การระบุเครื่องหมายเอสเอ็นพีในกุ้งกุลาดำ *Penaeus monodon*

นายอรุณ บัวกลิ่น

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IDENTIFICATION OF SNP MARKERS IN THE GIANT TIGER SHRIMP *Penaeus monodon*

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ค้นหา Single nucleotide polymorphism (SNP) ของยีนในกุ้งกุลาดำ โดยออกแบบไพร์เมอร์จากลำดับนิวคลีโอไทด์ของยีนที่ค้นหาจากวิธี EST จำนวน 101 ยีน และนำมาทดสอบกับดีเอ็นดีของกุ้งกุลาดำขนาดพ่อแม่พันธุ์ พบว่าสามารถให้ผลิตภัณฑ์พีซีอาร์ได้จำนวนยีนทั้งหมด 48 ยีน เมื่อนำผลิตภัณฑ์ของพีซีอาร์จากยีนต่าง ๆ มาวิเคราะห์รูปแบบทางพันธุกรรมด้วยเทคนิค SSCP พบความแปรผันทางพันธุกรรมใน 44 ยีน จึงทำการเลือกยีนที่มีหน้าที่สำคัญซึ่งประกอบด้วย ribophorin I และ receptor for activated protein kinase C (*RACK*), defender against cell death 1 (*DAD1*), thioredoxin peroxidase และ calponin 1 สำหรับการศึกษาต่อไป โดยทำการเปรียบเทียบผลการทดลองที่ได้จากก SSCP และการลำดับนิวคลีโอไทด์โดยตรง ซึ่งพบว่ามีประสิทธิภาพใกล้เคียงกัน จากนั้นทำการประยุกต์วิธีค้นหา SNP ด้วยเทคนิค PCR-RFLP และ PASA ทำการค้นหา full length cDNA ของยีน Ribophorin I และ *RACK* ด้วยเทคนิค RACE-PCR และพบค้นหา genomic full length ของ *DAD1* , thioredoxin peroxidase และ calponin 1 ด้วยวิธี genome walking เพื่อนำมาตรวจสอบ SNP ในส่วนของ 5′ UTR และ 3′ UTR

ตรวจสอบระดับการแสดงออกของยีนในรังไข่และเม็ดเลือดของยีน ribophorin I, *RACK*, *DAD1*, thioredoxin peroxidase และ calponin 1 ในรังไข่และอัณฑะของกุ้งกุลาดำด้วยวิธี semi-quantitative RT-PCR ซึ่งพบความแตกต่างของระดับการแสดงออกของยีนที่ศึกษาของกุ้งกุลาดำวัยเจริญพันธุ์อย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) แต่ไม่พบความแตกต่างของระดับการแสดงออกของยีน *RACK* ในรังไข่และอัณฑะของกุ้งกุลาดำพ่อแม่พันธุ์ ดังนั้นความสัมพันธ์ระหว่าง SNP กับระดับการแสดงออกของยีนดังกล่าวควรพิจารณาแยกกันระหว่างเพศผู้และเพศเมีย ทำการทดสอบผลของอุณหภูมิต่อระดับการแสดงออกของยีนในรังไข่และเม็ดเลือดของกุ้งวัยเจริญพันธุ์ พบความแตกต่างของระดับการแสดงออกของยีน thioredoxin peroxidase ในรังไข่เมื่อเปรียบเทียบกับสภาวะปกติและหลังจากเพิ่มอุณหภูมิ (33 องศาเซลเซียส, 6 ชั่วโมง) เป็นเวลา 6 ชั่วโมง ($P<0.05$) และความแตกต่างของระดับการแสดงออกของ *RACK* เปรียบเทียบกับกุ้งที่เลี้ยงที่อุณหภูมิปกติหลังจากเพิ่มอุณหภูมิไปแล้วเป็นเวลา 12 ชั่วโมง ($P<0.05$).

จากการศึกษาความสัมพันธ์ระหว่าง SNP กับระดับการแสดงออกของยีนทั้งในกุ้งขนาดพ่อแม่พันธุ์และกุ้งวัยเจริญพันธุ์ เนื่องจากจำนวนตัวอย่างที่จำกัดทำให้พบจำนวนจีโนไทป์ที่พบเป็นจำนวนมากที่พบในกุ้งเพียงตัวอย่างเดียวทำให้ไม่สามารถตรวจสอบความสัมพันธ์ของจีโนไทป์ดังกล่าวทางสถิติได้ ผลการศึกษาไม่พบความสัมพันธ์ระหว่าง SNP กับระดับการแสดงออกของยีนในตัวอย่างของกุ้งกุลาดำขนาดพ่อแม่พันธุ์และวัยเจริญพันธุ์อย่างมีนัยสำคัญทางสถิติ

ลายมือชื่อนิสิต.....อรุณ บัวกลิ่น.....

สาขาวิชา..หลักสูตรเทคโนโลยีชีวภาพ....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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ARUN BUAKLIN : IDENTIFICATION OF SNP MARKERS IN THE GIANT TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR: PROF. PIAMSAK MENASVETA, Ph.D., THESIS CO-ADVISOR: SIRAWUT KLINBUNGA, Ph.D, 212 pp. ISBN 974-14-1865-5

Single nucleotide polymorphism (SNP) by expressed sequence tags (SBE) of genes in functionally important genes of the giant tiger shrimp (*Penaeus monodon*) was examined. Primers were designed from 101 gene homologues initially isolated by the EST approach and tested against genomic DNA of wild *P. monodon*. Forty-eight gene homologues were successfully amplified and subjected to SSCP analysis. Forty-four of which were polymorphic. Five genes including ribophorin I, receptor for activated protein kinase C *(RACK)*, defender against cell death 1 *(DAD1)*, thioredoxin peroxidase and calponin 1 were chosen for further studies. SNP of these genes were confirmed by direct sequencing. Simplification of SNP detection was preliminary carried out by PCR-RFLP and PCR allele-specific amplification (PASA). RACE-PCR was carried out and successfully identified the full length of ribophorin I and *RACK*. In addition, the genomic full length of *DAD1*, thioredoxin peroxidase and calponin 1 were also successfully characterized by genome walk analysis. Polymorphism of 5′ UTR and 3′ UTR of each gene was successfully genotyped by SSCP analysis.

Semi-quantitative RT-PCR was carried out. Relative expression levels of ribophorin I, *RACK*, *DAD1*, thioredoxin peroxidase and calponin1 in ovaries and testes of juvenile *P. monodon* were significantly different ($P < 0.05$). However, the expression levels of *RACK* in male and female *P. monodon* broodstock were not significantly different. Therefore association analysis between SNP thorough SSCP and gene expression levels on male and female *P. monodon* should be considered separately. A time course response of these genes following the thermal stress was also examined. Only the expression level of thioredoxin peroxidase in ovaries and *RACK* in hemocytes of juvenile shrimps was significantly higher than that the normal level at 6 and 12 hours post thermal treatment, respectively ($P < 0.05$).

Correlations between SNP through SSCP and the corresponding gene expression in broodstock and juveniles of *P. monodon* were examined. Due to limited sample sizes, several SSCP genotypes carried by single individuals were therefore excluded from statistical analysis. Non-significant associations between SNP and gene expression levels were found in both broodstock and juvenile *P. monodon*.

Student's signature.....A. Buaklin.....
Field of study.....Biotechnology...Advisor's signature.....
Academic year.....2005.....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
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	revolution per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
Tris	tris(hydroxyl methyl) aminomethane
µg	microgram
µl	microlitre
µM	micromolar
UV	ultraviolet

CHAPTER I

INTRODUCTION

1.1 General introduction

The giant tiger shrimp (*Penaeus monodon*) is indeginous and one of the most economically important penaeid species in South East Asia. Farming of *P. monodon* has achieved a considerable economic and social importance in the region, constituting a significant source of income and employment.

In Thailand, *P. monodon* had been intensively cultured for more than two decades. Approximately 60% of the total shrimp production is from cultivation. Shrimp farms and hatcheries are scattered along the coastal areas of Thailand where southern provinces (Nakorn Sri Thammarat and Surat Thani) are the majority and those in the east (Chanthaburi) and central regions (Samut Sakhon and Samut Songkhram) comprise the minority in terms of number. The intensive farming system has been used for *P. monodon* farming activity resulting in consistent increase in the production (Department of Fisheries, 1999).

The increased demand of *P. monodon* in world markets has elevated the expansion of shrimp industry and activity of this important species. The culture of *P. monodon* is basically a two-step process composed of a broodstock-hatchery phase for producing seed or postlarvae and a grow-out phase usually in earthen culture ponds for on-growing of fry to marketable size. (sometimes a nursery for rearing of postlarvae to larger juveniles is incorporated.).

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

Thailand has become the world's leader in shrimp exports. The largest export markets for Thai Black Tiger Shrimp are the United States and Japan. The remaining important markets are Europe, Asian countries, Australia and others (Table 1.1).

However, the industry has consistently encountered production losses from infectious diseases, particularly from white spot syndrome virus (WSSV), yellow head virus (YHV) and *Vibrio* sp., environmental degradation and overexploitation of high quality natural female broodstock for seed production (Browdy, 1998). As a result, Thai shrimp export decreased from 181,050 in 2002 to only 69,412 in 2005 (Table 1.2).

Table 1.1 Giant Tiger Shrimp Export from Thailand to various countries between 2002 and 2003

Countries	**2002**		**2003**		**% different**	
	Quantity (MT)	**Value (MB)**	**Quantity (MT)**	**Value (MB)**	**Quantity (MT)**	**Value (MB)**
Asia	77,497.00	26,628.50	70,840.00	22,910.00	-8.59	-13.96
- China	2,640.00	519.00	2,794.00	693.00	5.83	33.53
- Japan	46,297.00	18,442.00	45,845.00	16,692.00	-0.98	-9.49
- Other	28,560.00	7,667.50	22,201.00	5,525.00	-22.27	-27.94
USA	107,622.00	39,267.00	132,394.00	40,577.00	23.02	3.34
Europe	6,065.00	1,518.00	4,874.00	1,278.00	-19.64	-15.81
Australia	6,466.00	1,821.00	7,866.00	1,969.50	21.65	8.15
Others	14,441.00	4,712.50	18,303.00	5,112.50	26.74	8.49
Total	212,091.00	73,947.00	234,277.00	71,847.00	10.46	-2.84

Source: The Customs Department

Table 1.2 Giant Tiger Shrimp Export 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

1.2 Genetic improvement of *P. monodon*

Farming of *P. monodon* relies entirely on wild caught broodstock for supply of juveniles because breeding of females in captivity is extremely difficult. The high demand on female broodstock leads to overexploitation of the natural populations of *P. monodon*. The lack of high quality wild and/or domesticated broodstock of *P. monodon* has possibly caused an occurrence of a large portion of stunted shrimps at the harvest time (3-5 g body weight at 4 month cultivation period) (Khamnamtong et al., 2005). As a result, the farmed production of *P. monodon* has decreased for the last few years.

A research concerning domestication of *P. monodon* to overcome this problem by production of high quality pond-reared *P. monodon* broodstock has been initiated (Withyachumnarnkul et al., 1998, Benzie, 1998). Subsequently, selective breeding programs of *P. monodon* will provide shrimps having desired phenotypes (specific pathogen free, SPF and/or high growth rate). This requires integrated biological disciplines (including genetics, reproductive biology, hormone regulation and nutrition in *P. monodon*).

Several factors should be concerned for improvement of *P. monodon* performance. These include elevation of breeding production and incorporation of advance biotechnology. More importantly, the production technology of *P. monodon* must be stable. Therefore, heritability of a particular trait can be readily estimated accurately.

Cumulative effects resulted from SBPs and hybridization should be used to resolve low production efficiency of *P. monodon*. Selection of appropriate founder populations is the most important step towards effective breeding programs in *P. monodon*. However, positive correlation between different commercial important traits should be considered at all steps of implementation.

The basic information about correlations between phenotypes and genotypes are crucial for genetic improvement of this species. Generally, molecular markers can be divided to those developed from the coding sequences of genes (type I) and those of noncoding genomic segments (type II). Among various type I markers, expressed

sequenced tags (EST) which is a single pass sequence generated from randomly selected cDNA clones is an effective method for identification of new genes and their expression patterns of genome under investigation. After characterization and annotation, cDNA or designed oligonucleotides of transcripts can be further used for microarray analysis. As well as the construction of genetic linkage maps and physical maps

At present, single nucleotide polymorphism (SNP) which is regarded as the simplest form of genetic variation has been applied to assist genetic improvement programmes in several species (Tao and Boulding, 2003). SNP is a single point variation occurring in the same position of the genome and usually found approximately one in every one thousand and three hundred bases in most organisms. SNP can be divided to noncoding (type II) SNP and coding SNP (type I) SNP. The former is composed of 5’ or 3’ nontranscribed regions and untranslated regions, introns and intergenic spacers The latter is composed of synonymous and nonsynonymous mutations of exons. SSCP is commonly used for identification of SNP because it is relatively simple and does not require expensive equipment.

SNP markers in candidate genes can be treated as similar as other molecular markers such as microsatellites or AFLP. However, the advantage of coding SNP is that they are located in DNA regions that code for functionally important proteins. Therefore, they are more likely to be near QTL that affect commercially important traits.

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*. Association analysis of SNP in functional important genes and their expression levels possibly opens 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.

1.3 Objectives of the thesis

The objectives of this research is to identify SNP in functionally important genes of *P. monodon* composing of ribophorin I, thioredoxin peroxidase, defender against apoptotic cell death 1 (*DAD1*), receptor for activated protein C kinase (*RACK*) and calponin 1 through SSCP analysis and to preliminary study association between SNPs in those genes and their expression levels in ovaries and hemocytes of *P. monodon*.

1.4 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): a member of Phylum Arthropoda; Subphylum Crustacea; Class Malacostraca; Subclass Eumalacostraca; Order Decapoda; Suborder Natantia; Infraorder Penaeidea; Superfamily Penaeoidea; Family Penaeidae Rafinesque, 1985; Genus Penaeus Fabricius, 1798; Subgenus *Penaeus*. The scientific name of this species is *Penaeus monodon* (Fabricius, 1798) and the common name is giant tiger prawn or black tiger shrimp.

1.5 Morphology

The external morphology of penaied shrimp is distinguished by a cephalothorax with a characteristic hard rosthum, and by a segmented abdomen (Fig. 1.1). Most organs are located in cephalothorax, while the body muscles are mainly in the abdomen. The internal morphology of penaeid shrimp is outlined by Fig. 1.2. Penaeids and other arthropods have an open circulatory system and, therefore, the blood and the blood cells are called hemolymph and hemocytes, respectively.

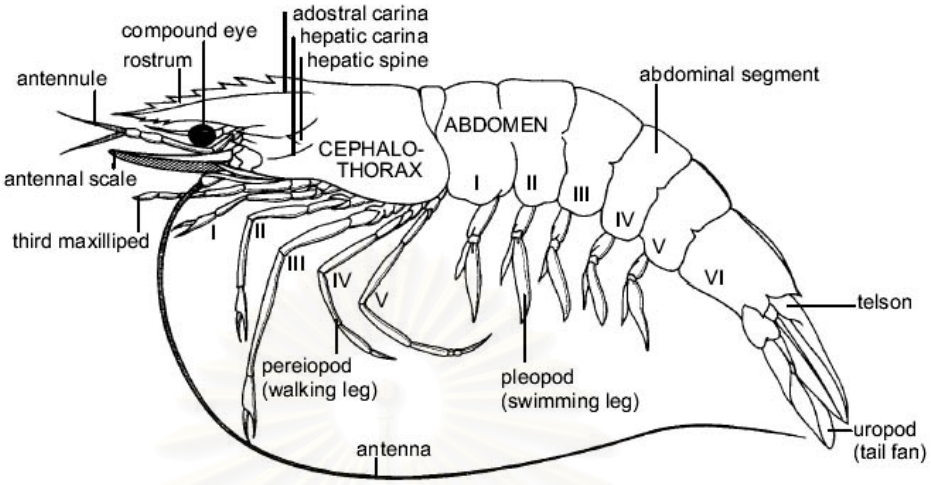

Figure 1.1 Lateral view of the external morphology of *P. monodon*. (Primavera, 1990)

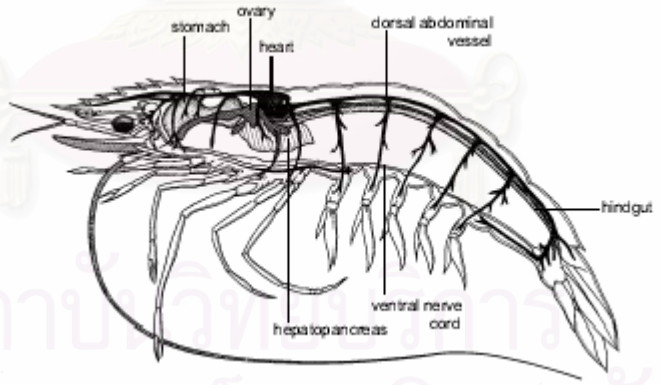

Figure1.2 Lateral view of the internal anatomy of a female *P. monodon.* (Primavera, 1990)

1.6 Molecular technique used for studies for SNP in this thesis

1.6.1 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, 17-30 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 temperature, and extension of the annealed primers by heat-stable DNA polymerase. The cycle is repeated for 30-40 times (Figure1.3). The amplification product is determined by agarose or polyacrylamide electrophoresis.

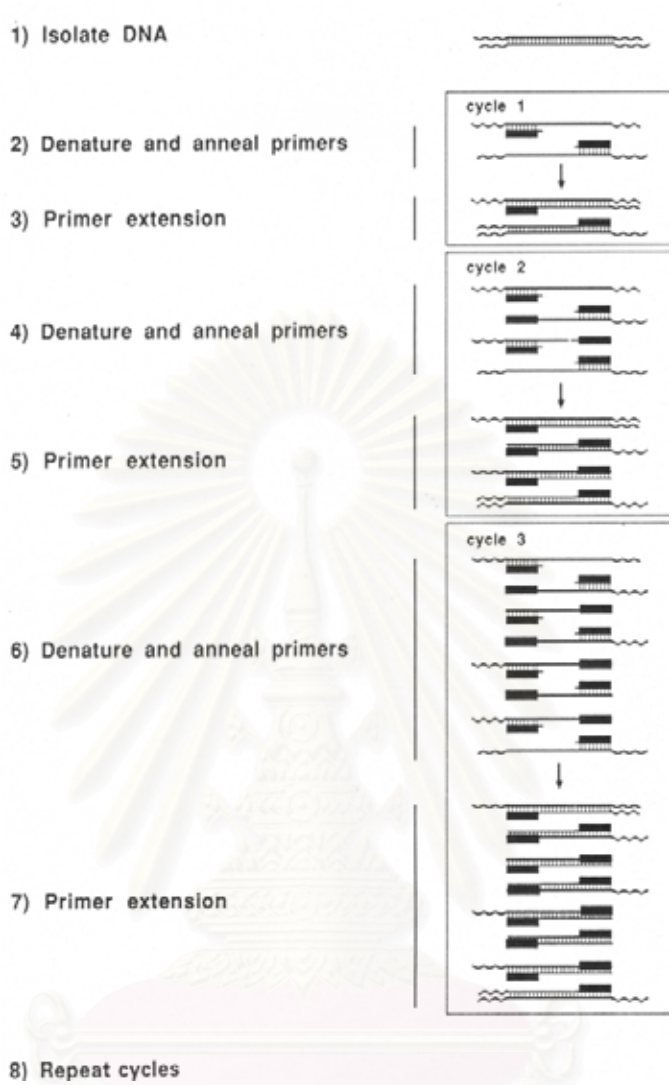

Figure 1.3 General illustration of the polymerase chain reaction (PCR) for amplifying DNA

1.6.2 PCR-SSCP

Single-stranded conformation polymorphism (SSCP) analysis was originally described by Orita et al. (1989) SSCP is one of the most widely used for the detection of mutations and variation of the DNA (deletions, insertions and single nucleotide polymorphism, SNP). The amplified PCR product (usually less than 400 bp in length) is denatured and loaded into low 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 Fig. 1.4.

The major advantage of SSCP is that polymorphism of PCR products 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 confirm the results by nucleotide sequencing. 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 low 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 complicate for estimation of allele frequencies precisely.

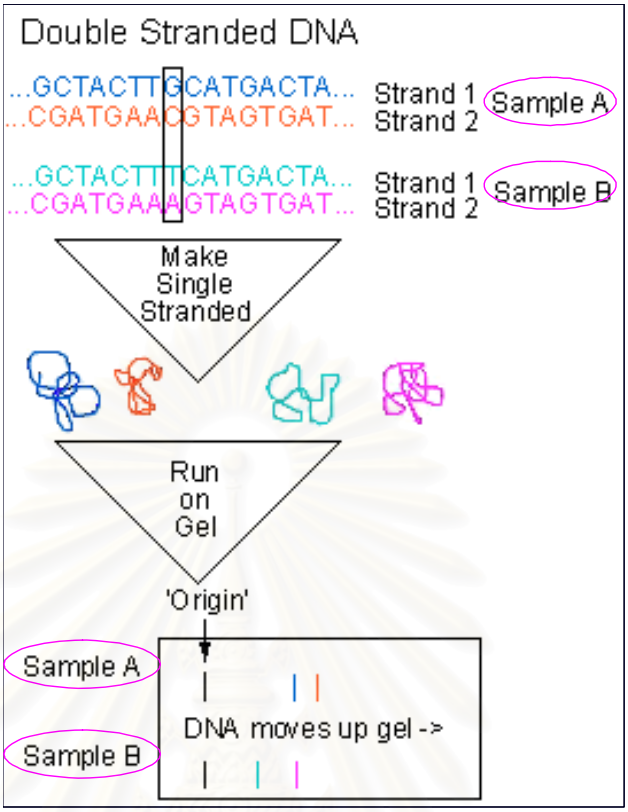

Figure 1.4 A schematic diagram of SSCP analysis for determination of polymorphism of DNA

1.6.3 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 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 (Fig1.5).

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

1.6.4 Genome walk analysis

Genome walk 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 (usually, *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′ Un-translated region (UTR) that required further characterization of SNPs at 3′ and 5’UTR.

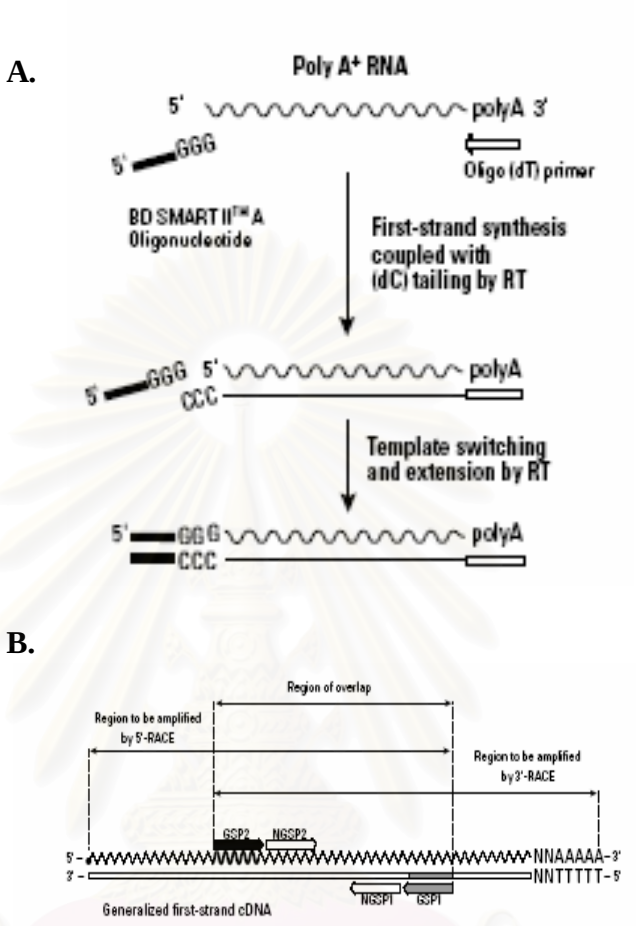

Figure 1.5 Mechanism of a SMART™ technology cDNA synthesis. First-strand synthesis is primed using a modified oligo (dT) primer (A). After Powerscript reverse transcriptase reaches the end of the mRNA template, it adds several dC residues. The SMART II A oligonucleotide anneals to the tail of the cDNA and serves as an extended template for PowerScript RT. The relationship of gene-specific primers to the cDNA template. This diagram shows a generalized first strand cDNA template (B).

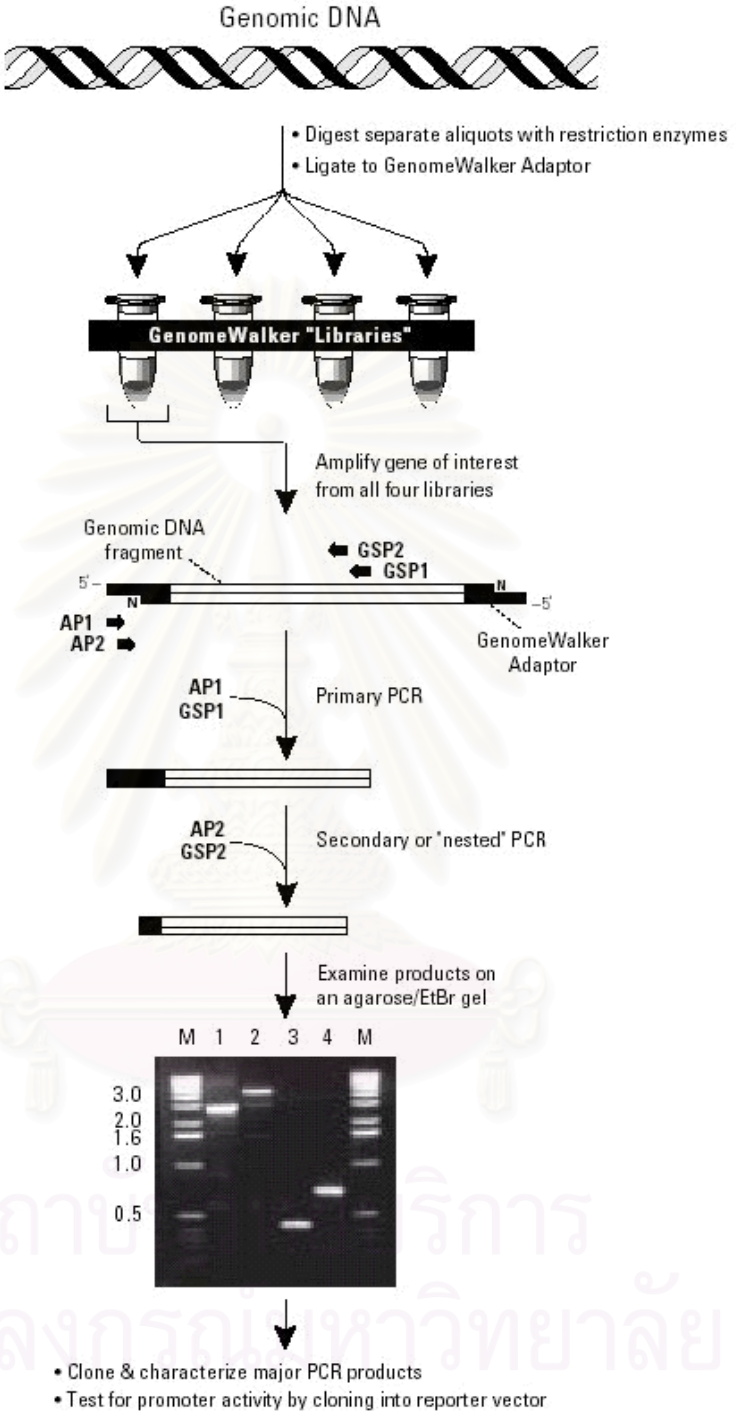

Figure 1.6 A flow chart illustrating the GenomeWalk analysis protocol.

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

DNA sequencing is the molecular biology technique for determined sequence of a piece of DNA. This technique provides high resolution and facilitating interpretation. However, sequencing of a large number of individuals using conventional method is extremely tedious and prohibitively possible. The sequencing method has been facilitated by the direct and indirect use of DNA fragments generated through PCR. At present, automatic DNA sequencing has been introduced and commonly used. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

1.6.6 RT-PCR (Reverse Transcription 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 template rather than genomic DNA was used as the template in amplification. This method contained 2 steps, in the first step first strand cDNA was synthesis using reverse transcriptase, which is made from a messenger RNA (mRNA). After that the cDNA was amplified using specific primer as same as amplified from genomic DNA.

RT-PCR is a comparable method of conventional PCR but the first strand cDNA rather than genomic DNA used as the template in the amplification reaction. It is a basic technique for determination of gene expression in a particular RNA population. The template for RT-PCR can be the first stranded cDNA synthesized from total RNA or poly A^+ RNA. Reverse transcription of total RNA can be performed with oligo(dT) or random primers using a reverse transcriptase. The product is then subjected to the second strand synthesis using a gene specific primer. The resulting product is used as 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.

1.7 Types of molecular markers

Generally, molecular markers can be divided to type I and type II. Type I marker are those developed from the coding sequences of genes. Under this definition, most RFLP markers are type I markers because they are identified during analysis of known genes (Liu and Cordes, 2004). Likewise, allozymes is also regarded as the type I markers. Type I markers are important and can be applied to various applications including as markers in population studies, comparative genomic studies, genome evolution and candidate gene identification. The type II markers are those of noncoding genomic segments. Under this definition, RAPD, microsatellites, AFLP and SNP in intervening sequences, 5′ and 3′ UTR are type II markers. Type II markers are very important in population genetic studies and aquaculture genetics particularly for species, strain and hybrid identification, inbreeding studies, and identification of markers linked to QTL (Liu and Cordes, 2004).

1.8 Single nucleotide polymorphism (SNP)

Single nucleotide polymorphism (SNP) is a single point variation (substitutions) occurring in the same genomic position of the DNA segments of different individual. SNP is usually found approximately one in every one thousand and three hundred bases in most organisms.

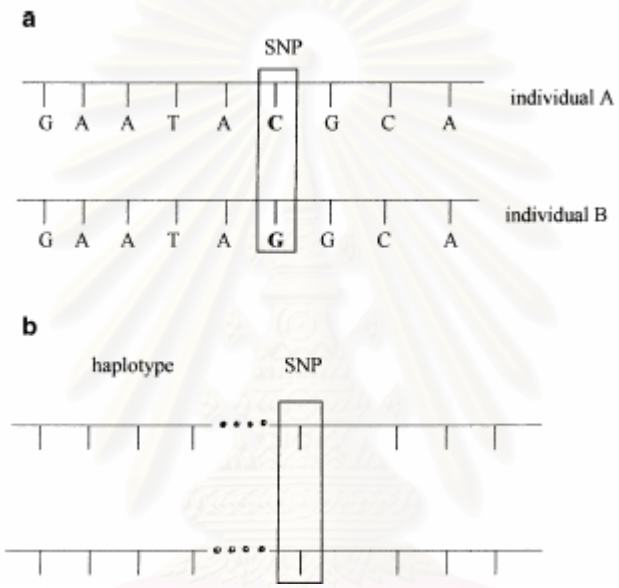

Figure 1.7 Single nucleotide polymorphism (SNP) is single-base variations among different individuals (A). Figure shows strings of nucleotides at which individuals A and B differ by just a single base. Individual A may respond to the drug positively, whereas individual B may show an adverse reaction or moderate response or no response at all. B A long stretch of DNA (e.g., 100,000 bases) with a distinctive pattern of SNPs at a given location of a chromosome is called haplotype. Haplotype diversity may be generated by new SNP alleles, which can arise because of mutations at different loci (Shastry, 2002).

1.8.1 EST

ESTs are 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. 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 species under investigation (Liu and Cordes, 2004)

-Identification of SNP by EST-derived markers

Generally, SNPs are less abundant in coding regions than in introns and other noncoding regions (Salisbury et al., 2003). Nevertheless, SNP in coding regions or transcripts of known genes may cause nonsynonymous (animo acid replacement) mutations, which may be play a direct or indirect role in phenotypic expression. Thus, SNP markers derived from EST or transcripts of known genes, can be applied to assist selective breeding programmes of *P. monodon* in the future.

1.8.2 Classification of SNP

1. 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.

2. 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.

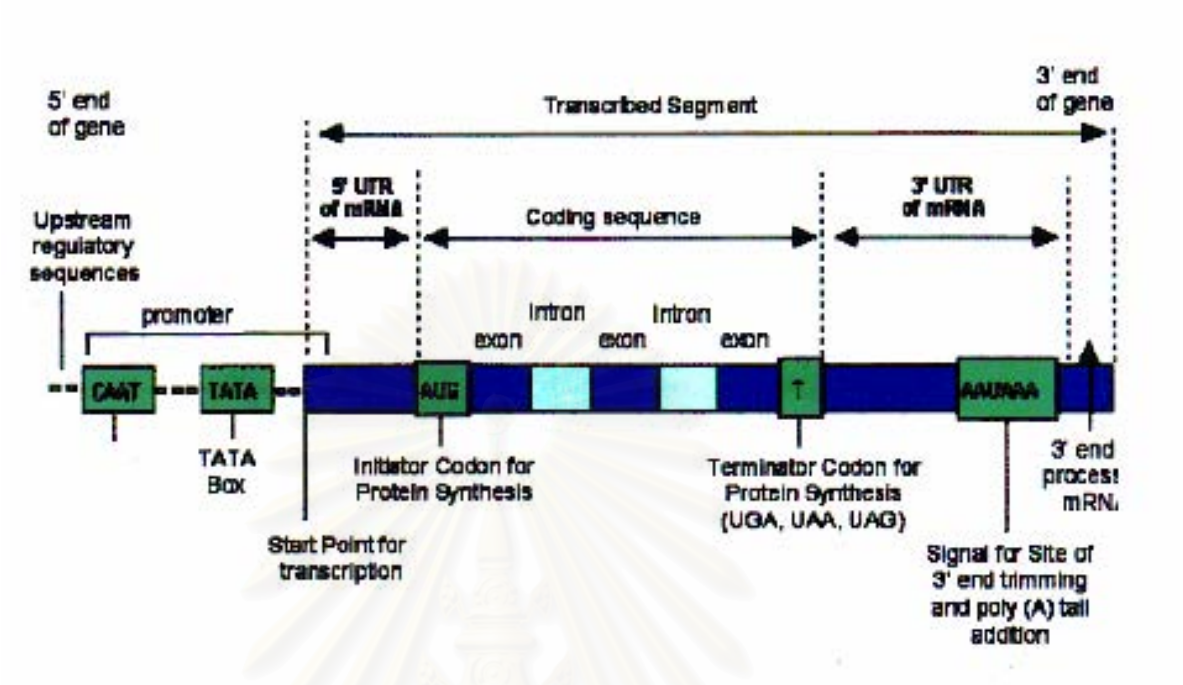

Figure 1.8 Diagram illustrating the structure of protein coding gene

1.8.3 SNP discovery

Discovery of SNP can be carried out by several molecular approaches including DNA sequencing and non-sequencing methods (Table 1.3). The former is the direct determination of SNP position in the DNA fragment whereas the latter is the indirect approach for determination of SNP. Generally, direct sequencing is applied for examination of genetic variation of different individuals. Nucleotide sequences are multiple aligned. True polymorphism and sequencing errors should be further confirmed (Figure1.9; Rafalski, 2002).

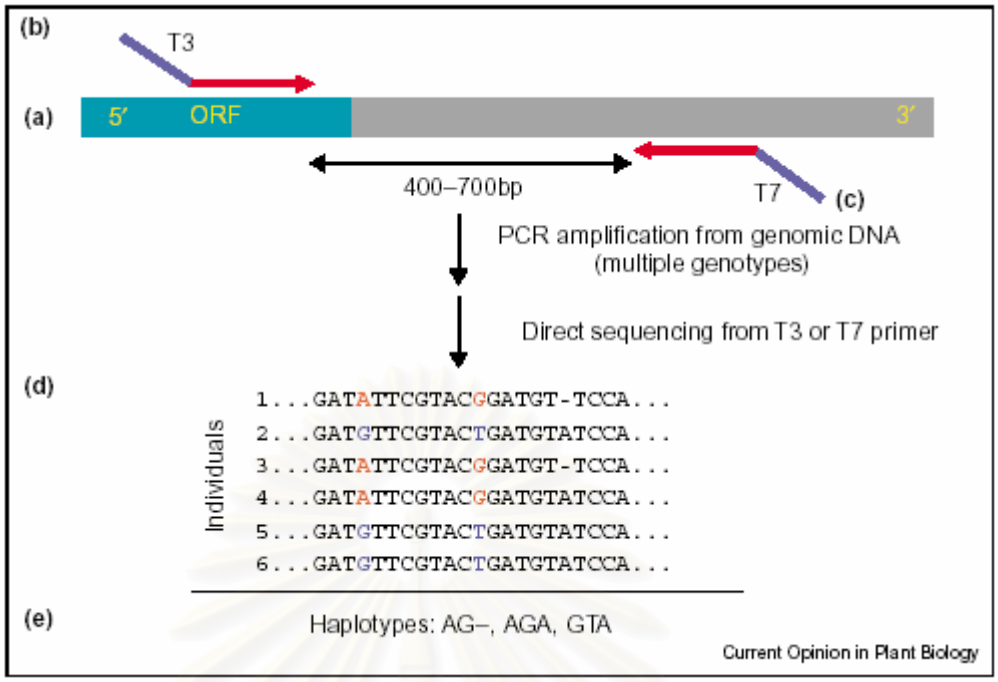

Figure 1.9 SNP discovery by a direct DNA sequencing method. A sequence in the vicinity of the 3′-UTR of an EST or a known gene is used to design a pair of amplification primers (a). Primers may contain T3/T7 promoter-homologous sequences to facilitate direct sequencing (b and c). After PCR amplification from a set of genetically diverse individuals, the PCR products are purified and directly sequenced. The resulting sequences are aligned and SNPs and insertion/deletion polymorphism are identified (d). In this hypothetical example, three haplotypes could be distinguished (Rafalski, 2002).

The non-sequencing based approaches for SNP discovery include single strand conformational polymorphisms (SSCP), denaturing gradient gel electrophoresis (DGGE), allele-specific amplification (ASA), Restriction fragment length polymorphisms (RFLP) and denaturing HPLC (DHPLC). Selection of a particular assay depends on many factors, including cost, high/low throughput, equipment needs, difficulty of assay, and potential for multiplexing (Table 1.3).

Table 1.3 A comparison 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

1.9 Studies of SNP in functionally important genes of various organisms

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 (Krawezak et al., 1992; Lohrer and Tangen, 2000; Pitarque et al., 2001; Spicker et al., 2001; Levan et al., 2001). Studies about

correlation between SNP and diseases have been extensively carried out in human (Table 1.4). Knowledge gain can be further applied in pharmacological studies particularly for response of medicines in different patients.

Table 1.4 A partial list of disorders associated with SNPs

Gene	Disorder	Reference
END 1 and NOS 1	Asthma	Immervoll et al., 2001
Mycocillin	POAG	Colomb et al., 2001
Fibrillin 1	Systemic sclerosis	Tan et al., 2001
MMP-1	Lung cancer	Zhu et al., 2001
KCNQ1	Arrhythmias	Kubota et al., 2001
MIF	Idiopathic arthritis	Donn et al., 2001
TAF1	Blood pressure	Koschinsky et al., 2001
MBL	Biliary cirrhosis	Matsushita et al., 2001
Syntaxin 1A	Type II diabetes	Tsunoda et al., 2001
Prolactin	Systemic lupus crythemalosus	Stevens et al., 2001
Melanocortin	Eating disorder	Adan and Vink. 2001
Insulin receptor	Migraine	McCarthy et al., 2001
Npps	Ossification	Koshizuka et al., 2001
P53	Lung cancer	Biros et al., 2001
Tau	Late-onset PD	Martin et al., 2001

SNPs, Single nucleotide polymorphisms; POAG, primary open-angle glaucoma; *MMP-1*, matrix metalloproteinase 1; *EDN 1*, endothelin 1; *NOS 1*, neuronal nitric oxide sythetase; *TAF1*, trombin activable fibrinolysis inhibitor; *KCNQ1*, potassium channel protein; *MIF*, macrophage inhibitory factor; PD, Parkinson disease

Table 1.5 Selected examples of polymorphisms associated with variable drug responses. (Benoit and Francois. 2000)

Protein	Drug	Polymorphism and consequence
CYP2C9	Warfarin	Increased risk of bleeding
CYP2C19	Omeprazole	Rapid metabolism and decreased efficacy in peptic ulcers
Dihydropyridine dehydrogenase	5-FU	Severe 5-FU toxicity due to 5' splice recognition-site mutation
UGP 1A1	Irinotecan	Metabolism and toxicity of Irinotecan (promoter polymorphism)
Thiopurine methyltransferase	6-Mercaptopurine (6MP)	Toxicity and efficacy of 6MP in leukemia
5-LO	ABT-761	Efficacy of ABT-761 in asthma (promoter polymorphism)
β2-Adrenergic receptor (ADBR2)	Salbutamol	Efficacy of salbutamol in asthma
Cholesteryl ester transfer protein (CETP)	Pravastatin	Efficacy of Pravastatin in coronary atherosclerosis
Stomelysin-1	Pravastatin	Speed of restenosis in voronary atherosclerosis (promoter polymorphism)
Serotonin receptor (5HT2A)	Clozapine	Long-term outcome of clozapine therapy in schizophrenia
Serotonin transporter (5HTT)	Fluvoxamine	Efficacy of fluvoxamine in delusional depression (promoter polymorphism)
Dopamine D3 receptor (DD3R)	Typical neuroleptics	Development of tardive dyskinesia in schizophrenic patients
α7-Nicotinic receptor (CHRNA7)	Acetylcholine	Affinity of acetylcholine and other agonists
Potassium channel (MiRP1)	Clarythromycin	Clarythromycin induced long QT-syndrome
α-Adducin	Hydrochlorothiazide	Efficacy of hydrochlorothiazide in the treatment of essential hypertension
Peroxisome proliferators-activated receptor (PPARγ2)	Insulin	Variation in sensitivity to insulin

In domestic species, association between SNP and economically important traits (growth and survival rates) or expression levels of the corresponding genes are useful and was applied to selective breeding programmes of several species.

Jiang et al. (2004) studied SNPs in the chicken pituitary-specific transcription factor gene (*PIT1*), encoded as the pituitary-specific transcription factor protein which binds to and trans-activates promoters of growth hormone (*GH*), prolactin (*PRL*) and thyroid-stimulating hormone chain (*TSHB*)-encoding gene. SNPs were detected by PCR-SSCP of ten chicken populations (*N* = 662) and further confirmed by DNA sequencing. A transversion (A980T) was found at the 980th position of the open reading frame (ORF) of *PIT1* cDNA. This SNP causes the replacement of asparagines (AAC, Asn) to isoleucine (ATC, Ile) at the 299th amino acid. The genotype A/A of this A980T significantly associated with the body weight at 8 weeks of age. This indicates that a *PIT1* SNP is a potential for comparison of the early growth rate in chicken.

Association between SNP in the growth hormone (*GH*) and growth hormone receptor (*GHR*) genes were studies in the Angus cattle. Genomic DNA was amplified using PCR primers encompassing the promoter region and exons 1 - of growth hormone genes. SNP in the PCR products was identified using the denaturing gradient gel electrophoresis (DGGE) method and sequencing. Three newly identified SNP were found in the promoter region of *GH* of the Aungus cattle. Polymerase chain reaction-based restriction fragment length polymorphism procedures were developed for rapid determination of the SNP genotypes in *GH* and *GHR* among Angus calves from lines divergently selected for high or low blood serum IGF-I concentration. The association between SNP and growth traits and serum insulin like growth factor-I (IGF-I) concentration were tested using the animal models. Association between SNP in promoter region of *GH* and concentration of IGF-1 was found on day 42 of the postweaning test (Ge et al., 2003)

Huang et al. (2002) reported effects of SNPs in the 5'flanking region of porcine heat shock protein 70.2 gene (*HSP70.2*) on semen quality in boars. PCR products amplified from 5' flanking region of *HSP70.2* of 51 individuals were subjected to direct DNA sequencing. Five SNP (at positions 44, 232, 250, 345 and

393) were identified. Semen quality was evaluated in terms of sperm motility, percentage of normal sperm, percentage of sperm with proximal plasma droplet, percentage of abnormal sperm, sperm concentration, semen volume per ejaculate and total sperm number per ejaculate. The effect of the SNPs on semen quality was evaluated based on breed-corrected data within a season, hot and cool season. For example, the sperm number per ejaculate of boars possess heterozygote genotype at the 232^{nd} and 250^{th} positions was significantly greater than that of boars exhibiting the AA genotype ($P < 0.05$) during the hot season. Additionally, boars possess TT and TC (345^{nd} position) genotypes had higher semen volume than those with the CC genotype ($P < 0.05$). Significant correlation between the AA/CC genotype (232^{nd} position) and phenotypes (sperm motility) was also observed in the cool season ($P < 0.05$). Results indicated association between the 5' flanking region of procine HSP70.2 and semen quality traits in the hot season. Therefore, identified SNPs may be used as a selection marker for improving the semen quality of boars.

Morganti et al. (2005) studies the relationships between promoter polymorphisms in the thymidylate synthase (*TS*) gene and mRNA levels in colorectal cancer patients. A polymorphic tandem repeat sequence (2 or 3 repeats; 2R and 3R) and a SNP, G>C, within the second repeat of the 3R alleles were found. The expression level of *TS* gene was semi-quantitatively estimated. The relationships between *TS* mRNA expression and genotypes were analyzed and statistically tested. Results indicated the relationship between tumoural *TS* mRNA expression levels and *TS* gene polymorphisms in the colonic mucosa of 48 colorectal cancer patients. The 3R/3R genotype was characterized by higher *TS* mRNA levels in the tumor than the 2R/2R-2R/3R genotypes ($P = 0.071$). Regarding the relationship with the SNP polymorphism, a statistically significant difference in *TS* gene expression between the 3RG/3RG genotype and the 2R/2R-2R/3RC-2R/3RC genotypes subset was observed ($P = 0.017$). But no statistically significant correlation was observed between experimental data and baseline clinical-pathological characteristics as well as clinical outcome in the relatively small patient series investigated. Results suggest that in 3R/3R patients, the G>C polymorphism may be an important factor in determining *TS* mRNA expression levels, and warrant further investigation of the role of *TS* promoter polymorphisms as predictors of sensitivity to 5-FU-based chemotherapy in larger case series.

The polymorphisms of 5′ flanking region of chicken prolactin (*cPRL*) gene were examined in several populations of Chinese chickens. Four SNPs were identified at position -2425(C/T), -2215(T/C), -2063(G/A) and -1967(A/G), a 24 bp indel and a poly A length polymorphism were identified from sequencing the 5′ flanking region (2638 bp) of *cPRL*. Realtime quantitative PCR and radioimmunology assay (RIA) was employed to investigate the potential association of the 24 bp indel locus with *cPRL* mRNA expression levels, plasma cPRL and brooding behaviors and observed that chickens with genotype AB (common genotype) had the highest *cPRL* mRNA levels, providing the possibility that this polymorphic site might be related to the broodiness in chickens via modulating the transcriptional level of *cPRL* gene. The dissociation among *cPRL* gene transcription, mRNA storage and hormone release was also observed. (Liang et al., 2005).

Wong et al. (2005) studied correlation between SNP and post-mRNA analysis of the 14-3-3 genes that encode Phosphoserine.theonine binding regulatory proteins in schizophrenia and bipolar disorder. Quantitative PCR was used to determine relative mRNA levels in dorsolateral prefrontal cortex (Brodmann's area 46) samples donated by the Stanley Medical Research Institute (SMRI). Selected SNPs of five 14-3-3 isoforms (β γ, ε, ζ, and η) were genotyped in all five isoforms for association analysis in both family and case control samples. No significant differences in 14-3-3 mRNA expression levels between the diagnostic groups were found. A significant genetic association with schizophrenia was found for 14-3-3ζ isoform in a subset of the case control sample ($P < 0.011$) and in a subset of the case control study.

Trakooljul et al. (2004), detected polymorphic in the porcine androgen receptor (*AR*) gene for association study and analysis of functional properties of the porcine *AR*. Eight SNPs, TG- and T-insertion/deletion polymorphisms upstream transcription initiation sites, three SNPs in the 5′UTR, one microsatellite $(CCTTT)_n$ in the intron of 5′UTR and a CAG-indel in exon 1 were detected. The *AR* mRNA expression levels determined by real-time RT-PCR in various tissues of female pigs showed the highest expression levels of the *AR* in ovaries (100%) followed by adrenal gland (83.8% relative to ovary), uterus (61.6%), liver (47.4%) and pituitary gland (1.3%). Detection of the *AR* mRNA transcrips in liver revealed that heterozygous males carrying the *AR* haplotype described from Berlin Miniature pig had higher

relative *AR* expressions than did those with the Duroc haplotype. Here they showed that the porcine *AR* is a highly polymorphic gene. Polymorphisms found in this gene affect the predicted amino acid sequence as well as consensus transcription factor binding sites and are associated with the allele-specific differences of the *AR* mRNA transcript level in liver, reinforcing *AR* as a potential candidate gene for traits related to pig reproduction and performance.

SNPs and expression of Netrin-G1 (*NTNG1*) and G2 (*NTNG2*) genes in Schizophrenia were studied (Suzuki et al., 2005). Twenty one SNPs in *NTNG1* (two novel SNPs) and ten SNPs in *NTNG2* (six novel SNPs) were analyzed in 124 schizopheric pedigrees. The expression levels of *NTNG1* and *NTNG2* were examined in the frontal (Brodmann's Area (BA)11 and BA46) and temporal (BA22) cortices from schizophrenic and control postmortem brains. The isoform-specific expression of *NTNG1* spice variants was assessed in these samples. The results showed association with schizophrenia, and concordantly, the expression levels were significantly different between schizophrenic and control brains. An association between *NTNG2* and schizophrenia was observed with SNPs and haploypes that clustered in the 5' region of the gene.

Correlation between SNP in ten candidate genes allied with the growth hormone axis and the age-specific growth rate (wet weight and fork length) were examined in the Arctic charr *(Salvelinus alpinus L.)*, The PCR product amplified from two backcrossed fullsib families (N = 217 and 95) analyzed for the existence of SNPs markers using PCR-RFLP or bidirectional amplification of specific alleles (Bi-PASA). A significant association between SNP alleles (*GHRH/PACAP2* A/G), was found for the locus containing the growth hormone-releasing hormone and pituitary adenylate cyclase-activating polypeptide genes (*GHRH/PACAP2*, P = 0.00001) and early juvenile growth rate of *S. alpinus* (Tao and Boulding., 2003).

He et al. (2003) studied SNP in interspecific hybrids of the channel catfish (*Ictalurus punctatus*) and the blue catfish (*Ictalurus furcatus*) which are closely related genetically. A total of 161 genes were examined and the average distribution of SNP was 1.32 per 100 bp. The majority of identified SNPs differed between two species. Accordingly, these SNPs are useful for mapping genes in channel catfish x

blue catfish interspecific resource families. The SNPs, that differed within species also observed and can be applied to genome scans in channel each species.

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 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 found to be significantly associated with BW of LV1 and LV2 populations.

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. These specimens were used for screening of genes illustrating polymorphic SSCP patterns. Moreover, broodstock-sized of *P. monodon* collected from Angsila (Chonburi) and juvenile of *P. monodon* from local farms were used for association analysis of SSCP patterns and levels of gene expression. Tissues of each shrimp (pleopods, testes and ovaries) were collected and kept at -80°C until required. Hemolymph was collected using 10% sodium citrate as an anticoagulant and centrifuged at 1000 *g* for 10 minutes. Hemocytes were then subjected to RNA extraction.

2.2 Nucleic acid extraction

2.2.1 Genomic DNA extraction

Genomic DNA was extracted from a piece of pleopod of each shrimp using a phenol-chloroform-proteinase K method (Klinbunga et al., 1999). A piece of pleopod tissue was dissected out from a frozen pleopod and placed in a prechilled microcentrifuge tube containing 500 µl of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl; pH 8.0) and briefly homogenized with a micropestle. SDS (10%) and RNase A (10 mg/ml) solutions were added to a final concentration of 1.0 % (w/v) and 100 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: chloroform: isoamylalcohol (25:24:1) was added and gently mix for 10 minutes. The solution was centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a newly sterile microcentrifuge tube. This extraction process

was then repeated once with phenol:chloroform:isoamylalcohol (25:24:1) and once with chloroform:isoamylalcohol (24:1). The aqueous phase was transferred into a sterile microcentrifuge. One-tenth volume of 3 M sodium acetate, pH 5.2 was added. DNA was precipitated by an addition of two volume of prechilled absolute ethanol and mixed thoroughly. The mixture was incubated at -80^{o}C for 30 minutes. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature and washed twice with 1 ml of 70% ethanol (5 minutes and 2 – 3 minutes, respectively). After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 50 – 80 μl of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The DNA solution was incubated at 37 ^{o}C for 1 – 2 hours and kept at 4 ^{o}C until further needed.

2.2.2 RNA extraction

Total RNA was extracted from ovaries, testes and hemocytes of *P. monodon* using TRI Reagent®. A piece of tissue was immediately placed in a mortar containing liquid nitrogen and ground to 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 and left for 5 minutes, before adding 0.2 ml of chloroform. The homogenate was vortexed for 15 seconds and left at room temperature for 2-15 minutes and centrifuged at 12000g for 15 minutes at 4 ^{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 minute and centrifuged at 12000g for 10 minutes at 4-25 ^{o}C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75 % ethanol centrifuged at 7500g for 5 minutes. 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 long storage.

2.3 Measuring concentrations of nucleic acids by spectrophotometry and electrophoresis

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 et al., 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}. 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 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 after 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

2.4.1 Design of primers from EST of *P. monodon*

Primer pairs were designed from EST sequences of homologues of known transcripts from hemocyte and ovarian cDNA libraries of *P. monodon* (Table 2.1).

Table 2.1 Sequences, length and the melting temperature of primers designed from EST of *P. monodon*

Gene	Sequence	Length	Tm
1.Whey acidic protein	F:5′TTGCTCTTGCTTTACCGAT3′	19	54
(*WAP*)*	R:5′TCACATCCCCGTCTGTCTTCA3′	21	64
2. Heat shock protein 60	F:5′GAAGGGCAAGGGTAATACTC3′	20	60
(*HSP60*)**	R:5′TAAGCAACGGAATGGACGTT3′	20	58
3. Survivin*	F:5′GGGAGGAGCACAAAAACCAT3′	20	60
	R:5′AAGTGAGGAGACAAGAACA3′	19	54
4. P109 protein**	F:5′AACCTGGGATTGTGTAAGCA3′	20	58
	R:5′AAGTCCAACTACTGAACGAG3′	20	58
5. Cyclophilin 18*	F:5′AAAAAGGTGTGGGCGGCTAA3′	20	60
	R:5′ACAACGGCATCTAACTGAAC3′	20	58
6. Chaperonin containing	F:5′CTCTGACTCCCTCCTCCATT3′	20	62
t-complex polypeptide	F5′AACCCCTCTGACATCTCCTA3′	20	60
(*CCTP*)***			
7. ProPO factor*	F:5′TTGGTCTTGCTTCCCTCTAC3′	20	60
	R:5′TTATTTTGTATCTCCTGCTCG3′	21	54
8. Clottable protein*	F:5′CTTTCCCACTACTAACTAACG3′	21	60
	R:5′GCTCTGTTGTAGGTTATGTG3′	20	58
9. Glutathione peroxidase	F:5′AACTGGCTTCCTCCGCTATC3′	20	60
(*GPX*)*	R:5′TGAGTTGGTTCATCAGGTGG3′	20	60
10. γ- glutamyltransferase	F:5′ACCTTCAACTCCAGCCAGC3′	19	60
(*GGT*)*	R:5′TAGCATTCTTCAAAATCCTTCC3′	22	60
11. Peptide poly cis trans	F:5′ATCTTCCATCGTGTGATTCC3′	20	58
isomer5 (*PPI*)*	R:5′TGTCCTTTCCAGCATTAGCC3′	20	60
12. Heat shock protein 10	F:5′TGGTTGCTGTAGGAGAGGG3′	19	60
(*HSP10*)***	R:5′TTTTACTCGTTCTTCATCTTGG3′	22	60
13. Superoxide	F:5′AATGTGGCTCTGGTGTAGG3′	19	58
dismusutase (*SOD*)**	R:5′TGGATTTAACCGAAGAGACTG3′	21	60
14. Casein kinase II beta	F:5′TGAACCAGATGAGGAGGAAGA3′	21	62
chain**	R:5′GGAAGCCAGTGCCGAAGTAA3′	20	62

Table 2.1 (cont.)

Gene	Sequence	Length	Tm
15. Chaperonin	F:5′CTATTCTGGGCTGGAGGAGGC3′	21	68
subunit 8**	R:5′CAACATCTGTGGGAGTGAGGC3′	21	70
16. Muskelin 1**	F:5′GTCACGATGGTCTTCCGATTC3′	21	64
	R:5′GTTCCAGTCCTCACAGCCTCC3′	21	68
17. Ovarian lipoprotein	F:5′CGGGATGAGTGCGAGAAGTGC3′	21	68
receptor**	R:5′CAGGGGCTCCGAGTCAAAGA3′	20	64
18. Ribophorin I**	F:5′CGACTTCCAGAGAGAGCACA3′	20	62
	R:5′GGTCTTCCATCCTCCAAACA3′	20	60
19. Innexin-2**	F:5′CCTTCACGATACCCTCCTTG3′	20	62
	F:5′ACCTTACCACCTTCCCAGAT3′	20	60
20. Thioredoxin**	F:5′CGAAGTGGTTGCTTGCTCTA3′	20	60
peroxidase**	R:5′CTGGCAGGTCATTGATTGTT3′	20	58
21. agCP13148**	F:5′CATACCTCGCATCATCAGTG3′	20	60
	R:5′CCTCAGGAGACGATACAAAGC3′	21	64
22. Asparaginyl-tRNA	F:5′GCTGTCCAACCCTGATGTGC3′	20	64
synthetase**	R:5′AGATGCCTTCTGTGCGTGA3′	19	58
23. Semaphorin 2A	F:5′CTTGGTGGTGATGAATGTAA3′	20	56
precursor**	R:5′GGATGGCTGTTCTGCTGGCT3′	20	64
24. Zeta1-cop**	F:5′TGGTGTCAGTGTTGAGTTGTCT3′	22	64
	R:5′GCTCTCCTCCAGCCTTAGTGC3′	21	68
25. Ferrochelatase**	F:5′GCTGCCATTCCAAGATTACAT3′	21	60
	R:5′TTTCAACTCCATCCTCCTCCA3′	21	62
26. Calcium independent phospholipaseA2 isoform 1**	F:5′TGTCCCACAGCACCAGTAAT3′	20	60
	R:5′CCAGCCTTCACCTCCTCTTC3′	20	64
27. HLA-B-associated	F:5′AGGCAGGAGTAGGAGAAACG3′	20	62
transcript 1A**	R:5′AAGGATGCTCAAAGCCACAG3′	20	60
28. Aldehyde dehydrogenase	F:5′CTGATGCCAACAAGGAAAGC3′	20	60
family 6, subfamily A1**	R:5′GGAGAAATAACAGGACCCAA3′	20	58

Table 2.1 (cont.)

Gene	Sequence	Length	Tm
29. Pre-B-cell colony-enhancing factor**	F:5′GAACGACGACCCTCTACTCC3′	20	64
	R:5′TCCCTCTGGCACAGCCTTGA3′	20	64
30. Carbonic anhydrase**	F:5′TGCGACGGTAACCTAACTGC3′	20	62
	R:5′TTGCCAACAACAGGAAACAT3	20	56
31. Chromobox protein**	F:5′TGGGAACCTGAATCTCATCTTG3′	22	64
	R:5′ACGAACATTTGCCTGCCTTG3′	20	60
32. Vacuolar-type H+-ATPase subunit A***	F:5′GACCTCACAGAGTCCATTTACA3′	22	64
	R:5′GGAGTTCTCAGGCACAATACCA3′	22	66
33. Dihydropteridine reductase**	F:5′CGTCTACGGAGGTCGTGGAG3′	20	66
	R:5′GAAGCAGCCTGAGCAGTGAT3′	20	58
34. Aminopeptidase**	F:5′TGGCTCGGCAAGGCTGGCTG3′	20	68
	R:5′CGTGGAGTGCGTGAAGAAAGG3′	21	66
35. Dolichyl-di-phosphooligosaccharide-protein glycotransferase**	F:5′TTCTGGCAACGGCAAAGTAG3′	20	60
	R:5′ATGGGTCAATGCGAACAAAG3′	20	58
36. Integrin beta 4 binding protein**	F:5′GCACTCGGAAATGTGGTTG3′	19	58
	R:5′CCTGGTTGGAAATGACTGAAT3′	21	60
37. 3-oxoacid CoA transferase**	F:5′CCTCCTACTTTGGCTCTGACG3′	21	66
	R:5′CACTGAATACGCACTTCTCTGT3′	22	64
38. Profilin***	F:5′GCTGATGGCTCTGGCTATG3′	19	60
	R:5′TCCTGCCTTCCCTTTCTTGC3′	20	62
39.Adenosylhomocys – teinase**	F:5′CCATCCAGACTGCTGTGCTC3′	20	64
	R:5′TCCCGTGGTGGTTTCTTCCG3′	20	64
40. COP9 subunit 6**	F:5′CAGTGATGTTAGCCCAGGAA3′	20	62
	R:5′CCAGCCAAGGAGGTCAAGGT3′	20	64
41. ATP/GTP-binding protein**	F:5′AGGTTGAGGGCAAGCAGGAT3′	20	62
	R:5′GCACAGCATAGTTGAGGAGAA3′	21	62
42. Protease**	F:5′GGAAGAAAGAAGCAAAGTC3′	19	54
	R:5′CCAGTCCTCCAATGTCAGCA3′	20	62

Table 2.1 (cont.)

Gene	Sequence	Length	Tm
43. Thiolase**	F:5′ATTCCCGAGTTGCCTGCTGT3′	20	62
	R:5′AACCCTGATGCCATTGTCTT3′	20	58
44. Aspartate	F:5′AGGAATGCCTATGCTGTGCG3′	20	62
aminotransferase**	R:5′CTGGATTACTAAGAATGGTGGA3′	21	60
45. Carnitine	F:5′CAAGAGCAGTGGCATCATCC3′	20	62
palmitoyltransferase II**	R:5′AGAACTATTCCTAAGGCGGTCAT3′	23	66
46. Receptor activating	F:5′AGACCACCCGTCGCTTTGAG3′	20	64
protein kinase C**	R:5′AACCTTACGCAGGACACCCA3′	22	66
47. Tetraspanin D107**	F:5′GTTCTTTGATGGCAAGTTCG3′	20	58
	R:5′CGTTCCACAGCAATGGTAGTTC3′	22	66
48. Methyl CpG binding	F:5′ATTTCCGCTTCAGACACTCCA3′	21	62
protein 2**	R:5′CATCATTCCATTATGTTTCCTT3′	22	58
49. Presenilin enhancer*	F:5′CAAGCCAAGCGACCATCCAT3′	20	62
	R:5′TAATCCAACGAGCCATACAA3′	20	56
50. Defender against cell	F:5′CGATGCCTACCTCTTCTACG3′	20	62
death 1*	R:5′GATGAAATCAGCAAAGCCTC3′	20	58
51. Glycogen	F:5′GGCTTCCTTGACCGTAACTT3′	20	60
phosphorylase*	R:5′CGAAATCCGTGCGAACCTGG3′	20	64
52. Nonclathrin coat	F:5′GTGTTGAGTTGTCTCTATGATGC3′	23	66
protein zeta*	R:5′ACAGTCTGGTCGTTGAATGG3′	20	60
53. NH2 non-histone	F: 5′AGGCAACACTCACCCAGAAG3′	20	62
chromosome protein 2-like*	R:5′TTGATACACCACAGGCACGG3′	20	62
54. FIV/2	F:5′CGTATGCCACATCCCACA3′	18	56
	R:5′TTCTTTTCTGAAGGAGGTCG3′	20	58
55. FIV20	F:5′GCCAAGCAGTAACAAAGACCA3′	21	62
	R:5′GGCTCAACCTCCAGGAACAG3′	19	62
56. FV(27)	F:5′GCCCTGGCACAGCACTTAG3′	19	62
	R:5′GTCCCAATCTTCCTCTTTCA3′	20	58

Table 2.1 (cont.)

Gene	Sequence	Length	Tm
57. FI/40	F:5′AATAACCGTTCTCAGCAGCA3′	20	58
	R:5′ATTCAAGGCGTTCACATCC3′	19	56
58. MII 51	F:5′CCTGATGAAATCGGGTCAAAAC3′	22	64
	R:5′ATACTCTCCTCTGCCGCTCG3′	20	64
59. FIII(4)R	F:5′GCAATCTCGCACAGCCAATACT3′	22	66
	R:5′CGGAAAGACAGGGCAGCAAC3′	20	64
60. 457(OP1)	F:5′CTTCTTATGTCTGTCCTTTGATGA3	24	66
	′ R:5′TTCTTAGGGAAACTGCTTGC3′	20	58
61. FI(1)	F:5′GTATTCCATCCTCAACAACTG3′	21	60
	R:5′ACTGGGAGCACTACCATCTT3′	20	60
62. 428(OPB17)R	F:5′CTCTGACTGGTGGAGGGAAT3′	20	62
	R:5′CTGGCTCGTGGGAGTGTAAT3′	20	62
63. FIII 39	F:5′ATCTCGCCAGGAGGAAATAA3′	20	58
	R:5′CCTTGTTCAGTTCTTGCCAC3′	20	60
64. FV-1	F:5′CGTATGCCACATCCCACAGA3′	20	62
	R:5′GAGCCCGTACCATTGAGAAA3′	20	60
65. FV 42	F:5′AAGTGACCTTGATATGAGTG3′	20	56
	R:5′ATCCCTTCGTTGTAAGTAGA3′	20	56
66. FIII 8	F:5′CCTCATAAACCAGGCACTAA3′	20	58
	R:5′AGAATCATCCCAGGAATCAC3′	20	58
67. MI 36	F:5′ATGTATGTTTGTGTATGTAGGTGTG3	25	66
	R:5′AGACGGCAAGGAAAGATGAG3′	20	60
68. FIV 33	F:5′TGGGACTGTTTGTTTCTTG3′	19	54
	R:5′TCTCTTGGTTAGGTGTTGGT3′	20	58
69. Nit protein 2*	F:5′TTGGTTGCCCTGCCTGAGTG3′	20	64
	R:5′CGGGTGATAGGACATCGGAC3′	20	64
70. Immunophilin FKBP	F:5′GAGAAGAAAGACGGAGGAGTG3′	21	64
52*	R:5′TATCAGGGTGGCATTTGGCG3′	20	62

Table 2.1 (cont.)

Gene	Primer sequence	Length	Tm
71. Calcium regulated heat stable protein*	F:5′CCAAACAAGGGCTTTCTGCTG3′	21	64
	R:5′CGCTTCGGTGGGATGGGACA3′	20	66
72. Dynein heavy chain 64C*	F:5′CAGTCTGCTGTTACTTGGCTATC3′	23	68
	R:5′GGTCTGTTTCTTCTCACCCTTG3′	22	66
73. Mapre 1 protein*	F:5′CAGTTGAGAACCTGAGCCGC3′	20	64
	R:5′TGGAGTGCCTTGAAGTTATTGA3′	22	62
74. Peroxinectin*	F:5′TGGACGCCATTACACGAAGC3′	20	62
	R:5′CACCAACCACGCACAGGAAG3′	20	64
75. Diphenol oxidase A2*	F:5′ACAATAATGAGGTAGCACGGT3′	21	60
	R:5′CGGAAAATCTGTCTCTCTGGGA3′	22	66
76. Dendritic cell protein***	F:5′AAGCCAGCAAAGTGATGATT3′	20	56
	R:5′CAAGAGGTCGTGGATGAGTTC3′	21	64
77. Vesicular integral-membrane protein**	F:5′GCCAATGTTATGCCGTCTGC3′	20	62
	R:5′ACCGCTTTCGCTGCTGCTCT3′	20	64
78. Short chain dehydrogenase*	F:5′GTGGCAGCAGCCGTAGATAG3′	20	64
	R:5′GGAGGAGAGATGTTCAAGATGTG3′	23	68
79. Female sterile**	F: 5′GCAATAACGGTGAACAAGGGA3′	21	62
	R: 5′GCAACCACATTAGTAGCCATA3′	21	60
80. Multicatalytic endopeptidase*	F: 5′GCCGAGTGTTCCAAGTAGAGT3′	21	64
	R: 5′ACATCAACGATAGCACGAGCA3′	21	62
81.Myosin regulatory light polypeptide 9*	F: 5′ATCCCGTAAGGCAGGAAAGA3′	20	60
	R: 5′CACCAAACAGAGTGAGGAACAT3′	22	64
82. Testes development related NYD SP19*	F:5′TTGCCGTTTACAAGCGACAC3′	20	60
	R:5′AGCACTCTCAAGGCTCCGTT3′	20	62
83. Motochondrial oxodicarboxylate*	F:5′GGAAGATGGCAAGAAATGAGG3′	21	62
	R:5′AAGGGTTTGACCAGGATAGC3′	20	60
84. Phospholipase C*	F:5′GTCTTACCAGAACTAGCAACCA3′	22	64
	R:5′ACATAATCTCCGACCTTCAAAT3′	22	58
85. Calcineurin B*	F:5′TGGGTAACGAAGCCTCATTG3′	20	60
	R:5′ATTCGGAAAGCAAAACGCAA3′	20	56

Table 2.1 (cont.)

Gene	Sequence	Length	Tm
86. Prefoldin subunit 2*	F:5′TCCAGAAGTTCCAGCAGATG3′	20	60
	R:5′TGGTCCCTATTGTTTGTGAGA3′	21	60
87. Calponin 1*	F:5′GCCGCCGAGTGCTTGGAATG3′	20	66
	R:5′CCTTCCTGCCCAGAGACT3′	18	58
88. Hydroxyacyl CoA	F:5′CGATTCCCTTCGTAATGTTG3′	20	58
dehydrogenase*	R:5′ATAGCTCACTGATGTCAAGTCA3′	22	62
89. Leucine rich repeat	F:5′GGCTCACATCACTCCCACTT3′	20	62
protein SHOC-2**	R:5′CCTCTAGATCCAACATCCGA3′	20	60
90. Cyclin A**	F:5′CAGCAAGTATGAGGTGGATTCT3′′	22	64
	R:5′TCTGCCCAACTCTGTAGGTATT3′	22	64
91. Phenylalanyl tRNA	F:5′TCAGCCATTCACTGCGGAGC3′	20	64
synthetase beta subunit*	R:3′TAGGAGGGACATCGACCACAA3′	21	64
92. Carbomoyl phosphate	F:5′TGTCACTTCACGCAGGATTG3′	20	60
synthetase 2**	R:5′ATAGTTGTCATTTGAGGCACC3′	22	62
93. Clathrin adaptor	F:5′TCCCAACTCCACTGAATACTT3′	21	60
protein AP50**	R:5′TGAGCTTCGACTCTAAACACCTT3′	23	66
94. Leukemia virus	F:5′GATGGCGGTCTTCAGGTGTC3′	20	64
receptor*	R:5′ATGGAAAGTGCGTGATGGGT3′	20	60
95. Endothelial cell growth	F:5′GGAGGTCGGCTGCTGTATCG3′R:5′	20	66
factor 1*	GCCACATCCACCATTTTCTT3′	20	58
96. NADH dehydrogenase	F:5′GAGGACAGGCTTGATTTATTG3′	21	60
subunit 5*	R:5′ATCCTAAACCATCCCACCCTA3′	21	62
97. 5 methylcytosin G/T	F:5′TTACCACGACCCTTGGAAACT3′	21	62
mismatch*	R:5′TCTCATGCAGCCCTAGACACT3′	21	64
98. Minute (2) 21AB	F:5′AGGAGAGGGACACCCAGACA3′	20	64
	R:5′TAGCATACCAAACATTAGACC3′	21	58
99. Myelodysplasia/	F:5′ATGTCTCCCTTCCCAGCACT3′	20	62
Myeloid leukemia	R:5′GTCTTGTTTCCCTTACACCACC3′	22	66
factor***			

Table 2.1 (cont.)

Gene	Sequence	Length	Tm
100. Thyroid hormone receptor associated protein**	F:5′GTAGGCTTGGTAGAATGGGC3′	20	62
	R:5′GAGCAGTAGCAGAAGTTGGGAA3′	22	66
101. Guanine nucleotide binding protein*	F:5′AGACCACCCGTCGCTTTGAG3′	20	64
	R:5′AACCTTACGCAGGACACCCA3′	20	62

*= Primers designed from EST found in the hemocyte cDNA library, ** primer pairs found in ovarian cDNA library and *** = primers designed from EST found in both ovarian and hemocyte cDNA libraries. Primer pairs numbers 54-68 were from RNA-arbitrary primed (RAP)-PCR.

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 a primer, 25 ng of genomic DNA of *P. monodon* and 1.0 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes). The amplification profiles were carried out following conditions described in Appendix A. The amplification products were electrophoretically analyzed through 1.2% agarose gels and visualized under a UV transilluminator after ethidium bromide staining.

2.5 Agarose gel electrophoresis (Sambrook and Rassell, 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. 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. To carry out agarose gel electrophoresis, one-fourth volume of the gel-loading dye (0.25% bromphenol blue and 25% ficoll) was added to each sample, mixed and loaded into the well. A 100-bp DNA ladder or

λ-*Hind* III was used as the standard DNA markers. Electrophoresis was carried out at 4 - 5 V/cm 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 under the UV light using a UV transilluminator.

2.6 Single strand conformational polymorphism (SSCP) analysis

The successful amplification products of various gene homologue 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.

2.6.1 Preparation of Glass Plates

The long glass plate was thoroughly wiped with 1 ml of 95% commercial grade ethanol in one direction with a tissue. This process was then repeated twice. Afterwards, the long glass plate was coated with 1 ml of freshly prepared Bind silane (10 μl of Bind silane, Amersaham Biosciences; 995 μl of 95% ethanol and 10 μl of 5% glacial acetic acid) and left for approximately 10 - 15 minutes. Excess binding solution was removed with a piece of tissue. The long glass plate was further cleaned with 95% ethanol for 3 times. The short glass plate was treated as described above with the exception that the binding solution was replaced by the Repel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The cleaned glass plates were assembled with a pair of 0.4 mM spacer.

Different concentration of low crosslink non-denaturing polyacrylamide gels (37.5:1 or 75:1 of acrylamide and bis-acrylamide) were prepared by dilution of a 40% stock solution to required 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 4 hours or overnight.

For SSCP analysis, 6 μl of the amplified PCR products were mixed with 24 μl of the SSCP loading dye (95% formamide,0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured in a boiling bath for 5 minutes and immediately cooled on ice for 3 minutes. The denatured products were electrophoretically analyzed

in native polyacrylamide gels at 250 – 300 volts for 14 – 18 hours at 4°C (see SSCP condition in Appendix A). Fractionated bands were visualized by silver staining

2.6.2 Silver staining

The gel plates were carefully separated. The long glass plate with the gel was placed in a plastic tray containing 1.5 litres of the fix/stop solution and agitated well for 25 – 30 minutes. The gel was soaked with shaking 3 times for 3 minutes with deionized water. The gel was lifted out from the tray between each wash and allowed the washed water draining out of the gel for 4 seconds. The gel was transferred to 0.1% silver nitrate (1.5 litres) and incubated with agitation at room temperature for 30 minutes. The gel was soaked in 1.5 litres of deionized water with shaking (10 forward and 10 backward steps) and immediately placed in the tray containing 1.5 litres of the chilled developing solution. This step is crucial and the time taken to soak the gel in the water and transfer it to chilled developing solution should be no longer than 5 – 10 seconds. The gel was well agitated until the first bands are visible (usually 1.5 – 2 minutes). The gel was then transferred to another tray containing 1.5 litres of chilled developer and shaked until bands from every lane were observed (usually 2 – 3 minutes). One litre of the fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was dried at 56 °C for 2-3 hours.

2.6.3 Direct DNA sequencing of PCR products

2.6.3.1 PCR and purification of the product

PCR products were purified using a HiYield™ Gel/PCR Mini Kit. Thirty microlitres of the PCR product were combined with twenty microlitres of TE in a microcentrifuge tube. Five volumes of DF buffer were added, mixed and vortexed. A DF column was inserted into a collection tube. The sample mixture was applied to the column. The tube was centrifuged at 6,000*g* (8,000 rpm) for 30 seconds. The flow-through was discarded. Five hundred microlitres of the wash buffer was added in the DF column and centrifuged at 6,000*g* (8,000 rpm) for 30 seconds. The flow-through was discarded. The DF column was placed in the collection tube and centrifuged at 14,000 rpm for 2 minutes to dry the column matrix. DNA was eluted out by the addition of 15 μl of the elution buffer or water into the center of column matrix and

left for 2 minutes. The purified PCR product was recovered by centrifugation at 14,000 rpm for 2 minutes.

2.6.3.2 DNA direct sequencing

Elute purified PCR products were direct sequenced using the automated DNA sequencer by MACROGEN (Korea) using the original forward or reverse primers of investigated genes as the sequencing primer.

2.7 Isolation and Characterization of the full length cDNA of ribophorin I and receptor for activated protein C kinase (*RACK*) using rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR)

2.7.1 First strand cDNA synthesis

Total RNA was extracted from ovaries of *P. monodon* using TRI Reagent. Messenger (m) RNA was purified using a QuickPrep micro mRNA Purification Kit (Amersham Biosciences). The RACE-Ready cDNA was synthesized using a BD SMART™ RACE cDNA Amplification Kit (BD Clontech) by combining 1.5 µg of ovarian mRNA with 1 µl of 5'CDS primer and 1 µl of 10 µm SMART II A oligonucleotide for 5'RACE-PCR and 1 µg of ovarian mRNA with 1 µl of 3'CDS primer A oligonucleotide for 3'RACE-PCR. The components were mixed and briefly centrifuged. The reaction was incubated at 70 ºC for 2 minutes and snap-cooled on ice for 2 minutes. The reaction tube was briefly centrifuged. After that, 2 µl of 5X First-Strand buffer, 1 µl of 20 mM DTT, 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 briefly centrifuged. The tubes were incubated at 42 ºC for 1.5 hours in an air incubator. The first strand reaction products were diluted with 125 µl of Tricine-EDTA Buffer and heated at 72 ºC for 7 minutes.

2.7.2 RACE-PCR

2.7.2.1 Primer design

A gene-specific primer (GSPs) was designed from ribophorin I (ovarian cDNA library) and *RACK* (hemocyte cDNA library) homologues

Table2.2 Primer sequences for the first strand cDNA synthesis and RACE-PCR

Primer	Sequence
SMART II A Oligonucleotide	5′-AAGCAGTGGTATCAACGCAGAGTACGC GGG-3′
3'-RACE CDS Primer A	5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ N-1N-3′ (N=A, C, G or T; N-1=A, G or C)
5'-RACE CDS Primer	5′-(T)25N-1N-3′ (N=A, C, G or T; N-1=A, G or C)
10X Universal Primer A Mix (UPM)	Long: 5′-CTAATACGACTCACTATAGGGCAA GCAGTGGTATCAACGCAGAGT-3′(0.4 μm) Short: 5′-CTAATACGACTCACTATAGGGC-3′ (2 μm)
Nested Universal Primer A (NUP)	5′-AAGCAGTGGTATCAACGCAGAGT-3′ (10 μm)

The same master mix for 5′- and 3′- RACE and control reactions was prepared. For each amplification reaction, 35.75 μl of deionized H_2O, 5 μl of 10X Advantage 2 PCR buffer, 1 μl of dNTP mix (10 μM each) and 1 μl of 50X Advantage 2 polymerase mix were combined. 5′-RACE-PCR and 3′-RACE-PCR were set up according to Table 2.4 and 2.5, respectively.

After characterization of primary RACE product, the Nested Gene Specific Primers (GSPs2) were designed for specific products.

Table 2.3 The gene specific primer (GSP1), their sequences and Tm of Ribophorin I and *RACK* gene.

Gene	Sequence	Length	Tm
1. 5′*RACK*	F:5′GAGAGAACCTTACGCAGGACACCCA3′	25	63.8
2.5'nested *RACK*	F:5′CACTGAAAGCAACAGAGAGCACATCC3′	26	62.1
3. 3′*RACK*	F:5′GCAAGACCACCCGTCGCTTTGAG3′	23	64.0
4.3′nested *RACK*	F:5′GTGTCTGGCTCCCGTGACAAAACC3′	24	63.9
5.5′Ribophorin I	F:5′AGTGGGTCTTCCATCCTCCAAACA3′	24	60.5
6.5′nested Ribophorin I	F:5′GGATGATGGTCTTGAAGGATTTGACAC3′	24	60.5
7.3′Ribophorin I	F:5′GTGTCTCACTGGGGCAATATTGCCGT3′	26	63.6
8.3′nested Ribophorin I	F:5′GATACGACTTCCAGAGAGAGCACA3′	24	60.5

The reaction was carried out for 20 cycles composing of a 94 ºC for 30 second, 68 ºC for 30 seconds and 72 ºC for 3 minutes. The primary 5′ and 3′ RACE-PCR products were electrophoretically analyzed. Nested PCR was performed using primers described in Table 2.3. The nested gene specific primers (nested GSP) for ribophorin I and *RACK* were designed for specific products. The primary PCR product was 50-fold diluted (1 µl of the product + 49 µl of TE). The amplification reaction was performed using 5 µl of the diluted PCR product as a template using the same condition for the first PCR for 15 cycles.

The positive amplification product of nested PCR was electrophoretically analyzed. The gel eluted product was cloned into pGEM-TEasy and further characterized by DNA sequencing.

Table2.4 Composition of 5′-RACE-PCR

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

Table2.5 Composition of 3′-RACE-PCR

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

2.8 Isolation and characterization of defender against cell death 1 (*DAD1*), thioredoxin peroxidase and calponin 1 using Genome walking analysis

2.8.1 Digestion of genomic DNA

The full length cDNA of *DAD1*, thioredoxin peroxidase and calponin 1 were already obtained from the cDNA library. These genes were further characterized using Genome walking analysis.

Two and a half microgrammes of genomic DNA of an individual of *P. monodon* were singly digested with 40 units of a blunt end generating restriction enzyme (*Alu* I, *Rsa* I, *Hae* III, *Dra* I, *Eco* RV, *Pvu* II, *Ssp* I or *Stu* I), 1X of appropriate restriction enzyme buffer and deionized H_2O in a reaction volume of 100 µl. 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.

2.8.2 Purification of digested DNA

An equal volume (95 µl) of buffer-equilibrated phenol was added. The mixture was vortexed at the low speed for 5-10 seconds and centrifuged for 5 minutes 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 (95 µl) of chloroform:isoamylalcohol was added, vortexed and centrifuged. The upper layer was transferred into a fresh 1.5 ml tube. One-tenth volume of 3 M NaOAc (pH 4.5) was added and mixed followed by 2.5 volume of ice cold absolute ethanol and thoroughly mixed. The mixture was kept at -80 ºC for 30 minutes. The digested DNA was recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature. The supernatant was discarded. The DNA pellet was briefly washed with ice-cols 70% ethanol and centrifuged at 12000 rpm for 5 minutes at room temperature. The supernatant was discarded. The pellet was air-dried. DNA was dissolved in 10 µl of TE (10 mM Tris, pH 8.0, 0.1 mM EDTA).

2.8.3 Ligation of genomic DNA to GenomeWalker 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.

2.8.4 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 $MgCl_2$, 0.2 µM each of Adaptor primer1 (AP1: 5′-GTA ATA CGA CTC ACT ATA GGG C-3′) and gene specific primer (Table 2.6), 1 µl of each DNA library of *P. monodon* and 1.0 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes).

The amplification reactions were carried out using the two-step cycle parameters including 7 cycles of a denaturing step at 94 ºC for 25 seconds and an annealing/extension step at 70 ºC for 3 minutes followed by 35 cycles of 94 ºC for 25 second, annealing at 65 ºC for 3 minutes and the final extension at 67 ºC for an additional 7 minutes.

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.

The primary PCR product was 50 fold diluted (1 µl of each primary PCR and 49 µl of deionized H_2O) and 1 µl of this was used as the DNA template for the secondary PCR. The PCR components of the secondary PCR were similar as those of the primary PCR with the exception that AP1 and GSP was replaced with 0.2 µM of AP2 primer (5′-ACT ATA GGG CAC GCG TGG T-3′) and 0.2 µM of nested GSP (gene specific primer 2, Table 2.6).

PCR was carried out composing of 5 cycles of a denaturing step at 94 ºC for 25 seconds and an annealing/extension step at 70 ºC for 3 minutes followed by 20 cycles of 94 ºC for 25 second and 65 ºC for 3 minutes. The final extension at 67 ºC was carried out for an additional 7 minutes. Five microlitres of the secondary 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.

Table 2.6 Sequences and Tm of gene specific primer (GSP1) and nested gene specific primer (GSP2) for genome walking analysis of thioredoxin peroxidase, *DAD1* and calponin 1.

Gene	Sequence	Length	Tm
Thioredoxin peroxidase	TPF:5′AAGCAGAATCTCCGTCAGGT3′	20	60
	TPR:5′CAAGCAACCACTCGCATCC3′	20	62
	Nested TPF:5′TAGATGAAACGCTGCGATTAGT3′	22	62
	Nested TPR:5′GGCAGACAAAGGTGAAATCC3′	20	60
DAD1	DAD1F:5′TTCCCCTTCAACTCCTTCCTGT3′	22	66
	DAD1R:5′CGATGCCTACCTCTTCTACG3′	20	62
	Nested DAD1F:5′GTGAGCTTGAGACTGCAGGCA3′	21	66
	Nested DAD1R:5′CTGATGAACCCAGACAGGAAGG3′	22	68
Calponin 1	CalF:5′GCCGCCGAGTGCTTGGAATG3′	20	66
	CalR:5′CCTTCCTGCCCAGAGACT3′	18	58
	Nested CalF:5′AACGCCTTTGTGGAGGGAGC3′	20	64
	Nested CalR:5′TGGAAGGTCTCCTGAGTGGGCA3′	22	70

2.9 Cloning of PCR-amplified DNA

2.9.1 Elution of DNA from agarose gels

The required DNA fragment was fractionated through agarose gels in duplication. One was run side-by-side with a 100 bp DNA markers and the other was loaded into the distal well of the gel. After electrophoresis, lanes representing the DNA standard and its proximal DNA sample were cut and 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.

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

2.9.2 Ligation of PCR product to pGEM-T easy vector

The ligation reaction was set up in the total volume of 10 µl containing 3 ul of the gel-eluted PCR product, 25 ng of pGEM-T easy vector, 5 µl of 2X rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10 % PEG 8000) and 3 Weiss units of T4 DNA ligase. The ligation mixture was gently mixed by pipetting and incubated at 4°C overnight.

2.9.3 Preparation of competent cells

A single colony of *E. coli* JM109 was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) with vigorous shaking at 37°C overnight. The starting culture was inoculated into 50 ml of LB broth and continued culture at 37°C with vigorous shaking to the OD_{600} of 0.5 – 0.8. The cells were chilled on ice for 10 minutes before centrifuged at 3,000 g for 10 minutes at 4°C. The pellets were resuspended in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged as above. The supernatant was

discarded and the pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ and divided into 200 μl aliquots. These competent cells could be used immediately or stored at –70°C for subsequent used.

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

The competent cells were thawed on ice for 5 minutes and divided to aliquots of 100 μl. Two microlitres of the ligation mixture was added and gently mixed by pipetting. The mixture was incubated on ice for 30 minutes. The reaction tube was then placed in a 42°C water bath for 45 seconds without shaking. The tube was then immediately snapped on ice for 2 – 3 minutes. One microlitre of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added to the tube. The cell suspension was incubated with shaking at 37°C for 1.5 hours. At the end on the incubation period, the cultured cell suspension was centrifuged at 12,000 rpm for 20 seconds at room temperature. The pellet was gently resuspended in 100 μl of SOC and spread on a LB agar plate containing 50 μg/ml of ampicillin, 25 μg/ml of IPTG and 20 μg/ml of X-gal. The plate was left until the cell suspension was absorbed and further incubated at 37°C overnight. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.9.5 Detection of recombinant clone by colony PCR

Colony PCR was performed in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 μM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 0.2 μM of pUC1 (5′-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.

2.9.6 Isolation and digestion of recombinant plasmid DNA

A recombinant clone was inoculated into 3 ml of LB broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 1.0 % NaCl) containing 50 μg/ml of amphicillin and incubated at 37°C with constant shaking at 250 rpm overnight. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 12,000 g for 1 min. The cell pellet was collected and resuspended with 250 μl of the buffer P1. The mixture was completely dispersed by vortexing. The mixture was then treated with 250 μl of the buffer P2, gently mixed and placed on ice for 10 min. Additionally, 350 μl of the buffer N3 was added and gently mixed.

To separate the cell debris, the mixture was centrifuged at 12,000 g for 10 minutes. The supernatant was transferred into the QIAprep column and centrifuged at 12,000 g for 30 - 60 seconds. The flow-through was discarded. The QIAprep spin column was washed by adding 0.75 ml of the buffer PE and centrifuged for 30 – 60 seconds. The flow-through was discarded. The spin tube was centrifuge for an additional 1 minute to remove the residual wash buffer. The QIAprep column was placed in a new 1.5 ml microcentrifuge tube and 40 μl of the EB buffer (10 mM Tris-Cl, pH 8.5) was added to elute the extracted plasmid DNA. The column was left at room temperature for 1 minute and centrifuge at 12,000 g for 1 minute.

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

2.9.7 Digestion of the amplified DNA insert

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

incubated at 37°C for 3 – 4 hours. Digestion patterns were analyzed by agarose gel electrophoresis.

2.9.8 DNA sequencing

Cloned DNA fragments from typical PCR, RT-PCR, RACE-PCR and genome walking analysis were sequenced by automated DNA sequencer using M13 forward and/or M13 reverse primer as the sequencing primer by MACROGEN (Korea)

2.10 Identification of SNP in 5′ and 3′ UTR of functionally important genes of *P. monodon*

2.10.1 Primer design

Primers were designed from 5′ and 3′ UTR of functionally important genes (*RACK*, Ribophorin I, *DAD1*, thioredoxin peroxidase and calponin1) of *P. monodon* with the amplification product of 180-350 bp.

For primers designed from 3′UTR, the reverse primers were normally located in the extreme of 3′end of the sequence (Table 2.7) where the forward primer was located approximately 50-80 bases upstream of the stop codon.

For primers designed from 5′UTR, the forward primers were normally located in the extreme of 5′end of the sequence and the reverse primers were located downstream from the start codon (Table 2.7).

2.10.2 PCR

PCR was performed 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 a primer, 25 ng of genomic DNA of *P. monodon* and 1 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes). The amplification profiles of 5′UTR and 3′UTR were set up according to Appendix B. The amplification products were electrophoretically analyzed through 1.2% agarose gels and visualized under a UV transilluminator after ethidium bromide staining and identified SNPs patterns using SSCP described previously. The SSCP conditions for

examination the amplification product of 5′UTR and 3′UTR of selected genes were illustrated in Appendix B.

Table 2.7 Primer pairs, primer sequences and Tm of functionally important gene used for identification of 5′ and 3′UTR of ribophorin I, thiredoxin peroxidase, *DAD1*, *RACK* and calponin I.

Gene	Sequence	Length	Tm
1.5′Ribophorin	F:5′GGGAGACTGAGGTTGAGCGT3′	20	64
I	R:5′CTGTCGTAGGCGGTGGCA3′	18	60
2.5′Thioredoxin	F:5′ATGTTAAGCATGAGTTGGAATT3′	22	58
peroxidase	R:5′TCAGTGGGGCAGACAAAGGT3′	20	62
3.5′*DAD1*	F:5′TGAGGAGGTGTAGTTGTTGTTTG3′	23	66
	R:5′CGTAGAAGAGGTAGGCATCG3′	20	62
4.5′*RACK*	F:5′CACTTGTGTAAGACGAATGATGGTT3′	25	70
	R:5′TCAGAGATTTGTCCCTGGAAGCA3′	23	68
5.5′Calponin 1	F:5′TTCCCCGTTTGTTGGCATCA3′	20	64
	R:5′CATTCCAAGCACTCGGCGGC3’	20	60
6.3′Ribophorin	F:5′AGACTCAGATCATCAGCGACCT3′	22	66
I	R:5′ATTTGCTCTCCTTTTCCTCCTAACT3′	25	70
7.3′Thioredoxin	F:5′GCACGCTTTCCAGTTCCCAG3′	20	64
peroxidase	R:5′TCAGTGGGGCAGACAAAGGT3′	20	62
8.3′*DAD1*	F:5′ACATCATCCTTCACCTTGTCAC3′	22	64
	R:5′CAAACTCCAATCGTTAAACCTT3′	22	60
9.3′*RACK*	F:5′CAACCGTTATTGGCTGTGTGC3′	21	64
	R:5′GCAGCACTCCAGAACATTCCCTT3′	23	70
10.3′Calponin 1	F:5′AGGGCTTCTGAGGGCATCGT3′	20	64
	R:5′TCCTTTTCTAGGCATACCACTG3′	20	60

2.10.3 SSCP and direct DNA sequencing of the amplified products

The PCR products were amplified. SSCP was carried out for the success amplification gene segment. For nucleotide sequencing, the amplification product *RACK* and 5′UTR of calponin 1 and *DAD1* was purified by a HiYield Gel/PCR DNA Fragment Extraction Kit using the protocol according to the manufacturer (Real Genomics). The PCR product of *RACK* and 5′UTR of *DAD1* were direct sequenced for both directions using the original primers as the sequencing primers by MACROGEN (Korea).

2.10.4 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of SNP

The PCR products 5′UTR of *DAD1* were amplified as describe in 2.10.2. The PCR product were separately digested with *Dra* I and *Hin*f I in a reaction volume of of 15 μl containing 1X appropriate restriction enzyme buffer (6 mM Tris-HCl; pH 7.5, 6 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT), 8 μl of the amplified product and 3 units of either *Dra* I or Hinf I. The reaction mixture was at incubated at 37°C for 3 – 4 hours. Digestion patterns were analyzed by agarose gel electrophoresis.

2.10.5 PCR-Allele Specific Amplification (PASA) of SNP

Primers were designed from 2 SNPs of 5'UTR of calponin 1 and 1 SNP of 5′UTR of *DAD1* at positions G123A, C221T and A276G, respectively. Two types of primers were designed, those with the common base at the terminal 3' end and those with the common base at the 3′end and a mismatched base at N-3 from the SNP position (Table 2.8)

Generally, PASA 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 $MgCl_2$, 0.25 μM of each primer, 25 ng of genomic DNA of *P. monodon* and 1.5 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes). The amplification profiles was carried out by predenaturing at 94 ºC for 3min and 10 cycles of a low stringent condition composing of 94 ºC for 30 second, 42 ºC for 1 minute and 72 ºC for 30 second followed by 35 cycles at a higher stringent condition

Table 2.8 Primer, primer sequences and position of SNP of 5′UTR of calponin 1 and 5′UTR of *DAD1*

Gene	Sequence	Position of SNP
1. 5′UTR of calponin 1: F1	F:5′CCTTCCTCCGTTGCTTTCTC**C**C**G**3′	G123A
2. 5′UTR of calponin 1: F2	F:5′CCTTCCTCCGTTGCTTTCTCTC**G**3′	G123A
3. 5′UTR of calponin 1: F3	F:5′CTCTCACCATCAGCTCTT**G**G**T**3′	C231T
4. 5′UTR of calponin 1: F4	F:5′CTCTCACCATCAGCTCTTTG**T** 3′	C231T
5. 5′UTR of *DAD1*: R1	R:5′AATTTCTGACAACCACCAATAGT**C**A**T**3′	A276G
6. 5′UTR of *DAD1*: R2	R:5′AATTTCTGACAACCACCAATAGTGA**T**-3′	A276G

composing of 94 ºC for 30 seconds, 50 ºC for 1 minute and 72 ºC for 30 seconds. The final extension was carried out at 72 ºC for 7 minutes. The amplification products were electrophoretically analyzed through 1.5% agarose gels and visualized under a UV transilluminator after ethidium bromide staining.

2.11 Semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Expression levels of calponin 1, *DAD1*, Ribophorin I, *RACK* and thioredoxin peroxidase were semiquatitatively determined. Elongation factor 1 alpha (EF1-α) was used as an internal control. The amplification conditions need to be optimized.

2.11.1 Primers

Primer pairs used for semiquantitative RT-PCR of calponin 1, *DAD1*, ribophorin I, *RACK* and thioredoxin peroxidase were illustrated in Table 2.1. Primer

for EF1-α were designed (F: 5′-ATG GTT GTC AAC TTT GCC CC-3′ and R: 5′-TTG ACC TCC TTG ATC ACA CC-3′).

2.11.2 Total RNA extraction and the first strand synthesis

Total RNA was extracted from hemocyte, testes and ovaries of broodstock-sized and juvenile *P. monodon* using TRI Reagent as previously described.

2.11.3 Determination of PCR conditions

Amplification was performed in a 25 µl reaction volume containing 0.1 µg of the first strand cDNA template, 1X PCR buffer (10mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% Triton X-100), 200 µM each of dNTP and 1 unit of Dynazyme™ DNA Polymerase (FINZYMES, Finland). PCR was carried out using the conditions described in Table 3.2.

2.11.4 Primer concentration

The optimal primer concentration for each primer pair (between 0 - 0.3 µM) was examined using the standard PCR conditions. The resulting product was electrophoretically analyzed. The primer concentration that gave specificity of the amplification product and clear results were selected for further optimization of PCR condition.

2.11.5 $MgCl_2$ concentration

The optimal $MgCl_2$ concentration of each primer pair (between 0 - 4 mM $MgCl_2$) was examined using the standard PCR conditions and the optimal primer concentration in 2.11.3. The concentration that gave the highest specificity was chosen.

2.11.6 Cycle number

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

2.11.7 Gel electrophoresis and quantitative analysis

The amplification product of genes under investigation and EF-1α were electrophoretically analyzed by the same gel. The intensity of interesting genes and that of EF-1α was quantifiedly from glossy prints of the gels using the Quantity One programme (BioRad)

2.12 Effects of temperature on expression of calponin 1, *DAD1*, ribophorin I, *RACK* and thioredoxin peroxidase

2.12.1 Temperature stress and semi-quantitative RT-PCR

Juvenile female *P. monodon* (approximately 4 month-old with the body weight of 21.07 ± 2.46 g were used in this experiments. They were acclimatized at the laboratory conditions (ambient temperature of 28 – 30 °C, salinity of 12 ppt) for 5 days. The experimental animals were fasted approximately 24 hours before the temperature treatment. They were treated at 33 ºC for 6 hours. Five shrimps were collected at 0, 6, 12 and 24 hours after treatment. Hemocyte and ovaries of each shrimp were collected and subjected to total RNA extraction. The first strand cDNA was synthesized and used as the template for semi-quantitative RT-PCR of calponin 1, *DAD1*, Ribophorin I, *RACK* and thioredoxin peroxidase between each shrimp groups (Table 3.2).

2.12.2 Data analysis

The expression level of each gene was normalized by that of EF-1α. 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 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 23.1 kb along with sheared DNA was obtained from pleopods of broodstock-sized shrimp kept at -30 °C since 1997 (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 contained RNA contamination as visualized by the smear at the bottom of gel and high ratio (> 2.0) of OD_{260}/OD_{280}.

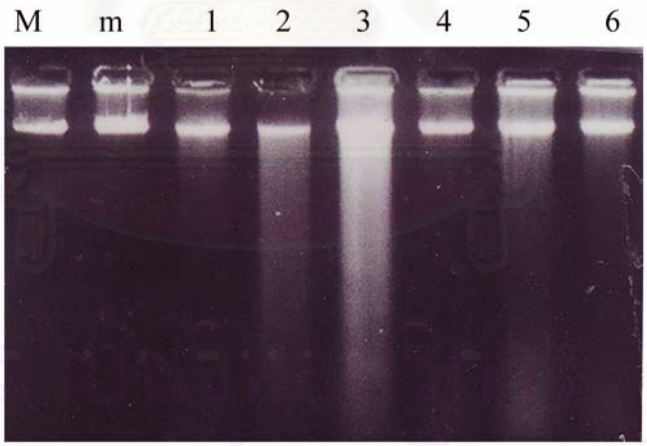

Figure 3.1 A 0.8% ethidium bromide-stained agarose gel showing the quality of genomic DNA extracted from the pleopod of *P. monodon*. Lanes M and m = 100 and 200 ng of undigested lambda DNA, respectively. Lanes 1 - 6 = Genomic DNA individually extracted from a piece of pleopod of *P. monodon*

3.2 Identification of polymorphic SSCP patterns of genes homologues of *P. monodon* cDNA.

A total of 101 primer pairs were designed from sequences of EST from ovarian and hemocyte cDNA libraries and used for amplification of genomic gene segments of wild *P. monodon*. Forty-eight genes homologues (accounting for 47.52% of overall primers,) were successfully amplified and 22 gene segments exhibited identical sizes with those expected from cDNA sequences. The remaining amplification gene segments showed larger product sizes suggesting the existence of an intron (s) in the amplification fragment.

The amplification product analyzed by agarose gel electrophoresis did not reveal large or indels heterozygotic(possible due to variation of allelic sizes, Figures 3.2 - 3.11) states were obvious. Gene homologues successfully amplified by designed primers were further analyzed by SSCP. Generally, SSCP analysis is more appropriate for fragments < 300 bp in length. Therefore, large amplification fragments were digested with appropriate restriction endonucleases before subjected to SSCP analysis. For example, ribophorin I was digested with *Hin*f I and polymorphic SSCP patterns were observed.

The migration rate of double strand DNA through low crosslink native polyacrylamide is faster than that of single strand DNA. Almost all of the amplified gene segments were polymorphic (Figure 3,12 – 3.21 and Table 2.1). Forty-four gene segment, for instance, homologues of *GGT*, *ADH*, ribophorin I, thioredoxin peroxidase, calponin 1, *DAD1* and *RACK* showed polymorphic SSCP patterns whereas survivin (Figure 3.13), peptide prolyl cis/tran isomerase, vacular-type H^+ ATPase and cyclin A (Figure 3.16) homologues were monomorphic when screened against genomic DNA of wild *P. monodon* originating from different geographic locations in Thai waters. Polymorphism may be resulted from SNP and/or small indels and reasonably high in several gene segments. Five gene homologues (calponin, *DAD 1*, ribophorin I, *RACK* and thioredoxin peroxidase) were chosen for further characterization and association analysis of SSCP patterns (SNP by EST, SBE) and their expression levels.

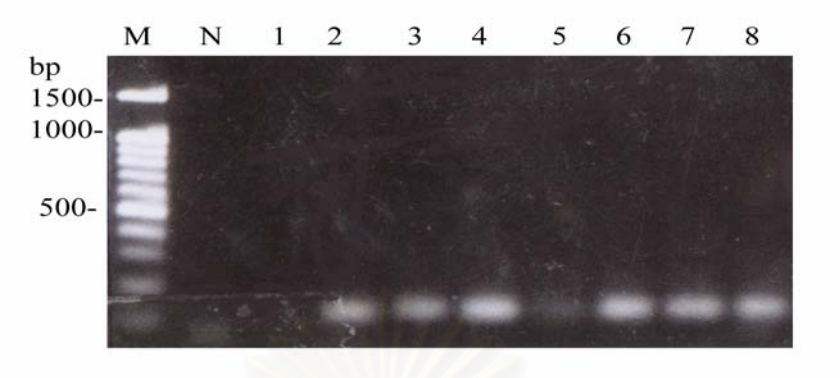

Figure 3.2 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologues of Gamma glutamyltransferrase *(GGT)* against genomic DNA of *P. monodon* from Chumphon (Lanes 1 - 4), Trang (Lanes 5 - 6), and Satun (Lanes 7 - 8). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

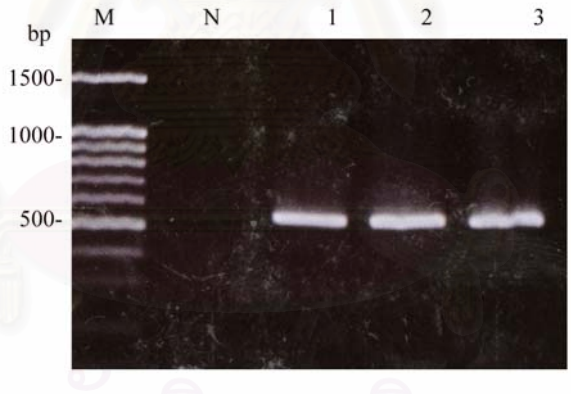

Figure 3.3 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of Survivin against genomic DNA of wild *P. monodon* (Lanes 1 - 3). Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

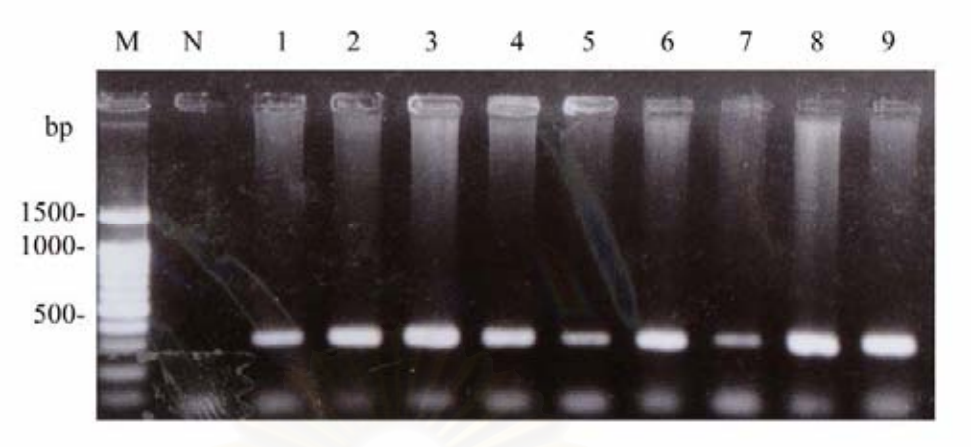

Figure 3.4 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of Aldehyde dehydrogenase family 6, subfamily A1 against genomic DNA of *P. monodon* from Chumphon (Lanes 1 - 4), Trang (Lanes 5 - 6), and Satun (Lanes 7 - 9). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

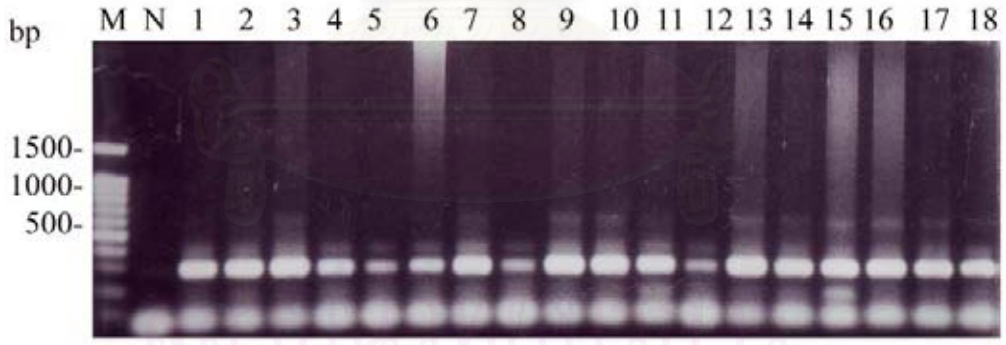

Figure 3.5 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of Vacuolar-type H^+-ATPase subunit A against genomic DNA of *P. monodon* from Chumphon (Lanes 1 - 5), Trang (Lanes 6 - 10), Satun (Lanes 11 - 15) and Phangnga (Lanes 16 - 18). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA tempate), respectively.

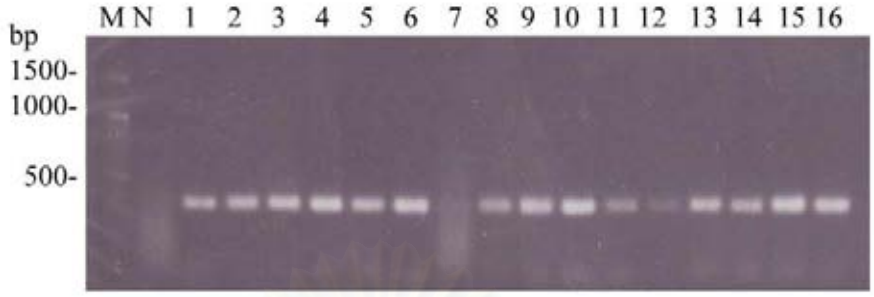

Figure 3.6 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of Cyclin A against genomic DNA of *P. monodon* from Chumphon (Lanes 1 - 5), Trang (Lanes 6 - 10), Satun (Lanes 11 - 14) and Phangnga (Lanes 15 - 16). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

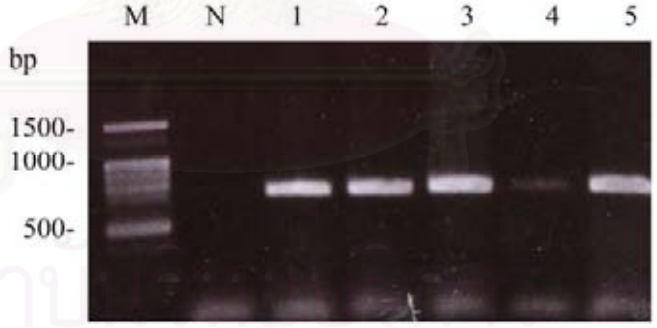

Figure 3.7 A 1.2% ethidium bromide stained agarose gel showing the amplification result of Ribophorin I against genomic DNA of *P. monodon* from Chumphon (Lanes 1 - 3) and Trang (Lane 5). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

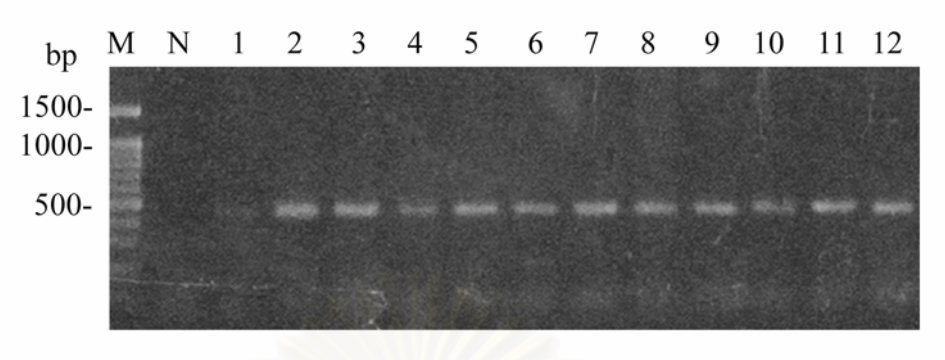

Figure 3.8 A 1.2% ethidium bromide stained agarose gel showing the amplification result of a homologue of Thioredoxin peroxidase against genomic DNA of *P. monodon* from Chumphon (Lanes 1 - 4), Trang (Lanes 5 - 6), Satun (Lanes 7 - 9) and Phangnga (Lanes 10 - 12). Lanes M and N are a 100 bp DNA DNA ladder 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 Calponin 1 against genomic DNA of *P. monodon* from Chumphon (Lanes 1 - 5), Trang (Lanes 5 - 7), Satun (Lanes 8 - 12) and Phangnga (Lanes 13 - 17). 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 amplification of *DAD1* in genomic DNA of each individual of *P. monodon* from Chumphon (Lanes 1-4), Trang (Lanes 5-8), Satun (Lanes 9-13) and Phangnga (Lanes 14-18). Lane 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 amplification of *RACK* in genomic DNA of each individual of *P. monodon* from Chumphon (Lanes 1 - 4), Trang (Lanes 5), Satun (Lanes 6 - 10) and Phangnga (Lanes 11 - 15). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

Figure 3.12 SSCP patterns of a *GGT* homologue from 17 non-related individuals of *P. monodon* broodstock. Four SSCP genotypes (pattern I, lanes 1, 2, 4 - 8, 10, 14 and 15; II, lanes 3, 9, 16 and 17; III, lanes 12, and IV, lanes 13) were observed. Lanes M and D are a 100 bp DNA marker and non-denatured PCR product (double strand control), respectively.

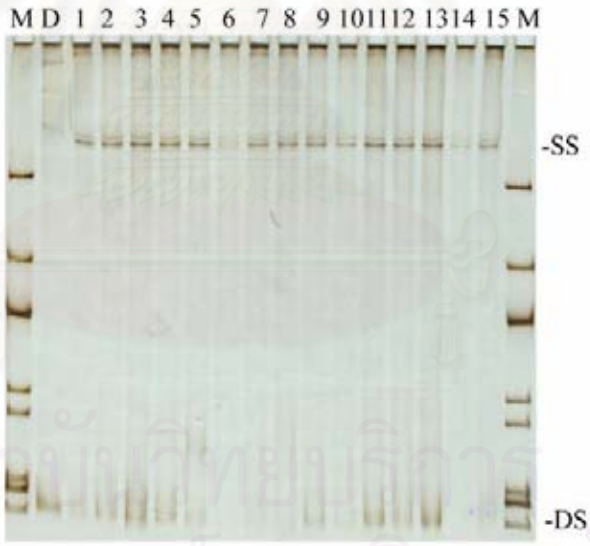

Figure 3.13 SSCP pattern of a survivin homologue from 15 non-related individuals of *P. monodon* broodstock. A monomorphic pattern was observed. Lanes M and D are a 100 bp DNA marker and non-denatured PCR product (double strand control), respectively.

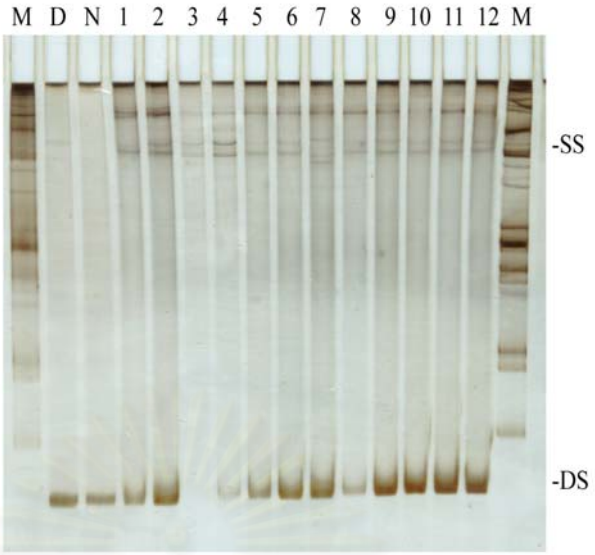

Figure 3.14 SSCP patterns of an Aldehyde dehydrogenase homologue from 12 non-related individuals of *P. monodon* broodstock. Four genotypes (pattern I, lanes 1; II, lanes 2, 5, 6, 8, 9, 10, 11 and 12; III, lanes 3 and 4, and IV, lanes 7) were observed. Lanes M, D and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and the negative control (without genomic DNA template), respectively.

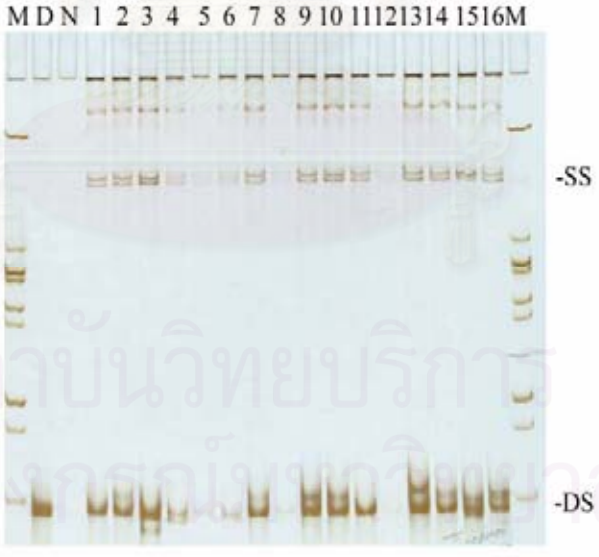

Figure 3.15 SSCP pattern of a homologue of Vacular-type H+ATPase subunit A from 16 non-related individuals of *P. monodon* broodstock. A monomorphic pattern was observed. Lanes M, D and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and the negative control (without genomic DNA template), respectively.

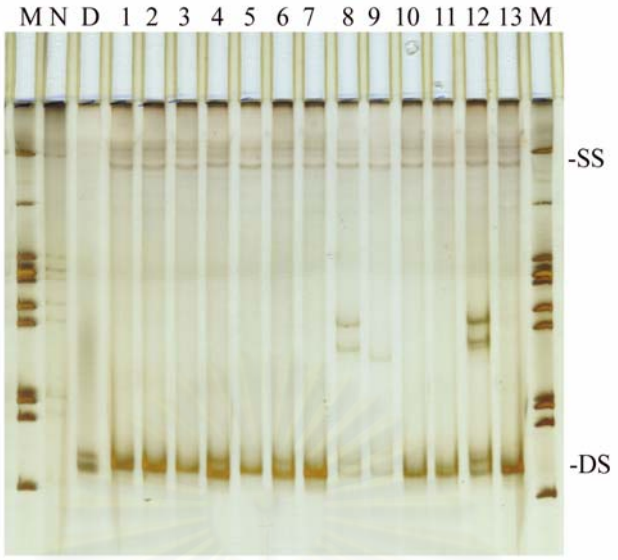

Figure 3.16 SSCP pattern of a Cyclin A homologue from 13 non-related individuals of *P. monodon* broodstock. A monomorphic pattern was observed. Lanes M, D and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and the negative control (without genomic DNA template), respectively.

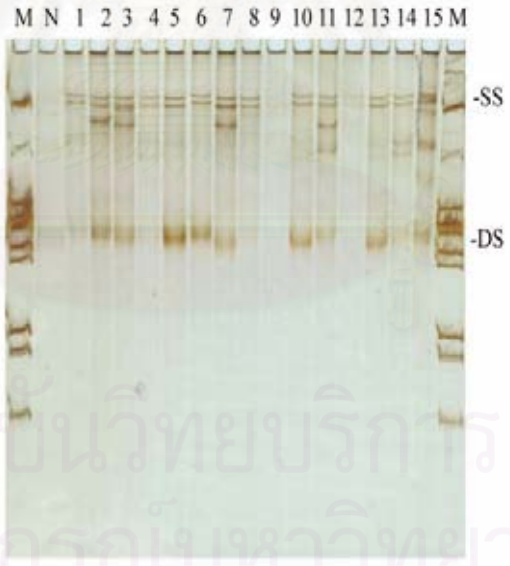

Figure 3.17 SSCP patterns of a Ribophorin I homologue (digesed with *Hin*f I before electrophoresis) from 15 non-related individuals of *P. monodon* broodstock. Eight genotypes (pattern I, lane 1; II, lane 2; III, lanes 3 and 12, and IV, lanes 4, 5 and 10; V, lanes 6, 11, 13 and 14; VI, lanes 7; X, lanes 8; and XI, lanes 15) were observed. Lanes M, D and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and the negative control (without genomic DNA template), respectively.

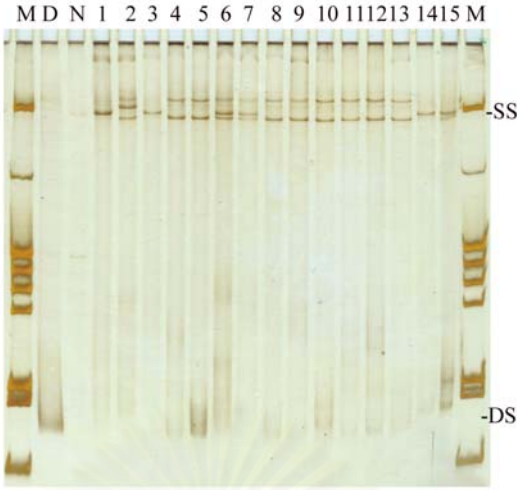

Figure 3.18 SSCP patterns of a Thioredoxin peroxidase homologue from 15 non-related individuals of *P. monodon* broodstock. Four genotypes (pattern I, lanes 1, 3, 14 and 15; III, lanes 4, 5, 8, 9, 10, 11, 12, and 13; V, lanes 2; and VI, lanes 6 and 7) were observed. Lanes M, D and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and the negative control (without genomic DNA template), respectively.

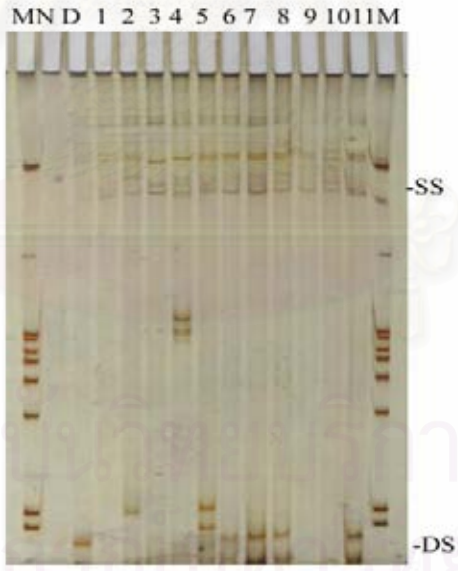

Figure 3.19 SSCP patterns of a Calponin I homologue from 11 non-related individuals of *P. monodon* broodstock. Six genotypes (pattern I, lanes 1 and 11; II, lanes 2; III, lanes 3, IV, lanes 4; V, lanes 5, 6, 7, 8 and 9; and VI, lanes 10) were observed. Lanes M, D and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and the negative control (without genomic DNA template), respectively.

Figure 3.20 SSCP patterns of a *DAD 1* homologue from 16 non-related individuals of *P. monodon* broodstock. Eight genotypes (pattern I, lanes 1, 8 and 10; II, lanes 2 and 11; III, lanes 3, 6, 15 and 16; IV, lanes 4; V, lanes 5; VI, lanes 7, 13 and 14; X, lanes 9; and XI, lanes 12) were observed. Lanes M, D and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and the negative control (without genomic DNA template), respectively.

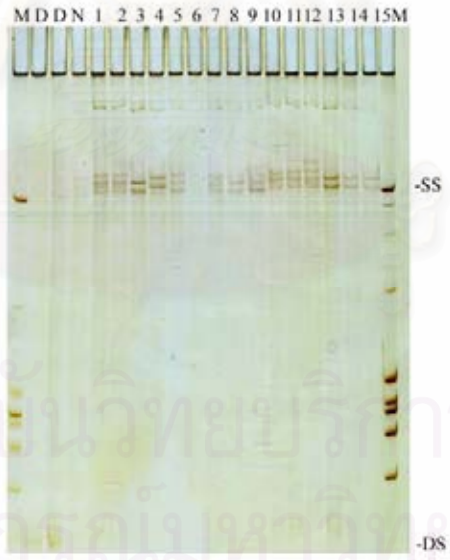

Figure 3.21 SSCP patterns of a homologue of *RACK* from 15 non-related individuals of *P. monodon* broodstock. Eight genotypes (pattern I, lanes 1, 2, and 7; II, lanes 3, 13, 14, and 15; III, lanes 4; IV, lanes 5 and 8; V, lanes 12; VI, lane 9, lanes 10 and 11) were observed. Lanes M, D and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and the negative control (without genomic DNA template), respectively.

Table 3.1 Expected size, observed size, full length and SSCP result in 101 genes

Gene	Expected size	Observed size	Full length cDNA(bp)	SSCP
1.Whey acidic protein (*WAP*)	291	-	564	ND
2. Heat shock protein 60 (*HSP60*)	295	295	719	Polymorphism
3. Apoptosis inhibitor survivin (Survivin)	320	500	600	Monomorphism
4. P109	183	-	548	ND
5. Cyclophilin 18	310	310	756	Polymorphism
6. Chaperonin cotaining t-complex polypeptide (*CCTP*)	262	600	836	Polymorphism
7.Prophenoloxidase activating factor (Propo factor)	154	200	568	Polymorphism
8. Clottable protein	152	152	5010	ND
9. Glutathione peroxidase	145	-	570	ND
10. Gamma glutamyltransferrase (*GGT*)	115	115	719	Polymorphism
11. Peptide prolyl cis trans isomerase (*PPI*)	166	300	1659	Monomorphism
12. Heat shock protein 10 (HSP 10)	176	800	665	Polymorphism
13. Superoxide dismutase (*SOD*)	166	400	908	ND
14. Casein kinase II beta chain	320	320	663	Polymorphism
15. Chaperonon subunit 8	323	-	1662	ND
16. Muskelin I	346	-	2205	ND
17. Ovarian lipoprotein receptor	354	-	3243	ND
18. Ribophorin I	190	700	1794	Polymorphism
19. Innexin 2	208	-	1077	ND
20. Thioredoxin peroxidase	233	500	588	Polymorphism
21. agCP 13148	212	700	2793	Polymorphism
22.Asparpginyl-tRNA synthetase	330	800	1644	Polymorphism
23. Semaphorin 2A precursor	256	-	2091	ND
24. Zeta1-cop	191	500	531	Polymorphism
25. Ferrochelatase	251	-	1227	ND
26. Calcium independent phospholipase A2 isoform 1	164	-	648	ND

Table 3.1 (cont.)

Gene	Expected size	Observed size	Full length cDNA(bp)	SSCP
27. HLAB associated trascript 1A;nuclear RNA helicase Bat1	150	150	1284	Polymorphism
28. Aldehyde dehydrogenase family 6, subfamily A1	202	350	1605	Polymorphism
29. pre B cell colony enhancing factor	239	-	1416	ND
30. Carbonic anhydrase	332	600	810	ND
31. Chromobox protein	303	-	555	ND
32. Vacuolar type H+ ATPase subunit A	150	300	1857	Monomorphism
33. Dihydropteridine reductase	327	-	705	
34. Aminopeptidase	174	400	711	Polymorphism
35. Dolichyl di phosphooligoccharideprotein glycotransferase	233	400	1323	Polymorphism
36. Integrin beta 4 binding protein	166	-	735	ND
37. 3-oxoacid CoA transfease	303	-	1560	ND
38. Profilin	229	-	378	ND
39. Adenosylhomocysteinase	314	-	1311	ND
40. COP9 subunit 6	316	-	972	ND
41. ATP/GTP-binding protein	360	-	1275	ND
42. Protease	293	700	1320	Polymorphism
43. Thiolase	231	-	1407	ND
44. Aspartate aminotransferase	334	-	1974	ND
45. Carnitine palmitoyltransferase II	334	-	1974	ND
46. Receptor activating protein kinase C	181	700	957	Polymorphism
47. Tetraspanin D107	316	-	717	ND
48. Methyl CpG binding protein 2	206	206	1509	ND
49. Presenilin enhancer	244	-	303	ND
50. Defender against cell death 1	214	400	339	Polymorphism

Table 3.1 (cont.)

Gene	Expected sized	Observed sized	Full length cDNA(bp)	SSCP
51. Glycogen phosphorylase	188	188	2532	Polymorphism
52. Nonclathrin coat protein zeta 2	203	600	519	Polymorphism
53. NH2 non-histone chromosome protein 2-like	246	700	384	Polymorphism
54. FIV/2	197	197	Not known	Polymorphism
55. FIV20	270	ND	Not known	ND
56. FV(27)	233	300	Not known	Polymorphism
57. FI/40	220	1000	Not known	ND
58. MII 51	123	123	Not known	Monomorphism
59. FIII(4)R	342	400	Not known	Polymorphism
60. 457(OP1)	324	324	Not known	Polymorphism
61. FI(1)	189	189	Not known	Polymorphism
62. 428(OPB17)R	238	238	Not known	Polymorphism
63. FIII 39	362	500	Not known	Polymorphism
64. FV-1	226	226	Not known	Polymorphism
65. FV 47	300	300	Not known	Polymorphism
66. FIII 8	333	333	Not known	Polymorphism
67. MI 36	182	182	Not known	Polymorphism
68. FIV 33	158	158	Not known	Polymorphism
69. Nit protein 2	292	-	693	ND
70. Immunophilin FKBP 52	312	-	1359	ND
71. Calcium regulated heat stable protein	263	-	441	ND
72. Dynein heavy chain 64C	192	-	13917	ND
73. Mapre 1 protein	205	320	813	Polymorphism
74. Peroxinectin	366	700	2334	Polymorphism
75. Diphenol oxidase A2	205	205	1482	Polymorphism
76. Dendritic cell protein	185	600	1122	Polymorphism
77. Short chain dehydrogenase	221	-	855	ND

Table 3.1 (cont.)

Gene	Expected size	Observed size	Full length cDNA(bp)	SSCP
78. Vesicular integral-membrane protein	187	600	1041	Polymorphism
79. Female sterile	296	-	5379	ND
80. Multicatalytic endopeptidase	217	-	765	ND
81.myosin regulatory light polypeptide 9	269	-	783	ND
82.Testes development related NYD SP19	193	-	1128	ND
83. Mitochondrial oxodicarboxylate	198	-	618	ND
84. Phospholipase C	188	-	3936	ND
85. Calcineurin B	319	-	510	ND
86. Prefoldin subunit 2	208	-	465	ND
87. Calponin	316	500	576	Polymorphism
88. Hydroxyacyl CoA dehydrogenase	244	-	1422	ND
89. Leucine rich repeat protein SHOC2	213	-	1746	ND
90. Cyclin A	256	400	1335	Monomorphism
91. Phenylalanyl tRNA synthetase beta subunit	221	700	1755	ND
92. Carbomoyl phosphate synthetase 2	202	-	2553	ND
93. Clathrin adaptor protein AP50	267	-	1275	ND
94. Leukemia virus receptor	230	-	2088	ND
95. Endothelial cell GF1	243	700	1413	ND
96. NADH dehydrogenase subunit 5	273	273	1722	Polymorphism
97. 5 methylcytosin G/T mismatch	181	-	1248	ND
98. Minute (2) 21AB	354	-	1224	ND
99. Myelodysplasia/Myeloid leukemia factor	271	-	927	ND
100. Thyroid hormone receptor associated protein	312	312	6598	Polymorphism
101. Guanine nucleotide binding protein	181	400	915	Polymorphism

- = no amplification product, ND = not determined.

A.

CGACTTCCAGAGAGAGCACAATACTTACTCTAGTGTCAAGTGAGTTTGACCAGAATTACTCATATTTAA
TATTTACAGGGCAATGTATGTCTATTGCTTGGTATGGCTCATAGTAAACAAGCAGTGAAGCATCAAAGT
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TTTGGAGGATGGAAGACC

B.

```
CDS             CGACTTCCAGAGAGAGCACAATACTTATTCTAGTGTCAA--------------------
RBR             CGACTTCCAGAGAGAGCACAATACTTACTCTAGTGTCAAGTGAGTTTGACCAGAATTACT
                ************************** ***********

CDS             ------------------------------------------------------------
RBR             CATATTTAATATTTACAGGGCAATGTATGTCTATTGCTTGGTATGGCTCATAGTAAACAA

CDS             ------------------------------------------------------------
RBR             GCAGTGAAGCATCAAAGTTTTACAAAGTAACCACTGCCATTGGAGGGAGAGGGAGATAAG

CDS             ------------------------------------------------------------
RBR             AGATAGATATAGATAGATAGATAGATAGATAGATAGATAATGAATAAAAGTATAGCAGAA

CDS             ------------------------------------------------------------
RBR             TTGCCAGTGTGAAACCATAGATAGCCATCTATTTCAGTGGCTATATGGGCAACAGGACTG

CDS             ------------------------------------------------------------
RBR             AGACATGAGACAGGCCCAATCCACTAAATTAGGGAGAGAGAAAAATGATGTATAGAATTT

CDS             ------------------------------------------------------------
RBR             TTGGAAGTGTTCAATGCTTATGGTATCATTTTCAGAGGTTGTTCATGCCATGATATCAAA

CDS             ------------------------------------------------------------
RBR             ATCATTATTATTATGTAGTATTATGTAGATTCCCAGGCCAAACGGTAGGACAGTTGTCAG

CDS             ------------------------------------------------------------
RBR             AAGCCTCTGGAGTCAGCGACATGAGAAATTTCTGATACCATCATTAATGGGTTAAAGAAA

CDS             ----------------ATCCTTCAAGACCATCATCCCAGCCTCAGCCAAGGATGTTTAC
RBR             ATCCTATTCATCTTCAGATCCTTCAAGACCATCATCCCAGCCTCAGCCAAGGATGTTTAC
                                 ****************************************

CDS             TACCGCGATGAGATCGGAAATATCTCCACCTCACACATGAGGATCCAGGATGATGCAGTG
RBR             TACCGCGATGAGATCGGAAATATCTCCACCTCACACATGAGGATCCAGGATGATGCAGTG
                ************************************************************

CDS             GAGCTGGACCTTAGGCCAAGGTTCCCCTTGTTTGGAGGATGGAAGACC
RBR             GAGCTGGACCTTAGGCCAAGGTTCCCCTTGTTTGGAGGATGGAAGACC
                *********************************************
```

Figure 3.22 A. Nucleotide sequence of a ribophorin I homologue. The positions of sequences primers were illustrated in boldface and underlined. **B.** Pairwise alignment between nucleotide sequences from coding sequence (CDS) and genomic DNA of ribophorin I. A new designed primer for further sequencing is italicized and underlined.

The amplified ribophorin I gene segment was approximately 700 bp in length which is larger than 190 bp expected from the cDNA sequence. This band was cloned and sequenced (Figure 3.22A). Pairwise alignment of sequences from genomic DNA fragment and EST confirm the existence of a 518 bp intron (Figure 3.22B). A new forward primer was designed and used to PCR amplificated of ribophorin I gene because it is theoretically expected that introns exhibit higher polymorphism than do exons. As expected, the new primer when combined with the former reverse primer provided scorable SSCP patterns but the extra step for digestion of the PCR product by *Hin*f I was eliminated (Figure 3.23).

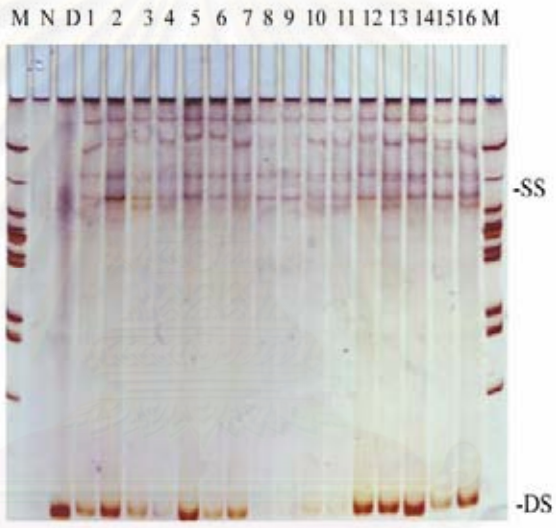

Figure 3.23 SSCP patterns of a ribophorin I homologue from 16 non-related individuals of *P. monodon* broodstock. Two genotypes (pattern I, lanes 1, 2, 5, 6, 8, 9, 12, 14, and 15; II, lanes 3, 4, 7, 10, 11, 13, and 16) were observed. Lanes M, D and N are a 100 bp DNA marker, non-denatured PCR product (double strand control) and the negative control (without genomic DNA template), respectively.

3.3 Isolation and Characterization of full length cDNA/genes of five functionally important genes

Polymorphic EST-derived markers were found from screening 101 primer pairs designed from nucleotide sequences of EST against genomic DNA of wild *P. monodon* by PCR and SSCP analyses. Five gene segments showing relatively high genetic diversity during the screening process were further characterized.

The partial sequences of ribophorin I and *RACK* were initially obtained from sequencing of the original clones. The full length of these gene homologues were then characterized by RACE-PCR. In contrast, the full length cDNA of, calponin 1, defender against cell death 1 *(DAD1)* and thioredoxin peroxidase was already identified. Therefore, genome walk analysis was carried out for examining genomic organization of these gene homologues.

3.3.1 Isolation and characterization of ribophorin I and *RACK* using RACE-PCR

3.3.1.1 RNA extraction and first strand synthesis

The quantity and quality of total RNA was spectrophotometrically electrophoretically. 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 3.24). The ovarian mRNA was purified and large amount of mRNA was obtained (30 - 50 per the purification tube). The purified mRNA was subjected to the synthesis of 5’ and 3’ RACE template.

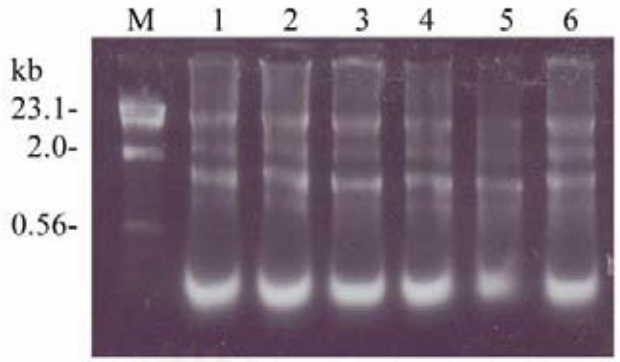

Figure 3.24 A 0.8% ethidium bromide-stained agarose gel showing the quality of RNA from ovaries (Lanes = 1 - 6) of *P. monodon.* Lanes M is λ/*Hin*d III marker.

3.3.1.2 Characterization of ribophorin I and receptor for activated protein C kinase *(RACK)*

The primary 5′and 3′ RACE-PCR were carried out and discrete amplification bands were obtained from RACE-PCR of both genes. The positive amplification product was obtained from typical PCR using 5′and 3′primers of both ribophorin I and *RACK*. Nevertheless, no amplification product was found from using 5′or 3′of each gene or UPM indicating that the primary product should not be non-specifically amplified. The sizes of the product was 1500 and 600 bp for 5′ RACE products and 1700 and 800 bp for 3′ RACE products of ribophorin I and *RACK*, respectively (Figure 3.25A).

Specific amplification products were obtained for the secondary PCR (nested PCR). The amplification bands of 1200, 400, 1500 and 700 bp were obtained (Figure 3.25B). These fragments were cloned and sequenced. Nucleotide sequences from original EST and 5′and 3′ RACE-PCR were combined. The full length cDNA of both ribophorin I and *RACK* were found.

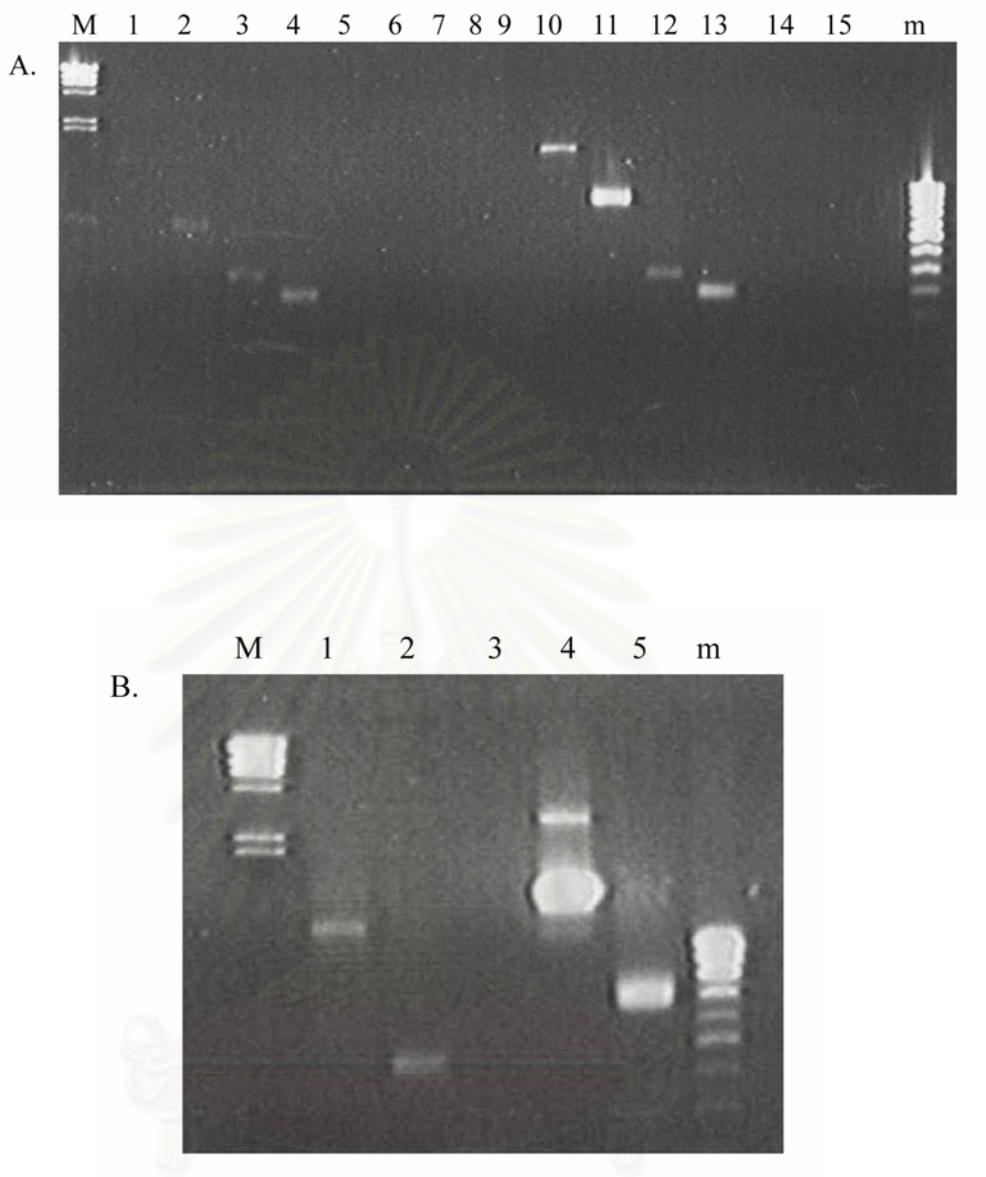

Figure 3.25 Primary 5′ and 3′ RACE-PCR of ribophorin I (lanes 2 and 10) and *RACK* (lanes 3 and 11). The typical PCR product of ribophorin I (lanes 4 and 12), *RACK* (lanes 5 and 13) and that from UPM (lanes 6 and 14), 5’ ribophorin I primer (lane 7), 5′RACK primer (lane 8), 3′ribophorin I primer (lane 15) and 3′ *RACK* primer (lane 16) alone were included (A). More intense PCR products were obtained by secondary 5′and 3′RACE-PCR of ribophorin I (lanes 1 and 4) and *RACK* (lanes 2 and 5) (B). λ-*Hin*d III (lanes M) and a 100 bp (lanes m) DNA ladder were used as the markers.

The combined nucleotides of ribophorin I was 2157 bp in length. The ORF of ribophorin I was 1806 bp encoding a polypeptide of 602 amino acids with 3 predicted *N*-link glycosylation at N_{215}, N_{293} and N_{504}, respectively (Figure 3.26). The 5′and 3′UTRs of ribophorin I were 150 and 175 bp (excluding the poly A tail).

```
GGAGACTGAGGTTGAGCGTCCTTTTTACGGCCCTCCCCCCACTCCCCGGCGCCCGTTTCC  60
CCCCGAAGGCTTCCCCGACAGGACGTTCTCCTCCTGACAGGTGTCGGCGGCCCGGCGTTG 120
CGGCTCGAGAGTCCCTCGGTGGCGTCCAGGATGGGTGTCCTCGGTGTCCTGTGTGTCCTG 180
                              M  G  V  L  G  V  L  C  V  L
GGTATCCTGGGGAGTGTTGTCCCCGGCGGCGCGTCTGCCACCGCCTACGACAGCGTGAAC 240
 G  I  L  G  S  V  V  P  G  G  A  S  A  T  A  Y  D  S  V  N
AAGGACCTCCTCCTGAAGAAGGTGGAGAGGAAGATCGACGTGGCGACGCAGGTCGTCAAG 300
 K  D  L  L  L  K  K  V  E  R  K  I  D  V  A  T  Q  V  V  K
ATCACGTCCAAGATCAGCCTGGAGAACGCTGGCTCGGGCGCCGTCGGGGCCTTCCTGCTG 360
 I  T  S  K  I  S  L  E  N  A  G  S  G  A  V  G  A  F  L  L
GCGCTCACGGAGGAGGAGAAGCGGAACCTCGCCTTCCTCAGTGCCCAGTCTGGCGGTACA 420
 A  L  T  E  E  E  K  R  N  L  A  F  L  S  A  Q  S  G  G  T
ACATTGCAGCTTTCTGAGAGCAAGGTGCATGAACATCAAGATATTGAATTCTTCAAGGTG 480
 T  L  Q  L  S  E  S  K  V  H  E  H  Q  D  I  E  F  F  K  V
GAACTTAAGGATGCTCTCCAGCCAGGCAAAATTGCTAATGTAGAGGTAGAATACTACCTT 540
 E  L  K  D  A  L  Q  P  G  K  I  A  N  V  E  V  E  Y  Y  L
ACTCATATGCTGGAGCCGTATCCTGCCGCCATTCAGCAAGGGGAGAAGCAGCTTGTACGA 600
 T  H  M  L  E  P  Y  P  A  A  I  Q  Q  G  E  K  Q  L  V  R
TACCTGGGCAACCACTACATTCTTACCCCCTACACCACCACAACCCAGACTACCACCGTC 660
 Y  L  G  N  H  Y  I  L  T  P  Y  T  T  T  T  Q  T  T  T  V
ACCCTCCCATCCCCAAATGTTGAATCCTACTCACGCCTGAAGCCAAGCTCCCACACTGAC 720
 T  L  P  S  P  N  V  E  S  Y  S  R  L  K  P  S  S  H  T  D
ACTTCTATCACTTATGGCCCCTATGATGGAGTTAAAACTTTCTCCCATGATGCAATGGCC 780
 T  S  I  T  Y  G  P  Y  D  G  V  K  T  F  S  H  D  A  M  A
ATTCACTACGAAAACAACTCCCCCTTCNTGACTGTGACAAGCTTGAAGCGTATGATTGAA 840
 I  H  Y  E  N  N  S  P  F  X  T  V  T  S  L  K  R  M  I  E
GTGTCTCACTGGGGCAATATTGCCGTTGAGGAAACTATTGATGTCCTCCATACGGGAGCC 900
 V  S  H  W  G  N  I  A  V  E  E  T  I  D  V  L  H  T  G  A
AAACTTAAGGGCTCTTTTTCAAGATACGACTTCCAGAGAGAGCACAATACTTACTCTAGT 960
 K  L  K  G  S  F  S  R  Y  D  F  Q  R  E  H  N  T  Y  S  S
GTCAAATCCTTCAAGACCATCATCCCAGCCTCAGCCAAGGATGTTTACTACCGCGATGAG 1020
 V  K  S  F  K  T  I  I  P  A  S  A  K  D  V  Y  Y  R  D  E
ATCGGAAATATCTCCACCTCACACATGAGGATCCAGGATGATGCAGTGGAGCTGGACCTT1080
 I  G  N  I  S  T  S  H  M  R  I  Q  D  D  A  V  E  L  D  L
AGGCCAAGGTTCCCCTTGTTTGGAGGATGGAAGACCCACTACAAGATTGGCTACAATGTC1140
 R  P  R  F  P  L  F  G  G  W  K  T  H  Y  K  I  G  Y  N  V
CCTTCATATGAATACCTTTACAACTATGGCGATGAATTCATATTAAAGATGAGGATCTTG1200
 P  S  Y  E  Y  L  Y  N  Y  G  D  E  F  I  L  K  M  R  I  L
GACCATGTCTTTGATGATGTGGTTGTGGACAATGTGATTGTGAAGGTGGTCTTGCCTGAA1260
 D  H  V  F  D  D  V  V  V  D  N  V  I  V  K  V  V  L  P  E
GGAGTACGCAATATGGAACTTGAGACGGCATACCCAGTAGAGCGCAAAGCCGACAGTTTG1320
 G  V  R  N  M  E  L  E  T  A  Y  P  V  E  R  K  A  D  S  L
CACTACACCTACTTAGATACCATTGGCCGCCCTATCATCACCATCAGTAAGAAAAACCTC1380
 H  Y  T  Y  L  D  T  I  G  R  P  I  I  T  I  S  K  K  N  L
GTGGAGAGTCACATCCAGGACTTCTCCCTCAAGTACAGCTTCCCATCAATCCTCATGCTG1440
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```
 V  E  S  H  I  Q  D  F  S  L  K  Y  S  F  P  S  I  L  M  L
CAGGAACCCCTCTTGGTGGTTGTGGCGTTCTTCATCCTCTTTATCACGGTTATCCTCTAT1500
 Q  E  P  L  L  V  V  V  A  F  F  I  L  F  I  T  V  I  L  Y
GTACGTCTTGACTTGACACTGAGCAAGGACGATGCCATTGAGGCCAAGATGCGCGTTTCC1560
 V  R  L  D  L  T  L  S  K  D  D  A  I  E  A  K  M  R  V  S
TCTCACTGTGAAATGATAGACTCCCACCATGACAAACGTGCCCGACAGTATGAGAAACTT1620
 S  H  C  E  M  I  D  S  H  H  D  K  R  A  R  Q  Y  E  K  L
GAAGAGGAGCTGCAGAAGTTGAAGGTGTCGAAGGATGTTAATCAGTCGCAGACTGCCACC1680
 E  E  E  L  Q  K  L  K  V  S  K  D  V  N  Q  S  Q  T  A  T
AAGAAGATCCTTCAGAGCATCCGTGATGAGACTCAGATCATCAGCGACCTGGGAGCCAAG1740
 K  K  I  L  Q  S  I  R  D  E  T  Q  I  I  S  D  L  G  A  K
ATTCGTTTGGACAACGCAGAGTATGCTGACAAAGTGGTTGAGCTCCAGAAGGCAGATAAG1800
 I  R  L  D  N  A  E  Y  A  D  K  V  V  E  L  Q  K  A  D  K
GTTCTGAGAGATAGCATTACACAGCAGCTGACCAACGTGGAGAAATTGGTGCTTGGGAAG1860
 V  L  R  D  S  I  T  Q  Q  L  T  N  V  E  K  L  V  L  G  K
ATGACCAAACAGCAGTACATGGATGCTGAAACTCCTCTACACAAAAAGAAGGAAGAGCAG1920
 M  T  K  Q  Q  Y  M  D  A  E  T  P  L  H  K  K  K  E  E  Q
TTGGAGAAAATGAAGACCATTTTGAGTGGCCTCTAAAAAGAAAGTAAATAGGATTTAGGG1980
 L  E  K  M  K  T  I  L  S  G  L  *
TTGGCTTGGTCAATTTTTCATATGTACTTCATAATGATGTATGATTCCTAAGTTATATAT2040
CAGTAACTTTTAAGTTAGGAGGAAAAGGAGAGCAAATTGACTTTATAATTTGCAAGATTC2100
ACACTAATAGTGCATTAAAGAAATTACCCACAAAAAAAAAAAAAAAAAAAAAAAA   2157
```

Figure 3.26 The full length cDNA sequences of ribophorin I of *P. monodon*. Start and stop codons are illustrated in boldface. Three putative *N*-linked glycosylation sites were found and are illustrated in bold-italicized.

The full length cDNA of ribophorin I was searched against data in the GenBank using Blast X and the closest homologues was ribophorin I of *Danio rerio* ($2e^{-161}$, accession number AF533661). The full length cDNA and amino acid of ribophorin I from various species was then retrieved and multiple aligned (Figures 3.27 and 3.28). Sequence divergence based on nucleotide and protein sequences were calculated. Large divergence was observed between ribophorin I of *P. monodon* and the remaining vertebrate species (Table 3.2). A neighbor-joining tree allocated ribophorin I from *Rattus norvegicus*, *Sus scrofa* and *Homo sapiens* into group I, that of *Danio rerio* to the other group and that of *P. monodon* to the last group (Figure 3.29).

```
H. sapiens        ATGGAGGCGCCAGCCGCCGGCTTGTTTC---TGCTCCTGTTGCTTGGGACTTGGGCCCCG
S. scrofa         ATGGAGGCGCCCGCAGTCTGCCTGCTACCGCTGCTGCTGTTGCTCTGGGCCTGGGCCCCG
R. norvegicus     ATGGAGGCGCCGATCGTC---TTGCTGC---TGCTGCTATGGCTCGGGGCCTTGGCCCCG
D. rerio          ------ATGTGGCGCGCCGGAGCGGTGA-------GTTTTGCGCTGATATGCTGTTTTTT
P. monodon        ATGGGTGTCCTCGGTGTCCTGTGTGTCCTGGGTATCCTGGGGAGTGTTGTCCCCGGCGGC
                            * *       *           *

H. sapiens        GCGCCGGGCAGCGCCTCCTCCGAGGCACCGCCG---CTGATCAATGAGGACGTGAAGCGC
S. scrofa         GCGCCGGGCCGCGCCTCCCCCGAGGCGCTGCCG---CTGGTCAACGAGGACGTGAAGCGC
R. norvegicus     ACGCCCGGCAGCGCCTCTTCGGAGGCTCCGCCG---CTGGTCAACGAGGACGTGAAGCGC
D. rerio          CTGCGGGTCGGTGTGCGCGGACGGGC-----------TGGTGAATGAAGATGTCAAGCGC
P. monodon        GCGTCTGCCACCGCCTACGACAGCGTGAACAAGGACCTCCTCCTGAAGAAGGTGGAGAGG
                    *   * *   *           *            *  *     *  * **  ** *

H. sapiens        ACAGTGGACCTAAGCAGCCACCTGGCTAAGGTGACGGCCGAGGTGGTCCTGGCGCACCTG
S. scrofa         ACGGTGGATCTGAGCAGCCACCTGGCTAAGGTGACGGCCGAGGTGGTCCTGGCGCACGCG
R. norvegicus     ACGGTGGACCTGAGCAGCCACCTAGCCAAGGTGACGGCTGAGGTGGTCCTGGCGCACCCG
D. rerio          ACGCTGGATCTCAGCTCTCACCTAGCCAAAATCACCGCCGAGAT----CCAGCTCGC--G
P. monodon        AAGATCGACGTGGCGACGCAGGTCGTCAAGATCACGTCCAAGATCAGCCTGGAGAACGCT
                  *   * **  *       **  * *  **  * **  *  ** *    *  *    *

H. sapiens        GGCGGCGGCTCCACGTCCCGAGCTACCTCTTTCCTGCTGGCTTTGGAGCCTGAGCTCGAG
S. scrofa         GGCAGCAGCTCCTCGCCCCGCGCCGCCTCCTTCCTACTAGCCCTGGAGCCCGAGCTGGAG
R. norvegicus     GGCGGCGGCTCTACAGCACGAGCCAGCTCTTTCGTTCTGGCCCTGGAGCCCGAACTGGAG
D. rerio          AACCGCGGATCC---TCCCGGGCCAACAGCTTCATCATCGGGCTGGAGGAGGAGCTCGCC
P. monodon        GGCTCGGGCGCC---------GTCGGGGCCTTCCTGCTGGCGCTCACGGAGGAGGAGAAG
                    *    *  *          *        *** *  * *   *   *   **

H. sapiens        GCCCGGCTGGCGCACCTGGGCGTGCAGGTAAAGGGAGAAGATGAGGAAGAGAACAATTTG
S. scrofa         GCCCGCTTGGCGCACCTCGGCGTGCAGGTGAAGGGAGAGGATGAGGAAGAGAACAATCTG
R. norvegicus     TCGCGACTTGCACACCTAGGCGTGCAGGTAAAGGGAGAAGATGAGGAAGACAACAACCTA
D. rerio          CCGCACCTGGCCTTCGTCGGCGCTTCTGTGAAGGGTGAAGAAGAGGAAGATGAACACCTC
P. monodon        CGGAACCTCGCCTTCCTCAGTGCCCAGTCTGGCGGTAC-----AACATTGCAGCTTTCTG
                         * **   * *  * *           **        *  *          *

H. sapiens        GAAGTACGTGAAACCAAAATTAAGGGTAAAAGTGGGAGATTCTTCACAGTCAAGCTCCCA
S. scrofa         GAAGTGAGAGAAACCAAAATTAAGGGTAAACGCGGGAGATTCTTCACAGTCACACTTCCA
R. norvegicus     GAAGTACGAGAAACCAAAATGAAGGGGAAAAGTGGGAGGTTTTTCACCGTCAAGCTCCCA
D. rerio          GAGCTGAAAGAGAAAACTGTTCATGGACAAAGTGGTAAATTCTTTGAGGCGCAGTTACCG
P. monodon        AGAGCAAGGTGCATGAACATC-------AAGATATTGAATTCTTCAAGGTGGAACTTAAG
                            *  *   *        **         ** **    *      *

H. sapiens        GTTGCTCTTGATCCTGGGGCCAAGATTTCAGTCATTGTGGAAACAGTCTACACCCATGTG
S. scrofa         GTTGCTCTTGATCCTGGGGCCAAGATTTCAGTCACTGTGGAAACCGTTTACACCCACGTG
R. norvegicus     GTTGCTCTTGATCCTGGGTCCAAGATCTCAATCGTTGTGGAAACTGTCCTACACCCATGTG
D. rerio          TCTTCTTTGGCTCCGGGGGCTAAGCTGCGGGTGAAGGTGGAGACTGTGTTCAGTCACGTC
P. monodon        GATGCTCTCCAGCCAGGCAAAATTGCTAATGTAGAGGTAGAATACTACCTTACTCATATG
                    * ** *    ** **    *          *    ** **          *  **  *

H. sapiens        CTTCATCCATATCCAACCCAGATCACCCAGTCAGAGAAACAGTTTGTGGTGTTTGAGGGG
S. scrofa         CTTCAGCCATATCCAACACAGATCACCCAATCAGAGAAACAGTTTGTGGTTTTCGAGGGG
R. norvegicus     CTTCATCCATACCCGACTCAGATAACTCAGTCAGAGAAACAGTTTGTGGTGTTTGAGGGC
D. rerio          CTGAAGCCGTTCCCCACACACATCACCCAGGCCGAGCGTCAGCTGGTGGTATTCCAGGGC
P. monodon        CTGGAGCCGTATCCTGCCGCCATTCAGCAAGGGGAGAAGCAGCTTGTACGATACCTGGGC
                  **  * ** * **  *    **    **    ***   *** * **    *    ***

H. sapiens        AACCATTATTTCTACTCTCCCTATCCAACGAAGACACAAACCATGCGTGTGAAGCTTGCC
S. scrofa         AACCATTATTTCTATTCTCCCTACCCAACCAAGTCACAAAGCATGCGTGTGAAGCTCGCC
R. norvegicus     AACCATTACTTCTACTCTCCCTATCCAACAAAGACCCAGACCATGCGAGTGAGACTTGCT
D. rerio          AACCACTACCTGTACTCCCCGTACCCGACCCGCTCCCAGACCACACACGGGTCCGACTGGCC
P. monodon        AACCACTACATTCTTACCCCCTACACCACCACAACCCAGACTACCACCGTCACCCTCCCA
                  ***** **  *     * ** **  * **     * ** *  *     **    **  *

H. sapiens        TCTCGAAATGTGGAGAGCTACACCAAGCTGGGGAACCCCACGCGCTCTGAGGACCTACTG
S. scrofa         TCCCGAAACGTGGAAAGCTACACCAAGCTGGGGAACCCCACGCGCTCTGAGGACCTCCTG
R. norvegicus     TCCCGAAATGTGGAGAGCCATACCAAGCTGGGGAACCCCTCAAGGTCTGAGGACATCCTG
D. rerio          TCTAAAACCGTGGAGAGCTACACTAAACTGGGCAACCCCACCAAGAGTGATGAGACCATT
P. monodon        TCCCCAAATGTTGAATCCTACTCACGCCTGAAGCCAAGCTCCCACACTGACACTTCTATC
                  **   **  ** **   * *  *    ***        * *      ***       *

H. sapiens        GATTATGGGCCTTTCAGAGATGTGCCTGCCTATAGTCAGGATACTTTTAAAGTACATTAT
S. scrofa         GATTACGGGCCTTTCCGAGATGTCCCTCCCTATAGTCAGGATACCTTTAAAGTACATTCC
R. norvegicus     GACTATGGGCCTTTTAAAGACATCCCTGCCTACAGTCAGGATACTTTCAAAGTACATTAT
D. rerio          GAGTATGGACCATTCAAAGACATTCCTCCCTTCAGCCAGGATGTGATGAAGATCCATTAT
P. monodon        ACTTATGGCCCCTATGATGGAGTTAAAACTTTCTCCCATGATGCAATGGCCATTCACTAC
                     ** ** ** *     *   *     * *     ** ***    *     * ** *
```

```
H. sapiens        GAGAACAACAGCCCTTTCCTGACCATCACCAGCATGACCCGAGTCATTGAAGTCTCTCAC
S. scrofa         GAGAACAACAGCCCCTTCCTGACCATCACCAGCATGACACGAGTCATTGAAGTCTCCCAC
R. norvegicus     GAGAACAATAGCCCTTTCCTGACCATCACCAGTATGACCCGGGTCATTGAGGTTTCTCAC
D. rerio          GAGAACAACTCGCCGTTCCTGACCATCAGCAGCATCACCCGCACCATCGAGGTCTCTCAC
P. monodon        GAAAACAACTCCCCCTTCNTGACTGTGACAAGCTTGAAGCGTATGATTGAAGTGTCTCAC
                  ** *****    ** *** ****  * *  **  * *  **    ** ** ** ** ***

H. sapiens        TGGGGTAATATTGCTGTGGAAGAAAATGTGGACTTAAAGCACACAGGAGCTGTGCTTAAG
S. scrofa         TGGGGTAATATTGCTGTGGAAGAAAATGTGGACCTGAAGCACACGGGGGCTGTGCTGAAG
R. norvegicus     TGGGGCAATATTGCTGTGGAAGAGAACGTGGACTTGAAGCATACAGGTGCGGTGCTGAAG
D. rerio          TGGGGAAACATCGCAGTGGAGGAGACCATCGACCTGAGGCACACCGGCGCTCACCTGAAG
P. monodon        TGGGGCAATATTGCCGTTGAGGAAACTATTGATGTCCTCCATACGGGAGCCAAACTTAAG
                  ***** ** ** ** ** ** ** *   * **  *    ** ** ** **    ** ***

H. sapiens        GGGCCTTTCTCACGCTATGATTACCAGAGACAGCCAGATAGTGGAATATCCTCCATCCGT
S. scrofa         GGGCCGTTTTCCCGCTATGACTATCAGAGACAGCCAGACAGTGGGATCTCCTCCATCCGT
R. norvegicus     GGACCTTTCTCCCGCTACGATTACCAGAGGCAGCCTGACAGTGGGATCTCCTCCATTCGT
D. rerio          GGCCCGTTCTCCCGCTACGACTACCAGAGGCAGTCTGACAGCGGCATTTCCTCCGTCAAA
P. monodon        GGCTCTTTTTCAAGATACGACTTCCAGAGAGAGCACAATACTTA---CTCTAGTGTCAAA
                  **  * ** **  * ** ** *  *****  **    * *        **     *

H. sapiens        TCTTTTAAGACCATCCTTCCTGCTGCTGCCCAGGATGTTTATTACCGGGATGAGATTGGC
S. scrofa         TCTTTTAAGACCATCCTTCCTGCTGCTGCCCAGGATGTTTATTACCGGGATGAGATTGGC
R. norvegicus     TCTTTTAAGACCATCCTTCCTGCTGCTGCCCAGGATGTGTATTACCGGGATGAAATCGGT
D. rerio          TCCTTCAAGACCATCCTCCCAGCCTCCGCTCAGGACGTGTACTACCGCGATGAGATCGGG
P. monodon        TCCTTCAAGACCATCATCCCAGCCTCAGCCAAGGATGTTTACTACCGCGATGAGATCGGA
                  ** ** ********* * ** **  * **  **** ** ** ***** ***** ** **

H. sapiens        AATGTTTCTACCAGCCACCTCCTTATTTTGGATGACTCTGTAGAGATGGAAATCCGGCCT
S. scrofa         AATGTTTCCACCAGCCACCTTCTTATTTTGGACGATTCTGTAGAGATGGAAATCCGGCCT
R. norvegicus     AATGTTTCCACCAGCCACCTCCTTATTTTGGATGACTCCGTGGAAATGGAAATCCGGCCT
D. rerio          AACATCTCCACCTCCCACCTCCAGGTTCTAGATGATTCTGTAGAGGTTGAAATTCGTCCC
P. monodon        AATATCTCCACCTCACACATGAGGATCCAGGATGATGCAGTGGAGCTGGACCTTAGGCCA
                  **  * ** ***   *** *    *    ** **  * ** **  * **  *  * **


H. sapiens        CGCTTCCCTCTCTTTGGCGGGTGGAAGACCCATTACATCGTTGGCTACAACCTCCCAAGC
S. scrofa         CGCTTCCCTCTTTTTGGCGGGTGGAAGACCCACTACATCGTTGGCTACAACCTCCCAAGC
R. norvegicus     CGATTTCCTCTCTTTGGAGGGTGGAAGACACACTACATCGTTGGTTACAACCTCCCAAGC
D. rerio          CGCTTCCCTCTGTTTGGTGGCTGGAAAACCCATTACATCATTGGTTATAATCTTCCCAGC
P. monodon        AGGTTCCCCTTGTTTGGAGGATGGAAGACCCACTACAAGATTGGCTACAATGTCCCTTCA
                   * ** **  * ***** ** ***** ** ** ****   **** ** **  * **

H. sapiens        TATGAGTACCTCTATAATTTGGGTGACCAGTATGCACTGAAGATGAGGTTTGTGGACCAT
S. scrofa         TACGAGTACCTCTATAATTTGGGAGACCAGTATGCACTGAAGATGAGGCTTGTGGACCAT
R. norvegicus     TATGAGTACCTCTATAATCTGGGTGACCAGTATGCACTGAAGATGCGGTTTGTAGACCAC
D. rerio          TACGAATACCTCTACAATCTAGGAGATCAGTATGCACTGAAGATGCGTCTGGTTGATCAT
P. monodon        TATGAATACCTTTACAACTATGGCGATGAATTCATATTAAAGATGAGGATCTTGGACCAT
                  ** ** ***** ** **    ** **  * *    * * ****** *  *  * ** **

H. sapiens        GTGTTTGATGAACAAGTGATAGATTCTCTGACTGTGAAGATCATCCTGCCTGAAGGAGCC
S. scrofa         GTGTTTGATGAGCAAGTGATAGACTCTCTGACTGTGAAGATCATCTTGCCTGAAGGGGCC
R. norvegicus     GTGTTTGATGAGCAAGTGATAGATTCTCTGACAGTGAAGATCATCCTTCCTGAGGGAGCC
D. rerio          GTCTATGATGACCAGGTTATTGDCCAGCTGACTGTCCGACTGATCCTCCCTGAAGGAGCC
P. monodon        GTCTTTGATGATGTGGTTGTGGACAATGTGATTGTGAAGGTGGTCTTGCCTGAAGGAGTA
                  ** * ******   **  * *  ***  **   *  ** * ***** ** *

H. sapiens        AAGAACATTGAAATTGATAGTCCCTATGAAATCAGCCGTGCCCCAGATGAGCTGCACTAC
S. scrofa         AAGAACATCCAGGTGGACAGTCCCTACGAGATCAGCCGAGCCCCTGACGAGCTGCACTAC
R. norvegicus     AAGAACATCCAGGTAGACAGTCCCTACGATATTAGCCGGGCCCCAGATGAGCTGCATTAC
D. rerio          AGAAACATCCATATGGACACCCCGTACCCCATCAGCCGCAGTCAGGACGAGCTGCACTAC
P. monodon        CGCAATATGGAACTTGAGACGGCATACCCAGTAGAGCGCAAAGCCGACAGTTTGCACTAC
                     ** **  *  * ** *   * **     *    **       **     **** ***

H. sapiens        ACCTATCTGGATACATTTGGCCGCCCTGTGATTGTTGCCTACAAGAAAAATCTGGTAGAA
S. scrofa         ACCTACCTGGACACGTTTGGCCGCCCCGTGATCGTGGCCCACAAGAAGAACCTGGTGGAA
R. norvegicus     ACCTACCTAGACACATTCGGCCGCCCGGTGATTGTTGCTTACAAGAAGAATCTAGTTGAA
D. rerio          ACTTACCTGGACACGTTTGGCAGACCTGTGCTGGTGGCCACCAAGAACAATCTGGTGGAG
P. monodon        ACCTACTTAGATACCATTGGCCGCCCTATCATCACCATCAGTAAGAAAAACCTCGTGGAG
                  ** **  * ** **  * *** * **  *  *          ***** ** ** ** **

H. sapiens        CAGCACATTCAGGACATTGTGGTCCACTACACGTTCAACAAGGTGCTCATGCTGCAGGAG
S. scrofa         CAGCATATTCAGGACATTGTGGTGCACTACACATTCAACAAGGTGCTCATGCTGCAGGAG
R. norvegicus     CAGCACATTCAGGACATTGTGGTGCACTACACATTCAACAAGGTGCTCATGCTGCAGGAG
D. rerio          CAGCACATTCAGGATGTTGTGGTTCACTACACCTTTAATAAGATCCTGATGCTGCAGGAG
P. monodon        AGTCACATCCAGGACTTCTCCCTCAAGTACAGCTTCCCATCAATCCTCATGCTGCAGGAA
                     ** ** *****  *     *  * ****  **        * ** ***********
```

```
H. sapiens        CCCCTGCTGGTGGTGGCGGCCTTCTACATCCTGTTCTTCACCGTTATCATCTATGTTCGG
S. scrofa         CCCCTGCTGGTGGTGGCCGCCTTTTACATCCTGTTCTTCACCGTCATTGTCTACGTCAGG
R. norvegicus     CCTCTGCTGGTTGTGGCCGCCTTCTACATCCTGTTCTTCACCGTCATCATCTACGTCCGT
D. rerio          CCGCTGTTAGTTGTGGGAGCATTCTATATCCTCTTCTTTACTGTCATCATCTACGTGCGC
P. monodon        CCCCTCTTGGTGGTTGTGGCGTTCTTCATCCTCTTTATCACGGTTATCCTCTATGTACGT
                  ** **  * ** ** *  ** ** *  ***** **  * ** ** **  **** **  *

H. sapiens        CTGGACTTCTCCATCACCAAGGATCCAGCCGCAGAAGCCAGGATGAAGGTAGCCTGCATC
S. scrofa         CTGGACTTCTCCATCACAAAGGATCCGGCTGCAGAGGCCAGGATGAAGGTGGCCTGCATC
R. norvegicus     CTAGACTTTTCCATCACCAAGGACCCAGCAGCAGAAGCCAGGATGAAAGTGGCCTGTATC
D. rerio          CTCGACTTCTCTATCACGAAGGATCCAGCAGCAGAGGTGCGCATGAAGGTGGCCTCCATC
P. monodon        CTTGACTTGACACTGAGCAAGGACGATGCCATTGAGGCCAAGATGCGCGT--TTCCTCTC
                  ** *****  *  * *  *****    **    ** *     ***   **        **

H. sapiens        ACAGAGCAGGTCTTGACCCTGGTCA--ACAAGAGAATAGGCCTTTACCGTCACTTTGACG
S. scrofa         ACAGAGCAGGTCTTGACCCTGGTGA--ACAAGAGAATAGGTCTCTACCGCCACTTTGATG
R. norvegicus     ACAGAGCAGGTCTTAACCCTGGTCA--ACAAGAGGTTAGGCCTCTACCGTCACTTTGATG
D. rerio          ACAGAGCAGGTCTTGACTCTGGTGA--ACAAGCGTCTGGGTCTCTACCGCCACATGGATG
P. monodon        ACTGTGAAATGATAGACTCCCACCATGACAAACGTGCCCGACAGTATGAGAAACTTGAAG
                  ** * * *    *  ** *     *  ****  *     * *  **     *  * ** *

H. sapiens        AGACCGTCAATAGGTACAAGCAATCCCGGGACAT--CTCCACCCTCAACAGTGGCAAGAA
S. scrofa         AGACGATCAATAGATACAAACAGTCCCGGGATGT--GTCCACCCTCAACAGCGGCAAGAA
R. norvegicus     AGACTGTCAATAGATACAAGCAGTCCCGGGACAT--CTCTACCCTCAATAGTGGCAAGAA
D. rerio          AGGTCGTGAACCGCTATAAGCAGTCGCGGGACAC--GGGAGCGCTAAACAGCGGTCGGAA
P. monodon        AGGAGCTGCAGAAGTTGAAGGTGTCGAAGGATGTTAATCAGTCGCAGACTGCCACCAAGA
                  **    *  *    *  **    **   ***               *  *       *

H. sapiens        GAGCCTGGAGACTGAACA-CAAGGCCTTGACCAGTGAGATTGCACTGCTGCAGTCCAGGC
S. scrofa         GAGCCTGGAGTTGGAGCA-CAAGGCCTTGACCAGTGAGGTGGCACTGCTACAGTCCAGGC
R. norvegicus     GAGCCTAGAGACAGAGCA-CAAAGCTGTGACCAGTGAGATTGCTGTGCTGCAGTCTAGGC
D. rerio          GACTCTGGAGGCTGAACA-TCGCACACTCACCAACGACATCACTGCCTTACAAACACGTC
P. monodon        AGATCC---TTCAGAGCATCCGTGATGAGACTCAGATCATCAGCGACCTGGGAGCCAAGA
                      *       ** **          **      *       *     *

H. sapiens        TGAAGACAGAGGGCTCTGATCTGTGCGACAGAGTGAGCGAAATGCAGAAGCTGGATGCAC
S scrofa          TGAAGACAGAAGGCTCTGACCTCTGTGACAAAGTAAGCGAAATGCAGAAACTGGACGCAC
R. norvegicus     TGAAGACGGAGGGCTCTGACCTGTGTGACAGGGTGAGCGAAATGCAGAAGCTGGACGCGC
D. rerio          TCAAAGCTGAGGGCTCCGATCTGGCAGAAAAGGTTGGAGAGATCCAGAAGCTGGACGGGC
P. monodon        TTCGTTTGGACAACGCAGAGTATGCTGACAAAGTGGTTGAGCTCCAGAAGGCAGATAAGG
                  *       **   * * **       ** *  **    **  * *****    **

H. sapiens        AGGTCAAGGAGCTGGTGCTGAAGTCGGCGGTGGAGGCTGAGCGCCTGGTGGCTGGCAAGC
S. scrofa         AGGTCAAGGAGCTGGTACTGAAGTCAGCGGTGGAGGCCGAGCGGCTGGTGGCTGGCAAGC
R. norvegicus     AGGTCAAGGAGCTGGTACTGAAGTCTGCGGTGGAGGCAGAGAGGCTGGTGGCTGGCAAGC
D. rerio          AGCTGAAGGATCTGGTGTGTCGCTCCTCTCTGGACGCCGAGAGGTTGGTGGCTGGAAAGG
P. monodon        TTCTGAGAGATAGCATTACACAGCAGCTGACCAACGTGGAGAAATTGGTGCTTGGGAAGA
                     * *  **     *                  * *  ***    *****  *** ***

H. sapiens        TCAAGAAAGACACGTACATTGAGAATGAGAAGCTCATCTCAGGAAAGCGCCAGGAGCTGG
S. scrofa         TCAAGAAAGACACATACATTGAGAATGAGAAGCTTATCTCGGGAAAGCGCCAGGAGCTGG
R. norvegicus     TCAAGAAGGACACATACATCGAGAATGAAAAGCTCAGCTCAGGAAAACGCCAGGAGCTGG
D. rerio          TGAAGAAGGACGCCTACATCGACTCTGATAAAAACCTGACAGGCAAGCGGCACGAGCTGG
P. monodon        TGACCAAACAGCAGTACATGGATGCTGAAACTCCTCTACACAAAAAGAAGGAAGAGCAGT
                  * *  **   *     ***** **  *** *           **     * **** *

H. sapiens        TCACCAAGATCGACCACATCCTGGATGCCCTGTAG
S. scrofa         TCACCAAGATCGACCACATCTTGGATGCACTGTAG
R. norvegicus     TCACCAAGATCGACCACATCCTGGACGCTCTGTAG
D. rerio          TCAACCGCATCGACAGCCTTCTGGACGCCTTGTAA
P. monodon        TGGAGAAAATGAAGACCATTTTGAGTGGCCTCTAA
                  *       **  *   * *  **   *   * **
```

Figure 3.27 Multiple alignments of ribophorin I nucleotide sequences of *P. monodon*, *Danio rerio* (accession number AF533661), *Rattus norvegicus* (BAE16987), *Sus scrofa* (CAC04096) and *Homo sapiens* (AAH10839).

```
H. sapiens        MEAPAAGLF-LLLLLGTWAPAPGSASSEAPPLINED-----VKRTVDLSSHLAKVTAEVV
S. scrofa         MEAPAVCLLPLLLLLWAWAPAPGRASPEALPLVNED-----VKRTVDLSSHLAKVTAEVV
R. norvegicus     MEAPIVLLL--LLWLGALAPTPGSASSEAPPLVNED-----VKRTVDLSSHLAKVTAEVV
D. rerio          MWRAGAVSF--ALICCFFC---GSVCADG--LVNED-----VKRTLDLSSHLAKITAEIQ
P. monodon        MGVLGVLCV--LGILGSVVP--GGASATAYDSVNKDLLLKKVERKIDVATQVVKITSKIS
                  *    .  .             * ... .   :*:*     *:*.:*:::::.*:*:::

H. sapiens        LAHLGGGSTSRATSFLLALEPELEARLAHLGVQVKGEDEEENNLEVRETKIKGKSG-RFF
S. scrofa         LAHAGSSSSPRAASFLLALEPELEARLAHLGVQVKGEDEEENNLEVRETKIKGKRG-RFF
R. norvegicus     LAHPGGGSTARASSFVLALEPELESRLAHLGVQVKGEDEEDNNLEVRETKMKGKSG-RFF
D. rerio          LANRG---SSRANSFIIGLEEELAPHLAFVGASVKGEEEEDEHLELKEKTVHGQSG-KFF
P. monodon        LENAG---SGAVGAFLLALTEEEKRNLAFLSAQSGG-----TTLQLSESKVHEHQDIEFF
                  * : *   :  . :*::.*  *   .**.:...  *       *:: *..:: : . .**

H. sapiens        TVKLPVALDPGAKISVIVETVYTHVLHPYPTQITQSEKQFVVFEGNHYFYSPYPTKTQTM
S. scrofa         TVTLPVALDPGAKISVTVETVYTHVLQPYPTQITQSEKQFVVFEGNHYFYSPYPTKSQSM
R. norvegicus     TVKLPVALDPGSKISIVVETVYTHVLHPYPTQITQSEKQFVVFEGNHYFYSPYPTKTQTM
D. rerio          EAQLPSSLAPGAKLRVKVETVFSHVLKPFPTHITQAERQLVVFQGNHYLYSPYPTRSQTT
P. monodon        KVELKDALQPGKIANVEVEYYLTHMLEPYPAAIQQGEKQLVRYLGNHYILTPYTTTTQTT
                   . *  :* **    : **   :*:*.*:*: * *.*:*:* : ****: :**.* :*:

H. sapiens        RVKLASRNVESYTKLGNPTRSEDLLDYGPFRDVPAYSQDTFKVHYENNSPFLTITSMTRV
S. scrofa         RVKLASRNVESYTKLGNPTRSEDLLDYGPFRDVPPYSQDTFKVHSENNSPFLTITSMTRV
R. norvegicus     RVRLASRNVESHTKLGNPSRSEDILDYGPFKDIPAYSQDTFKVHYENNSPFLTITSMTRV
D. rerio          RVRLASKTVESYTKLGNPTKSDETIEYGPFKDIPPFSQDVMKIHYENNSPFLTISSITRT
P. monodon        TVTLPSPNVESYSRLKPSSHTDTSITYGPYDGVKTFSHDAMAIHYENNSPFXTVTSLKRM
                   * *.* .***:::*  .:::: : ***: .: .:*:*.: :* ****** *::*:.*

H. sapiens        IEVSHWGNIAVEENVDLKHTGAVLKGPFSRYDYQRQPDSGISSIRSFKTILPAAAQDVYY
S. scrofa         IEVSHWGNIAVEENVDLKHTGAVLKGPFSRYDYQRQPDSGISSIRSFKTILPAAAQDVYY
R. norvegicus     IEVSHWGNIAVEENVDLKHTGAVLKGPFSRYDYQRQPDSGISSIRSFKTILPAAAQDVYY
D. rerio          IEVSHWGNIAVEETIDLRHTGAHLKGPFSRYDYQRQSDSGISSVKSFKTILPASAQDVYY
P. monodon        IEVSHWGNIAVEETIDVLHTGAKLKGSFSRYDFQREHNT-YSSVKSFKTIIPASAKDVYY
                  *************.:*: **** ***.*****:**: ::  **::*****:**:*:****

H. sapiens        RDEIGNVSTSHLLILDDSVEMEIRPRFPLFGGWKTHYIVGYNLPSYEYLYNLGDQYALKM
S. scrofa         RDEIGNVSTSHLLILDDSVEMEIRPRFPLFGGWKTHYIVGYNLPSYEYLYNLGDQYALKM
R. norvegicus     RDEIGNVSTSHLLILDDSVEMEIRPRFPLFGGWKTHYIVGYNLPSYEYLYNLGDQYALKM
D. rerio          RDEIGNISTSHLQVLDDSVEVEIRPRFPLFGGWKTHYIIGYNLPSYEYLYNLGDQYALKM
P. monodon        RDEIGNISTSHMRIQDDAVELDLRPRFPLFGGWKTHYKIGYNVPSYEYLYNYGDEFILKM
                  ******:****: : **:**:::*************** :***:******** **:: ***

H. sapiens        RFVDHVFDEQVIDSLTVKIILPEGAKNIEIDSPYEISRAPDELHYTYLDTFGRPVIVAYK
S. scrofa         RLVDHVFDEQVIDSLTVKIILPEGAKNIQVDSPYEISRAPDELHYTYLDTFGRPVIVAHK
R. norvegicus     RFVDHVFDEQVIDSLTVKIILPEGAKNIQVDSPYDISRAPDELHYTYLDTFGRPVIVAYK
D. rerio          RLVDHVYDDQVIQ-LTVRLILPEGARNIHMDTPYPISRSQDELHYTYLDTFGRPVLVATK
P. monodon        RILDHVFDDVVVDNVIVKVVLPEGVRNMELETAYPVERKADSLHYTYLDTIGRPIITISK
                  *::***:*: *:: : *:::****.:*:.:::.* :.*  *.********:***::.  *

H. sapiens        KNLVEQHIQDIVVHYTFNKVLMLQEPLLVVAAFYILFFTVIIYVRLDFSITKDPAAEARM
S. scrofa         KNLVEQHIQDIVVHYTFNKVLMLQEPLLVVAAFYILFFTVIVYVRLDFSITKDPAAEARM
R. norvegicus     KNLVEQHIQDIVVHYTFNKVLMLQEPLLVVAAFYILFFTVIIYVRLDFSITKDPAAEARM
D. rerio          NNLVEQHIQDVVVHYTFNKILMLQEPLLVVGAFYILFFTVIIYVRLDFSITKDPAAEVRM
P. monodon        KNLVESHIQDFSLKYSFPSILMLQEPLLVVVAFFILFITVILYVRLDLTLSKDDAIEAKM
                  :****.****. ::*:* .:********** **:***:***:*****:::** * *.:*

H. sapiens        KVACITEQVLTLVNKRIGLYRHFDETVNRYKQSRDISTLNSGKKSLETEHKALTSEIALL
S. scrofa         KVACITEQVLTLVNKRIGLYRHFDETINRYKQSRDVSTLNSGKKSLELEHKALTSEVALL
R. norvegicus     KVACITEQVLTLVNKRLGLYRHFDETVNRYKQSRDISTLNSGKKSLETEHKAVTSEIAVL
D. rerio          KVASITEQVLTLVNKRLGLYRHMDEVVNRYKQSRDTGALNSGRKTLEAEHRTLTNDITAL
P. monodon        RVSSHCEMIDSHHDKRARQYEKLEEELQKLKVSKDVNQSQTATKKILQSIRDETQIISDL
                  :*:.  * : :  :**   *.:::* ::: * *:* .  ::. *.:  . :  *. :: *

H. sapiens        QSRLKTEGSDLCDRVSEMQKLDAQVKELVLKSAVEAERLVAGKLKKDTYIENEKLISGKR
S. scrofa         QSRLKTEGSDLCDKVSEMQKLDAQVKELVLKSAVEAERLVAGKLKKDTYIENEKLISGKR
R. norvegicus     QSRLKTEGSDLCDRVSEMQKLDAQVKELVLKSAVEAERLVAGKLKKDTYIENEKLSSGKR
D. rerio          QTRLKAEGSDLAEKVGEIQKLDGQLKDLVCRSSLDAERLVAGKVKKDAYIDSDKNLTGKR
P. monodon        GAKIRLDNAEYADKVVELQKADKVLRDSITQQLTNVEKLVLGKMTKQQYMDAETPLHKKK
                   :::: :.:: .::* *:** *  ::: : :.  :.*:** **:.*: *:: :.    *:
```

```
H. sapiens        QELVTKIDHILDAL
S. scrofa         QELVTKIDHILDAL
R. norvegicus     QELVTKIDHILDAL
D. rerio          HELVNRIDSLLDAL
P. monodon        EEQLEKMKTILSGL
                  .* : ::. :*..*
```

Figure 3.28 Multiple alignments of ribophorin I protein sequences of *P. monodon*, *Danio rerio*, *Rattus norvegicus*, *Sus scrofa* and *Homo sapiens*.

Table 3.2 Sequence divergence of ribophorin I from different taxa based on nucleotide (below diagonal) and amino acids sequences (above diagonal)

	H. sapiens	***S. scrofa***	***R. norvegicus***	***D. rerio***	***P. monodon***
H. sapiens	-	0.0565	0.0548	0.3528	0.8589
S. scrofa	0.1063	-	0.0839	0.3634	0.8589
R. norvegicus	0.1246	0.1538	-	0.3581	0.8637
D. rerio	0.4285	0.4255	0.4211	-	0.8168
P. monodon	0.7550	0.7607	0.7962	0.7717	-

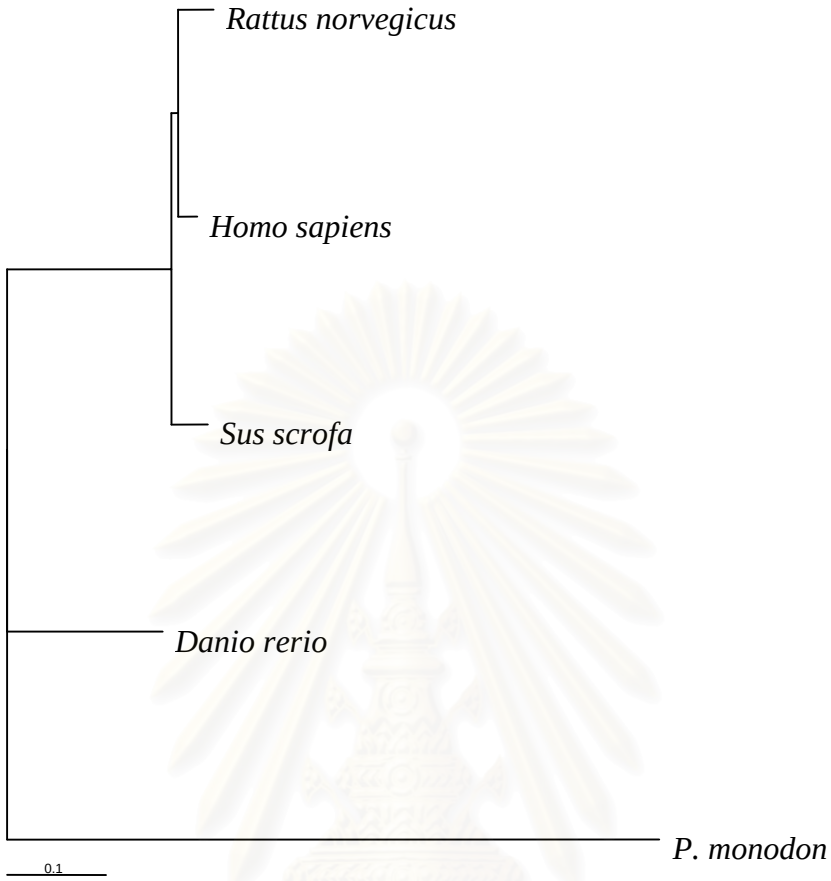

Figure 3.29 A neighbor-joining tree illustrating relations between ribophorin I of *P. monodon*, *Danio rerio*, *Rattus norvegicus*, *Sus scrofa* and *Homo sapiens.*

Nucleotide sequence of *RACK* is 1164 bp in length. The ORF of *RACK* was 957 bp. encoding a polypeptide of 319 amino acids. The poly A additional signal was found at eleven bases upstream from the poly A tail. Long 5′UTR of *RACK* (139 bp) was also identified and can be used to design primers for new SNP discovery (Figure 3.30).

```
GCTGTGTTGAAATAGTGGTTATACTGAGTGTATTACACTTGTGTAAGACGAATGATGGTT  60
AAGAGATACAGATAAATTCGATCGCGTTCGGCATTGTGTACAAAGCGGTTCCAGCCAGAT 120
AGATGACTAGAGAAACGTAATGAATGAGAGCTTACAGCTGCGCGGGACCCTGGTGGGCCA 180
                   M  N  E  S  L  Q  L  R  G  T  L  V  G  H
CAATGGCTGGGTCACACAGATCGCCACCAACAGGAATTTCCCTGACATGATCCTGTCTGC 240
 N  G  W  V  T  Q  I  A  T  N  R  N  F  P  D  M  I  L  S  A
TTCCAGGGACAAATCTCTGATTCTGTGGAAACTGACCCGTGAGGAGAACAACTATGGTGT 300
 S  R  D  K  S  L  I  L  W  K  L  T  R  E  E  N  N  Y  G  V
TCCCCAGAAACGTCTTCATGGCCACTCCCACTTCATCACTGATGTTGTTCTTTCTCTGGA 360
 P  Q  K  R  L  H  G  H  S  H  F  I  T  D  V  V  L  S  L  D
TGGTCACTTCGCCCTCTCTGGATCATGGGACAAGACCCTTCGTCTGTGGGATCTTGCAGC 420
 G  H  F  A  L  S  G  S  W  D  K  T  L  R  L  W  D  L  A  A
TGGCAAGACCACCCGTCGCTTTGAGGACCACACAAAGGATGTGCTCTCTGTTGCTTTCGT 480
 G  K  T  T  R  R  F  E  D  H  T  K  D  V  L  S  V  A  F  V
GGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAACCATCAAGCTGTGGAATAC 540
 A  D  N  R  Q  I  V  S  G  S  R  D  K  T  I  K  L  W  N  T
ACTTGCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGT 600
 L  A  Q  C  K  Y  T  I  Q  E  D  G  H  S  D  W  V  S  C  V
AAGGTTCTCTCCCTCCAACAGTAACCCCATCATTGTATCATGTGGATGGGACAAGGCTGT 660
 R  F  S  P  S  N  S  N  P  I  I  V  S  C  G  W  D  K  A  V
CAAGGTATGGAGCCTGACCAACTGCAAGCTCAAGACCAACCACTATGGACACACTGGTTA 720
 K  V  W  S  L  T  N  C  K  L  K  T  N  H  Y  G  H  T  G  Y
CTTGAATACAGTTACTGTCTCTCCTGATGGCTCCCTCTGTGCCTCTGGTGGCAAGGATGC 780
 L  N  T  V  T  V  S  P  D  G  S  L  C  A  S  G  G  K  D  A
TAAGGCCATGCTTTGGGATTTGAATGATGACAAGCATCTGTACACTTTGGACCACACTGA 840
 K  A  M  L  W  D  L  N  D  D  K  H  L  Y  T  L  D  H  T  D
TATCATCAACTCGCTGTGCTTCAGCCCCAACCGTTATTGGCTGTGTGCCGCTACTGGCCC 900
 I  I  N  S  L  C  F  S  P  N  R  Y  W  L  C  A  A  T  G  P
ATCTATCAAGATCTGGGATTTGGAAGGCAAGAATATGGTCGATGAATTGAAGCCCGATGT 960
 S  I  K  I  W  D  L  E  G  K  N  M  V  D  E  L  K  P  D  V
GATTACCCAGAACCCTAAGGCTGAACCCCCACAGTGTCTCTCCATGGCCTGGTCTGCAGA1020
 I  T  Q  N  P  K  A  E  P  P  Q  C  L  S  M  A  W  S  A  D
TGGGCAAACTCTGTTTGCTGGTTACAGTGACAGCAAGATCCGTGTCTGGCAGGTCAGCGT1080
 G  Q  T  L  F  A  G  Y  S  D  S  K  I  R  V  W  Q  V  S  V
TACTTCCCGTGCGTAAGGGAATGTTCTGGAGTGCTGCAATAAAGATTGCTTGATAAAAAA1140
 T  S  R  A  *
AAAAAAAAAAAAAAAAAAAAAAA                                    1164
```

Figure 3.30 The full length cDNA sequences of *RACK* of *P. monodon*. Start and stop codons are illustrated in boldface. The poly A additional signal is underlined.

The full length cDNA of *RACK* was searched against data in the GenBank using Blast X and the closest homologues was *RACK* of *Bombyx mori* (6e-161, ABD36343). The full length cDNA and amino acid of *RACK* of *B. mori*, *Plutella xylostella* (3e-159, BAD52259), *Heliothis virescens* (6e-156, AAK51552), *Oreochromis mossambicus* (3e-152, AAQ91574), *Paralichthys olivaceus* (7e-151, AAT35603), guanine nucleotide-binding protein of *Petromyzon marinus* (2e-154, AAM88904) and guanine nucleotide binding protein, beta polypeptide 2-like 1 of *Rattus norvegicus* (4e-153, NP_570090) were retrieved and multiple aligned with a homologue found in *P. monodon* (Figures 3.31 and 3.32).

Sequence divergence based on nucleotide and protein sequences were calculated. Large divergence was observed between *RACK* of *P. monodon* and the remaining species (Table 3.3). A neighbor-joining tree allocated *RACK* from *P. monodon* to be closely related with that of *B. mori*, *Plutella xylostella* and *Heliothis virescens* (Figure 3.33).

```
P. marinus (g)      ATGACGGAGCAGATGACCCTGCGCGGGACCCTCAAGGGCCACAACGGCTGGGTCACGCAA
R. norvegicus (g)   ATGACCGAGCAAATGACCCTTCGTGGGACCCTCAAGGGCCATAATGGATGGGTTACACAG
O. mossambicus (R)  ATGACCGAGCAGATGACAGTGAGGGGGACCCTGAAGGGGCACAGTGGATGGGTCACCCAG
P. olivaceus (R)    ATGACCGAGCAGATGACAGTGAGGGGGACCCTGAAGGGGCACAGCGGATGGGTCACCCAG
P. xylostella (R)   ATGACTGAAACTTTAAAGCTCAGGGGCACCTTGTGCGGCCACAATGGCTGGGTTACTCAG
H. virescens (R)    ATGACTGAAACTTTAAAGCTTAGAGGAACCCTCTGTGGCCACAATGGCTGGGTTACCCAA
B. mori (R)         ATGTCTGAAACATTAAAACTCCGAGGAACTCTTAGAGGCCACAATGGCTGGGTTACTCAA
P. monodon (R)      ATGAATGAGAGCTTACAGCTGCGCGGGACCCTGGTGGGCCACAATGGCTGGGTCACACAG
                    ***   **     *     *  * ** **  *    ** ** *  ** ***** ** **

P. marinus (g)      ATCGCCACAACCCCGCAGTTCCCCGACATGATCCTGTCCGCGTCGCGAGACAAGACGCTG
R. norvegicus (g)   ATCGCCACCACTCCGCAGTTCCCGGACATGATCCTGTCGGCGTCTCGAGACAAGACCATC
O. mossambicus (R)  ATCGCCACTACGCCCCAGTACCCCGATATGATCCTGTCGGCGTCCCGAGACAAGTCCATC
P. olivaceus (R)    ATCGCCACGACGCCCCAGTATCCCGACATGATTCTGTCGGCGTCCCGAGACAAGTCTATC
P. xylostella (R)   ATTGCCACCAATCCCAAGTACCCTGACATGATTTTGTCTTCTTCCCGAGACAAGACCCTC
H. virescens (R)    ATTGCGACCAACCCTAAATACCCTGACATGATTTTGTCTTCTTCTCGAGACAAAACCCTC
B. mori (R)         ATTGCAACTAATCCGAAATACCCGGACATGATCTTATCTTCCTCTCGAGACAAAACTCTC
P. monodon (R)      ATCGCCACCAACAGGAATTTCCCTGACATGATCCTGTCTGCTTCCAGGGACAAATCTCTG
                    ** ** ** *     * *  ** ** *****  * **  * **  * *****  *  *

P. marinus (g)      ATCATGTGGAAGCTGACCAGAGACGAGACCAACTACGGCATCCCACAGCGATCTCTGCAC
R. norvegicus (g)   ATCATGTGGAAGCTGACCAGGGATGAGACCAACTACGGCATACCACAACGTGCTCTTCGA
O. mossambicus (R)  ATCATGTGGAAACTGACCCGTGATGAAACCAACTACGGTATCCCCCAGCGCTCCCTGAAG
P. olivaceus (R)    ATCATGTGGAAGCTGACCCGTGATGAAACCAACTATGGCATCCCCCAGCGCTCCTTGAAG
P. xylostella (R)   ATCGTATGGAAACTGACCAGGGACGAGACCAACTACGGAATCCCCCAGAAGCGTCTGTAC
H. virescens (R)    ATCGTATGGAAGCTGACCAGAGACGAGACTAACTACGGTGTCCCGCAGAAGCGTCTGTAC
B. mori (R)         ATCGTGTGGAAGCTGACCAGAGACGAAAACAACTACGGTATACCTCAAAAGCGTTTATAC
P. monodon (R)      ATTCTGTGGAAACTGACCCGTGAGGAGAACAACTATGGTGTTCCCCAGAAACGTCTTCAT
                    **  * ***** ****** * ** ** *  ***** **  * ** **        *

P. marinus (g)      GGCCATGGGCATTTTGTGAGCGACGTGGTCATTTCCTCCGACGGCCAGTTCGCCCTCTCG
R. norvegicus (g)   GGTCACTCCCACTTTGTTAGCGATGTTGTCATCTCCTCTGATGGCCAGTTTGCCCTCTCA
O. mossambicus (R)  GGTCACTCTCACTTTGTGAGTGATGTTGTGATCTCCTCTGATGGACAGTTTGCCCTGTCC
P. olivaceus (R)    GGCCACTCTCACTTCGTGAGCGATGTTGTCATCTCCTCAGATGGACAGTTCGCCCTGTCC
P. xylostella (R)   GGCCACTCTCACTTTATCAGCGACGTGGTCCTGTCCAGCGACGGCAACTACGCTCTCTCT
H. virescens (R)    GGTCACTCTCACTTCATCTCGGACGTTGTGCTCTCCAGCGACGGCAACTACGCTCTGTCT
B. mori (R)         GGTCATTCGCACTTCATTTCTGATGTTGTGCTGTCTAGTGACGGTAATTACGCCCTTTCC
P. monodon (R)      GGCCACTCCCACTTCATCACTGATGTTGTTCTTTCTCTGGATGGTCACTTCGCCCTCTCT
                    ** **    ** **  *    ** ** **  * **    ** **  * *  ** ** **
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```
P. marinus (g)      GGCTCGTGGGATGGCACCCTTCGCCTCTGGGACCTCACCACGGGCACGACCACACGCCGT
R. norvegicus (g)   GGCTCCTGGGATGGAACCCTACGCCTCTGGGATCTCACAACGGGCACTACCACGAGACGA
O. mossambicus (R)  GGAGCCTGGGACGGGACCCTCCGCCTGTGGGATCTCACCACTGGTCTCACCACCCGCCGA
P. olivaceus (R)    GGCACCTGGGACAGTACCCTCCGCCTGTGGGATCTCACCACTGGCGTCACCACTCGCCAG
P. xylostella (R)   GGCTCGTGGGACAAGACCCTGCGTCTGTGGGACCTCGCCGCCGGCAAGACCACCAGGCGT
H. virescens (R)    GGCTCTTGGGACAAGACCCTGCGTCTGTGGGATCTTGCTGCCGGCAAGACCACCAGGCGT
B. mori (R)         GGTTCTTGGGACAAGACTTTGCGTTTGTGGGATCTCGCTGCAGGCAAGACTACCAGACGA
P. monodon (R)      GGATCATGGGACAAGACCCTTCGTCTGTGGGATCTTGCAGCTGGCAAGACCACCCGTCGC
                    **  * *****    **  * **  * ***** **  *  * **    ** **  * *

P. marinus (g)      CTCGTGGGCCACACCAAAGACGTGCTGAGCGTGGCGTTCTCCGCTGACAACCGTCAGATT
R. norvegicus (g)   TTTGTCGGCCACACCAAGGATGTGCTGAGCGTGGCTTTCTCCTCTGACAACCGGCAGATT
O. mossambicus (R)  TTCGTTGGACACACAAAGGATGTTTTGAGCGTGGCTTTCTCTGCTGATAACCGCCAGATT
P. olivaceus (R)    TTTGTTGGACACACAAAGGATGTTTTGAGTGTCGCCTTCTCTGCTGATAACCGCCAGATT
P. xylostella (R)   TTCGAAGACCATACCAAGGATGTCCTCTCCGTCGCCTTCTCTGTTGACAACCGTCAAATC
H. virescens (R)    TTCGAAGACCATACTAAGGATGTCCTCTCCGTGGCGTTCTCAGTTGACAACCGTCAGATT
B. mori (R)         TTCGAGGACCATACTAAGGATGTACTCTCGGTAGCCTTCTCAGTTGACAACCGTCAGATA
P. monodon (R)      TTTGAGGACCACACAAAGGATGTGCTCTCTGTTGCTTTCGTGGCTGATAACCGTCAGATT
                     * *  *  ** ** ** ** **  *    ** ** ***     *** ***** ** **

P. marinus (g)      GTGTCCGGCTCGCGTGACAAGACCATTAAGCTGTGGAACACCTTGGGTGTCTGCAAGTAC
R. norvegicus (g)   GTCTCTGGGTCCCGAGACAAGACCATTAAGTTATGGAATACTCTGGGTGTCTGCAAGTAC
O. mossambicus (R)  GTGTCTGGCTCCCGGGACAAGACCATCAAGCTGTGGAACACTCTTGGAGTCTGCAAGTAC
P. olivaceus (R)    GTGTCTGGATCCCGGGACAAGACCATCAAGCTATGGAACACTCTCGGAGTCTGCAAATAC
P. xylostella (R)   GTGTCTGGATCCCGAGACAAGACCATCAAGCTGTGGAACACCCTTGCCGAGTGCAAGTAC
H. virescens (R)    GTGTCTGGCTCTCGGGACAAGACCATCAAGCTGTGGAACACACTGGCTGAGTGCAAGTAC
B. mori (R)         GTGTCTGGTTCTCGCGACAAGACTATCAAACTCTGGAACACCCTTGCGGAGTGCAAGTAT
P. monodon (R)      GTGTCTGGCTCCCGTGACAAAACCATCAAGCTGTGGAATACACTTGCCCAGTGCAAATAC
                    ** ** ** ** ** ***** ** ** **  * ***** **  * *     ***** **

P. marinus (g)      ACCATCCAGGAGGATGGCCACACCGAGTGGGTGTCGTGTGTGCGCTTCTCTCCCAACAAT
R. norvegicus (g)   ACTGTCCAGGATGAGAGTCATTCAGAATGGGTGTCTTGTGTCCGCTTCTCCCCGAACAGC
O. mossambicus (R)  ACCATCCAGGATGAGGGCCACACTGAGTGGGTATCTTGTGTTCGCTTCTCCCCCAACAGC
P. olivaceus (R)    ACCATCCAGGATGAGGGCCACTCTGAGTGGGCGTCCTGTGTGCGCTTCTCCCCCAACAGC
P. xylostella (R)   ACCATCCAGGACGATGGCCACAGTGACTGGGTGTCCTGCGTGCGATTCTCACCGAACCAC
H. virescens (R)    ACCATCCAGGATGATGGCCACAGTGACTGGGTGTCCTGSGTCCGCTTCTCCCCAAACCAT
B. mori (R)         ACCATCCAAGATGATGGACACAGCGATTGGGTGTCATGTGTCAGATTCTCACCCAATCAT
P. monodon (R)      ACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGTAAGGTTCTCTCCCTCCAAC
                    **  **** ** **  * **    ** ****  ** ** **  * ***** **

P. marinus (g)      AACAATCCCATCATTGTCTCTTGTGGCTGGGACAAGCTGGTCAAGGTGTGGAATTTGACC
R. norvegicus (g)   AGCAACCCTATCATCGTCTCCTGCGGATGGGACAAGCTGGTCAAGGTGTGGAATCTGGCT
O. mossambicus (R)  AGCAACCCCATCATCGTCTCCTGTGGCTGGGACAAGATGGTTAAGGTGTGGAACCTGGCC
P. olivaceus (R)    AGCAACCCCATCATTGTCTCCTGTGGCTGGGACAAGCTGGTCAAGGTGTGGAACCTGGCC
P. xylostella (R)   GCCAACCCCATCATTGTGTCTGCTGGTTGGGACCGCACCGTGAAGGTCTGGCATCTGACC
H. virescens (R)    GCCAACCCCATCATTGTGTCTGCTGGTTGGGACCGCACTGTTAAGGTCTGGCACCTTACC
B. mori (R)         GCCAACCCCATTATTGTATCCTGTGGTTGGGACAGAACTGTCAAGGTCTGGCATCTCACT
P. monodon (R)      AGTAACCCCATCATTGTATCATGTGGATGGGACAAGGCTGTCAAGGTATGGAGCCTGACC
                      ** ** ** ** ** **    ** ******     ** ***** ***    *  *



P. marinus (g)      AACTGCAAGCTAAAGACCAACCACATTGGTCACACGGGCTACTTGAATACAGTGACCGTG
R. norvegicus (g)   AACTGCAAGCTAAAGACCAACCACATTGGCCACACTGGCTATCTGAACACAGTGACTGTC
O. mossambicus (R)  AACTGCAAGCTGAAGACCAACCACATTGGTCACACTGGCTACCTGAACACAGTGACCGTT
P. olivaceus (R)    AACTGCAAGTTGAAGACCAACCACATTGGCCACACTGGTTTCCTGAACACAGTGACTGTC
P. xylostella (R)   AACTGCAAGTTGAAGATCAACCACTTGGGCCACTCCGGCTACCTGAACACAGTGACTGTC
H. virescens (R)    AACTGCAAGCTCAAGATCAACCACCTTGGTCACTCTTGCTACCTGAACACAGTCACTGTT
B. mori (R)         AACTGTAAGCTCAAGATTAACCATTTGGGTCACTCTGGCTATCTGAACACTGTTACTGTA
P. monodon (R)      AACTGCAAGCTCAAGACCAACCACTATGGACACACTGGTTACTTGAATACAGTTACTGTC
                    ***** *** * ****  *****    ** *** *  * *   **** ** ** ** **

P. marinus (g)      TCTCCTGATGGTTCCCTGTGTGCATCTGGTGGGAAGGATGGGCAAGCCATGCTGTGGGAC
R. norvegicus (g)   TCTCCAGATGGATCCCTCTGTGCTTCTGGAGGCAAGGATGGCCAGGCTATGCTGTGGGAT
O. mossambicus (R)  TCTCCTGATGGCTCCCTGTGTGCATCTGGTGGAAAGGATGGCCAGGCCATGCTTTGGGAC
P. olivaceus (R)    TCTCCTGATGGCTCCCTGTGTGCATCTGGTGGAAAGGACGGCCAGGCCATGCTGTGGGAC
P. xylostella (R)   TCCCCCGATGGCTCGCTTTGCGCGTCCGGAGGCAAGGACATGAAGGCCATGCTCTGGGAC
H. virescens (R)    TTTTCTGACGGTTCTCTCTGCGCTTCCGGTGGCAAGGACATGAAGGCCATGCTCTGGGAC
B. mori (R)         TCACCTGATGGCTCTTTGTGTGCATCTGGTGGTAAGGATATGAAGGCGATGTTGTGGGAT
P. monodon (R)      TCTCCTGATGGCTCCCTCTGTGCCTCTGGTGGCAAGGATGCTAAGGCCATGCTTTGGGAT
                    *   * ** ** **  * ** ** ** ** ** *****     * ** *** * *****

P. marinus (g)      CTGAATGAGGGCAAGCATCTCTATACACTAGATGGAGGTGACACCATCAATGCGCTCTGC
R. norvegicus (g)   CTCAATGAAGGCAAGCACCTTTACACATTAGATGGTGGAGACATCATCAATGCCTTGTGC
O. mossambicus (R)  CTGAATGAAGGCAAGCACCTCTACACCCTGGACAGCGGTGATGTGATCAACGCCCTTTGC
P. olivaceus (R)    CTGAATGAGGGAAAGCACCTGTACACCCTGGATAGTGGTGACATGATCAGTGCCCTCTGC
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P. xylostella (R)  TTGAACGACGGCAAGCACCTGCACACGCTGGACCACAACGACATCATCACTGCTCTGTGC
H. virescens (R)   CTGAATGATGGCAAGCATCTGCACACCCTGGACCACAATGACATCATCACATCATTGTGC
Bmori              CTGAACGATGGCAAACACCTCCACACCTTAGACCACAATGATATCATCACGGCCTTGTGC
P. monodon (R)     TTGAATGATGACAAGCATCTGTACACTTTGGACCACACTGATATCATCAACTCGCTGTGC
                    * ** ** *  ** ** **  * **  * **       **    ****   *  * ***

P. marinus (g)     TTCTCACCAAACCGCTACTGGCTGTGTGCAGCCACGGGGCCCAGCATCAAGATCTGGGAT
R. norvegicus (g)  TTCAGCCCCAACCGCTACTGGCTCTGTGCTGCCACTGGCCCCAGTATCAAGATCTGGGAC
O. mossambicus (R) TTCAGCCCCAACCGTTACTGGCTCTGTGCCGCCACTGGACCCAGCATTAAGATCTGGGAT
P. olivaceus (R)   TTCAGCCCCAACCGTTACTGGCTGTGTGCCGCCACTGGCCCAAGCATCAAGATCTGGGAT
P. xylostella (R)  TTCTCGCCCAACAGATACTGGCTGTGCGCTGCCTTTGGACCCTCCATCAAGATCTGGGAT
H. virescens (R)   TTCTCACCCAACAGATACTGGCTGTGCGCTGCCTTCGGACCTTCCATCAAGATTTGGGAT
B. mori (R)        TTCTCACCCAACAGATACTGGCTATGTGCTGCTTTCGGACCTTCCATCAAGATCTGGGAT
P. monodon (R)     TTCAGCCCCAACCGTTATTGGCTGTGTGCCGCTACTGGCCCATCTATCAAGATCTGGGAT
                   ***   ** *** * ** ***** ** ** **    ** **    ** ***** *****

P. marinus (g)     CTTGAAGGCAAAGTGATTGTGGATGAGCTGAGGCAAGAAGTAATCAGCACAAGCAG----
R. norvegicus (g)  TTGGAGGGCAAGATCATGGTAGATGAACTGAAGCAAGAAGTTATCAGCACCAGCAG----
O. mossambicus (R) CTGGAGGGCAAGATCATTGTGGACGAGCTGAGACAGGAAGTGATCAGCACAAACAG----
P. olivaceus (R)   TTGGAGGGCAAGATCATTGTGGATGAGCTCAGACAGGAAGTGATCAGCACAAACAG----
P. xylostella (R)  CTGGAAAGCAAGGAGATGGTTGAGGAGCTCCGACCAGAGATCATCAACCAGACCCAGACC
H. virescens (R)   CTGGAAAGCAAGGAGATGGTTGAAGAGCTCAGGCCTGAGATCATCAACCAGACCCAGACT
B. mori (R)        CTGGAAAGCAAGGAGATGGTTGAAGAGCTCAGGCCTGAAATCATTAACCAAACGCAAACT
P. monodon (R)     TTGGAAGGCAAGAATATGGTCGATGAATTGAAGCCCGATGTGATTACCCAGAACCC----
                    * **  ****    ** ** ** **  *    *  **  * ** * *   *

P. marinus (g)     --CAAGGCTGAGCCTCCCCAGTGCATTTCCCTGGCCTGGTCTGCTGATGGACAGACTCTT
R. norvegicus (g)  --CAAGGCAGAGCCACCCCAGTGTACCTCTTTGGCTTGGTCTGCTGATGGCCAGACTCTG
O. mossambicus (R) --CAAGGCCGAACCCCCACAGTGTACTTCCCTGGCATGGTCTGCTGATGGACAGACCCTG
P. olivaceus (R)   --CAAGGCTGAACCCCCACAGTGTACTTCCCTGGCATGGTCTGCTGATGGACAGACCCTG
P. xylostella (R)  TCCAAGTCTGACCCTCCCCAGTGCTTGTCCCTTGCCTGGTCCACTGATGGACAGACCCTC
H. virescens (R)   TCCAAGTCAGACCCACCCCAGTGCCTGTCTCTGGCGTGGTCCACAGATGGCCAGACCCTC
B. mori (R)        TCCAAGACAGACCCTCCTCAGTGTCTCTCCCTAGCATGGTCTACTGATGGACAAACCTTA
P. monodon (R)     --TAAGGCTGAACCCCCACAGTGTCTCTCCATGGCCTGGTCTGCAGATGGGCAAACTCTG
                      *** * ** ** ** *****    **  * ** *****  * ***** ** **  *


P. marinus (g)     TTTGCTGGTTACACTGACAACTTCATCCGAGTTTGGCAGGTGACCATTGGCACTCGCTAA
R. norvegicus (g)  TTTGCTGGCTATACCGACAACTTGGTGCGTGTATGGCAGGTGACTATTGGTACCCGCTAA
O. mossambicus (R) TTTGCTGGCTACACTGACAACCTGATCAGAGTGTGGCAGGTCACCATTGGAACGCGATAA
P. olivaceus (R)   TTTGCTGGCTACACTGACAACCCGATCAGAGTGTGGCAAGTCACAGTTGGAACTCGATAA
P. xylostella (R)  TTTGCTGGTTACTCTGACAACATCATCCGAGTCTGGCAGGTGTCCGTCTCAGCCCGATAA
H. virescens (R)   TTCGCTGGCTACTCCGACAACATCATCAGAGTCTGGCAGGTGTCAGTCTCAGCACGATAA
B. mori (R)        TTTGCTGGTTACTCAGATAATACTATAAGAGTGTGGCAGGTTTCCATCTCAGCTCGATAA
P. monodon (R      TTTGCTGGTTACAGTGACAGCAAGATCCGTGTCTGGCAGGTCAGCGTTACTTCCCGTGCG
                   ** ***** **    ** *      *  * ** ***** **      *     * **

P. marinus (g)     ---
R. norvegicus (g)  ---
O. mossambicus (R) ---
P. olivaceus (R)   ---
P. xylostella (R)  ---
H. virescens (R)   ---
B. mori (R)        ---
P. monodon (R)     TAA
```

Figure 3.31 Multiple alignments of nucleotide sequences of *RACK* of *P. Monodon*, *Plutella xylostella*, *Bombyx mori*, *Heliothis virescens*, *Oreochromis mossambicus*, *Paralichthys olivaceus* and guanine nucleotide-binding protein of *Petromyzon marinus* and guanine nucleotide binding protein, beta polypeptide 2-like 1 of *Rattus norvegicus*.

```
O. mossambicus (R) MTEQMTVRGTLKGHSGWVTQIATTPQYPDMILSASRDKSIIMWKLTRDETNYGIPQRSLK
P. olivaceus (R)   MTEQMTVRGTLKGHSGWVTQIATTPQYPDMILSASRDKSIIMWKLTRDETNYGIPQRSLK
R. norvegicus (g)  MTEQMTLRGTLKGHNGWVTQIATTPQFPDMILSASRDKTIIMWKLTRDETNYGIPQRALR
P. marinus (g)     MTEQMTLRGTLKGHNGWVTQIATTPQFPDMILSASRDKTLIMWKLTRDETNYGIPQRSLH
P. xylostella (R)  MTETLKLRGTLCGHNGWVTQIATNPKYPDMILSSSRDKTLIVWKLTRDETNYGIPQKRLY
H. virescens (R)   MTETLKLRGTLCGHNGWVTQIATNPKYPDMILSSSRDKTLIVWKLTRDETNYGVPQKRLY
B. mori (R)        MSETLKLRGTLRGHNGWVTQIATNPKYPDMILSSSRDKTLIVWKLTRDENNYGIPQKRLY
P. monodon (R)     MNESLQLRGTLVGHNGWVTQIATNRNFPDMILSASRDKSLILWKLTREENNYGVPQKRLH
                   *.* : :**** **.********. ::******:****::*:*****:*.***:**: *
O. mossambicus (R) GHSHFVSDVVISSDGQFALSGAWDGTLRLWDLTTGLTTRRFVGHTKDVLSVAFSADNRQI
P. olivaceus (R)   GHSHFVSDVVISSDGQFALSGTWDSTLRLWDLTTGVTTRQFVGHTKDVLSVAFSADNRQI
R. norvegicus (g)  GHSHFVSDVVISSDGQFALSGSWDGTLRLWDLTTGTTTRRFVGHTKDVLSVAFSSDNRQI
P. marinus (g)     GHGHFVSDVVISSDGQFALSGSWDGTLRLWDLTTGTTTRRLVGHTKDVLSVAFSADNRQI
P. xylostella (R)  GHSHFISDVVLSSDGNYALSGSWDKTLRLWDLAAGKTTRRFEDHTKDVLSVAFSVDNRQI
H. virescens (R)   GHSHFISDVVLSSDGNYALSGSWDKTLRLWDLAAGKTTRRFEDHTKDVLSVAFSVDNRQI
B. mori (R)        GHSHFISDVVLSSDGNYALSGSWDKTLRLWDLAAGKTTRRFEDHTKDVLSVAFSVDNRQI
P. monodon (R)     GHSHFITDVVLSLDGHFALSGSWDKTLRLWDLAAGKTTRRFEDHTKDVLSVAFVADNRQI
                   **.**::***:* **::****:** *******::* ***:: .********** *****
O. mossambicus (R) VSGSRDKTIKLWNTLGVCKYTIQDEGHTEWVSCVRFSPNSSNPIIVSCGWDKMVKVWNLA
P. olivaceus (R)   VSGSRDKTIKLWNTLGVCKYTIQDEGHSEWASCVRFSPNSSNPIIVSCGWDKLVKVWNLA
R. norvegicus (g)  VSGSRDKTIKLWNTLGVCKYTVQDESHSEWVSCVRFSPNSSNPIIVSCGWDKLVKVWNLA
P. marinus (g)     VSGSRDKTIKLWNTLGVCKYTIQEDGHTEWVSCVRFSPNNNNPIIVSCGWDKLVKVWNLT
P. xylostella (R)  VSGSRDKTIKLWNTLAECKYTIQDDGHSDWVSCVRFSPNHANPIIVSAGWDRTVKVWHLT
H. virescens (R)   VSGSRDKTIKLWNTLAECKYTIQDDGHSDWVS-VRFSPNHANPIIVSAGWDRTVKVWHLT
B. mori (R)        VSGSRDKTIKLWNTLAECKYTIQDDGHSDWVSCVRFSPNHANPIIVSCGWDRTVKVWHLT
P. monodon (R)     VSGSRDKTIKLWNTLAQCKYTIQEDGHSDWVSCVRFSPSNSNPIIVSCGWDKAVKVWSLT
                   ***************. ****:*::.*::*.* *****.  ******.***: **** *:
O. mossambicus (R) NCKLKTNHIGHTGYLNTVTVSPDGSLCASGGKDGQAMLWDLNEGKHLYTLDSGDVINALC
P. olivaceus (R)   NCKLKTNHIGHTGFLNTVTVSPDGSLCASGGKDGQAMLWDLNEGKHLYTLDSGDMISALC
R. norvegicus (g)  NCKLKTNHIGHTGYLNTVTVSPDGSLCASGGKDGQAMLWDLNEGKHLYTLDGGDIINALC
P. marinus (g)     NCKLKTNHIGHTGYLNTVTVSPDGSLCASGGKDGQAMLWDLNEGKHLYTLDGGDTINALC
P. xylostella (R)  NCKLKINHLGHSGYLNTVTVSPDGSLCASGGKDMKAMLWDLNDGKHLHTLDHNDIITALC
H. virescens (R)   NCKLKINHLGHSCYLNTVTVFSDGSLCASGGKDMKAMLWDLNDGKHLHTLDHNDIITSLC
B. mori (R)        NCKLKINHLGHSGYLNTVTVSPDGSLCASGGKDMKAMLWDLNDGKHLHTLDHNDIITALC
P. monodon (R)     NCKLKTNHYGHTGYLNTVTVSPDGSLCASGGKDAKAMLWDLNDDKHLYTLDHTDIINSLC
                   ***** ** **: :****** .********** :*******:.***:***  * *.:**
O. mossambicus (R) FSPNRYWLCAATGPSIKIWDLEGKIIVDELRQEVI--STNSKAEPPQCTSLAWSADGQTL
P. olivaceus (R)   FSPNRYWLCAATGPSIKIWDLEGKIIVDELRQEVI--STNSKAEPPQCTSLAWSADGQTL
R. norvegicus (g)  FSPNRYWLCAATGPSIKIWDLEGKIMVDELKQEVI--STSSKAEPPQCTSLAWSADGQTL
P. marinus (g)     FSPNRYWLCAATGPSIKIWDLEGKVIVDELRQEVI--STSSKAEPPQCISLAWSADGQTL
P. xylostella (R)  FSPNRYWLCAAFGPSIKIWDLESKEMVEELRPEIINQTQTSKSDPPQCLSLAWSTDGQTL
H. virescens (R)   FSPNRYWLCAAFGPSIKIWDLESKEMVEELRPEIINQTQTSKSDPPQCLSLAWSTDGQTL
B. mori (R)        FSPNRYWLCAAFGPSIKIWDLESKEMVEELRPEIINQTQTSKTDPPQCLSLAWSTDGQTL
P. monodon (R)     FSPNRYWLCAATGPSIKIWDLEGKNMVDELKPDVI--TQNPKAEPPQCLSMAWSADGQTL
                   ********** **********.* :*:**: ::*  : ..*::**** *:***:*****
O. mossambicus (R) FAGYTDNLIRVWQVTIGTR-
P. olivaceus (R)   FAGYTDNPIRVWQVTVGTR-
R. norvegicus (g)  FAGYTDNLVRVWQVTIGTR-
P. marinus (g)     FAGYTDNFIRVWQVTIGTR-
P. xylostella (R)  FAGYSDNIIRVWQVSVSAR-
H. virescens (R)   FAGYSDNIIRVWQVSVSAR-
B. mori (R)        FAGYSDNTIRVWQVSISAR-
P. monodon (R)     FAGYSDSKIRVWQVSVTSRA
                   ****:*. :*****:: :*
```

Figure 3.32 Multiple alignments of amino acid sequences of *RACK* of *P. Monodon*, *Plutella xylostella*, *Bombyx mori*, *Heliothis virescens*, *Oreochromis mossambicus*, *Paralichthys olivaceus* and guanine nucleotide-binding protein of *Petromyzon marinus* and guanine nucleotide binding protein, beta polypeptide 2-like 1 of *Rattus norvegicus*.

Table 3.3 Sequence divergence of *RACK* and related gene homologue from different taxa based on nucleotide (below diagonal) and amino acids sequences (above diagonal)

	***P. marinus* (g)**	***R. norvegicus* (g)**	***O. mossambicus* (R)**	***P. olivaceus* (R)**	***P. xylostella* (R)**	***H. virescens* (R)**	***B. mori* (R)**	***P. monodon* (R)**
***P. marinus* (g)**	-	0.06948	0.07296	0.09419	0.24883	0.27211	0.24883	0.23578
***R. norvegicus* (g)**	0.2026	-	0.06257	0.07957	0.25323	0.27666	0.25323	0.24883
***O. mossambicus* (R)**	0.2033	0.1970	-	0.03889	0.26211	0.28583	0.26211	0.25766
***P. olivaceus* (R)**	0.2089	0.2068	0.0795	-	0.26658	0.29045	0.27561	0.26658
***P. xylostella* (R)**	0.3155	0.3725	0.3354	0.3340	-	0.01590	0.02229	0.19787
***H. virescens* (R)**	0.3402	0.3821	0.3472	0.3694	0.1216	-	0.03876	0.20272
***B. mori* (R)**	0.3782	0.4016	0.3732	0.4030	0.2157	0.1895	-	0.19377
***P. monodon* (R)**	0.3383	0.3375	0.3121	0.3119	0.3083	0.3099	0.3389	-

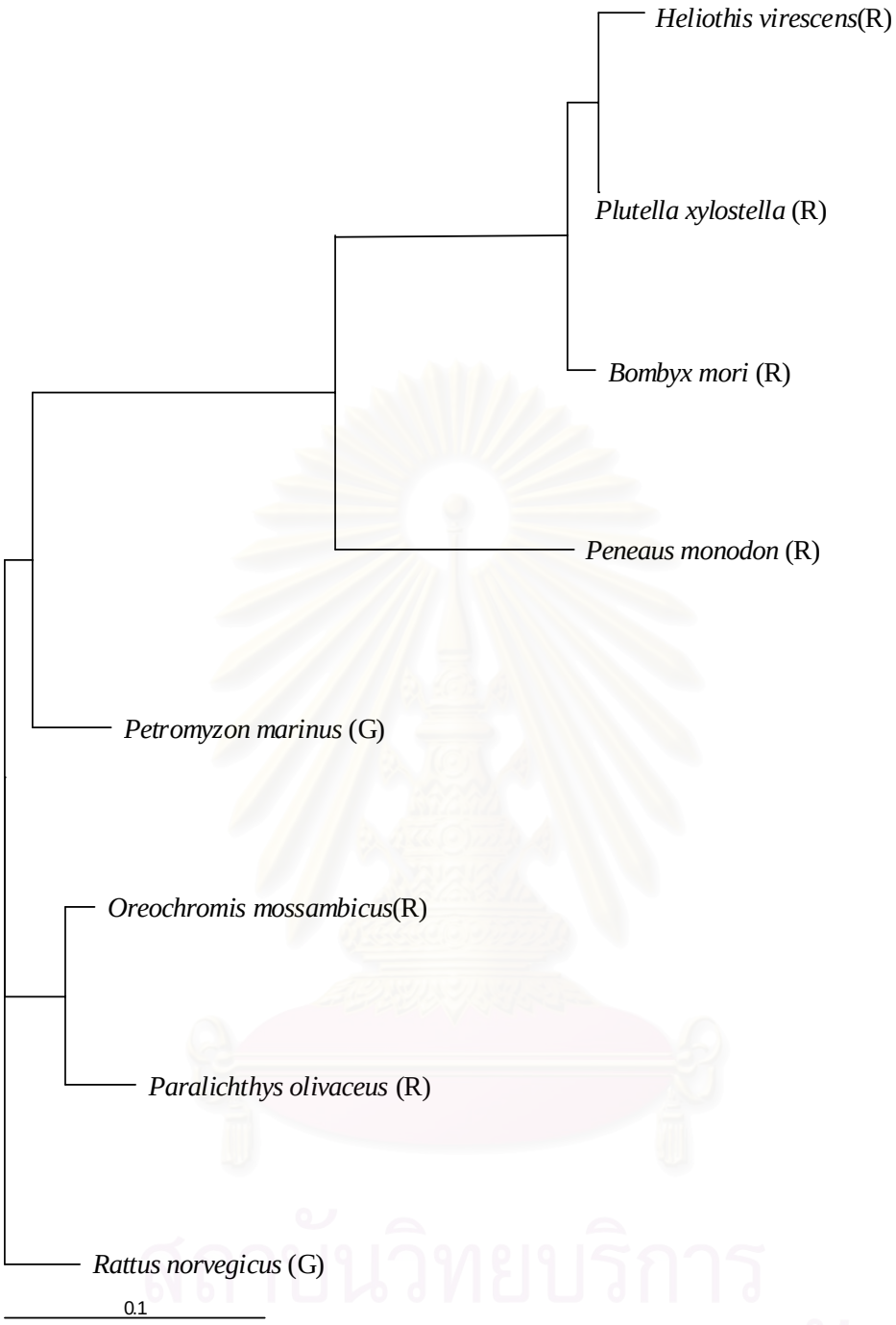

Figure 3.33 A neighbor-joining tree illustrating relations between *RACK* of *P. Monodon*, *Plutella xylostella*, *Bombyx mori*, *Heliothis virescens*, *Oreochromis mossambicus*, *Paralichthys olivaceus* and guanine nucleotide-binding protein of *Petromyzon marinus* and guanine nucleotide binding protein, beta polypeptide 2-like 1 of *Rattus norvegicus*, respectively.

3.3.2 Isolation and Characterization of Thioredoxin peroxidase, Calponin 1 and Defender against cell death 1 *(DAD1)* genes using Genome walking analysis

The full length cDNA of homologues of thioredoxin peroxidase, calponin 1 and *DAD1* were already isolated by EST analysis (Figure 3.34). Thus Genome walking analysis was applied to isolate the entire gene sequences of these functionally important transcripts. Generally, sequences of 5′ and 3′ genome walking fragments were combined with those of the PCR product generated by primers designed to flank the intron of each gene.

A.

GTCGTTGAGACAAGAGAATACCAAGACCACCACC**ATG**AGCAACACTGTTCCAGCTAT
TGGCAAACCTGCCCCTGTCTTCAAGGGCACTGCTGTTGTTGATGGGCAGTTCAAGGA
GATCTCCCTGGAAGATTACAAGGGCAAATATGTCATTTTCTTCTTCTACCCTTTGGA
TTTCACCTTTGTCTGCCCCACTGAAATTATCGCCTTCTCTGACCGTGTTGAAGAATT
CAGGAAAATTGGATGCGAAGTGGTTGCTTGCTCTACAGACTCCCACTTCTCCCACCT
TGCTTGGATCAACACTCCCCGCAAGGAAGGTGGTCTTGGTACGATGAAGATCCCTCT
TCTGGCTGACAAGTCAATGGAAGTTGCAAAGGCTTACGGAGTCCTTAAGGAGGATGA
AGGCATTGCTTTCAGAGGCCTCTTTGTTATTGATGGCAAGCAGAATCTCCGTCAGGT
AACAATCAATGACCTGCCAGTTGGGCGTGATGTAGATGAAACGCTGCGATTAGTACA
AGCCTTCCAGTTCACAGATGAGCATGGTGAAGTATGCCCTGCTGGCTGGAAACCCGG
AGCCAAGACAATGAAGGCCGATCCTACTGGCAGCAAAGAATACTTCCAGAATGAAAA
T**TAA**TATAACATCATCATTTAAAAAAAAAAAAAAAAA

B.

CCTGGGTGTACGCACGCTTTTCATCCATTCTTATCCTCCCCCTTGAACAAACCACGC
CAAA**ATG**AACCGTGCTACCAAGTCCGGAATCGCTGCCGAGGCTCAGGCTAAGGTCAA
CGCAAAGTACAGCGAAGAGCAGGCCGCCGAGTGCTTGGAATGGATCGCCATCATCAC
GAGCGCCGACATCAGCAAGTCTGGAGACGCCGACAATTTCTACGAGACCTTGAAGAA
TGGACAGCTGTTGTGCCAGGTGATTAACGCCCTCAAGCCCGGTCAGATCAAGAAGAT
CCAGACCTCCGCCATGGCATTCAAGTGCATGGAAAACATCAACGCCTTTGTGGAGGG
AGCTAAGGCCTGTGGGGTGCCCACTCAGGAGACCTTCCAGACCGTCGACCTCTGGGA
ACGACAGAACCTTAACTCTGTTGTTATCTGCTTGCAGTCTCTGGGCAGGAAGGGATC
TCAATTTGGAAAGCCTTCCATTGGCCCAAAAGAGTCTGAGAAGAATGTCCGTCACTT
CACCGAGGAGCAGCTCAGGGCTTCTGAGGGCATCGTCAACCTGCAGTATGGCTCCAA
CAAGGGTGCCACTCAGTCTGGCATGTCCTT

C.

GAGAAGACCAACGTTCTAAAGGCGATCGGTTTTTGCACGAAATATTTAGTGTAGATA
TTATAAAAGTAACCAACC**ATG**TCTTCAACATCACTATTGGTGGTTGTCAGAAATTTC
TATGATGAGTACATGAAGAAAACTCCCAAGAAATTAAAGATCGTCGATGCCTACCTC
TTCTACGTTCTGTTGACTGGGATCATCCAGTTTGTGTTTTGCTGTCTAGTTGGAACT
TTCCCCTTCAACTCCTTCCTGTCTGGGTTCATCAGTACAGTTGGCTGCTTTGTGTTA
GGGGTGAGCTTGAGACTGCAGGCAAACCCTCAGAACAAAATGCAGTTTGTTGGCATC
TCCCCAGAACGAGGCTTTGCTGATTTCATCTTTGCGCACATCATCCTTCACCTTGTC
ACTGTTAACTTCATTGGT**TAA**CAAAGGGACGATCAAGTATTTTGTTGGTTTTCCTTA
AGGATGTGAAATGTTCTAAGATCATGTGTAAAGGATATAAACATTCACTAAAGAACC
CATGTTATTTTCAAAGAAGCAGATTAATTTACATTCCCATGTGCGTGTAAGGTTTAA
CGATTGGAGTTTGTTCATGAAGTC

Figure 3.34 The full length cDNA of thioredoxin peroxidase (A), calponin I (B) and *DAD1* (C) homologues previously isolated and characterized by EST analysis.

3.3.2.1 The full length genomic DNA of thioredoxin peroxidase

The full length genomic DNA of thioredoxin peroxidase was generated by combining nucleotide sequences of 5′ and 3′ genome walking fragment, which both of them were generated from genome walking analysis, 2 fragment of PCR product (Figures 3.35 – 3.39). These fragments were cloned and sequenced. Nucleotide sequences were combined and edited.

The complete genomic sequence of thioredoxin peroxidase contained 3 exons (258, 162 and 171 bp) and 2 introns (89 and 111 bp). The ORF of thioredoxin peroxidase is 591 bp encoding a polypeptide of 197 amino acids (Figure 3.40).

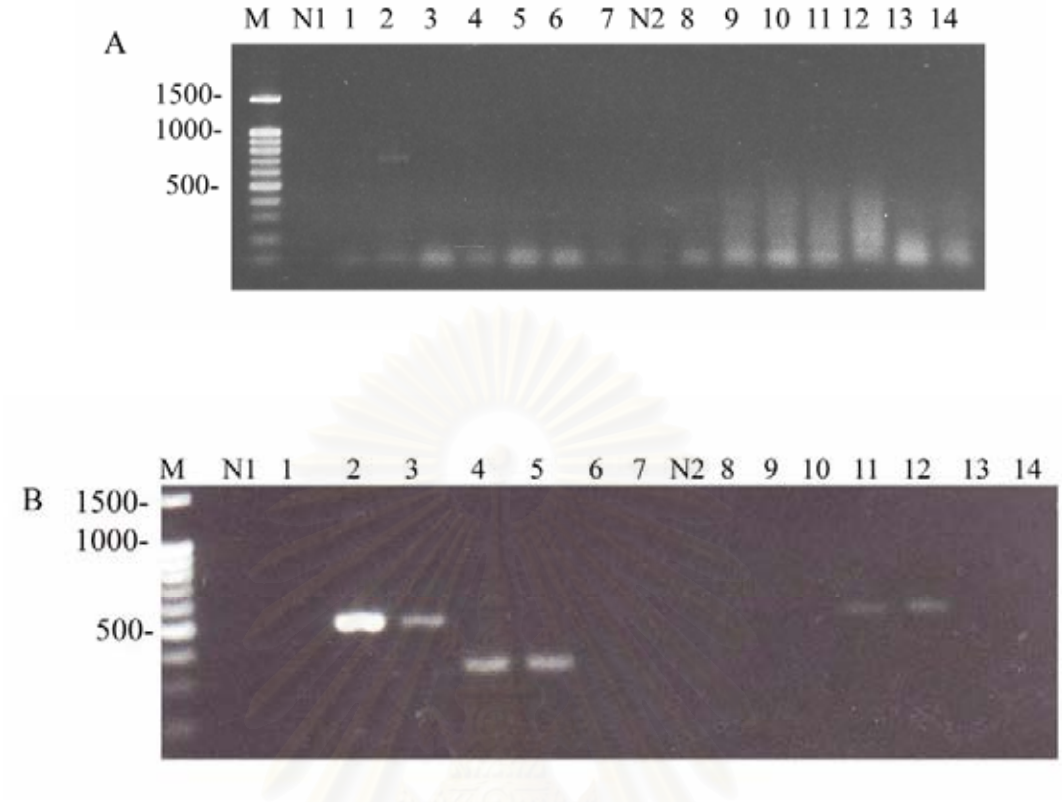

Figure 3.35 A. Primary PCR product of thioredoxin peroxidase. The *Alu* I mini-library of male (lane 1), *Dra* I mini-library of female and male (lanes 2 and 3), *Hae* III mini-library of female and male (lanes 4 and 5) and *Rsa* I mini-library of female and male (lanes 6 and 7) were amplified with the forward gene-specific primers and the adapter primer (AP1). The same template was amplified by the adapter primer (AP1) and the reverse gene-specific primer (lanes 8 – 14). **B.** Secondary PCR product of thioredoxin peroxidase. The primary products illustrated in lanes 1-7 (A) was diluted and amplified with a nested forward gene-specific primer and a nested adapter primer (AP2) (lanes 1 – 7, B. Likewise the primary products in lanes 8 – 14 (A) was amplified with a AP2 primer and nested reverse gene specific primer. Lanes M, N1 and N2 are a 100 bp DNA marker and the negative control (without DNA template), respectively.

TAGATGAACCGGTGTGATTAGTGCACGCTTTCCAGTTCCCAGAGGAGCATGCTGAAG
GTGAGGTTTCGTATGGCATTGTCAGGGAAAGTTCACGATTACATCAAGTCGAATCTG
AACTTTTTATTTTATGGCGATAATTTCTGTTCGAGGAAAGAGTAGTCAGTTTTCAGT
GAAAATAAGGCAATACAATTTGTCAGCAGTTGTATGGGTTAGCTAAGAGGTGATTTA
TATTTTGAATAAATGTATTCCCTTTCCCACATTTGGGCAGTGGGACGGCATGGCTGG
AAACCAGGAGTCACGTCAAAGAAGGCCGATCTAAGTGGCAGCAAAGAATACTTCCAG
AATGAAAATGAATATAGCATCCTCATTCCTCGGTTAGTATTTTTCTCGTATATATAA
AATAGTTAGGATTTGTCTTGTTCGAAAGAGTGGGCATTCAGGATAAGGCACTGGGTT
CATTTTGCTAACGATATGTCCATTTGTTAACCTGAAAGGGCGGTCG**ACCAGGAGTGC**
CTTATAGT

Figure 3.36 Nucleotide sequence of 3′UTR of thioredoxin peroxidase was generated by Genome Walking analysis using nested reverse primer and AP2, position of both sequences primers are illustrated in boldface and underlined.

ACTATAGGGCACGCGTGGTCGACGGCCCGGGCAGGTAAAGTCTTTGCTTGAAGATAT
TGAGAATCTGAAACTTTCAAATAAGACTGAAATTATTAGGGTATGTAGATAGGTAAA
TTAATAAAATGACTGGAATGTACTCTTAAGGTGGTAAGCTTTTTAACAATTTTAAGA
TTTTGCAACAAGTGGTTAATGAAAATCTGAAGGTTAATCCACATGAAGCATGTGTGC
ACATGTTAAGCATGAGTTGGAATTTCTGGAGACTATTATGTAATGTTTATGCCTAAC
CTGTTCCAATATTTTATTTCTAATCCCCCCCCCACCCCCACAGAATACCAAGACCAC
CACCATGAGCAACACTGTTCCAGCTATTGGCAAACCTGCCCCTGTCTTCAAGGGCAC
TGCTGTTGTTGATGGGCAGTTCAAGGAGATCTCCCTGGAAGATTACAAGGGCAAATA
TGTCATTTTCTTCTTCTACCCTTT**GGATTTCACCTTTGTCTGCC**

Figure 3.37 Nucleotide sequence of 5′UTR of thioredoxin peroxidase was generated by Genome Walking analysis using nested forward primer and AP2. Position of sequencing primers are illustrated in boldface and underlined.

TTGGATTTCACCTTTGTCTGCCCCACTGAAATTATCGCCTTCTCTGACCGTGTTGAA
GAATTCAGGAAAATTGGATGCGAAGTGGTTGCTTGCTCTACAGACTCCCACTTCTCC
CACCTTGCTTGGTAGGTATTAATGCTTTGTCCTAATTATTAAAAGTTTTTGAGAGAT
ATTAATTTTGATTTTTATTAATCCCCCATCCTCCACCTCACAGGATCAACACTCCCC
GCAAGGAAGGTGGTCTTGGTACGATGAAGATCCCTCTTCTGGCTGACAAGTCAATGG
AAGTTGCAAAGGCTTACGGAGTCCTTAAGGAGGATGAAGGCATTGCTTTCAGAGGCC
TCTTTGTTATTGATGGCAAGCAGAATCTCCGTCAGGTATGTCAAACTAGTGCTACAA
ATGAATGTTGTAATTTGGAAGGCGGTTTAGGCAAGCAATTTGAATTTTTTTCCCTTT
TTTTTTACGTTTCTTTTTTTTTTTTTTTTCAGGT**AACAATCAATGACCTGCCAG**

Figure 3.38 Nucleotide sequence of thioredoxin peroxidase generated by typical PCR. Gene specific primers are illustrated in boldface and underlined.

AAGCAGAATCTCCGTCAGGTATGTCAAACTAGTGCTACAAATGAATGTTGTAATTTG
GAAGGCCGTTTAGGCAAGCAATTTGAATTTTTTTCCCTTTTTTTGTACGTTTCTTTT
TTTTTTTTTTTTTTTACAGGTAACAATCAATGACCTGCCAGTTGGGCGTGATG**TAGA**
TGAAACGCTGCGATTAGT

Figure 3.39 Nucleotide sequence of thioredoxin peroxidase generated by PCR. Gene specific primers were illustrated in boldface and underlined.

```
AAAGTCTTTGCTTGAAGATATTGAGAATCTGAAACTTTCAAATAAGACTGAAATTATTAG  60
GGTATGTAGATAGGTAAATTAATAAAATGACTGGAATGTACTCTTAAGGTGGTAAGCTTT 120
TTAACAATTTTAAGATTTTGCAACAAGTGGTTAATGAAAATCTGAAGGTTAATCCACATG 180
AAGCATGTGTGCACATGTTAAGCATGAGTTGGAATTTCTGGAGACTATTATGTAATGTTT 240
ATGCCTAACCTGTTCCAATATTTTATTTCTAATCCCCCCCCCACCCCCACAGAATACCAA 300
GACCACCACC ATG AGCAACACTGTTCCAGCTATTGGCAAACCTGCCCCTGTCTTCAAGGG 360
          M  S  N  T  V  P  A  I  G  K  P  A  P  V  F  K  G
CACTGCTGTTGTTGATGGGCAGTTCAAGGAGATCTCCCTGGAAGATTACAAGGGCAAATA 420
 T  A  V  V  D  G  Q  F  K  E  I  S  L  E  D  Y  K  G  K  Y
TGTCATTTTCTTCTTCTACCCTTTGGATTTCACCTTTGTCTGCCCCACTGAAATTATCGC 480
 V  I  F  F  F  Y  P  L  D  F  T  F  V  C  P  T  E  I  I  A
CTTCTCTGACCGTGTTGAAGAATTCAGGAAAATTGGATGCGAAGTGGTTGCTTGCTCTAC 540
 F  S  D  R  V  E  E  F  R  K  I  G  C  E  V  V  A  C  S  T
AGACTCCCACTTCTCCCACCTTGCTTGG TAGGTATTAATGCTTTGTCCTAATTATTAAAA 600
 D  S  H  F  S  H  L  A  W
GTTTTTGAGAGATATTAATTTTGATTTTTATTAATCCCCCATCCTCCACCTCACAGG ATC 660
                                                           I
AACACTCCCCGCAAGGAAGGTGGTCTTGGTACGATGAAGATCCCTCTTCTGGCTGACAAG 720
 N  T  P  R  K  E  G  G  L  G  T  M  K  I  P  L  L  A  D  K
TCAATGGAAGTTGCAAAGGCTTACGGAGTCCTTAAGGAGGATGAAGGCATTGCTTTCAGA 780
 S  M  E  V  A  K  A  Y  G  V  L  K  E  D  E  G  I  A  F  R
GGCCTCTTTGTTATTGATGGCAAGCAGAATCTCCGTCAG GTATGTCAAACTAGTGCTACA 840
 G  L  F  V  I  D  G  K  Q  N  L  R  Q
AATGAATGTTGTAATTTGGAAGGCGGTTTAGGCAAGCAATTTGAATTTTTTTCCCTTTTT 900
TTTACGTTTCTTTTTTTTTTTTTTTCAG GTAACAATCAATGACCTGCCAGTTGGGCGT 960
                              V  T  I  N  D  L  P  V  G  R
GATGTAGATGAACCGGTGCGATTAGTGCACGCTTTCCAGTTCCCAGAGGAGCATGCTGAA1020
 D  V  D  E  P  V  R  L  V  H  A  F  Q  F  P  E  E  H  A  E
GGTGAGGTTTCGTATGGCATTGTCAGGGAAAGTTCACGATTACATCAAGTCGAATCTGAA1080
 G  E  V  S  Y  G  I  V  R  E  S  S  R  L  H  Q  V  E  S  E
CTTTTTATTTTATGGCGA TAA TTTCTGTTCGAGGAAAGAGTAGTCAGTTTTCAGTGAAAA1140
 L  F  I  L  W  R  *
TAAGGCAATACAATTTGTCAGCAGTTGTATGGGTTAGCTAAGAGGTGATTTATATTTTGA1200
ATAAATGTATTCCCTTTCCCACATTTGGGCAGTGGGACGGCATGGCTGGAAACCAGGAGT1260
CACGTCAAAGAAGGCCGATCTAAGTGGCAGCAAAGAATACTTCCAGAATGAAAATGAATA1320
TAGCATCCTCATTCCAAAAAAAAAAAAAAAAA                            1352
```

Figure 3.40 Coding nucleotides and deduced amino acids of each exon of *thioredoxin peroxidase* are capitalized. Introns are shown in capital and italicized. The start and stop codons are in boldface. The poly A additional signal site are underlined.

The full length cDNA of thioredoxin peroxidase was searched against data in the GenBank using Blast X and the closest homologues was 2cys thioredoxin peroxidase of *Aedes aegypti* at (9e-72, AAL37254). The full length cDNA and amino acid of 2cys thioredoxin peroxidase of *Aedes aegypti*, natural killer enhancing factor B of *Tetraodon nigroviridis* (8e-68, ABC59169), peroxiredoxin 2, isoform a of *Homo sapiens* (7e-67, AAH00452), peroxiredoxin 2 of *Bos Taurus* (3e-66, NP_777188) and thioredoxin peroxidase of *Branchiostoma belcheri tsingtaunese* (4e-66, AY737279) and *Myotis lucifugus* (1e-65, AAT79401) were retrieved and multiple aligned with a homologue found in *P. monodon* (Figures 3.41 and 3.42).

Sequence divergence based on nucleotide and protein sequences were calculated. Large divergence was observed between thioredoxin peroxidase of *P. monodon* and that of the remaining species (Table 3.4). A neighbor-joining tree allocated thioredoxin peroxidase of *P. monodon* to be separated from a homologue of other species (Figure 3.43).

```
H. sapiens (P)     ATGGCCTCCG---GTAACGCGCGCATCGGAAAGCCAGCCCCTGACTTCAAGGCCACAGCG
B. taurus (P)      ATGGCCTGCGTCTGCAAGGCGCACGTCGGAAAGCCCGCCCCGGAATTCCAGGCCACCGCG
T. nigroviridis(K) ATGTCTTCTG---GAAGCGCTAAGATCGGCCAGCCCGCCCCCGACTTCACCGCCAAAGCC
M. lucifugus (T)   ATGTCTTCAG---GAAATGCTAAAATCGGCCACCCTGCCCCCAACTTCAAAGCCACAGCT
B. belcheri  (T)   ATGTCTGCTG---GAAATGCCAAGCTCCAACACCCCGCTCCAAACTTCGAGAGCACGGCT
A. aegypti(2cys-T) ATGCCTGTCC---------CAGATCTGCAGAAGCCCGCCCCGAAATTCTCGGGAACCGCC
P. monodon (T)     ATGAGCAACAC---TGTTCCAGCTATTGGCAAACCTGCCCCTGTCTTCAAGGGCACTGCT
                   ***              *     *     * ** ** **    ***      *  **

H. sapiens (P)     GTGGTT---GATGGCGCCTTCAAAGAGGTGAAGCTGTCGGACTACAAAGGGAAGTACGTG
B. taurus (P)      GTGGTG---GATGGTGCCTTCAAGGAGGTGAAACTTTCCGACTACAAAGGGAAGTACGTG
T. nigroviridis(K) GTCGTG---GACGGACAGTTCAAGGACCTCAGACTCTCAGACTATAGAGGAAAGTATGTC
M. lucifugus (T)   GTTATGCCTGATGGTCAGTTCAAAGATATCAGCCTATCTGACTACAAAGGAAAATATGTG
B. belcheri  (T)   GTACTACCCTCTGGGGAGTTCAAGACCATAAAACTCTCGGACTATAAAGGAAAGTACTTG
A. aegypti(2cys-T) GTTGTG---AACGGTGCATTCAAAGAAATCAAACTGGAGGACTACGCCGGCAAGTACCTG
P. monodon (T)     GTTGTT---GATGGGCAGTTCAAGGAGATCTCCCTGGAAGATTACAAGGGCAAATATGTC
                   **  *       **    *****     *    **    ** **    ** ** **  *

H. sapiens (P)     GTCCTCTTTTTCTACCCTCTGGACTTCACTTTTGTGTGCCCCACCGAGATCATCGCGTTC
B. taurus (P)      GTCCTCTTTTTCTACCCGCTGGACTTTACCTTTGTGTGCCCCACGGAGATCGTAGCTTTC
T. nigroviridis(K) GTCTTCTTCTTCTACCCGCTGGACTTCACGTTTGTGTGTCCCACCGAGATCGTGGCCTTC
M. lucifugus (T)   GTGTTCTTCTTTTACCCTCTTGACTTTACCTTTGTGTGCCCCACGGAGATCATTGCTTTC
B. belcheri  (T)   GTCATCTTCTTCTACCCTCTGGATTTCACATTTGTGTGCCCGACAGAAATCATCGCCTTC
A. aegypti(2cys-T) GTGCTGTTCTTCTACCCCCTTGACTTCACCTTCGTCTGCCCGACCGAAATCATTGCCTTC
P. monodon (T)     ATTTTCTTCTTCTACCCTTTGGATTTCACCTTTGTCTGCCCCACTGAAATTATCGCCTTC
                    *  * ** ** *****  * ** ** ** ** ** ** ** ** ** ** **  * ** ***

H. sapiens (P)     AGCAACCGTGCAGAGGACTTCCGCAAGCTGGGCTGTGAAGTGCTGGGCGTCTCGGTGGAC
B. taurus (P)      AGCGACCGTGCTGCGGAGTTCCACAAGCTGAACTGCGAGGTGCTGGGCGTCTCGGTCGAC
T. nigroviridis(K) AGCGACAGGGTCCAGGACTTCCGCAGCATCAACTGCGAGGTTATTGGCTGCTCCATCGAC
M. lucifugus (T)   AGTGATAGGGCAGAAGAATTTAAGAAAATCAACTGCCAAGTGATTGGTGCTTCTGTGGAT
B. belcheri  (T)   AGCGATCGCGTGGAGGAGTTTCGTAAGATCAACTGCGAGGTGGTGGCGTGTTCAACAGAC
A. aegypti(2cys-T) TCGGACCGCGTCGAGGAGTTCGAGAAGATCGGCTGCTCTGTGATCGGCGTCTCGACCGAC
P. monodon (T)     TCTGACCGTGTTGAAGAATTCAGGAAAATTGGATGCGAAGTGGTTGCTTGCTCTACAGAC
                       *  * *     ** **    *   *    **    **  * *     **    **
```

```
H. sapiens (P)     TCTCAGTTCACCCACCTGGCTTGGATCAACACCCCCCGGAAAGAGGGAGGCTTGGGCCCC
B. taurus (P)      TCTCAGTTCACCCACCTGGCTTGGATCAACACTCCCAGGAAAGAGGGAGGCTTGGGCCCG
T. nigroviridis(K) TCCCACTTCACCCACTTGGCTTGGATTAACACTCCAAGAAAGGAGGGAGGTCTGGGTGAA
M. lucifugus (T)   TCTCACTTCTGTCACCTGGCATGGATCAACACACCCAAGAAACAAGGAGGACTGGGTCCC
B. belcheri  (T)   TCCCAATTCTCCCACTTGGCCTGGACGAACACCCCCAGAAAGCAGGGTGGACTGGGCCAG
A. aegypti(2cys-T) AGTCACTTCACCCATTTGGCCTGGATCAACACCCCGCGTAAGCAGGGCGGTCTCGGAGAG
P. monodon (T)     TCCCACTTCTCCCACCTTGCTTGGATCAACACTCCCCGCAAGGAAGGTGGTCTTGGTACG
                      ** ***   **  * ** ****  ***** **    **  * ** **  * **

H. sapiens (P)     CTGAACATCCCCCTGCTTGCTGACGTGACCAGACGCTTGTCTGAGGATTACGGCGTGCTG
B. taurus (P)      CTGAACATTCCCCTGCTGGCTGATGTAACCAGAAAGTTGTCCAGTGATTATGGCGTGCTG
T. nigroviridis(K) ATGAAGATCCCCCTCGTGGCCGACCTCACCAAGAGCATCTCCAAAGATTACGGCGTGCTG
M. lucifugus (T)   ATGAACATTCCTTTGGTGTCAGACCCCAAGCGCACCATTGCTCAGGACTACGGAGTCTTA
B. belcheri  (T)   ATGAAGATCCCAATCCTGGCCGACAAAGCGATGACCATATCCCGGGACTACGGCGTGTTG
A. aegypti(2cys-T) CTGCGAATTCCCCTGTTGGCCGACAAGTCCATGAAGATTTCCCGTGACTACGGAGTGCTC
P. monodon (T)     ATGAAGATCCCTCTTCTGGCTGACAAGTCAATGGAAGTTGCAAAGGCTTACGGAGTCCTT
                    **   ** **  *  *  * **              *  *    *  ** ** **  *

H. sapiens (P)     AAAACAGATGAGGGCATTGCCTACAGGGGCCTCTTTATCATCGATGGCAAGGGTGTCCTT
B. taurus (P)      AAGGAAGATGAGGGGATCGCCTACAGGGGCCTCTTTGTCATCGACGGCAAGGGTGTCCTT
T. nigroviridis(K) AAGGAAGACGACGGAATCGCCTACAGGGGCCTGTTTGTGATCGACGACAAGGGCGTCCTG
M. lucifugus (T)   AAGGCTGATGAAGGCATCTCATTCAGGGGCCTCTTTATCATTGATGATAAAGGTATCCTT
B. belcheri  (T)   ATGGAGCCTGAGGGCATCGCGTTCCGTGGTTTGTTCATCATTGACGACAAGGGTACCCTG
A. aegypti(2cys-T) CAGGAGGAGAGCGGTGTCCCATTCCGTGGACTGTTCGTCATCGATGGTAAGCAGAATCTC
P. monodon (T)     AAGGAGGATGAAGGCATTGCTTTCAGAGGCCTCTTTGTTATTGATGGCAAGCAGAATCTC
                             **  *  * * * * **  * **  * ** ** *  **       **

H. sapiens (P)     CGCCAGATCACTGTTAATGATTTGCCTGTGGGACGCTCCGTGGATGAGGCTCTGCGGCTG
B. taurus (P)      CGCCAGGTCACCATCAATGACTTGCCTGTGGGACGCTCTGTGGATGAGGCTCTGAGGCTG
T. nigroviridis(K) AGGCAGATCACCGTCAATGACTTGCCCGTGGGCCGCTCCGTAGACGAAACGCTGCGCTTG
M. lucifugus (T)   CGCCAGATCACTGTGAATGACCTTCCTGTTGGCCGCTCTGTGGATGAGACTCTGAGACTA
B. belcheri  (T)   CGCCAAATCACGATCAACGACCTGCCTGTCGGGCGTTCGGTCGACGAGACGCTGCGTCTG
A. aegypti(2cys-T) CGCCAGGTGACCGTCAATGATCTGCCCGTTGGACGCAGCGTCGATGAGACCCTCCGCCTG
P. monodon (T)     CGTCAGGTAACAATCAATGACCTGCCAGTTGGGCGTGATGTAGATGAACCGGTGCGATTA
                    * **  * **  * ** **  * ** ** ** **    ** ** **  *  *  *  *

H. sapiens (P)     GTCCAGGCCTTCCAGTACACAGACGAGCATGGGGAAGTTTGTCCCGCTGGCTGGAAGCCT
B. taurus (P)      GTCCAGGCTTTCCAGTACACAGATGAGCACGGGGAAGTCTGCCCCGCCGGCTGGACACCA
T. nigroviridis(K) GTCCAGGCCTTCCAGTTCACTGACAAATACGGAGAAGTGTGTCCCGCTGGCTGGAAACCT
M. lucifugus (T)   GTTCAGGCTTTCCAGTTCACTGACAAACATGGGGAAGTGTGCCCAGCTGGCTGGAAACCT
B. belcheri  (T)   GTTCAGGCCTTCCAGTTCACAGACAAACACGGGGAAGTGTGTCCTGCTGGCTGGAAGCCC
A. aegypti(2cys-T) GTGCAAGCATTCCAGTTCACCGATGAACACGGTGAGGTCTGCCCCGCCAACTGGAAGCCT
P. monodon (T)     GTACACGCTTTCCAGTTCCCAGAGGAGCATGCTGAAG-GTGAGGTTTCGTATGGCATTGT
                   ** ** ** ******* * * **  *  * *  ** *  **          ***

H. sapiens (P)     GGCAGTGACACGATTAAGCCCAACGTGGATGACAGCAAGGAATATTTCTCCAAACACAAT
B. taurus (P)      GGCAGTGACACAATCAAGCCCAATGTGGACGACAGCAAGGAATATTTCTCCAAACACAAC
T. nigroviridis(K) GGGAGCGATACCATCGTCCCAGATGTTGAGAAGAGCAAAACTTTCTTCTCCAAGCAAAAC
M. lucifugus (T)   GGCAGTGATACCATCAAGCCTGATGTCCAGAAGAGCAAAGAATATTTCTCTAAGCAGAAG
B. belcheri  (T)   GGTGCAGACACCATCAAACCCGACGTTAAGAACAGCAAAGAATATTTCTCCAAGCAGTAA
A. aegypti(2cys-T) GGATCCAAGACTATGGTCGCCGATCCGCAAAAGTCGAAGGAATACTTCAACGCCGCAAAC
P. monodon (T)     CAGGGAAAGTTCACGATT----ACATCAAGTCGAATCTGAACTTTTTATTTTATGGCGAT
                         *   *         *     *            *  **          *

H. sapiens (P)     TAG
B. taurus (P)      TAG
T. nigroviridis(K) TGA
M. lucifugus (T)   TGA
B. belcheri  (T)   ---
A. aegypti(2cys-T) TAA
P. monodon (T)     AA-
```

Figure 3.41 Multiple alignments of nucleotide sequences of thioredoxin peroxidase of *P. monodon*, *Myotis lucifugus* and *Branchiostoma belcheri tsingtaunese*, 2-Cys thioredoxin peroxidase of *Aedes aegypti*, Peroxidase of *Homo sapiens*, Peroxiredoxin 2 of *Bos taurus* and natural killer enhancing factor B of *Tetraodon nigroviridis*.

```
B. belcheri  (T)   -MSAGNAKLQHPAPNFESTAVLPSGEFKTIKLSDYKGKYLVIFFYPLDFTFVCPTEIIAF
M. lucifugus (T)   -MSSGNAKIGHPAPNFKATAVMPDGQFKDISLSDYKGKYVVFFFYPLDFTFVCPTEIIAF
H. sapiens (P)     -MASGNARIGKPAPDFKATAVVDG-AFKEVKLSDYKGKYVVLFFYPLDFTFVCPTEIIAF
B. taurus (P)      MACVCKAHVGKPAPEFQATAVVDG-AFKEVKLSDYKGKYVVLFFYPLDFTFVCPTEIVAF
T. nigroviridis(K) -MSSGSAKIGQPAPDFTAKAVVDG-QFKDLRLSDYRGKYVVFFFYPLDFTFVCPTEIVAF
A. aegypti(2cys-T) ---MPVPDLQKPAPKFSGTAVVNG-AFKEIKLEDYAGKYLVLFFYPLDFTFVCPTEIIAF
P. monodon (T)     -MSNTVPAIGKPAPVFKGTAVVDG-QFKEISLEDYKGKYVIFFFYPLDFTFVCPTEIIAF
                         . : :*** * ..**: .  ** : *.** ***:::***************:**

B. belcheri  (T)   SDRVEEFRKINCEVVACSTDSQFSHLAWTNTPRKQGGLGQMKIPILADKAMTISRDYGVL
M. lucifugus (T)   SDRAEEFKKINCQVIGASVDSHFCHLAWINTPKKQGGLGPMNIPLVSDPKRTIAQDYGVL
H. sapiens (P)     SNRAEDFRKLGCEVLGVSVDSQFTHLAWINTPRKEGGLGPLNIPLLADVTRRLSEDYGVL
B. taurus (P)      SDRAAEFHKLNCEVLGVSVDSQFTHLAWINTPRKEGGLGPLNIPLLADVTRKLSSDYGVL
T. nigroviridis(K) SDRVQDFRSINCEVIGCSIDSHFTHLAWINTPRKEGGLGEMKIPLVADLTKSISKDYGVL
A. aegypti(2cys-T) SDRVEEFEKIGCSVIGVSTDSHFTHLAWINTPRKQGGLGELRIPLLADKSMKISRDYGVL
P. monodon (T)     SDRVEEFRKIGCEVVACSTDSHFSHLAWINTPRKEGGLGTMKIPLLADKSMEVAKAYGVL
                   *:*. :*..:.*.*:. * **:* **** ***:*:**** :.**:::*    ::  ****

B. belcheri  (T)   MEPEGIAFRGLFIIDDKGTLRQITINDLPVGRSVDETLRLVQAFQFTDKHGEVCPAGWKP
M. lucifugus (T)   KADEGISFRGLFIIDDKGILRQITVNDLPVGRSVDETLRLVQAFQFTDKHGEVCPAGWKP
H. sapiens (P)     KTDEGIAYRGLFIIDGKGVLRQITVNDLPVGRSVDEALRLVQAFQYTDEHGEVCPAGWKP
B. taurus (P)      KEDEGIAYRGLFVIDGKGVLRQVTINDLPVGRSVDEALRLVQAFQYTDEHGEVCPAGWTP
T. nigroviridis(K) KEDDGIAYRGLFVIDDKGVLRQITVNDLPVGRSVDETLRLVQAFQFTDKYGEVCPAGWKP
A. aegypti(2cys-T) QEESGVPFRGLFVIDGKQNLRQVTVNDLPVGRSVDETLRLVQAFQFTDEHGEVCPANWKP
P. monodon (T)     KEDEGIAFRGLFVIDGKQNLRQVTINDLPVGRDVDEPVRLVHAFQFPEEHAEGEVSYGIV
                   .*:.:****:**.*  ***:*:*******.***.:***:***:.::::.*   :

B. belcheri (T)    GADTIKPDVKNSKEYFSKQ-
M. lucifugus(T)    GSDTIKPDVQKSKEYFSKQK
H. sapiens (P)     GSDTIKPNVDDSKEYFSKHN
B. taurus (P)      GSDTIKPNVDDSKEYFSKHN
T. nigroviridis(K) GSDTIVPDVEKSKTFFSKQN
A. aegypti(2cys-T) GSKTMVADPQKSKEYFNAAN
P. monodon (T)     RESSRLHQVESELFILWR--
                     .:   : ...   :
```

Figure 3.42 Multiple alignments of amino acid sequences of thioredoxin peroxidase of *P. monodon*, *Myotis lucifugus* and *Branchiostoma belcheri tsingtaunese*, 2-Cys thioredoxin peroxidase of *Aedes aegypti*, Peroxidase of *Homo sapiens*, Peroxiredoxin 2 of *Bos taurus* and natural killer enhancing factor B of *Tetraodon nigroviridis*.

Table 3.4 Sequence divergence of thioredoxin peroxidase and it related gene homologues from different taxa based on nucleotide (below diagonal) and amino acids sequences (above diagonal)

	H. sapiens **(P)**	***B. taurus*** **(P)**	***T. nigroviridis*** **(K)**	***M. lucifugus*** **(T)**	***B. belcheri*** **(T)**	***A. aegypti*** **(2cys-T)**	***P. monodon*** **(T)**
H. sapiens **(P)**	-	0.12056	0.28587	0.27855	0.34128	0.40998	0.54099
B. taurus **(P)**	0.1402	-	0.32355	0.34702	0.37361	0.36778	0.54099
T. nigroviridis **(K)**	0.3456	0.3307	-	0.26409	0.32558	0.40998	0.52098
M. lucifugus **(T)**	0.3426	0.3568	0.3413	-	0.24990	0.40998	0.55117
B. belcheri **(T)**	0.3909	0.3908	0.3440	0.3681	-	0.36194	0.51115
A. aegypti **(2cys-T)**	0.5107	0.4584	0.4947	0.5570	0.4940	-	0.47014
P. monodon **(T)**	0.5353	0.5800	0.5483	0.5271	0.5631	0.5528	-

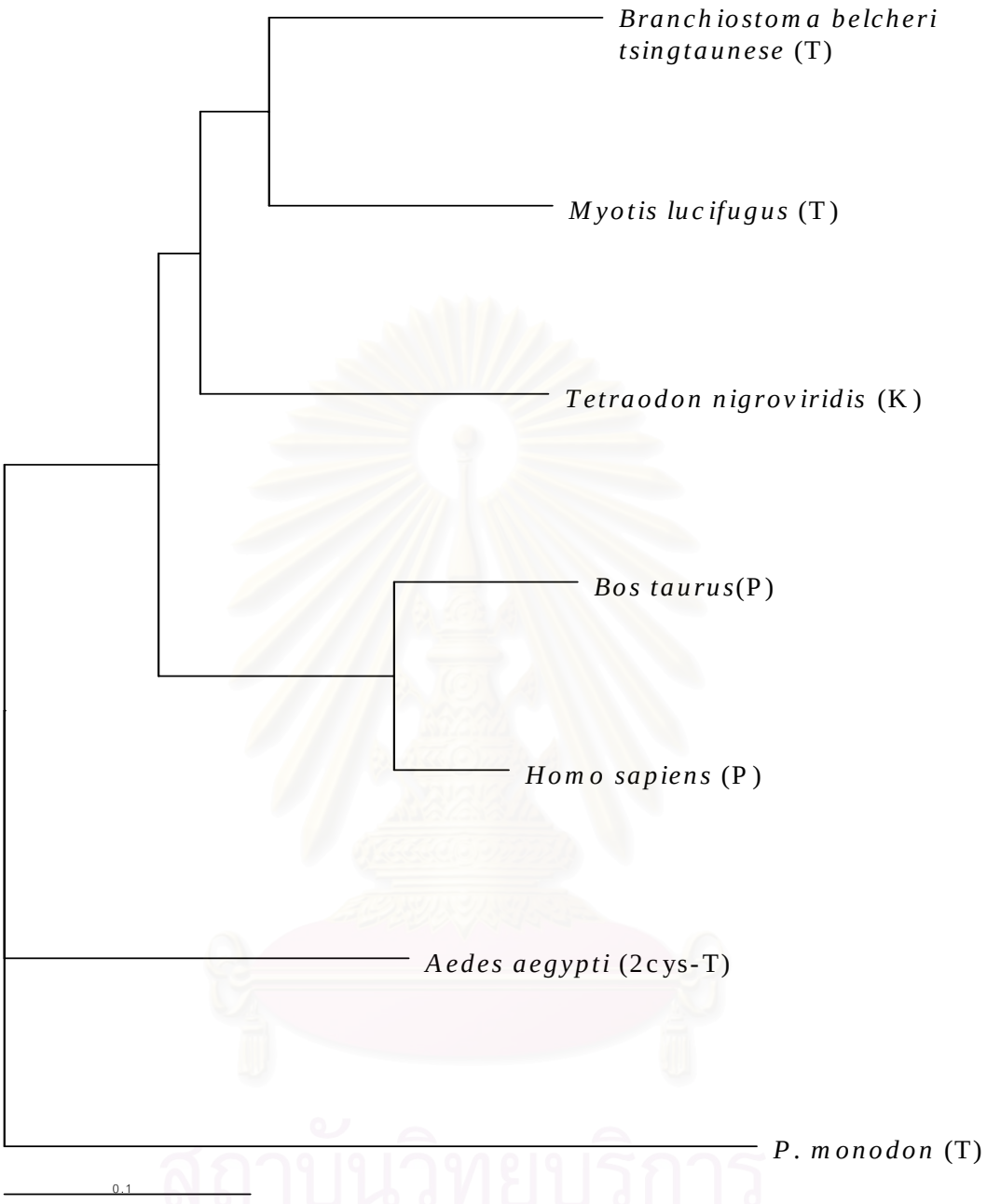

Figure 3.43 A neighbor-joining tree illustrating relations between thioredoxin peroxidase of *P. monodon*, *Myotis lucifugus* and *Branchiostoma belcheri tsingtaunese*, 2-Cys thioredoxin peroxidase of *Aedes aegypti*, Peroxidase of *Homo sapiens*, Peroxiredoxin 2 of *Bos taurus* and natural killer enhancing factor B of *Tetraodon nigroviridis*.

3.3.3.2 The full length genomic DNA of calponin 1

The full length genomic DNA sequence of calponin 1 was combined from sequences of 5′ and 3′ genome walking fragments (Figures 3.44 - 3.46) and those of a PCR product (Figure 3.47).

Calponin 1 is contained of 3 exons (185, 206 and 169 bp) and 2 introns (214 and 306 bp) with the ORF of 570 bp encoding a polypeptide of 190 amino acids. (Figure 3.48)

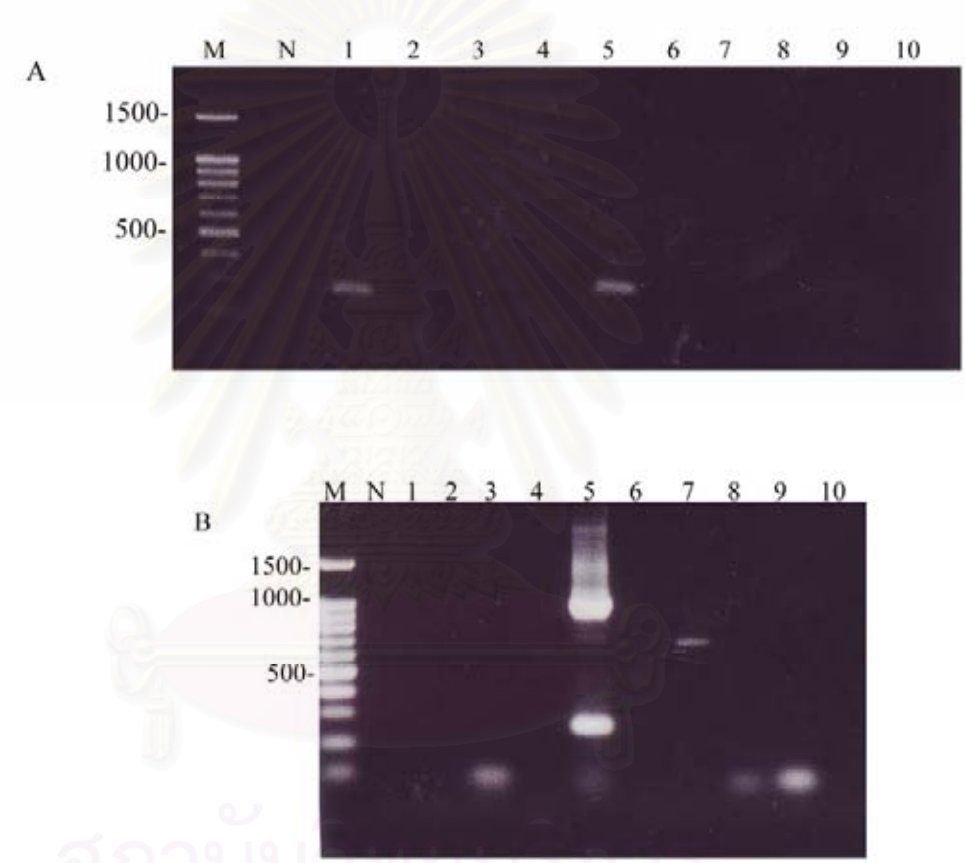

Figure 3.44 Primary (A) and secondary (B) PCR product of calponin 1. The product of *Alu* I, *Dra* I, *Hae* III *Stu* I, and *Rsa* I mini-libraries (lanes 1 – 5) amplified with forward gene-specific or nested gene-specific primers and the adapter primer (AP1 or AP2) and the adapter primer (AP1 or AP2) and the reverse gene-specific or nested reverse gene-specific primers (lanes 6 – 10). Lanes M and N is a 100 bp DNA ladder marker and the negative control (without genomic DNA template), respectively.

ACTATAGGGCACGCGTGGTCGACGGCCCGGGCAGGTAAAACCCTGTACAGTTATCTA
TACAATATGAAGCGTCAGATAAATGTTACCAGATATATTTTAACCTCCAGTCTGTTG
GAAGAATATATCTTGGCCGGAAATGTGAACCCAGACACACTACAGTTCGACCCTTGT
TGTTAAAGGTGCACTAGAGTAAAAGAAGGTTAATTTCCCCGTTTGTTGGCATCAGAT
TCCTTTGGTTTTGGGTTAAAGCAGATAGAGCACCATCTATGGATTTTCTTCCCCCAT
CTCTAGTGCACTCTTTTTGGGTCGACTTTCCTTCCTCCGTTGCTTTCTCTCGCGTAT
CTTCGAATTACTTCGTGCCATGTAATGCAATTCAGTGATAAGTTTGGGCATTCCTTT
TAGTATTGTATGTAACTCTCACCATTAGCTCTTTGTTTTCTTCTCAAATTCCTCAAT
ATATATTTTCTCCAACAGGTCAACGCAAAGTACAGCGAAGAGCAGGCCGCCGAGTGC
TTGGAATGGATCGCCATCATCACGAGCGCCGACATCAGCAAGTCTGGAGACGCCGAC
AATTTCTACGAGACCTTGAAGAATGGACAGCTGTTGTGCCAGTGAGTGTTTGGCGTC
GGCACGTTCGCCTTAATAATACGATTGAGGGTAGAGGACGCTTGATTTGTGGTCGGA
CTTGTATGTTTCGGTTCANAAGGCTGTTTTTTCTATTGACCTTTCTTTGCACTGGTA
TCTAGTAATGTGTTTGTTTTAAGTCTTCGTCGTCATCATCAGTAATTAGAGGGGGTT
CTCATTCATTTACTTTGTTCCCAACAGGGTGATTAACGCCCTCAAGCCCGGTCAGAT
CAAGAAGATCCAGACCTCCGCCATGGCATTCAAGTGCATGGAAAACATCAACGCCTT
TGTGGAGGGAGCTAAGGCCTGTGGGG**TGCCCACTCAGGAGACCTTCCA**

Figure 3.45 Nucleotide sequence of 5′genome walking fragment of calponin 1 generated by the AP2 and a nested reverse primer. The position of both sequences primers were illustrated in boldface and underlined.

AACGCCTTTGTGGAGGGAGCTAAGGCCTGTGGGGTGCCCACTCAGGAGACCTTCCAG
ACCGTCGACCTCTGGGAACGACAGAACCTTAACTCTGTTGTTATCTGCTTGCAGTCT
CTGGGCAGGAAGGTATGTAGCATTACTTGCTACCATTTGTAATTGTAGTCTGTATTT
TGGTTATCCATTTCTGTGAGGGAGAGTGTATAGTGATGACACTTTTACACTTGTCCT
GAGTATAATTCATTATTTGTAGAAATGTTTATTGGATAATAAATTTCTTATTCCCCT
TTATGGGCTTGCCATCAAGATATGCAGTATTTAATTCATTATGAATAGTATCAGTAT
GAATGATAAGTCTGTTGAAATTGTACTTGAGAGTGTAATTGGGCATGAAAGTGACAA
GTGCAATGATTTCTTTATTTTTTTGTGTTTCAGGGATCTCAATTTGGAAAGCCTTCC
ATTGGCCCAAAAGAGTCTGAGAAGAATGTCCGTCACTTCACCGAGGAGCAGCTCAGG
GCTTCTGAGGGCATCGTCAACCTGCAGTATGGCTCCAACAAGGGTGCCACTCAGTCT
GGCATGTCCTTCGGCAATACTCGCCACATGTAAAAGCAGTCTTTGTAGACTTTCACT
TTCACTTCATTTTTTAAAAAAAGTAGTTCAACATAATTCATCATGCTTCTAATATGT
TCCAATATATAATAGCGGGGAGGATTTCTTTTATATATAAAAATAAAAACTGAAAAA
AATGCATTGGCAGTGGTATGCCTAGAAAAGGAATTTTTACAACTGCAGTCTTAGGCA
AAGAAATGAATGTAAAAAAGGATGAAATCAGACATGTATCACTTGACCAATAGGTTG
CTACAATTTTTATTACATTGC

Figure 3.46 Nucleotide sequence of 3′genome walking fragment of calponin 1 generated by a nested forward primer and the AP2. The position of both sequences primers were illustrated in boldface and underlined.

GCCGCCGAGTGCTTGGAATGGATCGCCATCATCACGAGCGCCGACATCAGCAAGTCT
GGAGACGCCGACAATTTCTACGAGACCTTGAAGAATGGACAGCTGTTGTGCCAGTGA
GTGTTTGGCGTCGGCACGTTCGCCTTAATAATACGATTGAGGGTAGAGGACGCTTGA
TTTGTGGTCGGACTTGTATGTTTCGGTTCAAAAGGCTGTTTTTTCTATTGACCTTTC
TTTGCACTGGTATCTAGTAATGTGTTTGTTTTAAGTCTTCGTCATCATCATCAGTAA
TTAGAGGGGGTTCTCATTCATTTACTTTGTTCCCAACAGGGTGATTAACGCCCCCAA
GCCCGGTCAGATCAAGAAGATCCAGACCTCCGCCATGGCATTCAAGTGCATGGAAAA
CATCAACGCCTTTGTGGAGGGAGCTAAGGCCTGTGGGGTGCCCACTCAGGAGACCTT
CCAGACCGTCGACCTCTGGGAACGACAGAACCTTAACTCTGTTGTTATCTGCTTGC**A**
GTCTCTGGGCAGGAAGG

Figure 3.47 Nucleotide sequence of calponin 1 generated by typical PCR. Gene specific primers are illustrated in boldface and underlined.

```
AAAACCCTGTACAGTTATCTATACAATATGAAGCGTCAGATAAATGTTACCAGATATATT  60
TAACCTCCAGTCTGTTGGAAGAATATATCTTGGCCGGAAATGTGAACCCAGACACACTAC 120
AGTTCGACCCTTGTTGTTAAAGGTGCACTAGAGTAAAAGAAGGTTAATTTCCCCGTTTGT 180
TGGCATCAGATTCCTTTGGTTTTGGGTTAAAGCAGATAGAGCACCATCTATGGATTTTCT 240
TCCCCCATCTCTAGTGCACTCTTTTTGGGTCGACTTTCCTTCCTCCGTTGCTTTCTCTCG 300
CGTATCTTCGAATTACTTCGTGCCATGTAATGCAATTCAGTGATAAGTTTGGGCATTCCT 360
TTTAGTATTGTATGTAACTCTCACA**ATG**AACCGTGCTACCAAGTCCGGAATCGCTGCCGA 420
                          M  N  R  A  T  K  S  G  I  A  A  E
GGCTCAGGCTAAGGTCAACGCAAAGTACAGCGAAGAGCAGGCCGCCGAGTGCTTGGAATG 480
 A  Q  A  K  V  N  A  K  Y  S  E  E  Q  A  A  E  C  L  E  W
GATCGCCATCATCACGAGCGCCGACATCAGCAAGTCTGGAGACGCCGACAATTTCTACGA 540
 I  A  I  I  T  S  A  D  I  S  K  S  G  D  A  D  N  F  Y  E
GACCTTGAAGAATGGACAGCTGTTGTGCCA*GTGAGTGTTTGGCGTCGGCACGTTCGCCTT* 600
 T  L  K  N  G  Q  L  L  C  Q
*AATAATACGATTGAGGGTAGAGGACGCTTGATTTGTGGTCGGACTTGTATGTTTCGGTTC* 660
*AAAAGGCTGTTTTTTCTATTGACCTTTCTTTGCACTGGTATCTAGTAATGTGTTTGTTTT* 720
*AAGTCTTCGTCGTCATCATCAGTAATTAGAGGGGGTTCTCATTCATTTACTTTGTTCCCA* 780
*ACAG*GGTGATTAACGCCCTCAAGCCCGGTCAGATCAAGAAGATCCAGACCTCCGCCATGG 840
       V  I  N  A  L  K  P  G  Q  I  K  K  I  Q  T  S  A  M
CATTCAAGTGCATGGAAAACATCAACGCCTTTGTGGAGGGAGCTAAGGCCTGTGGGGTGC 900
 A  F  K  C  M  E  N  I  N  A  F  V  E  G  A  K  A  C  G  V
CCACTCAGGAGACCTTCCAGACCGTCGACCTCTGGGAACGACAGAACCTTAACTCTGTTG 960
 P  T  Q  E  T  F  Q  T  V  D  L  W  E  R  Q  N  L  N  S  V
TTATCTGCTTGCAGTCTCTGGGCAGGAAGG*TATGTAGCATTACTTGCTACCATTTGTAAT*1020
 V  I  C  L  Q  S  L  G  R  K
*TGTAGTCTGTATTTTGGTTATCCATTTCTGTGAGGGAGAGTGTATAGTGATGACACTTTT*1080
*ACACTTGTCCTGAGTATAATTCATTATTTGTAGAAATGTTTATTGGATAATAAATTTCTT*1140
*ATTCCCCTTTATGGGCTTGCCATCAAGATATGCAGTATTTAATTCATTATGAATAGTATC*1200
*AGTATGAATGATAAGTCTGTTGAAATTGTACTTGAGAGTGTAATTGGGCATGAAAGTGAC*1260
*AAGTGCAATGATTTCTTTATTTTTTTGTGTTTCAGG*GATCTCAATTTGGAAAGCCTTCCA1320
                                      G  S  Q  F  G  K  P  S
TTGGCCCAAAAGAGTCTGAGAAGAATGTCCGTCACTTCACCGAGGAGCAGCTCAGGGCTT1380
 I  G  P  K  E  S  E  K  N  V  R  H  F  T  E  E  Q  L  R  A
CTGAGGGCATCGTCAACCTGCAGTATGGCTCCAACAAGGGTGCCACTCAGTCTGGCATGT1440
 S  E  G  I  V  N  L  Q  Y  G  S  N  K  G  A  T  Q  S  G  M
CCTTCGGCAATACTCGCCACATG**TAA**AAGCAGTCTTTGTAGACTTTCACTTTCACTTCAT1500
 S  F  G  N  T  R  H  M  *
TTTTTAAAAAAAGTAGTTCAACATAATTCATCATGCTTCTAATATGTTCCAATATATAAT1560
AGCGGGGAGGATTTCTTTTATATATAAAAATAAAAACTGAAAAAAATGCATTGGCAGTGG1620
TATGCCTAGAAAAGGAATTTTTACAACTGCAGTCTTAGGCAAAGAAATGAATGTAAAAAA1680
GGATGAAATCAGACATGTATCACTTGACCAATAGGTTGCTACAATTTTTATTACATTGCA1740
TAGGAACTGGTAATAATGAAGCGAAGTCTCAAGGCCAGAGAAAATGCTTTAAAGTTCTCA1800
```

```
CTGAAACCAGAATTAAATATTTTAGTGCAAGCTGATGAGTAGCACCATTAGCTCATTTCC1860
AAATTGATGCATTTTCAATACACATCACATATTTGTTTTAACTGAAAACTGAAGGCGTAG1920
ATACATTATCAAAGAAAAATTATCCATCCAGGCTTTTTTCATATTTTACTAATTTGTAAG1980
CTTATTATAGTACAATTTATACAGATATAAGTGTTATACATTATGCAGTATAAAATGTAT2040
TTAAATAATGTTATTCTATGAAAAAGAAATTCATATATATGAAAAGTATTCCTTATTTAT2100
CATAGTTGCAGCTCATCTGTAGCAATATAGTGAAATGAATATTGTTTCATTTTTCTTTCC2160
TTTTTTTATTGGAGTCATATTCTCTTTATTTTACGTTACCATTGTATGTGTGGTATGAAG2220
TTTTATTTTCTTTTCCTAATAGAGAAATTATAGTCTTGTTTGAGCTGTCACATTCCAGTT2280
TGAGAGAAATTTTGTATGGAAATGAAATAAAAAGTTCAATACTAAAAAAAAAAAAAAAAA2340
AC                                                              2342
```

Figure 3.48 Coding nucleotides and deduced amino acids of each exon of calponin 1. Introns are italicized. The start and stop codons are illustrated in boldface. The poly A additional signal site are underlined.

The full length cDNA of calponin 1 was searched against data in the GenBank using Blast X and the closest homologues was *CPN-1* of *Caenorhabditis briggsae (*2e-39, AAF01687). The full length cDNA and amino acid sequences of *CPN-1* of *Caenorhabditis briggsae*, calponin of *Branchiostoma belcheri* (6e-26, BAC16745), *transgelin 2* of *Homo sapiens* (7e-24, CAI14604), transgelin 2 of *Mus musculus* (7e-24, AAH09076), Transgelin 2 *of Danio rerio* (2e-24, AAH44160) and mantle gene 2 of *Pinctada fucata (*4e-39, AAZ76256) were retrieved and multiple aligned (Figures 3.49 and 3.50).

Sequence divergence based on nucleotide and protein sequences were calculated. Large divergence was observed between calponin 1 of *P. monodon* and that of the remaining species (Table 3.5). A neighbor-joining tree allocated calponin 1 of *P. monodon* to be separated from other species. Phylogenetic analysis indicated distantly relationships between calponin 1 and trangelin 2. (Figure 3.51)

```
H. sapiens (T)     ATGTCCGCCTTTTCTTTGGCTTTGGCTTTGGTGAGCTCCCCGCAGCCACCGCCACCCATT
M. musculus (T)    ------------------------------------------------------------
D. rerio (T)       ------------------------------------------------------------
P. fucata (M)      ------------------------------------------------------------
C. briggsae (C)    ------------------------------------------------ATGTCGGCTTTC
P. monodon (C)     ------------------------------------------------------------
B. belcheri (C)    ------------------------------------------------------------


H. sapiens (T)     GGAATGGCCAACAGGGGACCTGCATATGGCCTGAGCCGGGAGGTGCAGCAGAAGATTGAG
M. musculus (T)    ---ATGGCCAACAGGGGACCTTCCTACGGCCTGAGCCGAGAGGTGCAGCAGAAGATTGAG
D. rerio (T)       ---ATGGCAAATAAAGGTCCGTCCTACGGTCTGAGTCGCGAGGTGCAGAGCAAGATAGAC
P. fucata (M)      ATGTCATCAACCAGAGCAACAAAATCAGGATTTGCCGCCGAAGCGCAGGCAAAGACTAAC
C. briggsae (C)    GCTTCTTCGGACCGCGCTGAAAAGTCTGGAATTGCCCTGGAGGCTCAACAAAAAATCTAC
P. monodon (C)     ------ATGAACCGTGCTACCAAGTCCGGAATCGCTGCCGAGGCTCAGGCTAAGGTCAAC
B. belcheri (C)    ------ATGAACCAAGGACCGACGTACGGATTGAGCGCTAAGGTTGCTCAAAAGATTGCG
                           *       *  **  *       * *       **


H. sapiens (T)     AAACAATATGATGCAGATCTGGAGCAGATCCTGATCCAGTGGATCACCACCCAGTGCCGA
M. musculus (T)    AAGCAGTACGACGCGGATCTGGAGCAGATCCTCATCCAGTGGATCACCACTCAGTGCCGC
D. rerio (T)       AAAAAGTATGACCCAGAGCTGGAGGGGCGGCTGGTACAGTGGATCGTCAGTCAGTGTGGA
P. fucata (M)      GCAAAATATGATCAAGATCATGCTGTACAGTGCCTTGAATGGATTGGCAGAAAGACAGGA
C. briggsae (C)    GAGAAATACGACAAGAATCTTGCAGGGGAAATTCTCCAATGGGTTCAGGATGTCACCGGG
P. monodon (C)     GCAAAGTACAGCGAAGAGCAGGCCGCCGAGTGCTTGGAATGGATCGCCATCATCACGAGC
B. belcheri (C)    GGGAAGAGGGACCC---CCAGAAAGAGGCGGAGGCCCAG---------GCTTGGATCGAG
                       *           *                 *


H. sapiens (T)     AAGGATGTGGGCCGGCCCCAGCCTGGACGCGAGAACTTCCAGAACTGGCTCAAGGATGGC
M. musculus (T)    GAGGACGTGGGCCAGCCCCAGCCTGGCCGTGAGAACTTCCAGAAGTGGCTCAAGGACGGC
D. rerio (T)       GAAGCTATAGGAAAACCTCAACCTGGAAAACAAGGCTTTCAGCAGTGGCTCAAGGATGGA
P. fucata (M)      GAACCAGTTAATACATGTGGA---GATCCTGAAAACTTCCACGAACAACTGAAGAATGGA
C. briggsae (C)    CTTTCATTCGACACTCAAGGA---GACGCTGATAATTTCGTCAAAGTTTTCCAAGACGGA
P. monodon (C)     GCCGACATCAGCAAGTCTGGA---GACGCCGACAATTTCTACGAGACCTTGAAGAATGGA
B. belcheri (C)    GAGCTGATTGGTGAGAAGTTTCCCGAGGGCGTGGCTTATGAGGACGCCCTGAAGGATGGC
                       *           *       *      *     *  *  * **


H. sapiens (T)     ACGGTGCTATGTGAGCTCATTAATGCACTGTACCCCGAGGGGCAGGCCCCAGTAAAGAAG
M. musculus (T)    ACGGTTCTGTGCAAGCTTATTAATTCACTGTATCCTGAGGGGCAGGCCCCAGTAAAGAAG
D. rerio (T)       TGTATTTTATGCGAGCTGATCAA--CAGTCTCTTCAAAGACTCAAAGCCT-GTGAAGAAG
P. fucata (M)      TATCTTCTGGCAAAACTTGCCAATGCAATTCAACCTGG---CTCAGTTAAAATTATGGGA
C. briggsae (C)    AGTGTTCTGTGCAATCTTGCAAACGCTCTCAAGCCAGG---AAGCGTCAAAA------AA
P. monodon (C)     CAGCTGTTGTGCCAGGTGATTAACGCCCTCAAGCCCGG---TCAGATCAAGA------AG
B. belcheri (C)    GTCATCCTGTGCAAGCTCATCAATGTGCTGGTTCCTGG---------TTCAGTCAAGAGG
                       *  *    *  *   **     *    *


H. sapiens (T)     ATCCAGGCCTCCACCATGGCCTTCAAGCAGATGGAG--CAGATCTCTCAGTTCCTGCAAG
M. musculus (T)    ATCCAGGCCTCTTCGATGGCCTTCAAGCAGATGGAG--CAGATCTCCCAGTTCCTGCAGG
D. rerio (T)       ATCCAGAGTTCTAGCATGGCTTTTAAACAGATGGAG--CAGATCTCTCAGTTTCTCACTG
P. fucata (M)      AACAAACCACCGACAATGGCCTTCAAACAGATGGAG--CTGATAGGGCAGT-TTGCTGAA
C. briggsae (C)    GTTAACACTTCTGCTATGGCTTTCAAGAAAATGGAG----AATATTTCATTCTTCCTGAA
P. monodon (C)     ATCCAGACCTCCGCCATGGCATTCAAGTGCATGGAA----AACATCAACGCCTTTGTGGA
B. belcheri (C)    ATCAATGCACAGAAGATGCCCTTCAAACAGATGGAG----AACATCGGGAACTTCCTGAC
                       *       *** * ** **    *****      *


H. sapiens (T)     CAGCTGAGCGCTATGGCATTAACACCACT--GACATCTTCCAAACTGTGGACCTCTGGGA
M. musculus (T)    CAGCCGAGCGCTATGGCATTAACACCACG--GACATCTTCCAGACTGTGGATCTCTGGGA
D. rerio (T)       CTGCCGAACGATACGGAATCACCAAATCT--GACATCTTTCAGACAGTCGACTTATGGGA
P. fucata (M)      TTTTGTAAGAAAATGGGACTAGATAA-T---GAGCTTTTCCAAACAGTAGACTTATATGA
C. briggsae (C)    GTTCGCAGAGGAATTCGTTCAAAAGTCT---GAACTCTTCCAGACCGTTGACCTGTATGA
P. monodon (C)     GGGAGCTAAGGCCTGTGGGGTGCCCACTCAGGAGACCTTCCAGACCGTCGACCTCTGGGA
B. belcheri (C)    GGCTGTGCAGGGTTACGGCGTCCCTGCCTCGGACCTGTTCCAGACCGTGGACCTGTACGA
                                                  **    ** ** ** ** **  * *  **


H. sapiens (T)     AGGAAAGAACATGGCCTGTGTGCAGCGGACGCTGATGAATCTGGGTGGGCTGGCAGTAGC
M. musculus (T)    AGGAAAGAACATGGCTTGTGTGCAGCGGACACTAATGAACCTGGGTGGGCTGGCAGTAGC
D. rerio (T)       GGGTAAGGACCTCGCTGCTGTGCAAATGACACTGTTGTCTTTGGGGAGTCTGGCTGTTAC
P. fucata (M)      GAGCCAGAATTTATCTAGTGTG-----GTTACTTGTATAACTGCATTAG-----------
C. briggsae (C)    AGGACAGGATCCGAATGCAGTGC-----TCATCTGCCTTGCTTCGTTGGCTCGTAAATCT
P. monodon (C)     ACGACAGAACCTTAACTCTGTT-----GTTATCTGCTTGCAGTCTCTGGGCAGGAAG---
B. belcheri (C)    GAGGCGAAACATCCCCGCGGTCACACAGTGTTTCTTCGCAATGGGTCGTGTGGCACAAAC
                     *     *          **
```

```
H. sapiens (T)     CCGAGATGATGGGCTCTTCTCTGGGGATC-----CCAACTGGTTCCCTAAGAAATCCAAG
M. musculus (T)    CAGGGACGATGGGCTCTTCTCTGGGGATC-----CCAACTGGTTTCCTAAGAAATCCAAG
D. rerio (T)       TAAGGATGACGGTTGTTACCGTGGTGACC-----CTGCTTGGTTCCCAAAGAAATCACAG
P. fucata (M)      -GAAGAAAGTTGGGA------------------GAATTAGGT--CCGAAAGAGTCAAAA
C. briggsae (C)    GAGAAACACTTTGGACGCT-------------CGGGACTTGGA--CCAAAAGAAGCACAG
P. monodon (C)     GGATCTCAATTTGGAAAGC-------------CTTCCATTGGC--CCAAAAGAGTCTGAG
B. belcheri (C)    AAAGCCAGACTATCGC-------GGGCCT-----ATATTTGGA--CCAAAGCCTTCGGAG
                                                      **   ** **     *  *

H. sapiens (T)     GAGAATCCTCGGAACTTCTCGGATAACCAGCTGCAAGAGGGCAAGAACGTGATCGGGTTA
M. musculus (T)    GAGAACCCTCGGAACTTCTCGGACAACCAGTTGCAAGAGGGCAAGAACGTGATTGGGTTG
D. rerio (T)       GAGAACAGGAGAGAGTTCTCTGAGGAGCAGATGAAAGAAGGCCAGAGTGTAATCGGCCTA
P. fucata (M)      GGACAGAAAAGAGAATGGACAGAAGAGCAAATGAAAGCAGGACAGAATATAATTGGTTTA
C. briggsae (C)    GGAGATCGTCGGGAATGGACTGAGGAGCAGCTGAAGGCTGGCCAAAATGTGATCGGGCTC
P. monodon (C)     AAGAATGTCCGTCACTTCACCGAGGAGCAGCTCAGGGCTTCTGAGGGCATCGTCAACCTG
B. belcheri (C)    GAAAACATCCGCTTGTTTACAGAAGACCAACTGATGGAGGGCAGGAAAGAGGTCAGCCTG
                       *     *    *   * **  * **  *    *               *     *

H. sapiens (T)     CAGATGGGCACCAACCGCGGGGCGTCTCAGGCAGGCATGACTGGCTACGGGATGCCACGC
M. musculus (T)    CAGATGGGCACCAACCGTGGAGCATCTCAGGCCGGCATGACCGGCTATGGGATGCCACGG
D. rerio (T)       CAGATGGGCACCAACAAAGGGGCATCGCAAGCAGGAATGACCGGTTACGGACGACCTCGG
P. fucata (M)      CAGATGGGAACAAACAAAGGAGCCAACCAATCAGGAATGAAT--ATTGGGAAATACACGT
C. briggsae (C)    CAAATGGGCAGCAATAAGGGTGCTACTGCCTCTGGACTCAAT--AT-GGGAAATACGCGA
P. monodon (C)     CAGTATGGCTCCAACAAGGGTGCCACTCAGTCTGGCATGTCC--TTCGG--AATACTCGC
B. belcheri (C)    CAGATGGGATCCAACAAGTTTGCCAACCAGTCAGGGCTGA---ACTTTGGTGTGAGACGC
                   **    **    **       **        * **  *       *  *        **

H. sapiens (T)     CAGATCCTCTGA--------------
M. musculus (T)    CAGATCCTCTGA--------------
D. rerio (T)       CAGATCCTAAACAACCAATAA-----
P. fucata (M)      CATATTGTAGATTGAGTCCATTTTAG
C. briggsae (C)    CACAT-GTAG----------------
P. monodon (C)     CACAT-GTAA----------------
B. belcheri (C)    CAAGTTACATAA--------------
                   **  *
```

Figure 3.49 Multiple alignments of nucleotide sequences of calponin 1 of *P. monodon*, *CPN-1* of *Caenorhabditis briggsae*, calponin of *Branchiostoma belcheri*, transgelin 2 of *Homo sapiens* , transgelin 2 of *Mus musculus*, transgelin 2 *of Danio rerio* and mantle gene 2 of *Pinctada fucata*.

```
H. sapiens (T)     MSAFSLALALVSSPQPPPPIGMANRGPAYGLSREVQQKIEKQYDADLEQILIQWITTQCR
M. musculus (T)    ----------------------MANRGPSYGLSREVQQKIEKQYDADLEQILIQWITTQCR
D. rerio (T)       ----------------------MANKGPSYGLSREVQSKIDKKYDPELEGRLVQWIVSQCG
B. belcheri (C)    -----------------------MNQGPTYGLSAKVAQKIAGKRDPQKEAEAQAWIE----
C. briggsae (C)    ----------------MSAFASSDRAEKSGIALEAQQKIYEKYDKNLAGEILQWVQDVTG
P. monodon (C)     -----------------------MNRATKSGIAAEAQAKVNAKYSEEQAAECLEWIAIITS
P. fucata (M)      --------------------MSSTRATKSG---AAAAKTNAKYDDH----AVCWIGR---
                                         :.   *    .  *   : . .       *:

H. sapiens (T)     KDVGRPQPGRENFQNWLKDGTVLCELINALYPEGQAPVKKIQASTMAFKQMEQISQFLQA
M. musculus (T)    EDVGQPQPGRENFQKWLKDGTVLCKLINSLYPEGQAPVKKIQASSMAFKQMEQISQFLQA
D. rerio (T)       EAIGKPQPGKQGFQQWLKDGCILCELINSLFKD-SKPVKKIQSSSMAFKQMEQISQFLTA
B. belcheri (C)    ELIGEKFPEGVAYEDALKDGVILCKLINVLVPG---SVKRINAQKMPFKQMENIGNFLTA
C. briggsae (C)    LSFDT-QGDADNFVKVFQDGSVLCNLANALKPG---SVKKVNTSAMAFKKMENISFFLKF
P. monodon (C)     ADISK-SGDADNFYETLKNGQLLCQVINALKPG---QIKKIQTSAMAFKCMENINAFVEG
P. fucata (M)      ------KTGVNTCGDNHKNGYAKANAIG--------SVKIMGN--------KTMAKMIGA
                                .  ::*   .:  .         :* :          : :  ::

H. sapiens (T)     AERYGINTTDIFQTVDLWEGKNMACVQRTLMNLGGLAVARDDGLFSGDPNWFPKKSKENP
M. musculus (T)    AERYGINTTDIFQTVDLWEGKNMACVQRTLMNLGGLAVARDDGLFSGDPNWFPKKSKENP
D. rerio (T)       AERYGITKSDIFQTVDLWEGKDLAAVQMTLLSLGSLAVTKDDGCYRGDPAWFPKKSQENR
B. belcheri (C)    VQGYGVPASDLFQTVDLYERRNIPAVTQCFFAMGRVAQTKPD--YRG-PIFGPKPSEENI
C. briggsae (C)    AEEF-VQKSELFQTVDLYEGQDPNAV---LICLASLARKSEK--HFGRSGLGPKEAQGDR
P. monodon (C)     AKACGVPTQETFQTVDLWERQNLNSV---VICLQSLGRKGSQ---FGKPSIGPKESEKNV
P. fucata (M)      CKKMGDNTVDYSNSS--------------VVTCITAGRKGGK---------------SKG
                    :       :  ::                ..     .    .               .

H. sapiens (T)     RNFSDNQLQEGKNVIGLQMGTNRGASQAGMTGYGMPRQIL
M. musculus (T)    RNFSDNQLQEGKNVIGLQMGTNRGASQAGMTGYGMPRQIL
D. rerio (T)       REFSEEQMKEGQSVIGLQMGTNKGASQAGMTGYGRPRQILNNQ
B. belcheri (C)    RLFTEDQLMEGRKEVSLQMGSNKFANQSGLN-FGVRRQVT
C. briggsae (C)    REWTEEQLKAGQNVIGLQMGSNKGATASGLN-MGNTRHM
P. monodon (C)     RHFTEEQLRASEGIVNLQYGSNKGATQSGMS-FGNTRHM
P. fucata (M)      KRWT---MKAG-NIIG--MGTNKGAN-SGMN-IGKYTSYCRS
                   : ::   :  .   :.   *:*: *. :*:.  *
```

Figure 3.50 Multiple alignments of amino acid sequences of calponin 1 of *P. monodon, CPN-1* of *Caenorhabditis briggsae*, calponin of *Branchiostoma belcheri*, transgelin 2 of *Homo sapiens* , transgelin 2 of *Mus musculus,* transgelin 2 *of Danio rerio* and mantle gene 2 of *Pinctada fucata*.

Table 3.5 Sequence divergence of calponin 1 from different taxa based on nucleotide (below diagonal) and amino acids sequences (above diagonal)

	H. sapiens **(T)**	***M. musculus*** **(T)**	***D. rerio*** **(T)**	***P. fucata*** **(M)**	***C. briggsae*** **(C)**	***P. monodon*** **(C)**	***B. belcheri*** **(C)**
H. sapiens **(T)**	-	0.03607	0.37941	2.11945	1.15088	1.21726	0.99725
M. musculus **(T)**	0.0993	-	0.35500	2.09986	1.11071	1.24013	0.96236
D. rerio **(T)**	0.4460	0.4495	-	2.05854	0.96236	1.22000	0.84987
P. fucata **(M)**	0.9301	0.9509	0.8168	-	1.58486	1.52062	2.14440
C. briggsae **(C)**	1.0050	0.9502	0.9938	0.6358	-	0.08057	1.21154
P. monodon **(C)**	0.9595	0.9426	1.0099	0.7798	0.7113	-	1.15291
B. belcheri **(C)**	0.8635	0.8596	0.9196	0.9477	1.0234	0.9779	-

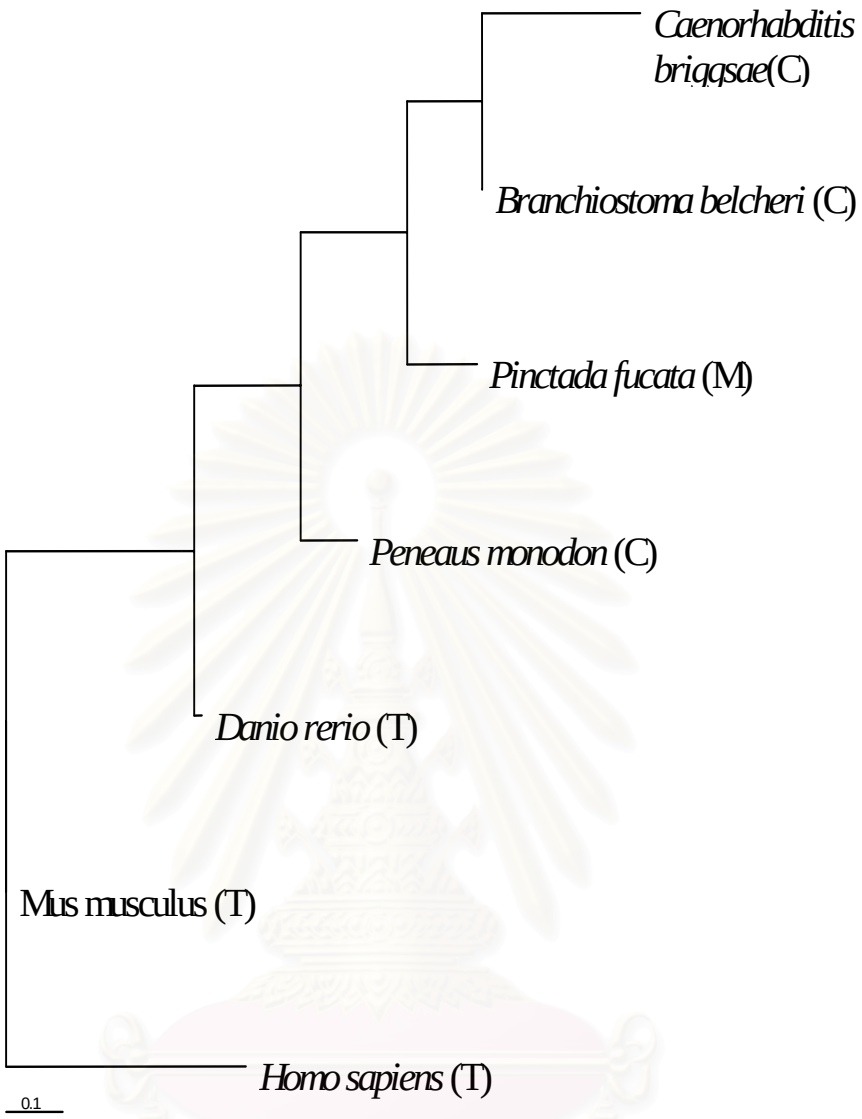

Figure 3.51 A neighbor-joining tree illustrating relations between calponin 1 of *P. monodon*, *CPN-1* of *Caenorhabditis briggsae*, calponin of *Branchiostoma belcheri*, transgelin 2 of *Homo sapiens* , transgelin 2 of *Mus musculus*, transgelin 2 *of Danio rerio* and mantle gene 2 of *Pinctada fucata*.

3.3.2.3 The full length genomic DNA of *DAD1*

The full length genomic DNA of *DAD1* was obtained by combining sequences of 5′ genome walking fragment (Figures 3.52 and 3.53) and a cloned PCR fragment (Figure 3.41). The other PCR product was amplified and direct sequenced to confirm the obtained sequence (data not shown).

DAD1 contained 2 exons (214 and 131 bp) and 1 intron (215 bp) with the ORF of 345 bp encoding a polypeptide of 115 amino acids (Figure 3.54).

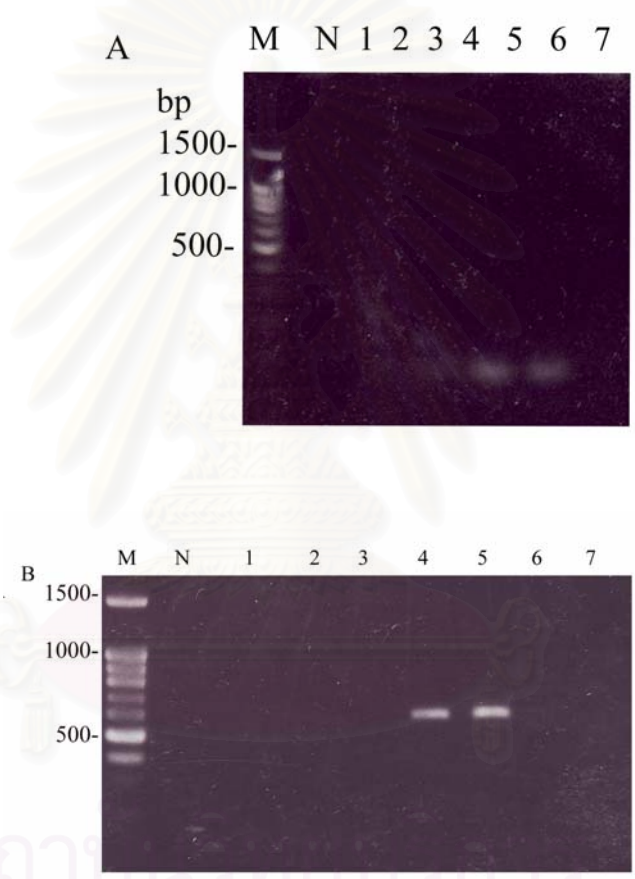

Figure 3.52 Primary (A) amd secondary (B) PCR product of *DAD1*. The template of *Alu* I the mini-library of male (lane 1), *Dra* I mini-library of female and male (lanes 2 and 3), *Hae* III mini-library of female and male (lanes 4 and 5) and *Rsa* I mini-library of female and male (lanes 6 and 7) was amplified with the adapter primer (AP1 or AP2) and gene specific reverse primers. Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

ACTATAGGGCACGCGTGGTCGACGGCCCGGGCAGGTCCAGGGTTAACCGCAGCACCA
GTGCCTCCACAGAGAGGTGAAGAATGGTGTGTCTAGCATGTGGACTTCTATTGTAAT
TTGTTTTGGAGCATTGTGTAGTAGCAAGGATAGATGTACTTGTATATTCCTTAGATT
TGTGGTGAGGAGGTGTAGTTGTTGTTTGTGTATGATTGTGTATTATTGCATGAAGAG
TAAGTGCTAACAGTTCACATAATGAGAATAAAAATTGCCATATTTTGACCTTTTAGG
AGTCTAAACGATGTCACATGTGGACATGGTGATATTGAATATGGTATAATATTATAA
TCTGGAAGGGTGAAATGCCCAAGGGTAATTTTTGTCTGTACCTAACCATTTATCAGA
TTGAAAAATCTAAATTTGTCCTCTCATTCCAGTAACCAACCATGTCTTCAACGTCAC
TATTGGTGGTTGTCAGAAATTTCTATGATGAGTACATGAAGAAAACTCCCAAGAAAT
TAAAGATCGTCGATGCCTACCTCTTCTACGTTCTGTTGACTGGGATCATCCAGTTTG
TGTTTTGCTGTCTAGTTGGAACTTTCCCCTTCAACT**CCTTCCTGTCTGGGTTCATCA**
G

Figure 3.53 Nucleotide sequence of 5′UTR of *DAD1* was generated by Genome Walking analysis using nested reverse primer and AP2. The positions of sequencing primers are illustrated in boldface and underlined.

CGATGCCTACCTCTTCTACGTTCTGTTGACTGGGATCATCCAGTTTGTGTTTTGCTG
TCTAGTTGGAACTTTCCCCTTCAACTCCTTCCTGTCTGGGTTCATCAGTACAGTTGG
CTGCTTTGTGTTAGGGGGTAAGTATCTATGGTTAATTGATGTTGGTTTGTAAAGTTA
GTTGGTGGTCATTTGTTTCACTGTCTTGAGTTCTCTTGAATGCATTATCAGTGATAA
TAAATTATGATTGTTTTTATTATAGCAATCATTACTGCTATTATTGTTACTGTGCTT
GTTTAATTATAATTTCTTTATTAATGTTTTTTAAATAATTATTTACCTTATTTTTCC
ACAGTGAGCTTGAGACTGCAGGCAAACCCTCAGAACAAAATGCAGTTTGTTGGCATC
TCCCCAGAAC**GAGGCTTTGCTGATTTCATC**

Figure 3.54 Nucleotide sequence of *DAD1* generated by PCR. Gene specific primers are illustrated in boldface and underlined.

```
CCAGGGTTAACCGCAGCACCAGTGCCTCCACAGAGAGGTGAAGAATGGTGTGTCTAGCAT  60
GTGGACTTCTATTGTAATTTGTTTTGGAGCATTGTGTAGTAGCAAGGATAGATGTACTTG 120
TATATTCCTTAGATTTGTGGTGAGGAGGTGTAGTTGTTGTTTGTGTATGATTGTGTATTA 180
TTGCATGAAGAGTAAGTGCTAACAGTTCACATAATGAGAATAAAAATTGCCATATTTTGA 240
CCTTTTAGGAGTCTAAACGATGTCACATGTGGACATGGTGATATTGAATATGGTATAATA 300
TTATAATCTGGAAGGGTGAAATGCCCAAGGGTAATTTTTGTCTGTACCTAACCATTTATC 360
AGATTGAAAAATCTAAATTTGTCCTCTCATTCCAGTAACCAACCATGTCTTCAACGTCAC 420
                                                  M  S  S  T  S
TATTGGTGGTTGTCAGAAATTTCTATGATGAGTACATGAAGAAAACTCCCAAGAAATTAA 480
 L  L  V  V  V  R  N  F  Y  D  E  Y  M  K  K  T  P  K  K  L
AGATCGTCGATGCCTACCTCTTCTACGTTCTGTTGACTGGGATCATCCAGTTTGTGTTTT 540
 K  I  V  D  A  Y  L  F  Y  V  L  L  T  G  I  I  Q  F  V  F
GCTGTCTAGTTGGAACTTTCCCCTTCAACTCCTTCCTGTCTGGGTTCATCAGTACAGTTG 600
 C  C  L  V  G  T  F  P  F  N  S  F  L  S  G  F  I  S  T  V
GCTGCTTTGTGTTAGGGGGTAAGTATCTATGGTTAATTGATGTTGGTTTGTAAAGTTAGT 660
 G  C  F  V  L  G
TGGTGGTCATTTGTTTCACTGTCTTGAGTTCTCTTGAATGCATTATCAGTGATAATAAAT 720
TATGATTGTTTTTATTATAGCAATCATTACTGCTATTATTGTTACTGTGCTTGTTTAATT 780
ATAATTTCTTTATTAATGTTTTTTAAATAATTATTTACCTTATTTTTCCACAGTGAGCTT 840
                                                        V  S  L
GAGACTGCAGGCAAACCCTCAGAACAAAATGCAGTTTGTTGGCATCTCCCCAGAACGAGG 900
 R  L  Q  A  N  P  Q  N  K  M  Q  F  V  G  I  S  P  E  R  G
CTTTGCTGATTTCATCTTTGCGCACATCATCCTTCACCTTGTCACTGTTAACTTCATTGG 960
 F  A  D  F  I  F  A  H  I  I  L  H  L  V  T  V  N  F  I  G
TTAACAAAGGGACGATCAAGTATTTTGTTGGTTTTCCTTAAGGATGTGAAATGTTCTAAG1020
 *
ATCATGTGTAAAGGATATAAACATTCACTAAAGAACCCATGTTATTTTCAAAGAAGCAGA1080
TTAATTTACATTCCCATGTGCGTGTAAGGTTTAACGATTGGAGTTTGTTCATGAAGTCAC1140
CAATAAATTTATAGTTAACGGAAAAAAAAAAAAAAAAAAAA                  1182
```

Figure 3.55 Coding nucleotides and deduced amino acids of each exon of *DAD1*. Introns are italicized. The start and stop codons are illustrated in boldface. The poly A additional signal site are underlined.

The full length cDNA of *DAD1* was searched against data in the GenBank using Blast X and the closest homologues was *DAD1* of *Anopheles gambiae* (5e-46, AAQ94040). The full length cDNA and amino acid sequences of *Anopheles gambiae*, *Argopecten irradians* (1e-43, AAX56947) at, *Ixodes scapularis* (1e-40, AAY66894), *Mus musculus* (1e-36, AAH53379) and oligosaccharyltransferase subunit homolog of *M. musculus* (1e-36, AAC53098) were retrieved and multiple aligned (Figures 3.56 and 3.57).

Sequence divergence based on nucleotide and protein sequences were calculated. Large divergence was observed between *DAD1* of *P. monodon* and that of the remaining species including oligosaccharyltransferase of *M. musculus* (Table 3.6).

A neighbor-joining tree allocated *DAD1* of *P. monodon* to be closely related with that of *Anopheles gambiae* (Figure 3.58).

```
M. musculus (D)    -----ATGTCGGCGTCTGTGGTGTCCGTCATCTCCCGGTTCCTGGAGGAGTACTTGAGCT
M. musculus (O)    -----ATGTCGGCGTCTGTGGTGTCCGTCATCTCCCGGTTCCTGGAGGAGTACTTGAACT
A. gambiae (D)     -----ATGAAGAACCTCACGGAA---GTGCTGCACAAGTTCTACGATGAGTACACGCACA
I. scapularis (D)  ATGGGTTCTACGAGCTTCTGGGA--CGTGGTGAAGCGCTTCTCGGAGGAGTACACCACGA
P. monodon (D)     --ATGTCTTCAACGTCACTATTGGTGGTTGTCAGAAATTTCTATGATGAGTACATGAAGA
A. irradians (D)   -----ATGCCTGATAGTCTGTTTTCAGTGGTGAAGAAATTCACAGATGAATACATAAGCA
                                       **  *       ***   ** ** ***

M. musculus (D)    CCACTCCGCAGCGGCTGAAGTTGCTGGACGCCTATCTCCTTTATATACTGCTGACCGGGG
M. musculus (O)    CCACTCCGCAGCGGCTGAAGTTGCTGGACGCCTATCTCCTTTATATACTGCTGACCGGGG
A. gambiae (D)     AAACGCCCAAGAAGCTGAAAATCGTCGACGCCTACTTGCTGTACATTCTGCTGACCGGCA
I. scapularis (D)  GCACGCCCAAGAAGATGAAGATCATCGACGCGTACCTGCAGTACGTGCTTCTCACGGGAG
P. monodon (D)     AAACTCCCAAGAAATTAAAGATCGTCGATGCCTACCTCTTCTACGTTCTGTTGACTGGGA
A. irradians (D)   GTACTCCGAAGAAATTGAAGATCGTAGATGCATACCTGTTCTACATATTGTTAACGGGCG
                     ** **  **    * **  *  * ** ** **  *    **  *  *  * ** **

M. musculus (D)    CGCTGCAGTTCGGCTACTGTCTCCTCGTGGGCACCTTCCCCTTCAACTCGTTCCTCTCTG
M. musculus (O)    CGCTGCAGTTCGGCTACTGTCTCCTCGTGGGCACCTTCCCCTTCAACTCGTTCCTCTCTG
A. gambiae (D)     TCATGCAGTTTGTTTACTGCTGCCTCGTTGGAACATTCCCGTTCAACTCCTTCCTGGCTG
I. scapularis (D)  TGGTGCAGTTCGTGTACTGCTGCATCGCCGGCACGTTCCCGTTCAACTCGTTCCTGTCGG
P. monodon (D)     TCATCCAGTTTGTGTTTTGCTGTCTAGTTGGAACTTTCCCCTTCAACTCCTTCCTGTCTG
A. irradians (D)   TCATCCAGTTCATGTATTGTGCGCTGGTTGGAACATTCCCTTTCAACTCTTTCCTGTCTG
                     * *****    *  **    * *  ** ** ***** ******** *****  * *

M. musculus (D)    GCTTCATCTCTTGTGTGGGCAGCTTCATCCTAGCGGTTTGCCTGAGAATACAGATCAACC
M. musculus (O)    GCTTCATCTCTTGTGTGGGCAGCTTCATCCTAGCGGTTTGCCTGAGAATACAGATCAACC
A. gambiae (D)     GGTTCATCAGCACGGTGAGCTGCTTCGTGCTGGGTGTGTGCCTTCGCCTCCAGTCGAACC
I. scapularis (D)  GCTTCATCACCTGCGTGGCGTCCTTCGTGCTGGGCGTCTGCCTGCGCCTCCAGGCCAACC
P. monodon (D)     GGTTCATCAGTACAGTTGGCTGCTTTGTGTTAGGGGTGAGCTTGAGACTGCAGGCAAACC
A. irradians (D)   GTTTCATCTCCAGTGTTGGTTCCTTCGTATTAGGAGTATGTTTGCGGCTACAAGTAAATC
                   * ******     **      ***  *  * *  **  *  *  *  * **    ** *

M. musculus (D)    CCCAGAACAAGGCGGACTTCCAAGGCATCTCTCCGGAGCGAGCCTTTGCTGACTTCCTCT
M. musculus (O)    CCCAGAACAAGGCGGACTTCCAAGGCATCTCTCCGGAGCGAGCCTTTGCTGACTTCCTCT
A. gambiae (D)     CCCAGAACAAGGAACAGTTCTTCGGAATCTCACCCGAGCGTGGATTCGCTGACTTTGTGT
I. scapularis (D)  CGCTGAACAAGGGACAGTTCTTCGGCATCAGCTCGGAACGGGCGTACGCCGACTTTATCT
P. monodon (D)     CTCAGAACAAAATGCAGTTTGTTGGCATCTCCCCAGAACGAGGCTTTGCTGATTTCATCT
A. irradians (D)   CACAGAATAAACATGACTTTACAGGAATTGGACCAGAAAGAGCTTTTGCTGATTTCATTT
                   * * *** **     * **    ** **     * **  * *  *  ** ** **  * *

M. musculus (D)    TTGCCAGCACGATCCTGCACCTTGTCGTCATGAACTTCGTTGGCTGA
M. musculus (O)    TTGCCAGCACGATCCTGCACCTTGTCGTCATGAACTTCGTTGGCTGA
A. gambiae (D)     TCGCACACATAATTCTGCACCTGGTCGTGGTCAACTTCAGCGGTTAA
I. scapularis (D)  TCGCACACGTCGTCTTGCATCTCGTCGTCATCAACTTCATTGGCTAG
P. monodon (D)     TTGCGCACATCATCCTTCACCTTGTCACTGTTAACTTCATTGGTTAA
A. irradians (D)   TTGCCCATATTATTTTACATTTGGTTGTCATCAACTTCATCGGATAG
                   * **     *  * **  * **     * ******   ** *
```

Figure 3.56 Multiple alignments of nucleotide sequences of *DAD1* of *P. monodon*, *Anopheles gambiae*, *Ixodes scapularis* and *Argopecten irradians* and *Mus musculus* and oligosaccharyltransferase subunit homolog of *M. musculus*.

```
A. gambiae (D)     --MKNLTEVLHKFYDEYTHKTPKKLKIVDAYLLYILLTGIMQFVYCCLVGTFPFNSFLAG
P. monodon (D)     MSSTSLLVVVRNFYDEYMKKTPKKLKIVDAYLFYVLLTGIIQFVFCCLVGTFPFNSFLSG
M. musculus (D)    -MSASVVSVISRFLEEYLSSTPQRLKLLDAYLLYILLTGALQFGYCLLVGTFPFNSFLSG
M. musculus (O)    -MSASVVSVISRFLEEYLNSTPQRLKLLDAYLLYILLTGALQFGYCLLVGTFPFNSFLSG
A. irradians (D)   -MPDSLFSVVKKFTDEYISSTPKKLKIVDAYLFYILLTGVIQFMYCALVGTFPFNSFLSG
I. scapularis (D)  MGSTSFWDVVKRFSEEYTTSTPKKMKIIDAYLQYVLLTGVVQFVYCCIAGTFPFNSFLSG
                       ..  *: .* :**  .**:::*::**** *:**** :** :* :.*********:*

A. gambiae (D)     FISTVSCFVLGVCLRLQSNPQNKEQFFGISPERGFADFVFAHIILHLVVVNFSG
P. monodon (D)     FISTVGCFVLGVSLRLQANPQNKMQFVGISPERGFADFIFAHIILHLVTVNFIG
M. musculus (D)    FISCVGSFILAVCLRIQINPQNKADFQGISPERAFADFLFASTILHLVVMNFVG
M. musculus (O)    FISCVGSFILAVCLRIQINPQNKADFQGISPERAFADFLFASTILHLVVMNFVG
A. irradians (D)   FISSVGSFVLGVCLRLQVNPQNKHDFTGIGPERAFADFIFAHIILHLVVINFIG
I. scapularis (D)  FITCVASFVLGVCLRLQANPLNKGQFFGISSERAYADFIFAHVVLHLVVINFIG
                   **: *..*:*.*.**:* ** ** :* **..**.:***:**  :****.:**
```

Figure 3.57 Multiple alignments of amino acid sequences of *DAD1* of *P. monodon, Anopheles gambiae*, *Ixodes scapularis* and *Argopecten irradians* and *Mus musculus* and oligosaccharyltransferase subunit homolog of *M. musculus*.

Table 3.6 Sequence divergence of *DAD1* from different taxa based on nucleotide (below diagonal) and amino acids sequences (above diagonal)

	***M. musculus* (D)**	***M. musculus* (O)**	***A. gambiae* (D)**	***I. scapularis* (D)**	***P. monodon* (D)**	***A. irradians* (D)**
***M. musculus* (D)**	-	0.00890	0.46596	0.49292	0.49292	0.35557
***M. musculus* (O)**	0.0029	-	0.46596	0.49292	0.49292	0.36975
***A. gambiae* (D)**	0.5223	0.5166	-	0.43415	0.24053	0.31792
***I. scapularis* (D)**	0.4966	0.4966	0.3686	-	0.37985	0.36975
***P. monodon* (D)**	0.5050	0.4996	0.3781	0.5044	-	0.28816
***A. irradians* (D)**	0.5534	0.5591	0.5035	0.5041	0.4356	-

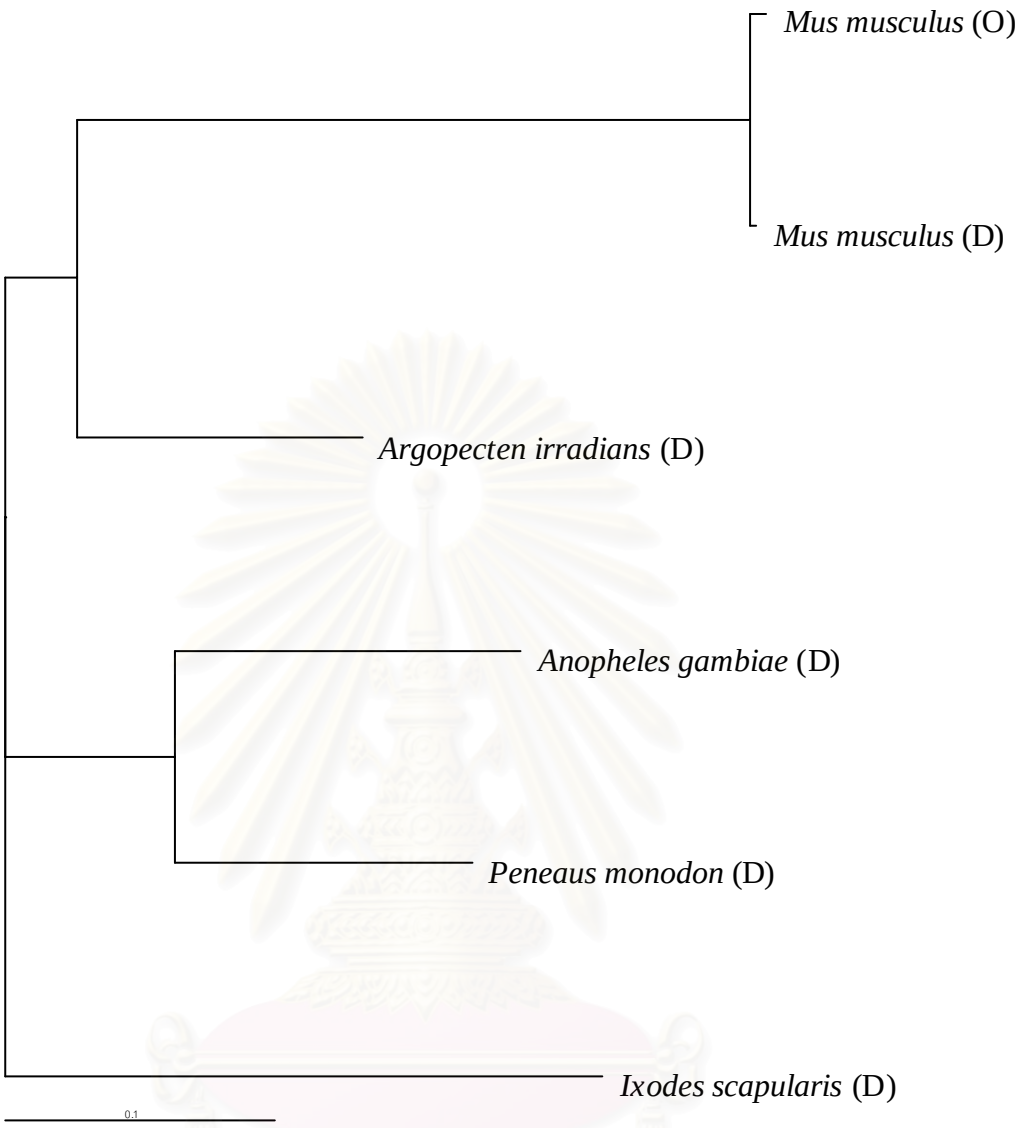

Figure 3.58 A neighbor-joining tree illustrating relations between *DAD1* of *P. monodon*, *Anopheles gambiae*, *Ixodes scapularis* and *Argopecten irradians* and *Mus musculus* and oligosaccharyltransferase subunit homolog of *M. musculus*.

3.4 Identification and Characterization of SNP in 5′ and 3′ untranslated region (UTR) of functionally important genes.

5′ and 3′-UTR of ribophorin I and *RACK* identified by RACE-PCR and those of for thioredoxin peroxidase, calponin 1 and *DAD1* identified by genome walk analysis were long enough to be analyzed by SSCP analysis for a new SNP discovery. A primer pair of each region was designed and tested for the amplification success (Figures 3.59 – 3.67).

The amplification of 5′ UTR of ribophorin I generated the faint band of 234 bp with smear nonspecific products (Figure 3.59) whereas the discrete amplification band (368 bp) was found from amplification of 3’ UTR of ribophorin I (Figure 3.60).

Likewise, the clear amplification product (277 bp) was observed from amplification of the 5′ UTR of thioredoxin peroxidase (Figure 3.61). Nevertheless, some specimens provided the faint amplification product whereas others did not provide the PCR product. The 3′UTR of this gene homologue was not successfully amplified in any investigated samples.

The fragments of 316 bp and 367 bp were obtained were obtained from amplification of the 5′ and 3′ UTR of calponin 1 (Figure 3.62 and 3.63). The amplification efficiency of both regions was consistent as revealed by comparable band intensity of each region across different individuals. Only a few individuals could not be successfully amplified at these gene regions.

The discrete fragments of 367 bp and 204 bp in length were found from amplification of the 5′ and 3′ UTR of *DAD1* across different individuals of wild *P. monodon* (Figure 3.64 and 3.65). A few individuals did not provide the amplification product. However, the band intensity of individuals generating the positive amplification results was quite comparable reflecting the consistent amplification success in these gene regions.

The intensity of the amplification product of 5′ (230 bp) and 3′ (250 bp) UTR of *RACK* was not as consistent as that of calponin 1 and *DAD1*. Some individuals provided the intense amplification band whereas others yielded the faint band. Only a few samples did not give the amplification product (Figure 3.66 and 3.67).

A.

AAGCAGTGGTATCAACGCAGAGTACGCGG**GGGAGACTGAGGTTGAGCGT**CCTTTTTACGGCCCTCCCCC
CACTCCCCGGCGCCCGTTTCCCCCCGAAGGCTTCCCCGACAGGACGTTCTCCTCCTGACAGGTGTCGGC
GGCCCGGCGTTGCGGCTCGAGAGTCCCTCGGTGGCGTCCAGG**ATG**GGTGTCCTCGGTGTCCTGTGTGTC
CTGGGTATCCTGGGGAGTGTTGTCCCCGGCGGCGCGTC**TGCCACCGCCTACGACAG**

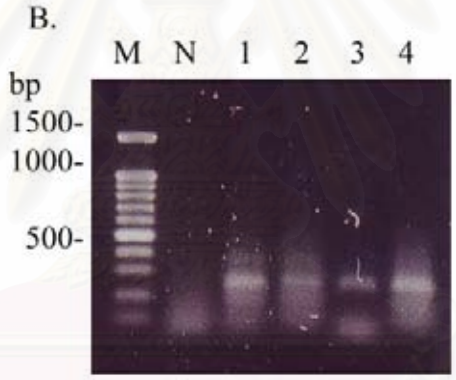

Figure 3.59 Positions of primer pairs for amplification of 5′-UTR of ribophorin I are illustrated in boldface and underlined (A). The resulting amplification product of shrimps from Chumphon (lanes 1 - 4) was electrophoretically analyzed by a 1.2% agarose gel (B). Lanes M and N are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

A.

AGACTCAGATCATCAGCGACCTGGGAGCCAAGATTCGTTTGGACAACGCAGAGTATGCTGACAAAGTGG
TTGAGCTCCAGAAGGCAGATAAGGTTCTGAGAGATAGCATTACACAGCAGCTGACCAACGTGGGAAATT
GGTGCTTGGGAAGATGACCAAACAGCAGTACATGGATGCTGAAACTCCTCTACACAAAAAGAAGGAAGA
GCAGTTGGAGAAAATGAAGACCATTTTGAGTGGCCTC**TAA**AAAGAAAGTAAATAGGATTTAGGGTTGGC
TTGGTCAATTTTTCATATGTACTTCATAATGATGTATGATTCCTAAGTTATATATCAGTAACTTTTA**AG**
TTAGGAGGAAAAGGAGAGCAAATTGACTTTATAATTTGCAAGATTCACACTAATAGTGCATTAAAGAAA
TTACCCACAAAAAAAAAAAAAAAAAAAAAAAAAA

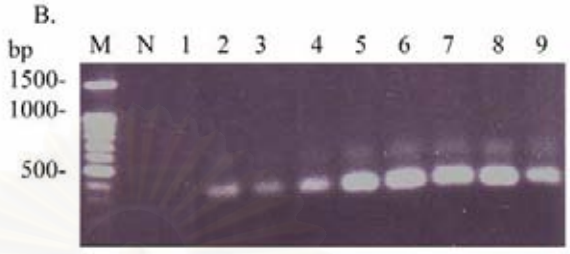

Figure 3.60 Positions of primer pairs for amplification of 3′-UTR of ribophorin I are illustrated in boldface and underlined (A). The resulting amplification product of shrimps from Chumphon (lanes 1 - 3), Trang (lanes 4 - 6), and Satun (lanes 7 - 9) was electrophoretically analyzed by a 1.2% agarose gel (B). Lanes M and N are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

A.

CGACGGCCCGGGCAGGTAAAGTCTTTGCTTGAAGATATTGAGAATCTGAAACTTTCAAATAAGACTGAA
ATTATTAGGGTATGTAGATAGGTAAATTAATAAAATGACTGGAATGTACTCTTAAGGTGGTAAGCTTTT
TAACAATTTTAAGATTTTGCAACAAGTGGTTAATGAAAATCTGAAGGTTAATCCACATGAAGCATGTGT
GCAC**ATGTTAAGCATGAGTTGGAATT**TCTGGAGACTATTATGTAATGTTTATGCCTAACCTGTTCCAAT
ATTTTATTTCTAATCCCCCCCCCACCCCCACAGAATACCAAGACCACCACC**ATG**AGCAACACTGTTCCA
GCTATTGGCAAACCTGCCCCTGTCTTCAAGGGCACTGCTGTTGTTGATGGGCAGTTCAAGGAGATCTCC
CTGGAAGATTACAAGGGCAAATATGTCATTTTCTTCTTCTACCCTTTGGATTTC**ACCTTTGTCTGCCCC**
ACTGA

Figure 3.61 A. Positions of primer pairs for amplification of 5′-UTR of thioredoxin peroxidase are illustrated in boldface and underlined (A). The resulting amplification product of shrimps from Chumphon (lanes 1 - 3), Trang (lanes 4 - 9) Satun (lanes 10 - 15) and Phangnga (lanes 16 – 17) was electrophoretically analyzed by a 1.2% agarose gel (B). Lanes M and N are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

A.

ACTATAGGGCACGCGTGGTCGACGGCCCGGGCAGGTAAAACCCTGTACAGTTATCTATACAATATGAAGCGTCAGA
TAAATGTTACCAGATATATTTAACCTCCAGTCTGTTGGAAGAATATATCTTGGCCGGAAATGTGAACCCAGACACA
CTACAGTTCGACCCTTGTTGTTAAAGGTGCACTAGAGTAAAAGAAGGTTAAT**TTCCCCGTTTGTTGGCATCA**GATT
CCTTTGGTTTTGGGTTAAAGCAGATAGAGCACCATCTATGGATTTTCTTCCCCCATCTCTAGTGCACTCTTTTTGG
GTCGACTTTCCTTCCTCCGTTGCTTTCTCTCGCGTATCTTCGAATTACTTCGTGCCATGTAATGCAATTCAGTGAT
AAGTTTGGGCATTCCTTTTAGTATTGT**ATG**TAACTCTCACCATTAGCTCTTTGTTTTCTTCTCAAATTCCTCAATA
TATATTTTCTCCAACAGGTCAACGCAAAGTACAGCGAAGAGCAG**GCCGCCGAGTGCTTGGAATG**

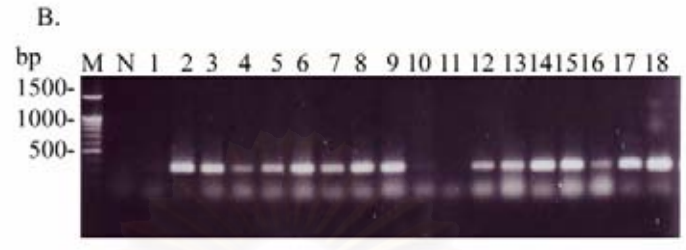

Figure 3.62 Positions of primer pairs for amplification of 5′-UTR of calponin 1 are illustrated in boldface and underlined (A). The resulting amplification product of shrimps from Chumphon (lanes 1 - 3), Trang (lanes 4 - 11), Satun (lanes 12 - 15) and Phangnga (lanes 16 – 18) was electrophoretically analyzed by a 1.2% agarose gel (B). Lanes M and N are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

A.

AGGGCTTCTGAGGGCATCGTCAACCTGCAGTATGGCTCCAACAAGGGTGCCACTCAGTCTGGCATGTCCTTCGGCA
ATACTCGCCACATGTAAAAGCAGTCTTTG**TAG**ACTTTCACTTTCACTTCATTTTTTAAAAAAAGTAGTTCAACATA
ATTCATCATGCTTCTAATATGTTCCAATATATAATAGCGGGGAGGATTTCTTTTATATATAAAAATAAAAACTGAA
AAAAATGCATTGG**CAGTGGTATGCCTAGAAAAGGA**ATTTTTACAACTGCAGTCTTAGGCAAAGAAATGAATGTAAA
AAAGGATGAAATCAGACATGTATCACTTGACCAATAGGTTGCTACAATTTTTATTACATTGC

Figure 3.63 Positions of primer pairs for amplification of 3′-UTR of calponin 1 are illustrated in boldface and underlined (A). The resulting amplification product of shrimps from Chumphon (lanes 1 - 3), Trang (lanes 4 - 9) Satun (lanes 10 - 15) and Phangnga (lanes 16 – 17) was electrophoretically analyzed by a 1.2% agarose gel (B). Lanes M and N are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

A.

ACTATAGGGCACGCGTGGTCGACGGCCCGGGCAGGTCCAGGGTTAACCGCAGCACCAGTGCCTCCACAG
AGAGGTGAAGAATGGTGTGTCTAGCATGTGGACTTCTATTGTAATTTGTTTTGGAGCATT*GTGTA*GTAG
CAAGGATAGATGTACTTGTATATTCCTTAGATTTGTGG**TGAGGAGGTGTAGTTGTTGTTTG**TGTATGAT
TGTGTATTATTGCATGAAGAGTAAGTGCTAACAGTTCACATAATGAGAATAAAAATTGCCATATTTTGA
CCTTTTAGGAGTCTAAACGATGTCACATGTGGACATGGTGATATTGAATATGGTATAATATTATAATCT
GGAAGGGTGAAATGCCCAAGGGTAATTTTTGTCTGTACCTAACCATTTATCAGATTGAAAAATCTAAAT
TTGTCCTCTCATTCCAGTAACCAACC**ATG**TCTTCAACGTCACTATTGGTGGTTGTCAGAAATTTCTATG
ATGAGTACATGAAGAAAACTCCCAAGAAATTAAAGATCGT**CGATGCCTACCTCTTCTACG**

Figure 3.64 Positions of primer pairs for amplification of 5′-UTR of *DAD1* are illustrated in boldface and underlined (A). The resulting amplification product of shrimps from Chumphon (lanes 1 - 3), Trang (lanes 4 - 9) Satun (lanes 10 - 14) and Phangnga (lanes 15 – 16) was electrophoretically analyzed by a 1.2% agarose gel (B). Lanes M and N are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

A.

CAAAATGCAGTTTGTTGGCATCTCCCCAGAACGAGGCTTTGCTGATTTCATCTTTGCGC**ACATCATCCT**
TCACCTTGTCACTGTTAACTTCATTGGT**TAA**CAAAGGGACGATCAAGTATTTTGTTGGTTTTCCTTAAG
GATGTGAAATGTTCTAAGATCATGTGTAAAGGATATAAACATTCACTAAAGAACCCATGTTATTTTCAA
AGAAGCAGATTAATTTACATTCCCATGTGCGTGT**AAGGTTTAACGATTGGAGTTTG**TTCATGAAGTC

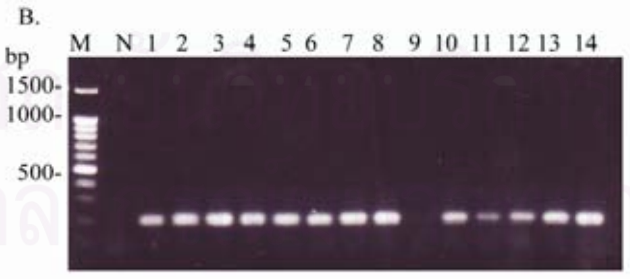

Figure 3.65 Positions of primer pairs for amplification of 3′-UTR of *DAD1* are illustrated in boldface and underlined (A). The resulting amplification product of shrimps from Chumphon (lanes 1 - 3), Trang (lanes 4 - 10), and Satun (lanes 11 - 18) was electrophoretically analyzed by a 1.2% agarose gel (B). Lanes M and N are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

A.

AAGCAGTGGTATCAACGCAGAGTACGCGGGGCTGTGTTGAAATAGTGGTTATACTGAGTGTATTA**CACT**
TGTGTAAGACGAATGATGGTTAAGAGATACAGATAAATTCGATCGCGTTCGGCATTGTGTACAAAGCGG
TTCCAGCCAGATAGATGACTAGAGAAACGTA**ATG**AATGAGAGCTTACAGCTGCGCGGGACCCTGGTGGG
CCACAATGGCTGGGTCACACAGATCGCCACCAACAGGAATTTCCCTGACATGATCCTGTC**TGCTTCCAG**
GGACAAATCTCTGATTCTG

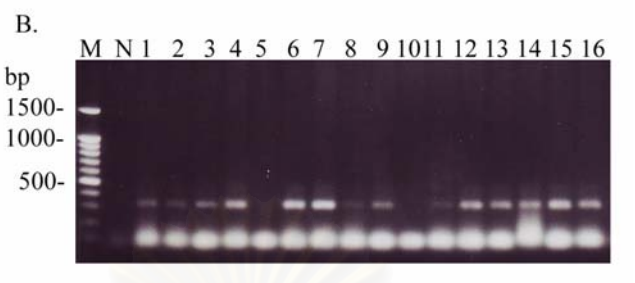

Figure 3.66 Positions of primer pairs for amplification of 5′-UTR of *RACK* are illustrated in boldface and underlined (A). The resulting amplification product of shrimps from Chumphon (lanes 1 - 3), Trang (lanes 4 - 8), and Satun (lanes 9 - 16) was electrophoretically analyzed by a 1.2% agarose gel (B). Lanes M and N are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

A.

CAACCGTTATTGGCTGTGTGCCGCTACTGGCCCATCTATCAAGATCTGGGATTTGGAAGGCAAGAATAT
GGTCGATGAATTGAAGCCCGATGTGATTACCCAGAACCCTAAGGCTGAACCCCCACAGTGTCTCTCCAT
GGCCTGGTCTGCAGATGGGCAAACTCTGTTTGCTGGTTACAGTGACAGCAAGATCCGTGTCTGGCAGGT
CAGCGTTACTTCCCGTGCG*T***AAGGGAATGTTCTGGAGTGCTGC**AATAAAGATTGCTTGATAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAA

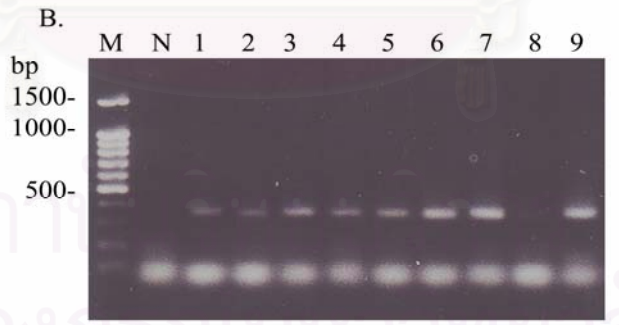

Figure 3.67 Positions of primer pairs for amplification of 3′-UTR of *RACK* are illustrated in boldface and underlined (A). The resulting amplification product of shrimps from Chumphon (lanes 1 - 3), Trang (lanes 4 - 7), and Satun (lanes 8 - 9) was electrophoretically analyzed by a 1.2% agarose gel (B). Lanes M and N are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

The amplification product of different gene regions in each gene homologue was analyzed by SSCP (Figures 3.68 – 3.72). Taken both the amplification success rate and polymorphic SSCP patterns of the amplified product into the account, the amplified exon of *DAD1*, *RACK* and thioredoxin peroxidase, the amplified exon/intron of ribophorin I and the amplified 5′UTR of calponin 1 were chosen for association analysis between SNP (through SSCP) and expression levels of these functionally important genes.

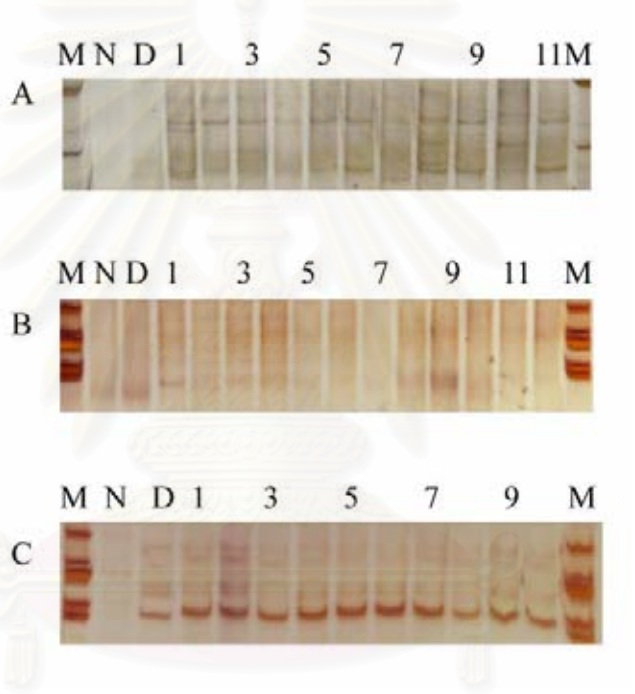

Figure 3.68 SSCP patterns of exon/intron (A) 5′-UTR (B) and 3′-UTR (C) of ribophorin I gene segments amplified from non-related individuals of wild *P. monodon*.

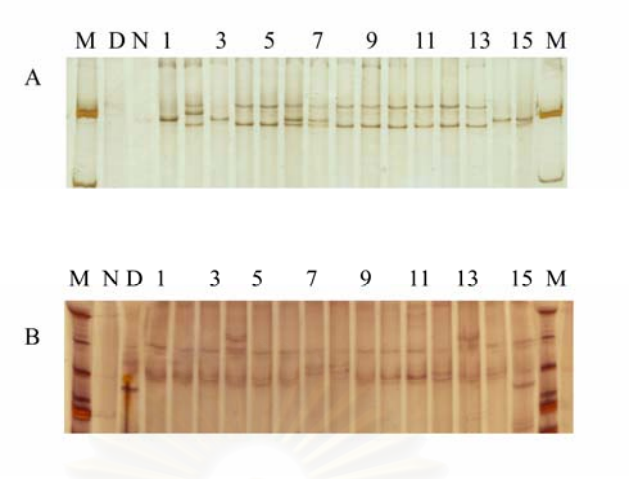

Figure 3.69 SSCP patterns of exon (A) and 5′-UTR (B) of thioredoxin peroxidase gene segments amplified from non-related individuals of wild *P. monodon*.

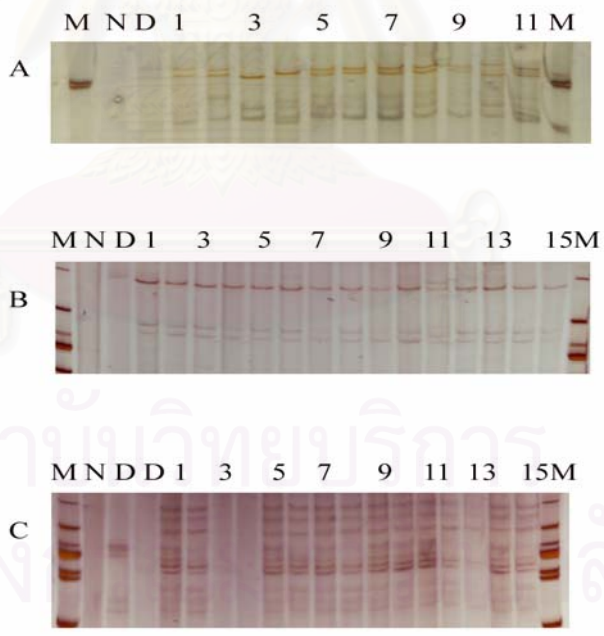

Figure 3.70 SSCP patterns of exon (A), 5′-UTR (B) and 3′-UTR (C) of calponin 1 gene segments amplified from non-related individuals of wild *P. monodon*.

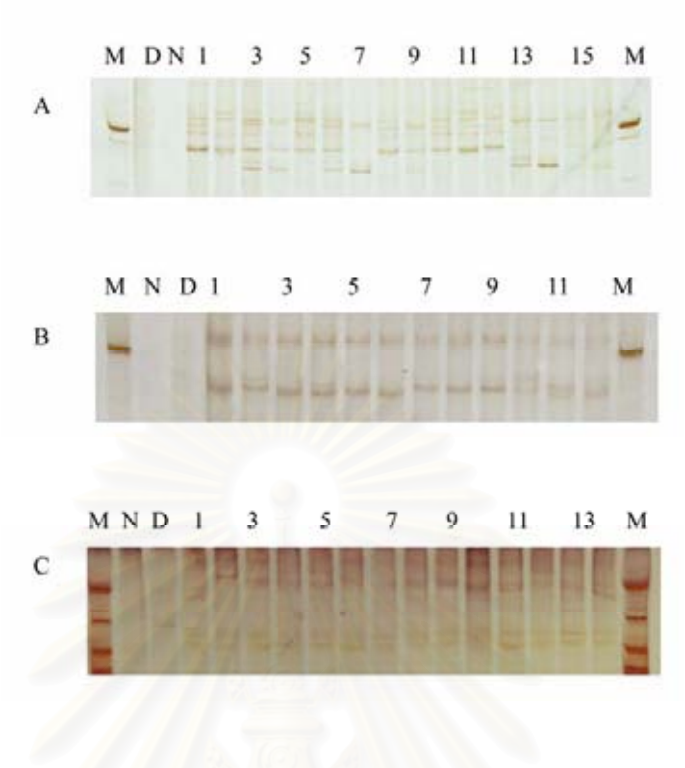

Figure 3.71 SSCP patterns of exon (A), 5′-UTR (B) and 3′-UTR (C) of *DAD1* gene segments amplified from non-related individuals of wild *P. monodon*.

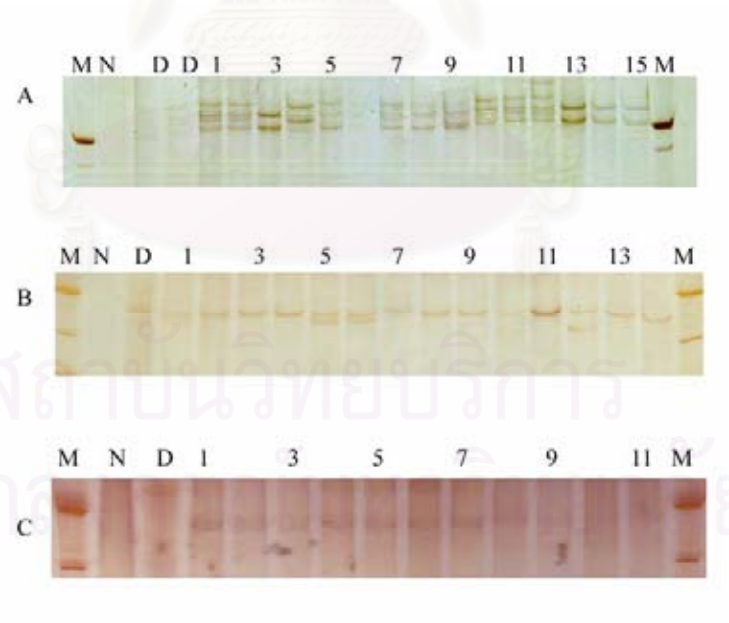

Figure 3.72 SSCP patterns of exon (A), 5′-UTR (B) and 3′-UTR (C) of *RACK* gene segments amplified from genomic DNA of non-related individuals of wild *P. monodon*.

3.5 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 of the same fragment. Accordingly, nucleotide of *P. monodon* exhibited different (or identical) SSCP patterns was compared.

Four SSCP patterns were found from polymorphism of the 5′ UTR of calponin 1. As can be seen in Figure 3.58, the SNP genotypes of PM1 (GGC-GGTT), PM2 (--G-GCTT), PM3 (GCCGGTTT) and PM4 (GCC-ACCG) are different. This implies that all different SSCP patterns of the 5′ UTR of calponin 1 represented different SNP (Figure 3.73).

Likewise, 7 SSCP patterns of 5'UTR of *DAD1* were initially observed from screening of wild *P. monodon*. A representative individual of each SSCP pattern was further analyzed by direct DNA sequencing. PM12 (ATGCTATCAATGATGT------GT), PM20 (ATGCTATCAATGGAGC------GT), PM22 (ATACTATCAATGATGCAAATTTGT), PM10 (GTACTATTAATAATAC------GT) and PM11 (GCAAGTTA---AATAC------CA) showed different DNA barcodes as detected by SSCP. Nevertheless, PM5 and PM1 which showed different SSCP patterns shared the same DNA barcode (GCAAGTTA---AATAC------CA) (Figure 3.74). This indicated the possible false positive of SSCP and the accuracy of the technique was reduced to 85.71%.

A large number of SSCP patterns were found from the *RACK* gene segment. Representatives of these genotypes (PM1, PM2 and PM7 for the pattern I, PM3, PM13, PM14 and PM15 for the pattern II, PM4 for the pattern III, PM5 and PM8 for the pattern IV, PM12 for the pattern V, PM9 for the pattern 9 for the pattern VI and PM10 and PM11 for the pattern VII) were further analyzed (Figure 3.75). Results indicated different DNA barcodes of shrimps exhibiting different SSCP genotypes. However, SSCP may underestimate levels of SNP variation for example, PM 1 (and PM2) and PM7 which share an identical SSCP pattern have different DNA barcodes. Likewise, PM13 (and PM14), PM3 and PM15 exhibiting the same SSCP genotype revealed different barcodes. Moreover, PM5 and PM8 (SSCP pattern IV) as well as

PM10 and PM11 (SSCP pattern VII) also showed different nucleotide sequences within groups.

```
PM1             TTCCCCGTTTGTTGGCATCAGATTCCTTTGGTTTTGGGTTAAAGCAGATAGAGCACCATC
PM2             TTCCCCGTTTGTTGGCATCAGATTCCTTTGGTTTTGGGTTAAAGCAGATAGA-CAC-ATC
PM3             TTCCCCGTTTGTTGGCATCAGATTCCTTTGGTTTTGGGTTAAAGCAGATAGAGCACCATC
PM4             TTCCCCGTTTGTTGGCATCAGATTCCTTTGGTTTTGGGTTAAAGCAGATAGAGCACCATC
                ************************************************ *** ***

PM1             TATGGATTTTCTTCCCCCA-TCTCTAGTGCACTCTTTTTGGGTCGACTTTCCTTCCTCCG
PM2             TATGGATTTTCTTCCCCCA-TCTCTAGTGCACTCTTTTTGGGTCGACTTTCCTTCCTCCG
PM3             TATGGATTTTCTTCCCGCAGTCTCTAGTGCACTCTTTTTGGGTCGACTTTCCTTCCTCCG
PM4             TATGGATTTTCTTCCCCCA-TCTCTAGTGCACTCTTTTTGGGTCGACTTTCCTTCCTCCG
                *************** **  ************************************

PM1             TTGCTTTCTCTCGCGTATCTTCGAATTACTTCGTGCCATGTAATGCAATTCAGTGATAAG
PM2             TTGCTTTCTCTCGCGTATCTTCGAATTACTTCGTGCCATGTAATGCAATTCAGTGATAAG
PM3             TTGCTTTCTCTCGCGTATCTTCGAATTACTTCGTGCCATGTAATGCAATTCAGTGATAAG
PM4             TTGCTTTCTCTCACGTATCTTCGAATTACTTCGTGCCATGTAATGCAATTCAGTGATAAG
                ************ ************************************************

PM1             TTTGGGCATTCCTTTTAGTATTGTATGTAACTCTCACCATCAGCTCTTTGTTTTCTTCTC
PM2             TTTGGGCATTCCTTTTAGTATTGTATGTAACTCTCACCATCAGCTCTTTGTTTTCTTCTC
PM3             TTTGGGCATTCCTTTTAGTATTGTATGTAACTCTCACCATTAGCTCTTTGTTTTCTTCTC
PM4             TTTGGGCATTCCTTTTAGTATTGTATGTAACTCTCACCATCAGCTCTCTGGTTTCTTCTC
                ************************************* ****** ** *********

PM1             AAATTCCTCAATATATATTTTCTCCAACAGGTCAACGCAAAGTACAGCGAAGAGCAGGCC
PM2             AAATTCCTCAATATATATTTTCTCCAACAGGTCAACGCAAAGTACAGCGAAGAGCAGGCC
PM3             AAATTCCTCAATATATATTTTCTCCAACAGGTCAACGCAAAGTACAGCGAAGAGCAGGCC
PM4             AAATTCCTCAATATATATTTTCTCCAACAGGTCAACGCAAAGTACAGCGAAGAGCAGGCC
                ************************************************************

PM1             GCCGAGTGCTTGGAATG
PM2             GCCGAGTGCTTGGAATG
PM3             GCCGAGTGCTTGGAATG
PM4             GCCGAGTGCTTGGAATG
                *****************
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Figure 3.73 Multiple alignments of 5′ UTR sequences of calponin 1. Positions of primers are highlighted. SNP (and indels) positions are illustrated in boldface and underlined.

```
PM12            TGAGGAGGTGTAGTTGTTGTTTGTGTATGATTATGTATTATTGCGTGAAGAGTAAGTGCT
PM20            TGAGGAGGTGTAGTTGTTGTTTGTGTATGATTATGTATTATTGCGTGAAGAGTAAGTGCT
PM22            TGAGGAGGTGTAGTTGTTGTTTGTGTATGATTATGTATTATTGCATGAAGAGTAAGTGCT
PM5             TGAGGAGGTGTAGTTGTTGTTTGTGTATGATTGTGTATTATTGCATGAAGAGTAAGTGCT
PM1             TGAGGAGGTGTAGTTGTTGTTTGTGTATGATTGTGTATTATTGCATGAAGAGTAAGTGCT
PM10            TGAGGAGGTGTAGTTGTTGTTTGTGTATGATTGTGTATTATTGCATGAAGAGTAAGTGCT
PM11            TGAGGAGGTGTAGTTGTTGTTTGTGTATGATTGTGTATTACTGCATGAAGAGTAAGTGAT
                ******************************* ******* *** ************* *
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PM12            AACAGTTCACATAATGAGAATAAAAATTGCCATATTTTGACCTTTTAGGAGTCTAAACGA
PM20            AACAGTTCACATAATGAGAATAAAAATTGCCATATTTTGACCTTTTAGGAGTCTAAACGA
PM22            AACAGTTCACATAATGAGAATAAAAATTGCCATATTTTGACCTTTTAGGAGTCTAAACGA
PM5             AACAGTTCACATAATGAGAATAAAAATTGCCATATTTTGACCTTATAGGAGTCTAAACGA
PM1             AACAGTTCACATAATGAGAATAAAAATTGCCATATTTTGACCTTATAGGAGTCTAAACGA
PM10            AACAGTTCACATAATGAGAATAAAAATTGCCATATTTTGACCTTTTAGGAGTTTAAACGA
PM11            AACAGTTCACATAAGGAGAATAAAAATTGCCATATTTTGTCCTTTTAGGAGTATAAACGA
                ************** ************************ **** ******* *******

PM12            TGTCACATGTGGACATGGTGATATTGAATATGGTATAATATTGTAATCTGGAAGGGTGAA
PM20            TGTCACATGTGGACATGGTGATATTGAATATGGTATAATATTGTAATCTGGAAGGGTGAA
PM22            TGTCACATGTGGACATGGTGATATTGAATATGGTATAATATTGTAATCTGGAAGGGTGAA
PM5             TGTCACATGTGGACATGGTGATATTGAATATGGTATAATATTATAATCTGGAAGGGTGAA
PM1             TGTCACATGTGGACATGGTGATATTGAATATGGTATAATATTATAATCTGGAAGGGTGAA
PM10            TGTCACATGTGGACATGGTGATATTGAATATGGTATAATATTATAATCTGGAAGGGTGAA
PM11            TGTCACATGTGGACATGGTGATATTGAATATGGTAT---ATTATAATCTGGAAGGGTGAA
                ************************************   ***  ****************

PM12            ATGCCCAAGGGTAATTTTTGTCTGTACCTAACCATTTATCAGATTGAAAAATCTAAATTT
PM20            ATGCCCAAGGGTAATTTTTGTCTGTACCTGACCATTTATCAGATTGAAAAATCTAAATTT
PM22            ATGCCCAAGGGTAATTTTTGTCTGTACCTAACCATTTATCAGATTGAAAAATCTAAATTT
PM5             ATGCCCAAGGGTAATTTTTGTCTGTACCTAACCATTTATCAGATTGAAAAATCTAAATTT
PM1             ATGCCCAAGGGTAATTTTTGTCTGTACCTAACCATTTATCAGATTGAAAAATCTAAATTT
PM10            ATGCCCAAGGGTAATTTTTGTCTGTACCTAACCATTTATCAGATTGAAAAATCTAAATTT
PM11            ATGCCCAAGGGTAATTTTTGTCTGTACCTAACCATTTATCAGATTGAAAAATCTAAATTT
                ****************************** ******************************

PM12            GTCCTCTCATTCCAGTAACCAACCATGTCTTCAACGTCATTATTGGTGGTTGTCAGAAAT
PM20            GTCCTCTCATTCCAGAAACCAACCATGTCTTCAACGTCACTATTGGTGGTTGTCAGAAAT
PM22            GTCCTCTCATTCCAGTAACCAACCATGTCTTCAACGTCACTATTGGTGGTTGTCAGAAAT
PM5             GTCCTCTCATTCCAGTAACCAACCATGTCTTCAACATCACTATTGGTGGTTGTCAGAAAT
PM1             GTCCTCTCATTCCAGTAACCAACCATGTCTTCAACATCACTATTGGTGGTTGTCAGAAAT
PM10            GTCCTCTCATTCCAGTAACCAACCATGTCTTCAACATCACTATTGGTGGTTGTCAGAAAT
PM11            GTCCTCTCATTCCAGTAACCAACCATGTCTTCAACATCACTATTGGTGGTTGTCAGAAAT
                *************** ******************** *** ********************

PM12            TT------CTATGATGAGTACATGAAGAAAACTCCCAAGAAATTAAAGATCGTCGATGCC
PM20            TT------CTATGATGAGTACATGAAGAAAACTCCCAAGAAATTAAAGATCGTCGATGCC
PM22            TTAAATTTCTATGATGAGTACATGAAGAAAACTCCCAAGAAATTAAAGATCGTCGATGCC
PM5             TT------CTATGATGAGTACATGAAGAAAACTCCCAAGAAATTAAAGATCGTCGATGCC
PM1             TT------CTATGATGAGTACATGAAGAAAACTCCCAAGAAATTAAAGATCGTCGATGCC
PM10            TT------CTATGATGAGTACATGAAGAAAACTCCCAAGAAATTAAAGATCGTCGATGCC
PM11            TT------CTATGATGAGTACATGAAGAAAACTCCCAACAAAATAAAGATCATCGATGCC
                **      ****************************** ***  ******************

PM12            TACCTCTTCTAG
PM20            TACCTCTTCTAG
PM22            TACCTCTTCTAG
PM5             TACCTCTTCTAG
PM1             TACCTCTTCTAG
PM10            TACCTCTTCTAG
PM11            TACCTCTTCTAG
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Figure 3.74 Multiple alignments of 5′UTR of *DAD1*. Positions of primers are highlighted. SNP (and indels) positions are illustrated in boldface and underlined.

```
PM1             GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM2             GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM11            GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM3             GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM10            GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM4             GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM9             GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM15            GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM7             GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGGTAGAAGAGGACGTTTCCCATTTGGTG
PM5             GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM14            GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM13            GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
PM12            GGAAGCTTTTTTGTTTTACATTTTTTTTTATTAGTTAGAAGAGGACGTTTCCCATTTGGTG
                **********************************************************

PM1             AGTATGATGCCTTGTTCACTTCATGGAAGNTAATTGTTTCAACTTGGNAGTCTCCAGTTA
PM2             AGTATGATGCCTTGTTCACTTCATGGAAGNTAATTGTTTCAACTTGGNAGTCTCCAGTTA
PM11            AGTATGATGCCTTGTTCACTTCATGGAAGNTAATTGTTTCAACTTGGNAGTCTCCAGTTA
PM3             AGTATGATGCCTTGTTCACTTCATGGAAGGTAATTGTTTCAACTTGGGAGTCTCCAGTTA
PM10            AGTATGATGCCTTGTTCACTTCATGGAAGGTAATTGTTTCAACTTGGNAGTCTCCAGTTA
PM4             AGTATGATGCCTTGTTCACTTCATGGAAGCTAATTGTTTCAACTTGGAAGTCTCCAGTTA
PM9             AGTATGATGCCTTGTTCACTTCATGGAAGCTAATTGTTTCAACTTGGAAGTCTCCAGTTA
PM15            AGTATGATGCCTTGTTCACTTCATGGAAGCTAATTGTTTCAACTTGGAAGTCTCCAGTTA
PM7             AGTATGATGCCTTGTTCACTTCATGGAAGCTAATTGTTTCAACTTGGAAGTCTCCAGTTA
PM5             AGTATGATGCCTNGTTCACTTCATGGAAGCTAATNGTTTCAACTTGGAAGTCTCCAGTTA
PM14            AGTATGATGCCTTGTTCACTTCATGGAAGCTAATTGTTTCAACTTGGAAGTCTCCAGTTA
PM13            AGTATGATGCCTTGTTCACTTCATGGAAGCTAATTGTTTCAACTTGGAAGTCTCCAGTTA
PM12            AGTATGATGCCTTGTTCACTTCATGGAAGNTAATTGTTTCAACTTGGNAGTCTCCAGTTA
                ************ **************** **** ************ ************

PM1             AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM2             AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM11            AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM3             AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM10            AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM4             AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM9             AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM15            AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM7             AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM5             AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM14            AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM13            AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
PM12            AGTCTTGTCTCCACCAAAAGTACAGTTTCTTGGTTCAGAAATGTCCTTTGCATGTTTGCC
                ************************************************************

PM1             ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM2             ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM11            ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM3             ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM10            ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM4             ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM9             ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM15            ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM7             ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM5             ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM14            ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM13            ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
PM12            ACTAATGAATTCCTTCCAGGTAATGGATTCCAGAACAAGATAATTTTTTTTCCAGGATGT
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PM1             GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM2             GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM11            GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM3             GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM10            GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM4             GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM9             GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM15            GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM7             GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM5             GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM14            GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM13            GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
PM12            GCTCTCTGTTGCTTTCAGTGCTGATAACCGTCAGATTGTGTCTGGCTCCCGTGACAAAAC
                ************************************************************

PM1             CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM2             CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM11            CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM3             CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM10            CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM4             CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM9             CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM15            CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM7             CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM5             CATCAAGGTAAGTTTACTAAAGTTTTGNATTGTTATCATTGAACATTTTGTAATGACCAT
PM14            CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM13            CATCAAGGTAAGTTTACTAAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
PM12            CATCAAGGTAAGTTTACTNAAGTTTTGAATTGTTATCATTGAACATTTTGTAATGACCAT
                ****************** ******** *********************************

PM1             GTAATACACAATACAAGTACACATTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
PM2             GTAATACACAATACAAGTACACATTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
PM11            GTAATACACAATACAAGTACACATTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
PM3             GTAATACACAATACAAGTACACATTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
PM10            GTAATACACAATACAAGTACACATTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
PM4             GTAATACACAATACAAGTACACATTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
PM9             GTAATACACAATACAAGTACACATTATAATTGGNTTCATTTTCAGCTGTGGAATACACTT
PM15            GTAATACACAATACAAGTACACATTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
PM7             GTAATACACAATACAAGTACACATTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
PM5             GTAATACACAATACAAGTACACATTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
PM14            GTAATACACAATACAAGTACACATTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
PM13            GTAATACACAATACAAGTACACATTATAATTGNCTTCATTTTCAGCTGTGGAATACACTT
PM12            GTAATACACAATACAAGTACACCTATAATTGGCTTCATTTTCAGCTGTGGAATACACTT
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PM1             GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGTAAGG
PM2             GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGTAAGG
PM11            GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGTAAGG
PM3             GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGTAAGG
PM10            GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGTAAGG
PM4             GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGACTGGGTGTCCTGCGTAAGG
PM9             GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGACTGGGTGTCCTGCGTAAGG
PM15            GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGACTGGGTGTCCTGCGTAAGG
PM7             GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGTAAGG
PM5             GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGTAAGG
PM14            GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGTAAGG
PM13            GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGATTGGGTGTCCTGCGTAAGG
PM12            GCCCAGTGCAAATACACAATCCAGGAGGATGGTCACTCTGACTGGGTGTCCTGCGTAAGG
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PM1             TT
PM2             TT
PM11            TT
PM3             TT
PM10            TT
PM4             TT
PM9             TT
PM15            TT
PM7             TT
PM5             TT
PM14            TT
PM13            TT
PM12            TT
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Figure 3.75 Multiple alignments of *RACK*. Positions of primers are highlighted. SNP (and indels) positions are illustrated in boldface and underlined.

Direct sequencing of ribophorin I and the 5′ UTR of thioredoxin peroxidase was unsuccessful. Presumably, the amplification product of each gene homologue may contain different target fragments having identical sizes but different sequences.

3.6 PCR-RFLP and PCR-allele specific amplification (PASA)

From direct sequencing of the 5′ UTR of *DAD1*, interesting SNP was found. The major allele C_{113} was detected along with the variant alleles, T_{113} or A_{113} the allele C can be distinguish from other by digestion with *Hin*f I (the expected product of 113 and 259 bp). Likewise the allele T can be differentiated by digestion with *Dra* I where the allele A (and G) could not be digested with those restriction enzymes. In addition, indels; AAATTT and AAT could be recognized by *Dra* I and *Ssp* I, respectively. This information opens the possibility to simplify the detection technique from SSCP to PCR-RFLP.

The preliminary results from digestion of the 5′ UTR of *DAD1* with *Dra* I indicated that 3 individuals (lanes 2 – 4, Figure 3.76A) could not be digested with this enzyme, therefore, these shrimps did not possess the T allele. Further digestion of the same specimens with *Hin*f I, only the 259 bp fragment was obtained indicating that these shrimps possessed the CC genotype (Figure 3.76B).

Likewise, one of the investigated specimens partially digested with *Dra* I (372 bp, 259 bp and possible 113 bp fragments (Figure 3.76A). Therefore, this shrimp was regarded as the heterozygote individual carrying T and the other alleles. Direct sequencing indicated that only T was found at this SNP position. The 5' UTR of *DAD1* revealed heterozygotic status of this individual by partial digestion with *Hin*f I. As a result, this shrimp should carry a TC allele at SNP_{113} (Figure 3.76B).

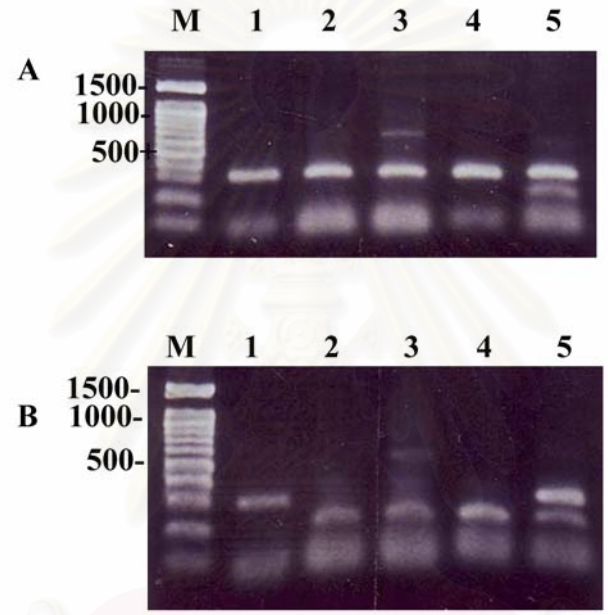


Figure 3.76 PCR-RFLP of the 5′UTR of *DAD1* digested with *Dra* I (A) and *Hin*f I (B) for simplification of SNP detection. Lanes M and 1 are a 100 bp DNA marker and undigested PCR product, respectively.

PASA of the 5′UTR of *DADI* and calponin1 was carried out by both typical and base mismatching approach. Primers were designed to detect A276G in the former and G123A and C231T in the latter. Nevertheless, results were not consistent and required further optimization of the amplification conditions. Therefore, simplification of SNP detection by PASA was not carried out further.

3.7 Dertermination of expression level of functionally important genes *in P. monodon* by semiquantitative RT-PCR

3.7.1 RT-PCR of five functionally important genes in *P. monodon* .

Calponin I, *DAD1* and *RACK* were originally isolated from the hemocyte cDNA library whereas thioredoxin peroxidase and ribophorin I were isolated from ovarian cDNA library. Therefore, the expression patterns of these transcripts were investigated in other tissues like ovaries and testes. All transcripts were expressed in hemocytes, ovaries and testes but ovaries showed greater levels of expression than testes and hemocytes. Therefore, expression levels of these functionally important transcripts were further semiquantitatively examined in ovaries and testes of juvenile and broodstock *P. monodon* (Figure 3.77 - 3.81).

3.7.2 Optimization of PCR conditions for Semi-quantitative RT-PCR

To carry out semiquantitative RT-PCR, several parameters of the amplification and PCR components required further optimization. As a result, primer and $MgCl_2$ concentrations and the number of amplification cycles were carefully optimized. Elongation factor 1α *(EF-1α)* was used as the internal control. The chosen parameter for each factor was that generating the highest specificity with the relatively intense product. The standard RT-PCR was carried out by using 100 ng of the first strand cDNA template from ovaries and testes of juvenile *P. monodon* (approximately 15 - 20 g body weight) at the annealing temperature of 55 °C, 1 U of Dynazyme DNA polymerase and 0.2 μM of each primer and various $MgCl_2$ concentrations for XX cycles. After the most suitable $MgCl_2$ concentration was chosen. RT-PCR was carried out using an optimized $MgCl_2$ concentration for further optimization of primer concentration. Finally, selected primer and $MgCl_2$ concentrations were included for optimization of the suitable number of amplification cycles. The number of cycles that still provided the PCR product in the exponential rage and did not reach a plateau level of amplification was chosen.

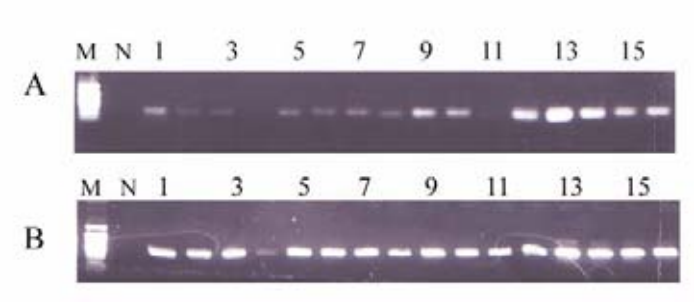


Figure 3.77 RT-PCR of ribophorin I (A) against the first strand cDNA of hemocytes of male (lanes 1-4) and female (lanes 5-8), testes (lanes 9-12) and ovaries (lanes 13-16) of juveniles *P. monodon. EF-1α* was included using the first strand cDNA template from the same individuals (B).

Figure 3.78 RT-PCR of thioredoxin peroxidase (A) against the first strand cDNA of hemocytes of male (lanes 1-4) and female (lanes 5-8), testes (lanes 9-12) and ovaries (lanes 13-16) of juveniles *P. monodon. EF-1α* was included using the first strand cDNA template from the same individuals (B).

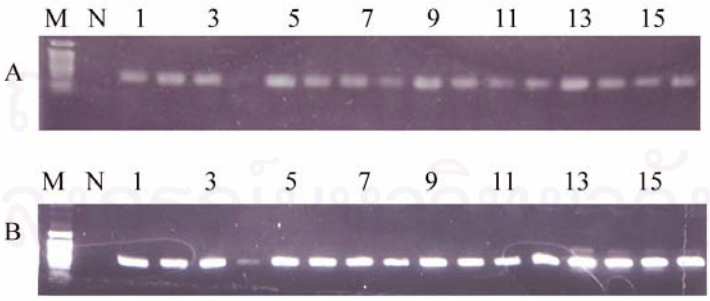


Figure 3.79 RT-PCR of calponin 1 (A) against the first strand cDNA of hemocytes of male (lanes 1-4) and female (lanes 5-8), testes (lanes 9-12) and ovaries (lanes 13-16) of juveniles *P. monodon. EF-1α* was included using the first strand cDNA template from the same individuals (B).

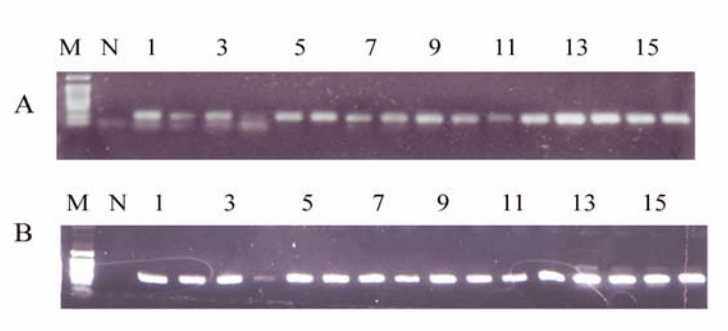


Figure 3.80 RT-PCR of *DAD1* (A) against the first strand cDNA of hemocytes of male (lanes 1-4) and female (lanes 5-8), testes (lanes 9-12) and ovaries (lanes 13-16) of juveniles *P. monodon. EF-1α* was included using the first strand cDNA template from the same individuals (B).

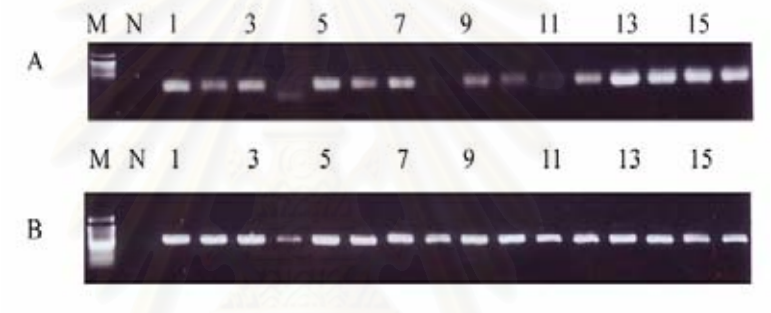


Figure 3.81 RT-PCR of *RACK* (A) RT-PCR of *ribophorin I* (A) against the first strand cDNA of hemocytes of male (lanes 1-4) and female (lanes 5-8), testes (lanes 9-12) and ovaries (lanes 13-16) of juveniles *P. monodon. EF-1α* was included using the first strand cDNA template from the same individuals (B).

The most suitable condition for amplification of *EF-1α* was 100 ng of the cDNA template at an annealing temperature of 55 °C, 1 U of Dynazyme DNA polymerase with optimized parameters including 1.5 mM of $MgCl_2$ and 0.2 μM of each primer. The amplification cycles included predenaturation at 94 °C for 5 minutes followed by 23 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 45 seconds. The final extension was carried out at 72°C for 7 minutes.

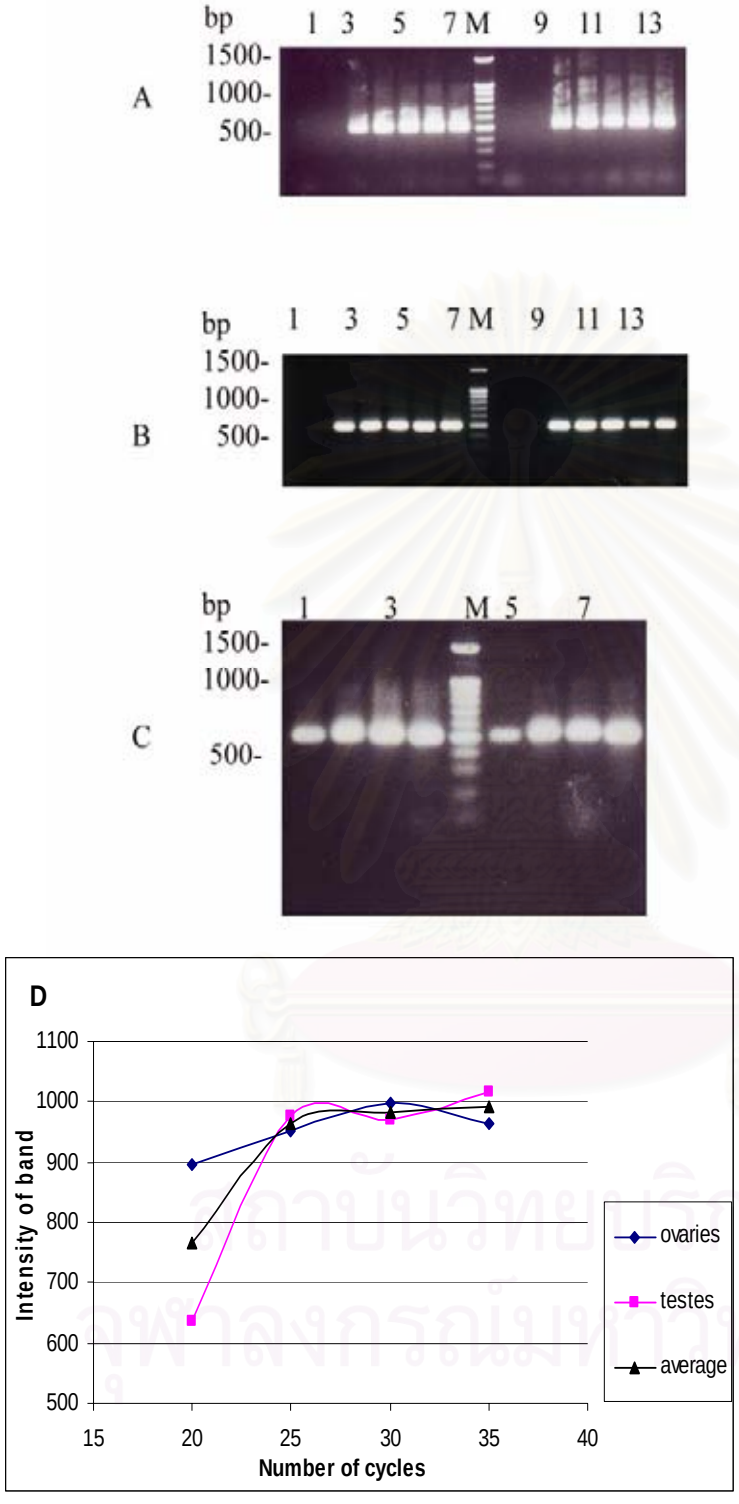


Figure 3.82 Optimization of the most suitable RT-PCR components for semiquantitative analysis of *EF 1α* (the internal control). RT-PCR was carried out using different concentrations of $MgCl_2$ (0, 1, 1.5, 2, 3, and 4 mM) against the template from ovaries (Lanes 2 – 7, A) and testes (Lanes 9 - 14, A) and different concentrations of primers (0, 0.1, 0.15, 0.2, 0.25 and 0.3 μM) against the cDNA template from ovaries (Lanes 1 – 7, B) and testes (Lanes 8 – 14, B). The number of RT-PCR cycles was carried out against the first strand cDNA of ovaries (lanes 1 - 4, C) and testes (lanes 5 – 8, C) for 20 (lanes 1 and 5, C), 25 (lanes 2 and 6, C), 30 (lanes 3 and 7, C) and 35 (lanes 4 and 8, C) cycles. Lands M are a 100 bp DNA ladder. Lanes 1 and 8 are the negative control (without the cDNA template). The intensity of amplified products was plotted against the number of amplification cycles (D).

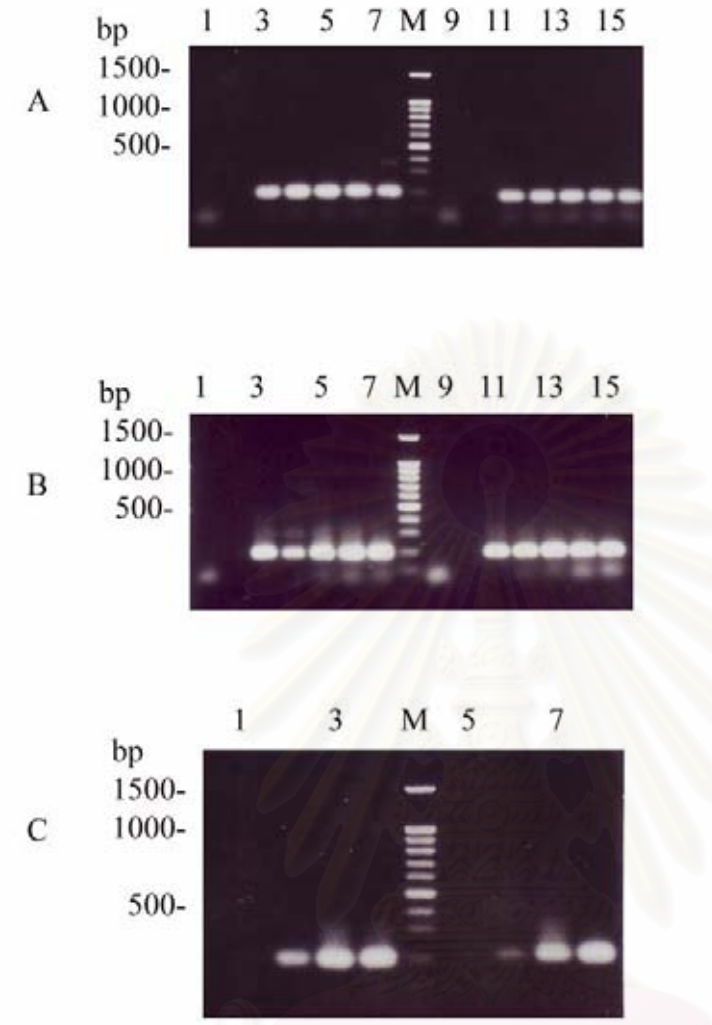
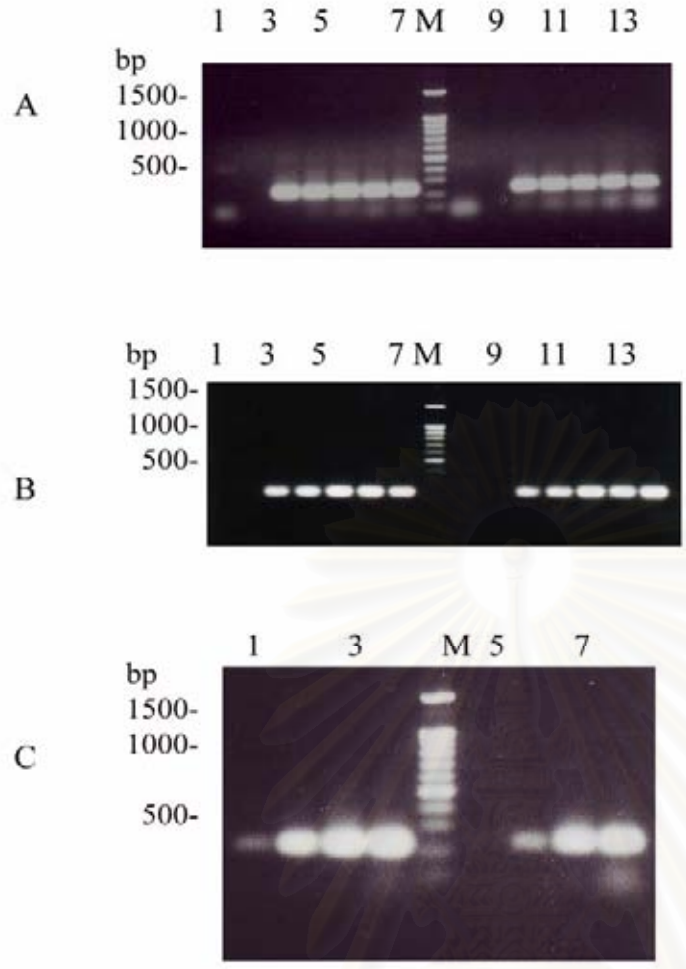


Figure 3.83 Optimization of the most suitable RT-PCR components for semiquantitative analysis of ribophorin I (exon). RT-PCR was carried out using different concentrations of $MgCl_2$ (0, 1, 1.5, 2, 3, and 4 mM) against the template from ovaries (Lanes 2 – 7, A) and testes (Lanes 9 - 14, A) and different concentrations of primers (0, 0.1, 0.15, 0.2, 0.25 and 0.3 μM) against the cDNA template from ovaries (Lanes 1 – 7, B) and testes (Lanes 8 – 14, B). The number of RT-PCR cycles was carried out against the first strand cDNA of ovaries (lanes 1 -4, C) and testes (lanes 5 – 8, C) for 20 (lanes 1 and, C), 25 (lanes 2 and 6, C), 30 (lanes 3 and 7, C) and 35 (lanes 4 and 8, C) cycles. Lands M are a 100 bp DNA ladder. Lanes 1 and 8 are the negative control (without the cDNA template). The intensity of amplified products was plotted against the number of amplification cycles (D).



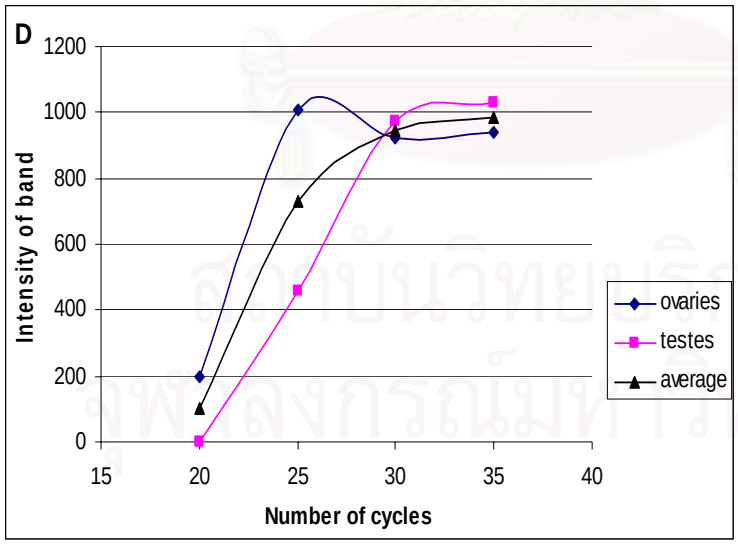


Figure 3.84 Optimization of the most suitable RT-PCR components for semiquantita -tive analysis of thioredoxin peroxidase (exon). RT-PCR was carried out using different concentrations of $MgCl_2$ (0, 1, 1.5, 2, 3, and 4 mM) against the template from ovaries (Lanes 2 – 7, A) and testes (Lanes 9 - 14, A) and different concentrations of primers (0, 0.1, 0.15, 0.2, 0.25 and 0.3 μM) against the cDNA template from ovaries (Lanes 1 – 7, B) and testes (Lanes 8 – 14, B). The number of RT-PCR cycles was carried out against the first strand cDNA of ovaries (lanes 1 -4, C) and testes (lanes 5 – 8, C) for 20 (lanes 1 and 5, C), 25 (lanes 2 and 6, C), 30 (lanes 3 and 7, C) and 35 (lanes 4 and 8, C) cycles. Lands M are a 100 bp DNA ladder. Lanes 1 and 8 are the negative control (without the cDNA template). The intensity of amplified products was plotted against the number of amplification cycles (D).

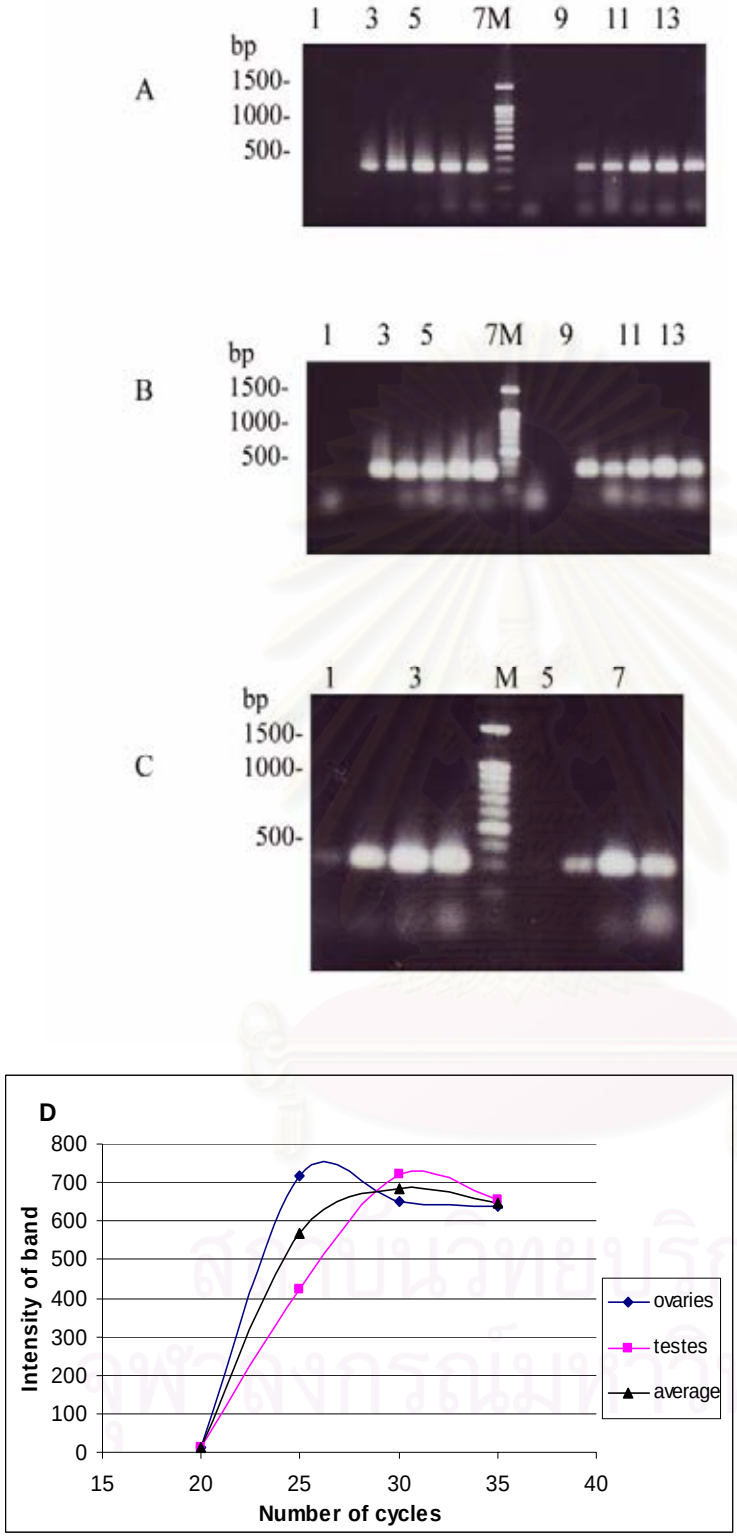


Figure 3.85 Optimization of the most suitable RT-PCR components for semiquantitative analysis of calponin 1 (exon) RT-PCR was carried out using different concentrations of $MgCl_2$ (0, 1, 1.5, 2, 3, and 4 mM) against the template from ovaries (Lanes 2 – 7, A) and testes (Lanes 9 - 14, A) and different concentrations of primers (0, 0.1, 0.15, 0.2, 0.25 and 0.3 μM) against the cDNA template from ovaries (Lanes 1 – 7, B) and testes (Lanes 8 – 14, B). The number of RT-PCR cycles was carried out against the first strand cDNA of ovaries (lanes 1 -4, C) and testes (lanes 5 – 8, C) for 20 (lanes 1 and 5, C), 25 (lanes 2 and 6, C), 30 (lanes 3 and 7, C) and 35 (lanes 4 and 8, C) cycles. Lands M are a 100 bp DNA ladder. Lanes 1 and 8 are the negative control (without the cDNA template). The intensity of amplified products was plotted against the number of amplification cycles (D).

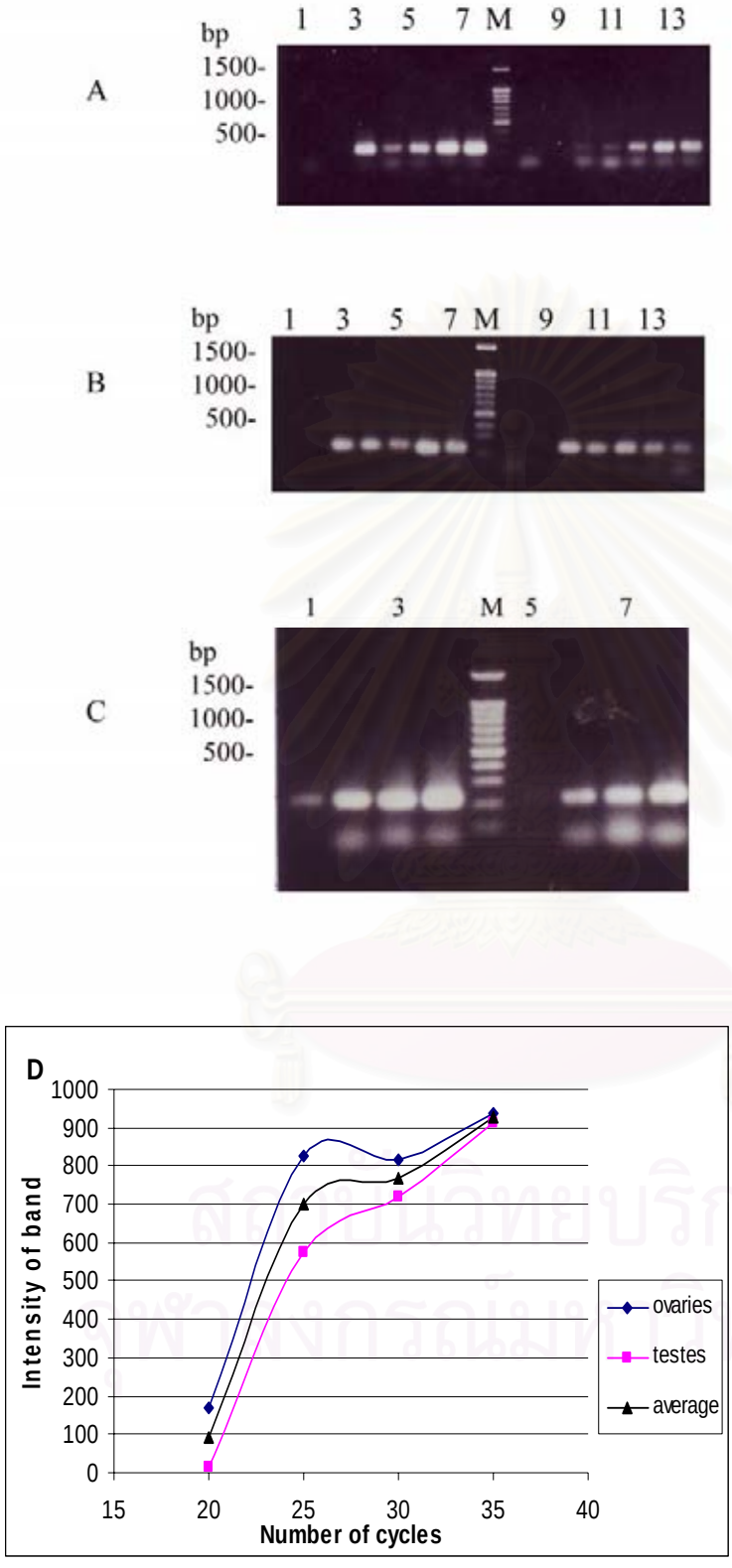


Figure 3.86 Optimization of the most suitable RT-PCR components for semiquantitative analysis of *DAD1* (exon) A standard RT-PCR was carried out using different concentrations of $MgCl_2$ (0, 1, 1.5, 2, 3, and 4 mM) against the template from ovaries (Lanes 2 – 7, A) and testes (Lanes 9 - 14, A) and different concentrations of primers (0, 0.1, 0.15, 0.2, 0.25 and 0.3 μM) against the cDNA template from ovaries (Lanes 1 – 7, B) and testes (Lanes 8 – 14, B). The number of RT-PCR cycles was carried out against the first strand cDNA of ovaries (lanes 1 -4, C) and testes (lanes 5 – 8, C) for 20 (lanes 1 and 5, C), 25 (lanes 2 and 6, C), 30 (lanes 3 and 7, C) and 35 (lanes 4 and 8, C) cycles. Lands M are a 100 bp DNA ladder. Lanes 1 and 8 are the negative control (without the cDNA template). The intensity of amplified products was plotted against the number of amplification cycles (D).

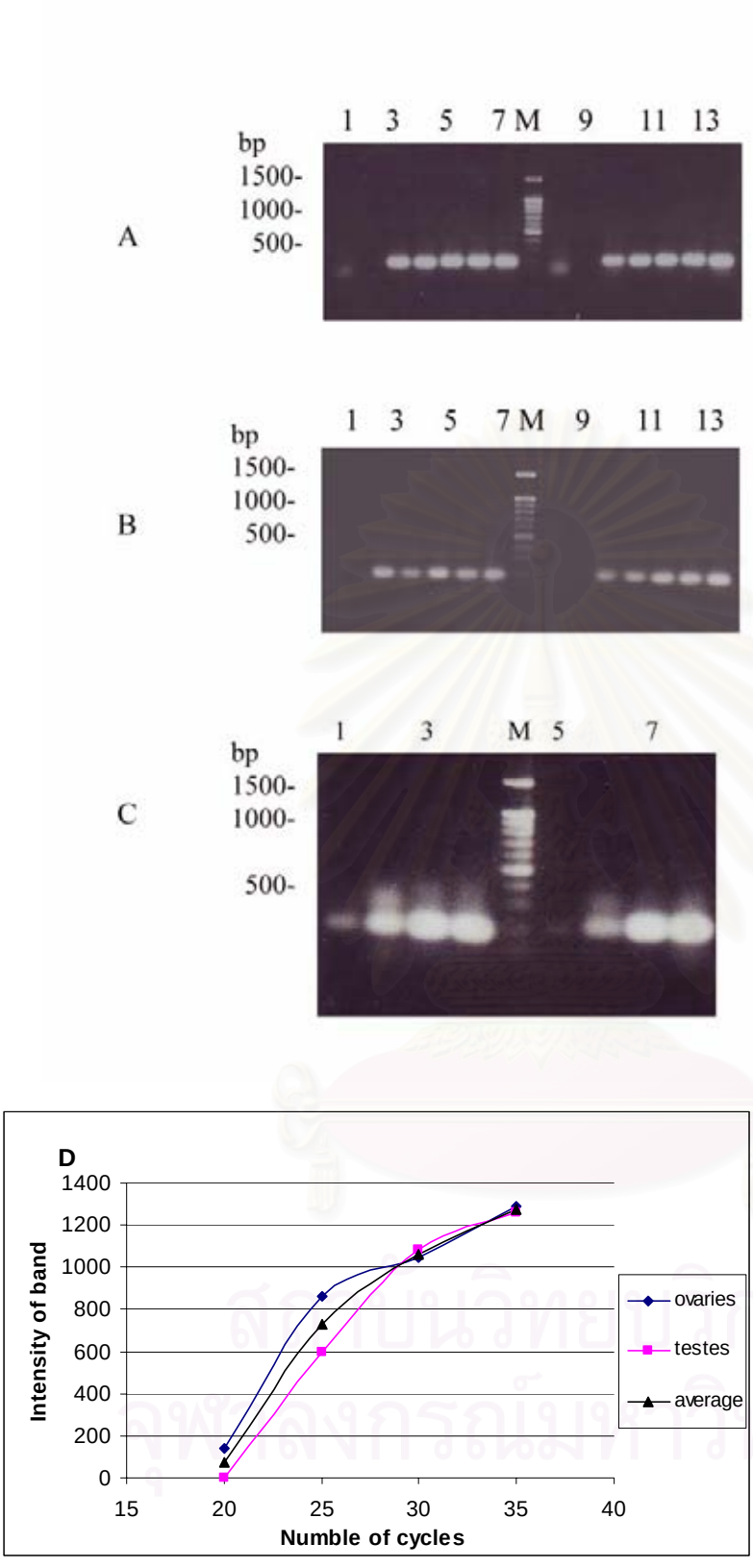


Figure 3.87 Optimization of the most suitable RT-PCR components for semiquantitative analysis of *RACK* (exon) RT-PCR was carried out using different concentrations of $MgCl_2$ (0, 1, 1.5, 2, 3, and 4 mM) against the template from ovaries (Lanes 2 – 7, A) and testes (Lanes 9 - 14, A) and different concentrations of primers (0, 0.1, 0.15, 0.2, 0.25 and 0.3 μM) against the cDNA template from ovaries (Lanes 1 – 7, B) and testes (Lanes 8 – 14, B). The number of RT-PCR cycles was carried out against the first strand cDNA of ovaries (lanes 1 -4, C) and testes (lanes 5 – 8, C) for 20 (lanes 1 and 5, C), 25 (lanes 2 and 6, C), 30 (lanes 3 and 7, C) and 35 (lanes 4 and 8, C) cycles. Lands M are a 100 bp DNA ladder. Lanes 1 and 8 are the negative control (without the cDNA template). The intensity of amplified products was plotted against the number of amplification cycles (D).

The optimized conditions for RT-PCR of the target genes (ribophorin I, thioredoxin peroxidase, calponin 1, *DAD1* and *RACK*) are illustrated by Figures 3.82 – 3.86 and Table 3.7. Generally, the expression pattern of all transcripts in ovaries was greater than that of testes.

Table 3.7 Optimized $MgCl_2$ and primer concentrations, number of amplification cycles and thermal profiles for semiquantitative RT-PCR of 5 functionally important gene

Gene	$MgCl_2$	Primer concentration	No. of cycles	PCR condition
1. Elongation factor 1 α	1.5 mM	0.2 µM	23	95°C for 5 min followed by optimized cycles of 94°C for 30 sec, 55°C for 45 sec and 72°C for 45 sec and 72°C for 7 min
2. Ribophorin I	2 mM	0.2 µM	28	
3. Thioredoxin peroxidase	2 mM	0.2 µM	28	As described in 1.
4. Calponin 1	2 mM	0.1 µM	28	As described in 1.
5. *DAD1*	2 mM	0.1 µM	28	As described in 1.
5. *RACK*	1.5 mM	0.2 µM	28	As described in 1.

3.7.3 Semiquantitative RT-PCR

Semiquantitative RT-PCR was carried out to determine whether the expression levels of ribophorin I, thioredoxin oeroxidase, calponin 1, *DAD1* and *RACK* were significantly different between ovaries and testes of juvenile *P. monodon*. The intensity of target genes was normalized by that of *EF-1α*. The relative expression levels was then statistically tested.

Figure 3.88 RT-PCR of ribophorin I (A) in ovaries (lanes 1-6) and testes (lanes 7-12) of juvenile *P. monodon*. *EF-1α* amplified from the same template (lanes 1-12, B) was included as the positive control.

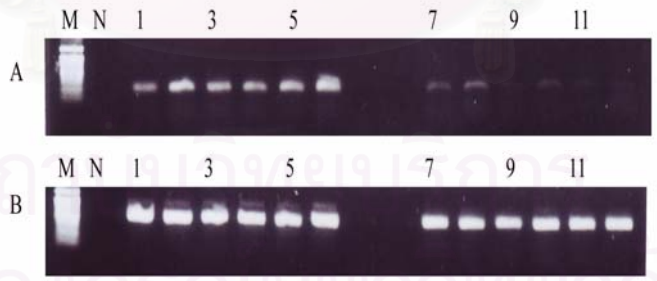


Figure 3.89 RT-PCR of thioredoxin peroxidase (A) in ovaries (lanes 1-6) and testes (lanes 7-12) of juvenile *P. monodon*. *EF-1α* amplified from the same template (lanes 1-12, B) was included as the positive control.

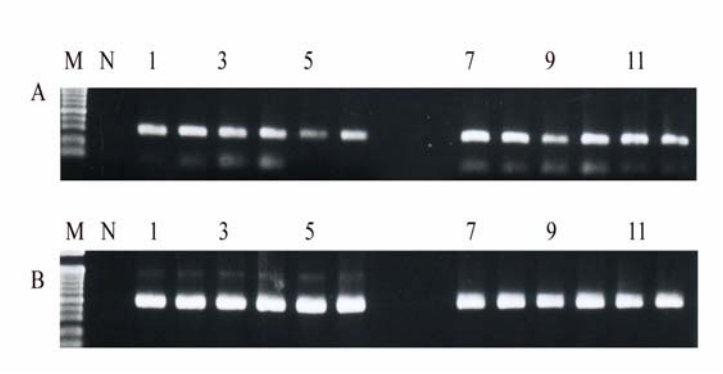


Figure 3.90 RT-PCR of calponin 1 (A) in ovaries (lanes 1-6) and testes (lanes 7-12) of juvenile *P. monodon*. *EF-1α* amplified from the same template (lanes 1-12, B) was included as the positive control.

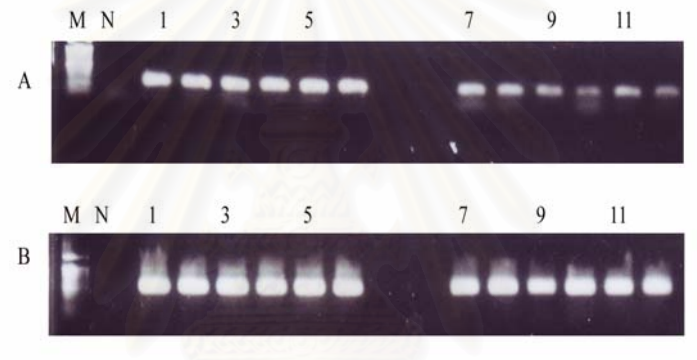


Figure 3.91 RT-PCR of *DAD1* (A) in ovaries (lanes 1-6) and testes (lanes 7-12) of juvenile *P. monodon*. *EF-1α* amplified from the same template (lanes 1-12, B) was included as the positive control.

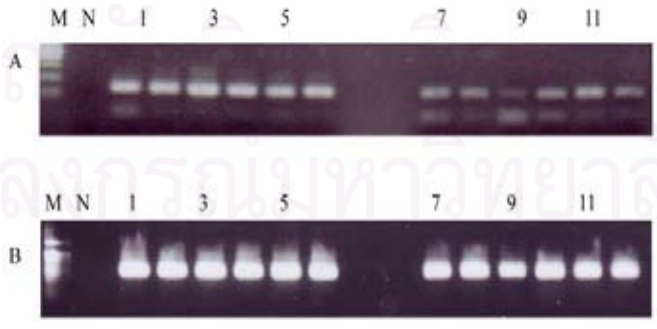


Figure 3.92 RT-PCR of *RACK* (A) in ovaries (lanes 1-6) and testes (lanes 7-12) of juvenile *P. monodon*. *EF-1α* amplified from the same template (lanes 1-12, B) was included as the positive control.

Relative expression levels of all transcripts in ovaries and testes were significantly different. The expression levels of ribophorin I, thioredoxin peroxidase, *DAD1* and *RACK* in ovaries were greater than those of testes. In contrast, the expression level of calponin 1 was more abundantly expressed in testes than ovaries of juvenile *P. monodon*.

Table 3.8 Relative expression level of ribophorin I, thioredoxin peroxidase, calponoin1, *DAD1* and *RACK* in ovaries and testes of *P. monodon*

Gene	**Relative Expression Level**	
	Ovaries (*N* = 6)	**Testes (*N*=6)**
1. Ribophorin I	1.48±0.27[a]	0.60±0.51[b]
2. Thioredoxin peroxidase	0.37±0.11[a]	0.13±0.05[b]
3. Calponin 1	0.50±0.07[a]	0.63±0.09[b]
4. *DAD1*	0.73±0.06[a]	0.34±0.14[b]
5. *RACK*	0.82±0.13[a]	0.60±0.22[b]

3.8 The association between temperature shock and their expression level

The function of almost all of the genes in this study involve with apoptosis. Although ribophorin I and *DAD1* were parts of the oligosacharyltransferase pathway, they also play important role as antiapoptotic agents. Thioredoxin peroxidase is an antioxidant (e.g. as the electron donor) to remove the reactive oxygen species (ROS), whereas *RACK* is functionally responsible for the intracellular localization of activated protein kinas C. Only calponin 1 which involves in the regulation of actomyosin interaction in several tissues may not involve in apoptotic processes. Therefore, thermal stress was applied to test effects on temperature on the expression levels of these genes in ovaries and hemocytes of juvenile *P. monodon*.

Results from semiquantitative RT-PCR of these genes in ovaries of juvenile *P. monodon* indicated that the relative expression levels of ribophorin I (Figure 3.93), calponin 1 (Figure 3.95), *DAD1* (Figure 3.96) and *RACK* (Figure 3.97) between different treatment were not significantly different. Nevertheless, the expression of thioredoxin peroxidase in ovaries (Figure 3.94) was significantly higher than the normal level at 6 hours post thermal treatment ($P < 0.05$). The expression levels was then decreased at 12 hours post thermal treatment and returned to the normal level at 24 hours after treatment (Table 3.9).

In contrast, results from semiquantitative RT-PCR of ribophorin I (Figure 3.98), thioredoxin peroxidase (Figure 3.99), calponin 1 (Figure 3.100) and *DAD1* (Figure 3.101) in hemocytes of juvenile *P. monodon* did not show statistically significant differences between different treatment. The expression of *RACK* (Figure 3.102) slightly increased at 0 and 6 hours post thermal treatment and significantly higher than the normal level at 12 hours post thermal treatment ($P < 0.05$). The expression levels decreased at 24 hours post thermal treatment (Table 3.9).

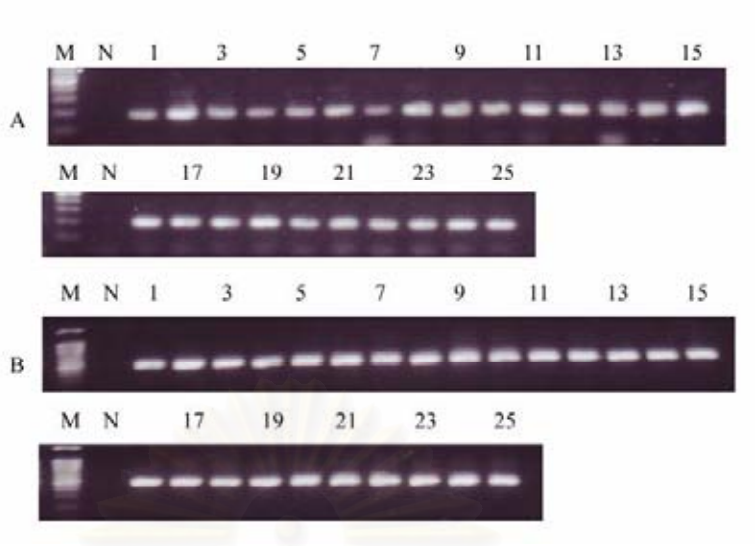


Figure 3.93 RT-PCR of ribophorin I (A) in ovaries of normal *P. monodon* (lanes 1 - 5) and that after heat stress for 0 hrs. (lanes 6 – 10), 6 hrs. (lanes 11 – 15), 12 hrs.(lanes 16 – 20) and 24 hrs. (lanes 21 – 25), respectively. *EF-1α* amplified from the same template was used to normalize the time course expression levels (lanes 1-25, B).

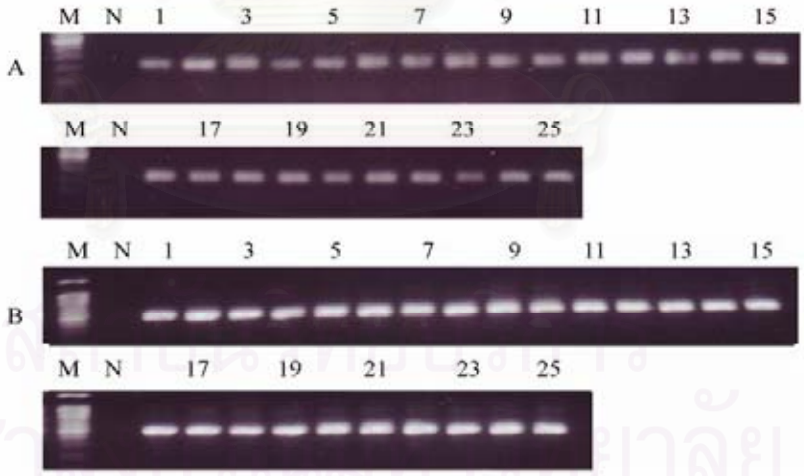


Figure 3.94 RT-PCR of thioredoxin peroxidase (A) in ovaries of normal *P. monodon* (lanes 1 - 5) and that after heat stress for 0 hrs. (lanes 6 – 10), 6 hrs. (lanes 11 – 15), 12 hrs.(lanes 16 – 20) and 24 hrs. (lanes 21 – 25), respectively. *EF-1α* amplified from the same template was used to normalize the time course expression levels (lanes 1-25, B).

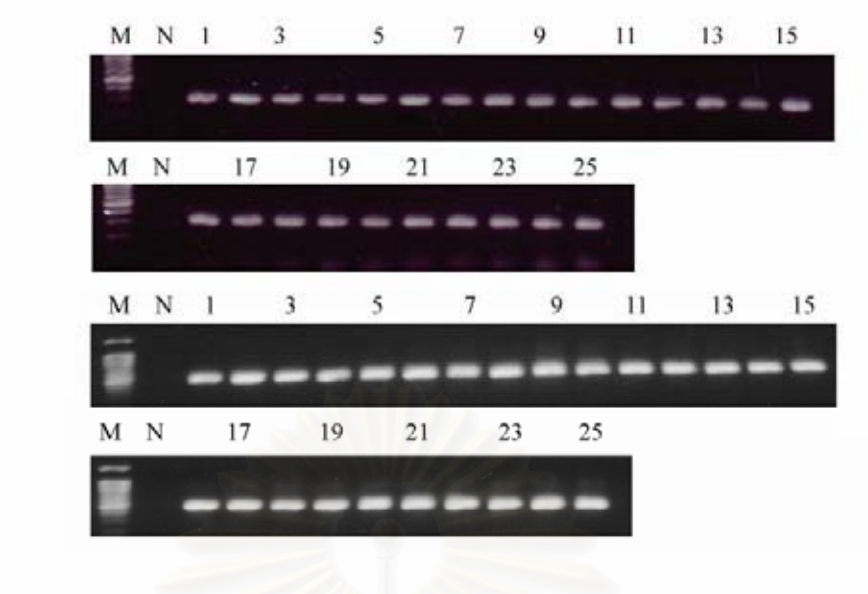


Figure 3.95 RT-PCR of calponin 1 (A) in ovaries of normal *P. monodon* (lanes 1 - 5) and that after heat stress for 0 hrs. (lanes 6 – 10), 6 hrs. (lanes 11 – 15), 12 hrs.(lanes 16 – 20) and 24 hrs. (lanes 21 – 25), respectively. *EF-1α* amplified from the same template was used to normalize the time course expression levels (lanes 1-25, B).

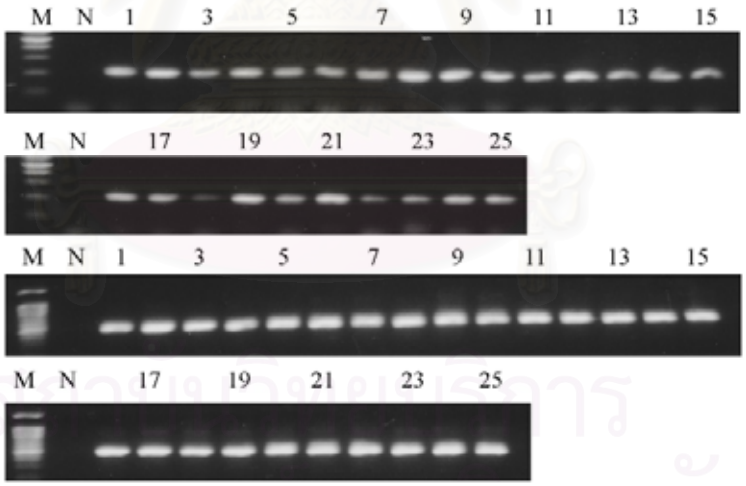


Figure 3.96 RT-PCR of *DAD1* (A) in ovaries of normal *P. monodon* (lanes 1 - 5) and that after heat stress for 0 hrs. (lanes 6 – 10), 6 hrs. (lanes 11 – 15), 12 hrs.(lanes 16 – 20) and 24 hrs. (lanes 21 – 25), respectively. *EF-1α* amplified from the same template was used to normalize the time course expression levels (lanes 1 - 25, B).

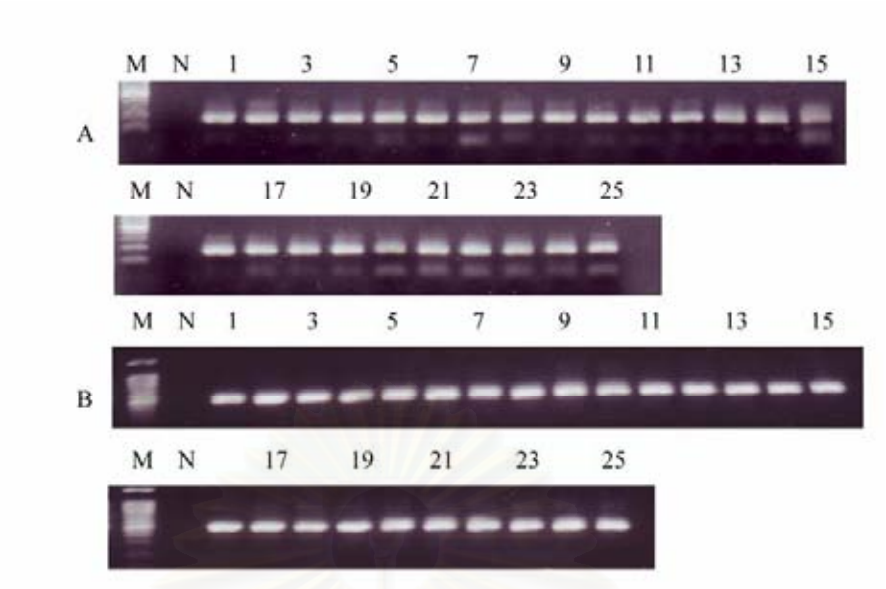


Figure 3.97 RT-PCR of *RACK* (A) in ovaries of normal *P. monodon* (lanes 1 - 5) and that after heat stress for 0 hrs. (lanes 6 – 10), 6 hrs. (lanes 11 – 15), 12 hrs.(lanes 16 – 20) and 24 hrs. (lanes 21 – 25), respectively. *EF-1α* amplified from the same template was used to normalize the time course expression levels (lanes 1-25, B).

Table 3.9 Time course relative expression levels of ribophorin I, thioredoxin peroxidase, calponoin 1, *DAD1* and *RACK* in ovaries of *P. monodon* treated with thermal stress

Gene	**Relative Expression Level**				
	Normal	**0 hrs.**	**6 hrs.**	**12 hrs.**	**24 hrs.**
1. Ribophorin I	0.95 ± 0.41^{a}	1.04 ± 0.23^{a}	1.33 ± 0.24^{a}	1.34 ± 0.44^{a}	1.05 ± 0.12^{a}
2. Thioredoxin peroxidase	0.51 ± 0.13^{a}	0.56 ± 0.04^{ab}	0.65 ± 0.08^{b}	0.56 ± 0.08^{ab}	0.49 ± 0.09^{a}
3. Calponin 1	0.67 ± 0.10^{a}	0.74 ± 0.08^{a}	0.71 ± 0.10^{a}	0.75 ± 0.07^{a}	0.72 ± 0.04^{a}
4. *DAD1*	0.61 ± 0.13^{a}	0.66 ± 0.13^{a}	0.56 ± 0.11^{a}	0.49 ± 0.23^{a}	0.48 ± 0.20^{a}
5. *RACK*	0.86 ± 0.11^{a}	0.80 ± 0.04^{a}	0.82 ± 0.13^{a}	0.82 ± 0.12^{a}	0.78 ± 0.02^{a}

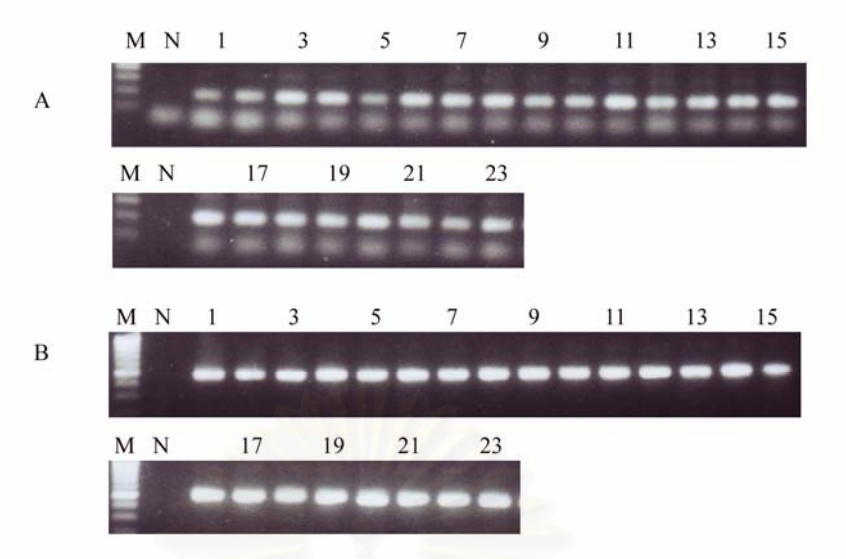


Figure 3.98 RT-PCR of ribophorin I (A) in hemocytes of normal *P. monodon* (lanes 1-3 and 16-17) and that after heat stress for 0 hrs. (lanes 4 – 6 and 18), 6 hrs. (lanes 7 – 9 and 19), 12 hrs.(lanes 10 – 12 and 20-21) and 24 hrs. (lanes 13 – 15 and 22 - 23), respectively. *EF-1α* amplified from the same template was used to normalize the time course expression levels (lanes 1-23, B).

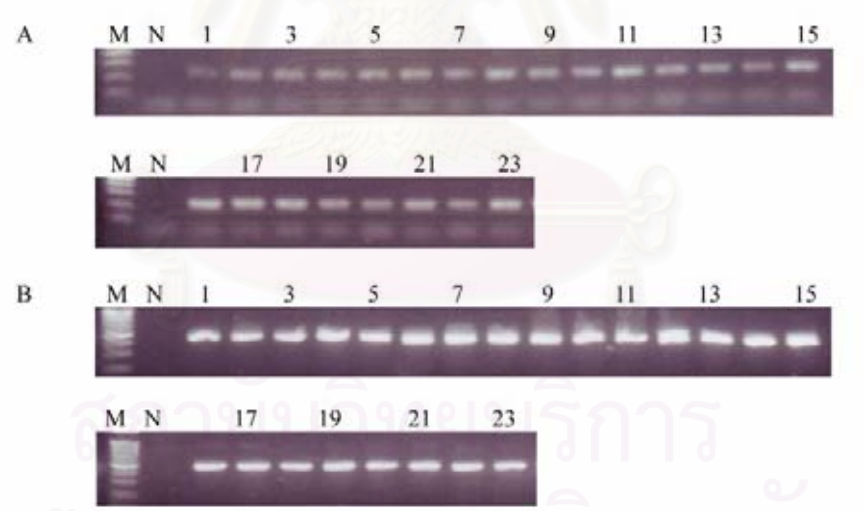


Figure 3.99 RT-PCR of thioredoxin peroxidase (A) in hemocytes of normal *P. monodon* (lanes 1-3 and 16-17) and that after heat stress for 0 hrs. (lanes 4 – 6 and 18), 6 hrs. (lanes 7 – 9 and 19), 12 hrs.(lanes 10 – 12 and 20-21) and 24 hrs. (lanes 13 – 15 and 22 - 23), respectively. *EF-1α* amplified from the same template was used to normalize the time course expression levels (lanes 1-23, B).

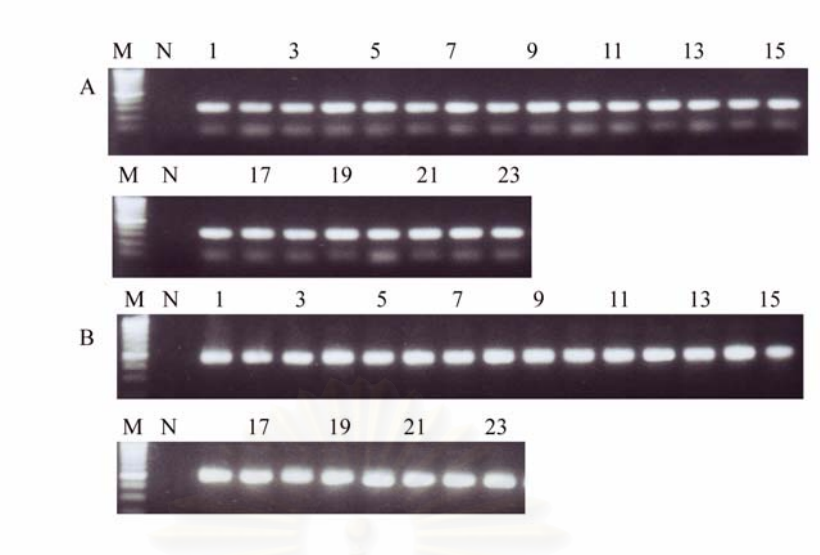


Figure 3.100 RT-PCR of calponin 1 (A) in hemocytes of normal *P. monodon* (lanes 1-3 and 16-17) and that after heat stress for 0 hrs. (lanes 4 – 6 and 18), 6 hrs. (lanes 7 – 9 and 19), 12 hrs.(lanes 10 – 12 and 20-21) and 24 hrs. (lanes 13 – 15 and 22 - 23), respectively. *EF-1α* amplified from the same template was used to normalize the time course expression levels (lanes 1-23, B).

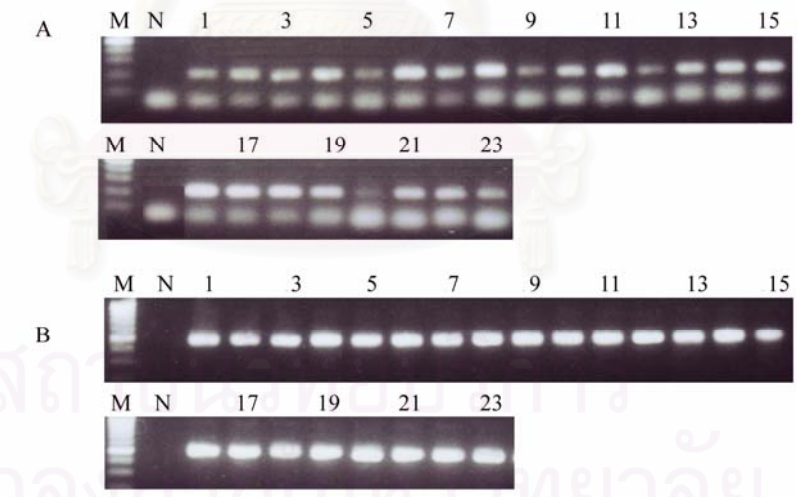


Figure 3.101 RT-PCR of *DAD1* (A) in hemocytes of normal *P. monodon* (lanes 1-3 and 16-17) and that after heat stress for 0 hrs. (lanes 4 – 6 and 18), 6 hrs. (lanes 7 – 9 and 19), 12 hrs.(lanes 10 – 12 and 20-21) and 24 hrs. (lanes 13 – 15 and 22 - 23), respectively. *EF-1α* amplified from the same template was used to normalize the time course expression levels (lanes 1-23, B).

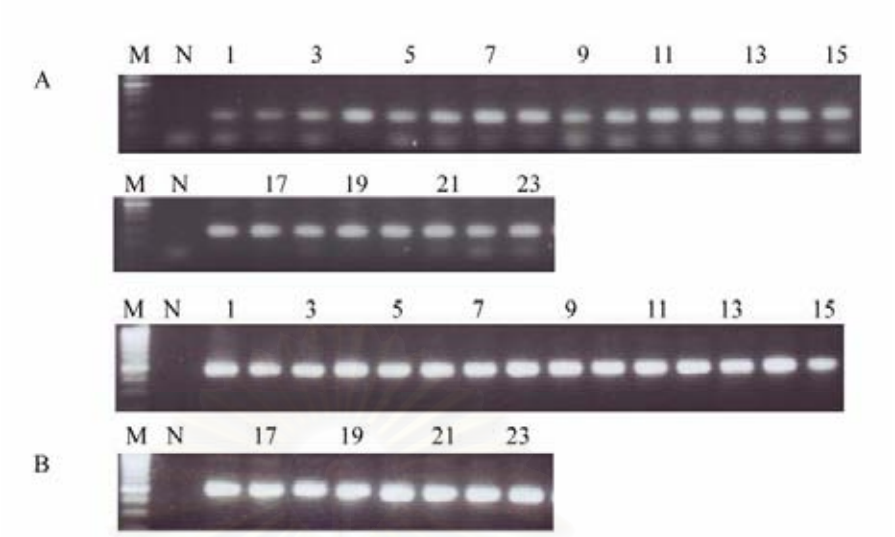


Figure 3.102 RT-PCR of *RACK* (A) in hemocytes of normal *P. monodon* (lanes 1-3 and 16-17) and that after heat stress for 0 hrs. (lanes 4 – 6 and 18), 6 hrs. (lanes 7 – 9 and 19), 12 hrs.(lanes 10 – 12 and 20-21) and 24 hrs. (lanes 13 – 15 and 22 - 23), respectively. *EF-1α* amplified from the same template was used to normalize the time course expression levels (lanes 1-23, B).

Table 3.10 Time course relative expression levels of ribophorin I, thioredoxin peroxidase, calponoin 1, *DAD1* and *RACK* in hemocytes of *P. monodon* treated with thermal stress

Gene	Relative Expression Level				
	Normal	**0 hrs.**	**6 hrs.**	**12 hrs.**	**24 hrs.**
1. Ribophorin I	0.59 ± 0.17^{a}	0.76 ± 0.14^{a}	0.67 ± 0.07^{a}	0.66 ± 0.10^{a}	0.67 ± 0.14^{a}
2. Thioredoxin peroxidase	0.15 ± 0.07^{a}	0.14 ± 0.06^{a}	0.14 ± 0.03^{a}	0.15 ± 0.04^{a}	0.16 ± 0.06^{a}
3. Calponin 1	0.68 ± 0.05^{a}	0.67 ± 0.10^{a}	0.72 ± 0.11^{a}	0.73 ± 0.09^{a}	0.70 ± 0.12^{a}
4. *DAD1*	0.54 ± 0.15^{a}	0.52 ± 0.19^{a}	0.50 ± 0.18^{a}	0.41 ± 0.23^{a}	0.54 ± 0.13^{a}
5. *RACK*	0.43 ± 0.24^{a}	0.56 ± 0.12^{ab}	0.63 ± 0.17^{ab}	0.70 ± 0.1^{b}	0.64 ± 0.06^{ab}

3.9 Association between SNP through SSCP patterns and expression levels of functionally important genes in *P. monodon* broodstock

SNP through SSCP patterns in of ribophorin I (exon/intron), thioredoxin peroxidase (exon), calponin 1 (5′UTR), *DAD1* (exon) and *RACK* (exon) were examined using 12 and 13 male and female broodstock of *P. monodon* originating from Chonburi. Relative expression levels of these genes were also evaluated. Association between SNP through SSCP patterns and levels of gene expression were considered.

Relative expression levels of all investigated genes in ovaries and testes were significantly different ($P < 0.05$). However, the expression levels of *RACK* in male and female *P. monodon* broodstock were not different. This indicated that association between expression levels and SNP through SSCP patterns in male and female *P. monodon* should be considered separately limiting the ability to test correlation in several genotypes.

When possible, statistical analysis was carried out and the results using limited sample sizes indicated non-significant association between SNP through SSCP genotypes and expression level. In addition, no correlations between ovarian stages (different GSI) and gene expression levels were observed.

Male *P. monodon* broodstock carrying genotypes 1 and 6 of the 5’ UTR of calponin 1 was able to statistically tested but the expression levels of these groups were not significantly different. Both male and female *P. monodon* broodstock exhibiting genotypes 1, 2, 5 and 7 and 1, 2 and 5 of the exon region of *calponin 1* did not reveal significant differences of the expression levels.

Likewise, both male and female *P. monodon* broodstock exhibiting genotypes 1, 2, 3 and 4 and 1, 2, 4 and 5 of *DAD1* could be statistically compared but did not reveal significant differences of the expression levels.

Association analysis between genotypes of males (1 and 7) and females (3, 5, 6 and 7) of broodstock-sized *P. monodon* were also not statistically significant.

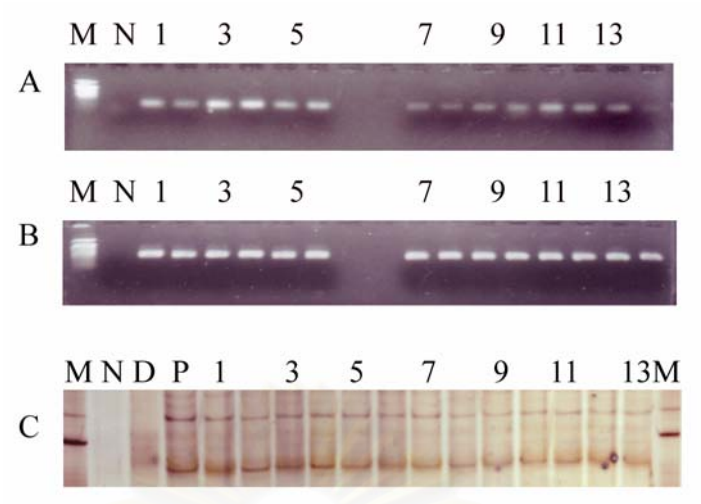


Figure 3.103 RT-PCR of ribophorin I (A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF1 - 6, A) and testes (lanes 7 – 14 = PMM1 - 8) of *P. monodon* broodstock and SSCP patterns (C) of ribophorin I amplified from genomic DNA of the same sample. Only one genotype was observed *from* ribophorin I was observed from these specimens.

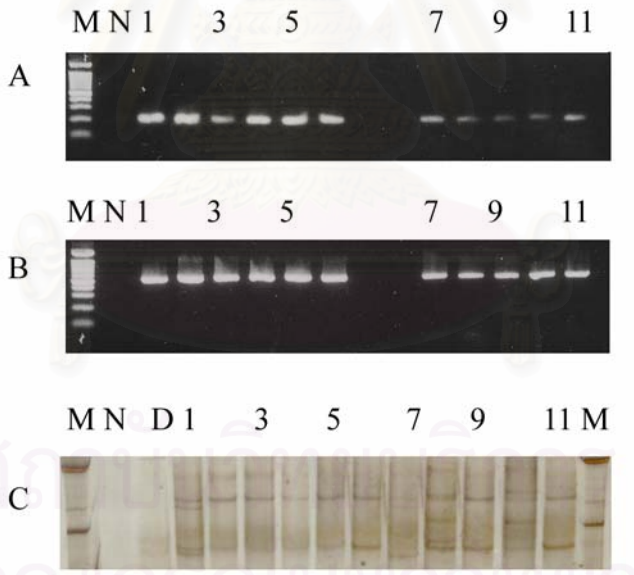


Figure 3.104 RT-PCR of ribophorin I (A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF7 - 12, A) and testes (lanes 7 – 11 = PMM9 - 13) of *P. monodon* broodstock and SSCP patterns (C) of ribophorin I amplified from genomic DNA of the same sample. Four genotypes (pattern I, lanes 2, 3, 4, 5, 6, and 7; II, lanes 1 and 9; III, lanes 11 and IV, lanes 8) were observed in these specimens.

Table 3.11 SSCP genotypes, gonadosomatic index (GSI) and relative expression levels of ribophorin I in ovaries and testes of *P. monodon* broodstock

Specimen	Genotype	GSI	Expression level*
PMF1	1	5.0	0.45
PMF2	1	2.4	0.55
PMF3	1	1.4	0.65
PMF4	1	2.0	0.65
PMF5	1	3.0	0.72
PMF6	1	2.1	0.61
PMF7	2	1.1	0.54
PMF8	1	0.87	0.63
PMF9	1	4.69	0.39
PMF10	1	1.89	0.80
PMF11	1	0.92	0.59
PMF12	1	0.65	0.57
PMM1	1	0.54	0.56
PMM2	1	0.59	0.42
PMM3	1	0.78	0.56
PMM4	1	0.61	0.45
PMM5	1	0.52	0.42
PMM6	1	0.67	0.33
PMM7	1	0.61	0.49
PMM8	1	0.84	0.29
PMM09	1	0.66	0.43
PMM10	4	1.1	0.36
PMM11	2	0.96	0.37
PMM12	1	0.78	0.34
PMM13	3	0.52	0.43

*intensity of ribophorin I/*EF*-1α

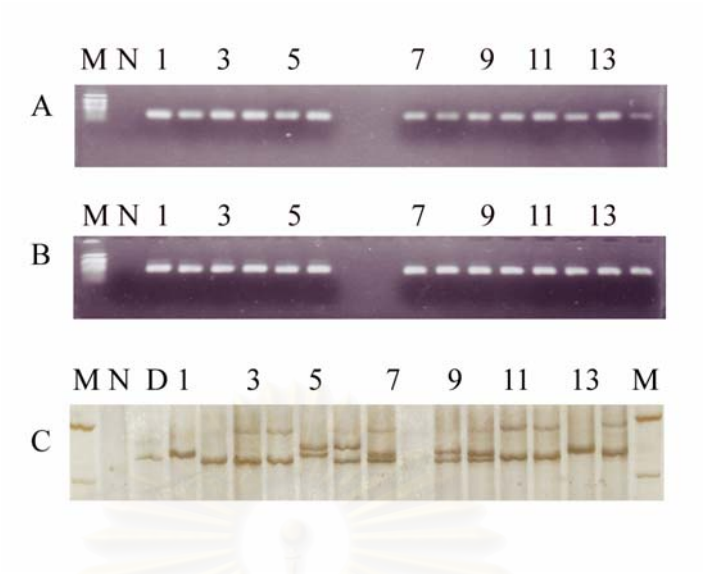


Figure 3.105 RT-PCR of thioredoxin peroxidase (A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF1 - 6, A) and testes (lanes 7 – 14 = PMM1 - 8) of *P. monodon* broodstock and SSCP patterns (C) of thioredoxin peroxidase amplified from genomic DNA of the same sample. Seven genotypes (pattern I, lanes 1, and 13; II, lanes 2; III, lanes 3, 4, 11, 12 and 14; IV, lanes 5, V, lanes 6, VI, lane 2, 13 and IX, lane 9) were observed in these specimens.

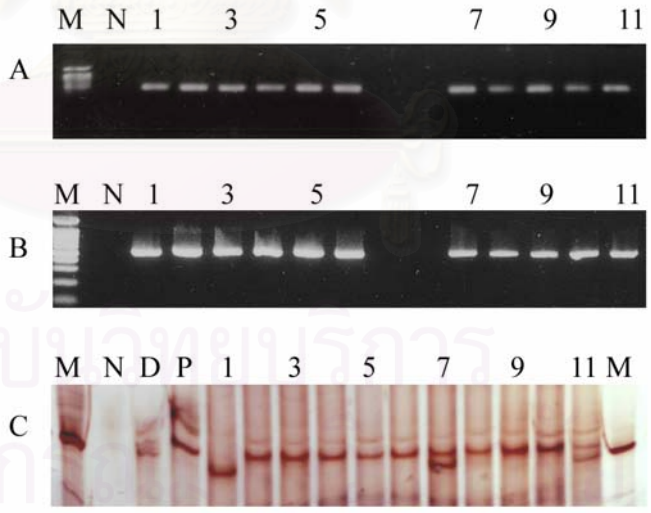


Figure 3.106 RT-PCR of thioredoxin peroxidase (A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF7 - 12, A) and testes (lanes 7 – 11 = PMM9 - 13) of *P. monodon* broodstock and SSCP patterns (C) of thioredoxin peroxidase amplified from genomic DNA of the same sample. Three genotypes (pattern III, lanes 1, 3, 4, 5, 6, 8, 9 and 10; VIII, lanes 2 and IX, lanes 7, and 11) were observed from these specimens.

Table 3.12 SSCP genotypes, gonadosomatic index (GSI) and relative expression levels of thioredoxin peroxidase in ovaries and testes of *P. monodon* broodstock

Specimen	Genotype	GSI	Expression level*
PMF1	7	5.0	0.73
PMF2	6	2.4	0.72
PMF3	3	1.4	0.84
PMF4	3	2.0	0.82
PMF5	1	3.0	0.74
PMF6	3	2.1	0.76
PMF7	8	1.1	0.19
PMF8	3	0.87	0.24
PMF9	3	4.69	0.24
PMF10	3	1.89	0.19
PMF11	3	0.92	0.28
PMF12	3	0.65	0.27
PMM1	1	0.54	0.60
PMM2	2	0.59	0.43
PMM3	3	0.78	0.64
PMM4	3	0.61	0.69
PMM5	4	0.52	0.72
PMM6	5	0.67	0.56
PMM7	6	0.61	0.71
PMM8	-	0.84	0.22
PMM09	9	0.66	0.27
PMM10	3	1.1	0.17
PMM11	3	0.96	0.23
PMM12	3	0.78	0.18
PMM13	9	0.52	0.24

*intensity of thioredoxin peroxidase/*EF-1α*

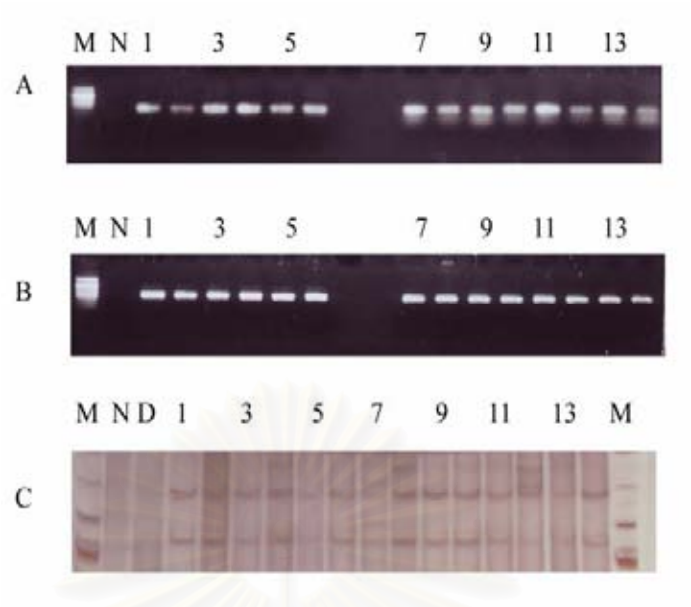


Figure 3.107 RT-PCR of calponin 1 (A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF1 - 6, A) and testes (lanes 7 – 14 = PMM1 - 8) of *P. monodon* broodstock and SSCP patterns (C) of 5' UTR of calponin 1 amplified from genomic DNA of the same sample. Five genotypes (pattern I, lanes 1, 2, 3, 4, 5, 6, 9, 10, 11 and 14; II, lanes7; III, lanes 8; IV, lanes 12 and V, lanes 13) were observed from these specimens.

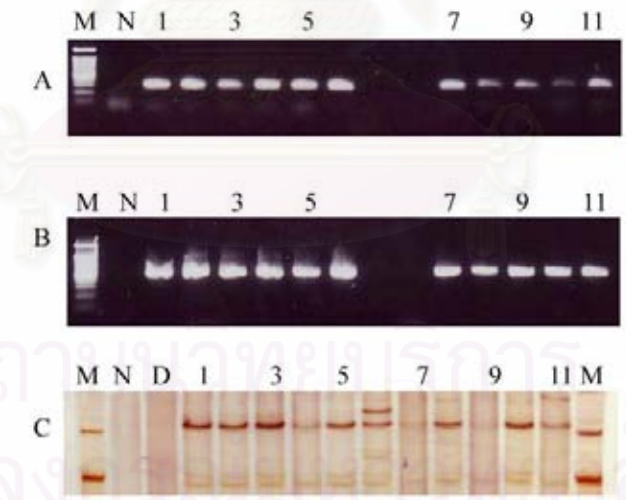


Figure 3.108 RT-PCR of calponin 1 (A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF7 - 12, A) and testes (lanes 7 – 11 = PMM9 - 13) of *P. monodon* broodstock and SSCP patterns (C) of 5' UTR of calponin 1 amplified from genomic DNA of the same sample. Five genotypes (pattern I, lanes 1, 2, 3, 4, 5; II, lanes 6;III , lanes 11; V, lane 11 and VI, lane 7, 8,and 9) were observed these specimens.

Table 3.13 SSCP genotypes (5′UTR), gonadosomatic index (GSI) and relative expression levels of calponin 1 in ovaries and testes of *P. monodon* broodstock

Specimens	Genotype	GSI	Expression level*
PMF1	1	5.0	0.73
PMF2	1	2.4	0.35
PMF3	1	1.4	1.00
PMF4	1	2.0	0.98
PMF5	1	3.0	0.75
PMF6	1	2.1	0.85
PMF7	1	1.1	0.70
PMF8	1	0.87	0.72
PMF9	1	4.69	0.59
PMF10	1	1.89	0.80
PMF11	1	0.92	0.87
PMF12	2	0.65	0.81
PMM1	2	0.54	0.75
PMM2	3	0.59	0.54
PMM3	1	0.78	0.62
PMM4	1	0.61	0.54
PMM5	1	0.52	0.98
PMM6	4	0.67	0.42
PMM7	5	0.61	0.43
PMM8	1	0.84	0.28
PMM09	6	0.66	0.72
PMM10	6	1.1	0.40
PMM11	6	0.96	0.39
PMM12	3	0.78	0.18
PMM13	5	0.52	0.61

*intensity of calponin 1/*EF-1α*

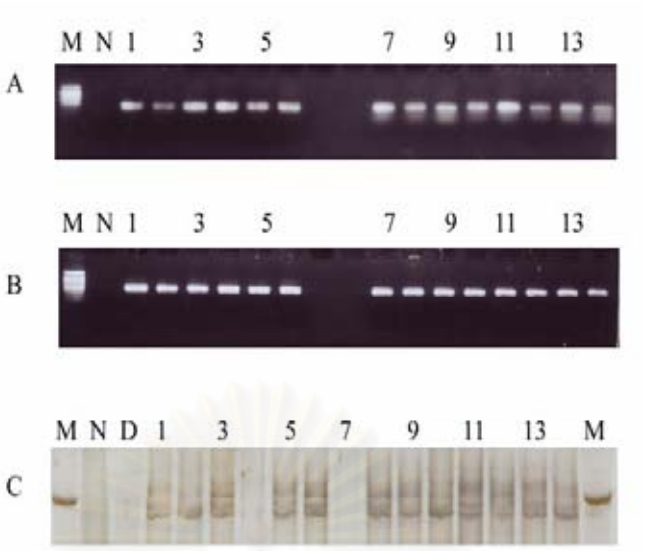


Figure 3.109 RT-PCR of calponin 1 (exon, A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF1 - 6, A) and testes (lanes 7 – 14 = PMM1 - 8) of *P. monodon* broodstock and SSCP patterns (C) of the exon of calponin 1 amplified from genomic DNA of the same sample. Five genotypes (pattern I, lanes 8 and 13; II, lanes 2, 3, 4, 6 and 8; V, lanes 5 and 9; VI, lane 11 and VII, lanes 12 and 14) were observed from these specimens.

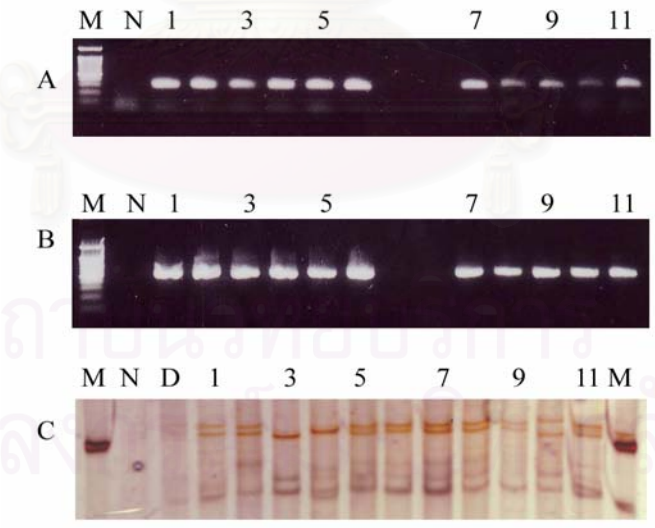


Figure 3.110 RT-PCR of calponin 1 (exon, A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF7 - 12, A) and testes (lanes 7 – 11 = PMM9 - 13) of *P. monodon* broodstock and SSCP patterns (C) of the exon of calponin 1 amplified from genomic DNA of the same sample. Six genotypes (pattern I, lanes 1 and 11; II, lanes 2;III, lanes 3; IV, lane 4; V, lane 5, 6, 7, 8,and 9; VI, lane 10) were observed from these specimens.

Table 3.14 SSCP genotypes (exon), gonadosomatic index (GSI) and relative expression levels of calponin 1 in ovaries and testes of *P. monodon* broodstock

Specimens	Genotype	GSI	Expression level*
PMF1	5	5.0	0.73
PMF2	2	2.4	0.35
PMF3	6	1.4	1.00
PMF4	7	2.0	0.98
PMF5	1	3.0	0.75
PMF6	7	2.1	0.85
PMF7	1	1.1	0.70
PMF8	2	0.87	0.72
PMF9	3	4.69	0.59
PMF10	4	1.89	0.80
PMF11	5	0.92	0.87
PMF12	5	0.65	0.81
PMM1	1	0.54	0.75
PMM2	2	0.59	0.54
PMM3	2	0.78	0.62
PMM4	2	0.61	0.55
PMM5	5	0.52	0.98
PMM6	2	0.67	0.42
PMM7	ND	0.61	0.43
PMM8	2	0.84	0.28
PMM09	5	0.66	0.72
PMM10	5	1.1	0.40
PMM11	5	0.96	0.39
PMM12	6	0.78	0.18
PMM13	1	0.52	0.61

*intensity of calponin 1/*EF-1α*

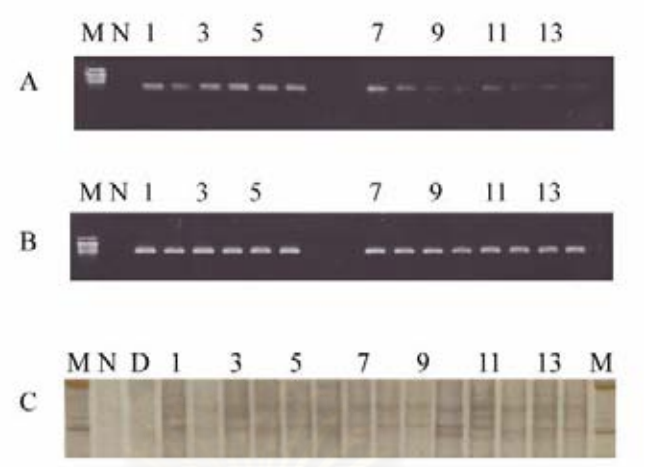


Figure 3.111 RT-PCR of *DAD1* (A) and EF-1α (B) in ovaries (lanes 1 – 6 = PMF1 - 6, A) and testes (lanes 7 – 14 = PMM1 - 8) of *P. monodon* broodstock and SSCP patterns (C) of 5' UTR of *DAD1* amplified from genomic DNA of the same sample. Six genotypes (pattern I, lanes 2, 4, 5, 9, 12and 14; II, lanes 1, 3, and 13; III, lane 11; IV, lanes 8 and 10; V, lane 6; and VI, lane 6) were observed from these specimens

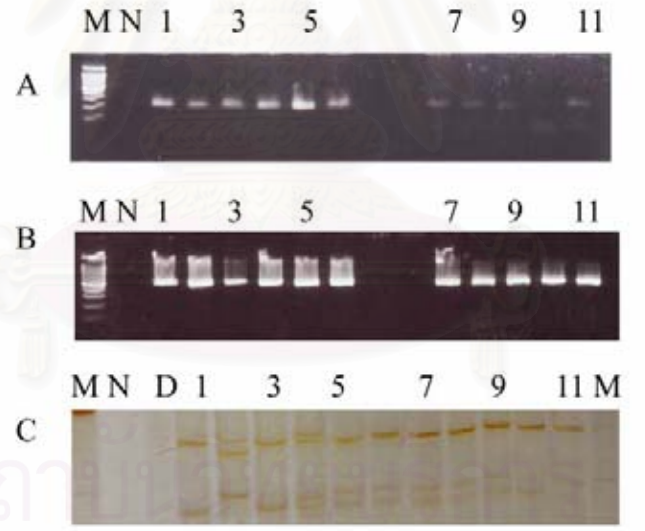


Figure 3.112 RT-PCR of *DAD1* (A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF7 - 12, A) and testes (lanes 7 – 11 = PMM9 - 13) of *P. monodon* broodstock and SSCP patterns (C) of *DAD1* amplified from genomic DNA of the same sample. Five genotypes (pattern I, lanes 1, 3 and 10; II, lanes 2;III, lanes 4; IV, lane 5, 6, 7 and 11; V, lane 6 and 9) were observed from these specimens.

Table 3.15 SSCP genotypes, gonadosomatic index (GSI) and relative expression levels of *DAD1* in ovaries and testes of *P. monodon* broodstock

Specimens	Genotype	GSI	Expression level
PMF1	1	5.0	0.95
PMF2	4	2.4	0.63
PMF3	3	1.4	1.178
PMF4	1	2.0	1.25
PMF5	2	3.0	1.30
PMF6	1	2.1	1.14
PMF7	1	1.1	0.70
PMF8	2	0.87	0.72
PMF9	1	4.69	0.59
PMF10	3	1.89	0.80
PMF11	4	0.92	0.87
PMF12	5	0.65	0.81
PMM1	2	0.54	1.29
PMM2	1	0.59	0.59
PMM3	2	0.78	0.21
PMM4	1	0.61	0.17
PMM5	1	0.52	0.40
PMM6	5	0.67	0.16
PMM7	6	0.61	0.20
PMM8	4	0.84	0.19
PMM09	4	0.66	0.72
PMM10	4	1.1	0.40
PMM11	5	0.96	0.39
PMM12	1	0.78	0.18
PMM13	4	0.52	0.61

*intensity of *DAD 1/EF-1α*

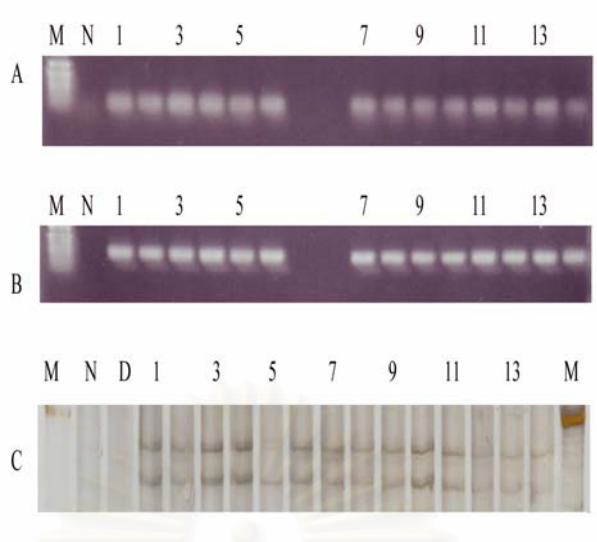


Figure 3.113 RT-PCR of *RACK* (A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF1 - 6, A) and testes (lanes 7 – 14 = PMM1 - 8) of *P. monodon* broodstock and SSCP patterns (C) of exon of *RACK* amplified from genomic DNA of the same sample. Three genotypes (pattern V, lanes 1 and 5; VI, lanes 2, 3, and 4; VII, lane 6, 7, 8, 9, 11, 12, 13 and 14) were observed from these specimens.

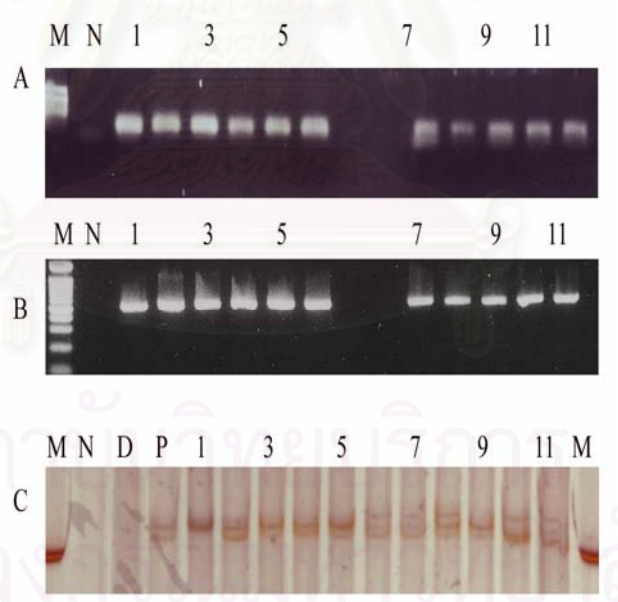


Figure 3.114 RT-PCR of *RACK* (A) and *EF-1α* (B) in ovaries (lanes 1 – 6 = PMF7 - 12, A) and testes (lanes 7 – 11 = PMM9 - 13) of *P. monodon* broodstock and SSCP patterns (C) of exon of *RACK* amplified from genomic DNA of the same sample. Four genotypes (pattern I, lanes 2, 3, 4, 5 and 9; II, lanes 1; III , lanes 6, 7, 8 and 11 and IV, lane 10) were observed from *RACK* polymorphism.

Table 3.16 SSCP genotypes (exon), gonadosomatic index (GSI) and relative expression levels of *RACK* in ovaries and testes of *P. monodon* broodstock

Specimens	Genotype	GSI	Expression level
PMF1	7	5.0	0.76
PMF2	6	2.4	0.76
PMF3	7	1.4	1.09
PMF4	7	2.0	0.83
PMF5	7	3.0	0.94
PMF6	7	2.1	1.00
PMF7	2	1.1	0.53
PMF8	1	0.87	0.66
PMF9	1	4.69	0.68
PMF10	1	1.89	0.60
PMF11	1	0.92	0.76
PMF12	3	0.65	0.69
PMM1	5	0.54	0.88
PMM2	6	0.59	0.70
PMM3	6	0.78	0.72
PMM4	6	0.61	0.73
PMM5	5	0.52	0.86
PMM6	7	0.67	0.68
PMM7	7	0.61	0.89
PMM8	7	0.84	0.54
PMM09	3	0.66	0.60
PMM10	3	1.1	0.64
PMM11	1	0.96	0.58
PMM12	4	0.78	0.62
PMM13	3	0.52	0.58

*intensity of *RACK/EF-1α*

3.10 Association analysis between SNP through SSCP patterns and the expression levels of functionally important genes in juvenile *P. monodon*.

Association analysis between SNP through SSCP patterns and the levels of gene expression was examined in juvenile *P. monodon*. As mentioned earlier, the expression levels of gene under investigation were significantly different between males (testes) and females (ovaries). As a result only female shrimps were included in the experiment. A total of 45 individuals were genotyped by SSCP but 16 of which was subjected to gene expression analysis using semiquantitative RT-PCR.

Amplification of thioredoxin peroxidase and *RACK* were not consistent. Expression analysis of these genes was successfully carried out (Figures 115 and 116 and Table 3.17). Accordingly, association analysis between genotypes and gene expression levels of these genes could not be determined

Figure 3.115 RT-PCR of thioredoxin peroxidase (A) and *EF-1α* (B) in ovaries (lanes 1 - 16) of juvenile *P. monodon.*

Figure 3.116 RT-PCR of *RACK* (A) and *EF-1α* (B) in ovaries (lanes 1 - 16) of juvenile *P. monodon.*

Table 3.17 Relative expression levels of thioredoxin peroxidase and *RACK* in ovaries of juvenile *P. monodon*

Specimens	Relative expression level	
	thioredoxin peroxidase	***RACK***
PJF1	0.94	0.45
PJF2	0.53	0.43
PJF3	0.69	0.44
PJF4	0.87	0.46
PJF5	0.80	0.47
PJF6	0.70	0.47
PJF7	0.95	0.48
PJF8	0.89	0.47
PJF9	0.72	0.49
PJF10	0.83	0.58
PJF11	0.50	0.52
PJF12	0.29	0.52
PJF13	0.19	0.53
PJF14	0.32	0.58
PJF15	0.49	0.56
PJF16	0.30	0.56

Several SSCP genotypes were found from SSCP analysis. Four individuals possessed the genotype 1 whereas three individuals each represented genotypes 2, 3 and 6. Only a single individual exhibited genotypes 4, 5 and 7. Statistical analysis did not reveal significant association between SNP through SSCP patterns (genotypes 1, 2, 3 and 6) and expression levels of ribophorin I in ovaries (Figure 3.117) of juvenile *P. monodon* (Table 3.18).

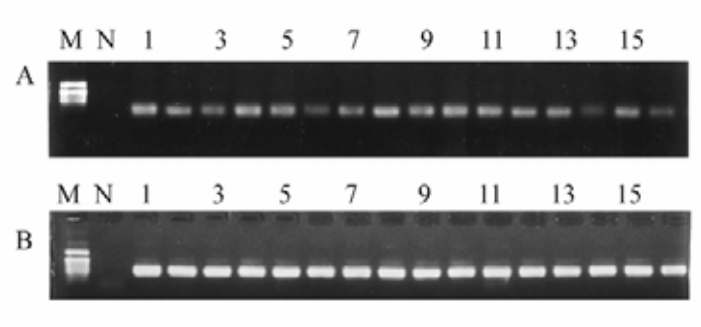


Figure 3.117 RT-PCR of ribophorin I (A) and *EF-1α* (B) in ovaries (lanes 1 - 16) of juvenile *P. monodon.*

Table 3.18 Relative expression levels of ribophorin I in ovaries of juvenile *P. monodon*

Specimens	Genotype	Expression levels
PJF1	1	1.37
PJF2	1	1.08
PJF3	1	0.81
PJF4	1	1.49
PJF5	2	1.17
PJF6	2	0.54
PJF7	2	0.93
PJF8	3	1.55
PJF9	3	1.25
PJF10	3	1.52
PJF11	4	1.30
PJF12	5	1.28
PJF13	6	1.15
PJF14	6	0.42
PJF15	6	1.01
PJF16	7	0.68

Amplification of the exon region of calponin 1 in juvenile *P. monodon* was not consistent. Therefore, the 5′UTR region was used for SSCP analysis (N = 45). Four SSCP patterns were observed in which the genotype 1 is the most common genotype found in 35 female individuals (77.78% of overall specimens). Only genotypes 1 and 2 were found in individuals analyzed by semiquantitative RT-PCR (Figure 3.118 and Table 3.19). Nevertheless, correlation between SSCP genotypes (5' UTR) and expression levels of calponin 1 was not significantly different.

Likewise, the exon region of *DAD1* was replaced by the 5' UTR region. SSCP analysis of 45 female individuals of juvenile *P. monodon*. Although the amplification product were column-purified to eliminate primer dimmers which can interfere SSCP results. Scoring of SSCP was then problematic at this gene segment. Six genotypes were found across overall specimens. Individuals carrying genotypes 1, 2, 3 and 5 were subjected to association analysis but did not show significant results.

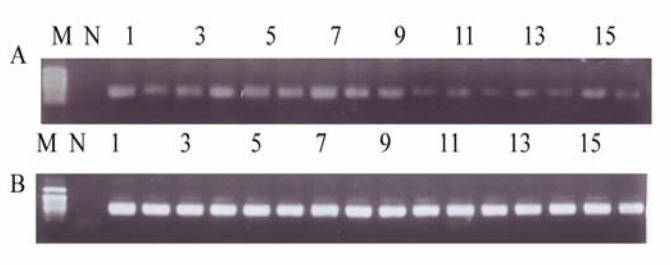


Figure 3.118 RT-PCR of calponin 1 (A) and *EF-1α* (B) in ovaries (lanes 1 - 16) of juvenile *P. monodon.*

Table 3.19 Relative expression levels of calponin 1 in ovaries of juvenile *P. monodon*

Specimens	Genotype	Expression levels
PJF1	1	0.28
PJF2	1	0.13
PJF3	1	0.19
PJF4	1	0.32
PJF5	1	0.28
PJF6	1	0.26
PJF7	1	0.36
PJF8	5	0.26
PJF9	5	0.24
PJF10	5	0.03
PJF11	1	0.09
PJF12	1	0.02
PJF13	ND	0.09
PJF14	ND	0.07
PJF15	ND	0.18
PJF16	1	0.07

ND = not determined.

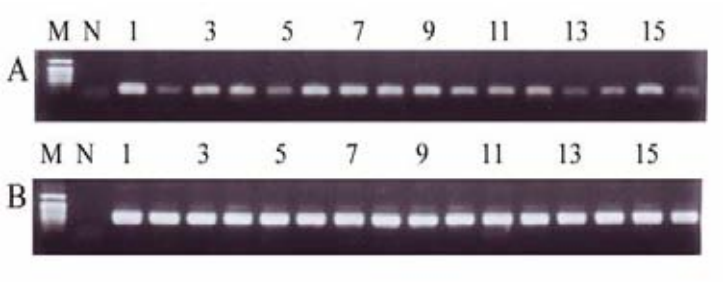


Figure 3.119 RT-PCR of *DAD1* (A) and *EF-1α* (B) in ovaries (lanes 1 - 16) of juvenile *P. monodon.*

Table 3.20 Relative expression levels of *DAD1* in ovaries of juvenile *P. monodon*

Specimens	Genotype	Expression levels
PJF1	1	0.66
PJF2	ND	0.17
PJF3	2	0.52
PJF4	2	0.49
PJF5	3	0.26
PJF6	ND	0.61
PJF7	ND	0.63
PJF8	5	0.56
PJF9	5	0.63
PJF10	2	0.433
PJF11	ND	0.40
PJF12	6	0.46
PJF13	5	0.15
PJF14	3	0.28
PJF15	3	0.51
PJF16	1	0.17

ND = not determined.

CHAPTER IV

DISCUSSION

Identification of genes exhibiting polymorphic SNP by SSCP analysis

Homologues of known transcripts from cDNA libraries of ovaries and temperature stress hemocytes of *P. monodon* were chosen for polymorphic studies. Approximately 47.52% (48/101) of primers yielded the amplification product. The failure to amplify the product in 52.48% of investigated primers possible due to the existence of intron (s) in the amplification region or alternatively, primers may prime at part of the region flanked by the intron. Some primers generated the larger fragment sizes than those expected from cDNA sequences due to the presence of the intron (s) located in the amplification regions. Large amplification fragments were restricted with appropriate restriction enzymes before genotyped by SSCP.

Liang et al. (2005) studied polymorphism of 5' flanking region in chicken prolactin (*cPRL*) and provided the possibility that polymorphic SNP site might be related to the broodiness in chickens via modulating the transcriptional level of *cPRL* The dissociation among *cPRL* gene transcription, mRNA storage and hormone was observed. Trakooljul et al. (2004) showed that the androgen receptor (*AR*) is highly polymorphic and polymorphism affect the predicted amino acid sequence and the transcription factor binding sites and are associated with allele-specific differences of the *AR* mRNA transcript level in liver of the porcine.

Likewise, polymorphism at the exon (and intron in gene homologues providing larger amplification sizes than expected) of various genes in *P. monodon* was examined. The giant tiger shrimp (*P. monodon*) has not yet domesticated therefore specimens from the same families and/or lineages are not available at present. Screening of polymorphic gene homologues were then carried out using non-related *P. monodon* broodstock originating from Satun, Trang, Phangnga, Chumphon and Trat. This was one of the major disadvantages of the experiments.

Correlations between genotypes (SNP through SSCP patterns) and phenotypes (levels of gene expression) of functionally important genes were searched and, if

significant, possibly applied for selective breeding programmes of *P. monodon*. SSCP patterns of characterized genes can be applied for different purposes. For example, polymorphic patterns of *CCTP*, *ProPO*, *GGT*, *HSP10* and ribiphorin I were observed but patterns cannot be scored as co-dominant markers (homozygotes and heterozygotes cannot be differentiated). Therefore, they may be used for identification of SNP and further studies for correlation between SNP and expression of functional important transcripts.

On the other hand, genes encoding thyroid hormone receptor associated protein, NADH dehydrogenase subunit 5, dentritic cell protein, FIII-4, nonclathrin coat protein zeta 2, vesicular integral membrane protein, *DAD1* and *RACK* act as co-dominant markers, and can be conveniently applied for both association analysis and construction of the genetic linkage maps in *P. monodon*.

Characterization of five functionally important genes in *P. monodon*

Several publications revealed significant association of SNP (and SSCP patterns) at the 5′UTR and phenotypes (e.g. growth rates) and/or levels of gene expression. Morganti et al. (2005) reported relationships between promoter polymorphism in the thymidylate synthase gene and mRNA levels in colorectal cancer. Aoki-Suzuki et al. (2005) revealed specific haplotypes encompassing alternatively spiced exons of the nitrin-G1 (*NTNG1*) were associated with schizophrenia. The mRNA of different isoform was significantly between schizophrenic and control brains. An association between *NTNG2* and schizophrenia was also observed with SNP and haplotypes of the 5' UTR.

Villette et al., (2002) studied SNP in the 3'UTR of membrane associated phospholipid hydroperoxide glutathione peroxidase (*GPX4*) of human. SNP was identified as a C/T variation at the 718th position. Individuals of different genotypes exhibited significant differences in the level of lymphocyte 5-lipoxygenase total products, with C718 showing increase levels of those products compared to T718 and T/C718 (36% and 44% increases, respectively). This suggests that SNP718 has functional effects and support the hypothesis that *GPX4* plays a regulatory role in leukotriene biosynthesis.

Likewise, 5 functionally important genes including ribophorin I, thioredoxin peroxidase, calponin 1, *DAD1* and *RACK* were chosen for further characterization and isolation of the full length of cDNA of ribophorin I and *RACK* and the genomic full length of thioredoxin peroxidase, calponin 1 and *DAD1*. SSCP patterns of the 5' and 3' UTR of these genes could then be further examined and compared with those from an exon of the same gene.

The ribophorin I gene encodes a rough endoplasmic reticulum (RER) specific membrance protein which is a subunit of oligosaccharyltransferase (Rajasekaran et al., 1995), and have an anti-apoptotic function.

RACK is functionally responsible for the intracellular localization of activated protein kinase C. Recently, it has been demonstrated that *RACK* also serves as an adaptor/scaffold for many signaling pathways, including interferon receptor-mediated activation of signal transducer and activator of translocation, insulin-like growth factor-dependent signaling and Src activation (Croze et al., 2000; Chen et al., 2004).

Thioredoxin peroxidase (*TPx*) is regarded as one of the members of peroxiredoxin (*Prx*) gene super family and functions as an antioxidant to remove the reactive oxygen species (ROS), O_2^- and H_2O_2 derived from normal cellular metabolism using thioredoxin as the electron donor.(Li et al., 2004)

Calponin 1 is the F-actin associated protein involved in the regulation of smooth muscle contraction or to be phosphorylated *in vitro* by protein kinase (PKC) and Ca^{2+} /calmodulin dependent protein kinase II (CaM kinase II).(Kaneko et al., 2000).

DAD1 encodes a protein possessing a major function as a negative regulator of programmed cell death (Kelleher and Gilmoe., 1997) found in human and mouse this genes map to chromosome 14q11-q12 and chromosome 14, respectively. The human *DAD1* cDNA contain an ORF of 685 bp, which encodes a polypeptide of 339 bp (Ylug et al., 1994). In spider (*Araneus ventricosus*), cDNA of *a DAD1* homologue contain a short ORF of 339 bp, which encodes a protein of 113 amino acid residues. The expression level of *DAD1* were particularly high when the spider was exposed at low (4ºC) and high (37ºC) temperatures, suggesting that the gene is responsive to temperature stimuli (Lee et al., 2003)

5′ and 3′ RACE-PCR was successfully used to isolate the full length cDNA of ribophorin I and *RACK* from the ovarian cDNA template. No sequence data of ribophorin I in crustaceans were found in the GenBank. Phylogenetic analysis indicated distantly related between ribophorin I of *P. monodon* and that of mammalian species (*H. sapiens, R. norvegicus* and *S. scrofa*) but closer relationship was observed when compared with fish (*D. rerio*).

A *RACK* homologue from *P. monodon* revealed phylogenetically close related with that of invertebrates (*Bombyx mori, Plutella xylostella, Heliothis virescens*) but distantly related with that of the tilapia (*Oreochromis mossambicus*) and *Paralichthys olivaceus* and guanine nucleotide-binding protein of *Petromyzon marinus* and guanine nucleotide binding protein, beta polypeptide 2-like 1 of *Rattus norvegicus*.

The full length cDNA of thioredoxin peroxidase (ovarian cDNA library), calponin 1 (hemocyte cDNA library) and *DAD1* (hemocyte cDNA library) were already obtained through EST analysis. The genomic full length of these three genes was further characterized by genome walking analysis. A neighbor-joining tree indicated that a homologue of thioredoxin peroxidase exhibited large divergence with members of the peroxiredoxin gene super family (antiapoptotic proteins) from 2-cys thioredoxin peroxidase of *Aedes aegypti*, natural killer enhancing factor *B* of *Tetraodon nigroviridis* , Peroxiredoxin 2, isoform a of *Homo sapiens*, peroxiredoxin 2 of *Bos Taurus* and thioredoxin peroxidase of *Branchiostoma belcheri tsingtaunese* and *Myotis lucifugus*

Phylogenetic analysis showed that calponin 1 of *P. monodon* closer related with *CPN-1* of *Caenorhabditis briggsae*, calponin of *Branchiostoma belcheri* and mantle gene 2 of *Pinctada fucata* and transgelin 2 *of Danio rerio* than transgelin 2 of *Homo sapiens* and *Mus musculus*. Transgelin is regarded as an actin cross-linking/gelling protein (by similarity), which involves in calcium interactions and contractile properties of the cells and contains 1 calponin-like repeat and shows less similarity with *P. monodon* calponin 1 than *CPN-1* and calponin from other species.

In mammalian species, *DAD1* is regarded as a subunit of the oligosaccharyltransferase (OST) system (Kelleher and Gilmore, 1997). A neighbor-joining tree indicated close relationships between *DAD1* of *P. monodon* and

Anopheles gambiae but distantly related with that of *A. irradiant, Ixodes scapularis, Mus musculus* and oligosaccharyltransferase subunit homologue of *M. musculus.* Moreover, *DAD1* and oligosaccharyltransferase subunit homologue of *M. musculus* is phylogenetically close related. The tree topology suggested that these genes were not born from the gene duplication process.

The full length cDNA and genomic DNA generated from RACE PCR and genome walking analysis provided the basic knowledge about gene organization. The obtained full length cDNA can be further used for protein expression *in vitro*. Using this technique, the complete gene sequence and the full length cDNA of vitellogenin (*PmVg1*) in *P. monodon* was successfully isolated. RT-PCR was carried out to examine *in vitro* effects of farnesoic acid and 20-hydroecdysone *PmVg1* expression and found that those neurohormones significantly stimulated the expression levels of *PmVt1* in an *in vitro* hepatopancreas culture (Tiu et al., 2005).

RT-PCR and semiquantitative RT-PCR of five functionally important genes

Ribophorin I and thioredoxin peroxidase were originally found in the ovarian cDNA library whereas calponin 1, *DAD1* and *RACK* were initially found in the hemocyte cDNA library. RT-PCR was carried out to determine whether these transcripts were expressed in gonads of *P. monodon*. These genes were not tissue-specific but ubiquitously expressed in hemocytes, ovaries and testes of juvenile *P. monodon.*

Under the laboratory conditions, semiquantitative PCR indicated significant differences on the expression levels in ovaries and testes of juvenile *P. monodon.* Only RACK did not show differential expression between ovaries and testes of *P. monodon* broodstock. Results critically suggested that association analysis between SNP and expression levels of these genes in ovaries and testes should be tested separately.

Under the thermal stress conditions, only thioredoxin peroxidase in ovaries revealed a greater expression level than did shrimps in the laboratory conditions at 6 hours after treatment. Additionally, the expression of *RACK* was significantly higher than the normal level at 12 hours after temperature stress ($P < 0.05$). Result did not

indicate the sensitive response of *DAD1* to temperature stimulation as previously reported in *A. ventricosus* (Lee et al., 2003).

Identified SNP at the 5′ UTR and coding region using direct sequencing

On the basis of previous studies, high genetic diversity of *P. monodon* has been reported based on both microsatellites (Supungul et al., 2000) and mtDNA (Klinbunga et al., 1999; 2001). Therefore, several SNP positions should be existence in the amplified gene segment. This may reduce the efficiency for detection of SNP by SSCP Moreover, simplification of SNP detection are required to deal with a large number of pedigreed specimens.

The full length sequences of ribophorin I, *RACK*, thioredoxin peroxidase, calponin and *DAD1* allow further characterization of SNP at the 5′ and 3′-UTR of each gene. New primer pairs were designed and tested. Only primers for amplification of the 3′ UTR of thioredoxin peroxidase did not generate the amplification product. The PCR product of the remaining genes were subjected to SSCP analysis and showed reasonable polymorphism when screened with genomic DNA of wild *P. monodon* broodstock.

Four SSCP patterns were observed from analysis of a 317 bp fragment (with 8 SNP/indels) of the 5′ UTR of calponin 1. Nucleotide sequences of individuals representing each SSCP genotypes were different indicating the accuracy of genotyping of SNP by SSCP analysis.

Likewise, seven SSCP patterns were found from analysis of the amplified 5′ UTR of *DAD1* (367 bp with 18 SNP/indels). However, two individuals representing different SSCP genotypes shared identical nucleotide sequences suggesting the existence of false positive results. This lowered the accuracy for genotyping of SSCP for the 5′ UTR of calponin 1.

Underestimation of SNP was occurred when SSCP was applied for genotyping a large amplification fragment (>500 bp with 11 SNP.indels) of *RACK*. Several individuals exhibiting the same SSCP genotype revealed different DNA barcodes..

Generally, it is accepted that the efficiency to detect single base substitutions by SSCP was approximately 80% of those verified by DNA sequencing (Shastry, 2002). Results from this thesis further indicated that SSCP is powerful for detection of small DNA fragment, for example 316 bp of calponin 1. The detection ability was slightly reduced when fragments is larger (e.g. 367 bp of *DAD1*) but extensive underestimation was found when the large fragment was analyzed (e.g. 500 bp of *RACK*).

The main advantage for species showing a large number of SNP/indels was that association between genotypes and phenotype (gene expression of economically important traits) is analyzed using string of SNP indels rather than a single position. This may result in ambiguous conclusions of obtained data. Several non-sequencing based methods can be used for identification of the exact SNP in the DNA fragments but the cost-effective approaches are PCR-RFLP and PCR allele-specific amplification (PASA as well as bidirectional-PASA).

The preliminary experiment for simplification of SSCP to PASA and PCR-RFLP were carried out.. Typical PASA developed in this study was not consistent. Genotyping of PASA was improved using primers modified to be mismatched at the position N-2 from the selective 3' terminal allele-specific bases. Nevertheless, PCR-RFLP of the 5' UTR of *DAD1* with *Dra* I and *Hin*f I showed promising results but needs further validation of the technique using pedigreed families of *P. monodon*.

Association between SNP through SSCP patterns and allele-specific expression levels of ribophorin I, *RACK*, thioredoxin peroxidase, calponin 1 and *DAD1*

Association analysis between genotypes of males and females of *P. monodon* broodstock and allele-specific expression level of investigated genes was not statistically significant. This was resulted from the large standard variation from original expression data. Limited numbers of individuals in each genotype class may interfere statistically analysis. Therefore, the experiment should be further carried out using larger sample sizes. Gene expression of normal, thermal-stress and viral-challenged shrimps may be compared.

Disregarding the large standard variation from RT-PCR and statistical tests, trends of correlation between different alleles (excluding those carried by single individuals) and gene expression levels were observed. The association can not be deduced from ribophorin I (broodstock) and thioredoxin peroxidase (juveniles) and *RACK* (juveniles) owing to the existence of only one common genotype in male and female broodstock and the failure to genotype female juveniles, respectively.

The average expression levels of ribophorin I in juveniles carrying genotype 3 (1.440, $N = 4$) was greater than those possessing genotype 1 (1.188, $N = 3$), 2 (0.880, $N = 3$) and 6 (0.86, $N = 3$).

Considering association of SNP and the expression levels of thioredoxin peroxidase in female and male *P. monodon* broodstock separately, the correlation figure in females could not be concluded due to the presence of one common genotype (genotype 3) and 4 singleton (genotypes 1, 6, 7 and 8) whereas males carrying genotypes 3 expressed greater levels of thioredoxin peroxidase (0.382, $N = 5$) than did males carrying genotype 9 (0.255, $N = 2$).

The average expression levels of the 5′ UTR of calponin 1 in male broodstock carrying genotype 3 (0.360, $N = 2$) was lower than those possessing genotype 5 (0.520, $N = 2$) and 6 (0.503, $N = 3$). Additionally, association between genotypes of the exon/intron region and the gene expression levels was also examined in male and female broodstock and female juveniles of *P. monodon*. The average expression levels of calponin 1 in female broodstock carrying genotype 7 (0.917, $N = 2$) was greater than those possessing genotype 5 (0.803, $N = 3$) and 2 (0.535, $N = 2$). In female juveniles, the average expression levels of calponin 1 in shrimps carrying genotype 1 (0.200, $N = 10$) was slightly greater than those possessing genotype 5 (0.177, $N = 2$).

Similarly, the average expression levels of *DAD1* in female broodstock carrying genotype 3 (0.990, $N = 2$) was greater than those possessing genotype 1 (0.926, $N = 5$) 2 (0.750, $N = 2$) and 4 (0.503, $N = 2$). Additionally, the average expression levels of *DAD1* in female broodstock carrying genotype 2 (0.750, $N = 2$) was greater than those possessing genotype 4 (0.480, $N = 4$), 1 (0.325, $N = 4$) and 5 (0.275, $N = 2$). In female juveniles, the average expression levels of *DAD1* in shrimps

carrying genotype 2 (0.480, *N* = 2) was slightly greater than those possessing genotype 5 (0.447, *N* = 3), 1 (0.415, *N* = 2) and 3 (0.350, *N* = 3).

The average expression levels of *RACK* in female broodstock carrying genotype 7 (0.924, *N* = 5) was greater than those possessing genotype 1 (0.675, *N* = 4) whereas the average expression levels of *RACK* in male broodstock carrying genotype 5 (0.870, *N* = 2) was slightly greater than those possessing genotype 6 (0.717, *N* = 3), 7 (0.703, *N* = 3) and 3 (0.607, *N* = 3).

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 their expression levels, this 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.

The genetic improvement and other biotechnological applications are crucial to the future sustainable development of *P. monodon* industry. Traditional selective breeding programmes can be immediately applied for long-term genetic improvement of this species. After stable breeding programmes have been established, the basic information about correlation between phenotypes (commercially important traits such as growth rates and disease resistance or expression levels of genes involving important functions) and genotypes (through genetic linkage maps or direct association) which is crucial for genetic improvement of this species, can be directly applied.

An understanding of shrimp genome is also important for studies about correlation between economically phenotypes and genotypes. EST libraries provide gene catalogues for further polymorphic studies. The EST-derived markers can be analyzed by several methods to identify SNP and indels. The availability of mapping populations will allow construction of the genetic linkage maps of *P. monodon* by type I markers. Sequencing of large inserts generated from BAC and/or FOSMID libraries will allow the construction of physical map of *P. monodon*. The information can be finally combined with other approach to merge the physical and genetic

linkage maps. Marker-assisted selection (MAS) will then be successfully identified and subsequently applied for genetic election of *P. monodon*.

CHAPTER V

CONCLUSIONS

1. Polymorphism of 101 gene homologues originally identified in cDNA libraries of ovaries and hemocytes of *P. monodon* were determined by SSCP analysis. A total of 48 gene segments were successfully amplified by PCR and 44 of which exbhibited polymorphism against genomic DNA of wild *P. monodon*.

2. RACR-PCR was carried out to identify and characterized the full length cDNA of ribophorin I and *RACK*. The ORF of the former was 1806 bp putative encoding a polypeptide of 602 amino acids with three predicted *N*-linked glycosylation whereas that of the latter was 957 bp encoding a polypeptide of 319 amino acid.

3. The full length genomic DNA of thioredoxin peroxidase, calponin 1 and *DAD1* homologues were characterized by genome walk analysis. Thioredoxin peroxidase contained 3 exons and 2 introns with the ORF of 591 bp encoding a polypeptide of 197 amino acid. Calponin 1 contained 3 exons and 2 introns with the ORF of 570 encoding a polypeptide of 190 amino acids. *DAD1* contained 2 exons and 1 intron with ORF of 345 bp encoding a polypeptide of 115 amino acids.

4. Semi-quantitative analysis of five investigated transcripts was developed. Relative expression levels of those transcripts in ovaries and testes of juvenile *P. monodon* were significantly different ($P < 0.05$). However, the expression levels of *RACK* in male and female *P. monodon* broodstock were not significantly different.

5. Following the thermal treatment, the expression level of thioredoxin peroxidase in ovaries and *RACK* in hemocytes of juvenile shrimps was significantly higher than that the normal level at 6 and 12 hours post-treatment, respectively ($P < 0.05$).

6. SNP of functionally important genes was analyzed through SSCP. Polymorphic SSCP patterns of calponin 1 (5′UTR), *DAD1* (5′UTR) and *RACK* (exon) were further characterized by direct DNA sequencing.

7. Correlation between SSCP patterns and the expression level of ribophorin I, thioredoxin peroxidase, calponin 1, *DAD1* and *RACK* in ovaries of juveniles and broddstock of *P. monodon* were not statistically significant.

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APPENDICES

APPENDIX A

PCR and SSCP conditions of 102 primer pairs

Genes	PCR conditions	SSCP conditions
1.Whey acidic protein (WAP)	94°C, 3 min for 1 cycle followed by 94°C, 1 min. 53°C, 1 min. 72°C, 2 min. for 40 cycles and 72°C, 7 min. for 1 cycle	-
2. Heat shock protein 60 (HSP60)	94°C, 3 min. for 1 cycle followed by 94°C, 1 min. 63°C, 1 min. decrease 2°C every 2 cycles (61, 59, 57,55°C) 72°C, 2 min. 94°C, 1 min. 53°C, 1 min. 72°C, 2 min. for 28 cycles and 72°C, 7 min. for 1 cycle	15%(acrylamide:bisacrylamide= 17.5:1), 180V ,19 hrs.
3. Survivin	As described in 1.	12.5%(acrylamide:bisacrylamide=75:1),180V ,18 hrs
4. P109 protein	As described in 1.	-
5. Cyclophilin 18	As described in 1.	15%(acrylamide:bisacrylamide=37.5:1),180V ,19 hrs.
6. Chaperonin containing t-complex polypeptide (CCTP)	As described in 2.	15%(acrylamide:bisacrylamide=37.5:1),180V ,19 hrs.
7. Propo factor	As described in 1.	17.5%(acrylamide:bisacrylamide=37.5:1),200V,21 hrs.

Genes	PCR conditions	SSCP conditions
8. Clottable protein	94°C, 3 min. for 1 cycle 94°C, 45 sec. 42°C, 1 min. 72°C, 45 sec. for 5 cycles 94°C, 45 sec. 53°C, 1 min. 72°C, 45 sec. for 35 cycles and 72°C, 7 min. for 1 cycle	-
9. Glutathione peroxidase (Gpx)	As described in 1.	-
10.Gamma glutamyltransferase(GGT)	As described in 1.	20%(acrylamide:bisacrylamide=75:1), 250V ,19.30 hrs.
11.Peptide polyl cis trans isomer5 (PPI)	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
12.Heat shock protein 10 (HSP10)	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
13. Superoxide dismusutase (SOD)	As described in 2.	-
14. Casein kinase II beta chain	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
15. Chaperonin subunit 8	As described in 1.	-
16. Muskelin 1	As described in 1.	-
17. Ovarian lipoprotein receptor	As described in 1.	-

Genes	PCR conditions	SSCP conditions
18. Ribophorin I	94°C, 3 min. for 1 cycle 94°C, 1 min. 63°C, 1 min. decrease 2°C every 2 cycles (61, 59, 57,55°C) 72°C, 2 min. 94°C, 1 min. 53°C, 1 min. 72°C, 2 min. for 28 cycles and 72°C, 7 min. for 1 cycle	12.5%(acrylamide:bisacrylamide=37.5:1),180V ,18 hrs
19. Innexin-2	As described in 1.	-
20. 2-Cys thioredoxin peroxidase	94°C, 3 min. for 1 cycle 94°C, 1 min. 55°C, 1 min. 72°C, 1.5 min. for 40 cycles and 72°C, 7 min. for 1 cycle	12.5%(acrylamide:bisacrylamide = 75:1),180V ,18 hrs
21. agCP13148	As described in 1.	12.5%(acrylamide:bisacrylamide = 75:1),180V ,18 hrs.
22. Asparaginyl-tRNA synthetase	94°C, 3 min. for 1 cycle 94°C, 45 sec. 65°C, 1 min. decrease 2°C every 2 cycles (63,61, 59, 57°C) 72°C, 2 min. 94°C, 45 sec 55°C, 1 min. 72°C, 1 min. for 30 cycles and 72°C, 7 min. for 1 cycle	10%(acrylamide:bisacrylamide = 75:1),180V ,17 hrs.

Genes	PCR conditions	SSCP conditions
23. Semaphorin 2A precurso	As described in 1.	
24. Zeta1-cop	As described in 22.	10%(acrylamide:bisacrylamide = 75:1),180V ,17 hrs.
25. Ferrochelatase	As described in 1.	
26. Calcium independent phospholipaseA2 isoform 1	As described in 1.	-
27. HLA-B-associated transcript 1A	94°C, 3 min. for 1 cycle 94°C, 45 sec. 45°C, 1 min. 72°C, 30 sec. for 5 cycles 94°C, 45 sec. 53°C, 1 min. 72°C, 30 sec. for 35 cycles and 72°C, 7 min. for 1 cycle	17.5%(acrylamide:bisacrylamide= 37.5:1),200 V ,21 hrs.
28. Aldehyde dehydrogenase family 6, subfamily A1	94°C, 3 min. 94°C, 45 sec. for 1 cycle 66°C, 1 min. decrease 3°C every 2 cycles (64,61, 59°C) 72°C, 45 sec 94°C, 45 sec 58°C, 1 min. 72°C, 45 sec. for 28 cycles 72°C, 7 min. for 1 cycle	15%(acrylamide:bisacrylamide=37.5:1),180V ,19 hrs.

Genes	PCR conditions	SSCP conditions
29. Pre-B-cell colony-enhancing factor	As described in 1.	-
30. Carbonic anhydrase	As described in 20	-
31. Chromobox protein	As described in 20	-
32. Vacuolar-type H+-ATPase subunit A	As described in 20	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
33. Dihydropteridine reductase	As described in 1.	-
34. Aminopeptidase	As described in 22	12.5%(acrylamide:bisacrylamide = 75:1),180V ,18 hrs.
35. Dolichyl-di-phosphooligosaccharide-protein glycotransferase	As described in 22	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
36. Integrin beta 4 binding protein	As described in 1.	-
37. 3-oxoacid CoA transferase	As described in 1.	-
38. Profilin	As described in 1.	-
39. Adenosylhomocys -teinase	As described in 1.	-
40.COP9 subunit 6	As described in 1.	-
41. ATP/GTP-binding protein	As described in 1.	-
42. Protease	As described in 20	10%(acrylamide:bisacrylamide = 75:1),180V ,17 hrs.
43. Thiolase	As described in 1.	-
44. Aspartate aminotransferase	As described in 1.	-
45. Carnitine palmitoyltransferase II	As described in 1.	-
46. Receptor activating protein kinase C	As described in 20.	10%(acrylamide:bisacrylamide = 75:1),180V ,17 hrs.

Genes	PCR conditions	SSCP conditions
47. Tetraspanin D107	94°C, 3 min. for 1 cycle 94°C, 45 sec. 42°C, 1 min. 72°C, 1.5 min. for 5 cycles 94°C, 45 sec. 52°C, 1 min. 72°C,1.5 min. for 35 cycles and 72°C, 7 min. for 1 cycle	-
48. Methyl CpG binding protein 2	94°C, 3 min. for 1 cycle 94°C, 45 sec. 42°C, 1 min. 72°C, 1.5 min. for 5 cycles 94°C, 45 sec. 52°C, 1 min. 72°C,1.5 min. for 35 cycles and 72°C, 7 min. for 1 cycle	-
49. Presenilin enhancer*	As described in 1.	-
50. Defender against cell death 1*	As described in 20.	12.5%(acrylamide:bisacrylamide = 75:1),180V ,18 hrs.
51. Glycogen phosphorylase*	As described in 20.	12.5%(acrylamide:bisacrylamide= 37.5:1),180 V, 18 hrs.
52. Nonclathrin coat protein zeta*	As described in 1.	12.5%(acrylamide:bisacrylamide= 37.5:1),180 V, 18 hrs.

Genes	PCR conditions	SSCP conditions
53. NH2 non-histone chromosome protein 2-like*	As described in 1.	10%(acrylamide:bisacrylamide = 75:1),180V ,17 hrs.
54. FIV/2	As described in 1.	17.5%(acrylamide:bisacrylamide= 37.5:1),200 V ,21 hrs.
55. FIV20	As described in 1.	-
56. FV(27)	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
57. FI/40	As described in 1.	-
58. MII 51	As described in 1.	20%(acrylamide:bisacrylamide=75:1), 250V ,19.30 hrs.
59. FIII(4)R	As described in 1.	-
60. 457(OP1)	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
61. FI(1)	As described in 1.	17.5%(acrylamide:bisacrylamide= 37.5:1),200 V ,21 hrs.
62. 428(OPB17)R	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
63. FIII 39	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
64. FV-1	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
65. FV 42	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
66. FIII 8	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.

Genes	PCR conditions	SSCP conditions
67. MI 36	As described in 1.	17.5%(acrylamide:bisacrylamide= 37.5:1),200 V ,21 hrs.
68. FIV 33	As described in 1.	17.5%(acrylamide:bisacrylamide= 37.5:1),200 V ,21 hrs.
69. Nit protein 2*	As described in 1.	-
70. Immunophilin FKBP 52*	As described in 1.	-
71. Calcium regulated heat stable protein*	As described in 1.	-
72. Dynein heavy chain 64C*	As described in 1.	-
73. Mapre 1 protein*	94°C, 3 min. 94°C, 45 sec. for 1 cycle 66°C, 1 min. decrease 3°C every cycles (63,60, 57°C) 72°C, 1 min. 94°C, 45 sec 55°C, 1 min. 72°C,1min. for 30 cycles 72°C, 7 min. for 1 cycle	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
74. Peroxinectin*	94°C, 3 min. 94°C, 45 sec. for 1 cycle 66°C, 1 min. decrease 3°C every cycles (63,60, 57°C) 72°C, 1 min. 94°C, 45 sec 55°C, 1 min. 72°C,1min. for 30 cycles 72°C, 7 min. for 1 cycle	10%(acrylamide:bisacrylamide = 75:1),180V ,17 hrs.

Genes	PCR conditions	SSCP conditions
75. Diphenol oxidase A2*	As described in 1.	17.5%(acrylamide:bisacrylamide= 37.5:1),200 V ,21 hrs.
76. Dendritic cell protein**	As described in 1.	10%(acrylamide:bisacrylamide = 75:1),180V ,17 hrs.
77. Vesicular integral-membrane protein	As described in 1.	10%(acrylamide:bisacrylamide = 75:1),180V ,17 hrs.
78. Short chain dehydrogenase*	As described in 1.	-
79. Female sterile	As described in 8.	-
80. Multicatalytic endopeptidase*	As described in 8.	-
81.Myosin regulatory light polypeptide 9*	As described in 8.	-
82. Testes development related NYD SP19*	As described in 8.	-
83. Motochondrial oxodicarboxylate*	As described in 8.	-
84. Phospholipase C*	As described in 8.	-
85. Calcineurin B*	As described in 8.	-
86. Prefoldin subunit 2*	As described in 8.	-
87. Calponin I*	As described in 18.	10%(acrylamide:bisacrylamide = 75:1),180V ,17 hrs.
88. Hydroxyacyl CoA dehydrogenase*	As described in 1.	
89. Leucine rich repeat protein SHOC-2	As described in 1.	
90. Cyclin A	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.+5%glycerol

Genes	PCR conditions	SSCP conditions
91. Phenylalanyl tRNA synthetase beta subunit*	As described in 1.	-
92. Carbomoyl phosphate synthetase 2	As described in 8.	-
93. Clathrin adaptor protein AP50	As described in 8.	-
94. Leukemia virus receptor*	As described in 8.	-
95. Endothelial cell growth factor 1*	As described in 8.	-
96. NADH dehydrogenase subunit 5*	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
97. 5 methylcytosin G/T mismatch*	As described in 8.	-
98. Minute (2) 21AB	As described in 8.	-
99. Myelodysplasia/ Myeloid leukemia factor**	As described in 8.	-
100. Thyroid hormone receptor associated protein	As described in 1.	15%(acrylamide:bisacrylamide = 37.5:1),180V ,19 hrs.
101. Guanine nucleotide binding protein*	As described in 1.	10%(acrylamide:bisacrylamide = 75:1),180V ,17 hrs.

* Primer pairs found in hemocyte cDNA library
** Primer pairs found both in ovarian and hemocyte cDNA library
Primer pairs in number 54-68 developed from RAP marker
Other primer pairs found in ovarian cDNA

APPENDIX B

PCR and SSCP conditions of 5 functionally important gene used for identification of 5'UTR and 3'UTR.

Gene	PCR condition	SSCP condition
1.5'Ribophorin I	94°C, 3 min. for 1 cycle 94°C, 1 min. 42°C, 1 min. 72°C, 45 sec. for 10 cycles 94°C, 1 min. 53°C, 1 min. 72°C, 45 sec. for 35 cycles and 72°C, 7 min. for 1 cycle	17.5%(acrylamide:bisacrylamide=37.5:1),200V,21 hrs.
2.5'Thioredoxin peroxidase	94°C, 3 min for 1 cycle followed by 94°C, 1 min. 53°C, 1 min. 72°C, 2 min. for 40 cycles and 72°C, 7 min. for 1 cycle	17.5%(acrylamide:bisacrylamide=37.5:1),200V,21 hrs.
3.5'DAD 1		15%(acrylamide:bisacrylamide=75:1),180V ,19 hrs
4.5'RACK-1	As described in 1.	15%(acrylamide:bisacrylamide=37.5:1),180V ,19 hrs
5.5'Calponin I	As described in 2.	15%(acrylamide:bisacrylamide=37.5:1),180V ,19 hrs
1.3'Ribophorin I	94°C, 3 min. for 1 cycle 94°C, 1 min. 42°C, 1 min. 72°C, 45 sec. for 10 cycles 94°C, 1 min. 53°C, 1 min.	17.5%(acrylamide:bisacrylamide=37.5:1),200V,21 hrs.

	72°C, 45 sec. for 35 cycles and 72°C, 7 min. for 1 cycle	
Gene	**PCR condition**	**SSCP condition**
2.3'Thioredoxin peroxidase	94°C, 3 min for 1 cycle followed by 94°C, 1 min. 53°C, 1 min. 72°C, 2 min. for 40 cycles and 72°C, 7 min. for 1 cycle	-
3.3'DAD 1	As described in 1.	17.5%(acrylamide:bisacrylamide=75:1),200V,21 hrs.
4.3'RACK-1	As described in 1.	17.5%(acrylamide:bisacrylamide=75:1),200V,21 hrs.
5.3'Calponin I	As described in 2.	15%(acrylamide:bisacrylamide=37.5:1),180V ,19 hrs.

APPENDIX C

Table 1 The expression levels of ribophorin I using ovaries and testes in *P. monodon* juveniles as template.

Specimens	Expression level	
	Ovaries	testes
1	1.197156	1.479234
2	1.127347	0.871416
3	1.786504	0.198745
4	1.483418	0.638279
5	1.542548	0.227475
6	1.753386	0.174262
Average	1.481727±0.27[a]	0.598235±0.51[b]

Table 2 The expression levels of thioredoxin peroxidase using ovaries and testes in *P. monodon* juveniles as template.

Specimens	Expression level	
	Ovaries	testes
1	0.214700	0.074842
2	0.433547	0.116110
3	0.336847	0.016060
4	0.315246	0.031793
5	0.397919	0.015011
6	0.522484	0.001421
Average	0.370124±0.11[a]	0.042539±0.04[b]

Table 3 The expression levels of calponin 1 using ovaries and testes in *P. monodon* juveniles as template.

Specimens	Expression level	
	Ovaries	testes
1	0.586151	0.69194
2	0.563590	0.670678
3	0.518696	0.480586
4	0.532612	0.633346
5	0.401898	0.731727
6	0.420079	0.590507
Average	0.503838±0.07[a]	0.633131±0.09[b]

Table 4 The expression levels of *DAD1* using ovaries and testes in *P. monodon* juveniles as template.

Specimens	Expression level	
	Ovaries	testes
1	0.680328	0.511440
2	0.687814	0.443467
3	0.654970	0.387800
4	0.793679	0.146985
5	0.812430	0.351169
6	0.731454	0.218697
Average	0.726779±0.06 [a]	0.34326±0.14 [b]

Table 5 The expression levels of *RACK* using ovaries and testes in *P. monodon* juveniles as template.

Specimens	Expression level	
	Ovaries	testes
1	0.775041	0.553899
2	0.714197	0.616051
3	1.037959	0.193014
4	0.745628	0.684165
5	0.716713	0.832197
6	0.916111	0.722531
Average	0.817608±0.13 [a]	0.60031±0.22 [b]

APPENDIX D

Table 1 Expression levels of ribophorin I in ovaries of *P. monodon* at different times of stress temperature.

Specimen	Expression level				
	Normal	0 hrs.	6 hrs.	12 hrs.	24 hrs.
1	0.750112	1.084831	1.425815	1.361882	1.146229
2	1.630085	0.724431	1.186264	1.516260	1.017885
3	1.010404	1.360521	1.200428	1.276840	0.945204
4	0.593855	1.02695	1.122738	1.862728	1.204263
5	0.777538	0.97856	1.707167	0.663939	0.958663
Average	0.952399 ±0.41 [a]	1.035058 ±0.23 [a]	1.328482 ±0.24 [a]	1.33633 ± 0.44 [a]	1.054449 ±0.12 [a]

Table 2 Expression levels of thioredoxin peroxidase in ovaries of *P. monodon* at different times of stress temperature.

Specimen	Expression level				
	Normal	0 hrs.	6 hrs.	12 hrs.	24 hrs.
1	0.460511	0.529538	0.607795	0.598622	0.548410
2	0.686368	0.585531	0.717088	0.536494	0.523497
3	0.608928	0.609096	0.555557	0.618978	0.323520
4	0.360531	0.515852	0.626377	0.601052	0.522380
5	0.425318	0.545270	0.735957	0.424838	0.533627
Average	0.508331 ±0.13 [a]	0.557058 ±0.04 [ab]	0.648555 ±0.08 [a]	0.555997 ±0.08 [ab]	0.490287 ±0.09 [b]

Table 3 Expression levels of calponin 1 in ovaries of *P. monodon* at different times of stress temperature.

Specimen	Expression level				
	Normal	0 hrs.	6 hrs.	12 hrs.	24 hrs.
1	0.743406	0.753550	0.772845	0.782681	0.674277
2	0.794073	0.729907	0.636754	0.757789	0.729903
3	0.679736	0.813204	0.755896	0.846190	0.756016
4	0.546727	0.703467	0.560146	0.712234	0.695174
5	0.595740	0.721740	0.804645	0.644781	0.750376
Average	0.671937 ±0.10[a]	0.744374 ±0.08[a]	0.706057 ±0.10 [a]	0.748735 ±0.07 [a]	0.721149 ±0.04 [a]

Table 4 Expression levels of DAD 1 in ovaries of *P. monodon* at different times of stress temperature.

Specimen	Expression level				
	Normal	0 hrs.	6 hrs.	12 hrs.	24 hrs.
1	0.684987	0.453293	0.502854	0.594761	0.754884
2	0.717717	0.650038	0.730173	0.493890	0.223245
3	0.457867	0.785451	0.472251	0.127198	0.374575
4	0.692275	0.740569	0.589794	0.781792	0.546255
5	0.480240	0.692122	0.498462	0.467441	0.483103
Average	0.606617 ±0.13 [a]	0.664295 ±0.13 [a]	0.558707 ±0.11 [a]	0.493016 ±0.23 [a]	0.476412 ±0.20 [a]

Table 5 Expression levels of RACK in ovaries of *P. monodon* at different times of stress temperature.

Specimen	Expression level				
	Normal	0 hrs.	6 hrs.	12 hrs.	24 hrs.
1	0.967935	0.811927	0.768527	0.941843	0.805039
2	0.857681	0.765626	0.741348	0.814634	0.770946
3	0.929475	0.752517	0.958007	0.914489	0.766940
4	0.886746	0.808196	0.961523	0.790211	0.802830
5	0.686085	0.859135	0.683092	0.630740	0.761756
Average	0.865584 ±0.11 [a]	0.79948 ±0.04 [a]	0.822499 ±0.13 [a]	0.818383 ±0.12 [a]	0.781502 ±0.02 [a]

APPENDIX E

Table 1 Expression levels of ribophorin I in hemocytes of *P. monodon* at different times of stress temperature.

Specimen	Expression level				
	Normal	0 hrs.	6 hrs.	12 hrs.	24 hrs.
1	0.335103	0.595286	0.648162	0.558514	0.678213
2	0.507934	0.366510	0.654350	0.767925	0.586902
3	0.700314	0.624195	0.507276	0.566558	0.880771
4	0.717635	0.700732	0.666358	0.760497	0.515893
5	0.713816	-	-	0.660217	0.672688
Average	0.594961 ±0.17 [a]	0.762241 ±0.14 [a]	0.666358 ±0.07 [a]	0.662742 ±0.10 [a]	0.666894 ±0.14 [a]

Table 2 Expression levels of thioredoxin peroxidase in hemocytes of *P. monodon* at different times of stress temperature.

Specimen	Expression level				
	Normal	0 hrs.	6 hrs.	12 hrs.	24 hrs.
1	0.058211	0.095035	0.113195	0.113349	0.123853
2	0.132180	0.115693	0.163842	0.172232	0.091591
3	0.123539	0.122816	0.122466	0.127224	0.236057
4	0.223820	0.236377	0.173119	0.134352	0.137139
5	0.235235	-	-	0.206557	0.216579
Average	0.154597 ±0.07 [a]	0.14248 ±0.06 [a]	0.143156 ±0.03 [a]	0.150743 ±0.04 [a]	0.161044 ±0.06 [a]

Table 3 Expression levels of calponin 1 in hemocytes of *P. monodon* at different times of stress temperature.

Specimen	Expression level				
	Normal	0 hrs.	6 hrs.	12 hrs.	24 hrs.
1	0.618282	0.665292	0.707147	0.674889	0.639757
2	0.656990	0.704589	0.616927	0.643175	0.527719
3	0.661036	0.546602	0.699272	0.697925	0.817112
4	0.714741	0.774932	0.870238	0.769876	0.791635
5	0.757687	-	-	0.861682	0.730703
Average	0.681747 ±0.05 [a]	0.672854 ±0.10 [a]	0.723396 ±0.11 [a]	0.729510 ±0.09 [a]	0.701385 ±0.12 [a]

Table 4 Expression levels of *DAD1* in hemocytes of *P. monodon* at different times of stress temperature..

Specimen	Expression level				
	Normal	0 hrs.	6 hrs.	12 hrs.	24 hrs.
1	0.338981	0.525611	0.507562	0.481311	0.524194
2	0.540407	0.248717	0.649477	0.641286	0.536368
3	0.460143	0.612140	0.244632	0.226178	0.733403
4	0.676208	0.697448	0.595757	0.107234	0.529788
5	0.684864	-	-	0.57405	0.375206
Average	0.540121 ±0.15 [a]	0.520979 ±0.19 [a]	0.499357 ±0.18 [a]	0.406012 ±0.23 [a]	0.539792 ±0.13 [a]

Table 5 Expression levels of *RACK* in hemocytes of *P. monodon* at different times of stress temperature.

Specimen	Expression level				
	Normal	0 hrs.	6 hrs.	12 hrs.	24 hrs.
1	0.158207	0.585021	0.708396	0.576231	0.697774
2	0.256273	0.426485	0.617981	0.686835	0.554148
3	0.375037	0.521173	0.401996	0.639020	0.676218
4	0.636599	0.700786	0.801499	0.769862	0.601394
5	0.707825	-	-	0.834188	0.685110
Average	0.426788 ±0.24 [a]	0.558367 ±0.12 [ab]	0.632468 ±0.17 [ab]	0.701227 ±0.10 [b]	0.642929 ±0.06 [ab]

APPENDIX F

Table 1 The expression levels of ribophorin I using ovaries and testes in *P. monodon* juveniles as template.

Specimens	Expression level	
	Ovaries	testes
1	1.197156	1.479234
2	1.127347	0.871416
3	1.786504	0.198745
4	1.483418	0.638279
5	1.542548	0.227475
6	1.753386	0.174262
Average	1.481727±0.27[a]	0.598235±0.51[b]

Table 2 The expression levels of thioredoxin peroxidase using ovaries and testes in *P. monodon* juveniles as template.

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2	0.433547	0.226110
3	0.336847	0.106060
4	0.315246	0.123170
5	0.397919	0.115011
6	0.522484	0.091421
Average	0.370124±0.11[a]	0.134436±0.05[b]

Table 3 The expression levels of calponin 1 using ovaries and testes in *P. monodon* juveniles as template.

Specimens	Expression level	
	Ovaries	testes
1	0.586151	0.69194
2	0.563590	0.670678
3	0.518696	0.480586
4	0.532612	0.633346
5	0.401898	0.731727
6	0.420079	0.590507
Average	0.503838±0.07[a]	0.633131±0.09[b]

Table 4 The expression levels of *DAD1* using ovaries and testes in *P. monodon* juveniles as template.

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4	0.793679	0.146985
5	0.812430	0.351169
6	0.731454	0.218697
Average	0.726779±0.06 [a]	0.34326±0.14 [b]

Table 5 The expression levels of *RACK* using ovaries and testes in *P. monodon* juveniles as template.

Specimens	Expression level	
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2	0.714197	0.616051
3	1.037959	0.193014
4	0.745628	0.684165
5	0.716713	0.832197
6	0.916111	0.722531
Average	0.817608±0.13 [a]	0.60031±0.22 [b]

Biography

Mr. Arun Buaklin was born on January 31, 1980 in Sukhothai Province, Thailand. He graduated with the degree of Bachelor of Science (Biotechnology) from Chiang mai University in 2002. He has studied for the degree of Master of Science (Biotechnology) at the Program of Biotechnology, Chulalongkorn University since 2003.

Publications during graduate study

1. Klinbunga, S., **Buaklin, A**., Aoki, T. and Menasveta, P. (2005). Determination of SSCP polymorphism and expression levels of ribophorin I and receptor for activated protein kinase C in the giant tiger shrimp (*Penaeus monodon*). Proceeding of The JSPS-NRCT International Symposium Joint Seminar 2005: Productivity techniques and effective utilization of aquatic animal resources into the new century. 19-21 December 2005. Kasetsat University, Bangkok, Thailand, p. 44-55.
2. **Buaklin, A**., Thumrungtanakit, S., Khamnamtong, B., Klinbunga, S. And Menasveta, P. 2005. SNPs by EST (SBE) and Expression Levels of Functionally Important Genes in the Giant Tiger Shrimp (*Penaeus monodon*) Biothailand 2005, Bangkok, Thailand (oral presentation).
3. **Buaklin, A**., Thumrungtanakit, S., Khamnamtong, B., Klinbunga, S. And Menasveta, P. 2005. Single nucleotide polymorphism and expression levels of functionally important genes in the giant tiger shrimp (*Penaeus monodon*).31th Congress on Science and Technology of Thailand (oral presentation).