA, 1.9 µl of 25 µM of GenomeWalker Adaptor (GenomeWalker Adaptor Forward (5′-GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCA GGT-3′) and GenomeWalker Adaptor Reverse (5′-PO_4-ACC TGC CC-NH_2-3′), 1.6 µl of 10X ligation buffer and 3 units of T4 DNA ligase. The reaction mixture was incubated at 16 ºC overnight. The reaction was terminated by incubation at 70 ºC for 5 minutes. The ligated product was ten fold diluted by an addition of 72 µl of TE (10mM Tris-HCl, pH 8.0, 0.1 mM EDTA).

2.10.4 PCR-based genomic DNA walking

PCR-based genomic DNA walking was carried out in a 25 µl reaction containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, EXT Taq 0.2 µM each of Adaptor primer1 (AP1: 5′-GTA ATA CGA CTC ACT ATA GGG C-3′) and gene specific primer (Table 2.5), 1 µl of each mini DNA library of *P. monodon* and 1.0 unit of DyNAzyme™ EXT DNA Polymerase

The amplification reactions were carried out using the thermal profile indicated in Table 2.6 Five microlitres of the primary PCR product was electrophoretically analyzed by a 1.2% agarose gel. A 100 bp ladder and λ–*Hin*d III was included as the DNA markers.

2.10.5 Cloning of fragments generated from genome walking analysis

The amplification product from genome walking analysis of each gene was electrophoresed and gel-eluted. The purified fragment was cloned into pGEM®-T Easy vector. Recombinant plasmid was extracted and sequenced as described previously

2.10.6 Colony PCR and digestion of the amplified insert

Colony PCR was performed in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 0.2 µM of pUC1 (5′-TCC GGC TCG TAT GTT GTG TGG A-3′) and pUC2 (5′-GTG GTG CAA GGC GAT TAA GTT GG-3′) primers and 0.5 unit of DyNAzyme™ II DNA Polymerase. A recombinant colony was picked up by the micropipette tip and mixed well in the amplification reaction. The PCR profiles was predenaturing at 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 60 seconds and 72 °C for 90 seconds. The final extension was carried out at 72°C for 7 minutes. The resulting PCR products were analyzed by agarose gel electrophoresis.

Clones showing corresponded DNA insert size were separately digested with *Hin*d III and *Rsa* I to verify whether a single insert contained only one type of

Table 2.5 Gene-specific primers (GSPs) used for genome walking analysis of functionally important genes in *P. monodon*

Gene specific primer	Sequence	Tm (°C)
Phosphatidylinositol -4 kinase		
5′ GW	F: 5′-CAACGCCATCAACTCCATCAC- 3′	64
Phosphatidyl serine receptor		
5′ GW	F: 5′-CAAGTGTGGAGAAGATAATGAAGG-3′	68
3′ GW	R: 5′-CCAGCGGTAAGGGGGGCGTC-3′	70
X-box binding protein		
5′ GW	F: 5′-TGATGAACTTCGGGACCTAA-3′	58
3′ GW	R: 5′-CCTCAACGACAACTGCTGCG-3′	64

Table 2.6 The amplification conditions for genome walking analysis of gene homologues of *P. monodon*

Gene homologue	Amplification condition
Phosphatidylinositol 4-kinase	
5′ GW	7 cycles of 94 °C for 25 s and 70 °C for 3 min
	35 cycles of 94 °C for 25 s, 65 °C for 3 min and the final extension at 65 °C for 7 min
Phosphatidyl serine receptor	
5′ GW	7 cycles of 94 °C for 25 s and 70 °C for 3 min
	35 cycles of 94 °C for 25 s, 65 °C for 3 min and the final extension at 65 °C for 7 min
3′ GW	As described in 5′ GW.
X-box binding protein	
5′ GW	7 cycles of 94 °C for 25 s and 70 °C for 3 min
	35 cycles of 94 °C for 25 s, 65 °C for 3 min and the final extension at 65 °C for 7 min
3′ GW	As described in 5′ GW.

sequence. Typically, the digestion reaction was set up in the total volume of 15 µl containing 1X of Buffer E (6 mM Tris-HCl; pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT) for *Hin*d III or Buffer C (10 mM Tris-HCl; pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT) for *Rsa* I, 5 µl of the amplified product and 2 units of either *Hin*d III or *Rsa* I. The reaction mixture was at incubated at 37°C for 3 - 4 hours. Digestion patterns were analyzed by agarose gel electrophoresis.

2.10.7 Extraction of recombinant plasmid DNA and restriction enzyme digestion

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

2.10.8 DNA sequencing

Nucleotide sequences of recombinant plasmids were examined by automated DNA sequencer using M13 forward and/or M13 reverse primer as the sequencing primer by MACROGEN (Korea).

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

2.11.1 Preparation of the 5′ and 3′RACE template

Total RNA was extracted from ovaries of *P. monodon* using TRI-REAGENT. The quality of extracted total DNA was determined by agarose gel electrophoresis. Messenger (m) RNA was purified using a QuickPrep *micro* mRNA Purification Kit (Amercham Phamacia Biotech). The RACE cDNA template was prepared by combining 1 µg of ovarian mRNA with 1 µl of 5-CDS primer and 1 µl of 10 µM SMART II A oligonucleotide for 5′ RACE-PCR or 1 µg of ovarian mRNA with 1 µl of 3′ CDS primer A for 3′ RACE-PCR (Table 2.7). The components were mixed and

spun briefly. The reaction was incubated at 70°C for 2 minutes and snap-cooled on ice for 2 minutes. The reaction tube was spun briefly. After that, 2 µl of 5x First-Strand buffer, 1 µl of 20 mM DDT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript reverse transcriptase were added. The reactions were mixed by gently pipetting and centrifuged briefly to collect the contents at the bottom of the tube.

The tube was incubated at 42 °C for 1.5 hours in a thermocycler. The first strand reaction product was diluted to 125 µl with Tricine-EDTA buffer (or TE buffer) and heated at 72°C for 7 minutes. The RACE template was stored at -20.

2.11.2 Primer designed for RACE-PCR and primer walking

Gene-specific primers (GSPs) were designed from ovaries and hemocyte libraries. The antisense primer (and nested antisense primer) was designed for 5′ RACE-PCR and the sense primer (and nested sense primer) for the 3′ RACE-PCR (Table 2.8).

For sequencing of genes that showed the full length from the 5′ direction, the product from colony PCR of the original EST clone was considered. If the insert was larger than that of the homologues, the 3′ direction was sequenced. Internal primers were designed for primer walking of the inserted cDNA (Table 2.9).

Table 2.7 Primer sequence for the first strand cDNA synthesis and RACE-PCR

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

Table 2.8 Gene-specific primers (GSPs) used for isolation of the full length cDNA of *rasputin* and *PI4K* in *P. monodon*

Gene	Sequence	Length	Tm
1. 5′*Rasputin*	F:5′-CTCCTCCACTTCACTTCCTCCCTCC-3′	25	80
2. 3′*Rasputin*	R:5′-CTCCCCAGACTCCTGTCACTCCTCA-3′	25	80
3. 5′*PI4K*	F:5′-GCGTGGTGATGGAGTTGATGGCGTTGT-3′	27	84

Table 2.9 Internal primers used for primer walking sequencing of the full length cDNA of functionally important genes in *P. monodon*

Internal primer	Sequence	Tm (°C)
LTB4DH		
5′ internal primer-I	F:5′-CCATTGTAGCAGTGTGAGGAAC-3′	66
3′ internal primer-I	R:5’-GCAGACACCACCGCCAAACT-3’	64
RUVB		
5′ internal primer-I	F: 5′-TTCCATCGGTGTGCGTATCAAG-3′	66
5′ internal primer-II	F: 5′-AAGGAGATTTTGAAGATTAGGTG-3′	62
3′ internal primer-I	R:5′-TGATGCACAAGTACAGTCTCGG-3′	66
3′ internal primer-II	R:5′-CCATCCCCATCAACATCATTCT-3′	64
3′ internal primer-III	R:5′-CAAAGTCATCATCACTGGTGTCCTC-3′	74
X-box binding protein		
5′ internal primer-I	F: 5′-TAGCGGACCATGACTATACC-3′	60
Rasputin		
5′ internal primer-I	F: 5′-AGGATGAACTGAAGGCGGAA-3′	60
5′ internal primer-II	F: 5′-GAAGAGGAAGGTTCTGTGTCAAAGG-3′	74
5′ internal primer-III	F: 5′-CCAAACATCTGCAAGAGGACTG-3′	66
5′ internal primer-IV	F: 5′-GTGCTTATTTCAGTTTCAACATCTA-3′	66
3′ internal primer-II	R: 5′-AGAAACACAATCTACATCAGGGGAG-3′	72
3′ internal primer-I	R: 5′-AAGTTCTCTTCTGATGTCCCTGTAA-3′	70

2.11.3 RACE-PCR

The master mix which is sufficient for 5′ and 3′ RACE-PCR and the control reactions was prepared. For each 25 µl amplification reaction, 14.0 µl sterile deionized H_2O, 2.5 µl of 10X Adventage® 2 PCR buffer, 0.5 µl of 10 uM dNTP mix and 0.5 µl of 50X Adventage® 2 polymerase mix were combined. The reaction was carried out for as described in Table 2.10.

For *Rasputin*, the reaction was carried out for 22 cycles composing of 94 ºC for 30s, 66 ºC for 1 min and 72 ºC for 3 min. The final extension was carried out at 72 ºC for 7 min. For *PI4K*, the reaction was initially carried out for 10 cycles composing of 94 ºC for 30s, 55 ºC for 1 min and 72 ºC for 3 minutes followed by 25 cycles of similar conditions with the exception that the annealing temperature was increased to 63 ºC. The primary 5′ and 3′ RACE-PCR products were electrophoretically analyzed.

The positive amplification product was electrophoretically analyzed. The gel-eluted product was cloned into pGEM-T Easy and further characterized by DNA sequencing.

Table 2.10 Compositions for amplification of the 5′ and 3′ ends of gene homologues using RACE-PCR

Component	5'-RACE Sample	3'-RACE Sample	UPM only (Control)
5′-RACE-Ready cDNA	1.5 µl	1.5 µl	1.5 µl
UPM(10X)	5.0 µl	5.0 µl	5.0 µl
5′ GSP(GSP1, 10µM)	5.0 µl	5.0 µl	-
H_2O	10.0 µl	10.0 µl	15.0 µl
Master Mix	17.5 µl	17.5 µl	17.5 µl
Final volume	25 µl	25 µl	25 µl

2.12 Semiquantitative RT-PCR of *LTB4DH*, *X-Box binding protein*, *RuvB like protein*, *phosphatidyllinosital-4-kinase* in ovaries of juvenile *P. monodon* following serotonin injection

2.12.1 Experimental animals

Female juveniles of *P. monodon* (approximately 4-month-old) were purchased from a local farm in Chonburi, eastern Thailand. The shrimp were acclimatized for 7 days at the laboratory conditions (28 ppt of seawater and the ambient temperature) and fed daily at approximately 5% of the body weight. Seven shrimp were placed in a fish tank (150 L) for each treatment (Appendix B) and were not fed at 24 hr before the treatment.

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

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

2.12.3 Experimental design

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

2.12.4 Total RNA extraction and the first strand cDNA synthesis

Ovaries were dissected from each shrimp immediately after specimens were collected. Total RNA was extracted from ovaries of *P. monodon* and 1.5 µg of total

RNA was reverse-transcribed using an ImProm- II™ Reverse Transcription System (Promega) as described previously.

2.12.5 Optimization of semiquantitative RT-PCR conditions

Initially, RT-PCR of *LTB4DH*, *X-Box binding protein*, *RuvB-like protein*, *phosphatidylinosital-4-kinase* (Table 2.1) and *elongation factor-1α* (F:5′-ATGGTTGTCAACTTTGCCCC-3′ and R:5′-TTGACCTCCTTGATCACACC-3′) were amplified in a 25 µl reaction volume following the standard RT-PCR for screening of gene expression described previously. The reaction contained 10 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl and 0.1 % Triton X – 100, 2 mM $MgCl_2$, 100 mM each of dATP, dCTP, dGTP and dTTP, 0.2 µM of each primer, 1 µl of 10-fold diluted first strand cDNA and 1 unit of Dynazyme™ DNA polymerase follow (FINNZYMES). PCR was carried out by predenaturation at 94°C for 3 min, followed by 25 cycles of 94°C for 30s, 53°C for 45s and 72 °C for 45s. The final extension was carried out at 72°C for 7 minutes. The resulting PCR products were analyzed by agarose gel electrophoresis. Afterwards, primer and $MgCl_2$ and the cycle numbers used for amplification were further optimized.

2.12.5.1 Optimization of primer concentrations

The optimal primer concentration for each primer pair (between 0, 0.1, 0.15, 0.2 and 0.25 µM) was examined using the standard PCR conditions. The resulting product was electrophoretically analyzed. The primer concentration that gave product specificity and clear results were selected for further optimization of the PCR conditions.

2.12.5.2. Optimization of $MgCl_2$ concentrations

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

2.12.5.3. Optimization of the number of amplification cycles

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

2.12.5.4. Semiquantitative RT-PCR, Gel electrophoresis and data analysis

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

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

CHAPTER III

RESULTS

3.1 DNA extraction

Genomic DNA was typically extracted from a piece of pleopod of each *P. monodon* using a phenol-chloroform-proteinase K method The quality of extracted genomic DNA was electrophoretically determined using a 0.8 % agarose gel. High molecular weight DNA at the similar size as that of undigested λ-DNA (approximately 50 kb) along with sheared DNA was obtained from the pleopods of broodstock-sized *P. monodon* kept at -30 °C for several years (Figure 3.1).

The ratio of OD_{260}/OD_{280} of extracted DNA ranged from 1.8 - 2.0 indicating that the quality of extracted DNA samples is acceptable for further used. Some DNA samples contaminated with RNA as visualized by the smear at the bottom of gel and the high ratio of $OD_{260}/OD_{280} \geq 2.0$ was found. However, RNA contamination did not affect the PCR amplification. Therefore, the extracted DNA can still be used.

Alternatively, a Chelex®-based method was also used for the rapid extraction of genomic DNA of *P. monodon* when a large number of specimens were used. The rapid extracted DNA was subjected to the amplification reaction as soon as possible after isolated.

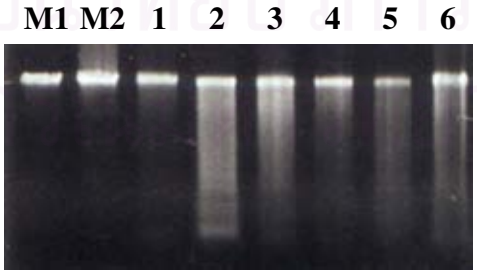

Figure 3.1 A 0.8% ethidium bromide-stained agarose gel showing the quality of genomic DNA (lanes 1 – 6) extracted from the pleopod of different individuals of *P. monodon*. Lanes M1 and M2 = 100 and 200 ng of undigested λDNA, respectively.

3.2 Identification of SSCP patterns of various genes homologues of *P. monodon*

In total, 108 primer pairs were designed from sequences of EST (cDNA) of ovarian, testis and hemocyte cDNA libraries and from RAP-PCR fragments. The primer pairs were tested against genomic DNA of wild *P. monodon* originating from Phangnga, Satun, Trang, Chumphon and Trat ($N = 3$ for each geographic sample)

Fifty-two primers pair did not generate the amplification product whereas five primer pairs yielded nonspecific amplification products. Fifty-six primer pairs provided the positive products. Thirty-one of which gave larger product sizes than those expected from the coding sequences (Table 3.1). This indicated the existence of intron(s) in the amplified fragments.

Size polymorphism among individuals possible due to variation of allelic sizes or indels was not observed when the amplification product was analyzed by agarose gel electrophoresis (Figures 3.2 - 3.11). Therefore an appropriate technique for identification of sequence polymorphism should be further applied.

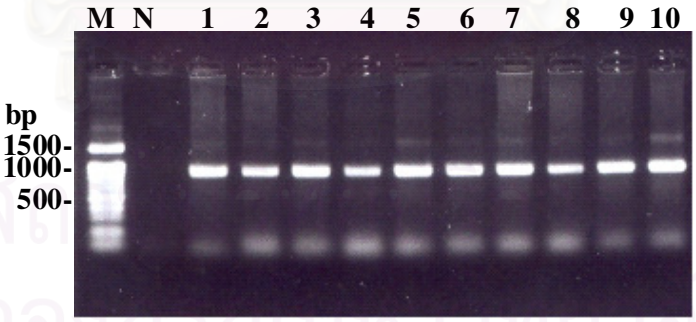

Figure 3.2 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of *LTB4DH* against genomic DNA of *P. monodon* from different geographic locations in Thai waters. (Lanes 1 - 10). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

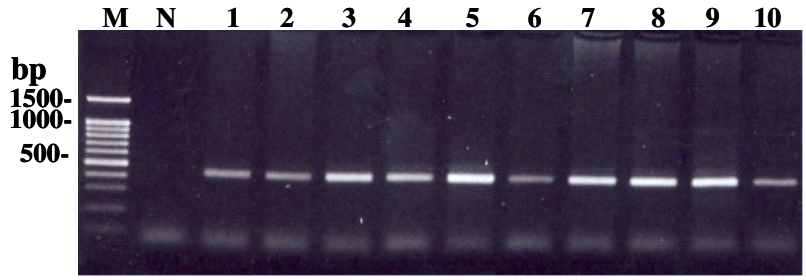

Figure 3.3 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of *phosphatidylserine receptor* against genomic DNA of *P. monodon* from different geographic locations in Thai waters (lanes 1 - 10). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

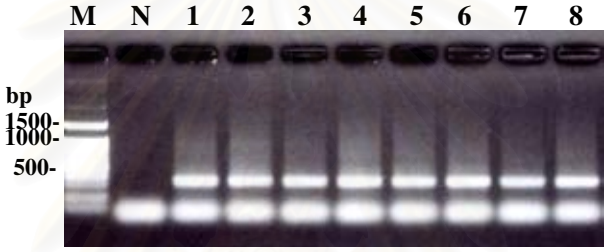

Figure 3.4 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of *phosphatidylinositol-4 kinase* against genomic DNA of *P. monodon* from different geographic locations in Thai waters (Lanes 1 - 8). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

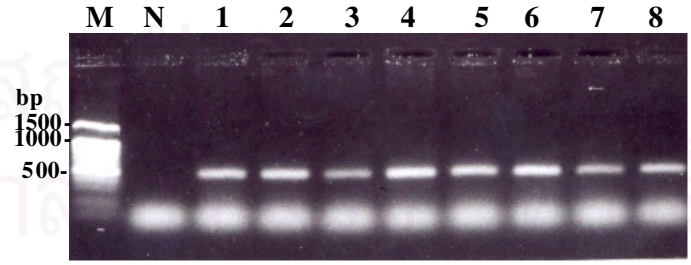

Figure 3.5 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of *RUVB-like protein* against genomic DNA of *P. monodon* from different geographic locations in Thai waters (lanes 1 - 8). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

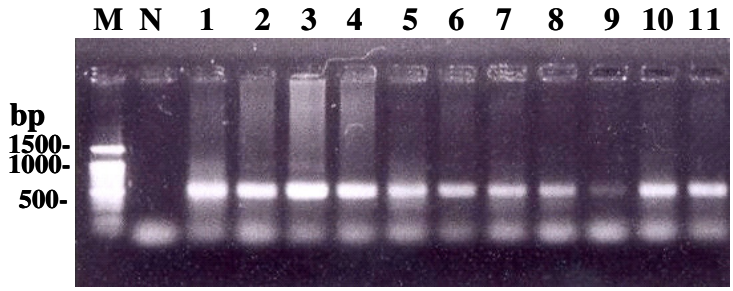

Figure 3.6 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of *Rasputin* against genomic DNA of *P. monodon* from different geographic locations in Thai waters (lanes 1 - 11). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

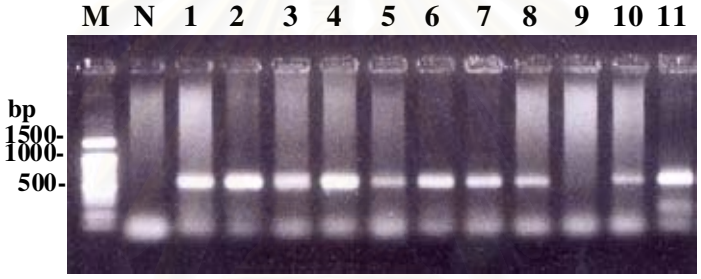

Figure 3.7 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of *laminin-β chain* against genomic DNA of *P. monodon* from different geographic locations in Thai waters (lanes 1 - 11). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

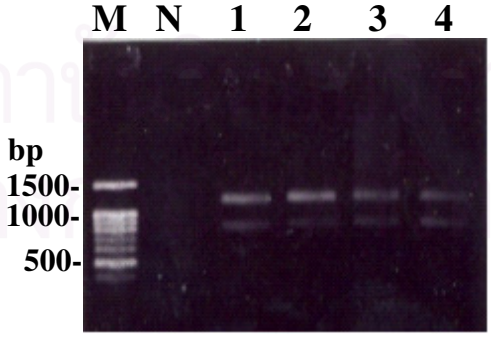

Figure 3.8 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of *laminin-β chain* against genomic DNA of *P. monodon* from different geographic locations in Thai waters (lanes 1 - 11). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

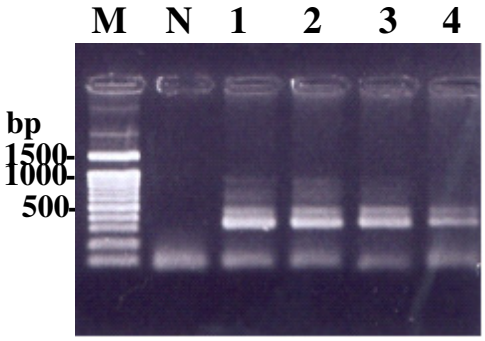

Figure 3.9 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of *vitellogenin* against genomic DNA of *P. monodon* from different geographic locations in Thai waters (lanes 1 - 11). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

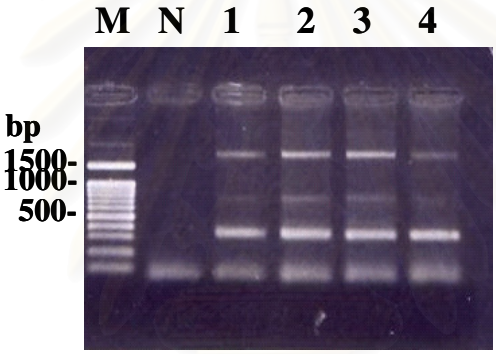

Figure 3.10 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of *BCS-2* against genomic DNA of *P. monodon* from different geographic locations in Thai waters (lanes 1 - 11). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

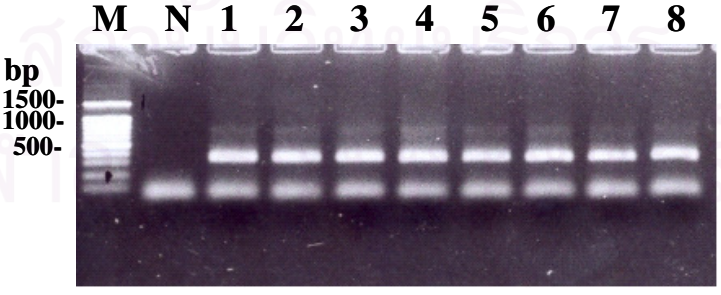

Figure 3.11 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of *RNA helicase* against genomic DNA of *P. monodon* from different geographic locations in Thai waters (Lanes 1 - 11). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

The main objective for this research is to identify single nucleotide polymorphism (SNP) of functionally important genes. SSCP which is convenient and cost-effective was used for genotying of wild *P. monodon* (N=15). The gene homologues exhibiting polymorphism can be further characterized by DNA sequencing.

The migration rate of the double strand DNA through low crosslink native polyacrylamide gels is faster than that of the single strand DNA. While *USO1* and *anilin* homologues were monomorphic (Figures 3.12 and 3.13), almost all of the amplified gene segments were polymorphic (Figure 3.14 – 3.21). Thirty-seven gene segments, for instance, homologues of *LTB4DH, RUVB-like protein, phosphatidylinositol-4 kinase* showed polymorphic SSCP patterns when screened against genomic DNA of wild *P. monodon* originating from different geographic locations in Thai waters.

The amplification product of various genes revealed sequence polymorphism reflecting from polymorphic SSCP patterns (Figure 3.12) Polymorphic patterns of *splicing factor 3a, subunit 1* (Figure 3.14) were observed but the patterns cannot be scored as co-dominant markers (homozygotes and heterozygotes cannot be differentiated). On the other hand, genes homologues of *phosphoglucose isomerase* (Figure 3.15), *solute carrier family 3 member 2* (Figure 3.16), *rasputin* (Figure 3.17), *RNA helicase* (Figure 3.18), *heterogeneous nuclear ribonucleoprotein 87F* (Figure 3.19), *laminin-β chain* (Figure 3.20) and *FV-42* (Figure 3.21) can be scored as co-dominant markers.

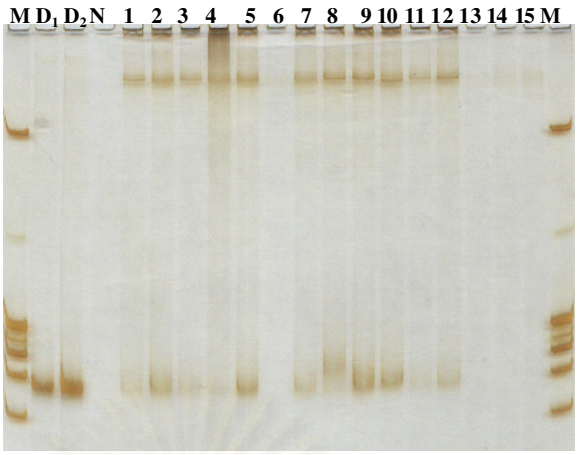

Figure 3.12 SSCP pattern of a *homologue* of *USO1* from of *P. monodon* broodstock originating from Chumphon (lanes 1-3), Phangnga (lanes 4-6), Satun (lanes 7-9), Trang (lanes 10-12) and Trat (lanes 13-15). A monomorphic pattern was observed. Lanes M, D_1 D_2 and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and negative control product respectively.

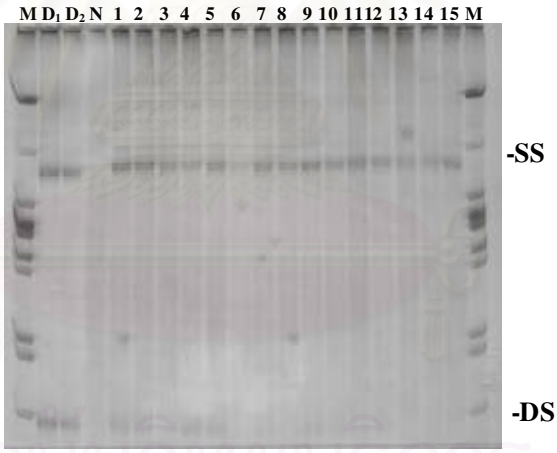

Figure 3.13 SSCP pattern of an *anilin* homologue of *P. monodon* broodstock originating from Chumphon (lanes 1-3), Phangnga (lanes 4-6), Satun (lanes 7-9), Trang (lanes 10-12) and Trat (lanes 13-15).... A monomorphic pattern was observed. Lanes M, D_1 D_2 and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and negative control product respectively.

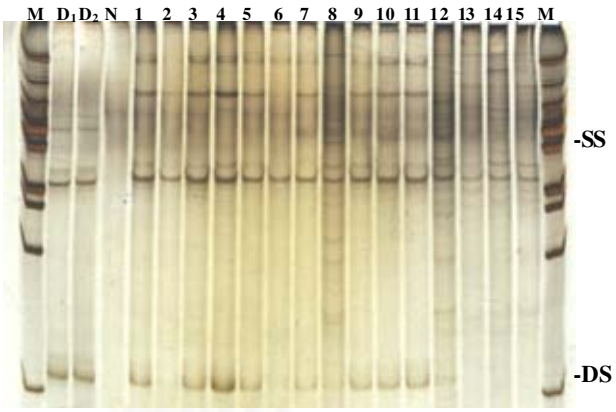

Figure 3.14 SSCP patterns of a *splicing factor 3a, subunit 1* homologue of *P. monodon* broodstock originating from Chumphon (lanes 1-3), Phangnga (lanes 4-6), Satun (lanes 7-9), Trang (lanes 10-12) and Trat (lanes 13-15). Four SSCP genotypes (pattern I, lanes 1-7, 9-11; II, lanes 8&12; III, lanes 13&15; IV, lanes 14) were observed. Lanes M, D_1 D_2 and N are a 100 bp DNA marker, the non-denatured PCR product (double strand control) and the negative control product respectively.

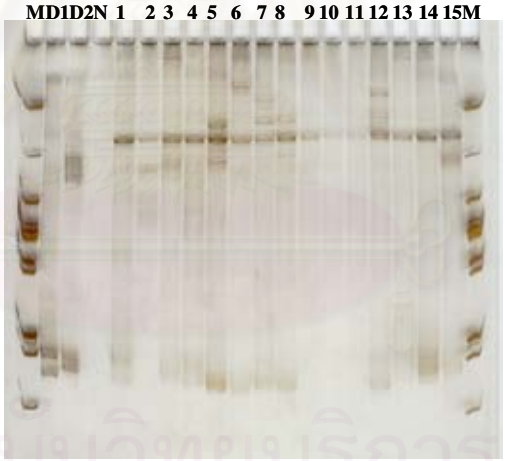

Figure 3.15 SSCP pattern of a homologue of *phosphoglucose isomerase* of *P. monodon* broodstock originating from Chumphon (lanes 1-3), Phangnga (lanes 4-6), Satun (lanes 7-9), Trang (lanes 10-12) and Trat (lanes 13-15). Eight SSCP genotypes (pattern I, lanes 1, 4, 9-11, 13-14; II, lane 2; III, lanes 3&15 ; IV, lane 5 ; V, lane 6 ; VI, lane 7 ; VII, lane 8; VIII, lane 12) were observed. Lanes M, D_1 D_2 and N are a 100 bp DNA marker, the non-denatured PCR product (double strand control) and the negative control product respectively.

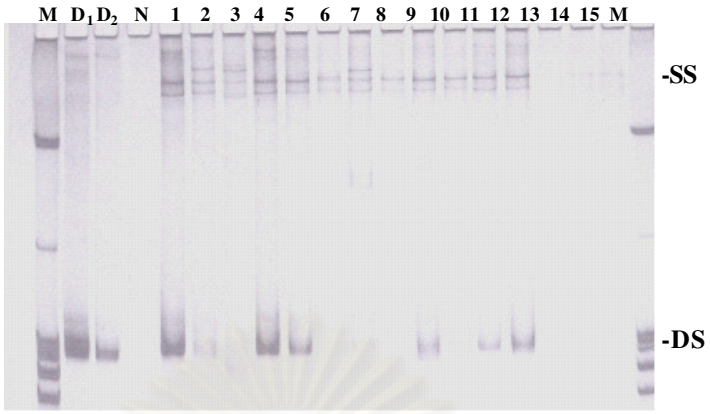

Figure 3.16 SSCP patterns of a *solute carrier family 3 member 2* homologue of *P. monodon* originating from Chumphon (lanes 1-3), Phangnga (lanes 4-6), Satun (lanes 7-9), Trang (lanes 10-12) and Trat (lanes 13-15). Five SSCP genotypes (pattern I, lanes 1& 4 and 5; II, lanes 2, 7, and 17; III, lane 3, IV, lane 6 and V, lanes 8 - 12) were observed. Lanes M, D_1 D_2 and N are a 100 bp DNA marker, the non-denatured PCR product (double strand control) and the negative control product respectively.

Figure 3.17 SSCP pattern of a *rasputin* homologue of *P. monodon* broodstock originating from Chumphon (lanes 1-3), Phangnga (lanes 4-6), Satun (lanes 7-9), Trang (lanes 10-12) and Trat (lanes 13-15).Two SSCP genotypes (pattern I, lanes 3-4, 6, 8, 10, 12; II, lanes 5, 14-15) were observed. Lanes M, D_1 D_2 and N are a 100 bp DNA marker, the non-denatured PCR product (double strand control) and the negative control product respectively.

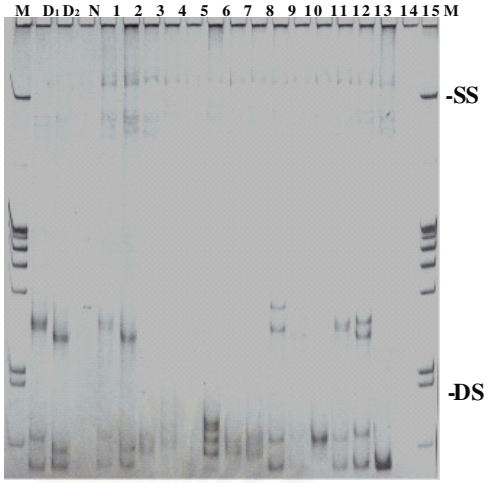

Figure 3.18 SSCP pattern of a *RNA helicase* homologue of *P. monodon* broodstock originating from Chumphon (lanes 1-3), Phangnga (lanes 4-6), Satun (lanes 7-9), Trang (lanes 10-12) and Trat (lanes 13-15). SSCP genotypes polymorphism were observed. Lanes M, D_1 D_2 and N are a 100 bp DNA marker,the non-denatured PCR product (double strand control) and the negative control product respectively.

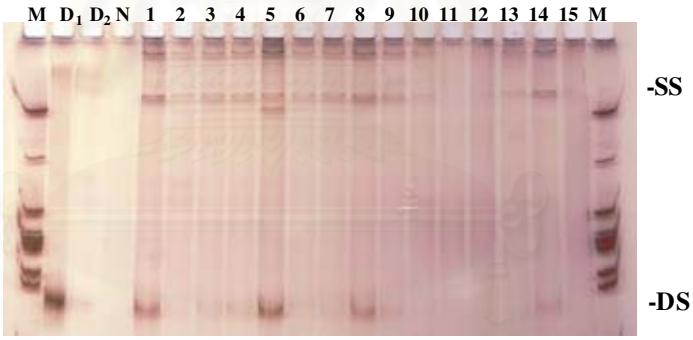

Figure 3.19 SSCP pattern of *a heterogeneous nuclear ribonucleoprotein 87F* homologue of *P. monodon* broodstock originating from Chumphon (lanes 1-3), Phangnga (lanes 4-6), Satun (lanes 7-9), Trang (lanes 10-12) and Trat (lanes 13-15). Two SSCP genotypes (pattern I, lanes 1-4, 6-10, and 13-14; II, lane 5) were observed. Lanes M, D_1 D_2 and N are a 100 bp DNA marker, the non-denatured PCR product (double strand control) and the negative control product respectively.

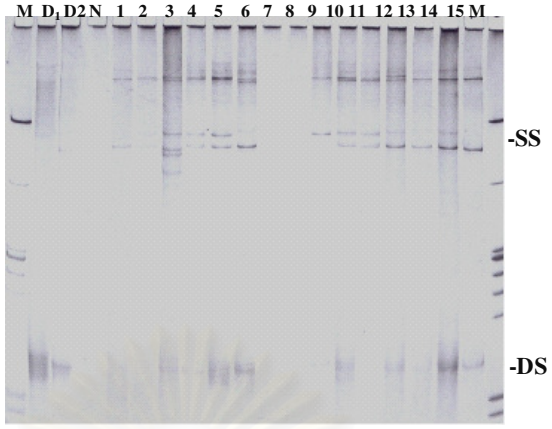

Figure 3.20 SSCP patterns of a *laminin-β chain* homologue of *P. monodon* broodstock originating from Chumphon (lanes 1-3), Phangnga (lanes 4-6), Satun (lanes 7-9), Trang (lanes 10-12) and Trat (lanes 13-15). Four genotypes (pattern I, lanes 1, 6, 13, and 15; II, lanes 2, 4-5, 10-12 and 14; III, lane 3; IV, lane 9) were observed. Lanes M, D_1 D_2 and N are a 100 bp DNA marker, the non-denatured PCR product (double strand control) and the negative control product respectively.

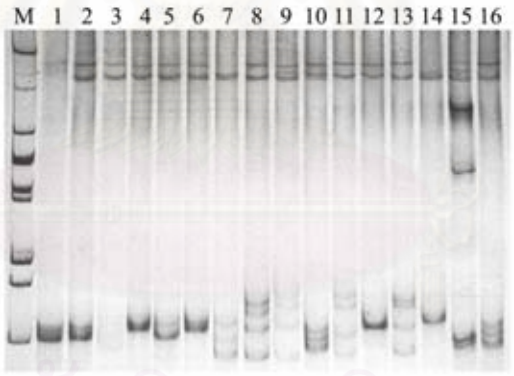

Figure 3.21 SSCP analysis of *FV-42* amplified from genomic DNA of *P. monodon* originating from Trat (lanes 2-4), Chumphon (lanes 5-7), Satun (lanes 8-10), Trang (lanes 11-13) and Phangnga (lanes 14-16) analyzed by a 17.5% (75:1) native polyacrylamide gel. Lane 1 is the non-denatured PCR product.

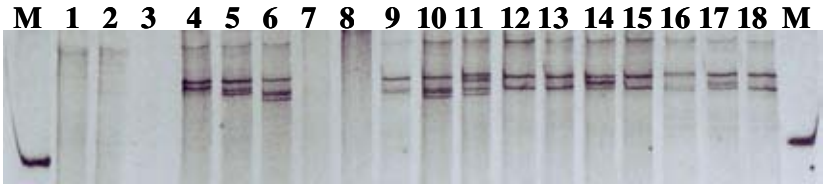

Figure 3.22 SSCP analysis of homologues of *NADP-dependent leukotriene B4 12-hydroxydehydrogenase* (*LTB4DH*) against genomic DNA of natural *P. monodon* (panel A) from Chumphon (lanes 1 – 3), Phangnga (lanes 4 - 6), Satun (lanes 7 - 9), Trang (lanes 10 - 12), Trat (lanes 13 - 15). The PCR product was denatured, size-fractionated through 10% (crosslink = 75:1) and silver-stained. Lanes M, 1 and 2 are a 100 bp DNA marker and the non-denatured PCR product (double strand DNA control), respectively.

Polymorphism of SSCP may be resulted from SNP and/or small indels and reasonably high in several gene segments. These polymorphic genes may be used for identification of SNP and further studies for correlations between SNP and economically important phenotypes like growth rates as well as expression of functional important transcripts. The ability to distinguish homo- and heterozygote states of several genes allowing their conveniently application for construction of the genetic linkage maps in *P. monodon*.

Table 3.1 Expected size, observed size, full length and SSCP results of 108 genes

Gene	Expected size (bp)	Observed size (bp)	Full length cDNA(bp)	SSCP
1. *Y-box protein**	435	NS	951	ND
2. *Hepatocarcinogenesis-related transcription factor* (*X-box binding protein)**	185	185	1116	Polymorphism
3. *Adipose differentiation related protein**	267	-	1275	ND
4. *ESO3 protein***	349	-	429	ND
5. *Exocyst complex component Sec6***	303	-	2214	ND
6. *Rasputin **	248	500	2070	Polymorphism
7. *Survival motor neuron**	245	-	849	ND
8. *Small nuclear ribonucleoprotein D2 – like protein***	288	-	357	ND
9.*Phosphatidylinositol -4 kinase**	335	335	1812	Polymorphism
10. *TATA –binding protein(TBP) -associated factor9***	307	-	729	ND
11. *Proactivator polypeptide precursor (Prosaposin)***	266	266	1575	ND
12. *Hypothetical protein (cyclic nucleotide gated channel beta subunit 1)**	270	270	4017	Polymorphism
13.*Carboxylesterase precursor***	282	-	1641	ND
14. *O-methyltransferase***	399	700	660	Polymorphism
15. *Rab-protein 10 CG17060-PA**	177	-	612	ND
16. *NADP-dependent leukotriene B4 12-hydroxydehydrogenase (LTB4DH)**	230	900	1047	Polymorphism
17. *Phosphoglucose isomerase***	225	450	1659	Polymorphism
18. *PEF- protein with a long N-terminal hydrophobic domain (peflin)***	314	-	852	ND
19. *Adenine nucleotide translocator 2**	239	500	510	Polymorphism
20. *TRAP-like protien precursor***	313	-	894	ND
21. *Hyaluronan receptor**	220	1100	1047	Polymorphism

Table 3.1 (cont.)

Gene	Expected size (bp)	Observed size (bp)	Full length cDNA(bp)	SSCP
22. *Keratinocyte associated protein2**	318	NS	438	ND
23. *Heterogeneous nuclear ribonucleo protein 87F***	298	480	1158	Polymorphism
24. *Laminin-β chain**	244	500	3504	Polymorphism
25. *Ras interacting protein RIPA**	263	1600	2172	ND
26. *U5 small nuclear ribonucleoprotein***	277	-	2199	ND
27. *Stromal membrane associated protein***	306	NS	Not known	ND
28. *Tissue specific transplantation antigen p35B like**	352	NS	963	ND
29. *RUVB- like protein 2**	257	500	1389	Polymorphism
30. *Phosphopyruvate hydratase***	223	223	1320	Polymorphism
31. *Microspherule protein 1***	317	-	1386	ND
32. *Solute carrier family 25, member 14 isoform UCP5S**	298	1000	966	Polymorphism
33. *CG1681-PA**	243	243	711	Polymorphism
34. *Carbonyl reductase1****	457	-	828	ND
35. *Contractile ring component anillin****	199	300	3348	Monomorphism
36. *NADP-ferrihemoprotein reductase; NADPH-cytochroome P450 reductase**	189	-	2037	ND
37. *Dynactin 4****	300	-	549	ND
38. *Cystathionine gamma-lyase****	145	-	1194	ND
39. *Splicing factor 3a,subunit 1***	216	216	2379	Polymorphism
40. *Phosphatidylserine receptor short form**	226	450	1317	Polymorphism
41. *BCS-2****	372	400	738	ND
42. *Proteasome (prosome, macropain)26Ssubunit,ATPase 2****	187	250	1425	ND

Table 3.1 (cont.)

Gene	Expected size (bp)	Observed size (bp)	Full length cDNA(bp)	SSCP
43. *USO1****	314	800	3585	Polymorphism
44. *Small androgen receptor-intercting protein**	345	-	719	ND
45. *Cdc2****	230	-	Not known	ND
46. *Cycilc AMP-regulated like protein***	264	500	Not known	Polymorphism
47. *Vitellogenin* (*VTG*)*	205	400	7758	Polymorphism
48. *SPAPC****	329	329	-	Polymorphism
49. *RNA helicase***	237	250	-	Polymorphism
50. *Death associated protein 5****	199	900	-	ND
51. *Nucleolar phosphatase**	365	365	-	Polymorphism
52. *Probable hormone receptor **	180	-	-	ND
53. *Solute carrier family 3 member 2***	139	NS	-	ND
54. *Mitotic checkpoint****	257	-	-	ND
55. *Serine palmitoyl transferase LCB2 subunit****	178	-	-	ND
56. *Small ubiquitin-like modifier****	362	-	-	ND
57. *Putative cold-induced protein****	372	-	624	ND
58. *Multiple inositol polyphosphate****	174	-	1359	ND
59. *Low molecular weight neurofilament protein XNF-L****	374	-	1449	ND
60. *Cell division protein kinase 7****	381	1000	1038	ND
61. *Adapter-related protein complex 1 beta 1 subunit (β-adeptin 1)****	290	-	2859	ND
62. *Gene flightless-I protein**	324	1000	3768	Polymorphism
63. *Salivary gland secretion 1****	336	-	870	ND
64. *TERA protein **	312	-	-	ND

Table 3.1 (cont.)

Gene	Expected size (bp)	Observed size (bp)	Full length cDNA(bp)	SSCP
65. *Spliceosome-association protein**	279	NS	-	ND
66. *Ovarian lipoprotein receptor**	250	250	-	Polymorphism
67. *Transposase****	390	-	1428	ND
68. *26S proteosome non-ATPase regulatory subunit 3****	140	140	1488	ND
69. *Oncoprotein nm23****	229	450	459	ND
70. *Voltage-dependent anion-selective channel protein 2**	280	800,450	849	ND
71. *Metaxin2****	212	1600	282	ND
72. *Acyl-CoA oxidase* (*ACX3*)***	250	-	-	ND
73. *Synaptobrevin like protein****	291	-	-	ND
74. *Proteasome* (*prosome,macropain*) *subunit,alpha type3****	Not - Know	-	-	ND
75. *Laminin-β chain****	Not - Know	NS	-	ND
76.*Ubiquitin carboxyl-terminal hydrolase14****	Not - Know	-	1497	ND
77. *U3 small nucleolar RNP protein IMP4****	Not - Know	-	876	ND
78. *Cell division protein kinase2****	Not - Know	500	-	ND
79. *SR-Protein kinase****	Not - Know	900	-	ND
80. *Multiprotein bridging factor****	Not - Know	-	-	ND
81. *Arginyl tRNA protein transferase1****	Not - Know	600	-	ND
82. *Ubiquitin isopeptidase ****	Not - Know	600	-	ND
83. *COP9 signalosome subunit5****	Not - Know	-	-	ND
84. *Programmed cell death7****	Not - Know	NS	-	ND
85. *Inhibitor of apoptosis protein****	Not - Know	1000	-	ND
86. *Nudix-type motif9 isofrom A****	Not - Know	500	1050	ND
87. *CyclophilinA ****	Not - Know	800	-	ND

Table 3.1 (cont.)

Gene	Expected size (bp)	Observed size (bp)	Full length cDNA(bp)	SSCP
88. *26S proteasome regulatory particle****	Not – Know	-	-	ND
89. *E1B-55kDa-associated protein5 *****	Not – Know	350	-	ND
90. *Polyadenylate binding protein II****	Not – Know	-	-	ND
91. *SRY-box 7; SOX7 transcription factor**	178	1000	-	Polymorphism
92. *Chk1 checkpoint kinase*	217	-	-	ND
93. *457/OPA01*	324	324	Not known	Polymorphism
94. *428/OPB17*	238	238	Not known	Polymorphism
95. *MI-36*	182	182	Not known	ND
96. *MII-51*	123	123	Not known	Polymorphism
97. *FI-1*	189	-	Not known	ND
98. *FI-40*	220	250	Not known	Polymorphism
99. *FI-6*	265	-	Not known	ND
100. *FII-17*	263	263	Not known	Monomorphism
101. *FIII-4*	342	500	Not known	Monomorphism
102. *FIII-8*	333	333	Not known	Monomorphism
103. *FIII-39*	362	500	Not known	ND
104. *FIV-2*	197	197	Not known	ND
105. *FIV-33*	158	-	Not known	ND
106. *FV-1*	226	300	Not known	Polymorphism
107. *FV-27*	223	223	Not known	Polymorphism
108. *FV-42*	207	207	Not known	Polymorphism

* = EST from ovarian cDNA library, ** = EST from a cDNA library of hemocytes of shrimp stressed with temperature, *** = EST from testis library cDNA library - = no amplification product, ND = not determined, NS = non specific.

3.3 Identification and characterization of SNP by direct sequencing of the PCR product

Direct sequencing of the amplification fragment was carried out to examine the efficiency of SSCP analysis in comparison to DNA sequencing of the same fragment. Accordingly, nucleotide sequences of *P. monodon* exhibited different (or identical) SSCP patterns of a particular gene were compared.

The PCR product of a representative individual representing each SSCP genotype of *NADP-dependent leukotriene-12-hydroxydehydrogenase* (*LTB4DH*), *RUVB-like protein 2, phosphatidylserine receptor short form, solute carrier family 25* was gel-eluted, direct sequenced and multiple aligned.

Six SSCP patterns were found from analysis of the *LTB4DH* segment against genomic DNA of wild *P. monodon* ($N = 15$). All SSCP types were directed sequenced but sequences were obtained from 5 SSCP types. As can be seen from Figure 3.23, several SNP were found and can be used to differentiate individuals possessing different SSCP genotypes exception between S2 and S11(figure 3.23). Based on the fact that the amplification product of this gene was approximately 900 bp in length, direct sequencing of this amplified gene product still did not cover the amplified fragment and polymorphic SNP may be existent in the unidentified portion of this gene segment.

Three SSCP patterns were found from genotyping of *phosphatidylserine receptor* gene segment (450 bp) of wild *P. monodon*. Representatives of these genotypes (C1, C3, S4) were direct sequenced and 380 bp sequences were obtained after multiple alignments (Figure 3.24). Results indicated that C1 and C3 which exhibited different SSCP patterns shared nucleotide sequences. Large sequence divergence was found between C1 & C3 from Chumphon and S4 from Satun.

Likewise, 4 SSCP patterns of *RUVB*-like protein (500 bp) were found from SSCP analysis. A representative individual of each SSCP pattern was further analyzed by direct DNA sequencing and 3 different types of sequences were obtained from multiple aligned 360 bp sequenced were obtained after multiple alignments (Figure 3.25).

A.

```
P7F             ATAGTGCCCTTTGGTTTGTATATTCCTATATGATATTCAATTACTTTTATCTGTTGATAA
S2F             ATAGTGCCCTTTGGTTTGTATATTCCTATATGATATTCAATTACTTTTATCTGTTGATAA
S11F            ATAGTGCCCTTTGGTTTGTATATTCCTATATGATATTCAATTACTTTTATCTGTTGATAA
S4F             ATAGTGCCCTTTGGTTTGTATATTCCTATATGATATTCAATTACTTTTATCTGTTGATAA
T9F             ATAGTGCCCTTTGGTTTGTATATTCCTATATGATATTCAATTACTTTTATCTGTTGATAA
                ************************************************************

P7F             TTATCATTGGTTTTTGTCTTTAGTAATTTAAGGCAATACCATTCCCACATAATGAAATAC
S2F             TTATCATTGGTTTTTGTCTTTAGTAATTTAAGGCAATACCATTCCCACATAATGAAATAC
S11F            TTATCATTGGTTTTTGTCTTTAGTAATTTAAGGCAATACCATTCCCACATAATGAAATAC
S4F             TTATCATTGGTTTTTGTCTCTAGTAATTTAAGGCAATACCATTCCCACATAATGAAATAC
T9F             TTATCATTGGTTTTTGTCTCTAGTAATTTAAGGCAATACCATTCCCACATAATGAAATAC
                ******************* ****************************************

P7F             AAAAAATCCTGTTATTCTAATGTTCTGGCAAATGATTAACTAACCTTTTTCAGTACTGCAA
S2F             AAGAATCCTGTTATTCTAATGTTCTGGCAAATGATTAACTAACCTTTTTCAGTACTGCAA
S11F            AAGAATCCTGTTATTCTAATGTTCTGGCAAATGATTAACTAACCTTTTTCAGTACTGCAA
S4F             AAGAAACCTGTTATTCTAATGTTCTGGCAAATGATTAACTAACCTTTTTCAGTACTGCAA
T9F             AAGAATCCTGTTATTCTAATGTTCTGGCAAATGATTAACTAACCTTTTTTCGTACTGCAA
                ** ** ****************************************  *********
P7F             TGTCACTAATGATTTACAAATGCTTTTGCAGATGTCATTATTGAAGCTGAATTTTTGAGT
S2F             TGTCACTAATGATTTACAAATGCTTTTGCAGATGTCATTATTGAAGCTGAATTTTTGAGT
S11F            TGTCACTAATGATTTACAAATGTTTTTGCAGATGTCATTATTGAAGCTGAATTTTTGAGT
S4F             TGTCACTAATGATTTACAAATGCTTTTGCAGATGTCATTATTGAAGCTGAATTTTTGAGT
T9F             TGTCACTAATGATTATCAAATGCTTTTGCAGATGTCATTGTTGACGCTCATCTTTGGAGT
                ************** ****** *************** **** *** *  *** ****

P7F             GTAGATCCATACATGCGGTACATGATCAAGCAAATCCCTCTTGATGTACCTGT
S2F             GTAGATCCATACATGCGGTACATGATCAAGCAAATCCCTCTTGATGTACCTGT
S11F            GTAGATCCATACATGCGGTACATGATCAAGCAAATCCCTCTTGATGTACCTGT
S4F             GTAGATCCATACATGCGGTACATGATCAAGCAAATCCCTCTTGATGTACCTGT
T9F             GTATATCCA-ACACGCGGTACATGAACAAGCAAATCCCTCTTGATGTACCTGT
                *** ***** *** *********** **************************
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B

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P7R             ATT-CTTCATCATAAC-TCTCCAAA-GTATTTGTATCACAAAGAAAATATGCTTACATGG
S2R             ATT-CTTCATCATAAC-TCTCCAAA-GTATTTGTATCACAAAGAAAATATGCTTACATGG
S11R            ATT-CTTCATCATAAC-TCTCCAAA-GTATTTGTATCACAAAGAAAATATGCTTACATGG
S4R             ATT-CTTCATCATAAC-TCTCCAAA-GTATTTGTATCACAAAGAAAATATGCTTACATGG
T9R             ATTGCTTCATCATAACATCTCCAAATGCATTTGTATCACAAAAAAAATATGCTTACATGG
                *** ************ ******** * ************** *****************

P7R             GAGA-GAACTAAAGGAAGAAGTACATAATGATTTACTAAACTTTTTGTTTGTACTTCCAG
S2R             GAGA-GAACTAAAGGAAGAAGTACATAATGATTTACTAAACTTTTTGTTTGTACTTCCAG
S11R            GAGA-GAACTAAAGGAAGAAGTACATAATGATTTACTAAACTTTTTGTTTGTACTTCCAG
S4R             GAGA-GAACTAAAGGAAGAAGTACATAATGATTTACTAAACTTTTTGTTTGTACTTCCAG
T9R             GAAAAGAACTAAAGGAAGAAGTACATAATGATTTACTAAACTTTTTGGTTGTACTTCCAG
                ** * ************************************** ************

P7R             TGTGCAATTGTTAATTTG-TCTATATA-TTTATGCAT-TTTTTA-AACAAAAATACATGG
S2R             TGTGCAATTGTTAATTTG-TCTATATA-TTTATGCAT-TTTTTA-AACAAAAATACATGG
S11R            TGTGCAATTGTTAATTTG-TCTATATA-TTTATGCAT-TTTTTA-AACAAAAATACATGG
S4R             TGTGCAATTGTTAATTTG-TCTATATA-TTTATGCAT-TTTTTA-AACAAAAATACATGG
T9R             TGAGCAATTGATCATTTGCTCTATATACTTTATGCATATTTTTACAACAAAAATACATGG
                ** ******* * ***** ******** ********* ****** ***************

P7R             CTCAATATTTACAATAATGTAGGACATTTCCTTTAGAGGGATTTTTTTTT
S2R             CTCAATATTTACAATAATGTAGGACATTTCCTTTAGAGGGATTTTTTTTT
S11R            CTCAATATTTACAATAATGTAGGACATTTCCTTTAGAGGGATTTTTTTTT
S4R             CTCAATATTCACAATAATGTAGGACATTTCCTTTAGAGGGATTTTTTTTT
T9R             CTCAATATTCACAATAATGAAGGACATTTCCTTTAGAGGGATTTTTTTTT
                ********* ********* ****************************
```

Figure 3.23 Multiple alignments of *LTB4DH* amplified from genomic DNA of representative individuals of *P. monodon* broodstock originating from Phangnga (P7), Satun (S2 and S11) and Trang (T9) which exhibit different SSCP genotypes using *LTB4DH* forward (F) and reverse (R) primers as the direct sequencing primers.

Figure 3.24 Multiple alignments of *phosphatidylserine receptor* amplified from genomic DNA of representative individuals of *P. monodon* broodstock originating from Chumphon (C1 and C3) and Satun (S4) which exhibit different SSCP genotypes using the original primer forward and reverse primers as the direct sequencing primer.

```
c1              AATGGTCTGGCCCAGTCTTTGGGCCCAGACACTCCTTTCACGGCTGTCTCTGCTTCAGAG
p10             AATGGTCTGGCCCAGTCTTTGGGCCCAGACACTCCTTTCACGGCTGTCTCTGCTTCAGAG
s4              AATGGTCTGGCCCAGTCTTTAGGCCCAGACACTCCTTTCACGGCTGTCTCTGCTTCAGAG
c3              AATGTTTGGGCCCATTTTTGGGGCCCAGCCATTCCTTTCAGGGCGGTTTTTGTTTCAGAG
                **** *  ****** * **  ******* ** ******** *** ** * ** *******

c1              ATTTACAGGCAGGAACTCTTTTTTTTTCTGTTGGTTTTTGATATGTTATAAAAAATGTAA
p10             ATTTACAGGCAGGAACTCTTTTTTTTTCTGTTGGTTTTTGATATGTTATAAAAAATGTAA
s4              ATTTACAGGCAGGAACTCTTTTTTTTTCTGTTGGTTTTTGATATGTTATAAAAAATGTAA
c3              ATTTCCAGCCAGGAATTTTTTTTTTTCTGTTGGTTTTTGATATGTTATAAAAAATGTAA
                **** *** ****** * ****************************************

c1              TAATCTGAGTAAAAAATTTTCCTGTCCCTCCATTATAGTAGGCCATAGAAGATTTTTCTT
p10             TAATCTGAGTAAAAAATTTTCCTGTCCCTCCATTATAGTAGGCCATAGAAGATTTTTCTT
s4              TAATCTGAGTAAAAAATTTTCCTGTCCCTCCATTATAGTAGGCCATAGAAGATTTTTCTT
c3              TAATCTGAGTAAAAAATTTTCCTGTCCCTCCATTATAGTAGGCCATAGAAGATTTTTCTT
                ************************************************************

c1              TTTCTTTGTTTCAAGGTTTTGATAGGCTTAACCACCCACAATATTTAAGTGTTCATAATG
p10             TTTCTTTGTTTCAAGGTTTTGATAGGCTTAACCACCCACAATATTTAAGTGTTCATAATG
s4              TTTCTTTGTTTCAAGGTTTTGATAGGCTTAACCACCCACAATATTTAAGTGTTCATAATG
c3              TTTCTTTGTTTCAAGGTTTTGATAGGCTTAACCACCCACAATATTTAAGTGTTCATAATG
                ************************************************************

c1              TAAAATTTTATGTTATTTGTGTCTGTTATATGAAACAAGTGTCTTTTTCTACAGCTTGGG
p10             TAAAATTTTATGTTATTTGTGTCTGTTATATGAAACAAGTGTCTTTTTCTACAGCTTGGG
s4              TAAAATTTTATGTTATTTGTGTCTGTTATATGAAACAAGTGTCTTTTTCTACAGCTTGGG
c3              TAAAATTTTATGTTATTTGTGTCTGTTATATGAAACAAGTGTCTTTTTCTACAGCTTGGG
                ************************************************************

c1              AATGTCTAAGAC
p10             AATGTCTAAGAC
s4              AATGTCTAAGAC
c3              AATGTCTAAGAC
                ************
```

Fig. 3.25 Multiple alignments of *RUVB-like protein* amplified from genomic DNA of representative individuals of *P. monodon* broodstock originating from Chumphon (C1 and C3), Phangnga (P10) and Satun (S4) which exhibit different SSCP genotypes using the original forward and reverse primers as the direct sequencing primers.

Five SSCP patterns were found from the *solute carrier family 25* gene segment (1000 bp). Representatives of these genotypes (C1, P28, S11) were further analyzed (Figure 3.26). Results indicated different types of DNA sequences of shrimps exhibiting different SSCP genotypes.

Results from the present study indicated variability of nucleotide sequences of a particular gene previously revealed by SSCP analysis. This suggested that SSCP analysis which is an alternative cost-effective technique of DNA sequencing and is a powerful technique to initially identify SNP in functionally important genes of *P. monodon*.

A.

```
c1/F            GTGGAGTCTTTTGTGGATACG-GTGTTGTAGTATAGGCGTTTGTAGCTGGGGGACGGTAG
S11/F           GTGGAGTCTTTTGTGGATACG-GTGTTGTAGTATAGGCGTTTGTAGCTGGGGGACGGTAG
P28/F           GTGGAGTCTTTTGTGGATACGCGTGTTGTAGTATAGGCATTTGTAGCTGGGGGACGGTAG
                ********************* *************** ********************

c1/F            CCTAGAGAGAGAGAGAATGCGGATATACTTTGTGCTACCTTTTGAATACGTTTAGTTAAG
S11/F           CCTAGAGAGAGATAGAATGCGGATATACTTTGTGCTACCTTTTGAATACGTTTAGTTAAG
P28/F           CCCAGAGAGAGAGAGAATGCGGATATCCTTTGCGCTACCTTTTGAATACGTTTAGTTAAG
                ** ********* ************* ***** ***************************

c1/F            TCAGCGAGGATGAAATAAAATGAAGAAAATGCAAAAGTGGTGGATATTGATTTTATTATT
S11/F           TCAGCGAGGATGAAATAAAATGAAGAAAATGCAAAAGTGGTGGATATTGATTTTATTATT
P28/F           TCAGCGAGGATGAAATAAAATGAAGAAAATGCAAAAGTGGTGGATATTGATTTTATTATT
                ************************************************************

c1/F            CTTTCATTGCAGTATTGCTCCAGCTGTCCTCCGTCAGGCAACATATGGGACCATCAAATT
S11/F           CTTTCATTGCAGTATTGCTCCAGCTGTCCTCCGTCAGGCAACATATGGGACCATCAAATT
P28/F           CTTTCATTGCAGTATTGCTCCAGCTGTCCTCCGTCAGGCAACATATGGGACCATCAAATT
                ************************************************************


c1/F            TGGCATTTATTACTCCCTGAAACAGATGCTAACAAAAAATCCCAATGAAGAGAGATTGGG
S11/F           TGGCATTTATTACTCCCTGAAACAGATGCTAACAAAAAATCCCAATGAAGAGAGATTGGG
P28/F           TGGCATTTATTACTCCCTGAAACAGATGCTAACAAAAAATCCCAATGAAGAGAGATTGGG
                ************************************************************

c1/F            CATCAATGTTTTTTGTGCAGTCACTGCAGGTATTGAAATACTCTACATTTGTCATATCAG
S11/F           CATCAATGTTTTTTGTGCAGTCACTGCAGGTATTGAAATACTCTACATTTGTCATATCAG
P28/F           CATCAATGTTTTTTGTGCAGTCACTGCAGGTATTGAAATACTCTACATTTGTCATATCAG
                ************************************************************

c1/F            ATAAAGATCTCTTTGCCTTGAATCATCCACAATACAAATGTAAAAAGTTACAAGTTAGAA
S11/F           ATAAAGATCTCTTTGCCTTGAATCATCCACAATACAAATGTAAAAAGTTACAAGTTAGAA
P28/F           ATAAAGATCTCTTTGCCTTGAATCATCCACAATACAAATGTAAAAAGTTACAAGTTAGAA
                ************************************************************

c1/F            TGATGTCATAATCTTCAGAATGACATGTAGTTCTAATGACATTCCAAATCCCTTGTGATT
S11/F           TGATGTCATAATCTTCAGAATGACATGTAGTTCTAATGACATACCAAATCCCTTGTGATT
P28/F           TGATGTCATAATCTTCAGAATGACATGTAGTTCTAATGACATTCCAAATCCCTTGTGATT
                ****************************************** *****************

c1/F            TGCATCAAAGAGGATATACACATCAGATGTAGTACAAACACCACATTACATAGTTGAATG
S11/F           TGCATCAAAGAGGATATACACATCAGATGTAGTACAAACACCACATTACATAGTTGAATG
P28/F           TGCATCAAAGAGGATATACACATCAGATGTAGTACAAACACCACTTTACATATTTGAATG
                ****************************************** ******* *******

c1/F            TGTGATAGTGAGTGACATAGCAAAGATCATTGCCTCATTCTCAACTTTAGAACTTTTTAG
S11/F           TGTGATAGTGAGTGACATAGCAAAGATCATTGCCTCATTCTCAACTTTAGAACTTTTTAG
P28/F           TGTGATAGTGAGTGACATAGCAAAGATCATTGCCTCATTCTCAACTTCAGAACTTTTTAG
                ************************************************ ************

c1/F            TATGCATACCATTAGACAATGTGACTGTGGTTGTCCTGGATACCATAAATATACTGATGA
S11/F           TATGCATACCATTAGACAATGTGACTGTGGTTGTCCTGGATACCATAAATATACTGATGA
P28/F           TATGCATACCATTAAACAATGTGACTGTGGTTGTCCTGGATACCATAAATATCCTGATGA
                ************** ************************************ *******

c1/F            ATATTATTCATAGGGGCATTGAATGGGAGAAGAGTGCATTAACCATTTATCCGAAAATGG
S11/F           ATATTATTCATAGGGGCATTGAATGGGAGAAGAGTGCATTAACCATTTATCCGAAAATGG
P28/F           ATATTATTCATTGGGGCCTTAAATGGGAAAAAAGTGCATTAACCCTTTATCCGAAAAGGG
                *********** ***** ** ******* ** ************ ************ **
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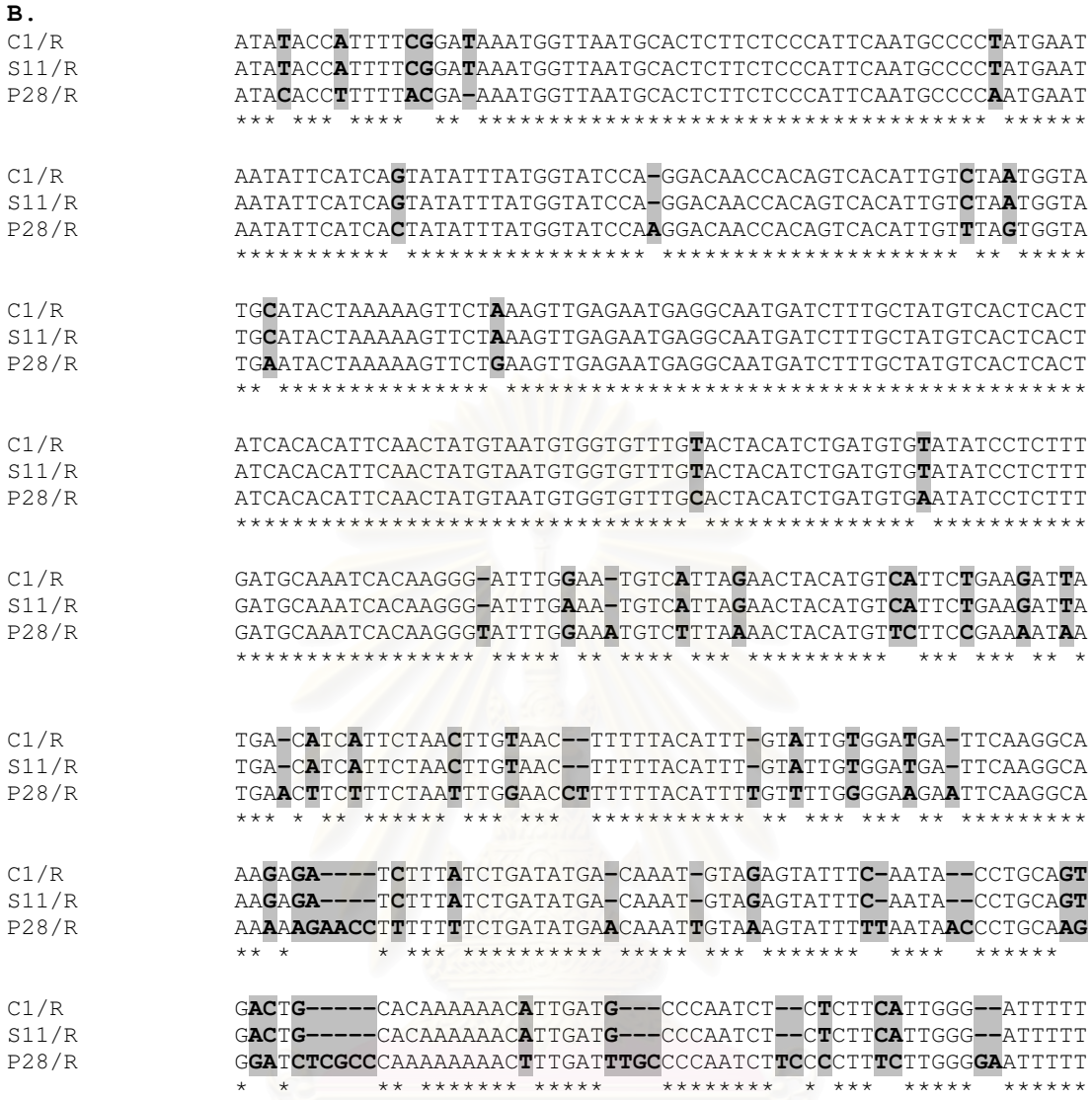

Figure 3.26 Multiple alignments of *solute carrier family 3 member 2* amplified from genomic DNA of representative individuals of *P. monodon* broodstock originating from Chumphon (C1), Phangnga (P28) and Satun (S11) which exhibit different SSCP genotypes using the original forward (F) and reverse (R) primers as the direct sequencing primers.

3.4 Association between SNP by SSCP of *NADP-dependent leukotriene B4 12-hydroxydehydrogenase* (also called *15-oxoprostaglandin 13-reductase*), *X-box binding protein* and *RUVB-like protein* and the growth rate of a G2 family of *P. monodon*

3.4.1 Secondary screening of polymorphic genes using genomic DNA of the G2 family of *P. monodon*

Five hundred individuals of the 2nd generation of a domesticated family of *P. monodon* (132 days of age, $\overline{X}$ = 18.8609 ± 4.64 g) were sampled. Shrimp exhibiting ten percent from the top (N = 50, $\overline{X}$ = 26.86 ± 3.33 g) and the bottom (N = 50, $\overline{X}$ = 10.71 ± 2.02 g) according to the body weight were selected and used for preliminary analysis of association between SSCP genotypes (also called SNP by SSCP) and the growth rate of juvenile *P. monodon*.

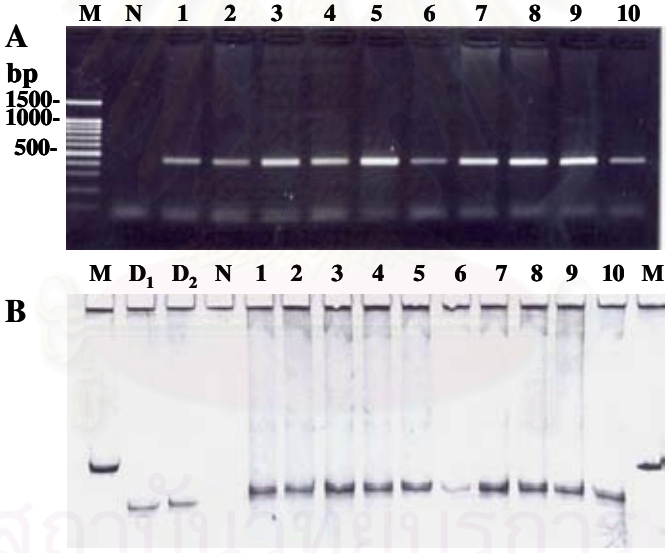

Figure 3.27 The amplified product (A) and its SSCP pattern (B) of a homologue of *phoshpatidylserine receptor* of a full-sib domesticated (G2) family of *P. monodon* exhibiting fast-growing (lanes 1 - 5) and slow-growing (lanes 6 - 10) rates. The PCR product was denatured, size-fractionated through 12.5% (crosslink = 37.5:1) and silver-stained. Lanes M, D1, D2 and N are a 100 bp DNA marker and the non-denatured PCR product (double strand DNA control) and the negative control, respectively.

Nineteen polymorphic gene segments previously screened with natural *P. monodon* (*N* = 15) were applied for genotyping of the G2 family of *P. monodon* (*N* = 5 each for presumably fast- and slow-growing shrimp at 132 days of age, Figure 3.27-3.32). Seven genes (*FII17*, *FIII4*, *FIII8*, *solute carrier family 3 member 2, DDPG*, *phosphatidylinositol 4 kinase* and *phosphatidylserine receptor short form*) did not revealed polymorphic patterns in these specimens whereas the remaining gene homologues showed lower polymorphic SSCP patterns in the G2 family than did the wild shrimp (Table 3.2).

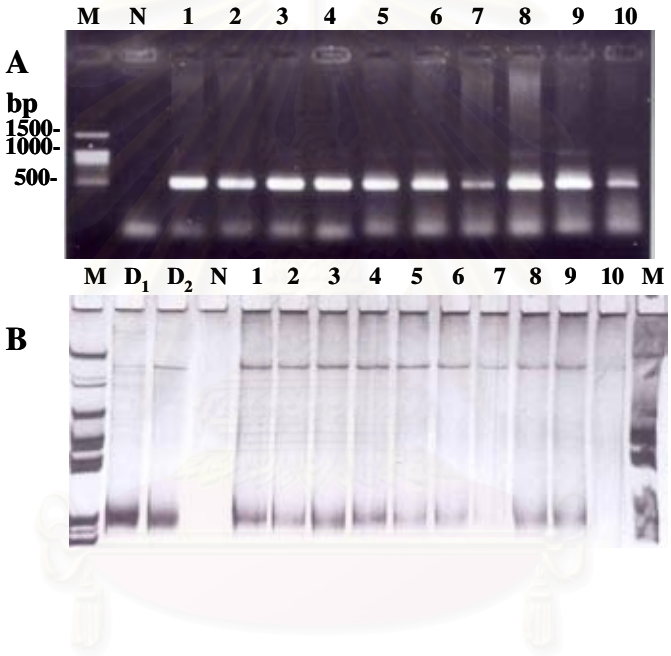

Figure 3.28 The amplified product (A) and its SSCP pattern (B) of *FIII-4* of a full-sib domesticated (G2) family of *P. monodon* exhibiting fast-growing (lanes 1 - 5) and slow-growing (lanes 6 - 10) rates. The PCR product was denatured, size-fractionated through 15% (crosslink = 37.5:1) and silver-stained. Lanes M, D1, D2 and N are a 100 bp DNA marker and the non-denatured PCR product (double strand DNA control) and the negative control, respectively.

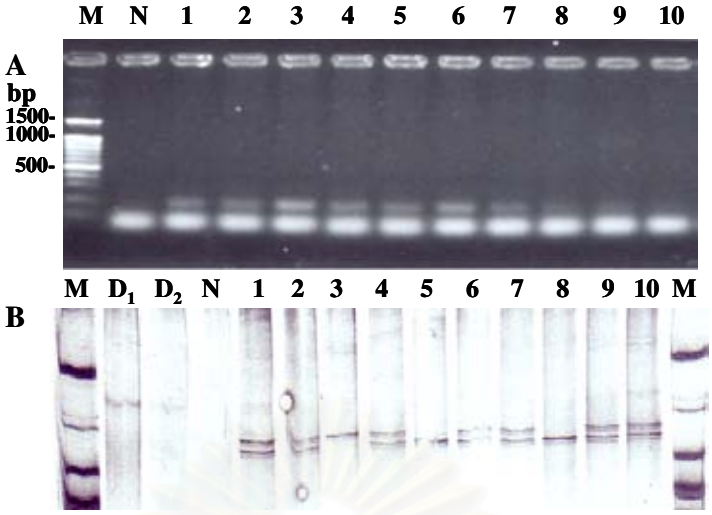

Figure 3.29 The amplified product (A) and its SSCP pattern (B) of a homologue of *X-box binding protein* of a full-sib domesticated (G2) family of *P. monodon* exhibiting fast-growing (lanes 1 - 5) and slow-growing (lanes 6 - 10) rates. The PCR product was denatured, size-fractionated through 17.5% (crosslink = 37.5:1) and silver-stained. Lanes M, D1, D2 and N are a 100 bp DNA marker and the non-denatured PCR product (double strand DNA control) and the negative control, respectively.

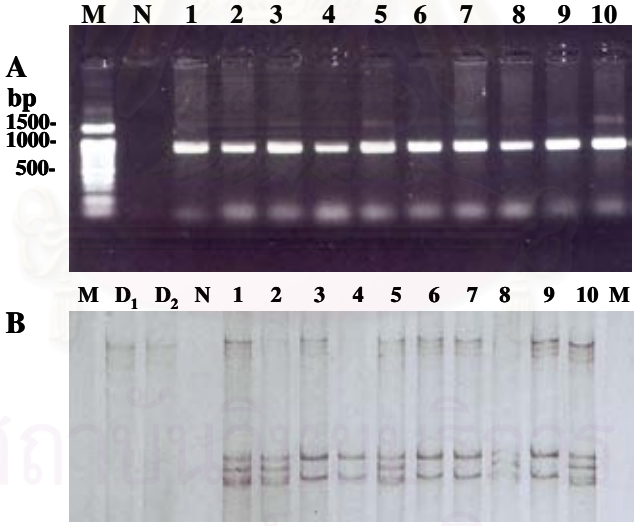

Figure 3.30 The amplified product (A) and its SSCP pattern (B) of a homologue of *LTB4DH* of a full-sib domesticated (G2) family of *P. monodon* exhibiting fast-growing (lanes 1 - 5) and slow-growing (lanes 6 - 10) rates. The PCR product was denatured, size-fractionated through 10% (crosslink = 75:1) and silver-stained. Lanes M, D1, D2 and N are a 100 bp DNA marker and the non-denatured PCR product (double strand DNA control) and the negative control, respectively.

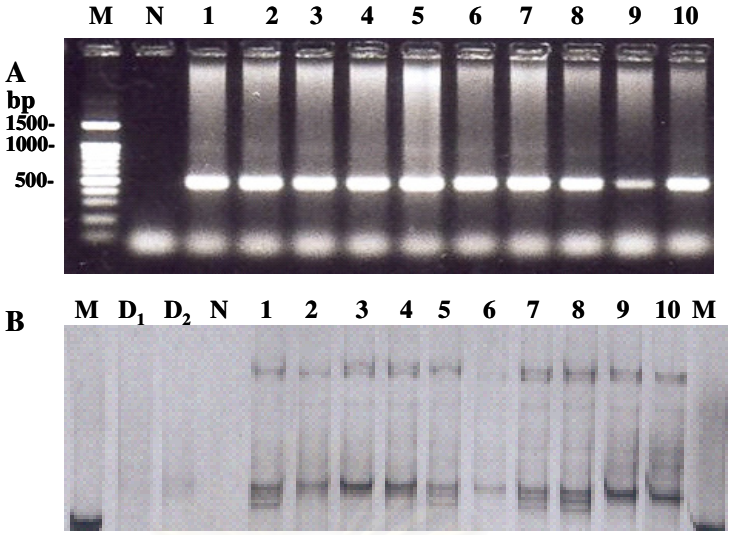

Figure 3.31 The amplified product (A) and its SSCP pattern (B) of a homologue of *RUVB-like protein* of a full-sib domesticated (G2) family of *P. monodon* exhibiting fast-growing (lanes 1 - 5) and slow-growing (lanes 6 - 10) rates. The PCR product was denatured, size-fractionated through 10% (crosslink = 75:1) and silver-stained. Lanes M, D1, D2 and N are a 100 bp DNA marker and the non-denatured PCR product (double strand DNA control) and the negative control,

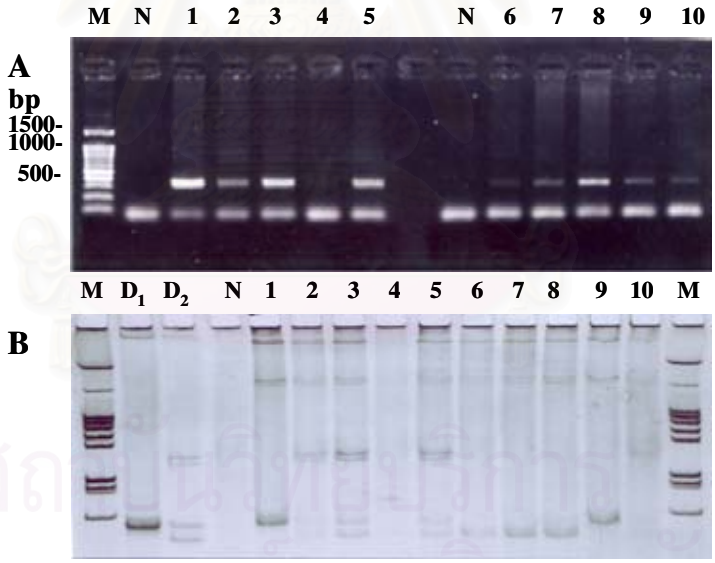

Figure 3.32 The amplified product (A) and its SSCP pattern (B) of a homologue of *phosphatidylinositol-4 kinase* of a full-sib domesticated (G2) family of *P. monodon* exhibiting fast-growing (lanes 1 - 5) and slow-growing (lanes 6 - 10) rates. The PCR product was denatured, size-fractionated through 12.5% (crosslink = 37.5:1) and silver-stained. Lanes M, D1, D2 and N are a 100 bp DNA marker and the non-denatured PCR product (double strand DNA control) and the negative control, respectively.

Table 3.2 Numbers of SSCP genotypes of gene homologues in wild and a G2 family of *P. monodon*

Gene	No. of SSCP genotypes	
	Wild (*N* = 15)	**G2 family (*N* = 10)**
Solute carrier family 3 member 2	4	1
Dolichyl diphosphooligocharide protein glycotransferase	3	1
NADP-dependent leukotriene B4 12-hydroxydehydrogenase (15-oxoprostaglandin 13-reductase)	5	2
Phosphatidylserine receptor short form	2	1
RUVB- like protein	4	3
X-box binding protein	7	3

Accordingly, *X-box binding protein* (Figure 3.29), *LTB4DH* (Figure 3.30)and *RUVB-like protein* (Figure 3.31) gene homologue are subjected to association analysis of SNP by SSCP and the age-specific growth rates of *P. monodon* (*N* = 60, 61 and 75, respectively).

Results did not indicate significant association between the body weight of 132-day-old *P. monodon* and SSCP genotypes of *LTB4DH* (2 genotype) and *X-box binding protein* (3 genotype). Nevertheless, a statistical significance between genotypes and phenotypes was found in presumably fast growing shrimps exhibiting genotypes 2 and genotype 1 of *RUVB-like protein* ($P < 0.05$, Table 3.3). A representative individual of each genotype of *RUVB-like protein* was direct sequenced and the nucleotide sequence confirmed sequence divergence identified by SSCP analysis (Figure 3.33).

Table 3.3 Association analysis between the body weight of juveniles of the second generation (G2) of *P. monodon* and SSCP genotypes of *LTB4DH*, *RUVB like protein* and *X-box binding protein*

Gene/genotype	Group	*N*	Average body weight ± SD	Average body weight ± SD
LTB4DH				
I	Slow-growing	17	10.5882 ± 2.4764[a]	
II	Slow-growing	13	10.7692 ± 1.3009 [a]	
I	Fast-growing	15		27.2667 ± 4.7580[b]
II	Fast-growing	15		28.0667 ± 2.9633[b]
X-box protein				
I	Slow-growing	18	11.3636 ± 1.1201[a]	
II	Slow-growing	11	10.1389 ± 2.3752[a]	
III	Slow-growing	9	11.6667 ± 1.0000[a]	
I	Fast-growing	4		27.2727 ± 3.4667[b]
II	Fast-growing	11		27.3043 ± 4.0388[b]
III	Fast-growing	23		26.0000 ± 1.8257[b]
RUVB*				
I	Slow-growing	13	10.8462 ± 2.0350[a]	
II	Slow-growing	17	11.2647 ± 1.1742[a]	
I	Fast-growing	15		26.1333± 4.5566[b]
II	Fast-growing	16		28.6875 ± 2.3564[c]

The same superscripts indicate that the body weight of shrimps possessing different genotypes were not significantly different (P > 0.05).

```
93R             TTTTGCAGGTTCGGCAGTTTTCCATGGTGGCCCACCCGGCCCCGGCCAGGCAACCATAGG
448R            TTTTGCAGGTCCGGCCGTTTTCCATTGTGGCCCACCCGGCCCCGGCCAGGCAACCCTAAG
                ********** **** ********* *************************** ** *

93R             TAATGGTTTGGGCCCGTTTTTGGGGCCCGACCCTCCTTTTACGGGTGTTTTTGGTTTAGA
448R            TAAAGGTTTGGGCCCGTTTTTTGGGCCCGACCCTCCTTTTCCGGGTGTTTTTGGTTTAGA
                *** ***************** ***************** ********************

93R             GATTTTCCGGCCGGGAATTTTTTTTTTTTTGTGGGGGTTTGAGATGTTATAAAAAATGTA
448R            GATTTTCCGGCCGGGAATTTTTTTTTTTTTGTGGGGGTTTGAGATGTTATAAAAAATGTA
                ************************************************************

93R             ATAATCTGAGTAAAAAATTTTCCTGTCCCTCCATTATAGTAGGCCATAGAAGATTTTTCT
448R            ATAATCTGAGTAAAAATTTTTCCTGTCCCTCCATTATAGTAGGCCATAGAAGATTTTTCT
                *************** ****************************************

93R             TTTTCTTTGTTTCAAGGTTTTGATAGGCTTAACCACCCACAATATTTAAGTGTTCATAAT
448R            TTTTCTTTGTTTCAAGGTTTTGATAGGCTTAACCACCCACAATATTTAAGTGTTCATAAT
                ************************************************************

93R             GTAAAATTTTATGTTATTTGTGTCTGTTATATGAAACAAGTGTCTTTTTCTACAGCTTGG
448R            GTAAAATTTTATGTTATTTGTGTCTGTTATATGAAACAAGTGTCTTTTTCTACAGCTTGG
                ************************************************************

93R             GAATGTCTAAGACGCGAGGTATTAACACAA-GCTTTCT-GACGTTCCATCGGGAATGTTC
448R            GAATGTCTAAGAC-CGAGGTATTAACACANAGCTTTCTAGACGATCCATCCGGAATGTCT
                ************* *************** ******* **** ****** *******

93R             --GATAGTGGCG--TATCTT-CTGCAGTGCGAGGG-
448R            CGGATAGTGGAGGGTATCATACTGCAGTGTGATGGG
                  ******** *  **** * ******** ** **
```

Figure 3.33 Alignments of direct sequenced *RUVB-like protein* amplified from genomic DNA of representative individuals of juvenile *P. monodon* (G2) exhibiting SSCP genotypes I (93R) and II (448R) usind .

3.4.2 Association between SNP by SSCP of *RUVB like protein* and the growth rate of commercially cultured P. *monodon* juveniles

The G2 family used in the experiments was established from the inbred founders limiting the ability to examine correlation between the age-specific growth rate and genotypes of several genes that exhibit polymorphism during the primary screening with natural *P. monodon*. A new sample set of juvenile *P. monodon* (*N* = 359, the average body weight = 17.39 ± 4.36 g) was collected from a commercial farm in Chonburi. Genotypes of juvenile shrimp were identified at *CUPmo13* and *CUPmo02*

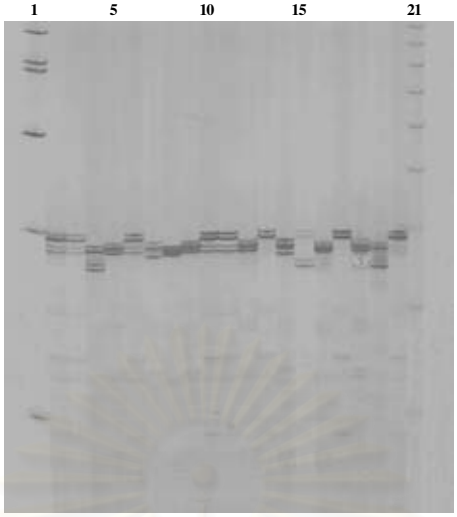

Figure 3.34 Microsatellite analysis of commercially cultured *P. monodon* juveniles at the *CUPmo13* locus (lanes 2 - 19). The PCR product was denatured, size-fractionated through 4.5% denaturing polyacrylamide gel and silver-stained. Lanes 1 and 21 are a 100 bp DNA marker.

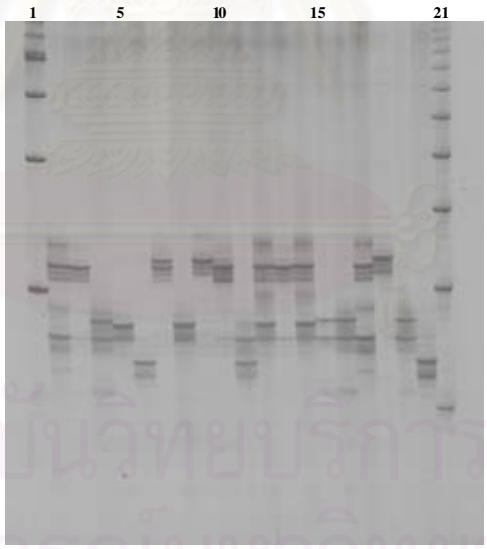

Figure 3.35 Microsatellite analysis of commercially cultured *P. monodon* juveniles at the *CUPmo02* locus (lanes 2 - 19). The PCR product was denatured, size-fractionated through 4.5% denaturing polyacrylamide gel and silver-stained. Lanes 1 and 21 are a 100 bp DNA marker.

loci. A total of seven and eight genotypes were found from 16 individuals examined (Figures 3.34 and 3.35) indicating that more than at least 2 families were contributed in the cultured pond. The precise number of contributed families can be evaluated if a larger number of juveniles are genotyped.

The amplification product of *RUVB-like protein* of these specimens was genotyped by SSCP and four different patterns were found (N = 335, Figure 3.36). The frequencies of SSCP patterns were tested to determine whether they are statistically different.

Results indicated significantly differences in allele frequencies between shrimp carrying genotypes A and B (the body weight of 19.2768 ± 3.6402 and 19.2929 ± 4.5477 with N = 34 and 78, respectively) and those carrying genotypes C and D (the body weight of 16.5277 ± 3.8466 and 16.3645 ± 4.3780 with N = 94 and 129, respectively) (P <0.05). This indicated significant correlation between the body weight (growth rate) of juvenile *P. monodon* and SSCP genotypes of *RUVB-like protein* (Table 3.4).

The PCR product of *RUVB-like protein* of a representative individual of each genotype was cloned and sequenced for both directions. Three SNP positions ($G \rightarrow A_{81}$, $A \rightarrow T_{196}$ and $G \rightarrow T_{248}$) were found from aligned nucleotide sequences of *RUVB-like protein* (Figure 3.37) SSCP and DNA sequencing are equally potential for differentiation of *RUVB-like protein* genotypes.

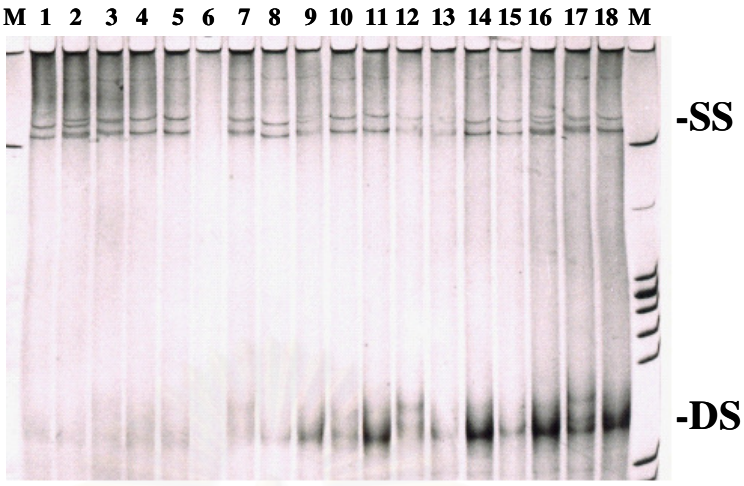

Figure 3.36 SSCP patterns of a homologue of *RUVB-like protein* of commercially cultured *P. monodon* juveniles (lanes 1 - 18). The PCR product was denatured, size-fractionated through 10% (crosslink = 75:1) and silver-stained. Lane M is a 100 bp DNA marker.

Table 3.4 Simple association analysis between the body weight of juvenile P. *monodon* from commercial farm and SSCP genotypes of *LTB4DH*, *RUVB- like protein* and *X-box binding protein*

Gene/genotype	*N*	Average body weight ± SD	Average body weight ± SD
RUVB-like protein*			
A	34	19.2768 ± 3.6402[a]	
B	78	19.2929 ± 4.5477[a]	
C	94		16.5277 ± 3.8466 [b]
D	129		16.3645 ± 4.3780[b]

The same superscripts indicate that the body weight of shrimps possessing different genotypes were not significantly different (P > 0.05).

```
RUVB5-1         TTGGCAGGTCAGGCAGTTCTCATTGCTGGCCAGCCTGGCACAGGCAAGACAGCCATAGCT
RUVB16-1        TTGGCAGGTCAGGCAGTTCTCATTGCTGGCCAGCCTGGCACAGGCAAGACAGCCATAGCT
RUVB17-1        TTGGCAGGTCAGGCAGTTCTCATTGCTGGCCAGCCTGGCACAGGCAAGACAGCCATAGCT
RUVB1-1         TTGGCAGGTCAGGCAGTTCTCATTGCTGGCCAGCCTGGCACAGGCAAGACAGCCATAGCT
                ************************************************************

RUVB5-1         AATGGTCTGGCCCAGTCTTTAGGCCCAGACACTCCTTTCACGGCTGTCTCTGCTTCAGAG
RUVB16-1        AATGGTCTGGCCCAGTCTTTAGGCCCAGACACTCCTTTCACGGCTGTCTCTGCTTCAGAG
RUVB17-1        AATGGTCTGGCCCAGTCTTTGGGCCCAGACACTCCTTTCACGGCTGTCTCTGCTTCAGAG
RUVB1-1         AATGGTCTGGCCCAGTCTTTGGGCCCAGACACTCCTTTCACGGCTGTCTCTGCTTCAGAG
                ******************* ****************************************

RUVB5-1         ATTTACAGGCAGGAACTCTTTTTTTTTCTGTTGGTTTTTGATATGTTATAAAAAATGTAA
RUVB16-1        ATTTACAGGCAGGAACTCTTTTTTTTTCTGTTGGTTTTTGATATGTTATAAAAAATGTAA
RUVB17-1        ATTTACAGGCAGGAACTCTTTTTTTTTCTGTTGGTTTTTGATATGTTATAAAAAATGTAA
RUVB1-1         ATTTACAGGCAGGAACTCTTTTTTTTTCTGTTGGTTTTTGATATGTTATAAAAAATGTAA
                ************************************************************

RUVB5-1         TAATCTGAGTAAAAATTTTTCCTGTCCCTCCATTATAGTAGGCCATAGAAGATTTTTCTT
RUVB16-1        TAATCTGAGTAAAAATTTTTCCTGTCCCTCCATTATAGTAGGCCATAGAAGATTTTTCTT
RUVB17-1        TAATCTGAGTAAAAATTTTTCCTGTCCCTCCATTATAGTAGGCCATAGAAGATTTTTCTT
RUVB1-1         TAATCTGAGTAAAAATTTTTCCTGTCCCTCCATTATAGTAGGCCATAGAAGATTTTTCTT
                *************** ****************************************

RUVB5-1         TTTCTTTGTTTCAAGGTTTTGATAGGCTTAACCACCCACAATATTTAAGTGTTCATAATG
RUVB16-1        TTTCTTTGTTTCAAGGTTTTGATAGGCTTAACCACCCACAATATTTAAGTGTTCATAATG
RUVB17-1        TTTCTTTGTTTCAAGGTTTTGATAGGCTTAACCACCCACAATATTTAAGTGTTCATAATG
RUVB1-1         TTTCTTTTTTTCAAGGTTTTGATAGGCTTAACCACCCACAATATTTAAGTGTTCATAATG
                ******* ****************************************************

RUVB5-1         TAAAATTTTATGTTATTTGTGTCTGTTATATGAAACAAGTGTCTTTTTCTACAGCTTGGG
RUVB16-1        TAAAATTTTATGTTATTTGTGTCTGTTATATGAAACAAGTGTCTTTTTCTACAGCTTGGG
RUVB17-1        TAAAATTTTATGTTATTTGTGTCTGTTATATGAAACAAGTGTCTTTTTCTACAGCTTGGG
RUVB1-1         TAAAATTTTATGTTATTTGTGTCTGTTATATGAAACAAGTGTCTTTTTCTACAGCTTGGG
                ************************************************************

RUVB5-1         AATGTCTAAGACCGAGGTATTAACACAAGCTTTCCGACGTTCCATCGGTGTGCGTATCAA
RUVB16-1        AATGTCTAAGACCGAGGTATTAACACAAGCTTTCCGACGTTCCATCGGTGTGCGTATCAA
RUVB17-1        AATGTCTAAGACCGAGGTATTAACACAAGCTTTCCGACGTTCCATCGGTGTGCGTATCAA
RUVB1-1         AATGTCTAAGACCGAGGTATTAACACAAGCTTTCCGACGTTCCATCGGTGTGCGTATCAA
                ************************************************************

RUVB5-1         GGAAGAAACAGAAATAATTGAAGGAGAGGTTGTAGAGGTGCAGATTGACCGTCCTGCCAC
RUVB16-1        GGAAGAAACAGAAATAATTGAAGGAGAGGTTGTAGAGGTGCAGATTGACCGTCCTGCCAC
RUVB17-1        GGAAGAAACAGAAATAATTGAAGGAGAGGTTGTAGAGGTGCAGATTGACCGTCCTGCCAC
RUVB1-1         GGAAGAAACAGAAATAATTGAAGGAGAGGTTGTAGAGGTGCAGATTGACCGTCCTGCCAC
                ************************************************************

RUVB5-1f        TGGA
RUVB16-1        TGGA
RUVB17-1f       TGGA
RUVB1-1         TGGA
                ****
```

Figure 3.37 Multiple alignments of *RUVB-like protein* amplified from genomic DNA of representative individuals of commercially cultured *P. monodon* juveniles exhibiting genotypes A (RUVB5-1), B (RUVB16-1), C (RUVB17-1) and D (RUVB1-1)Exons are illustrated in boldface.

3.5 Isolation and characterization of the full length cDNA of homologues of *RUVB-like protein, NADP-dependent leukotriene B4 12-hydroxydehydrogenase, X-box binding protein* and *rasputin* using RACE-PCR and 3′ sequencing of the original EST clone

The partial cDNA sequence of *rasputin* were initially obtained from EST analysis. The full length of this gene homologue was then further identified and characterized by RACE-PCR. In addition, the amplified insert sizes of *RUVB-like protein*, *LTB4DH* and *X-box binding protein* suggested that the original EST clones should contain the full length cDNA of these gene homologues. The 3' end sequencing and primer walking were then applied.

3.5.1 RNA extraction and the first strand synthesis

The quantity and quality of total RNA was spectrophotometrically and electrophoretically analyzed. The ratio of OD_{260}/OD_{280} of extracted RNA ranged from 1.8 – 2.0 indicating that RNA samples were relatively pure. Agarose gel electrophoresis indicated smear total RNA with a few discrete bands implying the accepted quality of extracted total RNA (Figure 3.38). The ovarian mRNA was purified and subjected to the synthesis of 5′ and 3′ RACE template.

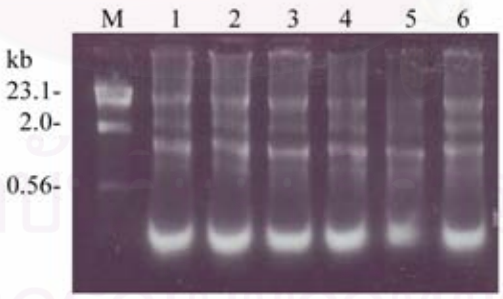

Figure 3.38 A 0.8% ethidium bromide-stained agarose gel showing the quality of RNA from ovaries of different individuals of *P. monodon* (lanes = 1 - 6). Lanes M is λ-*Hin*d III marker.

3.5.2 Isolation and characterization of the full length cDNA of a homologue of *rasputin*

The primary 5′and 3′RACE-PCR were carried out using the RACE-PCR template from ovaries of *P. monodon* broodstock. A single discrete amplification bands of approximately 600 bp was obtained from 5′ RACE-PCR whereas three discrete bands of approximately 3.0, 2.5 and 1.9 kb were obtained from 3′ RACE-PCR (Fig 3.36). The single fragments for 5′RACE-PCR and the smallest band of 3′ RACE-PCR were cloned and sequenced. In addition, the original EST clone exhibited a large insert size. Primer walking was then further applied for sequencing of the large insert size from 3′RACE-PCR and the 3' end of the original EST clone.

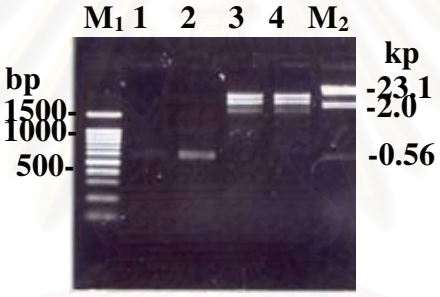

Figure 3.39 The primary 5′ and 3′RACE-PCR of *rasputin* (lanes 1 and 2 for 5′ RACE-PCR and lanes 3 and 4 for 3′RACE-PCR). A 100 bp (lanes M_1) DNA ladder and λ-*Hin*d III (lanes M_2) were used as the DNA markers.

Nucleotide sequences of 5′ and 3′ EST and RACE-PCR fragments were assembled and annotated. The full length cDNA of *P. monodon rasputin* was 4049 bp in length. The ORF of the transcript was 1659 bp encoding a polypeptide of 552 amino acids. The 5′and 3′ UTRs of *rasputin* were 132 and 2305 bp excluding the poly A tail, Figures 3.40 and 3.41). The best hit for this transcript was *rasputin* of *Drosophila melanogaster* (E-value = 1e-73). The calculated pI and MW of *P. monodon rasputin* were 6.73 and 58998.6 daltons, respectively.

The signal peptide was not found in this presumably nonsecretory protein. A NTF2 domain (E-value = 1.30e-43) was found at amino acids positions 11 - 128. Two additional domains (extension 2 and RRM) were also found at lower similarity (9.60e-02 and 5.90e-03) (Fig 3.40).

Domain	Begin	End	E-value
Pfam:NTF2	11	128	1.30e-43
Pfam:Extensin_2	146	416	9.60e-02
Pfam:RRM_1	407	470	5.90e-03

Figure 3.40 Diagram illustrating the full length cDNA of *rasputin* in *P. monodon*. The NTF2 domain was found in this transcript. The scale bar is 200 bp in length.

```
CCCTATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGACTACGCGGGACAACAG   60
CCATATTACCACAGCCAAATACGTCATCTCTTTAGCCAAATCCTTATTCGCTCGCAAAGA  120
GTTTAACAGAGGATGGTCATGGAAACTCCATCACCGCAATGCGTTGGCCTTGAGTTTGTG  180
            M  V  M  E  T  P  S  P  Q  C  V  G  L  E  F  V
CGCCAGTACTACACGGTGCTCAACAAGGCCCCCATGCACCTTCATCGTTTTTTCAGTCAC  240
 R  Q  Y  Y  T  V  L  N  K  A  P  M  H  L  H  R  F  F  S  H
AACTCCTCATTTATGCATGGGGGCCATAACTCCATGAGTGAACCCATTATCGGCCAAGCA  300
 N  S  S  F  M  H  G  G  H  N  S  M  S  E  P  I  I  G  Q  A
GACATACATAAAATGATCATGAGCCTTAACTTCCGTGATTGTCATGCTAAGATCCGAATG  360
 D  I  H  K  M  I  M  S  L  N  F  R  D  C  H  A  K  I  R  M
GTTGATGCTCAAGCTACACTCGGTAATGGAGTTGTCATCCAGGTTACTGGTGAGCTCTCC  420
 V  D  A  Q  A  T  L  G  N  G  V  V  I  Q  V  T  G  E  L  S
AACAATGGAATGCCCATGCGACGATTCATGCAGACTTTTGTACTAGCGCCTCAGACCCCC  480
 N  N  G  M  P  M  R  R  F  M  Q  T  F  V  L  A  P  Q  T  P
AAGAAGTTCTACGTGCACAATGACATTTTCCGGTACCAAGACGAGGTTTTCAGTGATGAA  540
 K  K  F  Y  V  H  N  D  I  F  R  Y  Q  D  E  V  F  S  D  E
GAGGGTGCAGAGGAGGGAGGAAGTGAAGTGGAGGAGGACTTGGAAGGCCCCGTCTCCACA  600
 E  G  A  E  E  G  G  S  E  V  E  E  D  L  E  G  P  V  S  T
CAAGGCTTCTCAGCACCCTCTGCTGCTGTCAATGGATCTGCCCATCCGCCTGAGCCTGAA  660
```

```
 Q  G  F  S  A  P  S  A  A  V  N  G  S  A  H  P  P  E  P  E
CCCACTCCACCTGCTG***CTCCCCAGACTCCTGTCACTCCTCA***AGTCCCAACACCTGTACCT  720
 P  T  P  P  A  A  P  Q  T  P  V  T  P  Q  V  P  T  P  V  P
ACTGTTCCAGTACCTGCTCCAGGAAATTCATCAAATGCCGCCACCACAGCTCCTGTTGCC  780
 T  V  P  V  P  A  P  G  N  S  S  N  A  A  T  T  A  P  V  A
ACTGCCCCTCTTTCTCCAACACCTCCAGCAATAACACCCACAGAACCTGTACCAGAAAGT  840
 T  A  P  L  S  P  T  P  P  A  I  T  P  T  E  P  V  P  E  S
CCACCCACTCCAGAAAATGATGAGCAGGGCTGGGGTACTCCAGGCACAGATGGATGGGGT  900
 P  P  T  P  E  N  D  E  Q  G  W  G  T  P  G  T  D  G  W  G
GCTCCTGCAGAGCCTCAGGGTTCTTGGGATGAGCCAGCAGTACCTCAGAAGGAGACACCA  960
 A  P  A  E  P  Q  G  S  W  D  E  P  A  V  P  Q  K  E  T  P
GATCCAGACCCCCCACAACCAGAACCTGTTCAGTTGCCATCAAATGAGCCGAAGACTTTT 1020
 D  P  D  P  P  Q  P  E  P  V  Q  L  P  S  N  E  P  K  T  F
GCCAATCTCTTCAAAAATTCCCAAGGTTTCCCTGCAGCTCCAGCACCTGCTGCCTCACAG 1080
 A  N  L  F  K  N  S  Q  G  F  P  A  A  P  A  P  A  A  S  Q
CAATCCTCTCATGCCCAGCCAACTCCTCCAGCACAGCAACAGCCTCCACAGAATCGAAGT 1140
 Q  S  S  H  A  Q  P  T  P  P  A  Q  Q  Q  P  P  Q  N  R  S
GACAACCGAACCCAGGCAAGCACCACCACTCCAGCTGTGAGTCAGGCTCACTCTACTGGT 1200
 D  N  R  T  Q  A  S  T  T  T  P  A  V  S  Q  A  H  S  T  G
GGCTTCACCCAACGACCACCAAGGAATGCACAAGGTCGAGGGGCACCGCGTGATCGTCCT 1260
 G  F  T  Q  R  P  P  R  N  A  Q  G  R  G  A  P  R  D  R  P
TATAGGCCCAACAGTGAAGACAATTCAGAATTAGAGAGCCTAGGTGGGGAAGAAGGAAGG 1320
 Y  R  P  N  S  E  D  N  S  E  L  E  S  L  G  G  E  E  G  R
AGAGCTCCTCCTGTACCAGACGGTCAGCAGATCTTTGTTGGTAACCTTCCACACACAGCT 1380
 R  A  P  P  V  P  D  G  Q  Q  I  F  V  G  N  L  P  H  T  A
GGAGAGGATGAACTGAAGGCGGAATTCTCAAGATTTGGGAAAATTATGGATATCCGTATT 1440
 G  E  D  E  L  K  A  E  F  S  R  F  G  K  I  M  D  I  R  I
AATACTGCTAAAGGAAAAAACTGGGTCCTAAAGGTCAGGTCCCCAACTTCGGTTTCATCA 1500
 N  T  A  K  G  K  N  W  V  L  K  V  R  S  P  T  S  V  S  S
CATTTGAAGAGGAAGGTTCTGTGTCAAAGGCTCTCAACTCTAGGCCAATCCATCTTAATG 1560
 H  L  K  R  K  V  L  C  Q  R  L  S  T  L  G  Q  S  I  L  M
GCGAGCATCGGATTGAATGTTGAAGAGAAGAAGGCTCGTCTAAGGATGGATGGAGGTGGT 1620
 A  S  I  G  L  N  V  E  E  K  K  A  R  L  R  M  D  G  G  G
ATGGGCCGTCCCAACAGTGGCCGTGGAGGAATGGGAGGCAGCGGTGGCATGCGTCAAGGC 1680
 M  G  R  P  N  S  G  R  G  G  M  G  G  S  G  G  M  R  Q  G
CCCCCTAGTGGCTTACAGGGACGAGGTGGAGGACGACCAGGCTTTAATCGTGACAGTAAT 1740
 P  P  S  G  L  Q  G  R  G  G  G  R  P  G  F  N  R  D  S  N
CGAGGTGGACCAGGAGGACCCAGAGGTAGTTTTGCACCGCGACGT**TAG**GCTAACCAATTA 1800
 R  G  G  P  G  G  P  R  G  S  F  A  P  R  R  *  A  N  Q  L

TAGTCACCATGGCCTACTCTCTTACTACTCATAGGAAACCTCTGTCCAGTGCAGCCATTC 1860
TCAACAGATGGCTGCAGTGTAAGTGGTCCAAACATCTGCAAGAGGACTGCTCAAGTTAAA 1920
TCTGCAGTAGAGCCATTGTACAACTTGATGGAATGGTGTGCTTCTCAGTGAATGTTGCTG 1980
TTTTGATAATGTAACTCGTCAATGGCCACTTGAAAATACTGGTGCTTGAGGTGATGATGA 2040
TGAAACTTCAGTGATATGGTAAACAAACTTCACAATAACATAAGTGGCTTCTTTTGTAAT 2100
TTAGTTACTCCCACTTCTCTAACTGTATTAGAGTTATCAGATTGATTTCCATTTCGTGAA 2160
CACAACAATTTTTCCACTGATGACGTGTACTTAGACATAGTGTCTCCCCTGATGTAGATT 2220
GTGTTTCTATGAGTAAATAAAAGAACCCTCCTTTTTTGGATGGTTGTGATGGAATTCAAT 2280
CTCTAAATCTTGGACAACAATTACTCTTTTATTATTTGGTAATAATAAAACATGTTATTG 2340
TGATTACCAAAATCATTCCCTTTTTAACAGCTACTAGTCAGGATTGAAACAAAGTTTTTT 2400
TTTGTGCTTATTTCAGTTTCAACATCTAAAAGGTCCTTGAAATTATATTGGAAATATAGA 2460
AGAAGTGGATTAAAATGGGTACTGCTTGAGGTCATTGATTTATGTACTGGTATTTGATTC 2520
CAGTGATGTTTTTGTATGTTAGAATTTTTTAACTTATTTTTGAAACTTCAGCTGAGACTG 2580
ATGTACATTTTGTACCTGGCAACAGAAGATTGGTGAGGTTTAGTCCACTTTTTCCAGTGA 2640
TCAGCATGCAGACACAGTAATCCTCAGAGATGTCTTATCTGACTTGGCCCACATCCATGC 2700
TCACTGTTTTGACGTTACATTTCCAGCACTGCTGTCATACTGGGCATTACACATACATAA 2760
AATGTTAACTTGTTACATGTAGTTCACAAGGCCAAGTACAGATCAGCATCTGCTAATAGC 2820
TGATTCCATTTTTTTATTTGATGTGGGTGAAGTTGCCAATATCCGTTAATATTGACAACT 2880
TCACATTATCACAGAAGTACTTTAACTGGAGGAGCGAAGAGGAAACCAGTGTCCAAGGTC 2940
ACATTCTCGTCTATTTTGTAGTAAATCACTTTGATCACTACTCAAAGATTATGCTTGATT 3000
CAACCTCTTTCATATGTTGTAGTGTGCTAGTTTCTCCGAATGGGAAATGAATAATAGAAT 3060
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GGAGCATTTATATTTCACAAGTGTGAGTTGTAAACACCCCAAAGCATTTCTATATTAAAA 3120
AAAAGAAAAAAAAACTGTGCGAAAGAGGGAATAAAATAGCCTTATCACCACCAACACTGT 3180
TGCAACTACTTTCCCCATGTTTGTTTCATTCCAAGGGATTAGATATGAAAGATGTCTTAG 3240
CATGCAATAATTTTCAGAACTGGTAAAATGGAATATTGTTTTTTATATTATCATCGTAAG 3300
TTTTAGTATTAAATTTTTCATTGTGTAAACTATGTACAAATCAGTATTACTTTTTTTTCT 3360
TTTTTTTTTCCTCTTCTTTTATTTCGTTTTTCTTTTTTTATGCACAGTTTGTTACTGGTT 3420
GTGTTACAATGGAAGCTCTCACACCACAGTATGATGAGCAGAAATGTGATATTAGTTTAG 3480
ATAGTGGGCAAGCAGACACAGGGCAGATCTGAAAGTCATTGCATGTCAGGATAGCTTTTG 3540
AAACTGTCCTTTCAAATTGTTATAAAAAAAGGGCATTAGATAATTGCCCACACCTGGAGC 3600
TAGATGAACCTCTGGGAATTGTTACAGGGACATCAGAAGAGAACTTAGCTCACAGGTCAT 3660
GTGAAGGGATATGCTGTAGTTGCAGACTTTATACATTCTGTTCAGGATCTATTGCATTGG 3720
TAAATTAATTATGGGTTACTGTTTTTCTTAAGCATTTTGAAAAATAATTCAGATTTAAAA 3780
AGTCTAGACCCCTCGAACTTCAGCTATTCCAAATCCCATAGCTTACATTATCATTGGAGC 3840
TCCTGGGAGCATGGCTAGCATGTGATCAGTTTTAATTTGATTTAAAAGTTTTGAAAAAGG 3900
TTTGAAAATTGCTTTTGCTTTAACTTCCTTTAAGGTCTGTTCTTGCAGCTCCTCCTGGTC 3960
AGAAACTTTGTGATGTCAAATACTGAGGAATGAAAGTGAGAAATAAAAGCCTCTTTGCAA 4020
AAAAAAAAAAAAAAAAAAAAAAAAAAAA..............................  4049
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Figure 3.41 The full length cDNA sequences of a homologue of *rasputin* of *P. monodon* Start and stop codons are illustrated in boldface and underlined. The 5′ and 3′ RACE-PCR primers are underlined, boldfaced and italicized. The internal sequencing primers are underlined. The polyA additional signal is boldfaced.

3.5.3 Isolation and characterization of the full length cDNA of a homologue of *RUVB-like protein*

The partial sequence of *RUVB-like protein* was obtained from sequencing of the 5' end of the EST from ovarian cDNA library. The size of colony PCR product of this EST revealed that the full length cDNA should be covered by the cDNA clone. Therefore, the 3' end sequencing and primer walking were applied for isolation and characterization of this gene homologue.

The full length cDNA of nucleotides of *RUVB-like protein* was 4244 bp in length. The ORF of this gene was 1395 bp encoding a polypeptide of 464 amino acids with the 5′ and 3′UTRs of 436 and 2413 bp (excluding the poly A tail, Figures 3.42 and 3.43). The closest similarity of *RUVB-like protein* of *P. monodon* was *RUVBB-like protein 2* (p47 protein) of *Apis mellifera* (E-value = 0.0).

The calculated pI and MW of *RUVB-like protein* of *P. monodon* were 5.48 and 51618.3 daltons, respectively. The signal peptide was not found in this presumably nonsecretory protein. An AAA domain (E-value = 3.01e-11) was found at amino acids positions 69 – 361(Fig 3.42).

Domain	Begin	End	E-value
AAA	69	361	3.01e-11

Figuer 3.42 Diagram illustrating the full length cDNA of *RUVB-like protein*. The AAA domain was found in this transcript. The scale bar is 200 bp in length.

```
CGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGC   60
CTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGA  120
AAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGG  180
CTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTC  240
ACACAGGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGG  300
AACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGC  360
TGCAGGAATTCGGCACGAGGCCTCGTGCCGCTCGCCTCCGCGTTGGTAGTCTCTCGAAAC  420
GCATGTTGTTTTGATCATGGCGGCGGTTAGTCTGACAAGTGAGGAACATGAGGTCACAAG  480
                 M  A  A  V  S  L  T  S  E  E  H  E  V  T  R
GATTGAGCGCATTGGTGCTCATTCCCACATCAGAGGGTTGGGCCTCACGGATGAGCTTAC  540
 I  E  R  I  G  A  H  S  H  I  R  G  L  G  L  T  D  E  L  T
TCCGAAACCCTCGGCTCAAGGCATGGTCGGGCAGATTAAGGCAAGACGAGCTGCTGGTGT  600
 P  K  P  S  A  Q  G  M  V  G  Q  I  K  A  R  R  A  A  G  V
GGTGCACAACATGGTAAAGGAAGGCAAGCTGGCAGGTCAGGCAGTTCTCATTGCTGGCCA  660
 V  H  N  M  V  K  E  G  K  L  A  G  Q  A  V  L  I  A  G  Q
GCCTGGCACAGGCAAGACAGCCATAGCTAATGGTCTGGCCCAGTCTTTAGGCCCAGACAC  720
 P  G  T  G  K  T  A  I  A  N  G  L  A  Q  S  L  G  P  D  T
TCCTTTCACGGCTGTCTCTGCTTCAGAGATTTACAGCTTGGGAATGTCTAAGACCGAGGT  780
 P  F  T  A  V  S  A  S  E  I  Y  S  L  G  M  S  K  T  E  V
ATTAACACAAGCTTTCCGACGTTCCATCGGTGTGCGTATCAAGGAAGAAACAGAAATAAT  840
 L  T  Q  A  F  R  R  S  I  G  V  R  I  K  E  E  T  E  I  I
TGAAGGAGAGGTTGTAGAGGTGCAGATTGACCGTCCTGCCACTGGCACAGGAGCCAAAGT  900
 E  G  E  V  V  E  V  Q  I  D  R  P  A  T  G  T  G  A  K  V
TGGGAAGCTCACTCTCAAGACCACAGAAATGGAAACCATTTATGATCTTGGCAACAAGCT  960
 G  K  L  T  L  K  T  T  E  M  E  T  I  Y  D  L  G  N  K  L
TATAGAAGCTCTAACAAAGGAGAAGGTTCAAGCTGGAGATGTAATCACCATTGATAAGGC 1020
 I  E  A  L  T  K  E  K  V  Q  A  G  D  V  I  T  I  D  K  A
CACAGGCAAGATCACGAAGCTGGGAAGGTCTTTCACACGTGCTCGAGACTACGATGCTAC 1080
 T  G  K  I  T  K  L  G  R  S  F  T  R  A  R  D  Y  D  A  T
AGGCCCCCAAACTAGGTTTGTACAGTGTCCAGAAGGTGAGCTACAGAAAAGGAAAGAAGT 1140
 G  P  Q  T  R  F  V  Q  C  P  E  G  E  L  Q  K  R  K  E  V
TGTACACACGGTCACGTTGCATGAAATTGATGTCATCAACAACCGAACACAAGGCTTCAT 1200
 V  H  T  V  T  L  H  E  I  D  V  I  N  N  R  T  Q  G  F  M
GGCTCTCTTCTCAGGTGATACAGGTGAAATAAAAGGTGAGGTAAGGCAACAGATCAATGC 1260
 A  L  F  S  G  D  T  G  E  I  K  G  E  V  R  Q  Q  I  N  A
AAAAGTTGCTGAATGGCGTGAAGAAGGCAAGGCTGAGCTGGTTCCAGGTGTTCTCTTCAT 1320
 K  V  A  E  W  R  E  E  G  K  A  E  L  V  P  G  V  L  F  I
TGATGAGGTACACATGTTGGACATTGAGTGTTTCTCCTTTATTAACCGGGCCCTGGAAGA 1380
 D  E  V  H  M  L  D  I  E  C  F  S  F  I  N  R  A  L  E  D
TGACATGGCCCCTGTGGTGATTGTGGCTACAAACCGCGGAATCACACGCATCAGAGGCAC 1440
 D  M  A  P  V  V  I  V  A  T  N  R  G  I  T  R  I  R  G  T
```

```
GCGCAACCTCTCTCCTCATGGTATTCCCATTGACATGCTGGACAGAATGATCATTATTAA 1500
 R  N  L  S  P  H  G  I  P  I  D  M  L  D  R  M  I  I  I  K
GACTACTCCATATCAGGAAAAAGAGATCAAGGAGATTTTGAAGATTAGGTGTGAAGAAGA 1560
 T  T  P  Y  Q  E  K  E  I  K  E  I  L  K  I  R  C  E  E  E
GGATTGTGAAATTCAAGACGATGCCCTGGTGGTATTGACCAAAATAGGTCTTGAAACTCT 1620
 D  C  E  I  Q  D  D  A  L  V  V  L  T  K  I  G  L  E  T  L
TGAAACCTCCCTCCGCTATGCCATCCAACTCATTACTTTGGCAAATTTGGCAGCGATGAA 1680
 E  T  S  L  R  Y  A  I  Q  L  I  T  L  A  N  L  A  A  M  K
GAGAAAAGAAAAACAGATTGCAATTGTTGATGTGAAGAAGGTATACCAACTCTTTATTGA 1740
 R  K  E  K  Q  I  A  I  V  D  V  K  K  V  Y  Q  L  F  I  D
TGAACAAAGATCACAGAAGTTCCTTAAAGAATATGAAGATGAATTTATGTTTGATGAAGG 1800
 E  Q  R  S  Q  K  F  L  K  E  Y  E  D  E  F  M  F  D  E  G
TACTGAAACTGCGATGGAGACAAACTAAACGCTAAAGATTATGCCTCAATAGAATAAAAG 1860
 T  E  T  A  M  E  T  N  *  T  L  K  I  M  P  Q  *  N  K  S

TATTTCTTCAGTGAAAATAGGAAGGAGTTCTTTATATAGGTCAAATCTCAAAAAAACAAA 1920
ACAAAATATAGCCAGTGTAAATCTCCCACAGAAGTATTCCATTATGTTAAGCTAATCATA 1980
AATATATATGTAAAAACCCTCGTGCCGAATTCGGCACGAGGGGTACACTGGTGATGGCTT 2040
TGCCTGCCAAGATATCGATGAATGCTTGGTTGCCAACGGTGGCTGTGGTACAAATGCCTT 2100
GTGTAGCAATACTCCAGGCAGCCGAGACTGTACTTGTGCATCAGGTTACACTGGGGATGG 2160
CTTTACTTGCTCTGATATCGATGAGTGCCTTGTTAACAATGGAGGCTGTGATGCAAATGC 2220
AGGGTGTCAAAACACAGCTGGTTCAAGAACCTGCACATGTCTCCCAGGCTTCTCTGGGAA 2280
TGGTGTTACTTGCTCTGATGTGGACGAGTGCTTGGTTGCCAACGGTGGTTGTGATGCAAA 2340
TGCCCTGTGTAGCAATACTCCAGGCAGCCGTGACTGCACCTGCACATCAGGGTTCACTGG 2400
GAATGGCCTAGCTTGCACAGATGTGGACGAGTGTCTGGTGGCTAATGGTGGGTGCCATGC 2460
TAATGCTCAGTGCACCAATACTGCTGGTAGCAGAACCTGCTCTTGCCTTGCTGGCTATAC 2520
TGGTGATGGACAAGTGTGCACACTCCTCCAGTGTCCTGTTGGCTTTGCTGGTCAAGGTCA 2580
AGACTGTGCTCCAGACTCTGACCTTGATGGCTTCCCAGACACTGAACTGAGCTGTTCAAG 2640
CAAATACTGCCGTAAGGATAACTGTGTCAACCGACCAAATTCTGGGCAAGAGGATGCCGA 2700
TGGTGACGGGATTGGTGATGCTTGTGATACTGATGCTGATGGAGATGGACTTCTCGATAC 2760
TTCGGATAACTGTCCACTAATAGCCAACCCTGGGCAACAAGATGGAGACTCGGATACACA 2820
TGGAGATGTATGTGACAACTGCCCTGTAGTCTCCAACCCCAGCCAGAATGATGTTGATGG 2880
GGATGGTACAGGCGACAAATGCGACGATGATATTGACAATGATGGCTTGCTAAATACTGC 2940
TGACAACTGTCCAAAGACTGCAAATGGCAACCAAGCTGATGGTGATGGAGATGGCTTTGG 3000
AGACTTGTGTGACAATTGCCCCCAGCATACCAATCCTGGGCAAGAAGATGCAGATGAAGA 3060
TCTACTAGGGGATGCATGTGATGATAACATCGACTCTGACAGTGATGGTGTGGAGGACAG 3120
TGTGGACAATTGTCCAAATGTAGCCAATGGTGACCAGCAGGACGTAGATGGTGATGGGTT 3180
AGGTGATGCCTGTGATTCAGACAGTGACAACGATGGTATTGATGATACGACTGACAACTG 3240
CTTGCTAATCCCAAATTCGGACCAGAAGGATACCGACGGGAATGGCCGTGGCGATGCTTG 3300
CACCTCTGACTTTGATGGTGACAAAATTGTAGACTCGGATGATAACTGCATTGCCAACCC 3360
AAATGTGCATGCCACCGATTTCAGGCAACTCCAAATGGTGGCACTAGACCCACAAACGGC 3420
GTCTACTCCTCCAGTATGGATTGTCTACGACAATGGTGCAGAAATTCATCAGACGTTGAA 3480
CTCGGACCCAGCTATAGCAGTGGGTGACCATACACTTGTGGATGTCGACTTTGAAGGGAC 3540
CTTCTTCATAGAGGACACCAGTGATGATGACTTTGTTGGCTTTATCTTCAGCTACCAAAG 3600
CAATTCCAAGTTCTATGTCATGATGTGGAAGAAGGGACCACAAAGCTGGTTTGGCCAAGC 3660
AGAGAGAGGAGTTACTCTGAAGCTAATTGACTCTGCTACAGGCCCTGGCACGGCACTTCG 3720
GGATGCTTTGTGGCTCACTGGCTCTACAACAAACCAGGCAACCTTGCTCTGGCATGATAG 3780
CAGTATTGGATGGACCCCTAAAGTTGCCTACCGCTGGCTGCTTCACCACCGGCCTGATAT 3840
TGGTACCATGAGGTTCTACTTGTACCAGGGTAACAACCAAGTCATTGACTCTGGAAACAT 3900
ATATGACAGCACCCTAAAAGGAGGCAGGCTAGGTCTCTTCTGCTTCTCCCAGAAGCAAAT 3960
TATATGGTCCAATGTGAAGTATTCTTGTTCAGATGATGTACCCCAAGACATGTTTAATGA 4020
CCTGCCCACTAACCTACAGACGCAAGTCCTGACTTCATAAGGACTTGCTTTTTGCACAAA 4080
CTAAAGCGTCTTGCACTTGGACACGACGCATCTTGAAGATTGGTCTTGATACTGTAAGAC 4140
GCTAGTCTATATAAAATTTTATATTAAATAATTTTAAATTAAAAAAAAAAAAAAAAAAA 4200
AAAA.................................................... 4244
```

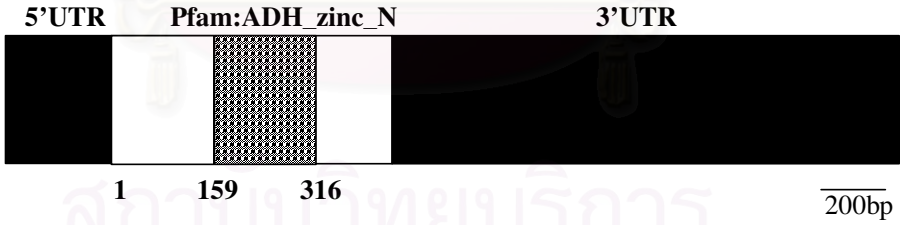
Figure 3.43 The full length cDNA sequence of *RUVB-like protein.* Start and stop codons are illustrated in boldface and underlined. The internal sequencing primers are underlined.

3.5.4 Isolation and characterization of the full length cDNA of a homologue of *NADP-dependent leukotriene B4 12-hydroxydehydrogenase* (*LTB4DH*)

The partial sequence of *LTB4DH* of *P. monodon* (also called *15-oxoprostaglandin reductase*) was initially obtained from sequencing of the 5′ end of the EST from ovarian cDNA library. The size of colony PCR product of this EST revealed that the full length cDNA should be covered by the original EST clone. Therefore, the 3' end sequencing and primer walking were applied for isolation and characterization of this gene homologue.

The full length cDNA of nucleotides of *LTB4DH* of *P. monodon* was 2548 bp in length. The ORF of this gene was 1038 bp encoding a polypeptide of 345 amino acids with the 5′ and 3′UTRs of 165 and 1384 bp (excluding the poly A tail, Figures 3.44 and 3.45). The best hit of *LTB4DH* of *P. monodon* was *NADP-dependent leukotriene B4 12-hydroxydehydrogenase* of *Tribolium castaneum* (E-value = 8e-78).

The calculated pI and MW of *LTB4DH* of *P. monodon* were 5.62 and 39326.4 daltons, respectively. The signal peptide was not found in this presumably nonsecretory protein. An ADH zinc N domain (E-value = 4.50e-11) was found at amino acids positions 159 – 316 (Fig 3.44).



Domain	Begin	End	E-value
Pfam:ADH_zinc_N	159	316	4.50e-11

Figuer 3.44 Diagram illustrating the full length cDNA of *NADP-dependent leukotriene B4 12-hydroxydehydrogenase* (*LTB4DH*). The ADH zinc N domain was found in this transcript. The scale bar is 200 bp in length.

```
CCGATGAAGATTCGCCAGCTCGAATTACCCTCACTAAGGGAACAAAAGCTGGAGCTCCAC   60
CGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGG  120
CCTCCCTGAGAGAAATCTGTAGTCCCTAGCATCCCAGAAGCCAAAATGGTGACAGCAAAG  180
                                                 M  V  T  A  K
GTGTGGAAGCTAGCCAAGCGGCCAGAGGGGATGCCCAAGAAGGAAGACTTTGTGTGTGTG  240
 V  W  K  L  A  K  R  P  E  G  M  P  K  K  E  D  F  V  C  V
GAAGAAGAGCTGGCTTGTGAAGATGGAGATGTCATTATTGAAGCTGAATTTTTGAGTGTA  300
 E  E  E  L  A  C  E  D  G  D  V  I  I  E  A  E  F  L  S  V
GATCCATACATGCGGTACATGATCAAGCAAATCCCTCTTGATGTACCTGTCACCGGTACT  360
 D  P  Y  M  R  Y  M  I  K  Q  I  P  L  D  V  P  V  T  G  T
CAGGTTGCAAAAGTGATTGAGAGTAAGAACCCAGAGTGGCCAGTGGGTACGTACGTTGTT  420
 Q  V  A  K  V  I  E  S  K  N  P  E  W  P  V  G  T  Y  V  V
TCACCGTCTGGCTGGCGCTCCCACACCCGACTCACTGAGAAAGACTTCAAGGCTGATAAC  480
 S  P  S  G  W  R  S  H  T  R  L  T  E  K  D  F  K  A  D  N
TTTTTCCAGAAGCCCATGAAGCTGCCAGAGATGGGAGACTTGCCTAAGAGTCTTGGCTTA  540
 F  F  Q  K  P  M  K  L  P  E  M  G  D  L  P  K  S  L  G  L
GGGATTCTGGGCATGCCGGGGAATACAGCATATTTTGGATTTCTTGAAATCTGCCGACCG  600
 G  I  L  G  M  P  G  N  T  A  Y  F  G  F  L  E  I  C  R  P
AAAGCAGGAGACACTGTGTTGGTAAATGCTGCTGCTGGAGCAGTTGGAAGTGCTGTGATT  660
 K  A  G  D  T  V  L  V  N  A  A  A  G  A  V  G  S  A  V  I
CAGATAGCCAAAATCAAAGGGTGTAAGGTAATTGCCTTTTCGGGATCTGATGAAACAGGT  720
 Q  I  A  K  I  K  G  C  K  V  I  A  F  S  G  S  D  E  T  G
AGCGTGGACCAAAGACCTTGGGGCAGACCATGCCTTCAACTACAAAACAGCAAACATTGG  780
 S  V  D  Q  R  P  W  G  R  P  C  L  Q  L  Q  N  S  K  H  W
ACAGTTCACTGCTGGGAGGCACTCAGTCAAGCAGCGCCTGATAAGATTAACTGCTTCTTT  840
 T  V  H  C  W  E  A  L  S  Q  A  A  P  D  K  I  N  C  F  F
GAAAATGTTGGAGGACAGTTCACTGCTGAAGCTCTACCACACATGGCTGAGAGGGGCAGA  900
 E  N  V  G  G  Q  F  T  A  E  A  L  P  H  M  A  E  R  G  R
ATGGCAATTTGTGGTGTGATTTCAGAGTACAATGATGACGACAAGGATTCTGGTGTGGTG  960
 M  A  I  C  G  V  I  S  E  Y  N  D  D  D  K  D  S  G  V  V
GCAATGACTAGTCCGTTGAATCCAGCTACGATACTTTGGAAGCAACTTGCAGTGGAAGGC 1020
 A  M  T  S  P  L  N  P  A  T  I  L  W  K  Q  L  A  V  E  G
TTCATTGTTACAAGGTGGTTTGATCGCTGGATGGAAGGAATTGATCAGATGAAGCAGTGG 1080
 F  I  V  T  R  W  F  D  R  W  M  E  G  I  D  Q  M  K  Q  W
ATCAGTGAGGGTAAAGTCAAGTACAAGGAAACTGTTGTAAAGGGATTTGACAAAATGCCT 1140
 I  S  E  G  K  V  K  Y  K  E  T  V  V  K  G  F  D  K  M  P
GAAGCATTCATTGGCCTTTTCCTCGGTGAAAATACAGGCAAGGCCATTGTAGCAGTGTGA 1200
 E  A  F  I  G  L  F  L  G  E  N  T  G  K  A  I  V  A  V  *

GGAACCTTGTGTGGAATGAAAAGATCAAAACAATCTACTTTATTTTCATATTTTCTTAAT 1260
TTAATAGGGAACCACAACCTAAAAATATAACTTGATAAATTCATCTGTTGAAACTTATTA 1320
TGGTGGAAAATTGTGCAGCATTGGCAAACATTTTCTTTGTTTTGGATGTTAAAAATATTT 1380
CTATAATAGCTGCATGCACTGGAAAATACTCTTACTTTACAAGTAGGCCAGTACGTCTAT 1440
TTCACATCTTCATTTTTGAAGATGCTATTTCATATATATGCAGACCTGTTTATTGCTTTT 1500
CCATTCTGTAATTTATCTATCTTCCATTCTTCTAAATTATCCTTGTTGAGGCATTGCCAA 1560
CTTCAAATAAAGTGCAAACCTCGTGCCGAATTCGGCACGAGGGTCAAATACTTTCTTTGT 1620
TGTGTTGGCCCTGGGGTTAGCCCTTGTTGCAGCCAAAGAGGACATCCGAAGCGAACGCAG 1680
CGTTACTGCAGACAACCACCCTTACTCGAAGCTATGTGAGAAACAACCTGACAAATTCAT 1740
TTGCGGCAACTGCAAGACCTTGATCCAGTGTGTGAAGGGACAGGCCTTCACTCGCCACTG 1800
CATTGAGGACCACTTCTGTTCGGAGAGGCCCCAGTTTGGCGGTGGTGTCTGCTACCCAAA 1860
TGAACCTCTGGACTGCACCTGCGTGAAGGCCAACGAGTTCCGAGTGGACCCCTACGACTC 1920
TCAGAGGTTCTTCTCTTGCAAGGCTGTTGGCTCCACCCCCGAGAACTACAAGTGCCCAGA 1980
TGGTATGGTCTTCGATGAAGGCTCGGCACAGTGCCAGACGGCAAGTGGCCTGCCTCCATG 2040
CGTGGTGGCGGGTACTTTTGCCAACCCAAGCAACTGCAGTGAATACTACTCGTGCATTAG 2100
CCTGCGCAGTGGATGGCTGCAGAAGTCGTTCATGTGCACCAATGACATGATGTACAACGA 2160
ACAAAAGGATGCATGTGAAGATCCGTGCATATACCAGTTCGTGTGCCAGCAGGAAGGCCG 2220
TTACCCTGACCTTCTGAACAAGCAGAATTACTTTGAATGCTACATGCTTGGTGGCGTGTT 2280
GCAGCAGCTACGTTACAGCTGCCCTGAGAGCTACAGGTGGGATATCGTGTCTCCAGGTGT 2340
GGGCCAGTGCGTGGAGGACCATGGAGATAAAGATTCTAACTATGCCTTCGGGCAGTGCGA 2400
CATCCCTGACAACCTGTGTCCTGGCCCGTGAGAACTTGTTTACTAATAAGGGCAACCCTG 2460
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TCGCAACACCACTAAATGGAATAGGGGCCAGACCTCGACTTCTGTCTTAAATAAATGTTG 2520
CTTTTTAAAAAAAAAAAAAAAAAAAAAA.............................. 2548
```

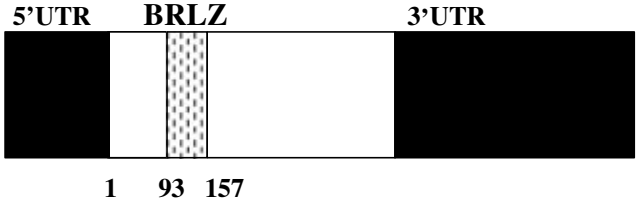
Figure 3.45 The full length cDNA sequences of *NADP-dependent leukotriene B4 12-hydroxydehydrogenase (LTB4DH* also called *15-oxoprostaglangin reductase).* Start and stop codons are illustrated in boldface and underlined. The polyA additional signal site is in boldface. The internal sequencing primers are underlined. Note that a full length cDNA of peritrophin (ORF = 954 bp with two stop codon at positions 1547 - 1549 and 1668 - 1670 of the entire *LDB4DH* ORF, bold-italicized) were found in the 3′ UTR of *LTB4DH*.

3.5.5 Isolation and characterization of the full length cDNA of a homologue of *X-box binding protein*

The partial sequence of *X-box binding protein* of *P. monodon* was initially obtained from sequencing of the 5' end of the EST from ovarian cDNA library. The size of colony PCR product of this EST revealed that the full length cDNA should be covered by the original EST clone. Therefore, the 3' end sequencing was applied for isolation and characterization of this gene homologue.

The full length cDNA of nucleotides of *X-box binding protein* of *P. monodon* was 1921 bp in length. The ORF of this gene was 858 bp encoding a polypeptide of 285 amino acids with the 5′and 3′UTRs of 265 and 278 bp (excluding the poly A tail, Figures 3.46 and 3.47). The best hit of *X-box binding protein* of *P. monodon* was *XBP1 protein* of *Strongylocentrotus purpuratus* (E-value = 4e-20).

The calculated pI and MW of *LTB4DH* of *P. monodon* were 7.65 and 30661.1 daltons, respectively. The signal peptide was not found in this presumably nonsecretory protein. A BRLZ domain (E-value = 2.32e-10) was found at amino acids positions 93 – 157 (Fig 3.46).



Domain	Begin	End	E-value
BRLZ	93	157	2.32e-10

Figuer 3.46 Diagram illustrating the full length cDNA of *X-box binding protein* of *P. monodon*. The BRLZ domain was found in this transcript. The scale bar is 200 bp in length.

```
CGATGACGATCGCCAGCTCGAATTACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACC   60
GCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGG  120
GACGCAGGAAGACGTAAGATGTAAGAAGCTCCCGAGACCCCACGCCGCTCACACCCAATC  180
CTCGATCTCGACCATCATCATCTTGGAAGCGGACCAGGACCACCGCCTCGAGTTGAGAGA  240
GATTAGAGAGAGAGAGACGAGACCCATGGCCAAGACGATCGTGATAACGCTCCCGAAGGG  300
                         M  A  K  T  I  V  I  T  L  P  K  G

GCTGGGCGACGGGGCCCGAGTGGCGGCTCCGGCGGCCCTCGCCACGGCCTCGCTCGCCTC  360
 L  G  D  G  A  R  V  A  A  P  A  A  L  A  T  A  S  L  A  S
GGACGAGCCGCAGCAGGGGTTCCAGACGCCAGGGTTCCCGCTGGAGGAGGAGGAGGAGGA  420
 R  Q  L  G  S  L  F  A  A  G  N  F  G  S  L  V  H  I  I  K
CAGGCAGCTCGGCTCCCTCTTCGCCGCCGGGAATTTCGGGAGTCTGGTCCACATCATCAA  480
 D  E  P  Q  Q  G  F  Q  T  P  G  F  P  L  E  E  E  E  E  E
GGTGCTGGCGCCGGGCGAGGACAAACCCCCCGCCAGGAAGAGGCAGAGGCTCGACCACCT  540
 V  L  A  P  G  E  D  K  P  P  A  R  K  R  Q  R  L  D  H  L
CAGCATGGAGGAGAAAATCATGAGGAGAAAATTAAAGAATAGAGTTGCCGCACAGACAGC  600
 S  M  E  E  K  I  M  R  R  K  L  K  N  R  V  A  A  Q  T  A
CCGTGACAGGAAGAAGCAGAGAATGGATCAGCTTGAAGCGCAGATTGATGAACTTCGGGA  660
 R  D  R  K  K  Q  R  M  D  Q  L  E  A  Q  I  D  E  L  R  D
CCTAACAGCTGTTTTATCTGAACAGAACACTTGTCTTGCAGAAGAAAATGCAGCACTGAA  720
 L  T  A  V  L  S  E  Q  N  T  C  L  A  E  E  N  A  A  L  K
GGAGATGTTGACAAAATGTACGTGTGGACAGGGCAGCACAGAGAGCAATGTCAACGAAGC  780
 E  M  L  T  K  C  T  C  G  Q  G  S  T  E  S  N  V  N  E  A
CCCTGTCAACAACCCCGCTGGTGGATCAGCAGTATCCCAACCTCAGCAGAAGGCAGTGGG  840
 L  N  V  S  C  D  D  T  H  N  A  A  V  V  V  E  E  V  T  I
CTTGAATGTCAGCTGTGACGACACGCACAACGCAGCAGTTGTCGTTGAGGAAGTCACGAT  900
 P  V  N  N  P  A  G  G  S  A  V  S  Q  P  Q  Q  K  A  V  G
CAACAGTCAAGTCAGCATTTTGCAAACCCTTCCAGTCAAGAAGAGAACTGCAAAGTGGTG  960
 M  L  A  V  L  R  I  M  V  L  S  T  L  C  S  Q  W  V  N  T
AGCTGCCTTGGCAGTGTTAGCAAACAGAACTCTCGGAACCCTTCCCAGTTTACCTGCCAG 1020
 A  A  L  A  V  L  A  N  R  T  L  G  T  L  P  S  L  P  A  S
GATGTTGGCAGTGCTCCGGATCATGGTTCTCTCAACACTCTGCTCACAGTGGGTCAATAC 1080
 N  S  Q  V  S  I  L  Q  T  L  P  V  K  K  R  T  A  K  W  W
GGGACCACACCAACAGTCCTGGAATCCAACTGGGATGTAGCGGACCATGACTATACCAAA 1140
 G  P  H  Q  Q  S  W  N  P  T  G  M  *  R  T  M  T  I  P  K
```

```
CGGACCATGACTATACCAAAGGAAGTCACACAAAGAACGACTTAAACTTGAGCCTTACGC 1200
CTGCTGAGGACGACCTTATCACACAACTGACGAGAGCCATGGACCAGGTTCCTGAAATCA 1260
GCGTGAAGGACACATCACCAGAAGGCGTCACCATTTCTTTAGACGACGCAGCACCTCAGA 1320
CAGACAGCCTCGCTACACCAGACATTTTAAAGGAACTTCTAGATGGCTGGTTTGAGAGTA 1380
ATGTTGAAGCACAGGAAGGAAGTCAGGGTGCCAATCAGGAGCTGAACACTTCACCAGTGA 1440
AGGAGGAGAACCTCCAACCCACAAATGAGAGAGCTGCATCACCCATCACCAAGGAACAGA 1500
TCCTGGATTTCCTGCAAGACAAGATGCAGAGTCCCAGTCATGGATCCTCCACAGAGTCTG 1560
AGTCTGGTTATGATTCCATCACATCTCCAAGGTCCTTGGGGTCGCCAGAGATGATTGACC 1620
TGACATCCCCAAGGTCTTTGGGGTCGCCAGAGCCACTCGACCAGGAGATGGAACTGGACG 1680
ACTCTTTCAGTGAATTATTCCCATCACTGTTTTAGCTTGTTCAGTATTTAAAAGCACCTT 1740
GTACCTGTGTTTAATATAAGAATGTGTACTTTAGTCAGTTGAATTTTAAGGTTGTAAATA 1800
GTATTTGTAAGGCTTAAGCTTAATGTTTGAAGTGTGATTGCTATTAAAAAGAGATATTTT 1860
CAAAAAAAAAAAAAAAAAAACTCGAGGGGGGGCCCGTACCCAATCGCCTTAGTATCATAA 1920
A..................................................... 1921
```

Figure 3.47 The full length cDNA sequences of *X-box binding protein*. Start and stop codons are illustrated in boldface and underlined. The internal sequencing primers are underlined and boldfaced.

3.6 Identification of introns in the 5′end of *phosphatidylserine receptor* genes using genome walking analysis

The full length cDNA of homologues of *phosphatidylserine receptor* were already isolated by Miss Rachanimuk Preechaphol using RACE-PCR analysis (Fig 3.48).

Genome walking analysis was then applied to isolate the 5′ and 3′ genomic sequence of this gene. The amplification product was obtained from 5′ but not 3′ genome walking analysis (Figure 3.49).

A 1000 bp fragment was cloned and sequenced. Three introns (37, 277 and 212 bp) were found within the amplification product which contained 3 exons 213, 153 and 107 bp). Nevertheless, the obtained sequences indicated that introns of *phosphatidylserine receptor* of *P. monodon* did not follow a GT/AG rule (Figure 3.50). The partial genomic sequence of this gene can be used for identification of SNP in across different individuals.

```
TTAACATGATTACGCCACCTCGAATTTAAACCTCACTAAAGGGAACAAACAGCTGGAGCT   60
CCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGCTTTCCTCCT  120
GTTTTTGTCAAGCCCATGGATCACAGGTGCCGGAAGAGAGTGAGAGAAGCCAAAAAGAAA  180
GCGAGACCAGAATTATGTTTAGAAAAGGGCGTGTGGACAAAACATGACTACAAGAACAAG  240
TTTGACTGCTCTATAGACATTGTTCCAGATACAGTAGAACGCATTCATGCAAATGAGGTC  300
CCAATTGAAGAGTTCATCGAGCGATATGAGAAGCCTTACAAACCAGTTGTTATTGAAGCG  360
GTCACAGACAACTGGAAGGCTCGATATAAGTGGACCCTAGAGAGGTTATACAAGAAATAC  420
AGAAATCAGAAATTCAAGTGTGGAGAAGATAATGAAGGCTACAGCGTAAAGATGAAGATG  480
AAGTATTACATTGAATATATGAAGACAACAGATGATGATAGTCCACTCTATATATTTGAT  540
AGCAGTTTTGGTGAGCATGTCCGTCGTAAAAAGCTTCTAGAAGATTATGATGTGCCGAAA  600
TATTTTCGAGATGATTTATTTAAGTATGCCGGTGAGGATAGACGCCCCCCTTACCGCTGG  660
TTCGTCATGGGTCCTGCTCGTTCAGGGACCGGCATTCACATAGATCCTTTAGGAACTAGT  720
GCTTGGAATACGCTTCTCCGTGGTCACAAAAGATGGTGCATGTTCCCTACAGATACTCCC  780
AAGGAGCTTATTAAGGTGACTTCTGCAGAAGGAGGGAAGCAAGTAGATGAAGCTATAACA  840
TGGTTCTCCATTATTTATCCTCGTACGAAACTTCCAAATTGGCCAGAGAAATATAAGCCG  900
CTGGAGTTGGTGCAGCATCCTGGTGAAACAGTATTTGTGCCTGGAGGCTGGTGGCATGTT  960
GTCATTAACCTTGACAATACAATTGCCATCACTCAGAACTTTGCTTCATGCACAAACTTC 1020
CCTGTTGTGTGGCACAAGACTGTGCGGGGCCGTCCAAAGCTTTCCAAAAAGTGGCTTCGA 1080
ATCTTAAGGGAGAAGAAGCCAGACCTAGCAGTTGTAGCAGATTCAATTGATATGAGCAAA 1140
AAAAAGAAGAGGAGGAAGACACACAACTCTCCTCCGAGGTCTTGAGGGAGCCAGTCCAGC 1200
GATGAATCCTCATCCTCTGAGAGTGTTTCAGACAGTGGTCAGGAGAGCTTACCAGAACAT 1260
AGCTGTGGAATGGCATCTGACTCCTCATCAGATTCATCTTCATCGTCTTCCTCGTCTTCT 1320
AGCGCTCTTGAAGACGGTGCTGCTGCTTCCGACTCTCCACTCCCCTCTATCCTTCCAACA 1380
CCACCACCAAGGAATGTTTCTCCAGCTTGTGTCATGTCTGCTTGTAATGCACATTGGTTC 1440
TCACCTCGCATTCTAGGCATACAACAAAGACATTGATACGATGAATTCTCAGAGAGCATG 1500
TAGGATATACATATATATTTATATTACTGTCCACATTTTTTACTTTTTTATTGTATTACC 1560
CCAGAGCTTATATACCATCGGAATAACGTATACTATGTATTTGGTGAAAACTTTTAAGTG 1620
GAAGTATTAGATATTAATACATGTAACAGAAAAAAGGGTAAAAATATTTCCTGAAACACA 1680
GGATGATAGATCAGATGTTTTGATTTATATATCTTGGTTGCTTATGTCAGCAAAAGAAAT 1740
AAATCATAAAAAAAAACAATAAAATAAACAAAATGACAAAAAAAAAAAAAAAAAAAAAAA 1800
AAA..................................................... 1803
```

Figure 3.48 The full length cDNA of a homologue of *phosphatidylserine receptor* previously isolated and characterized by RACE-PCR analysis. The multiple polyA additional site is boldfaced.

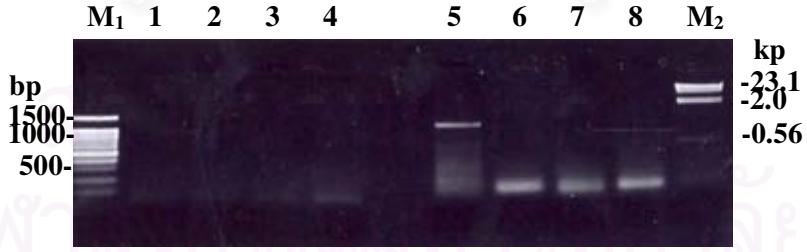


Figure 3.49 The primary PCR product of *phosphatidylserine receptor*. The *Alu* I mini-library of male (lane 1) and *Dra* I mini-library (lanes 2 and 3), *Hae* III mini-library (lanes 4 and 5) and *Rsa* I mini-library of female and male (lanes 6 and 7) were amplified with the forward gene-specific primers and the adapter primer (AP1) (lanes 1 - 4) and the reverse gene-specific primer and the AP1 primer (lanes 5 - 8), respectively. A 100 bp DNA ladder (lane M1) and λ–*Hin*d III were used as the DNA markers.

*AAACAACTCTTTTTTTCATAATGGTTATTTTTACTTT*CAGAATTATGTTTAGAAAAG
GGCGTGTGGACAAAACATGACTACAAGAACAAGTTTGACTGCTCTATAGACATTGTT
CCAGATACAGTAGAACGCATTCATGCAAATGAGGTCCCAATTGAAGAGTTCATCGAG
CGATATGAGAAGCCTTACAAACCAGTTGTTATTGAAGCGGTCACAGACAACTGGAAG
GCTCGATATAAGTGGACCCTAG*AGGTACAGCAGACATCTGAGTATGGCAATTATTGT*
AAATAAAGTTTATTTTTTAGATTGATATTGAGGTAGGAAGGAAAAAGTTCAAGAATT
GGGATTTGATTAAGGTAAAAATCATAGTTGTATTATATATGTAACATTTACAATTAT
ATAATGTGAAAGTAGATGGATTGAACCCAAAATTTGGTCATATCAGCTAGCATATTG
AGACTAATATAGACATTAAAAAGTCTTATGATTTAATTATTTAATTATATCAATGTT
*CATGTACCTTTTAC*AGAGGTTATACAAGAAATACAGAAATCAGAAATTCAAGTGTGG
AGAAGATAATGAAGGCTACAGCGTAAAGATGAAGATGAAGTATTACATTGAATATAT
GAAGACAACAGATGATGATAGTCCACTCTATATATTTGATAGCAGTTTTGGTG*AGGT*
GAGAACCCTTTTCTGGGTACTTAGTTCCTGGTTACTCTAAGTTTACGTATTAGTTTG
AGTAAAATGTTATTTTAGTACCAGTAGGTTTTCAGTGTATTGCAGTTTATTTAATTC
ATATATTTTCCTTTTTTATTCCATAATGCTTGTGTCCGTAATACTTTTGATTTTTAA
*TTTATGGCACACACTATTCATGTACTGCCTCGCTTTC*AGCATGTCCGTCGTAAAAAG
CTTCTAGAAGATTATGATGTGCCGAAATATTTTCGAGATGATTTATTTAAGTATGCC
GGTGAGGATA**GACGCCCCCCTTACCGCTGG**

Figure 3.50 Nucleotide sequence obtained from the 5′ genome walking fragment of *phosphatidylserine receptor*. Introns are italicized and underlined. Genome walking primers are illustrated in boldface and underlined.

3.7 Determination of the expression level of functionally important genes *in P. monodon* by end point RT-PCR

LTB4DH also recognized as *15-oxoprostaglandin 13-reductase* may functionally involve in reproductive maturation and ovarian development of female *P. monodon*. The expression patterns of this transcript in ovaries and testes of broodstock-sized *P. monodon* were then examined. Results indicated preferential expression levels of *15-oxoprostaglandin 13-reductase* in ovaries of female *P. monodon* ($N = 6$ for each sex, Figure 51).

Likewise, expression patterns of *PI4K*, *X-box binding protein*, *RUVB-like protein* were examined and revealed a greater expression levels in ovaries than testes of *P. monodon* broodstock (Figures 3.52 – 3.54).

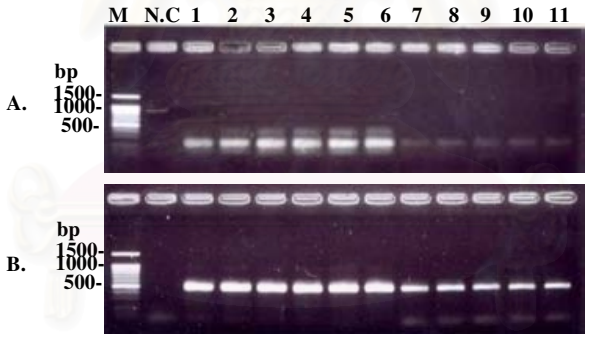


Figure 3.5.1 RT-PCR of a *LTB4DH* homologue (*15-oxoprostaglandin 13-reductase*, panel A) using the first strand cDNA of ovaries (lanes 1 - 6) and testes (lanes 7 - 11) of *P. monodon* broodstock. *EF-1-α* was included as the positive control and successfully amplified with all specimens (lanes 1 - 11, panel B). Lanes M and N are a 100 bp DNA marker and the negative control, respectively.

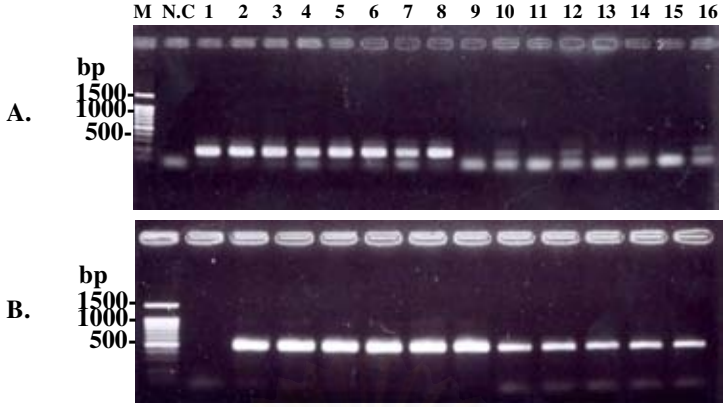


Figure 3.52 RT-PCR of a homologue of *X-box binding protein* (panel A) using the first strand cDNA of ovaries (lanes 1 - 8) and testes (lanes 9 - 16) of *P. monodon* broodstock. *EF-1-α* was included as the positive control and successfully amplified with all specimens (lanes 1 - 16, panel B). Lanes M and N are a 100 bp DNA marker and the negative control, respectively.

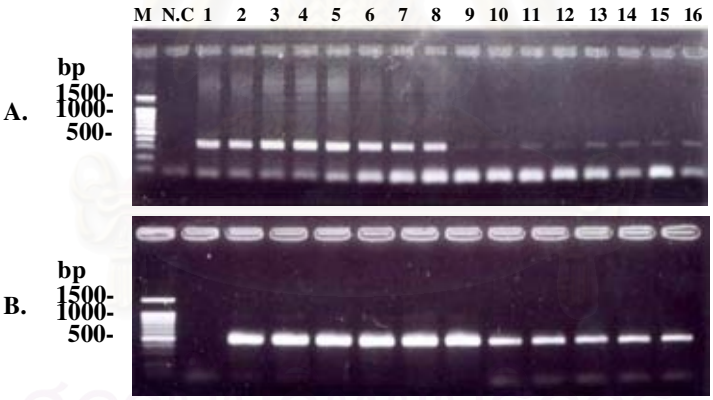


Figure 3.53 RT-PCR a of homologue of *phosphatidylinositol-4 kinase* (panel A) using the first strand cDNA of ovaries (lanes 1 - 8) and testes (lanes 9 - 16) of *P. monodon* broodstock. *EF-1-α* was included as the positive control and successfully amplified with all specimens (lanes 1 - 16, panel B). Lanes M and N are a 100 bp DNA marker and the negative control, respectively.

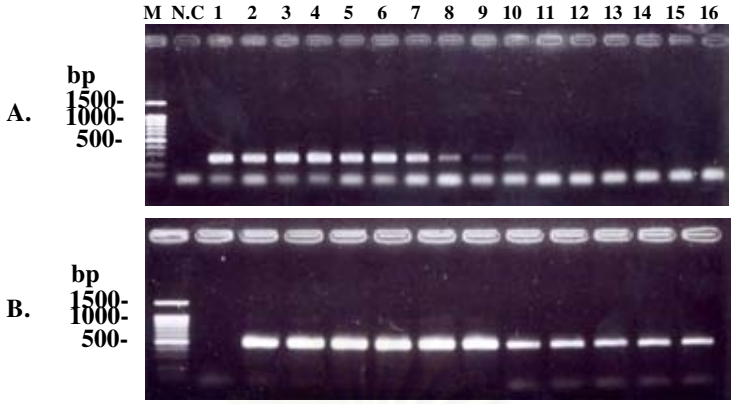


Figure 3.54 RT-PCR of a homologue of *RUVB-like protein* (panel A) using the first strand cDNA of ovaries (lanes 1 - 8) and testes (lanes 9 - 16) of *P. monodon* broodstock. *EF-1-α* was included as the positive control and successfully amplified with all specimens (lanes 1 - 16, panel B). Lanes M and N are a 100 bp DNA marker and the negative control, respectively.

3.8 Semiquantitative RT-PCR of *LTB4DH*, *X-box binding protein* and *RUVB-like protein* upon induction by 5-HT treatment

Gene homologues in this study are functionally involved with reproductive biology and/or growth of *P.monodon*. Effects of 5-HT which is reported as one of the neurotransmitters playing an important role in reproductive maturation of shrimp were examined by semiquantitative RT-PCR.

3.8.1 Optimization of semi-quantitative RT-PCR conditions

The first strand cDNA of ovaries of juvenile shrimp injected with the normal saline (NS) and 5-HT for 12, 24, 48 and 72 hours (A12, A24, A48 and A72, respectively; $N = 5$ for each group) and those of juvenile shrimp repeatedly injected with 5-HT for 12, 24, 48 and 72 hours (B12, B24, B48 and B72, respectively; $N = 5$ for each group) after the first injection, were used as template for a time-course analysis of homologues of *LDB4DH, X-box binding protein* and *RUVB-like protein* using semiquantitative RT-PCR analysis. This technique requires optimization of

several parameters including concentrations of primers, $MgCl_2$, and the number of PCR cycles.

Primers for the target genes were designed. *EF-1α* was used as the control. The end-point RT-PCR was carried out by using 100 ng of 1μl of 10 fold dilution of the first strand cDNA template from ovaries of a female of *P. monodon* juvenile and the annealing temperature of 53°C as previously used for analysis of gene expression in ovaries and testes of *P. monodon* broodstock.

3.8.1.1 Optimization of primer concentrations

RT-PCR of each gene was carried out with fixed components except primer concentrations (0, 0.10, 0.15, 0.20 and 0.25 μM). Lower concentrations may result in non-quantitative amplification whereas higher concentrations of primer may leave a large amount of unused primers which could give rise to non-specific amplification products. The suitable concentration of primers for each gene is shown by Table 3.5.

3.8.1.2 Optimization of $MgCl_2$ concentrations

The optimal concentration of $MgCl_2$ (between 0, 1.0, 1.5, 2.0 and 3.0 mM) for each primer pair was carefully examined using the amplification conditions with the optimal primer concentration. The concentration of $MgCl_2$ that gave the highest yields and specificity for each PCR product was chosen (Table 3.5).

3.8.1.3 Optimization of cycle numbers

The number of amplification cycles was important because the product reflecting the expression level should be measured quantitatively before reaching a plateau amplification phase. At the plateau stage, transcripts initially present at different levels may give equal intensity of the amplification products.

In this experiment, RT-PCR of each gene was performed using the conditions that primers and $MgCl_2$ concentrations were optimized for 18, 20, 23, 25 and 30 cycles. The number of cycles that gave the highest yield before the product reached a plateau phase of amplification was chosen (Table 3.5).

Table 3.5 Optimal primer and $MgCl_2$ concentrations and the number of PCR cycles for semiquantitative analysis of genes in *P. monodon*

Transcript	Expected amplicons (bp)	Primer concentration (μM)	$MgCl_2$ concentration (mM)	PCR cycles
LTB4DH	230	0.15	1.5	22
X-box binding protein	185	0.15	2.0	22
RuvB like protein	257	0.15	1.5	25

3.9 Semiquantitative RT-PCR

3.9.1. *LTB4DH*

The expression levels of *LDB4DH* in juvenile *P. monodon* upon single injection with 5-HT (group A) was comparable to that of double injection (group B) and both treatment was significantly higher than the control ($P < 0.05$).

Within the single injection group, the expression level of *LDB4DH* was initially up-regulated at 12 hour post treatment (12 hpt; 0.689 ± 0.030, $P < 0.05$) and further increased at 24 hpt (0.706 ± 0.031, $P < 0.05$). The highest expression of female sterile was observed at 72 hpt (0.873 ± 0.061, $P < 0.05$). The low expression level at 48 hpt (0.571 ± 0.041 compared to 0.558 ± 0.087 of the control, $P > 0.05$) should have resulted from the experimental errors rather than the actual effect of 5-HT (Figures 3.55 and 3.56; Table 3.6).

Within the double injection group, the expression of *LDB4DH* was significantly up-regulated at 12 and 24 hpt (0.876 ± 0.079 and 0.802 ± 0.099, $P < 0.05$). The expression of *LDB4DH* was reduced to the normal level at 48 and 72 hpt (0.604 ± 0.085 and 0.604 ± 0.041, $P > 0.05$) (Figures 3.55 and 3.56; Table 3.6).

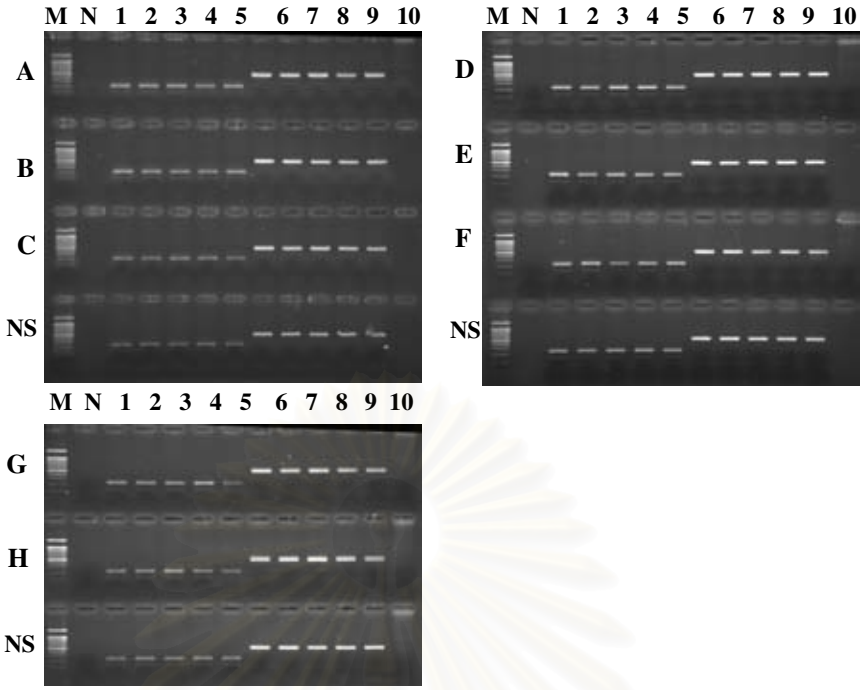


Figure 3.55. A 1.8% ethidium bromide-stained agarose gel showing the expression level of *LDB4DH* (lanes 1 - 5) and *EF-1α* (lanes 6 - 10) of single (A - D) and double (E - H) injection of 5-HT for 12 (A and E), 24 (B and F), 48 (C and G) and 72 (D and H) hours post injection. The positive control (0.85% NaCl, NS) was also included. Lane M = 100 bp ladder DNA.

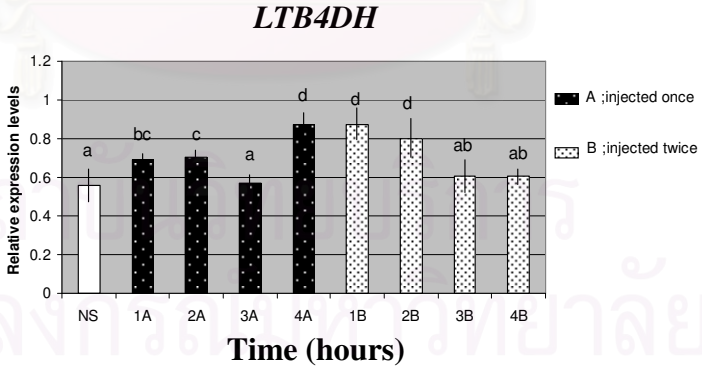


Figure 3.56 Histograms showing the time-course relative expression levels of *LDB4DH* for 12, 24, 48 and 72 hours post treatment of 5-HT. The positive control (0.85% NaCl, NS) was also included. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.9.2 *X-box binding protein*

The expression levels of *X-box binding protein* in juvenile *P. monodon* upon single injection with 5-HT (group A) was comparable to that of double injection (group B) and both treatment was significantly higher than the control (P <0.05).

Within the single injection group, the expression level of *X-box binding protein* was initially up-regulated at 12 and 24 hpt (0.746 ± 0.044 and 0.702 ± 0.077, P <0.05) and seemed to be returned to the normal level at 48 hpt (0.623 ± 0.035 compared to 0.562 ± 0.052 of the control, P >0.05). The highest expression of *X-box binding protein* was observed at 72 hpt (0.859 ± 0.095, P <0.05) but the expression level should be resulted from the experimental errors rather than the actual effect of 5-HT (Figure 3.57 and 3.58; Table 3.6).

Within the double injection group, the expression of *X-box binding protein* was significantly up-regulated at 12 hpt (0.748 ± 0.037, P <0.05). The stimulating effects were still prolonged through 24, 48 and 72 hpt (0.682 ± 0.046, 0.715±0.053 and 0.708 ± 0.091, P <0.05) (Figures 3.55 and 3.56; Table 3.8).

3.9.3 *RUVB-like protein*

The expression levels of *RUVB-like protein* in juvenile *P. monodon* upon single injection with 5-HT (group A) was lower than that of double injection (group B) and both treatment was significantly higher than the control (P <0.05).

Within the single injection group, the expression level of *RUVB-like protein* was significantly up-regulated at 12 hpt (0.911 ± 0.119, P <0.05) and returned to the normal level at 24, 48 and 72 hpt (0.752 ± 0.104, 0.659 ± 0.106 and 0.714 ± 0.0124 compared to 0.653 ± 0.264, P >0.05). (Figure 3.59 and 3.60; Table 3.6).

Within the double injection group, the expression of *RUVB-like protein* was not singnificantly different from the normal level at 12 and 24 hpt (0.697 ± 0.052 and 0.646 ± 0.118, P >0.05) but the expression levels were up-regulated at 48 and 72 hpt (1.049 ± 0.015 and 1.044 ± 0.066, P <0.05).

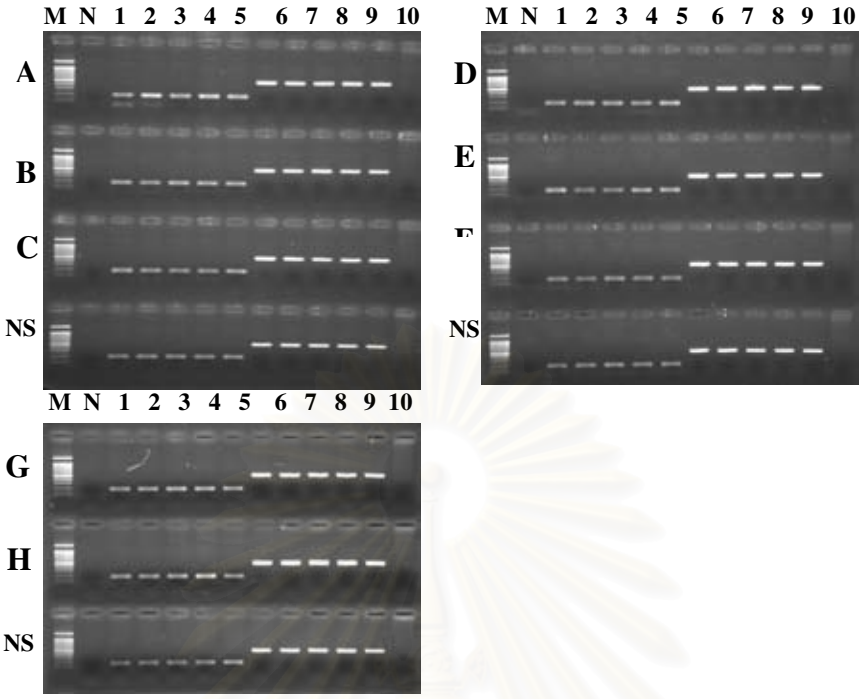


Figure. 3.57 A 1.8% ethidium bromide-stained agarose gel showing the expression level of *X-box binding protein* (lanes 1 - 5) and *EF-1α* (lanes 6 - 10) of single (A - D) and double (E - H) injection of 5-HT for 12 (A and E), 24 (B and F), 48 (C and G) and 72 (D and H) hours post injection. The positive control (0.85% NaCl, NS) was also included. Lane M = 100 bp ladder DNA.

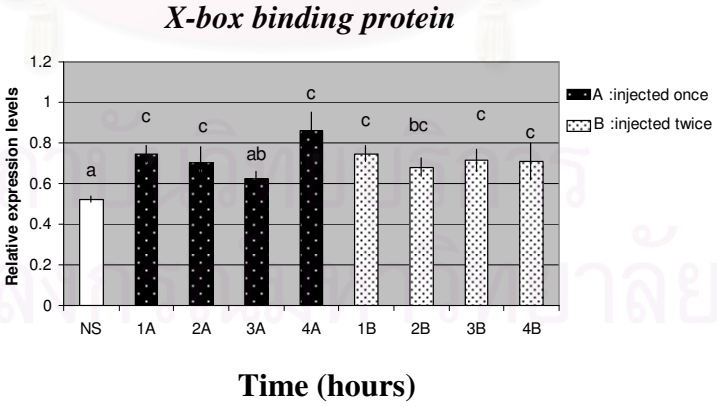


Figure 3.58 Histograms showing the time-course relative expression levels of *X-box binding protein* for 12, 24, 48 and 72 hours post treatment of 5-HT. The positive control (0.85% NaCl, NS) was also included. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

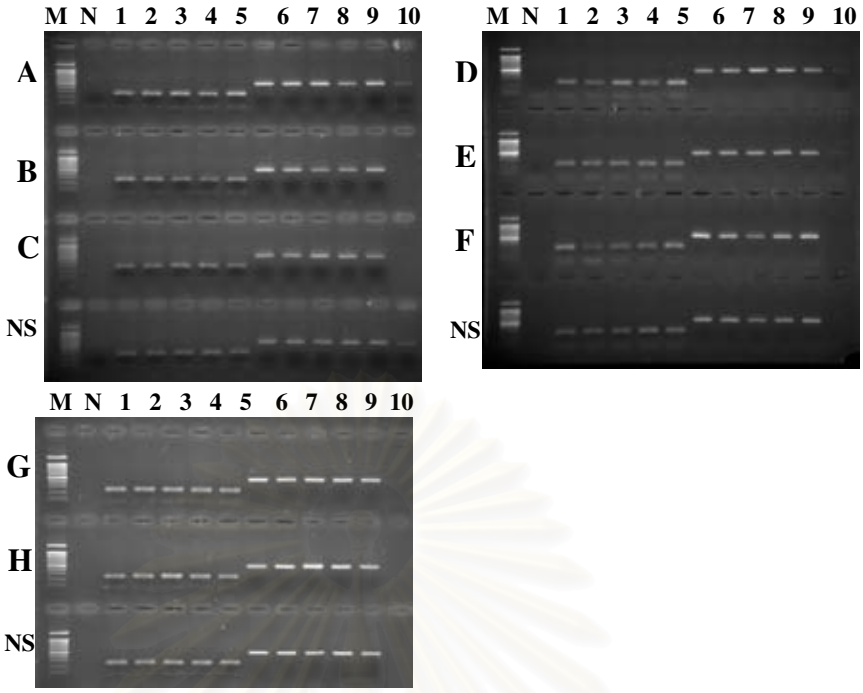


Figure 3.59 A 1.8% ethidium bromide-stained agarose gel showing the expression level of *RUVB-like protein* (lanes 1 - 5) and *EF-1α* (lanes 6 - 10) of single (A - D) and double (E - H) injection of 5-HT for 12 (A and E), 24 (B and F), 48 (C and G) and 72 (D and H) hours post injection. The positive control (0.85% NaCl, NS) was also included. Lane M = 100 bp ladder DNA.

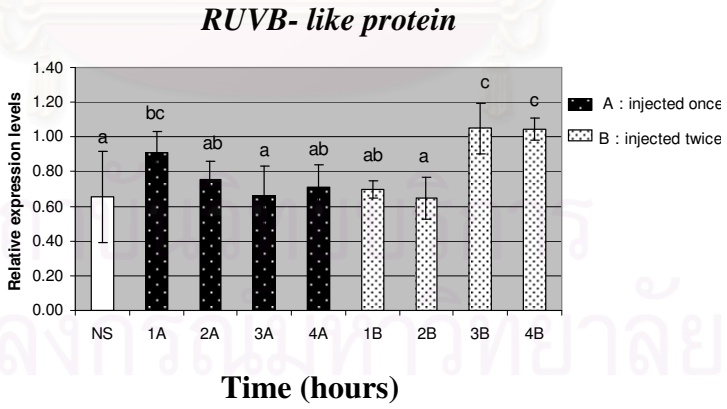


Figure 3.60 Histograms showing the time-course relative expression levels of *RUVB-like protein* for 12, 24, 48 and 72 hours post treatment of 5-HT. The positive control (0.85% NaCl, NS) was also included. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.6 A time-course analysis of *LDB4DH*, *X-box binding protein* and *RUVB-like protein* expression levels using semiquantitative RT-PCR. The same superscripts between different time interval data are not significantly different ($P > 0.05$)

Gene	Mean Relative Expression Level*								
	Control	**12A**	**24A**	**48A**	**72A**	**12B**	**24B**	**48B**	**72B**
LTB4DH	0.558±0.087^{a}	0.689±0.030$^{b\,c}$	0.706±0.031^{c}	0.571±0.041^{a}	0.873±0.061^{d}	0.876±0.079^{d}	0.802±0.099^{d}	0.604±0.085^{ab}	0.604±0.041^{ab}
Xbp	0.562±0.052^{a}	0.746±0.044^{c}	0.702±0.077^{c}	0.623±0.035^{ab}	0.859±0.095^{d}	0.748±0.037^{c}	0.682±0.046^{bc}	0.715±0.053^{c}	0.708±0.091^{c}
RUVB	0.653±0.264^{a}	0.911±0.119^{bc}	0.752±0.104^{ab}	0.659±0.106^{a}	0.714±0.0124^{ab}	0.697±0.052^{ab}	0.646±0.118^{a}	1.049±0.015^{c}	1.044 ±0.066^{c}

*The expression of *EF-1α* was normalized to 1.00. The expression of the target genes was determined as the signal ratio between the target gene and *EF-1α*.

CHAPTER IV

DISCUSSION

Identification of genes exhibiting polymorphic SNP by SSCP analysis

Several approaches have been used for SNP discovery. These include DNA sequencing (Paul Hunt et al., 2005), SSCP analysis (Hecker et al., 1999), heteroduplex analysis (Sorrentino et al., 1992), allele specific oligonucleotide (ASO) (Malmgren et al., 1996), denaturing gradient gel electrophoresis (DDPG)(Cariello et al., 1988), ligation chain reaction (LCR)(Kalin et al., 1992) and mass spectroscopy (Sasayama et al., 2006). Each approach has its advantages and limitations, but all are still useful for SNP genotyping. Large-scale analysis of SNP, however, requires the availability of expensive, cutting-edge equipment. SSCP was chosen for analysis of gene polymorphism in this study owing to its simple and cost-effective than other method but results of this method should be further confirmed by DNA sequencing due to the false positive/negative of the technique.

Homologues of known transcripts from cDNA libraries of ovaries, testes, hemocytes and RAP-PCR fragments of *P. monodon* were chosen for polymorphic studies using SSCP. Approximately 51.85% (56/108) of primers yielded the amplification product. The failure to amplify the product in 48.15% of investigated primers is possible due to the priming of primers at part of the region flanked by the intron at one or both priming sites or alternatively, the existence of a large intron (s) in the amplification region to be amplified by typical PCR. The other possibility is that several primers used may not be appropriate and new primers should be re-designed.

Several primers generated the larger fragment size than that expected from cDNA sequences due to the presence of the intron (s) located within the amplification region. SSCP is effectively identified 80% of a single nucleotide polymorphism (SNP) when the analyzed fragments are less than 300 bp in length (Shastry, 2002). Typically, large amplification fragment should be digested with restriction enzyme

before subjected to SSCP analysis. Nevertheless, large fragments (up to 900 bp) could be successfully analyzed in this study by simply lowering the percentage of crosslink between acrylamide and bis-acrylamide and of the gels.

Primers were initially tested against genomic DNA of wild *P. monodon* because a lack of an appropriate based population (families). Among 42 gene homologues screened, 37 gene exhibited different degrees of polymorphism (88.09%). SSCP patterns of *splicing factor 3a, subunit 1*, *prosaposin, O-metyltransferase, USO1, gene flightless* and *hyaluronan receptor* were polymorphic but the patterns was scored as dominant markers (homozygotes and heterozygotes cannot be differentiated from one another).

In contrast, homologues of *phosphoglucose isomerase*, *solute carrier family 3 member 2*, *rasputin*, *RNA helicase*, *heterogeneous nuclear ribonucleoprotein 87F*, *laminin-β chain*, *phosphatidylinositol-4-kinase*, *adenine nucleotide translocator 2*, *RUVB like protein2, arginyl-tRNA-protein transferase, SRY-box 7* (*SOX7*) and *FV-42* can be scored as co-dominant markers.

Polymorphic SSCP patterns reflecting the availability of SNP in the gene fragments are applicable to various applications. Klinbunga et al (2006) analyzed SSCP patterns of *disulfide isomerase* (*DSl*), *zinc .finger protein* (*ZFP*), *PMO920*, and *PMT1700* for population genetic studies of *P. monodon* in Thai waters. The number of codominant alleles per locus for overall samples was 6 for *PMO920*, 5 for *PMT1700*, and 12 for *ZFP*, and there were 19 dominant alleles for *DSI*. The observed heterozygosity of each geographic sample was 0.3043 - 0.5128 for *PMO920*, 0.3462 - 0.4643 for *PMT1700*, and 0.5000 - 0.8108 for *ZFP*. Linkage disequilibrium analysis indicated that genotypes of these loci segregate randomly ($P > 0.05$). Low genetic distance was found between pairs of geographic samples (0.0077 - 0.0178). The neighbor-joining tree constructed from the average genetic distance of overall loci allocated the Andaman samples (Satun, Trang, and Phangnga) into one cluster, and Chumphon and Trat into other clusters. Geographic differentiation between Satun-Trat and Satun-Phangnga was found only at the *ZFP* locus ($P < 0.05$), suggesting low degrees of genetic subdivision of Thai *P. monodon*.

Single nucleotide polymorphism by expressed sequence tags (SNP by EST, SBE) were recently developed by amplification of 102 previously identified ESTs in ovaries and hemocytes of *P. monodon*. A total of 48 successfully amplified genes were further characterized by SSCP analysis and 44 of which were polymorphic. The full length of *ribophorin I* and *receptor for activated protein kinase C* (*RACK*) were successfully characterized by RACE-PCR. Semi-quantitative RT-PCR of *ribophorin I* and *RACK* were carried out. Significantly different expression levels of *ribophorin I* ($P < 0.05$) but not in *RACK* ($P > 0.05$) were observed in ovaries and testes of *P. monodon* broodstock. Although the preliminary study did not reveal significant association between SNP (SSCP patterns) and levels of *ribophorin I* and *RACK* expression in the limited sample size of *P. monodon* broodstock, that approach demonstrates the possibility to further test for association between SNP of candidate genes allied with growth and reproduction (degrees of gene expression by the wild type/mutant alleles, growth rate and reproductive performance) of *P. monodon* (Klinbunga et al., 2006).

SSCP derived from AFLP analysis was also applied for identification of genomic sex determination marker in *P. monodon*. Bulked segregant analysis (BSA) and AFLP were used. A total of 256 primer combinations were tested against 6 - 10 bulked genomic DNA of *P. monodon*. Five and one candidate female- and male-specific AFLP fragments were identified. Female-specific fragments were cloned and further characterized. SCAR markers derived from FE10M9520, FE10M10725.1, FE10M10725.2 and FE14M16340 provided the positive amplification product in both male and female *P. monodon*. Further analysis of these markers using SSCP and genome walk analysis indicated that they were not sex-linked (Khamnamtong et al., 2006).

The PCR product of *LTB4DH*, *RUVB-like protein 2*, *phosphatidylserine receptor short form*, *and solute carrier family 25* was gel-eluted, direct sequenced and multiple aligned. Reasonable polymorphism when screened with genomic DNA of wild *P. monodon* broodstock was found.

Results from DNA sequencing indicated over-estimation of polymorphism by SSCP. For example, 5, 4, and 3 SSCP genotypes previously found in *NADP-dependent leukotriene-12-hydroxydehydrogenase* (*LTB4DH*), *RUVB-like protein 2*

and *phosphatidylserine receptor short form* revealed 4, 3 and 2 different type of sequences. In contrast, 3 different types of nucleotide sequences were obtained from 3 SSCP genotypes of *solute carrier family 25*. This indicated that the false positive ratter than the false negative may be obtained from polymorphic studies using SSCP analysis.

Nevertheless, sequencing errors from direct sequencing of the PCR product should be taken into account because numbers of substitutions and indels between SSCP genotypes showing different DNA sequences were too large. For instance a 523 bp aligned nucleotides of 5′and 3′ of *LTB4DH* contained 43 SNP/indels, a 380 bp aligned nucleotides of *phosphatidylserine receptor* contained 67 SNP/indels and a 312 bp aligned nucleotides of *RUVB-like protein* contained 21 SNP/indels. To overcome the possibility of the sequencing errors, the PCR product representing different SSCP genotypes of each gene should be cloned and subsequently sequenced.

Generally, the efficiency to detect single base substitutions by SSCP was approximately 80% of those verified by DNA sequencing (Shastry, 2002). Results from this study indicated that SSCP is a simply powerful technique for detection of polymorphism of DNA segments when used with cautions.

Association between SNP through SSCP patterns of functionally important genes and the growth rates of *P. monodon*

SNP markers in candidate genes can be treated as similar as other bi-allelic co-dominantly segregated DNA markers. The advantage of coding SNP is that they are located in regions that code for functionally important proteins. Therefore, they are more likely to be near QTL that affect commercially important traits. Several SNP positions should be existent in the amplified gene segment of *P. monodon*. This promotes the efficiency for detection of SNP by SSCP.

Correlations between genotypic and phenotypic variations in shrimp are still not understood. Therefore, effects of SNP and expression levels and phenotypes of functional important genes in *P. monodon* should be carried out. Analysis of gene-based SNP is possibly one of the efficient approaches for discovery of genes which are important in complex traits in *P. monodon*.

To preliminary screen for association between SNP through SSCP genotypes and the growth rate of *P. monodon*, the G2 domesticated family of *P. monodon* (132 days of age, $\overline{X}$ = 18.8609 ± 4.64 g, N = 500) were collected. Based on the fact that the primary objective for collecting those specimens was the construction of genetic linkage maps of *P. monodon*, only shrimp exhibiting the 10% top (N = 50, $\overline{X}$ = 26.86 ± 3.33 g) and the 10% bottom (N = 50, $\overline{X}$ = 10.71 ± 2.02 g) of the body weight were sampled. Therefore, the power of the test was reduced due to the discontinuous distribution of the body weight rather than random samples were used for identifying quantitative trait loci (QTL) like those linked with the growth rates of *P. monodon*.

Nineteen polymorphic gene segments were applied for genotyping of the G2 family of *P. monodon*. Seven genes (*FII-17*, *FIII-4*, *FIII-8* from RAP-PCR and *solute carrier family 3 member 2, DDPG*, *phosphatidylinosiol-4 kinase* and *phosphatidylserine receptor short form*) did not revealed polymorphic patterns in these specimens.

Nine RAP-PCR fragments (*457/OPA01*, *428/OPB17*, *MI-36*, *MII-51*, *FI-40*, *FIV-33*, *FV-1*, *FV-27* and *FV-42*) were polymorphic. Nevertheless, these transcripts were identified by randomly amplified of the expressed transcripts in ovaries of *P. monodon*. After annotation, they were all regarded as unknown transcripts. The full length cDNA of these transcripts were still not isolated and their functions are not known. Therefore, they were not included for further analysis.

In contrast, homologues of *X-box binding protein*, *LTB4DH*, *RUVB-like protein* also revealed polymorphic patterns in the G2 family of *P. monodon* (N =10) and acted as co-dominant markers. These gene homologues were chosen for further association analysis between SNP by SSCP patterns and the growth rates of the G2 family of *P. monodon*.

Results indicated no correlation between the body weight of 132-day-old *P. monodon* and SSCP genotypes of *LTB4DH* (2 genotypes) and *X-box binding protein* (3 genotypes). Nevertheless, a statistical significance between genotypes (SSCP patterns) of *RUVB-like protein* and phenotypes (the body weight of a 132-day-old shrimp) was found in presumably the fast growing shrimp exhibiting the genotype II (ADG = 0.22) and the genotype I (ADG = 0.20) of *RUVB-like protein* ($P < 0.05$).

Although presumably slow growth shrimp exhibiting the genotype II (ADG = 0.09) possessed a slightly greater body weight than those possessed the genotype I (ADG = 0.08). The result in shrimp exhibiting the slow growth rates was not statistically significant ($P > 0.05$). This may be resulted from a limited sample size of investigated shrimp ($N = 13 – 17$ for each group).

Poor quality of genomic DNA extracted from the G2 shrimp was obtained due to inappropriate storage of specimens in the domesticated facilities. Therefore, the success amplification of this sample set was relatively low. In addition, SSCP patterns of *RUVB-like protein* in some individuals were not clear and not scorable. This decreased the number of investigated individuals from 13 and 17 individuals for shrimp carrying genotypes I and II in the slow growth group and 15 and 16 individuals for those in the presumably fast growing group rather than a total of 25 individuals per group. The G2 family used in the experiment was established from inbred founders (G0) limiting the ability to examine correlation between the age-specific growth rate and genotypes of *solute carrier family 3 member 2, DDPG* and *phosphatidylserine receptor short form* that exhibit polymorphism during the primary screening with natural *P. monodon*.

Gupta (2007) examined SNP in the exons 4 and 5 of the growth hormone gene using PCR-SSCP in the Black Bengal goats, a prolific meat breed of India. SSCP showed 7 and 5 haplotypes in caprine GH gene exon-4 and exon-5 in Black Bengal. All haplotypes revealed novel sequences. In exon-4, codons 6, 36 and 54 were polymorphic. At codon 6, AA arginine (R) changed to histidine (H) and proline (P), showing 6RR, 6HH and 6PP genotypes. At codons 36 three genotypes DD, VV and DV were observed due to SNP showing changed from aspartic acid (D) to valine (V). At codon 54, AA change from arginine to tryptophan (W) and 54RR and 54WW genotypes were observed. SNPs were also observed at codon 23 (serine to threonine) and at 37 (arginine to proline) in 8% of goats. In exon-5 nucleotide substitution (G/A) at codon 10 and (A/G) at 14 respectively changed AA from glycine (K) to glutamic acid (E).

In chicken, genes that are part of the somatotropic axis play a crucial role in the regulation of growth and development. A total of 75 pairs of primers were designed and four chicken breeds, significantly differing in growth and reproduction

characteristics, were used to identify SNP using the denaturing high performance liquid chromatography (DHPLC). A total of 283 SNP were discovered in 12 growth-related genes The observed average distances in bp between the SNP in the 5′UTR, coding regions (non- and synonymous), introns and 3′UTR were 172, 151 (473 and 222), 89 and 141 respectively. Fifteen non-synonymous SNP altered the translated precursors or mature proteins of *GH*, *GHR*, *ghrelin*, *IGFBP-2*, *PIT-1* and *SS*. Fifteen indels of no less than 2 bps and 2 poly (A) polymorphisms were also observed in 9 genes. Fifty-nine PCR-RFLP markers were found in 11 genes. The SNP discovered in that study provided suitable markers for association studies of candidate genes for growth related traits in chickens (Nie et al., 2005).

SNP in the coding sequence of the bovine transferrin gene was determined. Two alleles (SSCP1 and SSCP2) were detected by SSCP analysis and the mutation point was identified and confirmed by direct sequencing of the PCR products. The relationship between protein and DNA polymorphism was established. Protein variants A, D1 and E correspond to SSCP allele 1 and variant D2 corresponds to SSCP allele 2. DNA sequences from genotypes AA, AE, AD2, D1E, D2E and D2D2 reveal an A/G substitution at position 1455 of the cDNA which causes a Gly/Glu substitution which could be responsible for the mobility difference between D1 and D2 variants. Because of the number of variants, this suggests that other SNPs also exist in the bovine transferrin gene (Laurent and Rodellar, 2001).

Analysis of gene-based SNP is one of the efficient approaches for discovery of genes which are significantly contributed in complex traits of *P. monodon*. Although larger sample sizes are required for association analysis of SNP in functional important genes and age-specific growth rates, this preliminary study demonstrated the possibility to locate major loci responding for quantitative traits of *P. monodon* where the information on correlations of genotypes and phenotypes through genetic linkage maps in this species are not available at present.

Glenn et al. (2005) studied association analysis of SNP of alpha-amylase (*AMY2*) and cathepsin-L (*CTSL*). The *AMY2* gene segment contained 4 intronic polymorphism at nucleotides 340, 351, 415 and 501 in *L. vannamei* but not in *P. monodon* sequences. The *CTSL* gene segment contained one intronic polymorphism at nucleotides 681 in *L. vannamei* and 178 in *P. monodon* sequences. PCR-RFLP was

developed to detect SNP of those genes (G351A of *AMY2* by *Sca* I, C618G of *CTSL* by *Pvu* II and G178C of *CTSL* by *Pst* I) using 2 populations of *L. vannalei* (LV1 and LV2, $N = 75$ and 30 with the mean BW of 0.35 ± 0.06 and 2.52 ± 0.30 g, respectively) and a mapping population of *P. monodon* ($N = 41$) of investigated shrimps. SNP genotypes were carried out using PCR-RFLP of *AMY2* with *Sca I* and *CTSL* with *Pvu* II. Neither polymorphism of *AMY2* and *CTSL* were found to be significantly associated with the body weight of LV1 and LV2 populations.

It is not possible to obtain appropriate families of *P. monodon* for SNP identification at present. Accordingly, a new sample set of juvenile *P. monodon* ($N =$ 359, the average body weight = 17.39 ± 4.36 g) was collected from a commercial farm. Microsatellite analysis indicated that specimens could be from several families of wild *P. monodon*.

Unlike the G2 family, the amplification success of *RUVB-like protein* against genomic DNA of the second sample set was nearly 100%. The body weight of juvenile shrimp carrying genotype A and B was greater than those exhibiting genotype C and D for approximately 17.27% ($P < 0.05$). This is the first report for the correlation of SNP and the growth rate of penaeid shrimp.

The amplification product of a shrimp exhibiting each genotype of *RUVB-like protein* (484 nucleotides after multiple alignments) were cloned and sequenced. Three SNP positions ($G \rightarrow A_{81}$, $A \rightarrow T_{196}$ and $T \rightarrow_{G248}$) were unambiguously differentiated 4 SSCP genotypes of this gene homologue.

The $G \rightarrow A_{81}$ caused a synonymous mutation and differentiated the fast growing shrimp (genotypes A and B) from the slow growing shrimp (genotypes C and D) communally cultured in the same pond. The intronic $A \rightarrow T_{196}$ differentiated shrimp exhibiting the genotype A from those exhibiting other genotypes. The intronic $T \rightarrow_{G248}$ SNP differentiated shrimp possessing the genotype D from those possessing other genotypes.

Significant association of SNP (SSCP patterns) and phenotypes (e.g. growth rates) of aquatic species was also reported. Recently, Xu and Zhu (2006) characterized two *parvalbumim* genes and their association with the growth traits in

the Asian sea bass (*Lates calcarifer)*. Expression of *PVALB2* was detected only in muscle, brain, and intestine, was up to 10-fold lower than *PVALB1* expression. A $(CT)_{17}$ microsatellite was identified in the 3′-untranslated region of *PVALB1* and three SNP were identified in the third intron of *PVALB2*. The microsatellite in *PVALB1* was significantly associated with body weight and body length at 90 days post-hatch (P <0.01) whereas the SNPs in *PVALB2* were not associated with these traits (P >0.05).

An understanding of shrimp genome is important for studies about correlations between economically phenotypes and genotypes. A large number of gene homologues identified through an EST approach provide gene catalogues for further polymorphic studies. SNP and indels in EST-derived markers can be analyzed by several methods. The availability of appropriate families with a particular selected trait in the domestication programmes of *P. monodon* will allow the direct application of SNP association analysisto assist genetic selection of *P.monodon*.

Characterization of the full length cDNA of homologues of *rasputin*. *X-box binding protein, NADP-dependent leukotriene B4 12-hydroxydehydrogenase,* and *RUVB-like protein 2*

The full length cDNA of 4 functionally important genes including *X-box protein*, *LTB4DH* and *RUVB-like protein* and *rasputin* were successfully identified and characterized.

The *X-box binding protein* encodes a bZIP-containing transcription factor that plays a key role in the unfolded protein response (UPR), an evolutionarily conserved signalling pathway activated by an overload of misfolded proteins in the endoplasmic reticulum (ER).

Souid et al. (2007) isolated *XBP-1* in *Drosophila* and illustrated that is ubiquitously transcribed, and high levels are found in embryonic salivary glands and in the ovarian follicle cells committed to the synthesis of the respiratory appendages. Loss of function of *Dxbp-1* induced a recessive larval lethality, thus, revealing an essential requirement for this gene. The *Dxbp-1* gene appears to play an essential role during the development of *Drosophila*, hypothetically by stimulating the folding capacities of the ER in cells committed to intense secretory activities.

PMXBP-1 was isolated in this study (ORF of 858 bp encoding a polypeptide of 285 amino acids). It contained three putative *N*-linked glycosylation sites and a BRLZ domain (also called the basic-leucine zipper, bZIP) which is a basic region mediating sequence-specific DNA-binding followed by a leucine zipper region required for dimerization.

LTB4DH (also called 15-oxoprostaglandin 13-reductase; PGR/LTB4DH), possesses two enzymatic activities appreciated for their roles in the metabolism of prostaglandins and leukotriene B4, (LTB4). (Clish et al., 2000). LTB4, a metabolite of arachidonic acid, is a potent chemotactic factor stimulating polymorphonuclear leukocytes, macrophages, and eosinophils through G-protein-coupled receptors (leukotriene B4 receptor; BLT), and plays important roles in inflammatory responses and host defense mechanisms. LTB4 also acts as a regulator of transcription by binding to a peroxisome proliferator-activated receptor alpha. The primary structure of PGR, which catalyses the conversion of 15-keto-PG into 13,14-dihydro 15-keto-PG, was reported to be identical to LTB4 12-HD. PGs mediate a wide range of physiological processes, including ovulation, homeostasis, platelet aggregation, control of water balance, and immune response. PGR is a critical enzyme that irreversibly inactivates all types of PGs (Yamamoto et al., 2001).

PMLDB4DH (ORF of 1038 bp encoding a polypeptide of 345 amino acids with the 3′UTRs of 1387 bp). An ADH zinc *N* domain was found in this gene. This region is the C-terminal domain of the Zinc-binding alcohol dehydrogenases. The prostaglandin reductase activity suggested its involvement in reproductive biology of *P. monodon*. Interestingly, a full length cDNA of peritrophin (ORF = 954 bp with two stop codon that need to be confirmed) were found in the 3′ UTR of *LTB4DH*.

Peritrophin, a major component of cortical rods and is the precursor of the jelly layer of the shrimp eggs, is highly expressed during oocyte development of marine shrimp (Khayat *et al*., 2001). Synthesis of *peritrophin* in ovaries of *P. semisulcatus* is inhibited by crustacean hyperglycemic hormone (CHH) purified from the sinus gland extract of *M. japonicus* (Avarre *et al*., 2001). *Peritrophin* was not differentially expressed between ovaries and testes of *P. monodon* broodstock (Leelatanawit *et al*., 2004) and between different stages of ovarian development of *M. japonicus* after eyestalk ablation (Okumura *et al*., 2006).

RUVB-like protein (also called a *TATA-binding protein*, *TBP*) plays a critical role in maintaining gene diversification through chromosomal rearrangement and also genome stability through the repair of DNA damage and is essential for growth in eukaryotes (Xiao-Bo Qiu et al., 1998). Recently, a 49-kD TBP-interacting protein (TIP49) as isolated in human. The human TIP49 was highly homologous to bacterial *RUVB* proteins that function as a DNA helicase to promote branch migration of the Holliday junction. (Makino et al, 1998) *RUVB* is an ATPase transforming chemical energy into mechanical force necessary to pull DNA through a complex of two RUVA tetramers (Makini et al., 1998).

PMRUVB was successfully isolated and characterized (ORF of 1395 bp encoding a polypeptide of 464 amino acids with the 3′UTRs of 2416 bp). Two putative *N*-linked glycosylation sites and an AAA domain where AAA-ATPases associate with a variety of cellular activities were found.

The *Drosophila* homologue of *Ras-GTPase activating protein SH3 domain binding protein* (*G3BP*) was reported and named *Rasputin*. It interacts with members of the *Ras* signalling pathway. *Rasputin* encodes a protein possessing a central function during many cellular responses, including proliferation and differentiation and regarded as the growth promoting factor.

5′ and 3′ RACE-PCR was used to isolate the full length cDNA of *rasputin* from the ovarian cDNA template of *P. monodon*. The full length cDNA of *P. monodon rasputin* contain an ORF of 1659 bp, which encodes a glycopolypeptide of 552 amino acid residues. Four predicted *N*-linked glycosylation sites were found. A nuclear transport factor 2 (NTF2) domains which is a homodimer of approximately 14kDa subunits and stimulates the efficient nuclear import of a protein was found.

All characterized gene homologues are non-secretory proteins because the predicted signal peptide was not found in their ORF. The full length ORF and 5′ UTR of *rasputin* and *RUVB-like protein* were relatively long allowing the possibility to design additional primers to further used for association analysis between their genotypes and commercially important genotypes of *P. monodon*. Genome walking analysis may be applied for identification of introns in these full transcripts and further used for SNP identification.

The expression of *LTB4DH*, *X-box binding protein* and *RUVB-like protein* are up-regulated by serotonin (5-HT) administration

LTB4DH, PI4K, X-box binding protein, RUVB-like protein were originally isolated from the ovarian cDNA library**.** RT-PCR indicated their preferential expression in ovaries than testes of *P. monodon*. Result critically suggested that these genes may play an importance role in reproductive maturation and ovarian development of female *P. monodon*.

Serotonin (5-HT) stimulates the release of several crustacean hormones including the hyperglycemic hormone (Keller et al., 1985), the red pigment dispersing hormone (Rao and Fingerman, 1975), the molt inhibiting hormone (Mattson and Spaziani, 1985). It has been demonstrated that 5HT injection induced ovarian maturation in the crayfish *Procambarus clarkii* (Kullkarni et al., 1992; Sarojini et al., 1995) and the white Pacific shrimp *L. vannamei* (Vaca and Alfaro, 2000), but at rates lower than the unilateral eyestalk ablation. Recently Alfaro et al. (2004) reported that injection of combined 5HT and dopamine antagonist, spiperone, in *L. stylirostris* and *L. vannamei*, stimulated ovarian maturation, spawning and the release of maturation promoting pheromones.

Recently, effects of exogenous 5-HT on the reproductive performance of *P. monodon* was examined. 5-HT solution was injected into domesticated *P. monodon* broodstock at 50 μg g^{-1} body weight and ovarian maturation and spawning were recorded. The levels of 5HT in ovaries were measured by ELISA. The 5-HT-injected *P. monodon* developed ovarian maturation and spawning rate at the level comparable to that of unilateral eyestalk-ablated shrimp. Hatching rate and the amount of nauplii produced per spawner were also significantly higher in the 5-HT-injected shrimp, compared to the eyestalk-ablated shrimp. 5-HT-positive reactions were found in the follicular cells of pre-vitellogenic oocytes, in the cytoplasm of early vitellogenic oocytes and on the cell membrane and cytoplasm of late vitellogenic oocytes. 5-HT in the ovary was present at 3.53 ± 0.26 ng/mg protein level in previtellogenic stage and increased to 17.03 ± 0.57 ng/mg protein level in the mature stage of the ovaries (Wongprasert et al., 2006).

The splicing of 26 nucleotides in the coding region of the *X-box binding protein-1* (*XBP-1*) transcript to generate a mature active transcription factor is a part of the unfolded protein response to intracellular endoplasmic reticulum stress. In mice, serotonergic activation by an intraperitoneal (i.p.) injection of 80 mg/kg 5-hydroxy-L-tryptophan (Try) with 1.2 mg/kg clorgyline (Try 80/Clrgy group) significantly increased *XBP-1* splicing in all brain regions examined (Toda et al., 2006). Therefore, serotonin direct affects the activity of *X-box binding protein-1*.

In the present study, effects of 5-HT on expression levels of various genes in ovaries of juvenile *P. monodon* (approximately 30 g body weight, injected with 50 μg g^{-1} body weight) were examined by semiquatitative RT-PCR. 5-HT significantly elevated the transcription levels of *LTB4DH, X-box binding protein* and *RUVB-like protein* ($P < 0.05$).

All of the gene homologues ($P < 0.05$) were up-regulated upon single injection of at 50 μg g^{-1} body weight of juvenile shrimp. Based on the mean relative expression levels of the target gene and the positive control (*EF-1α*), *LDB4DH* did not require the repeat injection of 5-HT as the second injection adverse the positive effect of the first injection at 48 and 77 hpt ($P < 0.05$). Nevertheless, repeat injection of 5-HT extended its effects on the expression level of *X-box binding protein* and *RUVB-like protein*

Practically, *P. momodon* broodstock rather than juvenile shrimp should have used in the experiment to investigate effects of 5-HT on expression of genes in ovaries of *P. monodon*. However, a large number of individuals are required for the appropriately designed experiments and wild *P. monodon* are extremely expensive. Therefore, juvenile *P. monodon* was used instead.

At present, domestication of *P. monodon* have been initiated to produce fast-growing and specific pathogen free (SPF) broodstock and seed for the shrimp industry. The genetic improvement of *P. monodon* cannot be achieved without knowledge on the control of growth and reproduction of *P. monodon.* The long term objectives for domestication and genetic selection of *P. monodon*, require comparison of important traits in different stocks/lines of wild and domesticated *P. monodon* using QTLs as marker assisted selection (MAS). Improvement of shrimps suitable for

culture can then be selected appropriately. The basic knowledge from this thesis (e.g. association between SSCP patterns of *RUVB-like protein* and the growth rate of juvenile *P. monodon* and significant induction of gene-related with reproductive biology of *P. monodon*) can be applied to assist domestication and breeding programmes of this economically important species more effectively.

CHAPTER V

CONCLUSIONS

1. Polymorphism of 108 gene homologues originally identified in cDNA libraries of hemocytes, ovaries and testes of *P. monodon* by an EST approach was examined by SSCP analysis. A total of 56 gene segments were successfully amplified by PCR and 37 of 42 investigated genes exhibited SSCP polymorphism against genomic DNA of wild *P. monodon*.

2. Nucleotide sequences of an individual representing each SSCP genotype of *X-box binding protein*, *LTB4DH*, *PI4K*, *RUVB-like protein*, *phosphatidylserine receptor* and *solute carrier family 3 member 2* were examined and showed compatible results with those from SSCP analysis.

3. Association between SNP by SSCP of *X-box binding protein* ($N = 76$), *LTB4DH* ($N = 60$) and *RUVB-like protein* ($N = 61$) and the body weight of a *P. monodon* G2 family (132 day olds, $N = 76$) was determined. Results only indicated significant association between SSCP genotypes I and II of *RUVB-like protein* and the body weight of the fast growing group ($P < 0.05$) but not in the slow growing group ($P > 0.05$).

4. Significant association between SSCP genotypes of *RUVB-like protein* and the body weight of *P. monodon* was also found when commercially cultured *P. monodon* juveniles were examined ($N = 335$, $P < 0.05$).

5. RACR-PCR was carried out to identify and characterize the full length cDNA of a homologue of *rasputin*. The full length cDNA of *LTB4DH*, *X-box binding protein* and *RUVB-like protein* was obtained from sequencing of the 3′ end and primer walking of the original EST clones.

6. Semiquantitative RT-PCR indicated significant up-regulation of *LTB4DH*, *X-box binding protein* and *RUVB-like protein* in ovaries of juvenile *P. monodon* upon induction of 5-HT ($P < 0.05$).

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APPENDICES

APPENDIX A

PCR and SSCP conditions of 102 primer pairs

Genes	PCR conditions	SSCP conditions
1. Y-box protein*	94°C, 3 min for 1 cycle followed by 94°C, 45 sec. 53°C, 1 min. 72°C, 2 min. for 35 cycles and 72°C, 7 min. for 1 cycle	-
2. *hepatocarcinogenesis-related transcription factor* (X-box protein)*	94°C, 3 min for 1 cycle followed by 94°C, 45 sec. 53°C, 1 min. 72°C, 1 min. for 35 cycles and 72°C, 7 min. for 1 cycle	17.5%(acrylamide:bisacrylamide= 37.5:1), 200V ,14 hrs.
3. Adipose –differentiation related protein*	As described in 1.	-
4 ESO3 Protein**	As described in 1.	-
5. *Exocyst complex component Sec6***	As described in 1.	-
6. *rasputin* *	As described in 1.	12.5%(acrylamide:bisacrylamide=37.5:1),250V ,15.32 hrs.
7. *survival motor neuron**	As described in 1.	-.

Genes	PCR conditions	SSCP conditions
8. Small nuclear ribonucleoprotein D2 – like protein**	As described in 1.	-
9.*Phosphatidyllinositol -4 kinase**	As described in 1.	12.5%(acrylamide:bisacrylamide=37.5:1),250V ,15 hrs.
10. TATA –binding protein(TBP) -associated factor9 **	As described in 1.	-
11. *Proactivator polypeptide precursor (Prosaposin)***	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,13.30hrs.
12. hypothetical protein (cyclic nucleotide gated channel beta subunit 1)*	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,13.30hrs.
13.*Carboxylesterase precursor***	As described in 1.	-
14.*Predicted O-methyltransferase***	94°C, 3 min for 1 cycle followed by 94°C, 45 sec. 55°C, 1 min. 72°C, 1 min. for 35 cycles and 72°C, 7 min. for 1 cycle.	10%(acrylamide:bisacrylamide = 75:1),200V ,13 hrs.
15. Rab-protein 10 CG17060-PA(OV)	As described in 1.	-
16. *NADP-dependent leukotriene B4 12-hydroxydehydrogenase (LTB4DH)**	As described in 14.	10%(acrylamide:bisacrylamide = 75:1),200V ,13 hrs.
17.*Phosphoglucose isomerase***	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,13.30hrs
18. *PEF- protein with a long N-terminal Hydrophobic domain (peflin)***	As described in 1.	-

Genes	PCR conditions	SSCP conditions
19. *Adenine nucleotide translocator 2**	94°C, 3 min for 1 cycle followed by 94°C, 30 sec. 55°C, 1 min. 72°C, 45 sec. for 35 cycles and 72°C, 7 min. for 1 cycle	10 %(acrylamide:bisacrylamide = 75:1),200V ,17.30 hrs.
20. TRAP-like protien precursor**	As described in 1.	-
21. Hyaluronan receptor*	As described in 1.	10 %(acrylamide: bisacrylamide = 75:1), 200V, 17.30 hrs.
22. Keratinocyte associated protein2*	As described in 1.	-
23. Heterogeneous nuclear ribonucleo protein 87F**	As described in 1.	12.5 %(acrylamide: bisacrylamide = 37.5:1), 250V, 14.16hrs.
24. laminin beta chain *	As described in 1.	12.5%(acrylamide:bisacrylamide=37.5:1),250V ,15.32 hrs
25. ras interacting protein RIPA*	As described in 1.	-
26. U5 small nuclear ribonucleoprotein**	As described in 1.	-
27. stromal membrane associated protein**	As described in 1.	-
28.tissue specific transplantation antigen p35B like*	As described in 1.	-

Genes	PCR conditions	SSCP conditions
29.RuvB like protein 2*	94°C, 3 min. 94°C, 30 sec. for 1 cycle 63°C, 1 min. decrease 2°C every cycles (61,59, 57,55°C) 72°C, 45 sec. 94°C, 30 sec 53°C, 1 min. 72°C, 45 sec. for 30 cycles 72°C, 7 min. for 1 cycle	10%(acrylamide:bisacrylamide = 75:1),250V ,14.30hrs
30.phosphopyruvate hydratase**	As described in 14.	15%(acrylamide:bisacrylamide = 37.5:1),300V ,16 hrs.
31. microspherule protein 1**	As described in 1.	-
32. *solute carrier family 25, member 14 isoform UCP5S* *	As described in 2.	10%(acrylamide:bisacrylamide = 75:1),200V ,15hrs
33.CG1681-PA*	94°C, 3 min. for 1 cycle 94°C, 30 sec. 45°C, 1 min. 72°C, 45 sec. for 10 cycle 94°C, 30 sec 53°C, 1 min. 72°C, 45 sec. for 30 cycles 72°C, 7 min. for 1 cycle	15%(acrylamide:bisacrylamide = 37.5:1),250V ,14.30hrs
34.carbonyl reductase1***	As described in 1.	-
35.contractile ring component anillin***	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,15 hrs.
36.NADP-ferrihemoprotein reductase; NADPH-cytochroome P450 reductase*	As described in 1.	-

Genes	PCR conditions	SSCP conditions
37.similar to dynactin 4***	As described in 1.	-
38.cystathionine gamma-lyase***	As described in 1.	-
39.splicing factor 3a,subunit 1**	As described in 19.	17.5%(acrylamide:bisacrylamide = 37.5:1),300V ,16hrs
40.phosphatidylserine receptor short form *	As described in 19.	12.5%(acrylamide:bisacrylamide = 37.5:1),200V ,15hrs.
41.*BCS-2****	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,15 hrs
42.proteasome (prosome, macropain)26Ssubunit,ATPase2***	As described in 1.	-
43.similar to USO1***	94°C, 3 min for 1 cycle followed by 94°C, 45 sec. 55°C, 1 min. 72°C,2 min. for 35 cycles and 72°C, 7 min. for 1 cycle.	10%(acrylamide:bisacrylamide = 75:1),200V ,15 hrs.
44.small androgen receptor-intercting protein*	As described in 1.	-
45.Cdc2 homologue***	As described in 1.	-
46.cycilc AMP-regulated protein like protein**	As described in 1.	12.5%(acrylamide:bisacrylamide= 37.5:1),250 V ,13.30 hrs
47.vitellogenin VTG*	As described in 14.	15%(acrylamide:bisacrylamide= 37.5:1),300 V ,21 hrs
48.similar to SPAPC***	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,14.30hrs
49.RNA helicase**	As described in 29.	12.5%(acrylamide:bisacrylamide = 75:1),200V ,15 hrs.

Genes	PCR conditions	SSCP conditions
50.death associated protein 5***	As described in 1.	-
51. nucleolar phosphatase*	As described in 33.	12.5%(acrylamide:bisacrylamide = 75:1),200V ,15 hrs.
52.probable hormone receptor **	As described in 1.	-
53.solute carrier family 3 member 2 **	As described in 1.	-
54.mitotic checkpoint***	As described in 1.	-
55.serine palmitoyl transferase LCB2 subunit***	As described in 1.	-
56. small ubiquitin-like modifier***	As described in 1.	-
57.putative cold-induced protein***	As described in 1.	-
58.multiple inositol polyphosphate***	As described in 1.	-
59.low molecular weight neurofilament protein XNF-L***	As described in 1.	-
60.cell divition protein kinase 7***	As described in 1.	-
61. adapter-related protein complex 1 beta 1 subunit (beta-adeptin 1)***	As described in 1.	-
62. gene flightless-I protein*	As described in 1.	10%(acrylamide:bisacrylamide = 75:1),200V ,15 hrs.
63.salivary gland secretion 1***	As described in 1.	-
64. TERA protein (chromosome 12 open reading frame 14)*	As described in 1.	-
65.splceosome-association protein9*	As described in 1.	-
66.ovarylipoprotein *	As described in 1.	10%(acrylamide:bisacrylamide = 75:1),250V ,15 hrs.

Genes	PCR conditions	SSCP conditions
67.transposase***	As described in 1.	-
68.Probable 26s proteasome non-ATPase regulatory subunit 3 ***	As described in 1.	-
69.oncoprotein nm23***	As described in 1.	-
70.Voltage-dependent anion-selective channel protein 2*	As described in 1.	-
71. similar to metaxin2***	As described in 1.	-
72. acyl-coA oxidase (ACX3)***	As described in 1.	-
73.synaptobrevin like protein***	As described in 1.	-
74.proteasome (prosome,macropain) subunit,alpha type3.***	As described in 1.	-
75.laminin beta chain***	As described in 1.	-
76.ubiquitin carboxyl-terminal hydrolase14***	As described in 1.	-
77.putative U3 sno RNP protein IMP4***	As described in 1.	-
78.cell divition protein kinase2 ***	As described in 1.	-
79.SRPK2 protein***	As described in 1.	-
80.multiprotein bridging factor***	As described in 1.	-
81.similar to arginyl-tRNA-protein transferase1***	As described in 1.	-
82.ubiquitin isopeptidase ***	As described in 1.	-
83.similar to COP9 signalosome subunit5***	As described in 1.	-
84.similar to programmed cell death7***	As described in 1.	-

Genes	PCR conditions	SSCP conditions
85.Inhibitor of apoptosis protein***	As described in 1.	-
86.nudix-type motif9 isofrom A***	As described in 1.	-
87.cyclophilinA_ORF ***	As described in 1.	-
88.26s proteasome regulatory particle***	As described in 1.	-
89.E1B-55kDa-associated protein5 ***	As described in 1.	-
90.polyadenylate binding proteinII***	As described in 1.	-
91. similar to SRY-box 7; SOX7 transcription factor*	As described in 29.	10%(acrylamide:bisacrylamide = 75:1),250V ,14.30hrs
92. Chk1 checkpoint kinase	As described in 1.	-
93. 457/OPA01	As described in 2.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,15 hrs
94. 428/OPB17	As described in 2.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,15 hrs
95. MI(36)	As described in 2.	17.5%(acrylamide:bisacrylamide = 37.5:1),180V ,15 hrs.
96.MII(51)	As described in 2.	17.5%(acrylamide:bisacrylamide = 37.5:1),200V ,15 hrs.
97.FI(1)	As described in 2.	-
98.FI(40)	As described in 2.	15%(acrylamide:bisacrylamide = 75:1),200V ,16hrs
99.FI(6)	As described in 2.	-
100.FII(17)	As described in 2.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,16hrs.

Genes	PCR conditions	SSCP conditions
101.FIII(4)	As described in 2.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,16hrs
102.FIII(8)	As described in 2.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,16hrs
103.FIII(39)	As described in 2.	-
104.FIV(2)	As described in 2.	-
105.FIV(33)	As described in 2.	17.5%(acrylamide:bisacrylamide = 37.5:1),200V ,15 hrs.
106.FV(1)	As described in 2.	15%(acrylamide:bisacrylamide = 37.5:1),250V ,16hrs
107.FV(27)	As described in 2.	15%(acrylamide:bisacrylamide = 75:1),200V ,16hrs
108.FV(42)	As described in 2.	17.5%(acrylamide:bisacrylamide = 37.5:1),180V ,15 hrs

APPENDIX B

The body weigh of juvenile *of P.monodon* female used for serotonin (5-HT) treatment.

No. Tank	Weigh of shrimp (grams)											
	1	2	3	4	5	6	7	8	9	10	11	12
1	33.9	26.4	32.7	27.4	43.9	27.3	35.0	25.7	30.0	25.7	35.0	22.7
2	35.0	25.0	30.5	28.0	41.3	29.8	34.8	23.7	36.3	29.7	41.4	20.4
3	31.9	24.8	34.9	26.0	44.3	27.1	31.6	23.7	32.5	26.1	42.7	21.8
4	33.3	23.4	30.4	25.2	40.3	26.4	30.0	26.9	32.3	23.5	37.0	21.5
5	33.3	21.8	32.0	27.7	43.1	25.8	34.1	27.1	33.9	29.0	38.0	21.1
6	30.6	21.2	32.4	26.0	45.1	22.2	31.7	21.6	31.2	20.5	27.7	23.0
7	35.2	27.7	30.1	21.1	36.6	-	36.6	22.8	30.0	21.0	27.6	21.4
8	33.2	28.7	31.4	23.2	32.4	-	32.4	20.9	30.1	24.0	27.0	21.0

Biography

Miss. Sirikan Prasertlux was born on November 13, 1981 in Bangkok, Thailand. She graduated with the degree of Bachelor of Science (Biotechnology) from Ramkhamhaeng University in 2003. She has studied for the degree of Master of Science (Biotechnology) at the Program in Biotechnology, Chulalongkorn University since 2004.

Publications related with this thesis

1. Sittikankeaw, K., Prasertlux, S., Thumtungtanakit, S., Klinbunga, S. and Menasveta, P. (2005). Identification of sex-specific expressed genes and their single nucleotide polymorphism (SNP) in ovaries of the giant tiger shrimp *Penaeus monodon.* 31st Congress on Science and Technology of Thailand, 18–20 October 2005, Nakornratchasrima, Thailand (Oral presentation).

2. Prasertlak, S., Klinbunga, S. and Menasveta, S. (2006). Identification of single nucleotide polymorphism (SNP) of genes in hemocytes, ovaries and testes off the gisnt tiger shrimp *Penaeus monodon*. 32nd Congress on Science and Technology of Thailand, 10–12 October 2006, Queen Sirikit National Convention Hall, Bangkok, Thailand (Oral presentation).

3. Prasertlux, S., Klinbunga, S. and Mensveta, P. (2007). Association between SNP by SSCP and age-specific growth rate of the giant tiger shrimp (*Penaeus monodon*). 6th National Symposium on Marine Shrimp. 18-20 March 2007, NSTDA, Pathumthani, Thailand (Oral presentation).