การหาและวิเคราะห์เครื่องหมายพันธุกรรมที่จำเพาะในการจำแนกประชากรกุ้งกุลาดำ *Penaeus monodon* ในประเทศไทย

นางสาวทิฐิมา นวลบุญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

หลักสูตรเทคโนโลยีชีวภาพ

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2543

ISBN 974-13-0883-3

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

IDENTIFICATION AND CHARACTERIZATION OF POPULATION-SPECIFIC MARKER OF *Penaeus monodon* IN THAILAND

Miss Thitima naunboon

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biotechnology

Program of Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2000

ISBN 974-13-0883-3

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Thesis Title IDENTIFICATION AND CHARACTERIZATION OF POPULATION- SPECIFIC MARKER OF *Penaeus monodon* IN THAILAND

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ทิฐิมา นวลบุญ : การหาและวิเคราะห์เครื่องหมายพันธุกรรมที่จำเพาะในการจำแนกประชากรกุ้งกุลาดำ *Penaeus monodon* ในประเทศไทย(IDENTIFICATION AND CHARACTERIZATION OF POPULATION-SPECIFIC MARKER OF *Penaeus monodon* IN THAILAND) อ.ที่ปรึกษา : รศ.ดร. อัญชลี ทัศนาขจร, อ.ที่ปรึกษาร่วม : ผศ.ดร.วิเชียร ริมพาณิชยกิจ, 102 หน้า, ISBN 974-13-0883-3

ทำการตรวจหาแถบดีเอ็นเอที่ให้ความแตกต่างระหว่างกุ้งกุลาดำจากชายฝั่งทะเลอันดามัน และทะเลฝั่งอ่าวไทยด้วยเทคนิค Randomly Amplified PolymorphicDNA (RAPD) โดยการคัดเลือกเบสไม่จำเพาะทั้งสิ้น 11 ไพรเมอร์ ซึ่งมี 2 ไพรเมอร์คือ ไพรเมอร์ OPB 08 และ OPB15 ให้แถบดีเอ็นเอที่พบเฉพาะในกุ้งกุลาดำจากทะเลฝั่งอ่าวไทย กล่าวคือไพรเมอร์ OPB 08 ให้แถบดีเอ็นเอขนาดประมาณ 850 คู่เบส และไพรเมอร์ OPB 15 ให้แถบดีเอ็นเอขนาด 1,000 คู่เบส เมื่อเพิ่มจำนวนกุ้งกุลาดำทดสอบ 2 ไพร์เมอร์นี้พบว่าทั้ง 2 ไพรเมอร์ สามารถให้แถบดีเอ็นเอในกุ้งจากฝั่งอันดามันเช่นกัน ฉะนั้นจึงนำไพรเมอร์ 428 ที่มีรายงานมาก่อนว่าให้แถบดีเอ็นเอขนาดประมาณ 950 คู่เบสที่พบเฉพาะในกุ้งที่มาจากทะเลอันดามัน คือ จากสตูล-ตรังเป็นเครื่องหมายในการจำแนกกลุ่มประชากรจากฝั่งทะเลอันดามันและอ่าวไทยต่อไป

ทำการแยกชิ้นดีเอ็นเอ 950 คู่เบส นำไปโคลนเข้าพลาสมิด pUC 18 แล้วทรานฟอร์มเข้าสู่เซลล์แบคทีเรีย XL1-Blue พบโคลน 3 แบบ (คือโคลน A, B และ C) ที่ให้ขนาดชิ้นดีเอ็นเอ 900, 950 (เมื่อตัดด้วยเอนไซม์ *Bam* HI จะให้ดีเอ็นเอ 2 ขนาด คือ 650 และ 350 คู่เบส) และ 350 คู่เบส ตามลำดับ และทำการตรวจสอบว่าทั้ง 3 โคลนมาจากดีเอ็นเอขนาด 950 คู่เบส ที่แยกกุ้งทั้งสองฝั่งได้ โดยการทำSouthern Hybridizationด้วยดีเอ็นเอติดตามทั้ง 3 ขนาด (900, 650 และ 350 คู่เบส) พบว่าดีเอ็นเอติดตามขนาด 900 คู่เบส ให้แถบดีเอ็นเอที่ ปรากฏในกุ้งกุลาดำทั่ง 2 ฝั่ง ขณะที่ เมื่อใช้ดีเอ็นเอขนาด 650 คู่เบส เป็นตัวติดตามจะให้แถบดีเอ็นเอเพียงแถบเดียวเฉพาะกุ้งกุลาดำจากฝั่งอันดามัน และเมื่อใช้ดีเอ็นเอขนาด 350 คู่เบส ก็จะให้ผลเช่นกัน จากนั้นนำชิ้นดีเอ็นเอทั้ง 3 ขนาดไปหาลำดับนิวคลีโอไทด์และนำไปเปรียบเทียบกับยีนอื่นๆ ที่มีรายงานไว้ใน GenBank ด้วยโปรแกรม BLAST พบว่าดีเอ็นเอขนาด 900 คู่เบส คล้ายกับยีน Asparagine Synthetase ส่วนดีเอ็นเอขนาด 950 และ 350 คู่เบส พบว่าไม่มีความคล้ายคลึงกับยีนใดๆ จากลำดับนิวคลีโอไทด์ที่ได้นำมาทำการออกแบบไพรเมอร์ เพื่อนำไปใช้เพิ่มปริมาณดีเอ็นเออย่างจำเพาะด้วยเทคนิคพีซีอาร์ แต่ไม่สามารถใช้แยกกุ้งจาก 2 ฝั่งทะเลได้

ดังนั้นวิธีที่จำเพาะในแยกความแตกต่างของกุ้งกุลาดำทั้งสองฝั่งได้ ก็คือการนำเทคนิค Randomly Amplified Polymorphic DNA (RAPD) มาใช้คู่ไปกับการทำ Southern Hybridization โดยใช้ดีเอ็นเอติดตามขนาด 650 และ 350 คู่เบส ซึ่งให้แถบดีเอ็นเอเพียง 1 แถบ ขนาด 950 คู่เบส ซึ่งพบเฉพาะในกุ้งจากฝั่งอันดามัน

หลักสูตร… …..เทคโนโลยีชีวภาพ……………………ลายมือชื่อนิสิต……………………………………
สาขาวิชา …….เทคโนโลยีชีวภาพ……………………ลายมือชื่ออาจารย์ที่ปรึกษา……………………………
ปีการศึกษา ………2543……………………………ลายมือชื่ออาจารย์ที่ปรึกษาร่วม…………………………

4072265023 : MAJOR BIOTECHNOLOGY

KEY WORD : *Penaeus monodon* / RAPD / POPULATION-SPECIFIC MARKER

THITIMA NAUNBOON: IDENTIFICATION AND CHARACTERIZATION OF POPULATION-SPECIFIC MARKER OF *Penaeus monodon* IN THAILAND. THESIS ADVISOR: ASSOC..PROF. ANCHALEE TASSANAKAJON, Ph,D. THESIS CO-ADVISOR : ASSIST. PROF. VICHIEN RIMPHANITCHAYAKIT, Ph,D. 102 pp. ISBN 974-13-0883-3

Randomly amplified polymorphic DNA (RAPD) analysis was used to identify a specific marker which could distinguish between the wild populations of *Penaeus monodon* from the Andaman Sea and the Gulf of Thailand. The eleven selected RAPD primers were screened. Two primers, OPB 08 and OPB15, showed a DNA band with size about 850 bp and 1,000 bp, respectively, which consistenly appeared only in the sample from the Gulf of Thailand. By increasing the number of samples in each group using primer OPB 08 and OPB 15, these bands also appeared in the samples from the Andaman Sea. Therefore, the primer 428 which has been previously reported to provide a 950 bp specific to the population of the Andaman Sea was used in this study. The primer 428 appeared to identify a more variable region among the samples of Thai *P.monodon* from the Andaman Sea so a band with size about 950 bp which present in all of Satun Trang, but absent in samples from Trad was used as a population-specific marker

The 950 bp fragment was cloned and transformed into *E.coli*: XL1-Blue. Three different types of recombinant clones, A, B and C were obtained with the insert fragment size of 900 , 950 bp (which upon digestion with *Bam* HI providing two bands with size about 650 and 350 bp) and 350 bp, respectively. To ensure that the insert fragment of the 3 clones were from the 950 bp RAPD marker, Southern blot hybridization was performed using the three insert fragments (900 bp, 650 bp and 350 bp) as DNA probes. A single specific band was appeared only in the samples form the Andaman Sea when using the 650 and 350 bp fragments while a positive band was found in all samples when using the 900 bp fragment. The insert fragment of the 3 clones were sequenced by using the ABI-PRISM automated sequencer and then aligned to other genes in the GenBank using BLAST program. Comparison of the 900 bp sequence showed similarity with the sequence of asparagine synthetase while the 650 and the 350 bp insert fragment were not sinificantly similar to any sequence in the GenBank. The specific primers were designed from the nucleotide sequences of the 350 bp fragment but could not distinguish the population of *P.monodon* from the Andaman Sea and the Gulf of Thailand.

Therefore, a method which can be to distinguish the two populations was to amplified the genomic DNA using primer 428, followed by Southern blot hybridization with the 650 or 350 bp fragments. This method yielded a single band of 950 bp specific to the population of the Andaman Sea.

Department……………...Biotechnology……………Student' s signature……………………………………….
Field of study …………...Biotechnology…………...Advisor ' s signature………………………………………
Academic year……………….2000…………………Co-advisor ' s signature……………………………………

ACKNOWLEDEMENT

I would like to express my deepest gratitude and sincere to my advisor, DR. Anchalee Tassanakajon, for her valuable advice, encouragement and kindness throughout the course of this study.

I am very grateful to Dr.Vichien Rimphanitchayakit for his grate helps, guidances, and suggestion in laboratory techniques. I am also very grateful to Dr.Sirawut Klinbunga for participating in the thesis committee.

Special thank are given to all members in 707, 708 and 709 laboratories, Department of Biochemistry, Chulalungkorn University, for their helps and friendships during my study.

Finally, I would like to express my deepest appreciation to my parents and members of family for their unlimited love, understanding and encouragement.

Thitima Nuanboon

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LIST OF ABBREVIATIONS

bp	=	base pair
°C	=	degree Celcius
dATP	=	deoxyadenosine triphosphate
dCTP	=	deoxcytosine triphosphate
dGTP	=	deoxyguanine triphosphate
dTTP	=	deoxythymine triphosphate
DNA	=	deoxyribonucleic acid
Fig	=	figure
M	=	molar
mg	=	milligram
min	=	minute
ml	=	millilitre
mM	=	millimolar
MT	=	metric ton
mtDNA	=	mitochondrial DNA
ng	=	nanogram
nm	=	nanometre
O.D.	=	optical density
PCR	=	polymerase chain reaction
ppm	=	part per million
RAPD	=	randomly amplified polymorphic DNA
RFLP	**=**	**restriction fragment length polymorphism**
sec	=	second
μg	=	microgram
μl	=	microlitre
μM	=	micromolar

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Chapter I

Introduction

At present, aquaculture is the world's fastest growing food-production sector, providing an acceptable, protein rich supplement to, and substitute for wild aquatic animals and plants. Over the last decade, aquatic production from capture fisheries and aquaculture has increased steadily, reaching 120.7 million mt in 1995, an increase of around 15.6 millon mt since 1989. Much of this increase is attributable to aquaculture. The proportion of total aquatic production attributable to aquaculture (including plants), increased form 14.4% in 1989 to 23% in 1995 (FAO, 1997: cited in Subasinghe et al., 1998).

The cultured shrimp subsector grew at an annual percent rate (APR) of 16.8 between 1984 and 1995. This increase was principally due to culture of penaeid shimp species, which accounted for 96.3% of all cultured shrimp in 1995. Penaeid production, notably of giant tiger shrimp (*Penaeus monodon*) and other *Penaeus* species, increased from 31% or 54,000 metric-tons (mt) and 12% or 21,000 metric-tons (mt), respectively, in 1984 to 54% or 503,000 metric-tons (mt) and 18% or 165,000 metric-tons (mt) in 1995 (Subasinghe et al., 1998). In 1999, the world's shrimp farmers produced an estimated 814,250 mt of whole shrimp (Table1.1). In Southeast Asia, Thailand produced 31.1% of the eastern's production, the largest production in the world (Table 1.2). Since 1991, the export revenue of fresh and frozen shrimp of Thailand was among the top ten of aquatic production, reaching 62,684.4 million bahts (56.58%) in 1996 to 75,699.3 million bahts (54.61%) in1997 (Table 1.3). Fresh and frozen shrimps from Thailand were exported to Japan, USA, EU, Asean,Twaiwan, etc.(Fig1.1,Table 1.4).

Table 1.1 World Prawn Farming in 1999

Area of Prawn Farming	% of World production	Heads-on Production (metric-tons)	Hectares in production	Kilograms per Hectare	Number per Hatcheries	Number of Farms
Western Hemispher	21	171,500	137,400	1,248	289	1,907
Eastern Hemispher	79	642,750	1,114,050	577	5,488	374,006
Total	100	814,250	1,251,450	1,825	5,777	375,913

Source: World Prawn Farming in 1999

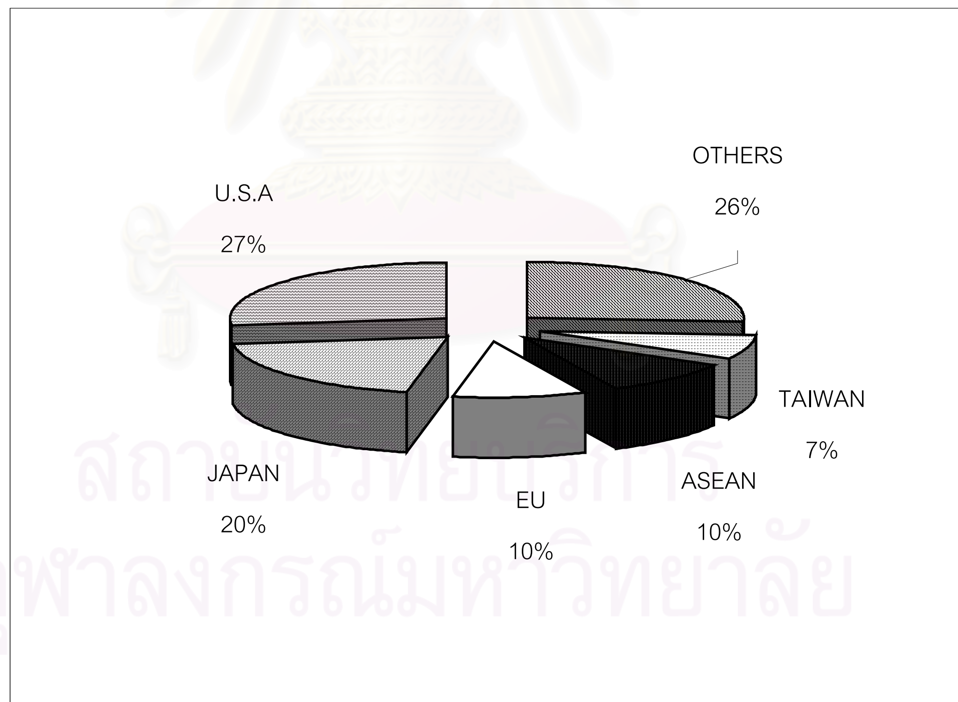

Fig 1.1 Export Quantity of Fresh and frozen Shrimp by country 1997

Table 1.2 Eastern Hemispher Farming in 1999

Country	Percent of Production	Heads-on Production (metric-tons)	Hectares in production	Kilograms per Hectare	Number per Hatcheries	Number of Farms
Thailand	31.1	200,000	80,000	2,500	1,000	20,000
China	17.1	110,000	180,000	611	2,000	10,000
Indonesia	15.6	100,000	350,000	286	300	225,000
India	10.9	70,000	130,000	538	225	100,000
Other	10.6	50,000	100,00	500	500	5,000
Philippines	7.8	40,000	60,000	667	120	4,000
Vietnam	6.2	40,000	200,000	200	1,000	6,000
Taiwan	6.2	20,000	5,000	4,000	220	3,000
Malaysia	3.1	6,000	4,000	1,500	100	800
Iran	0.4	2,500	4,000	625	10	150
Australia	0.4	2,400	600	4,000	8	45
New Caledonia	0.3	1,850	450	4,111	5	11
Total	100	642,750	1,114,050	577	5,488	374,006

Source: World Prawn Farming in 1999

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Table 1.3 Fisheries Export by Group of Commodity 1996-1997

	1996		1997	
Group of Commodity	Quantity (ton)	Value (MB)	Quantity (ton)	Value (MB)
Shrimp	230,739	62,683.4	211,998	75,699.3
Fresh&Frozen	161,462	43,402.4	137,080	47,183.8
Airtight Container	67,248	180,707.0	73,493	28,115.4
Salt&Dried	1,295	323.5	1,125	300.7
Smoke&Boiled	788	250.5	300	99.4
Others	916,155	48,097.9	969,257	916,155
Total	1,146,948	110,781.3	1,181,255	138,624.0

Source: International Fisheries Trade Sub-Division

Table 1.4 Fresh and Frozen Shrimp in Main Country 1993-1997

Quantity (ton)

	Year				
Country	1993	1994	1995	1996	1997
Japan	53,873	66,082	50,738	35,575	27,803
USA	46,034	53,332	44,385	41,812	37,991
EU	15,027	17,502	20,712	18,195	13,383
Asean	10,731	11,649	14,018	14,681	13,787
Taiwan	8,338	11,649	11,743	10,034	8,955
Others	14,883	26,462	33,495	41,465	35,163
Total	148,886	187,886	175,091	161,462	137,082

Source: International Fisheries Trade Sub-Division

1.1Taxonomy of *P. monodon*

Penaeid shrimp belong to the largest phylum in the Animal Kingdom, the Arthropoda, characterized by jointed appendages and exoskeleton is periodically molted.

Phylum Arthropoda

Subphylum Crustacea

Class Malacostraca

Subclass Eumalacostraca

Order Decapoda

Suborder Natantia

Infraorder Penaeidea

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1985

Genus Penaeus Fabricius, 1798

Subgenus Penaeus

Species monodon

Specific name: *Penaeus monodon* (Fabricius), 1798

Common name: giant tiger prawn (or shrimp) or black tiger prawn (or shrimp)

FAO name: giant tiger prawn

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1.2 Morphology

Externally, the prawn can be basically divided into the thorax and abdomen (Fig1.2). The thorax (or head) is covered by a single, immobile carapace which protects internal organs and support muscle origins. The eyestalks and eyes, the sensory antennae (all paired) arise rostrally. The walking legs or pereopods are the thoracic appendages. The abdomen has the obvious segmentation of invertebrate. A pair of swimming legs or

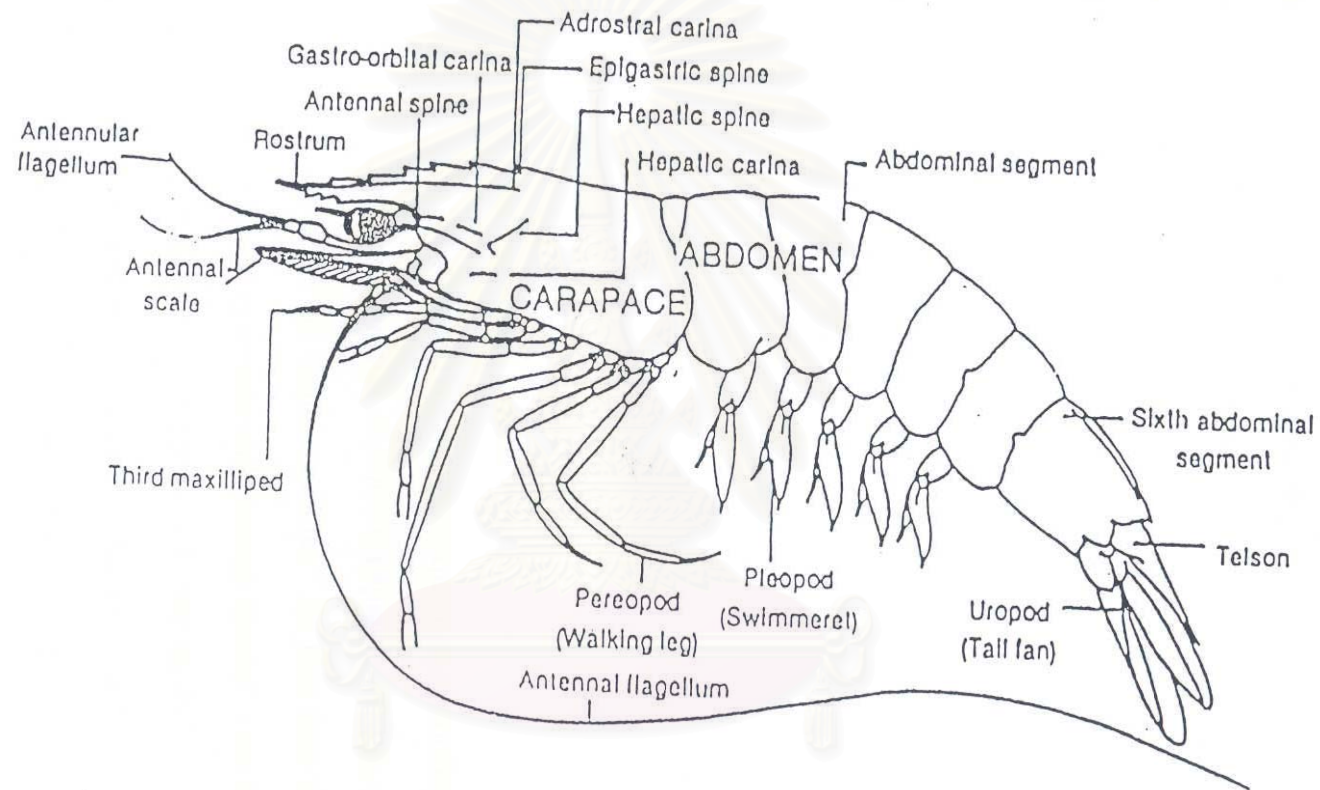

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Fig 1.2 Lateral view of *P. monodon* showing important parts.

pleopods arises from each of the six abdominal segments. A tail fan comprising a telson, which bears the anus, and two uropods attaches to the last (6 th)abdominal segment. A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of prawns (Anderson,1993).

Penaeids are dioecious and the external structures of the genital system are the major dimorphic features. The male has two pairs of modified abdominal appendages on the first and second abdominal segments (the petasma and appendix musculina) (Bailey-Brock and Shaun, 1992). The petasma is a pair of endopod of the 1st pleopod and the appendix musculina is a pair of endopod of the 2 nd pleopod, that deliver sperm to the female'external receptacle (the thylecum) located between of the fifth pereopod. The petasma, the appendix musculina and thelycum are located on the ventral surface (Anderson,1993).

1.3 Reproduction

Penaeids usually mate and spawn at sea. The timing of mating in relation to moulting and spawning follows one of the two sequences according to the structure of the thelycum. The thelycum is a special seminal receptacle for the storage of spermatozoa. The mating occurs just after the female moults. At this time the male can insert the spermatophore (encapsulated spermatozoa) through the soft cuticle of the thelycum. The spermatophore is attached to the exterior of the thelycum of a hard-cuticle, fully mature female only hours before she spawns. Penaeid spawn directly into the sea water, and the eggs are fertilised by the stored spermatozoa at the moment of spawning (Bailey-Brook and Moss, 1992).

1.4 Distribution

The black tiger prawn is principally distributed in the Indo-West Pacific region; the East and Southeast Africa, the Red sea and Arabian Gulf, the Indian subcontinent and throughout the Malasian Archipelago to Northern Australia and Japan (Fig 1.3).

Penaeus monodon is extensively cultured for food in tropical and subtropical water, but not yet to be domesticated (Grey et al., 1989). One difficulty facing those wishing to select animals for improved commercial traits, such as growth rate or size, is the high coefficient of variation for these characters (Benzie et al., 1992).

1.5 Exploitation

Black tiger prawn, *Penaeus monodon* is an economically important species because *P.monodon* is the marine animal with the high nutrient and fine flavor. Therefore, *P.monodon* is wanted for the international markets. Black tiger prawn industry in Thailand has rapidly expanded. The increasing of *P.monodon* farming is the cause to require the increasing of wild broodstocks due to the farming cycle has yet to be completed as the seed *P.monodon* used in the shrimp industry comes entirely form wild broods. Rapid expansion of *P.monodon* farming has led to heavy exploitation of the female broodstock in natural populations (Klinbunga et al., 1999). Present, Farming production of *P.monodon* must has the good broodstocks for the high survival which is the main problem. The world's shrimp farmers rely on wild shrimp for production of seedstock. They either capture wild postlavae, which are stocked into nursery and growout ponds, or they spawn wild females at hatchery (World shrimp framing, 1999). At present the farming production of *P.monodon* is a leveling-off, and may even drop slightly in production (Withyachumnarnkul et al., 1998). Therefore, the farmers must have good genetic management to maintain genetic variation, to increase the prawn

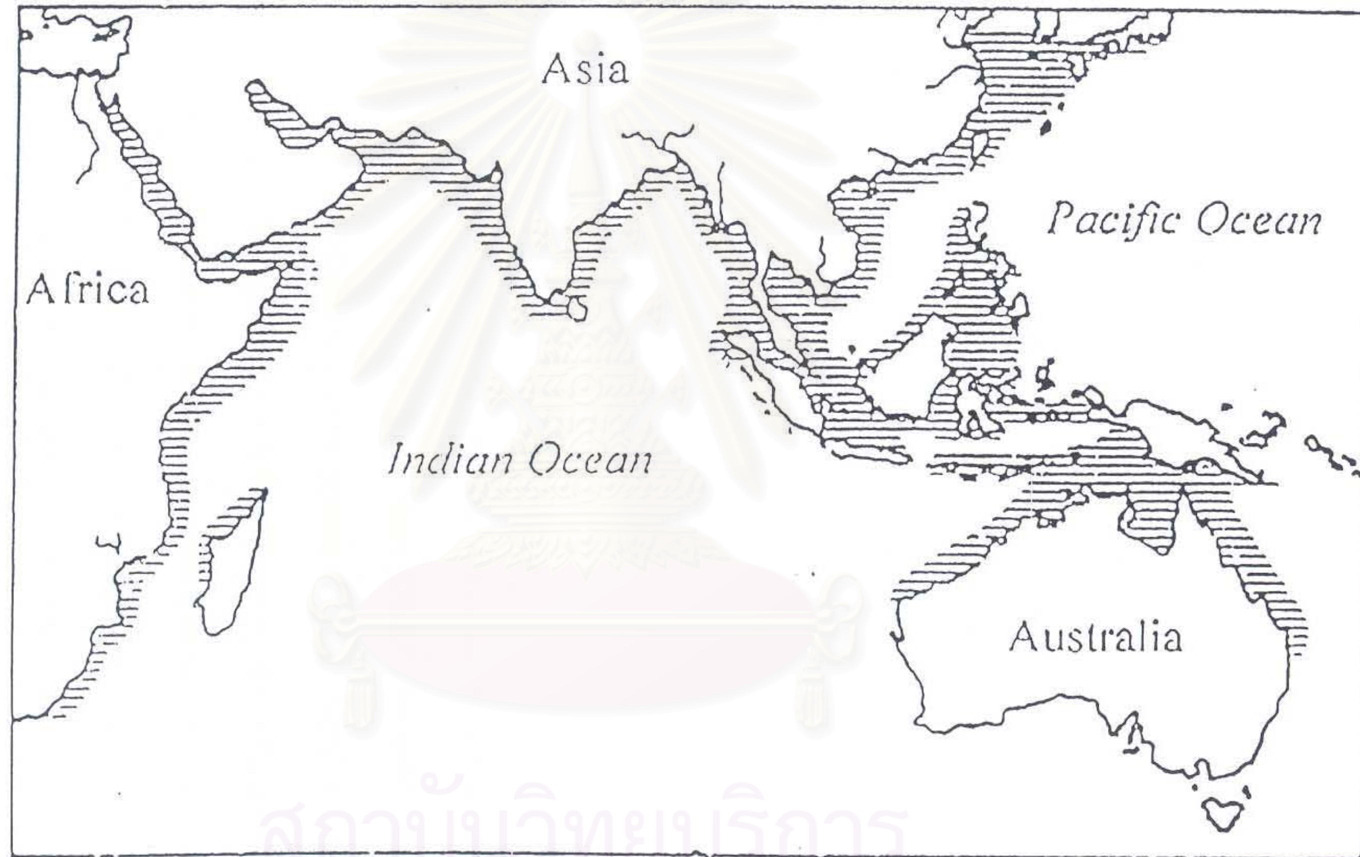

Fig 1.3 Geographic distribution of *P. monodon* in Indo-West Pacific region (Grey et al., 1983)

productions and to solve the other problems (Pongsoomboon, 1996).

During the past decade, classical strategies of evaluating genetic variability such as comparative anatomy, morphology, embryology and physiology had been increasingly complemented by molecular techniques. The development of so-call "molecular marker" is based on polymorphism found in protein or DNA molecule. The properties generally desirable for molecular marker are (1) highly polymorphic behavior, (2) codominant inheritance, (3) frequent occurance in the genome, (4) even distribution thoughout the genome, (5) selectively neutral behavior, (6) easy access, (7) easy and fast assay, (8) high reproducibility and (9) easy exchange of data between laboratories. No molecular marker is found to yet fulfil all of these criteria. A suitable molecular marker can be chosen form a variety of marker system, each of which combines at least some of above mentioned properties (Weising et al., 1995).

1.6 Protein marker

The resolution of protein electrophoresis is not always adequate for detecting differences between populations or individuals. Because of redundancy in the DNA that dictates protein sequences all charge of protein electrophoresis. Furthermore, protein electrophoresis is limited to detecting change that affect genes (Carvalho et al., 1995).
Protein electrophores is technique of allozyme and isozyme which are to examine genetic variation in fisheries and aquaculture. Because the differences of encoded loci, the term "allozyme" is related to different allelic forms of nuclear-encoded enzyme in the same loci, whereas "isozyme" is related to different loci. Isozymes are functionally similar but separable form of enzymes encoded by more than one locus (Market and Moller, 1959).

Several studies had found that genetic variation in enzyme (or protein) loci was relatively low. In penaeid shrimps, examination of isozyme and allozyme variation indicated that both isozyme and allozyme showed low levels of genetic variation (Hedgecock, 1997; Lester, 1983; Sunden, 1991).

Allozyme

Allozyme arise from heritable, eletrophoretically detectable difference in the amino acid composition of enzymes that share a common substate (Carvalho et al., 1995). They provide not only estimators of genetic differentiation and reproductive isolation, but also provide data on mating pattern in relation to the Hardy-Weinberg paradigma (Richardson et al., 1986). Also, allozyme electrophoresis remains the predominant tool used for studying genetic variation limited by the small number of polymorphic loci that can be examined (Garcia et al., 1994). The use of allozyme marker for describing population structure implicity assume that these markers are selectively neutral and that genetic drift is responsible for population differentiation. In addition, the advantages of allozyme electrophoresis primarily relate to its speed and relatively low cost; however, disadvantages are the strict requirement of fresh or frozen (Carvalho et al., 1995). However, allozyme analysis showed a low level of sensitivity in Penaeid prawn (Nelson et al., 1982: cited in Meruane et al., 1997). The lack of allozyme variation observed in prawns meant that it was not clear whether the lack of spatial variation in most studies reflected high dispersal throughout species range, or lack of sensitivity of the technique (Benzie et a., 1992).

The low level of allozyme variability in the prawn population would not be enough to establish a marker assisted in selective breeding program. DNA markers, such as RFLP and RAPD, are more sensitive than allozymes.

1.7 DNA marker

Molecular markers at the DNA level as opposed to morphological characters or allozymes have several advantages. First, since the genotype of organism is examined directly, environmental and development influences on the phenotype are not a concern. Second, since different regions of DNA evolve at different, appropriate regions may be chosen for a given study. Third, DNA marker are not restricted to coding regions, an almost unlimited number of detectable polymorphisms exists. Finally a variety of techniques have been developed; each of which has the potential to provide suitable markers for a particular problem (Vernon, Jones, and Noble 1995; Weising et al., 1995).

Restriction fragment length polymorphisms (RFLPs)

Restriction fragment length polymorphisms (RFLPs) are base on differences in fragment length obtained by digesting the DNA samples with restriction endonucleases (Yu, Deynze and Pauls, 1993). Digestion of a particular DNA fragment with enzyme result in a reproducible set of fragments of defined lengths. Point mutation within the recognition sequence as well as insertion or deletion will result in an altered pattern of restriction fragments (Weising et al., 1995). The most extentive use of restriction enzyme in population studies has been for the survey of allelic diversity and population differentiation in animal mitochondrial DNA. Large molecule such as chromosomal DNA cannot be analyzed by restriction enzymes directly because there are too many enzymatic cleavage sites for interpretable banding patterns to be observed (Parker et al., 1998). Moreover, RFLPs were widely used for genomic mapping and population studies. The mode of inheritance of RFLP maker is codominant (Caetano and Gresshoff, 1997).

Microsatellite

Microsatellites are short streches (ten to hundreds of base pair) of DNA composed of di-, tri-, or tetranucleotide repeats arrays in tandem. Microsatellite can also be composed of different types of repeats (e.g. a GT repeat adjacent to or interposed with GA repeats). Each microsatellite locus (i.e. tandemly arrayed repeat) is flanked by unique sequences. If the sequences flanking the microsatellites are known, primers can be synthesized complementary to these flanking sequences such that the tandem array of microsatellite locus can be amplified by the polymerase chain reaction (Wright and Bentze, 1991: cited in Carvalho et al., 1995). Furthermore, microsatellites have been employed in fisheries and aquaculture for determining relatedness among individual (including parent offspring identification), stock identification, selective breeding program and genome mapping (Park et al., 1994). Microsatellites isolated from penaeid prawns with great difficulty are characterized by large size (100 repeats or more), and degenerated ends making the design of effective primers difficult, and their application to mapping likely to be limited in the short-term (Moore et al, 1997, Tassanakajon et al., 1998).

Randomly amplified polymorphic DNA (RAPD)

The RAPD technique was invented by Williams et al (1990). It was the technically simplest variation of the arbritary primed PCR methods. Primers are ten nucleotides in length with a GC content of 50% to 80% and do not contain palindromic sequence. The amplification products were separated on an agarose gel and detected by staining with ethidium bromide. Primers with lower GC content usually do not yield amplification products. Because of the order of nucleotide in the 10 mer primers was

arbitary, no prior knowledge of DNA sequence was need, and the primer can be universally used for eukaryotes and prokaryotes.

To obtain an amplification product with only one primer there must be two identical (or highly similar) target sequences in close vicinity to each other: one site on one strand and the other site on the other strand, in the opposite direction. The distance between both sites should not exceed a few kilobases (Weising et al.,1995). A subset of the elongation products in the first cycle can serve as templates for the following cycles because it has the second sites for primer annealing within the distance suitable for amplification.

The RAPD products were finally detected as DNA fragment length polymorphism in multiple sites by the presence or absence of DNA bands after gel electrophoresis. DNA polymorphicsm revealed by this method may result from chromosome rearrangements within the amplified sequence, deletion of priming sites, insertion or deletion of the sequence between the priming sites and base substitutions in priming sites (Chalsrisook, 1994).

A particular RAPD fragment is usually present (allele A) or absent (allele a). This allele distribution was typical for a dominant marker. A fragment was seen in the homologus (AA) as well as in the heterozygous (Aa) situation, and only the absence of the fragment reveals the underlying genotype (aa). Therefore, the RAPD fragment cannot be estimated since the homozygote (AA) cannot be discerned from the heterozygote (Aa) in population genetic.

The main advantages of RAPD analysis were deem to be its sensitivity and speed. One of the most important uses of RAPD was the detection of population or family specific marker (Garcia et al., 1994). Like other molecular markers, RAPD can be use to tag chromosome and genes, to fingerprint the genome and to produce genomic

map. Another the advantage of RAPD was non-radioactive very small amount of genomic DNA as sufficient. Multiplex detection of polymorphism can be clone.This is no need for expensive equipment beyond a thermocycler and a transilluminator (Caetano and Gresshoff, 1997).

The RAPD-PCR method presents some limitations such as sensitivity to reaction condition, dominance, occasionally non-reproducible amplificattion products and the possible co-migration of amplified fragments (Yu et al., 1993; Calejas and Ochando, 1998). The major disadvantage of RAPD marker was the non-reproducible amplification patterns among different laboratories. The PCR amplification process that produces RAPD involves competition among fragments that can be affected by small variations in the reaction conditions. Fastidious attention to reagents and amplification conditions were required to ensure that the same marker resolved today can be recovered in the future in the same or different laboratory (Caetano and Gresshoff, 1997). The RAPD analysis and allozyme variation give the coincident patterns. Phylogenetic relationship elucidated by both molecular techniques were similar, but RAPD shows greater differentiation than allozyme variation. A great part of RAPD variation was likely to result from non-coding and repetitive DNA, in contrast, allozymes represent variation in the functional gene products, Therefore, natural selection may act more strongly on allozyme coding regions than the RAPD marker (Callejas and Ochando, 1998). The advantage of the RAPD marker technique over RFLP technique was its ability to detect extensive polymorphism rapidly with only a very small amount of genomic DNA (Chalsrisook, 1994). Also, RAPD analysis can provide more information on genetic variation and is less expensive and more rapid than RFLP analysis.

In Penaeid shrimp, Garcia et al.(1994) had evaluated the genetic diversity of *P.vannamei* shrimp using three molecular genetic techniques, namely, restriction

fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD) and allozyme variation. They reported a high level of polymorphisms with RAPD in *Penaeus vannamei*, being higher than the level of polymorphisms with allozyme markers. In 1995, Garcia and Benzie suggested that RAPD markers would be useful in providing markers for breeding programs of *P.monodon*. They reported that the levels of variation were similar to those observed in other taxa, and were likely to be adequate to obtain markers to assist selective breeding programs.

RAPD analysis was used to amplify the genome of black tiger prawns (*Penaeus monodon*) to detect DNA markers and assess the utility of the RAPD method for investigating genetic variation in wild *Penaeus monodon* in Thailand. The results suggested the existence of genetic population differentiation between the Andaman sea and the Gulf of Thailand of Thai *P. monodon.*

Tassanakajon et al. (1997, 1998a and 1998b) studied RAPD analysis of Thai *P. monodon* from five geographic locations (Chumpon, Trad, Phangnga, Satun and Trang) and revealed different levels of genetic variability among the samples. The Andaman Sea *P. monodon* was significantly different from that of the Gulf of Thailand (*P values* between 0.0000 and 0.0387) when they used five microsatellite loci. Klinbunga et al., 1999) examined the genetic variation and population structure in *P. monodon* collected from Satun (the Andaman Sea) and Surat and Trad (the Gulf of Thailand) using mitochondrial DNA restriction fragment length polymorphism (mt DNA-RFLP). The results showed geographic heterogeneity indicating population differentiation between *P.monodon* from the Andaman Sea and the Gulf of Thailand ($P < .0001$).

The objective of this thesis was to identify and characterize the population-specific marker which can distinguish between the populations of Thai *P. monodon* from the Andaman Sea and Gulf of Thailand.

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Materials and Methods

2.1 Equipments

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
- A -20 ºC Freezer
- A -80 ºC Freezer (Bara Laboratories Co., Ltd)
- Automatic micropipettes P10, P20, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)
- Gene pulser (Bio-RAD Laboratories, U.S.A.)
- Heating block BD 1761G-26 (Sybron Thermermolyne Co., USA)
- Hybridization oven (Hybaid, USA)
- Incubator 37 ºC (Kallenkamp, England)
- Microcentrifuge tube 0.5, 1.5 ml (Bio-RAD Laboratories., USA)
- PCR Thermal cycler PCR system 2400 (Perkin Elmer)
- PCR Thin wall microcentrifuge tube 0.2 ml (Perkin Elmer)
- PCR Workstation Model # P-036 (Scientific Co., USA)
- Pipette tips 10, 20, 200 and 1000 µl (Bio-RAD Laboratories, USA)
- Power supply: Power PAC 3000 (Bio-RAD Laboratories, USA)
- Refrigerated microcentrifuge: Kubota 1300 (Kubota, Japan)
- Spectrophotometer DU 650 (Beckman, USA)
- Southern blotter (Hybaid, USA)
- White / UV Transilluminator: UVP Image Store 7500 (Mitshubichi Electric Corporation, Japan)

2.2 Chemical reagents

- Absolute ethanol (Merck, Germany)
- Agarose gel type 1-A Low (Sigma Chemical Co., USA)
- Ammonium acetate (Merck,Germany)
- Bacto-agar (Difco, USA)
- Bacto-yeast extract (Difco, USA)
- Bacto-tryptone (Difco, USA)
- Boric acid (Merck, Germany)
- Bromophenol blue (Merck, Germany)
- Chloroform (Merck, Germany)
- Ethidium bromide(Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- 100 mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medision, Wisconsin)
- Gene Amp PCR core reagent (Gibco BRL Life Tecnologies, Inc.,U.S.A)

  :10X PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl)

  :25 mM $MgCl_2$
- Glucose (Merck, Germany)
- Hydrochloric acid (Merck, Germany)
- Isoamyl alcohol (Merck, Germany)
- Nylon menbrane filter (Whatman International Ltd., England)
- Oligonucleotide primers: 10-mer (Operon Technologies Co. Ltd., University of British Columbia)
- Phenol crystal (Fluka Germany)
- Sodium acetate (Merck, Germany)

- Sodium choride (Merck, Germany)
- Sodium dodecyl sulfate (Sigma Chemical CO., USA)
- Sodium hydroxide (Merck, Germany)
- Tris-(hydroxy methyl)-aminomethane (Fluka Chemika-Bio Chemika, Switzerland)
- Whatman 3 MM paper (Whatman International Ltd., England)
- Xylene cyanol (Sigma, USA)

2.3 Enzymes

- Proteinase K (Gibco BRL Life Technologies, Inc., USA)
- RNase A (Sigma Chemical Co., USA)
- Ampli Taq DNA polymerase (Gibco BRL Life Technologies, Inc., USA)
- *Bam*HI (Promega Corporation Medison, Wisconsin)
- T4 DNA ligase (Phamacia, USA)
- *Eco*RI (New England Bio Labs)

2.4 Bacterial strain

- *Escherichai* coli: strain XL1 Blue

(F':: Tn*10 proA*$^{+}$ *B*$^{+}$ *lacI*^{q} *Δ(lacZ) M15/recA1 end A1 gyrA 96(Nal*^{r} *) thi hsdR17* $(r_k^- m_k^+)$ *supE44 relA Δ lac*)

2.5 Cloning vector

pUC18 / BamHI / BAP (Phamacia, USA)

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2.6 Samples

The black tiger shrimp broodstocks (*P.monodon*) were wild-caught alive from Satun, Trang located in the Andaman Sea and from Trad located in the Gulf of Thailand during December 1997 - February 1998 (Fig 2.1). Pleopods were excised from freshly killed *P.monodon* individuals and immediately placed on dry ice. Alternatively, dissected pleopods or the whole post larvae from the hatcheries were immediately placed into the tube containing an enough amount of absolute ethanol and transported back to the laboratory at the Department of Biochemistry, Faculty of Science, Chulalongkorn University. Specimens were stored at -80 ºC until required.

2.7 DNA extraction

Genomic DNA was extracted from a pleopod of each *P.monodon* individual using a phenol-chloroform modified from that of Davis et al. (1986). As soon as possible after removing form a –80 ºC freezer, a pleopod was transferred into a 1.5 ml microcentrifuge tube containing 400 µl of pre-chilled extraction buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 200 mM sucrose, 50 mM EDTA, pH 8.0) and briefly homogenized with a pre-chilled glass homogenizer. A 40% SDS solution was added to a final concentration of 1.0 % (w/v). The resulting mixture was then incubated at 65 ºC for 1 hour following by an addition of 10 µl of a proteinase-K solution (30mg/ml) and 5 µl of a RNase A solution (10 mg/ml).

The mixture was further incubated at the same temperature for 3 hours. To remove protein, ninety-one microliters of 5 M potassium acetate was added, thoroughly mixed and incubated at 4 ºC for 10 min prior to centrifugation at 12,000 rpm for 10 min at 4ºC. The supernatant was decanted to a sterile microcentrifuge tube. An equal volume of buffer-equilibrated phenol-chloroform-isoamyl alcohol (25:24:1 v/v) was added and

Fig 2.1 Map of Thailand illustrating sample collection sites including Satun, Trang and Trad.

gently mixed. The upper aqueous phase was carefully transferred to a new microcentrifure tube. One tenth volume of 3 M sodium acetate pH 5.5 was added. DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and kept at -20 ºC overnight to ensure complete precipitation. The precipitated DNA pellet was recovered using a cut tip and briefly washed twice with 70 % ethanol. The pellet was air-dried and resuspended in 300µl of TE buffer (10 mM Tri-HCl, pH 7.4 and 1 mM EDTA). The DNA solution was incubated at 37 ºC for 1-2 hours for complete dissolution and kept at 4 ºC until further needed.

2.8 Spectrophotometric determination of DNA concentration

DNA concentration is estimated by measuring the OD_{260} . An optical density (OD) is equivalent to 50 ng double-stranded DNA per ml. DNA sample concentration is estimated in µg/ml by OD_{260} x dilution factor x 50. An estimation of the purity of a sample can be obtained by calculating the ratio of O.D. at 260 and 280 nm. For a pure preparation of DNA, $OD_{260/280}$ should be $\geq$ 1.8 (Kirby, 1992).

2.9 Primer screening and PCR condition

Arbitrary primers purchased from Operon Technologies and University of British Columbia were used to amplify genomic DNA by polymerase chain reaction adapted from Williams et al. (1990). The optimal RAPD-PCR program parameters for reproducible amplification of *Penaeus monodon* genomic were 35 cycles of 5 sec at 94 º C (denaturation), 45 sec at 36 ºC (annealing) and 90 sec at 72 ºC (extention) then finished with a final extention at 72 ºC for 10 min. Amplification reactions were carried out in a final volume of 25 µl containing to 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$,

100 mM each dATP, dCTP, dGTP, dTTP, 0.2 μM primer, 50 ng genomic DNA and 1 unit of Ampli Taq DNA polymerase. Reactions were performed in a Perkin-Elmer Cycler Modle 2400. The primers that were able to amplify shrimp genome were used for further screening to ensure scorable and reproducible RAPD patterns of 6 shrimp samples per primer. Selected primers were used to examine different RAPD patterns among the wild populations of *Penaeus monodon* from Andaman Sea and the Gulf of Thailand.

These primers from University of British Columbia and Operon Technologies were primarily selected according to Pongsomboon (1996) and Hunsonti (1998).

2.10 Agarose gel electrophoresis

DNA was analyzed by using sub-marine gel electrophoresis. Agarose was mixed with Tris-Borate-EDTA(TBE) buffer (8.9 mM Tris HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) to make the concentration of 1.6% (W/V) and 0.7% (W/V) for detection of the amplification products and the quality of extracted genomic DNA, respectively. The calculated amount of agarose was dissolved in 1XTBE buffer by heating until complete solubilization. The solution was allowed to cool at room temperature and poured into a chamber set with comb. After the gel hardened, the comb was carefully withdrawn and the seal was removed from the ends of the platform. Sufficient 1XTBE buffer was added to cover the gel. An appropiate amount of RAPD-amplified DNA samples or extracted genomic DNA was mixed with 1/4 volume of the gel-loading dye (0.25% bromphenol blue, 0.25 xylene cyanol FF and 15% ficoll) and loaded into the well. The low molecular weight 100 bp DNA ladder (Promega) and Lamda *Hin*d III fragments were used as standard DNA markers. The electrophoresis was carried out in 1XTBE buffer from cathode to anode at 100 volts until bromphenol blue marker dye migrated

almost out of the gel. After finishing, the gel was stained in 2.5 μg/ml ethidium bromide (EtBr) solution for 5 min and destained (to remove excessive EtBr) by submerged in an excessive water. The DNA band were detected under UV transilluminator and photographed through a red filter using Kodak Tri-X-Pan 400 flim. The exposure time was usually about 15-20 seconds.

2.11 Detection of RAPD patterns between the Andaman Sea and the Gulf of Thailand *P. monodon.*

Samples from the Andaman Sea (Satun, Trang) and the Gulf of Thailand (Trad) which were prepared by Pongsomboon (1996) and by Supungul (1998), were amplified using each of the selected primer. Consequently, the primer 428 gave a specific band, which was found only in Satun-Trang samples; its size was about 950 bp (Pongsomboon (1996)). To confirm that RAPD marker could differentiate between these two groups, a number of samples (6-10 individuals) each were tested. The DNA band at 950 bp which only in the samples from the Andaman Sea was isolated and further characterized.

2.12 Isolation and characterizaton of RAPD marker

2.12.1 DNA elution

The RAPD band from 2.11 was eluted from agarose gel using Qiaquick gel extraction (Qiagen Ltd.). The DNA band was excised with a razor blade and transferred into a 1.5 ml mirocentrifuge tube. The agarose was weighed to determine the added volumes of buffer QC (supplied by the manufacture). The mixture was incubated at 50 ^{o} C for 10 min or the gel slice has completely dissolved. After, the gel slice has completely dissolved, applied the sample to the QIA quick column inserted in a 2 ml collection tube and centrifuged for 1 min. The flow-through solution was discharged and placed the QIA

quick column back in the same collection tube. An another 0.5 ml of buffer QG was added to QIA quick column and centrifuged for 1 min. The flow-through solution was discarded and centrifuged the QIA quick column for additional 1 min at 12,000 rpm. The QIA quick column was placed into a clean 1.5 ml microcentrifuge tube and added 50 μl buffer EB (10 mM Tris HCl, pH 8.5) or H_2O to the center of the QIA quick membrane and let the column stand for 5 min before centrifuged at 12,000 rpm for 60 second.

2.12.2 Purification of eluted DNA

After *in vitro* amplification of DNA, Taq DNA polymerase remains bound to DNA molecules and therefore interferes digestion of PCR amplified DNA with restriction endonucleases. Digestion of PCR products with protein are prior to the subsequent digestion with restriction enzymes significantly increase cloning efficiency by several times (Crowe, 1991). As a result, a proteinase K solution was added to eluted DNA to 50 μg/ml final concentration in the presence of 0.5% SDS. The mixture was incubated for 60 min at 65ºC. After cooling to temperature, the mixture was extracted once with phenol-chloroform and once with chloroform. DNA was recovered by ethanol precipitation.

2.13 Preparation of marker flanked by *Bam*HI sites

The primer 428 (decamer) that gave a specific band was found only in the population of Andaman Sea, was joined with a 12 base sequence containing *Bam*HI sites (5' CGCGGATCCGCG 3'). The resulting 22 base oligonucleotide primer was synthesized by Gibco BRL Comstom Primer. This primer was used to amplify the DNA fragment. The amplification profile was identical to that when the primer without the *Bam*HI sequence was used. The amplified product was eluted from the agarose gel

and digested with *Bam*HI. The digested DNA was isolated and ligated to the pUC18/*Bam*HI/ BAP.

2.14 Ligation of PCR products with plasmids

The mixture of sticky-end ligation must contained a suitable amount between DNA insert at the ratio 1:3. The 10 µl ligation reaction was composed of 1µl of 10x DNA ligase buffer, 5-10 unit of T_4 ligase (Pharmacia), 50 ng of pUC18 / *Bam*HI / BAP and 150 ng of DNA insert. Content was mixed, quick spun for 30 seconds and incubated at 16 ºC for overnight.

2.15 *E.coli* competent cell preparation.

A single colony of *E.coli* XL1 Blue was picked up using a loop and transferred into 2 ml of LB-broth. The overnight culture was incubated in a 37ºC shaker for overnight. One percent of the overnight culture was inoculated into 100 ml of L-Broth (1% Bactotryptone, 0.5% Bacto yeast extract, 0.5% NaCl) and further incubated at the same temperature for 3-5 hours until the optical density at 600 nm (O.D.600) of the cell reached 0.5-0.8. The cells were removed into cold falcon tube 200l and chilled on ice for 15-30 min and harvested by centrifugation at 4,000 rpm for 15 min at 4 ºC. The supernatant (medium) was discarded. The cell pellets were resuspended 1 liter of ice cold water and centrifuged at 4,000 rpm for 15 minutes. The cell pellets were resuspended by adding 0.5 liters of ice cold water and centrifuged at 4,000 rpm for 15 minutes. The washing was repeated with 20 ml of ice cold 10% glycerol. Finally, the was resuspended in of 2 or 3 ml in ice cold 10% glycerol. The competent cell suspension was divided into 40 µl aliquots and stored at -80 ºC for at least 6 months under these conditions.

2.16 Electrotansformation (Dower et al., 1988)

The ligated mixture was transformed into *E.coli* XL1 Blue competent cells. They were thawed on ice for 3 min. Next, mixed 40 µl of the cell suspension with 1 or 2 µl of DNA and let them on ice for 1 min. The mixture was electroporated in a precilled 0.2 cm cuvette using the Gene Pluser (Bio-Rad) with setting parameters of 25 µl F 200Ω and 2.5 KV. The time constant should be between 4 and 5 msec. After electrophoration, the mixture was immediately removed from the cuvette and added to a new tube containing 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM $MgSO_4$, 20 mM glucose) and incubated in a 37 ºC Shaker (225 rpm) for 1 hour The time constant should be between 4 and 5 msec. Finally, plated on selective LB agar plates (50µl/ml of ampicilin, 25µg/ml of IPTG, 20 µg/ml of X-Gal) and further incubated at 37 ºC for overnight (Sambrook et al, 1989).

2.17 Plasmid DNA isolation

A white colony was inoculated into a tube containing 2 ml LB-broth supplemented with 50 µl/ml of ampicilin and incubated at 37 ºC with shaking overnight. The culture was transferred into 1.5 ml microcentrifuge tube and spun at 12,000 rpm for 45 seconds. The supernate was discarded. One hundred and eighty microlitres of STET buffer (8% (w/v) sucrose, 0.5% (v/v) triton X-100, 50 mM EDTA pH 8.0, 50 mM Tris HCl, pH8.0) and 20 µl of lysozyme (10 mg/ml) were added to the pellet. The mixture was thoroughly mixed by pipetting up and down and incubated at room temperature for 75 seconds. Next, the cell were lysed by boiling for 75 seconds and centrifuged at 11,000 rpm for 10 min. The white debris was discarded. The plasmid DNA was washed with an equal volume of cold isopropanol, mixed and placed at -80 ºC for 10 min, centrifuged at 11,000 rpm for 10 min and air-dried. The plasmid DNA was dissolved in 30 µl of TE

resuspended by incubating at 65 ºC for 15 min.

2.18 Detection of the desired recombinant plasmid

*Bam*HI was used to digest the recombinant plasmid DNA in 20 µl reaction. The reaction contained 2 µl of 10X buffer, 1 unit of *Bam*HI (Promega) and 200 ng of recombinant plasmid DNA. The mixture was mixed, quick spun and incubated at 37 ºC for 3 hours or overnight. After this step, the digested recombinant plasmid DNA was analyzed by agarose gel electrophroresis. The size of DNA insert is estimated by comparing with restriction enzyme digested pUC18 and 100 bp ladder.

2.19 DNA labeling by the random prime

The 50 µl of labeling reaction was composed of the interested DNA fragment which was diluted to a concentration of 50 ng/µl in either distilled water or TE buffer. It was denatured by heating for 5 min in a boiling water bath, then chilled on ice. The 10 µl of nucleotide mixture (5x stock solution of fluorescein-11-dUTP (Fl-dUTP, dATP, dCTP, dGTP and dTTP in Tris-HCl, pH 7.8, 2-mercaptoethanal and $MgCl_2$), 5 µl of primers (random nonamers) and 1 µl of enzyme solution (5 units/µl exonuclease-free Klenow, in bufffer solution, pH 6.7) was added, mixed, quick spun to collect the contents at the bottom of the tube and incubated at 37 ºC for 1 hour. The reaction mixture was stopped by adding 20 mM EDTA, pH 8.0 and probe can then be stored in a freezer at -15 ºC to -30 ºC in the dark for at least 12 months.

2.20 Southern blot hybridization

2.20.1 DNA transfer by vacuum blotting

Size fraction of the DNA was carried out by agarose gel electrophoresis. Before transferring to a hybridization membrane, the DNA in the agarose must be treated to ensure efficient transfer and to generate single strand DNA suitable for hybridization. The DNA was depurinated by soaking the gel in the depurinating solution (0.25 M HCl) for 15 min at room temperature with shaking. The purination solution was replaced twice with denaturing solution (0.5M NaOH) for 30 min and gentle shaking. After removing the denaturing solution, neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl, pH7.4) for 15 min was treated twice with gently shaking. The DNA was transferred to a hybridization membrane cut to the size of the gel. The transfer was carried out by vacuum blotting and completed in 1.5 hr.

The white porous supporting screen was placed within the base of the apparatus. Whatman 3 MM ^{TM} filter paper was cut larger than the gel and then prewet with 10xSSC and placed over the membrane. The rubber mask should have a template cut such that the window was 2-5 mm smaller than the gel or enough to provide for a good seal. The gel was carefully transferred into position over the opening in the rubber mask in contact with the membrane and the lid was placed on the base of the unit. The transfer buffer (10xSSC) was gently poured onto the surface of the gel. The gel should contain sufficient buffer for complete transfer. When completed the pump was turn off and the remaining transfer was removed. The membrane was soaked in 2xSSC for 5 min and air-dried between two sheet of filter paper.

2.20.2 Hybridization and detection

The DNA was fixed to the membrane by baking for 2 hours at 80 ºC and was

ready for DNA hybridization and detection by using non-radioactive system. The membrane which did not used immediately could be store between sheets of Whatman 3 MM ™ paper in sealed plastic bag at 4 ºC.

2.21 Hybridization

The membrane was prehybridized in a prehybridization solution (5xSSC, 0.1% (w/v) SDS, 5%(w/v) dextran sulfate, 20 fold dilution of liquid block, 100 µg/ml denature sheared heterologous DNA). The membrane was then incubated at 60 ºC for at least 30 min. The labeled DNA was denatured by boiling for 5 min and snap cooled on ice. The denatured labeled DNA was added to the prehybridization solution and further overnight incubated at 60 ºC with gentle shaking. The prehybridization solution was removed and the membrane was washed with 1xSSC, 0.1%(w/v) SDS at 60 ºC for a minimum of 15 min with gentle agitation and carried out a further washed in 0.5xSSC, 0.1%(w/v) SDS at 60 ºC for 15 min. The membrane could be air-dried and kept for later use or used directly for hybridization.

2.22 Immunological detection

After briefly washing in buffer A (100 mM Tris-HCl, 600 mM NaCl, pH 7.4) for 1 min, the membrane was then blocked in the diluted liquid block (20 fold of liquid block in buffer A) and incubated for 30 min at room temperature with constant agitation. The diluted anti-fluorescein-HRP conjugate (1000 fold in freshly prepare, 0.5%(w/v) bovine serum albumin (BSA) in buffer A) was added. The membrane was then incubated at room temperature for 30 min with gentle shaking. The unbound conjugate was removed by washing for 3x10 min in excess 0.1% (w/v) Tween™-20 in buffer A. The mixture of

detection solution I and II at the ratio 1:1 was added. The membrane was incubated for 1 min at room temperature and exposed with X-ray films.

2.23 DNA sequencing and analysis

The clone containing desired DNA fragment was sequenced by using the ABI-PRISM automated sequencer at Mahidol University, Salaya Campus and Bioservice Unit (BSU). The sequence of desired DNA fragment was analyzed by sequence alignment to other DNA sequence deposited in the GenBank using BLAST (Basic local Alignment Search Tool) at the website: http:www.ncbi.nlm.nih.gov (Atschu et al., 1997).

2.24 PCR Amplification of DNA fragment specific to the population of *P.monodon*

2.24.1 Primer Designation

Forward and reverse primers for the amplification of specific fragment were designed from the partial nucleotide sequences of the 950 bp and 350 bp inserted nucleotide sequences using Oligo 4.0s program. Furthermore, by techniques of allele specific amplification, forward and reverse primers for the amplification of specific fragment were designed from the partial nucleotide sequence of the 350 bp insert fragment and the nucleotide sequence of the 350 bp PCR product using Oligo 4.0s program.

2.24.2 Detection of PCR specific fragment in *P. monodon*

The parameters, to effect reveal amplification, e.g. activity of Ampli*Tag* DNA polymerase, Mg^{2+} concentration, primer concentration, template concentration and annealing temperature for the primers, were optimized. The performance of PCR, the number of cycles, the choice of temperature and time at temperature for each step in the

cycle, were also determined. Basically, the PCR profile as35 cycles of 15 sec at 94 ºC, 30 sec at 52 ºC and 30 sec at 72 ºC. The 20 µl PCR reaction contained 25 ng of genomic DNA, 2 µM of each primers, 200 µM of each dNTPs, 2.5 mM of $MgCl_2$, and 0.5 unit of *Tag* DNA polymerase.

2.24.3 Allele Specific Amplification.

The parameters, to effect reveal amplification, e.g. activity of Ampli*Tag* DNA polymerase, Mg^{2+} concentration, primer concentration, template concentration and annealing temperature for the primers, were optimized. The performance of PCR, the number of cycles, the choice of temperature and time at temperature for each step in the cycle, were also determined. Basically, the PCR profiles were 35 cycles of 5 sec at 94 ºC, 120 sec at 36 ºC and 90 sec at 72 ºC. The 25 µl PCR reaction contained 25 ng of genomic DNA, 10 mM Tris-HCl, pH 8.3 and 50 mM KCl, 0.05 M of each primers, 25µM of each dNTPs, 1.5 mM of $MgCl_2$, and 0.3 unit of *Tag* polymerase.

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Chapter III

Results

3.1 DNA extraction

Total DNA was extracted from the pleopods by using proteinase K-phenol-chloroform extraction method. The quality and quantity of DNA were determined by electrophoresis on a 0.7 % agarose gel comparing to a standard DNA marker (λ DNA/*Hin*dIII). The extracted DNA consisted of high molecular weight DNA greater than 23.1 kb (Fig 3.1). The concentration of DNA was spectrophotometrically determined by measuring the absorbance at 260 nm (1 OD_{260} unit was equivalent to 50 μg DNA/ml)(Sambrook et al., 1989). The ratio of $OD_{260/280}$ was higher than 1.8 indicating that the of quality extracted DNA sample was good for PCR amplification (Kirby, 1992).

3.2 Primer screening and selection

The arbitary decanucleotide primers (10 bases long) were purchased form the University of British Columbia (UBC) and Operon Technologies, Inc. The eleven primers, 2 from UBC and 8 from Operon Tecnologies were selected according to Pongsomboon (1996) and Hunsonti (1998) Table 3.1. These primers have been shown to successfully amplify *P.monodon* DNA producing polymorphic DNA banding. The primer 428 (5' GGCTGCGGTA 3') produced a specific band which has been found only in the Satun-Trang sample (Pongsomboon, 1996).

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Table 3.1 Sequences of arbitary primers used for primer screening

primers	Sequences (5' to 3')
University of British Columbia	
268	AGG CCG CTT A
428	GGC TGC GGT A
Operon Technologies	
OPA 02	TGC CGA GCT G
OPA 04	AAT CGG GCTG
OPA 16	AGC CAG CGA A
OPB 04	GGA CTG GAG T
OPB 06	TGC TCT GCC C
OPB 07	GGT GAC GCA G
OPB 08	GTC CAC ACG G
OPB 15	GGA GGG TGT T
OPB18	CCA ACG CAG T

3.3 Determination of the population-specific marker(s)

The RAPD analysis of the two groups, the Andaman Sea and the Gulf of Thailand *P.monodon*, (3 individuals each) using the eleven selected primers produced scorable bands ranging in size form 200 to more than 1,500 bp (Figs. 3.2-3.6). Two selected primers, OPB 08 and OPB 15, showed DNA bands which consistently appeared only in the samples from the Gulf of Thailand. Using OPB 08, a DNA band of about 850 bp existed only in the samples from the Gulf of Thailand (Fig. 3.7). Using primer OPB 15, DNA band with of about 1,000 bp appeared only in the samples from the Gulf of

Thailand (Fig. 3.8). To confirm whether these bands can be used as DNA markers to differentiate between the two samples, the number of samples tested was increased. The 850 bp DNA was existed in both the Andaman Sea and the Gulf of Thailand *P. monodon* with slight variations in band intensity (Fig. 3.9). Likewise, the 1,000 bp was existed in 4 of 6 the samples of the Gulf of Thailand but faint band was also found in the samples form the Andaman Sea (Fig. 3.10). In other word, these two primers could not distinguish the wild populations between the Andaman Sea and the Gulf of Thailand.

Primer 428 appeared to identify a more variable region among the samples of Thai *P.monodon* from the Andaman Sea. A band with size about 950 bp which present in all samples of Satun-Trang, but absent in samples from Trad can be used as a population-specific marker (Pongsomboon, 1996) Figs. 3.11, 3.12.

3.4 Cloning of a 950 bp RAPD marker

The RAPD band of 950 bp fragment was cloned using a *Bam* HI sticky end adapter method (Vincent et al., 1993). After electrophoresis, a 950bp DNA fragment was excised and recovered from the gel by Qiaquick gel extraction (Qiagen Ltd.). The purified DNA was reamplified by using the primer 428 containing a *Bam* HI linker (5' CGCGGATCCGCGGGCTGCGGTA 3') Fig.3.13. The reamplified fragment was eluted and purified by a proteinase K/phenol/chloroform extraction, digested with *Bam* HI and further ligated into pUC18/*Bam* HI/BAP vector. The ligation mixture was transformed into XL1-Blue competent cells. Recombinant plasmids were then prepared from the white colonies. To determine the size of DNA insert recombinant clones were digested with *Bam* HI and analyzed with 1 % agarose gel. The results are shown in Fig 3.14. Three different types of recombinant clone, clones A, B and C, were found with the insert fragment sizes of 900 bp, 950 bp (which upon digestion with *Bam* HI gave two

bands with sizes about 650 and 350 bp) and 350 bp respectively.

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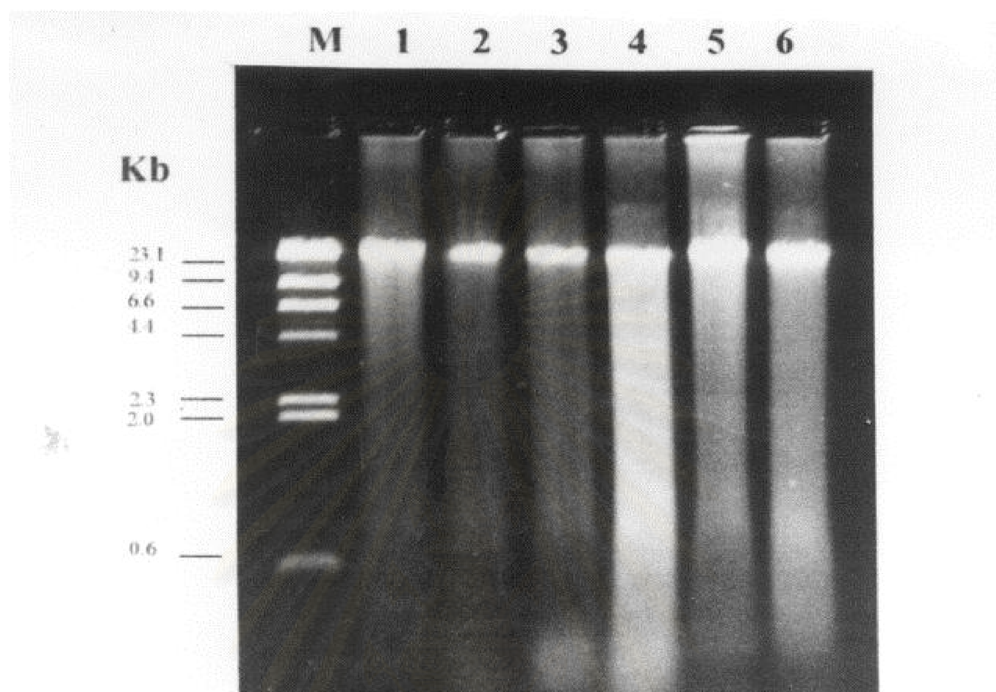

Figure 3.1 Ethidium bromide staining of 0.7 % agarose gel showing DNA extracted from the pleopods of *P. monodon*

lane M = λ DNA / *Hin*dIII standard marker

lanes 1 - 6 = genomic DNA of 6 individuals of *P. monodon*

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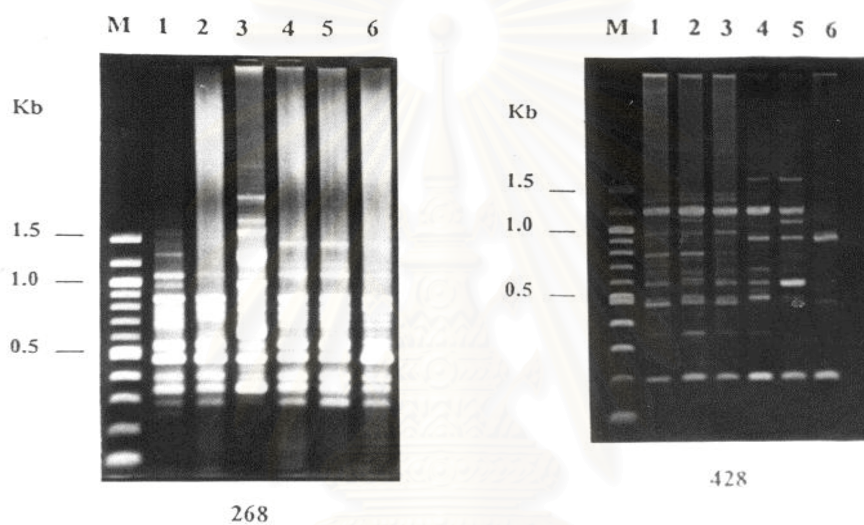

Figure 3.2 RAPD patterns using primer 268 and primer 428

lane M = 100 bp DNA ladder

lanes 1 - 3 = individuals collected Satun-Trang

lanes 4 - 6 = individuals collected from Trad

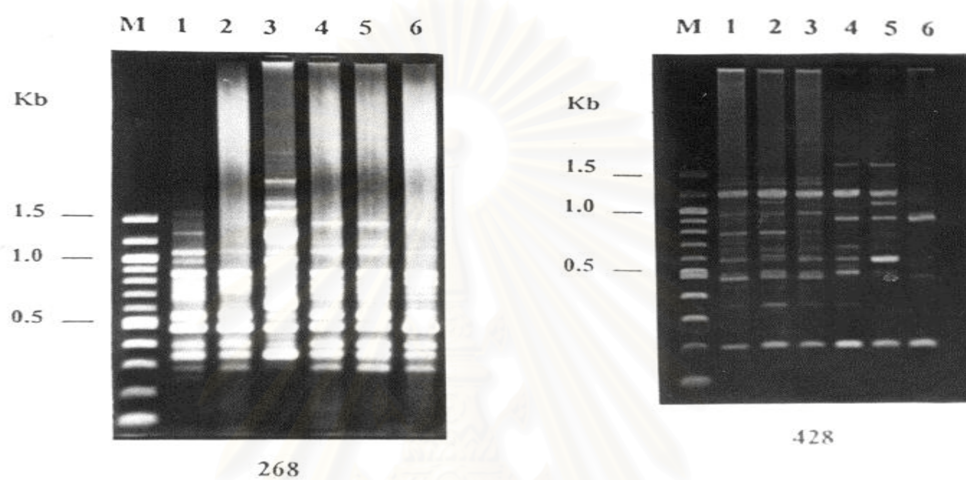

Figure 3.3 RAPD patterns using primer OPA 02 and primer OPA 04

lane M = 100 bp DNA ladder

lanes 1 - 3 = individuals collected from Satun-Trang

lanes 4 - 6 = individuals collected from Trad

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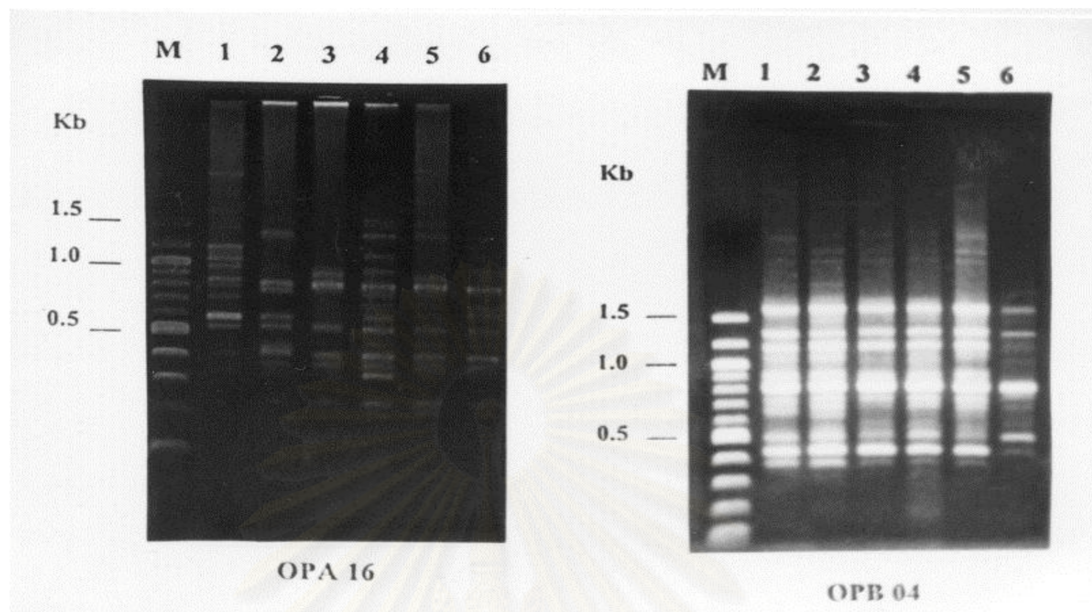

Figure 3.4 RAPD patterns using primer OPA 16 and primer OPB 04

lane M = 100 bp DNA ladder

lanes 1 - 3 = individuals collected from Satun-Trang

lanes 4 - 6 = individuals collected from Trad

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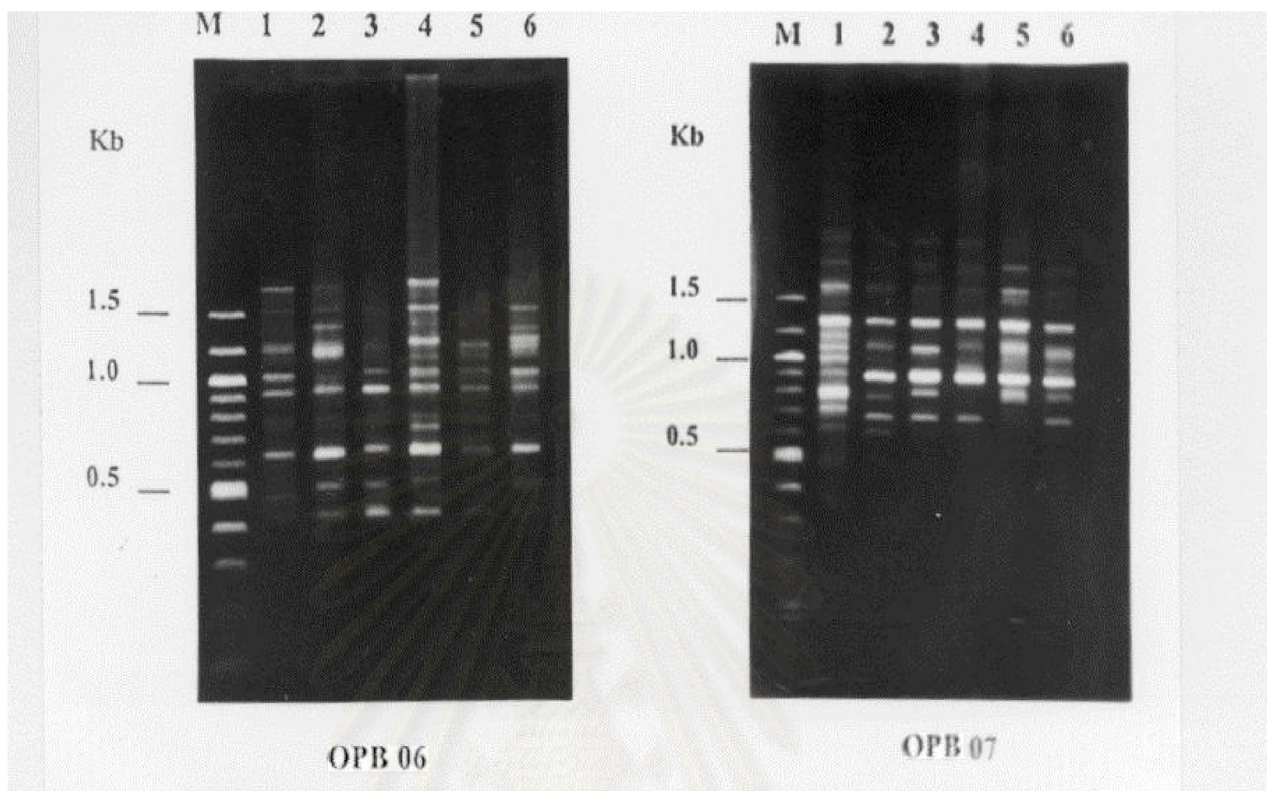

Figure 3.5 RAPD patterns using primer OPB 06 and primer OPB 07

lane M = 100 bp DNA ladder

lanes 1 - 3 = individuals collected from Satun-Trang

lanes 4 - 6 = individuals collected from Trad

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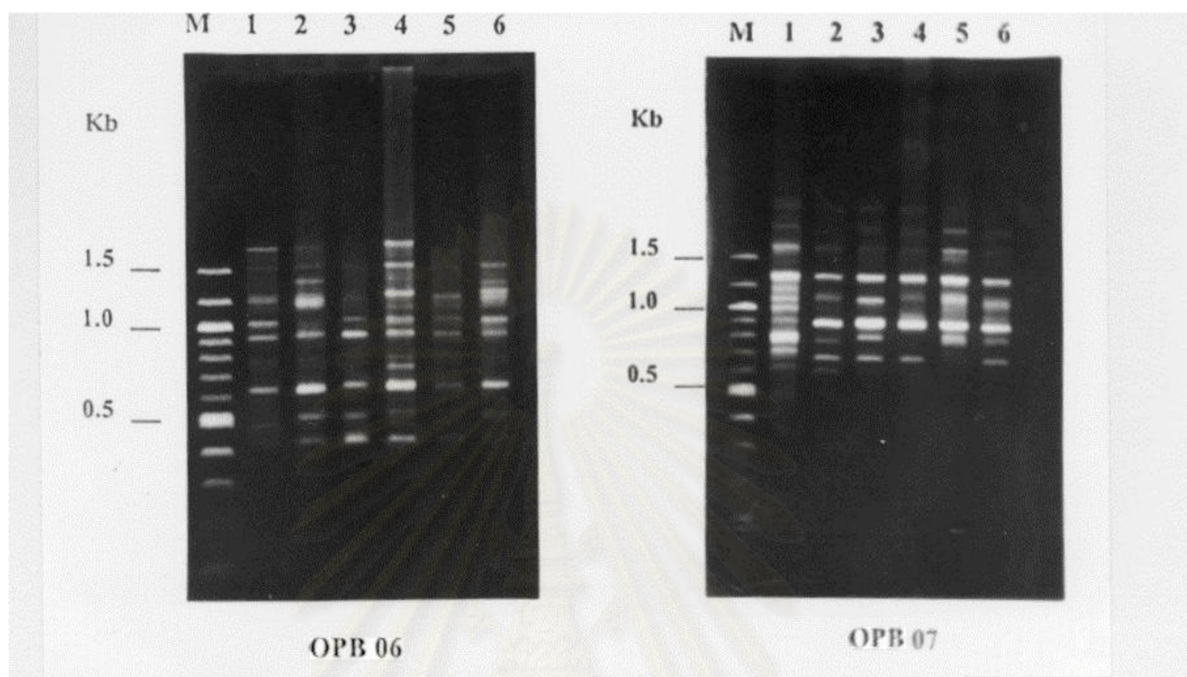

Figure 3.6 RAPD patterns using primer OPB 15

lane M = 100 bp DNA ladder

lanes 1 - 3 = individuals collected from Trad

lanes 4 - 6 = individuals collected from Satun-Trang

RAPD patterns using primer OPB 18

lanes 1 - 3 = individuals collected from Satun-Trang

lanes 4 - 6 = individuals collected from Trad

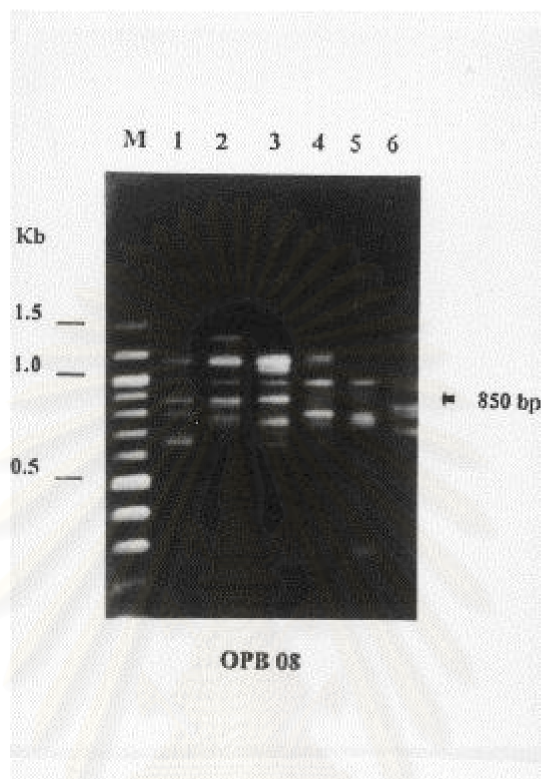

Figure 3.7 RAPD patterns using primer OPB 08

lane M = 100 bp DNA ladder

lanes 1 - 3 = individuals collected from Trad

lanes 4 - 6 = individuals collected from Satun-Trang

⬅ indicates a DNA band found only in Trad samples

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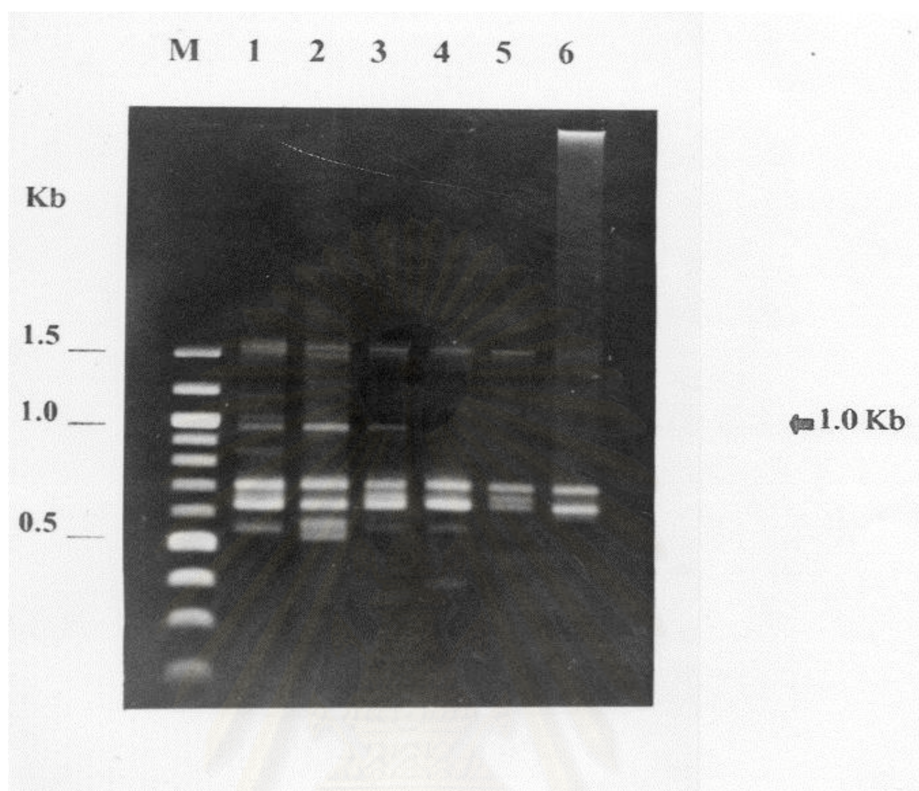

Figure 3.8 RAPD patterns using primer OPB 15

lane M = 100 bp DNA ladder

lanes 1 - 3 = individuals collect from Trad

lanes 4 - 6 = individuals collected from Satun-Trang

⬅ indicates a DNA band found only in Trad samples

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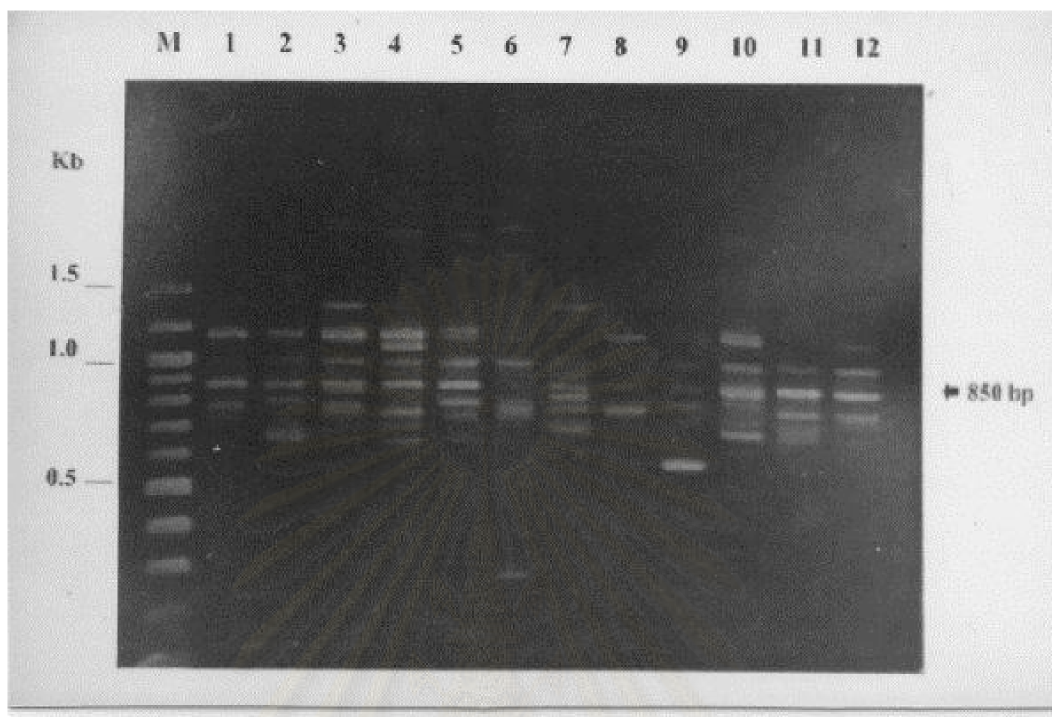

Figure 3.9 RAPD patterns using primer OPB 08

lane M = 100 bp DNA ladder

lanes 1 - 6 = individuals collected from Trad

lanes 7 - 12 = individuals collected from Satun-Trang

⬅ indicates a DNA band found only in Trad samples

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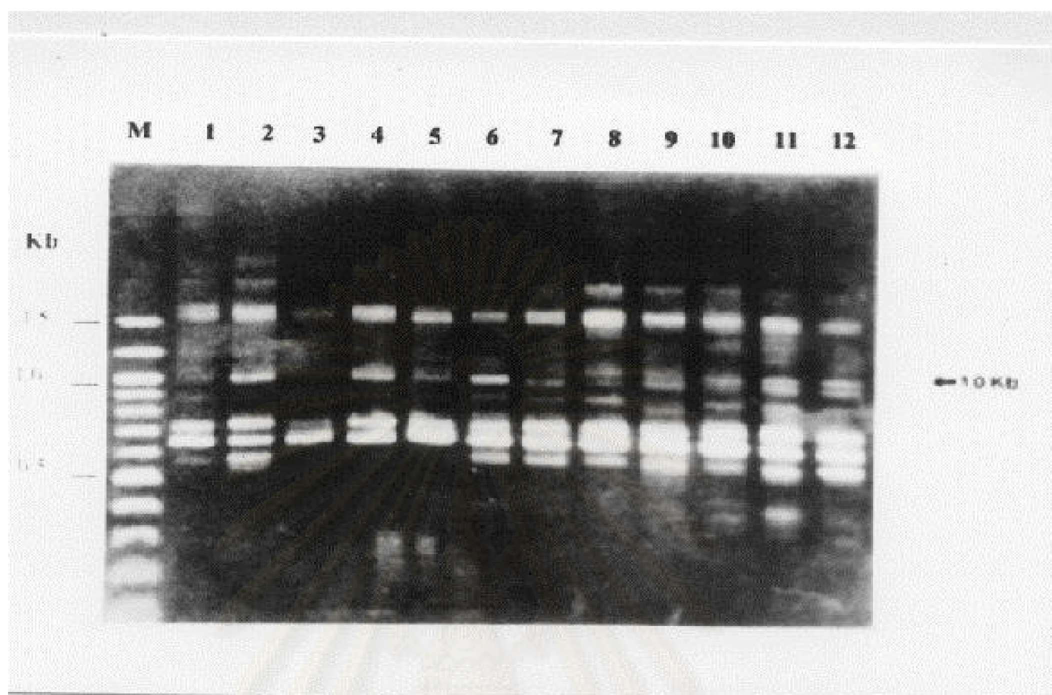

Figure 3.10 RAPD patterns using primer OPB 15

lane M = 100 bp DNA ladder

lanes 1 - 6 = individuals collected from Trad

lanes 7 - 12 = individuals collected from Satun-Trang

⬅ indicates a DNA band found only in Trad samples

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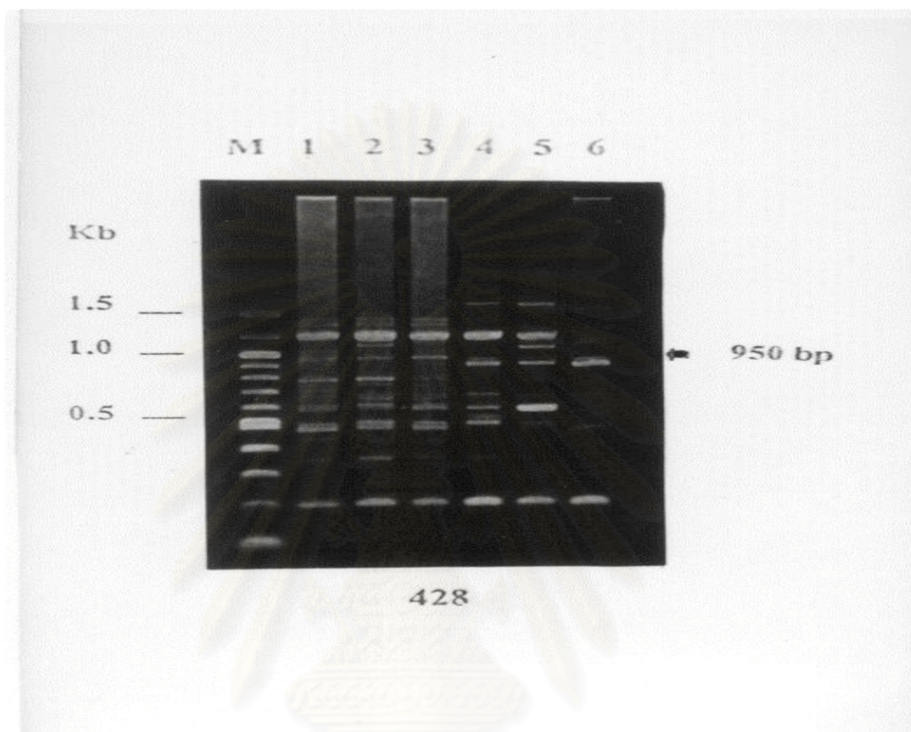

Figure 3.11 RAPD patterns using primer 428

lane M = 100 bp DNA ladder

lanes 1 – 3 = individuals collected from Satun-Trang

lanes 4 - 6 = individuals collected from Trad

⬅ indicates a DNA band found only in Satun-Trang samples

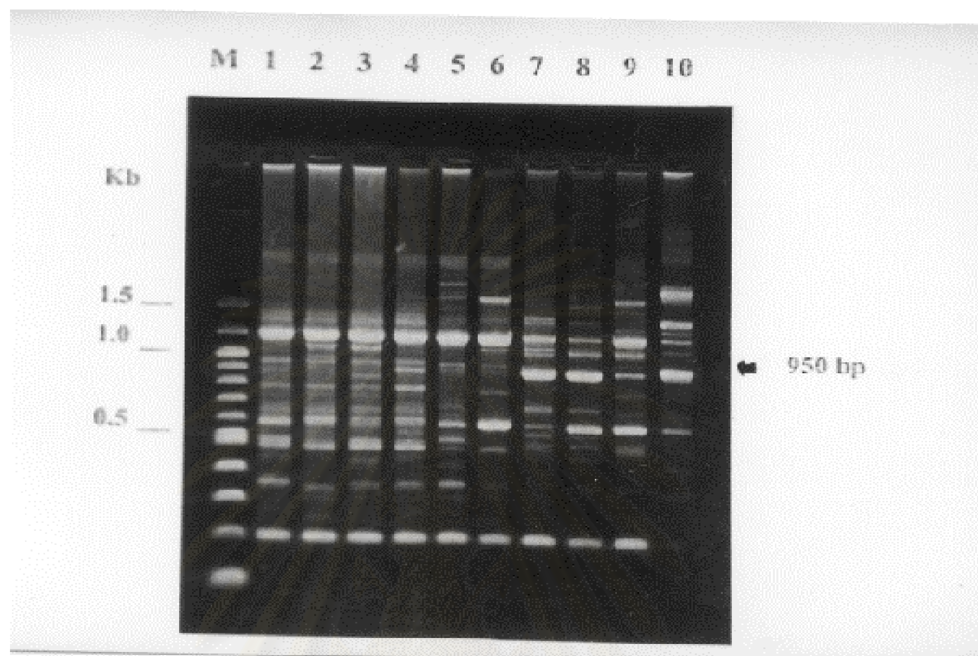

Figure 3.12 RAPD patterns using primer 428

lane M = 100 bp DNA ladder

lanes 1 - 5 = individuals collected from Satun-Trang

lanes 6 - 10 = individuals collected from Trad

⬅ indicates a DNA band found only in Satun-Trang samples

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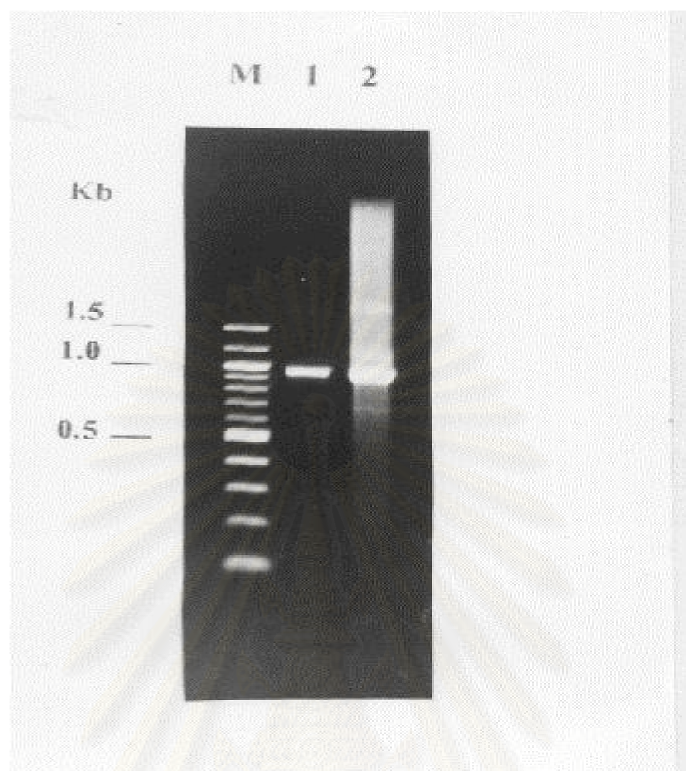

Figure 3.13 RAPD patterns of reamplification of 950 bp eluted fragment using oligonucleotide primer containing *Bam* HI site
(5' CGCGGATCCGCGGGCTGCGGTA 3')

lane M = 100 bp DNA ladder

lane 1 = a 950 bp eluted fragment (control)

lane 2 = products of reamplified 950 bp fragment

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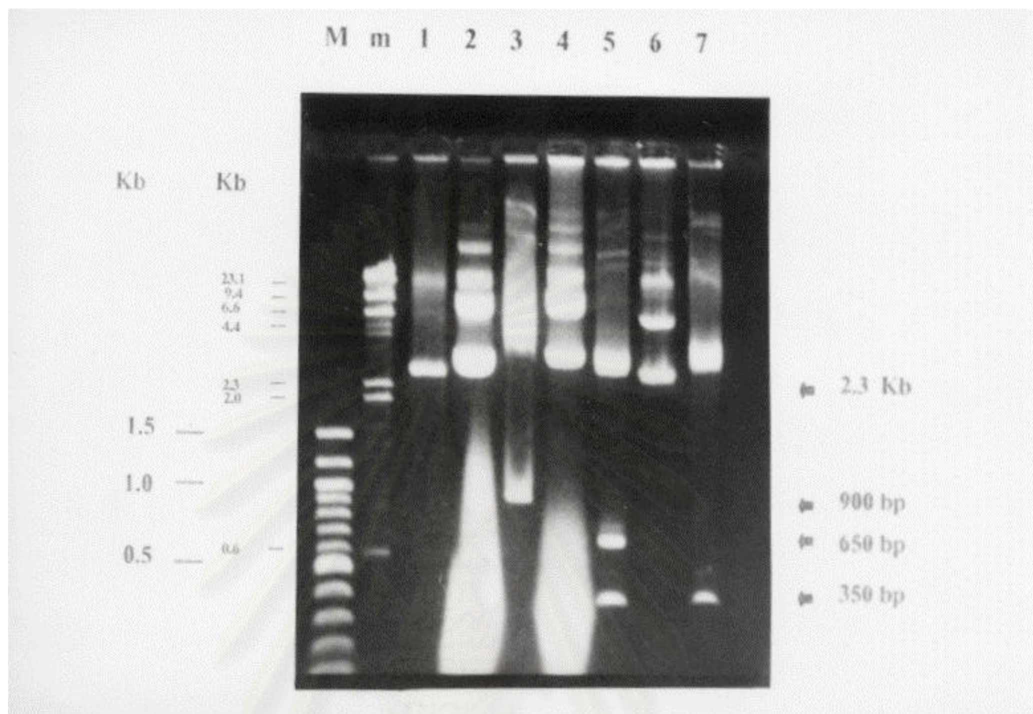

Figure 3.14 Ethidium bromide staining of recombinant clones on 1 % agarose gel

lane M = 100 bp DNA ladder

lane m = λ DNA / *Hin*dIII standard marker

lane 1 = digest pUC 18 vector

lane 2 = undigested recombinant clone A

lane 3 = *Bam* HI digest recombinant clone A

lane 4 = undigested recombinant clone B

lane 5 = *Bam* HI digest recombinant clone B

lane 6 = undigested recombinant clone C

lane 7 = *Bam* HI digest recombinant clone C

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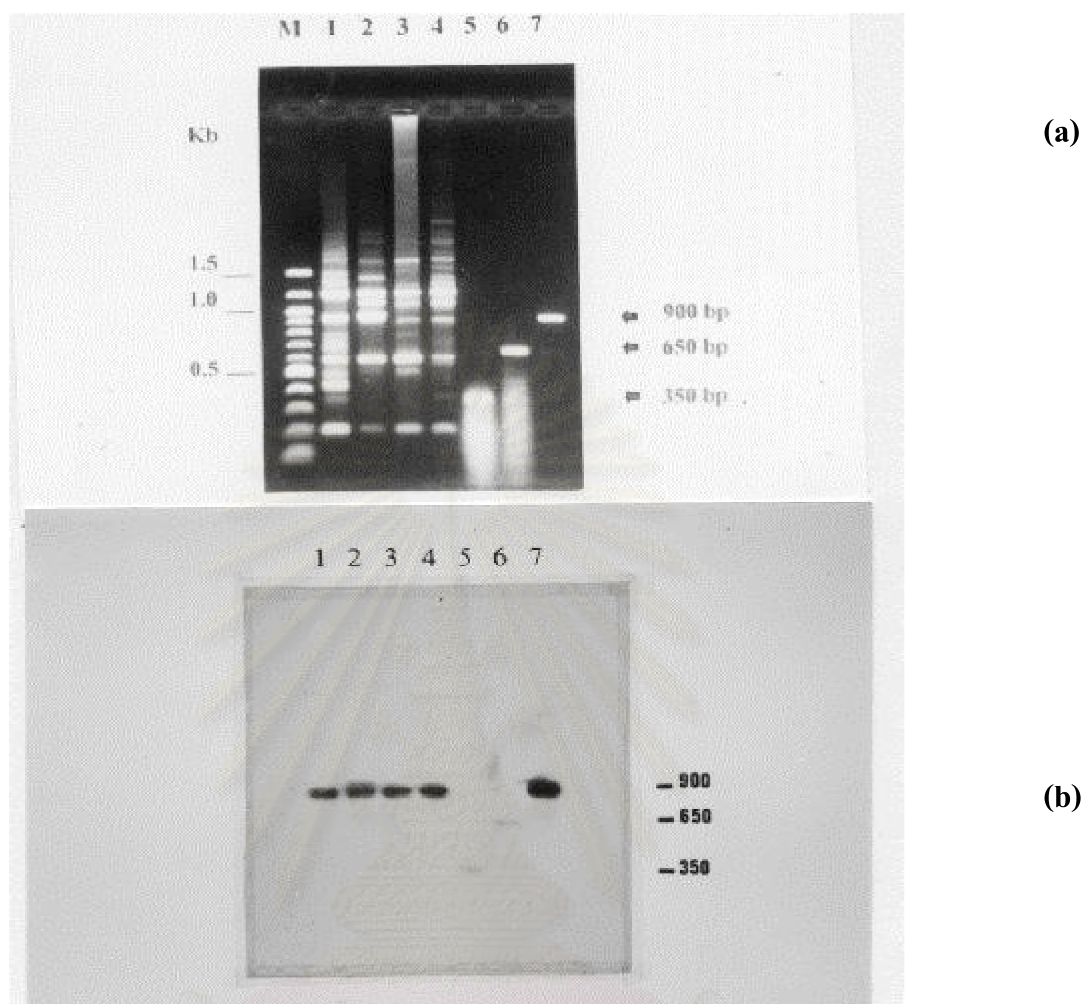
3.5 Southern blot analysis

To ensure that the insert fragments of the 3 clones were from the 950 bp RAPD marker, the recombinant clone were digested with *Bam* HI and the insert fragments were labelled by the random prime labelling method as described in section 2.19. Next, the RAPD patterns which amplified by primer 428 were subjected to agarose gel electrophoresis and then analyzed by Southern blot hybridization technique as described in section 2.20. A 900 bp inserted fragment of the clone A used as a DNA probe through Southern analysis gave positive signal within all samples of the two populations (Fig. 3.15). On the other hand, a 650 bp insert fragment of clone B gave positive signal within the particular samples of the Andaman Sea and the insert of clone C (Fig. 3.16). Also, a 350 bp insert fragment of clone C with the particular samples of the Andaman Sea and the insert of clone B (Fig. 3.17).

3.6 DNA sequence analysis

The inserts of the 3 recombinant clones (clones A, B, C) were sequenced by the ABI-PRISM automated sequencer (Figs. 3.18-3.20 and Appendix A). The sequences were compared to other DNAs in the GenBank using BLAST program as described in section 2.23. Comparison of the 900 bp sequence of clone A with those deposited in the GenBank showed similarity with the sequence of asparagine synthetase (Fig. 3.21). Also, the nucleotide sequence was converted into amino acid sequence which was compared to other proteins in the GenBank using BLAST program. The result as shown in Fig. 3.22 revealed the similarity to asparagine synthetase within 71 % identity. However, the sequence of the 650 bp inserted fragment of clone B and the 350 insert fragment of clone C were not significantly similar to any other DNA in the GenBank. In addition, the comparison between the sequence of clone B and the sequence of clone C by using



(a)

(b)

Figure 3.15 Analysis of the 900 bp, 950 bp and 350 bp DNA fragments by Southern blot hybridization using a DNA probe of 900 bp (clone A)

lane M = 100 bp DNA ladder

lanes 1-2 = RAPD patterns of individuals collected from Satun-Trang

lanes 3-4 = RAPD patterns of individuals collected from Trad

lane 5 = *Bam* HI digest recombinant clone C

lane 6 = *Bam* HI digest recombinant clone B

lane 7 = *Bam* HI digest recombinant clone A

(a) Ethidium bromide stained gel **(b)** Southern blot hybridization of (a)

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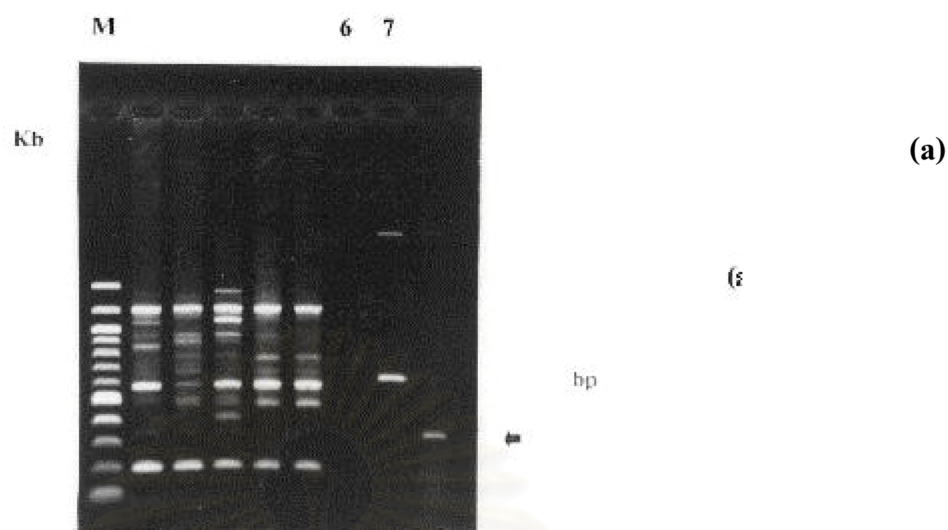

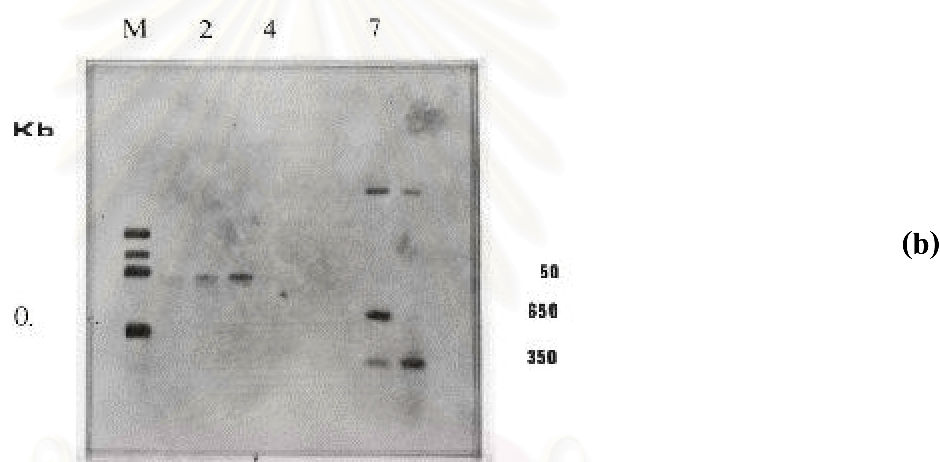

Figure 3.16 Analysis of the 950 bp and 350 bp DNA fragments by Southern blot hybridization using a DNA probe of 950 bp (clone B)

lane M = 100 bp DNA ladder

lanes 1-3 = RAPD patterns of individuals collected from Satun-Trang

lanes 4-5 = RAPD patterns of individuals collected from Trad

lane 7 = *Bam* HI digest recombinant clone B

lane 8 = *Bam* HI digest recombinant clone C

(a) Ethidium bromide stained gel **(b)** Southern blot hybridization of (a)

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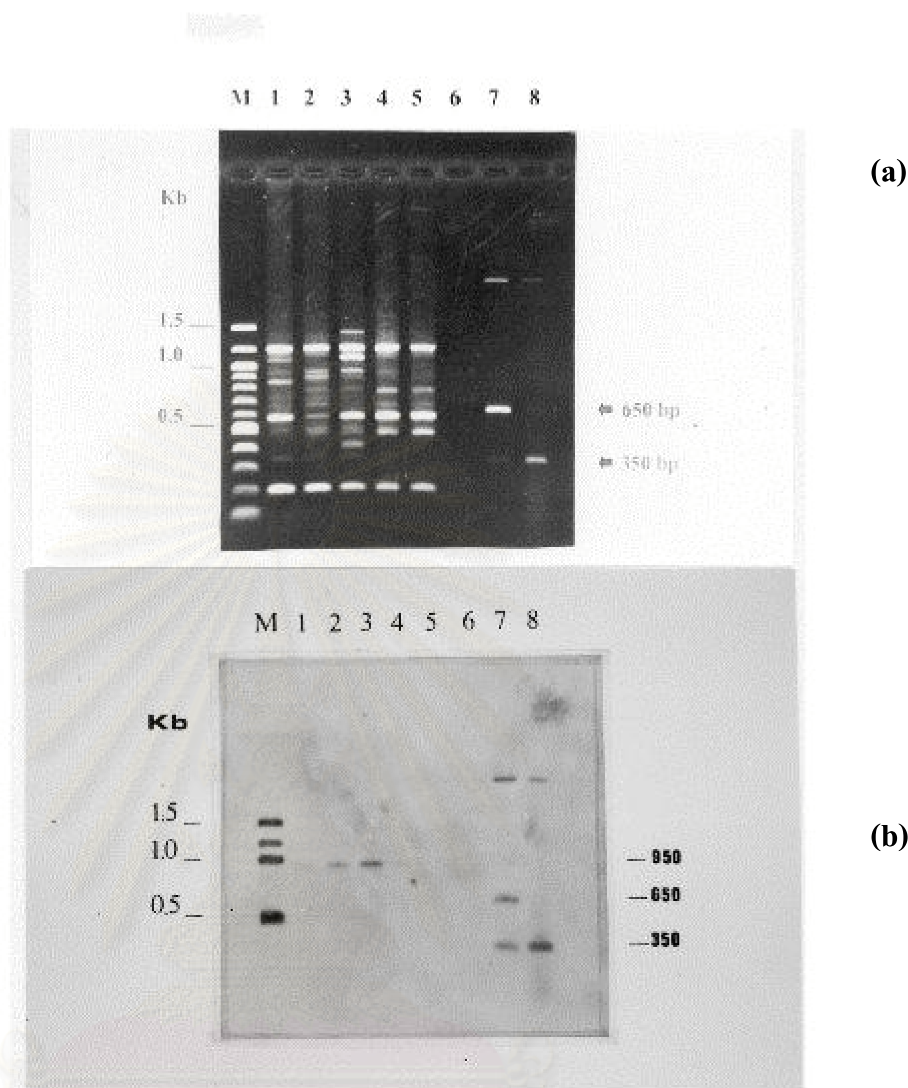

Figure 3.17 Analysis of the 950 bp and 350 bp DNA fragments by Southern blot hybridization using a DNA probe of 350 bp (clone C)

lane M = 100 bp DNA ladder

lanes 1-3 = RAPD patterns of individuals collected from Satun-Trang

lanes 4-5 = RAPD patterns of individuals collected from Trad

lane 7 = *Bam* HI digest recombinant clone B

lane 8 = *Bam* HI digest recombinant clone C

(a) Ethidium bromide stained gel **(b)** Southern blot hybridization of (a)

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GGCTGCGGTACACAGGCAATTAATGAGCGATGTACCTTATGGTGTGTTACTGTCTGGTGGTTTAGATTC
TTCGGTTACTTCTGCGATCGCAAAAAAGTATGCGCAAAAACGTATTGAAAGCGATGATACTTCCGATGC
TTGGTACCCGCAGCTGCACTCCTTTTCAGTAGGGTTAGAAGGCTCACCAGATTTGGCAGCAGCTCAAA
AAGTTGCCGATTACATTGGCACCATTCACCACGAAATAAAATTTACCATACAAGAAGGCTTAGATGCCA
TTCGAGATGTAATTTATAATATAGAAACCTACGATATTACTACAATTCGTTCATCTACACCCATGTATTTA
ATGGCTCGTGTAATTAAATCCATGGGAATTAAAATGGTGTTGTCGGGAGAAGGAGCAGACGAAATTTTT
GGTGGTTATTTATACTTTCATAAAGCTCCAAATGCAAAAGAATTTCACCAAGAAACCGTACGTAAGTTAG
ATAAACTTCACATGTACGATTGCTTAAGAGCCAATAAAAGTTTAATGGCTTGGGGAATTGAgGGCAGAG
TACCATTTTTAGATAAAGAATTTATGGATGTTGCCATGCGAATAAATNCAAAAGACAAAATGATAAATGA
CGAACGCATGGNAAAATGGGTAATTCGAAAAGCTTTTGAAGACTTACTGGCAGAAAGTGTAACATTGG
AGACCAAAAGAACAATTTACCGATGGGGTTGGCTTTAGTTGGATAGATCCCCTTAAGAGGTTAGTTATT
AANAGGTAACCGCCAACCANTGGGAAAAGCCAAATTCCGAATTCCAATCCAACCCCAACCCATTAGG

Figure 3.18 Nucleotide sequence of the 900 bp fragment using the ABI-PRISM automated sequencer.

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Primer B-F

GGATCCGCGGATCCGCG**GGCTGCGGTACGTTGTTG**CCCATGCTCCCGAGCG
CTTACCTTTTCGAGCATATGGGTCAAGATAGAACAAGCCAATGTGCTCACCTG
AAGTTTATCTTTAACTTCCCACACACGAACATCTTCATGAAATACAGGAACA
GAGCCGTCTGTAATTTCTACGAAGTCGTAGTTAAATAAGCGTCCCGATACGTA
GAACATTGCTTCTTGTAGTTTTTCAACCTGTAGGTATTTTTTCACTTCGCTTGA
ATCCAGTGCGTATTTGTCCATACGTACTTTTTCTGCGTAATAGCGATAATCCC
ACGGCTTAAATTCTTTGATGCCATCTTTAGCGGCAATTTCCATCATATCGGCA
ACTTCTTCGTCAACACGCGCAATTGCGGCAGGCCATACTTTTTCCATCAATGC
GATTGCATTTTCAGGTTTCTTTGCCTGCGATCTTCTAGACGCCACGAGGCATA
ATTGTCATAACCTAACAAGCCTCACGTTCATGACAAAGCGTAAGATCTCTTTA
ATAATTGCATTATTGNCAAATTCATCACCCGTTATCACCACGATTATAATAAG
GTTNCCAAACCTTCTTACCAGCTCGCGATCNGTAGAGTATGTTAAGAATGGAT
CC

Figure 3.19 Nucleotide sequence of the 950 bp insert fragment of clone B. The locations and sequences of P. monodon specific forward primer (primer B-F) are labeled in bold face and underlined.

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Primer C-F

GGATCCGCGGATCCGCGGGCTGCGGTATACAACGGCGAAGAAATGAAAT

CACTTCGTAAAGATCAGCAGCGATTAGTGTGGCTAACTTATAATGGCTTTG

CTCGAAATGGTGCCACGTTAGAAGGTGATAAGAAAAAGCGTTACGCTGAA

Primer C-F 2

ATTAATCAACGCCTTGCTGAGCTTCACACCAAATTTGGCAATAATGTACTT

GCTGATGAAGAAAACTACGTGTTGTTTTGGACGAATCGCAATTGGGTGGT

Primer C-R

TGACTGATTCAATCAAAAGTGCTGCTGCATCAGCTGCTAAAGAGCGTGGT

CAGGAAGGTAAGTATGCAATTACTAATACGCGCTCTTCAATGGATCC

Figure 3.20 Nucleotide sequence of the 350 bp insert fragment of clone C. The locations and sequences ofv P. monodon specific forward primer (primer C-F 1 and primer C-F 2) and those complementary to reverse primer (primer C-R 1)

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BLAST program showed that the sequence of clone C were significantly similar to the sequence of clone B but the sequence are the inverse complementary sequence (Fig 3.23). However, the sequence of clone A was not similar to these two sequences.

3.7 Primer Design & Specificity testing

Specific primers (forward and reverse) were designed from the nucleotide sequences of clone B and clone C (Table 3.2) and used for amplifcation of *P.monodon* genomic DNA. Size of PCR products were identical to those expected from their DNA sequences (950 bp and 300 bp respectively).

Table 3.2 Sequences of oligonucleotide primers designs from recombinant clones carrying a population-specific fragment of *P.monodon*

Clone	Primer Sequence
B (950 bp)	Primer B-F : 5'-GGC TGC GGT ACG TTG TTG C-3'
C (350 bp)	Primer C-F 1 : 5'-TAC ACG GCG AAG AAA TGA AAT CAC-3' Primer C-R 1 : 5'-TGG TCA GGA AGG TAA GTA TGC AAT-3'

From PCR optimization, the PCR profiles of the 300 bp were 35 cycles of 5 sec 94 ºC, 120 sec 62 ºC, 90 sec 72 ºC and the PCR profile of the 950 bp were similar except that the annealing were 120 sec 50 ºC. The PCR was performed with a total volume of 25 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 mM each dATP, dCTP, dGTP, dTTP, 0.2 µM primer, 50 ng genomic DNA and 1 unit of Ampli *Tag* DNA polymerase.

To determine whether a 300 bp DNA fragment was specific to population from the Andaman Sea, 5 individuals in each of the Andaman Sea and Gulf of Thailand were tested. The result showed that a 300 bp DNA fragment presented in both populations (Fig. 3.24). Also, to determine whether a 950 bp DNA fragment was specific to the population from the Andaman Sea, 2 individuals in each group were tested but the 950 bp DNA fragment did not appear in any of the populations (Fig. 3.25).

3.8 Specificity test of the PCR product

The PCR product of the 300 bp obtained from amplification of *P.monodon* from the Gulf of Thailand were sequenced by the ABI-PRISM automated sequencer (Fig. 3.26). Comparison of this sequence with the sequence of clone C by using BLAST programs showed similarity of the two sequences but only one base difference (Fig. 3.27). To determine whether this one difference is really exist between the two populations, the technique of allele specific amplification was used to design primers. Specific primers (forward and reverse), 14 base and 15 base in length were designed from the nucleotide sequences of the 300 bp fragment of clone C and the nucleotide sequence of PCR product using Oligo 4.0s program as shown in Table 3.3

Table 3.3 Sequence of oligonucleotide primers designs from the nucleotide sequences of the 300 bp fragment of clone C and the nucleotide sequence of PCR product,

Primer Sequence
C-F 2 5' TGA GCT TCA CAC C**A** 3'
C-F 3 5' TGA GCT TCA CAC C**G** 3'
C-R 2 5' TTA CCT TCC TGA CCA 3'

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Amplification of *P.monodon* DNA using these specific primers yield a PCR product with size about 130 bp. The PCR profiles were 35 cycles of 5 sec at 94 ºC, 120 sec at 36 ºC and 90 sec at 72 ºC. The 25 µl PCR reaction contained 25 ng of genomic DNA, 10 mM Tris-HCl, pH 8.3 and 50 mM KCl, 0.05 M of each primers, 25 µM of each dNTPs, 1.5 mM of $MgCl_2$, and 0.3 unit of *Tag* polymerase.

To determine whether a 130 bp DNA fragment was specific to population from the Andaman Sea, 3 individuals in each of the Andaman Sea and Gulf of Thailand were tested. The result of primer C-F 2 showed that a 130 bp DNA fragment did not presented in both populations. On the other hand, the result of primers C-F 3 showed that a 130 bp DNA fragment did not presented in the population of the Andaman Sea and exist in 1 out of 3 of the Gulf of Thailand (Figs. 3.28-3.30)

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Query= (828 letters)

Database: nt 844,200 sequences; 3,084,938,205 total letters

Sequences producing significant alignments:

(bits) Value

gb|AE004180.1|AE004180 Vibrio cholerae chromosome I, sectio... 82 6e-13

dbj|AB025342.1|AB025342 Moritella marina genes, complete cd... 76 4e-11

emb|Z98547.1|PFMAL3P3 Plasmodium falciparum MAL3P3, complet... 62 6e-07

emb|AL022117.1|SPBC119 S.pombe chromosome II cosmid c119 54 1e-04

gb|U77678.1|GMU77678 Glycine max asparagine synthetase 2 (A... 46 0.033

gb|AF095452.1|AF095452 Arabidopsis thaliana asparagine synt... 44 0.13

emb|AL360334.1|ATF18D22 Arabidopsis thaliana DNA chromosome... 44 0.13

emb|AL356332.1|ATT31P16 Arabidopsis thaliana DNA chromosome... 44 0.13

gb|AF057294.1|AF057294 Streptococcus pneumoniae 23F capsula... 42 0.52

gb|AF030373.1|AF030373 Streptococcus pneumoniae strain SP-2... 42 0.52

dbj|AB050900.1|AB050900 Raphanus sativus Asn1 mRNA for aspa... 42 0.52

gb|AC002541.1|AC002541 Human BAC clone CTB-43K6 from 7q21-q... 40 2.0

emb|AL136305.14|AL136305 Human DNA sequence from clone RP1-... 40 2.0

emb|AL360354.1|VYIVD10 Plasmodium vivax telomeric YAC clone... 40 2.0

emb|AL161583.2|ATCHRIV79 Arabidopsis thaliana DNA chromosom... 40 2.0

dbj|AP000924.6|AP000924 Homo sapiens genomic DNA, chromosom... 40 2.0

dbj|AP001883.5|AP001883 Homo sapiens genomic DNA, chromosom... 40 2.0

emb|AL035678.1|ATF17M5 Arabidopsis thaliana DNA chromosome ... 40 2.0

emb|Z72354.1|VFAS1 V.faba mRNA for asparagine synthetase 40 2.0

Figure 3.21 Comparison of nucleotides of the 900 bp fragment using the ABI-PRISM automate sequencer with those deposited in the GenBank.

```
gb|AE004180.1|AE004180 Vibrio cholerae chromosome I, section 88 of 251 of the complete
            chromosome
          Length = 10377

Score = 81.8 bits (41), Expect = 6e-13
Identities = 167/209 (79%)
Strand = Plus / Minus


Query: 269  gatgccattcgagatgtaatttataatatagaaacctacgatattactacaattcgttca 328
            ||||| ||||| ||||| |||||  | || ||||| || ||| | ||||| |||||| |
Sbjct: 7600 gatgcgattcgtgatgtgatttaccacatcgaaacttatgatgtcactacgattcgtgct 7541


Query: 329  tctacacccatgtatttaatggctcgtgtaattaaatccatgggaattaaaatggtgttg 388
            ||||| |||||||  || ||||  |||  ||| ||| ||||||| || ||||||||| |
Sbjct: 7540 tctactcccatgttcttgatggggcgtaaaatcaaagccatgggcatcaaaatggtgctt 7481


Query: 389  tcgggagaaggagcagacgaaattttggtggttatttatactttcataaagctccaaat 448
            || || || ||||||||||||||||| ||||| ||| | ||||| |||||||| |||||
Sbjct: 7480 tccggtgagggagcagacgaaatttcggtggctatctctacttccataaagcgccaaac 7421


Query: 449  gcaaaagaatttcaccaagaaaccgtacg 477
            || ||||| |||||| | |||||||||||
Sbjct: 7420 gctaaagagtttcacgaggaaaccgtacg 7392

Score = 46.1 bits (23), Expect = 0.033
Identities = 29/31 (93%)
Strand = Plus / Minus


Query: 564  tagataaagaatttatggatgttgccatgcg 594
            |||||||||||||||| |||||||| |||||
Sbjct: 7305 tagataaagaatttattgatgttgcgatgcg 7275



dbj|AB025342.1|AB025342 Moritella marina genes, complete cds, similar to eicosapentaenoic
            acid synthesis gene cluster
          Length = 41587

Score = 75.8 bits (38), Expect = 4e-11
Identities = 146/182 (80%)
Strand = Plus / Minus


Query: 299  gaaacctacgatattactacaattcgttcatctacacccatgtatttaatggctcgtgta 358
            |||||||||||| | || ||||||||| |||| || || |||||  ||||||| |||  |
Sbjct: 2959 gaaacctacgatgtaacaacaattcgtgcatcaacccctatgtacctaatggcacgtaaa 2900


Query: 359  attaaatccatgggaattaaaatggtgttgtcgggagaaggagcagacgaaattttggt 418
            |||||| ||||||| ||||| |||||| | || || ||||| || || ||| | || |||
Sbjct: 2899 attaaagccatggggattaagatggtgctttctggtgaaggtgctgatgaactgttcggt 2840
```

Figure 3.21 (continued)

```
Query: 419  ggttatttatactttcataaagctccaaatgcaaaagaatttcaccaagaaaccgtacgt 478
            ||||| || ||||| || ||||| |||||||| |||||||| ||  |||| || ||||||
Sbjct: 2839 ggttacttgtacttccacaaagcgccaaatgcgaaagaattccatgaagagacggtacgt 2780


Query: 479  aa 480
            ||
Sbjct: 2779 aa 2778

emb|Z98547.1|PFMAL3P3 Plasmodium falciparum MAL3P3, complete sequence
          Length = 114736

Score = 61.9 bits (31), Expect = 6e-07
Identities = 55/63 (87%)
Strand = Plus / Plus


Query: 281   gatgtaatttataatatagaaacctacgatattactacaattcgttcatctacacccatg 340
             |||||||||||| |||||||||| || |||||||| || || ||| |||||||||| |||
Sbjct: 74212 gatgtaatttatcatatagaaacgtatgatattaccactatacgtgcatctacacctatg 74271


Query: 341   tat 343
             |||
Sbjct: 74272 tat 74274

emb|AL022117.1|SPBC119 S.pombe chromosome II cosmid c119
          Length = 40699

Score = 54.0 bits (27), Expect = 1e-04
Identities = 48/55 (87%)
Strand = Plus / Plus


Query: 375   ttaaaatggtgttgtcgggagaaggagcagacgaaatttttggtggttatttata 429
             |||||||||| ||||| || || ||| | |||||||| |||||||||||||||||
Sbjct: 23679 ttaaaatggttttgtctggtgagggatctgacgaaatctttggtggttatttata 23733

gb|U77678.1|GMU77678 Glycine max asparagine synthetase 2 (AS2) mRNA, complete cds
          Length = 2143

Score = 46.1 bits (23), Expect = 0.033
Identities = 44/51 (86%)
Strand = Plus / Plus


Query: 699  aaagaacaatttaccgatggggttggctttagttggatagatccccttaag 749
            ||||||||||| |  ||||| ||||||| ||||||||| ||| ||||||||
Sbjct: 1502 aaagaacaattcagtgatggagttggctatagttggattgatgcccttaag 1552

gb|AF095452.1|AF095452 Arabidopsis thaliana asparagine synthetase (ASN3) mRNA, complete
cds
          Length = 1986

Score = 44.1 bits (22), Expect = 0.13
Identities = 34/38 (89%)
Strand = Plus / Plus


Query: 407  gaaatttttggtggttatttatactttcataaagctcc 444
            ||||||||||| || ||||| ||||| |||||||||||
Sbjct: 1039 gaaatttttggaggatatttgtacttccataaagctcc 1076
```

Figure 3.21 (continued)

```
emb|AL360334.1|ATF18D22 Arabidopsis thaliana DNA chromosome 5, BAC clone F18D22 (ESSA
project)
          Length = 57180

Score = 44.1 bits (22), Expect = 0.13
Identities = 34/38 (89%) Strand = Plus / Minus


Query: 407   gaaattttggtggttatttatactttcataaagctcc 444
             ||||||||||| || ||||| ||||| |||||||||||
Sbjct: 18991 gaaattttggaggatattgtacttccataaagctcc 18954

emb|AL356332.1|ATT31P16 Arabidopsis thaliana DNA chromosome 5, BAC clone T31P16 (ESSA
project)
          Length = 80088

Score = 44.1 bits (22), Expect = 0.13
Identities = 34/38 (89%)
Strand = Plus / Minus


Query: 407   gaaattttggtggttatttatactttcataaagctcc 444
             ||||||||||| || ||||| ||||| |||||||||||
Sbjct: 78893 gaaattttggaggatattgtacttccataaagctcc 78856

gb|AF057294.1|AF057294 Streptococcus pneumoniae 23F capsular polysaccharide locus,
complete
             sequence
          Length = 22306

Score = 42.1 bits (21), Expect = 0.52
Identities = 21/21 (100%)
Strand = Plus / Plus


Query: 432   ttcataaagctccaaatgcaa 452
             |||||||||||||||||||||
Sbjct: 15895 ttcataaagctccaaatgcaa 15915

gb|AF030373.1|AF030373 Streptococcus pneumoniae strain SP-264 alpha, 1-6-glucosidase
(dexB)
             gene, complete cds; capsular polysaccharide biosynthetic
             locus, complete sequence; and oligopeptide binding protein
             (aliA) gene, complete cds
          Length = 24722

Score = 42.1 bits (21), Expect = 0.52
Identities = 21/21 (100%)
Strand = Plus / Plus


Query: 432   ttcataaagctccaaatgcaa 452
             |||||||||||||||||||||
Sbjct: 16354 ttcataaagctccaaatgcaa 16374


dbj|AB050900.1|AB050900 Raphanus sativus Asn1 mRNA for asparagine synthetase, complete
cds
          Length = 2088

Score = 42.1 bits (21), Expect = 0.52
Identities = 27/29 (93%)
Strand = Plus / Plus
```

Figure 3.21 (continued)

```
Query: 491  cttcacatgtacgattgcttaagagccaa 519
            ||||||| ||||||||| |||||||||||
Sbjct: 1178 cttcacaagtacgattgtttaagagccaa 1206

gb|AC012306.11|AC012306 Homo sapiens clone RP11-395A13, complete sequence
          Length = 153617

Score = 40.1 bits (20), Expect = 2.0
Identities = 23/24 (95%)
Strand = Plus / Minus


Query: 418   tggttatttatactttcataaagc 441
             ||||||| ||||||||||||||||
Sbjct: 29413 tggttatatatactttcataaagc 29390

gb|AE004124.1|AE004124 Vibrio cholerae chromosome I, section 32 of 251 of the complete
            chromosome
          Length = 11069

Score = 40.1 bits (20), Expect = 2.0
Identities = 20/20 (100%)
Strand = Plus / Plus


Query: 259  agaaggcttagatgccattc 278
            ||||||||||||||||||||
Sbjct: 2244 agaaggcttagatgccattc 2263

gb|AC018513.5|AC018513 Homo sapiens chromosome 14 clone RP11-58H3 map 14q31, complete
sequence
          Length = 190773

Score = 40.1 bits (20), Expect = 2.0
Identities = 29/32 (90%)
Strand = Plus / Minus


Query: 359    attaaatccatgggaattaaaatggtgttgtc 390
              |||||| | |||| |||||||||||||||||
Sbjct: 108992 attaaagctatggaaattaaaatggtgttgtc 108961 Database: nt

gb|AC002541.1|AC002541 Human BAC clone CTB-43K6 from 7q21-q22, complete sequence [Homo
             sapiens]
          Length = 166983

Score = 40.1 bits (20), Expect = 2.0
Identities = 20/20 (100%)
Strand = Plus / Minus


Query: 559   atttttagataaagaattta 578
             ||||||||||||||||||||
Sbjct: 28779 atttttagataaagaattta 28760

emb|AL136305.14|AL136305 Human DNA sequence from clone RP1-259A10 on chromosome 6p22.1-23
             Contains the gene for an ssDNA binding protein (SEB4D),
             ESTs, STSs, GSSs and a CpG island, complete sequence [Homo
             sapiens]
          Length = 150520
Score = 40.1 bits (20), Expect = 2.0
Identities = 20/20 (100%)
Strand = Plus / Minus
```

Figure 3.21 (continued)

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```
Query: 326   tcatctacacccatgtattt 345
             ||||||||||||||||||||
Sbjct: 24056 tcatctacacccatgtattt 24037

emb|AL360354.1|VYIVD10 Plasmodium vivax telomeric YAC clone, complete finished sequence
          Length = 155711

Score = 40.1 bits (20), Expect = 2.0
Identities = 20/20 (100%)
Strand = Plus / Minus


Query: 439    agctccaaatgcaaaagaat 458
              ||||||||||||||||||||
Sbjct: 149380 agctccaaatgcaaaagaat 149361

emb|AL161583.2|ATCHRIV79 Arabidopsis thaliana DNA chromosome 4, contig fragment No. 79
          Length = 199536

Score = 40.1 bits (20), Expect = 2.0
Identities = 23/24 (95%)
Strand = Plus / Plus


Query: 796   ttccgaattccaatccaaccccaa 819
             |||| |||||||||||||||||||
Sbjct: 81534 ttcccaattccaatccaaccccaa 81557

dbj|AP000924.6|AP000924 Homo sapiens genomic DNA, chromosome 11q, clone:RP11-758F15,
complete
              sequence
          Length = 172315

Score = 40.1 bits (20), Expect = 2.0
Identities = 20/20 (100%)
Strand = Plus / Minus


Query: 281    gatgtaatttataatataga 300
              ||||||||||||||||||||
Sbjct: 131915 gatgtaatttataatataga 131896


Posted date:  Apr 19, 2001 10:43 PM
Number of letters in database: 3,084,938,205
Number of sequences in database:  844,200

Lambda     K      H
    1.37    0.711     1.31

Gapped
Lambda     K      H
    1.37    0.711     1.31
```

Figure 3.21 (continued)

Query= (828 letters)

Database: nr 673,391 sequences; 211,820,757 total letters

Sequences producing significant alignments: (bits) Value

dbj|BAA89376.1| (AB025342) ORF2 [Moritella marina] 363 e-105

gb|AAG54996.1|AE005245_5 (AE005245) asparagine synthetase B... 348 e-100

sp|P22106|ASNB_ECOLI ASPARAGINE SYNTHETASE B [GLUTAMINE-HYD... 347 e-100

pdb|1CT9|A Chain A, Crystal Structure Of Asparagine Synthet... 347 e-100

pir||H82255 asparagine synthetase B, glutamine-hydrolyzing ... 355 5e-97

sp|P31752|ASNS_ASPOF ASPARAGINE SYNTHASE [GLUTAMINE-HYDROLY... 306 5e-87

emb|CAA67889.1| (X99552) asparagine synthetase [Asparagus o... 306 5e-87

gb|AAF02775.1|AF190728_1 (AF190728) asparagine synthetase [... 305 9e-87

pir||D82846 asparagine synthase B XF0118 [imported] - Xylel... 317 2e-85

sp|O24661|ASNS_TRIVS ASPARAGINE SYNTHETASE [GLUTAMINE-HYDRO... 300 5e-85

sp|P49078|ASNS_ARATH ASPARAGINE SYNTHETASE [GLUTAMINE-HYDRO... 300 5e-85

gb|AAF02776.1|AF190729_1 (AF190729) asparagine synthetase [... 300 6e-85

sp|P49091|ASNS_BRAOL ASPARAGINE SYNTHETASE [GLUTAMINE-HYDRO... 298 1e-84

dbj|BAB17726.1| (AB050900) asparagine synthetase [Raphanus ... 298 1e-84

emb|CAA08913.1| (AJ009952) asparagine synthetase type II [P... 299 1e-84

dbj|BAA96251.1| (AB035247) asparagine synthetase [Astragalu... 298 2e-84

pir||T50812 asparagine synthetase ASN3 - Arabidopsis thalia... 298 2e-84

pir||T50028 asparagine synthetase (ASN3) - Arabidopsis thal... 298 2e-84

gb|AAC72837.1| (AF095453) asparagine synthetase [Arabidopsi... 297 2e-84

Figure 3.22 Comparison of aminoacid sequence of the 900 bp fragment using the ABI-PRISM automate sequencer with those deposited in the GenBank.

```
dbj|BAB34127.1| (AP002552) asparagine synthetase B [Escherichia coli O157:H7]
          Length = 554

Score =  348 bits (892), Expect(2) = e-100
Identities = 165/232 (71%), Positives = 198/232 (85%)
Frame = +2

Query: 5   AVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEGS 184
           +V   LMSDVPYGVLLSGGLDSS+ SAI KKYA +R+E  + S+AW+PQLHSF+VGL GS
Sbjct: 219 SVKSHLMSDVPYGVLLSGGLDSSIISAITKKYAARRVEDQERSEAWWPQLHSFAVGLPGS 278

Query: 185 PDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVIK 364
           PDL AAQ+VA+++GT+HHEI FT+QEGLDAIRDVIY+IETYD+TTIR+STPMYLM+R IK
Sbjct: 279 PDLKAAQEVANHLGTVHHEIHFTVQEGLDAIRDVIYHIETYDVTTIRASTPMYLMSRKIK 338

Query: 365 SMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWGI 544
           +MGIKMVLSGEG+DE+FGGYLYFHKAPNAKE H+ETVRKL  LHMYDC RANK++ AWG+
Sbjct: 339 AMGIKMVLSGEGSDEVFGGYLYFHKAPNAKELHEETVRKLLALHMYDCARANKAMSAWGV 398

Query: 545 EGRVPFLDKEFMDVAMRINXKDKMINDERMXKWVIRKAFEDLLAESVTLETK 700
           E RVPFLDK+F+DVAMRIN +DKM  + +M K ++R+ FE  L  SV    K
Sbjct: 399 EARVPFLDKKFLDVAMRINPQDKMCGNGKMEKHILRECFESYLPASVAWRQK 450
Score = 41.6 bits (96), Expect(2) = e-100
Identities = 16/22 (72%), Positives = 19/22 (85%)
Frame = +3

Query: 690 WRPKEQFTDGVGFSWIDPLKRL 755
           WR KEQF+DGVG+SWID LK +
Sbjct: 447 WRQKEQFSDGVGYSWIDTLKEV 468
>sp|P22106|ASNB_ECOLI ASPARAGINE SYNTHETASE B [GLUTAMINE-HYDROLYZING]
pir||AJECN asparagine synthase (glutamine-hydrolyzing) (EC 6.3.5.4) -
           Escherichia coli
gb|AAA23498.1| (J05554) asparagine synthetase B [Escherichia coli]
dbj|BAA35317.1| (D90706) Asparagine synthase (glutamine-hydrolyzing) (EC 6.3.5.4)
           [Escherichia coli]
gb|AAC73768.1| (AE000171) asparagine synthetase B [Escherichia coli K12]

Figure 3.22 (continued)
Length = 554

Score =  347 bits (890), Expect(2) = e-100
Identities = 165/232 (71%), Positives = 198/232 (85%)
Frame = +2

Figure 3.22 (continued)
Query: 5   AVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEGS 184
           +V   LMSDVPYGVLLSGGLDSS+ SAI KKYA +R+E  + S+AW+PQLHSF+VGL GS
Sbjct: 219 SVKSHLMSDVPYGVLLSGGLDSSIISAITKKYAARRVEDQERSEAWWPQLHSFAVGLPGS 278

Query: 185 PDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVIK 364
           PDL AAQ+VA+++GT+HHEI FT+QEGLDAIRDVIY+IETYD+TTIR+STPMYLM+R IK
Sbjct: 279 PDLKAAQEVANHLGTVHHEIHFTVQEGLDAIRDVIYHIETYDVTTIRASTPMYLMSRKIK 338

Query: 365 SMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWGI 544
           +MGIKMVLSGEG+DE+FGGYLYFHKAPNAKE H+ETVRKL  LHMYDC RANK++ AWG+
Sbjct: 339 AMGIKMVLSGEGSDEVFGGYLYFHKAPNAKELHEETVRKLLALHMYDCARANKAMSAWGV 398

Query: 545 EGRVPFLDKEFMDVAMRINXKDKMINDERMXKWVIRKAFEDLLAESVTLETK 700
           E RVPFLDK+F+DVAMRIN +DKM  + +M K ++R+ FE  L  SV    K
Sbjct: 399 EARVPFLDKKFLDVAMRINPQDKMCGNGKMEKHILRECFEAYLPASVAWRQK 450
Score = 41.6 bits (96), Expect(2) = e-100
Identities = 16/22 (72%), Positives = 19/22 (85%)
Frame = +3

Query: 690 WRPKEQFTDGVGFSWIDPLKRL 755
           WR KEQF+DGVG+SWID LK +
Sbjct: 447 WRQKEQFSDGVGYSWIDTLKEV 468
>pdb|1CT9|A Chain A, Crystal Structure Of Asparagine Synthetase B From Escherichia
```

Figure 3.22 (continued)

```
Coli

pdb|1CT9|B Chain B, Crystal Structure Of Asparagine Synthetase B From Escherichia Coli

pdb|1CT9|C Chain C, Crystal Structure Of Asparagine Synthetase B From Escherichia Coli

pdb|1CT9|D Chain D, Crystal Structure Of Asparagine Synthetase B From Escherichia Coli
          Length = 553

Score =  347 bits (890), Expect(2) = e-100
Identities = 165/232 (71%), Positives = 198/232 (85%)
Frame = +2


Query: 5 AVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEGS 184
           +V   LMSDVPYGVLLSGGLDSS+ SAI KKYA +R+E  + S+AW+PQLHSF+VGL GS
Sbjct: 218 SVKSHLMSDVPYGVLLSGGLDSSIISAITKKYAARRVEDQERSEAWWPQLHSFAVGLPGS 277

Query: 185 PDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVIK 364
           PDL AAQ+VA+++GT+HHEI FT+QEGLDAIRDVIY+IETYD+TTIR+STPMYLM+R IK
Sbjct: 278 PDLKAAQEVANHLGTVHHEIHFTVQEGLDAIRDVIYHIETYDVTTIRASTPMYLMSRKIK 337

Query: 365 SMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWGI 544
           +MGIKMVLSGEG+DE+FGGYLYFHKAPNAKE H+ETVRKL  LHMYDC RANK++ AWG+
Sbjct: 338 AMGIKMVLSGEGSDEVFGGYLYFHKAPNAKELHEETVRKLLALHMYDCARANKAMSAWGV 397

Query: 545 EGRVPFLDKEFMDVAMRINXKDKMINDERMXKWVIRKAFEDLLAESVTLETK 700
           E RVPFLDK+F+DVAMRIN +DKM  + +M K ++R+ FE  L  SV    K
Sbjct: 398 EARVPFLDKKFLDVAMRINPQDKMCGNGKMEKHILRECFEAYLPASVAWRQK 449
Score = 41.6 bits (96), Expect(2) = e-100
Identities = 16/22 (72%), Positives = 19/22 (85%)
Frame = +3

Query: 690 WRPKEQFTDGVGFSWIDPLKRL 755
           WR KEQF+DGVG+SWID LK +
Sbjct: 446 WRQKEQFSDGVGYSWIDTLKEV 467

>pir||H82255 asparagine synthetase B, glutamine-hydrolyzingC0991 [imported] -
           Vibrio cholerae (group O1 strain N16961)
 gb|AAF94152.1| (AE004180) asparagine synthetase B, glutamine-hydrolyzing [Vibrio
           cholerae]
          Length = 554

 Score =  355 bits (910), Expect = 5e-97
 Identities = 172/245 (70%), Positives = 207/245 (84%), Gaps = 3/245 (1%)
 Frame = +2

Query: 2   AAVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEG 181
           AAV RQLM+DVPYGVLLSGGLDSS+TSAIAK++A  RIE D+ S AW+PQLHSF++GLEG
Sbjct: 218 AAVKRQLMTDVPYGVLLSGGLDSSITSAIAKRFAAMRIEDDEKSAAWWPQLHSFAIGLEG 277

Query: 182 SPDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVI 361
           +PDL AA++VA+ IGT+HHE+ +TIQEGLDAIRDVIY+IETYD+TTIR+STPM+LM R I
Sbjct: 278 APDLKAAREVAEKIGTVHHEMTYTIQEGLDAIRDVIYHIETYDVTTIRASTPMFLMGRKI 337

Query: 362 KSMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWG 541
           K+MGIKMVLSGEGADEIFGGYLYFHKAPNAKEFH+ETVRKL  L+++DC RANKSL AWG
Sbjct: 338 KAMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHEETVRKLLALNLFDCARANKSLAAWG 397

Query: 542 IEGRVPFLDKEFMDVAMRINXKDKMINDERMXKWVIRKAFEDLLAESVTLETKRTI---Y 712
           +EGRVPFLDKEF+DVAMR+N  DKM  + +M K ++R+ FE  L ES+    K
Sbjct: 398 VEGRVPFLDKEFIDVAMRLNPADKMCGNGKMEKHILRECFEHYLPESIAWRQKEQFSDGV 457
```

Figure 3.22 (continued)

```
Query: 713 RWGWL 727
            +GW+
Sbjct: 458 GYGWI 462
 Score = 39.7 bits (91), Expect = 0.050
 Identities = 15/20 (75%), Positives = 17/20 (85%)
 Frame = +3

Query: 690 WRPKEQFTDGVGFSWIDPLK 749
           WR KEQF+DGVG+ WID LK
Sbjct: 447 WRQKEQFSDGVGYGWIDTLK 466
>sp|P31752|ASNS_ASPOF ASPARAGINE SYNTHASE [GLUTAMINE-HYDROLYZING] (AS)
 pir||S25165 asparagine synthase (glutamine-hydrolyzing) (EC 6.3.5.4) - garden
           asparagus
 emb|CAA48141.1| (X67958) asparagine synthase (glutamine-hydrolysing) [Asparagus
           officinalis]
          Length = 590

 Score =  306 bits (785), Expect(2) = 5e-87
 Identities = 145/223 (65%), Positives = 184/223 (82%), Gaps = 2/223 (0%)
 Frame = +2

Query: 5   AVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEGS 184
           AV ++LM+DVP+GVLLSGGLDSS+ +A+  ++     +     ++ W  QLHSF VGLEGS
Sbjct: 217 AVIKRLMTDVPFGVLLSGGLDSSLVAAVTARH----LAGSKAAEQWGTQLHSFCVGLEGS 272

Query: 185 PDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVIK 364
           PDL AA++VA+Y+GT+HHE  FT+Q+G+DAI DVI++IETYD+TTIR+STPM+LMAR IK
Sbjct: 273 PDLKAAKEVAEYLGTVHHEFHFTVQDGIDAIEDVIFHIETYDVTTIRASTPMFLMARKIK 332

Query: 365 SMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWGI 544
           S+G+KMV+SGEG+DEIFGGYLYFHKAPN +EFH ET RK+  LH YDCLRANK+  AWG+
Sbjct: 333 SLGVKMVISGEGSDEIFGGYLYFHKAPNKEEFHHETCRKIKALHQYDCLRANKATSAWGL 392

Query: 545 EGRVPFLDKEFMDVAMRINXKDKMINDE--RMXKWVIRKAFED 667
           E RVPFLDKEFMDVAM I+ + KMI  +  R+ KWV+RKAF+D
Sbjct: 393 EARVPFLDKEFMDVAMSIDPESKMIKPDLGRIEKWVLRKAFDD 435
 Score = 37.4 bits (85), Expect(2) = 5e-87
 Identities = 15/20 (75%), Positives = 18/20 (90%)
 Frame = +3

Query: 690 WRPKEQFTDGVGFSWIDPLK 749
           +R KEQF+DGVG+SWID LK
Sbjct: 447 YRQKEQFSDGVGYSWIDGLK 466
>emb|CAA67889.1| (X99552) asparagine synthetase [Asparagus officinalis]
          Length = 590

 Score =  306 bits (785), Expect(2) = 5e-87
 Identities = 145/223 (65%), Positives = 184/223 (82%), Gaps = 2/223 (0%)
 Frame = +2

Query: 5   AVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEGS 184
           AV ++LM+DVP+GVLLSGGLDSS+ +A+  ++     +     ++ W  QLHSF VGLEGS
Sbjct: 217 AVIKRLMTDVPFGVLLSGGLDSSLVAAVTARH----LAGSKAAEQWGTQLHSFCVGLEGS 272

Query: 185 PDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVIK 364
           PDL AA++VA+Y+GT+HHE  FT+Q+G+DAI DVI++IETYD+TTIR+STPM+LMAR IK
Sbjct: 273 PDLKAAKEVAEYLGTVHHEFHFTVQDGIDAIEDVIFHIETYDVTTIRASTPMFLMARKIK 332

Query: 365 SMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWGI 544
           S+G+KMV+SGEG+DEIFGGYLYFHKAPN +EFH ET RK+  LH YDCLRANK+  AWG+
Sbjct: 333 SLGVKMVISGEGSDEIFGGYLYFHKAPNKEEFHHETCRKIKALHQYDCLRANKATSAWGL 392
```

Figure 3.22 (continued)

```
Query: 545 EGRVPFLDKEFMDVAMRINXKDKMINDE--RMXKWVIRKAFED 667

           E RVPFLDKEFMDVAM I+ + KMI   +  R+ KWV+RKAF+D
Sbjct: 393 EARVPFLDKEFMDVAMSIDPESKMIKPDLGRIEKWVLRKAFDD 435
 Score = 37.4 bits (85), Expect(2) = 5e-87
 Identities = 15/20 (75%), Positives = 18/20 (90%)
 Frame = +3

Query: 690 WRPKEQFTDGVGFSWIDPLK 749
           +R KEQF+DGVG+SWID LK
Sbjct: 447 YRQKEQFSDGVGYSWIDGLK 466
>gb|AAF02775.1|AF190728_1 (AF190728) asparagine synthetase [Helianthus annuus]
          Length = 591

 Score =  305 bits (782), Expect(2) = 9e-87
 Identities = 147/223 (65%), Positives = 184/223 (81%), Gaps = 2/223 (0%)
 Frame = +2

Query: 5   AVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEGS 184
           AV ++LM+DVP+GVLLSGGLDSS+ ++I  +Y     +     +  W  QLHSF VGLEGS
Sbjct: 219 AVIKRLMTDVPFGVLLSGGLDSSLVASITARY----LAGTKAAKQWGAQLHSFCVGLEGS 274

Query: 185 PDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVIK 364
           PDL AA++VADY+GT+HHE  FT+Q+G+DAI DVIY+IETYD+TTIR+STPM+LM+R IK
Sbjct: 275 PDLKAAREVADYLGTVHHEFHFTVQDGIDAIEDVIYHIETYDVTTIRASTPMFLMSRKIK 334

Query: 365 SMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWGI 544
           S+G+KMV+SGEG+DEIFGGYLYFHKA N +EFHQET RK+  LH YDCLRANKS  AWG+
Sbjct: 335 SLGVKMVISGEGSDEIFGGYLYFHKALNREEFHQETCRKIKALHQYDCLRANKSTSAWGL 394

Query: 545 EGRVPFLDKEFMDVAMRINXKDKMIN--DERMXKWVIRKAFED 667
           E RVPFLDKEF++VAM I+ + KMIN   +R+ KWV+R+AFED
Sbjct: 395 EARVPFLDKEFINVAMSIDPEAKMINMDQKRIEKWVLRRAFED 437
 Score = 37.7 bits (86), Expect(2) = 9e-87
 Identities = 19/34 (55%), Positives = 22/34 (63%)
 Frame = +3

Query: 648 FEKLLKTYWQKV*HWRPKEQFTDGVGFSWIDPLK 749
           FE      Y  K   +R KEQF+DGVG+SWID LK
Sbjct: 435 FEDEEHPYLPKHILYRQKEQFSDGVGYSWIDGLK 468
>pir||D82846 asparagine synthase B XF0118 [imported] - Xylella fastidiosa
           (strain 9a5c)
 gb|AAF82931.1|AE003865_8 (AE003865) asparagine synthase B [Xylella fastidiosa]
          Length = 563

 Score =  317 bits (811), Expect = 2e-85
 Identities = 149/249 (59%), Positives = 202/249 (80%), Gaps = 8/249 (3%)
 Frame = +2

Query: 5   AVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEGS 184
           AVHRQLM+DVPYGVLLSGGLDSS+ +A+A +YA+ RIE++D S+AW+P+LHSF++GL+ S
Sbjct: 222 AVHRQLMTDVPYGVLLSGGLDSSLVAAVAARYARHRIETNDQSEAWWPRLHSFAIGLKDS 281

Query: 185 PDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVIK 364
           PDL+AA    A+ + T+HH  ++T+QEGLD + +VI +IETYD+TTIR+STPM+L+AR IK
Sbjct: 282 PDLSAANVAAEALNTVHHGFEYTLQEGLDVLPEVIRHIETYDVTTIRASTPMFLLARRIK 341

Query: 365 SMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWGI 544
           +MG+KMVLSGEG+DEIFGGYLYFHKAPNA+EFH+E VRKL+ L+ YDCLRANK++MAWG+
Sbjct: 342 AMGVKMVLSGEGSDEIFGGYLYFHKAPNAREFHEELVRKLNALYYYDCLRANKAMMAWGV 401

Query: 545 EGRVPFLDKEFMDVAMRINXKDKMIN-----DERMXKWVIRKAFEDLLAESVTLETKRTI 709
           E RVPFLD+EF+DVAMR++ + KM++       ++M K ++R AF+  L  S+    K
Sbjct: 402 EPRVPFLDREFLDVAMRMDAQHKMVDKTSDGPQQMEKGILRAAFDGYLPPSILWRQKEQF 461
```

Figure 3.22 (continued)

```
Query: 710 ---YRWGWL 727
               +GW+
Sbjct: 462 SDGVGYGWI 470
 Score = 39.3 bits (90), Expect = 0.065
 Identities = 15/20 (75%), Positives = 17/20 (85%)
 Frame = +3

Query: 690 WRPKEQFTDGVGFSWIDPLK 749
           WR KEQF+DGVG+ WID LK
Sbjct: 455 WRQKEQFSDGVGYGWIDGLK 474
>sp|O24661|ASNS_TRIVS ASPARAGINE SYNTHETASE [GLUTAMINE-HYDROLYZING] (GLUTAMINE-DEPENDENT
           ASPARAGINE SYNTHETASE)
 gb|AAD05033.1| (AF014055) asparagine synthetase [Triphysaria versicolor]
 gb|AAD05034.1| (AF014056) asparagine synthetase [Triphysaria versicolor]
 gb|AAD05035.1| (AF014057) asparagine synthetase [Triphysaria versicolor]
          Length = 586

 Score =  300 bits (767), Expect(2) = 5e-85
 Identities = 141/223 (63%), Positives = 183/223 (81%), Gaps = 2/223 (0%)
 Frame = +2

Query: 5   AVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEGS 184
           AV ++LM+DVP+GVLLSGGLDSS+ +A+  ++     +      +  W  QLHSF VGLEGS
Sbjct: 217 AVIKRLMTDVPFGVLLSGGLDSSLVAAVTARH----LAGTKAAKRWGSQLHSFCVGLEGS 272

Query: 185 PDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVIK 364
           PDL A ++VADY+GT+HHE  FT+Q+G+DAI DVIY+IETYD+TTIR+STPM+LM+R IK
Sbjct: 273 PDLKAGKEVADYLGTVHHEFLFTVQDGIDAIEDVIYHIETYDVTTIRASTPMFLMSRKIK 332

Query: 365 SMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWGI 544
           S+G+KMV+SGEG+DEIFGGYLYFHKAPN +EFH+ET RK+  LH YDCLRANK+  AWG+
Sbjct: 333 SLGVKMVISGEGSDEIFGGYLYFHKAPNKEEFHRETCRKIKALHQYDCLRANKATSAWGL 392

Query: 545 EGRVPFLDKEFMDVAMRINXKDKMINDE--RMXKWVIRKAFED 667
           E RVPFLDKEF+++AM I+ + KMI  +  R+ KW++RKAF+D
Sbjct: 393 EARVPFLDKEFVNLAMSIDPEAKMIKPDQGRIEKWILRKAFDD 435
 Score = 37.7 bits (86), Expect(2) = 5e-85
 Identities = 18/34 (52%), Positives = 23/34 (66%)
 Frame = +3

Query: 648 FEKLLKTYWQKV*HWRPKEQFTDGVGFSWIDPLK 749
           F+    + Y  K   +R KEQF+DGVG+SWID LK
Sbjct: 433 FDDEERPYLPKHILYRQKEQFSDGVGYSWIDGLK 466
>sp|P49078|ASNS_ARATH ASPARAGINE SYNTHETASE [GLUTAMINE-HYDROLYZING] (GLUTAMINE-DEPENDENT
           ASPARAGINE SYNTHETASE)
 pir||T12989 asparagine synthase (glutamine-hydrolyzing) (EC 6.3.5.4) -
           Arabidopsis thaliana
 gb|AAA74359.1| (L29083) glutamine-dependent asparagine synthetase [Arabidopsis
           thaliana]
 emb|CAB51206.1| (AL096860) glutamine-dependent asparagine synthetase [Arabidopsis
           thaliana]
          Length = 584

 Score =  300 bits (767), Expect(2) = 5e-85
 Identities = 142/223 (63%), Positives = 183/223 (81%), Gaps = 2/223 (0%)
 Frame = +2

Query: 5   AVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEGS 184
           AV ++LM+DVP+GVLLSGGLDSS+ ++I  ++     +      +  W PQLHSF VGLEGS
Sbjct: 217 AVIKRLMTDVPFGVLLSGGLDSSLVASITARH----LAGTKAAKQWGPQLHSFCVGLEGS 272

Query: 185 PDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVIK 364
           PDL A ++VA+Y+GT+HHE  F++Q+G+DAI DVIY++ETYD+TTIR+STPM+LM+R IK
Sbjct: 273 PDLKAGKEVAEYLGTVHHEFHFSVQDGIDAIEDVIYHVETYDVTTIRASTPMFLMSRKIK 332
```

Figure 3.22 (continued)

```
Query: 365 SMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWGI 544
           S+G+KMVLSGEGADEIFGGYLYFHKAPN KEFHQET RK+  LH YDCLRANKS   A+G+
Sbjct: 333 SLGVKMVLSGEGADEIFGGYLYFHKAPNKKEFHQETCRKIKALHKYDCLRANKSTSAFGL 392

Query: 545 EGRVPFLDKEFMDVAMRINXKDKMINDE--RMXKWVIRKAFED 667
           E RVPFLDK+F++ AM ++ + KMI  E  R+ KWV+R+AF+D
Sbjct: 393 EARVPFLDKDFINTAMSLDPESKMIKPEEGRIEKWVLRRAFDD 435
 Score = 37.7 bits (86), Expect(2) = 5e-85
 Identities = 18/34 (52%), Positives = 23/34 (66%)
 Frame = +3

Query: 648 FEKLLKTYWQKV*HWRPKEQFTDGVGFSWIDPLK 749
           F+    + Y  K   +R KEQF+DGVG+SWID LK
Sbjct: 433 FDDEERPYLPKHILYRQKEQFSDGVGYSWIDGLK 466
>gb|AAF02776.1|AF190729_1 (AF190729) asparagine synthetase [Helianthus annuus]
          Length = 558

 Score =  300 bits (767), Expect(2) = 6e-85
 Identities = 143/223 (64%), Positives = 185/223 (82%), Gaps = 2/223 (0%)
 Frame = +2

Query: 5   AVHRQLMSDVPYGVLLSGGLDSSVTSAIAKKYAQKRIESDDTSDAWYPQLHSFSVGLEGS 184
           AV ++LM+DVP+GVLLSGGLDSS+ +A+A ++    +   +    W  QLH+F +GL+GS
Sbjct: 217 AVIKRLMTDVPFGVLLSGGLDSSLVAAVASRH----LVDSEAYCQWGSQLHTFCIGLKGS 272

Query: 185 PDLAAAQKVADYIGTIHHEIKFTIQEGLDAIRDVIYNIETYDITTIRSSTPMYLMARVIK 364
           PDL AA++VADY+GT HHE  FT+QEG+DA+ +VIY+IETYD+TTIR+STPM+LM+R IK
Sbjct: 273 PDLVAAREVADYLGTRHHEFYFTVQEGIDALEEVIYHIETYDVTTIRASTPMFLMSRKIK 332

Query: 365 SMGIKMVLSGEGADEIFGGYLYFHKAPNAKEFHQETVRKLDKLHMYDCLRANKSLMAWGI 544
           S+G+KMVLSGEG+DEIFGGYLYFHKAPN +EFH+ET RK+  LH+YDCLRANKS  AWG+
Sbjct: 333 SLGVKMVLSGEGSDEIFGGYLYFHKAPNKEEFHEETCRKIKALHLYDCLRANKSTSAWGL 392

Query: 545 EGRVPFLDKEFMDVAMRINXKDKMINDE--RMXKWVIRKAFED 667
           E RVPFLDKEF++VAM I+ K KMI+ +  R+ KWV+R AF+D
Sbjct: 393 EARVPFLDKEFINVAMSIDPKWKMIDRDNGRIEKWVLRNAFDD 435
 Score = 37.4 bits (85), Expect(2) = 6e-85
 Identities = 17/29 (58%), Positives = 21/29 (71%)
 Frame = +3

Query: 663 KTYWQKV*HWRPKEQFTDGVGFSWIDPLK 749
           K Y  K   +R KEQF+DGVG+SWID L+
Sbjct: 438 KPYLPKHILYRQKEQFSDGVGYSWIDGLR 466


Database: nr
    Posted date:  Apr 19, 2001 11:46 PM
  Number of letters in database: 211,820,757
  Number of sequences in database:  673,391

Lambda     K      H
   0.318    0.135    0.401

Gapped
Lambda     K      H
   0.267   0.0410    0.140
```

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Figure 3.22 (continued)

```
Score =  225 bits (117), Expect = 1e-56
Identities = 224/262 (85%), Gaps = 8/262 (3%)
 Strand = Plus / Minus
```

Sequence 1	lcl|seq_1	Length	343	(1 .. 343)
Sequence 2	lcl|seq_2	Length	1005	(1 .. 1005)

```
Query: 88   ggctttgctcgaaatggtgccacgttagaaggtgataagaaaaa-gcgttacgctgaaat 146
             |||||||||| ||| ||  ||| ||| | |||||||||| ||||| | ||| |||||||||
Sbjct: 888  ggctttgctcnaaagggnnccccgtaa-aaggtgataanaaaaaagggttncgctgaaat 830

Query: 147  taatcaacgcctt-gctgagcttcacaccaaatttggc-aataatgtacttgctgatgaa 204
             ||||| ||||| | | | |||||||| || | |||||| ||||||| |||||||||  ||
Sbjct: 829  taatccacgccntngttaagcttcacnccgattttggccaataatgnacttgctgaataa 770

Query: 205  gaaaactacgtg-ttgtttt-ggacgaatcgcaattgggtggtttgactgattcaatcaa 262
               |||||||| | ||||||| ||||||||||| |||||||||||||||  |  ||||||||
Sbjct: 769  agaaactacggggttgttttnggacgaatcgccattgggtggtttgacntaatcaatcaa 710

Query: 263  aagtgctgctgcatcagctgctaaagagcgtggtcaggaaggtaagtatgcaattactaa 322
             |||||||||||||||||||||||| ||| |||  ||| ||||||||||||||||||||||
Sbjct: 709  aagtgctgctgcatcagctgctaa-gagggtgtccagaaaggtaagtatgcaattactaa 651

Query: 323  tacgcgctcttc-aatggatcc 343
             |||||||||||| |||||||||
Sbjct: 650  tacgcgctcttccaatggatcc 629

Score = 46.8 bits (24), Expect = 0.008
Identities = 24/24 (100%)
 Strand = Plus / Plus

Query: 22   tacaacggcgaagaaatgaaatca 45
             ||||||||||||||||||||||||
Sbjct: 982  tacaacggcgaagaaatgaaatca 1005

Score = 41.1 bits (21), Expect = 0.43
Identities = 21/21 (100%)
 Strand = Plus / Plus
```

Figure 3.23 The comparison between the nucleotide sequences of the 950 fragment and the 350 fragment using BLAST program.

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```
Query: 1  gcggatccgcgggctgcggta 21
          |||||||||||||||||||||
Sbjct: 7  gcggatccgcgggctgcggta 27

CPU time:     0.03 user secs.          0.04 sys. secs             0.07 total
secs.

Gapped
Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 10
Number of Sequences: 0
Number of extensions: 10
Number of successful extensions: 9
Number of sequences better than 10.0: 1
length of query: 343
length of database: 3,084,938,205
effective HSP length: 24
effective length of query: 319
effective length of database: 3,084,938,181
effective search space: 984095279739
effective search space used: 984095279739
T: 0
A: 30
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 19 (37.2 bits)
```

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Figure 3.23 (continued)

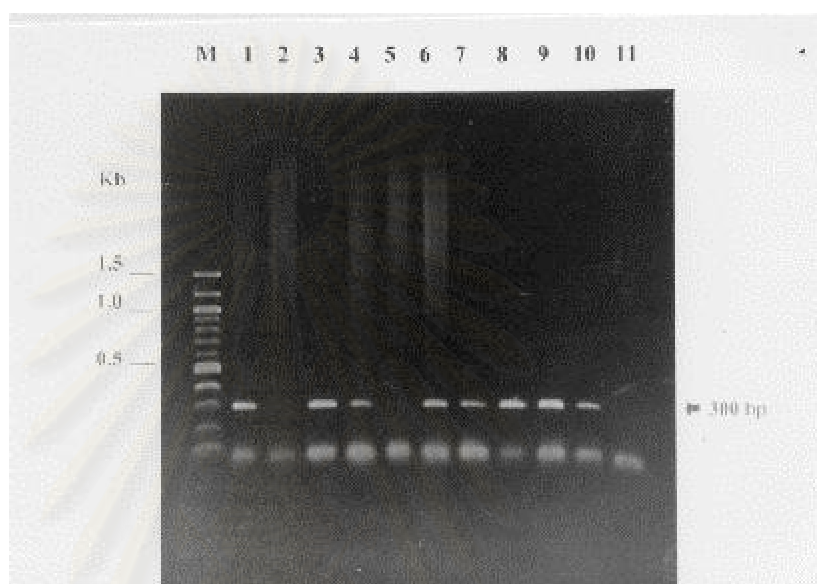

Figure 3.24 Ethidium bromide staining of 300 bp PCR product

lane M = 100 bp DNA ladder

lanes 1 - 5 = individuals collected from Satun-Trang

lane 6 –10 = individuals collected from Trad

lane 11 = negative control

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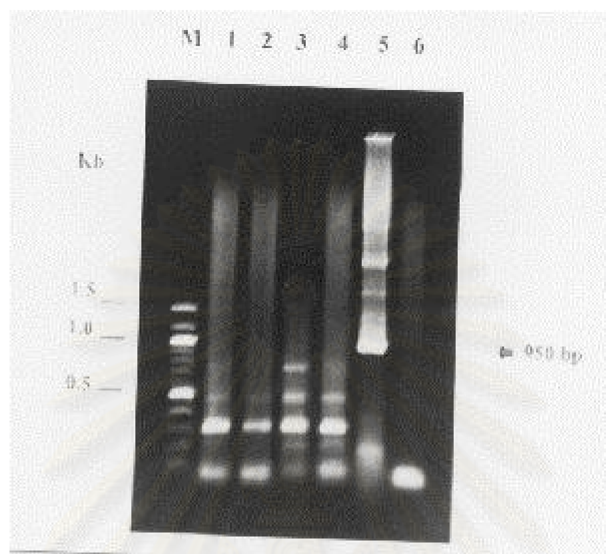

Figure 3.25 Ethidium bromide staining of 950 bp PCR product

lane M = 100 bp DNA ladder

lanes 1 - 2 = individuals collected from Satun-Trang

lanes 3 – 4 = individuals collected from Trad

lane 5 = positive control containing from clone B

lane 6 = negative control

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GGATCCGCGGATCCGCGGGCTGCGGTATACAACGGCGAAGAAATGAAAT

CACTTCGTAAAGATCAGCAGCGATTAGTGTGGCTAACTTATAATGGCTTTG

CTCGAAATGGTGCCACGTTAGAAGGTGATAAGAAAAAGCGTTACGCTGAA

Primer C-F 3

ATTAATCAACGCCTTGCTGAGCTTCACACCGAATTTGGCAATAATGTACTT

GCTGATGAAGAAAACTACGTGTTGTTTTGGACGAATCGCAATTGGGTGGT

Primer C-R 2

TGACTGATTCAATCAAAAGTGCTGCTGCATCAGCTGCTAAAGAGCGTGGT

CAGGAAGGTAAGTATGCAATTACTAATACGCGCTCTTCAATGGATCC

Figure 3.26 Nucleotide sequence of the 300 bp PCR product using ABI-PRISM automate sequencer. The locations and sequences of *P.monodon* specific forward primer (primer C-F 3) and those complementary to reverse primer (primer C-R 2) are labelled in bold face and underlined.

```
Sequence 1          lcl|seq_1          Length      715   (1 .. 715)

Sequence 2          lcl|seq_2          Length      343   (1 .. 343)

Query: 1   gtaaagatcagcagcgattagtgtggctaacttataatggctttgctcgaaatggtgcca 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 50  gtaaagatcagcagcgattagtgtggctaacttataatggctttgctcgaaatggtgcca 109


Query: 61  cgttagaaggtgataagaaaaagcgttacgctgaaattaatcaacgccttgctgagcttc 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 110 cgttagaaggtgataagaaaaagcgttacgctgaaattaatcaacgccttgctgagcttc 169


Query: 121 acaccgaatttggcaataatgtacttgctgatgaanaaaactacntgttgtttttggacg 180
           ||||| ||||||||||||||||||||||||||||| |||||||| ||||| |||||||||
Sbjct: 170 acaccaaatttggcaataatgtacttgctgatgaagaaaactacgtgttg-tttggacg 228


Query: 181 aatcgcaattgggtggtttgactgattcaatcaaaagtgctgctgcatcanctgctaaag 240
           |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct: 229 aatcgcaattgggtggtttgactgattcaatcaaaagtgctgctgcatcagctgctaaag 288


Query: 241 agcgtggtcaggaaggtaagtatgcaat 268
           ||||||||||||||||||||||||||||
Sbjct: 289 agcgtggtcaggaaggtaagtatgcaat 316

CPU time:     0.04 user secs.     0.03 sys. secs      0.07 total secs.

Gapped
Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12
```

Figure 3.27 Comparison of nucleotide of the 300 bp PCR with that of the 350 bp insert fragment using BLAST program.

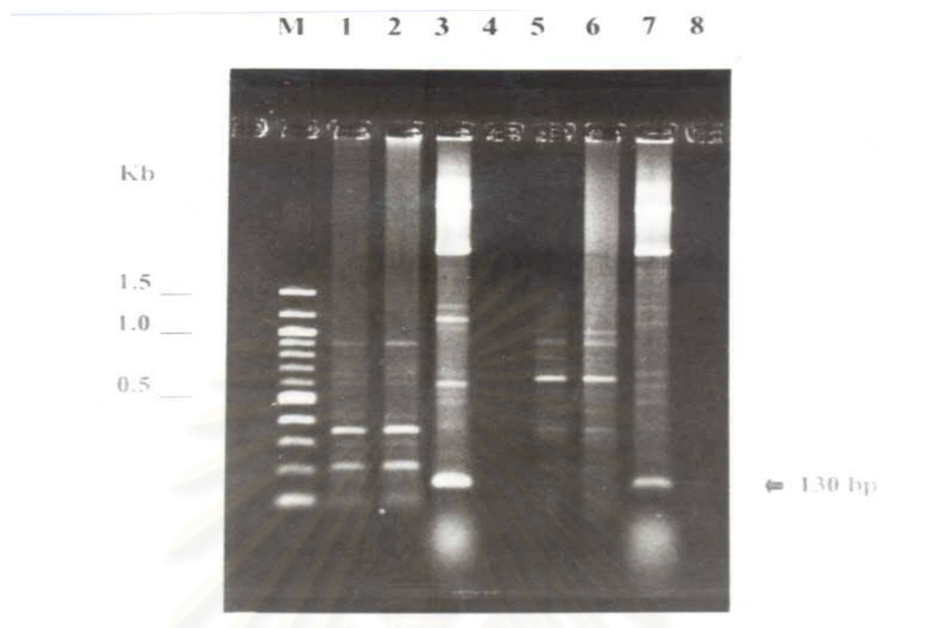

Figure 3.28 Ethidium bromide staining of 130 bp PCR product of shrimp using primer C-F 2 and primer C-F 3

lane M = 100 bp DNA ladder

lanes 1 – 2 = individuals collected from Satun-Trang

lane 3 = positive control containing DNA from clone B using primers C-F 2

lane 4 = negative control

lanes 5 - 6 = individuals collected from Trad

lane 7 = positive control containing DNA from clone B using primers C-F 3

lane 8 = negative control

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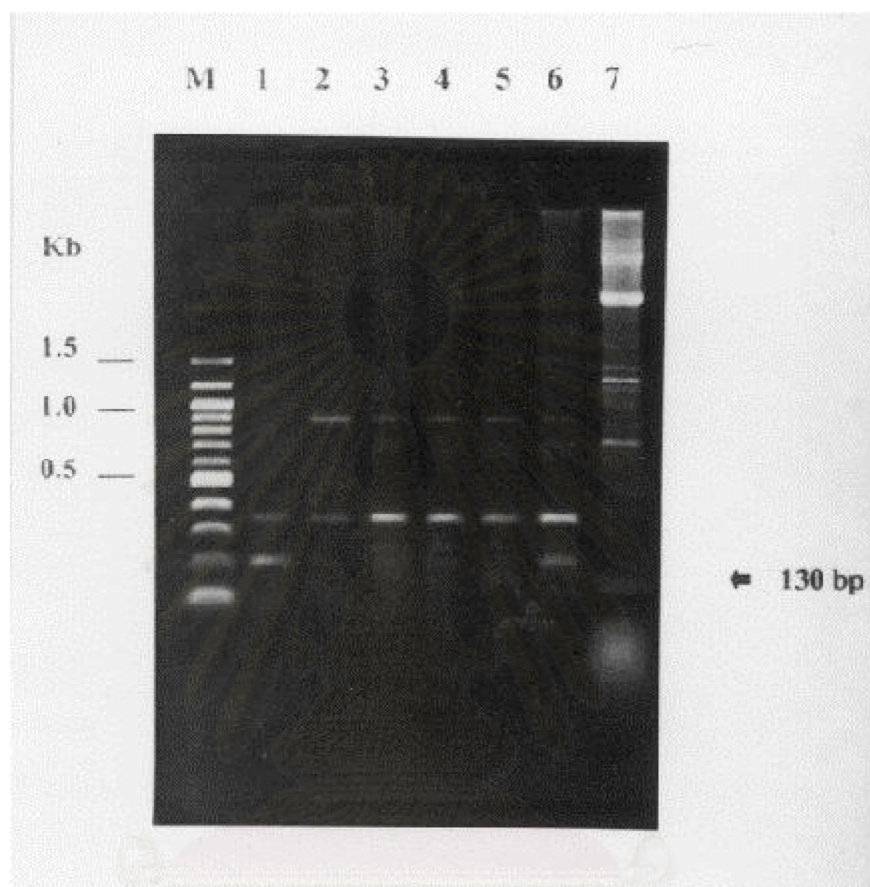

Figure 3.29 Ethidium bromide staining of 130 bp PCR product of shrimp using primer C-F 2 and primer C-R 2.

lane M = 100 bp DNA ladder

lanes 1 - 3= individuals collected from Satun-Trang

lanes 4 - 6 = individuals collected from Trad

lane 7 = positive control containing DNA from clone B

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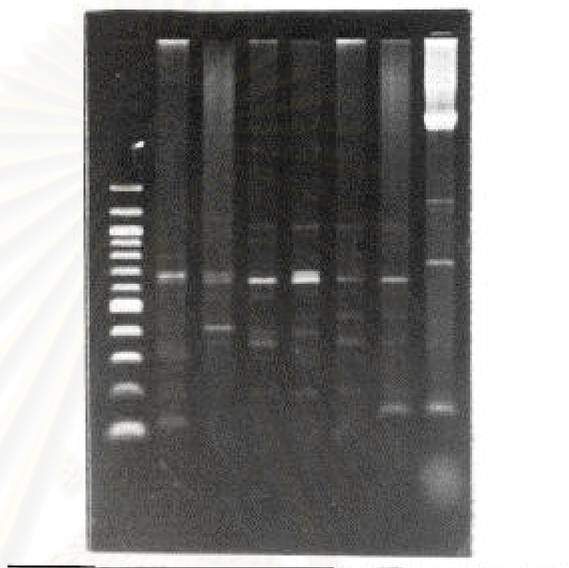

Figure 3.30 Ethidium bromide staining of 130 bp PCR product of shrimp using primer C-F 3 and primer C-R 2.

lane M = 100 bp DNA ladder

lanes 1 - 3 = individuals collected from Satun-Trang

lanes 4 - 6 = individuals collected from Trad

lane 7 = positive control containing DNA from clone B

Chapter IV

Discussion

The two population of *P.monodon* in Thailand, the Andaman Sea and the Gulf of Thailand, have been shown to be genetically different (Tassanakajon et al., 1998; Supungun et al., 2000; Klinbunga et al., 1999). The specific marker could distinguish between the two populations would provide a useful tool for further genetic studies and identification of populations in selective breeding program of Thai *P.monodon* (Jarayabhand et al., 1999). The RAPD technique, developed by Williams et al (1990) and Welsh and McClalland (1990) has been used successfully in genetic studies of *P.monodon* (Tassanakajon et al., 1997) and *P.vanamei* (Garcia et al., 1994). The eleven RAPD primers were screened for genetic polymorphism between *P.monodon* form the Andaman Sea and the Gulf of Thailand. On the basis of a preliminary results primer OPB 08 and primer OPB 15 gave the DNA bands which consistently appeared only in the samples from the Gulf of Thailand. By increasing the number of samples, the DNA bands showed in all 2 populations so they could not be used to distinguished between the two populations. Primer 428 previously by Tassanakajon et al. (1997) appeared to identify a more variable region among the samples of Thai *P monodon*. A band with size about 950 bp was found in the samples form the Andaman Sea but absence in those form the Gulf of Thailand, therefore, this DNA fragment was selected for further analysis. Similarity, Klinbunga et al (2000) used Randomly amplified polymorphic DNA (RAPD) to identify species-specific markers of 5 oyster species in Thailand. Cloning of the 950 bp fragment was performed by reamplification of the DNA fragment and ligation of the amplified fragment into pUC 18. The PCR product is notorious for being difficult to clone. Similarity, Hadjeb and Berkowits (1996) reported low efficiency of

transformation when cloning PCR product and many false positive clone were found. Because there are two primary problems related to the activity of *Taq* DNA polymerase. First, *Taq* DNA polymerase tends to add an extra base to the 3' end of the fragment, and this base is almost exclusively an adenosine (Clark et al., 1988). Second, there is some indication that the Taq DNA polymerase may remain bound to the DNA and thereby inhibit endonuclease activity. The first problem interferes with bluntend cloning, while the second interferes with cutting restriction site in a vector molecule (Jung et al., 1990). In this study, we used the method which has the adapter RAPD primer linkage. A new oligonucleotide primer containing 12 base at *Bam* HI adapter and 10 bases of RAPD primer (primer 428) was synthesized. This primer was used to reamplified the 950 bp DNA fragment and ligated into pUC 18/*Bam* HI/BAP as described in 2.14. The method yielded three different types of recombinant clones: A, B and C which contained the inserted fragments of 900 bp, 950 bp (which upon digestion with *Bam* HI providing two bands with size about 650 and 350 bp) and 350 bp, respectively.

Southern hybridization of RAPD patterns of *P.monodon* samples with the 900 fragment showed a single band of 950 bp in all samples while the 650 and 350 fragments yield positive results only in the sample of the Andaman Sea. The result of BLAST program revealed that the sequence of the 900 bp fragment, showed similarity with the sequence of asparagine synthetase. Apparently, this fragment is a coding region of *P.monodon* and present in all populations. Upon cloning, this DNA fragment was eluted with the 950 bp fragment and ligated into the vector. The 950 bp fragment contained the restriction sites *Bam* HI within the sequences, therefore, digestion of clone B with *Bam* HI resulted in the 650 and 350 bp fragment. Form nucleotide sequences of clone B and C, the 350 insert fragment of clone C was infact part of the insert fragment of clone B but they were inserted in opposite orientation.

The result of BLAST showed that the sequences were not significantly similar to any other DNA in the GenBank. These sequence may be the introns non-coding regions of the shrimp genome. Benzie (1998) reported the gemome size of four species of penaeid shrimp was approximately 70 % that of human genome.. The size of introns, normally found in eukaryotes, are broadly distribute from < 50 to >200,000 bp (80 % of structural gene).

The specific primers designed from the DNA sequence of RAPD marker providing a more specific detection han the DNA analysis. The use of single short arbitary primers and low annealing temperature in the RAPD results in non-repoducible DNA pattern. Klinbunga et al. (2000) reported a specific method for species identification in the tropical oyster (Crassostrea belcheri) by cloning, sequencing and designing species-specific primers of RAPD markers. To specific identify the population of Thai *P.monodon* the specific primers were design from the insert fragment of clone B and the insert fragment of clone C using Oligo4.0 software program. The primer of clone C-F 1 and primer clone C-R1 showed a 300 bp DNA fragment presented in both populations. While the primer clone B-F 1 and C-R1 did not yeild PCR product in any group of the populations but positive in the control plasmid. This may be occurred from the difference in the sequence of genomic DNA and clone B; therefore, the 950 bp DNA band appeared only in the positive control. In any case, the two pair of specific primers could not be use to identify the population of *P. monodon* between the Andaman and the Gulf of Thailand. Consequently, the PCR product of the 300 bp obtained from amplification of *P.monodon* from the Gulf of Thailand were sequenced by the ABI-PRISM automated sequencer. Comparison of this sequence with the sequence of the 350 fragment by using BLAST programs showed similarity of the two sequences but only one base difference.

To determine whether this one difference is really exist between the two population, the technique of allele specific amplification (ASA) was used to design primers. This method are now available for the detection of single base changes in a given DNA fragment and search for the DNA sequence are manyfold; (1) explanation of the mechanism of the disease, (2) elucidation of evolution relationship between species, (3) investigation and understanding of the mechanism mutagenic substances, (4) detection of disease – related genes for the diagnosis of certain diseases, (5) investigation of certain pathogenicity traits of various causative agents, (6) comprehension of the basis of resistance development against chemotherapeutic substance by microorganisms, (7) genetic linkage studies the PCR technique has brought considerable experimental simplifications into this methods for detect of point mutations. The basis of this procedure is the hypothesis that a mismatch at the 3 end of one or both of the used digonucleotides-with high probability-prevents the 3' elongation of the primer by *Taq* Polymerase. Of course, only DNA polymerase that do not exhibit proofreading activity most be used for such an assay (Rolfs et al., 1992). In 1989, Wu et al used the ASA for detecting of the normal or sickle - cell globin allele in genome DNA. Sommer (1991) have shown this biallelelic polymorphic sites of the human dopamine D2 receptor. In this study, the result of ASA showed a 130 bp PCR product presented only in the positive control of the recombinant clone C but absence in all 2 population and there, cannot be use to distinguish the populations of *P.monodon* from the Andaman Sea and the Gulf of Thailand. These one base difference may be ocurred result the polymorphism at the region of shrimps, or error of *Tag* polymerase. In other word, the primers which designed from the sequences of RAPD fragment did not provide a specific marker for identification the populations of *P. monodon* between the Andaman Sea and the Gulf of Thailand.

Inconclution, Southern blot hybridization and RAPD patterns by the 428 RAPD primer) probing with the 650 and 350 fragments provided the most specific and reliable method to detect the population of Thai P.monodon. This method yeild a single band of 950 appeared only in the Andaman Sea samples. Apparently, this method is better thand the RAPD analysis suffer from non reproducible RAPD patterns (Harding et al., 1997). Moreover, the RAPD patterns consisted of many DNA bands which made a more complex DNA pattern for distinguishing the population of the Andaman Sea form those of the Gulf of Thailand. Nevertheless, Southern hybridization requires multiple steps for analysis so it is less simple than the RAPD. It is suggested that this specific method is use to confirm the results of the RAPD analysis when ambiguous results are contain.

Charpter V

Conclusion

1. Screening of RAPD primers show that amplification of shrimp genomic DNA using primer 428 yeild a 950 bp DNA band only in the samples of *P.monodon* from the Andaman Sea.

2. Cloning of the 950bp DNA fragment yeild three different types of recombinant clones: A, B and C which contained the insert fragments of 900 bp, 950 bp (which upon digestion with *Bam* HI providing two bands with size about 650 and 350 bp) and 350 bp, respectively.

3. Southern hybridization of RAPD patterns of *P.monodon* samples with the 900 fragment showed a single band of 950 bp in all samples while the 650 and 350 fragments yielded positive results only in the sample of the Andaman Sea.

4. The sequence of the 900 bp fragment showed similarity with the sequence of asparagine synthetase but the sequences of the 650bp fragment and the 350 bp fragment were not significantly similar to any other DNA in the GenBank..

5. Specific primer design form the sequence of the 350 bp fragment amplified the genomic DNA of the two populations of Thai *P.monodon*.

6. In this study, the specific method for population identification of Thai *P.monodon.* obtained from the RAPD analysis using primer 428, followed by Southern hybridization with the 650or 350 bp fragment.

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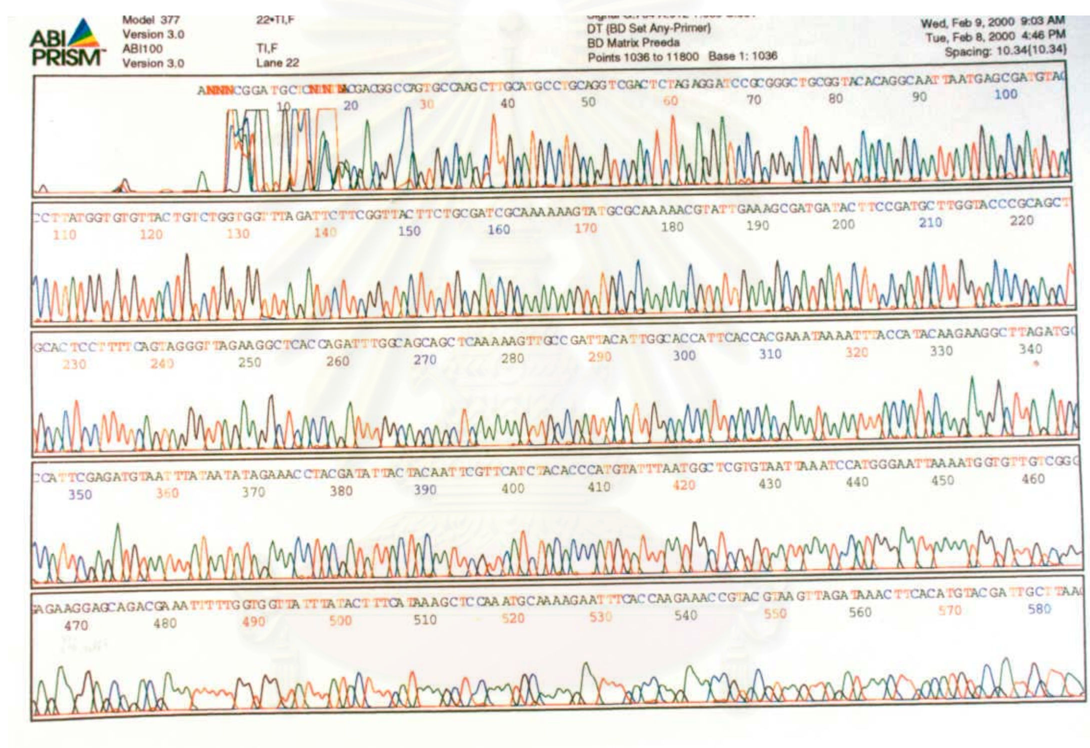
APPENDIX

The sequencing profiles of clone A, B and C and 300 bp product.



Clone A

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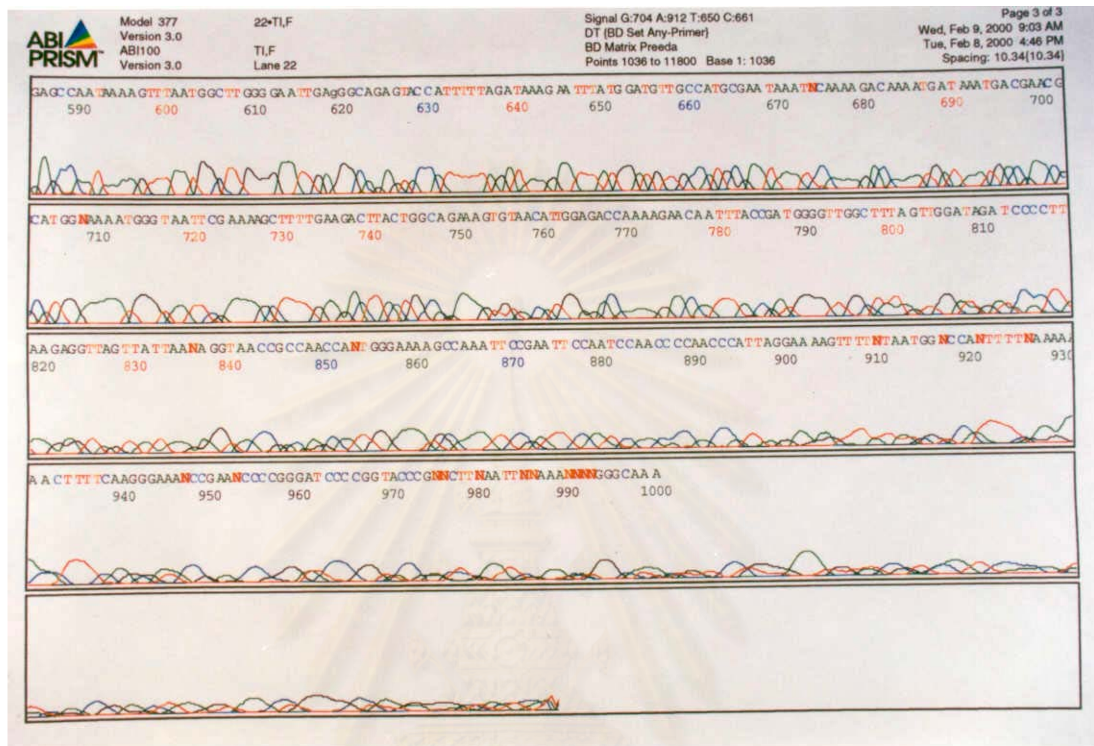

Clone A (continued)

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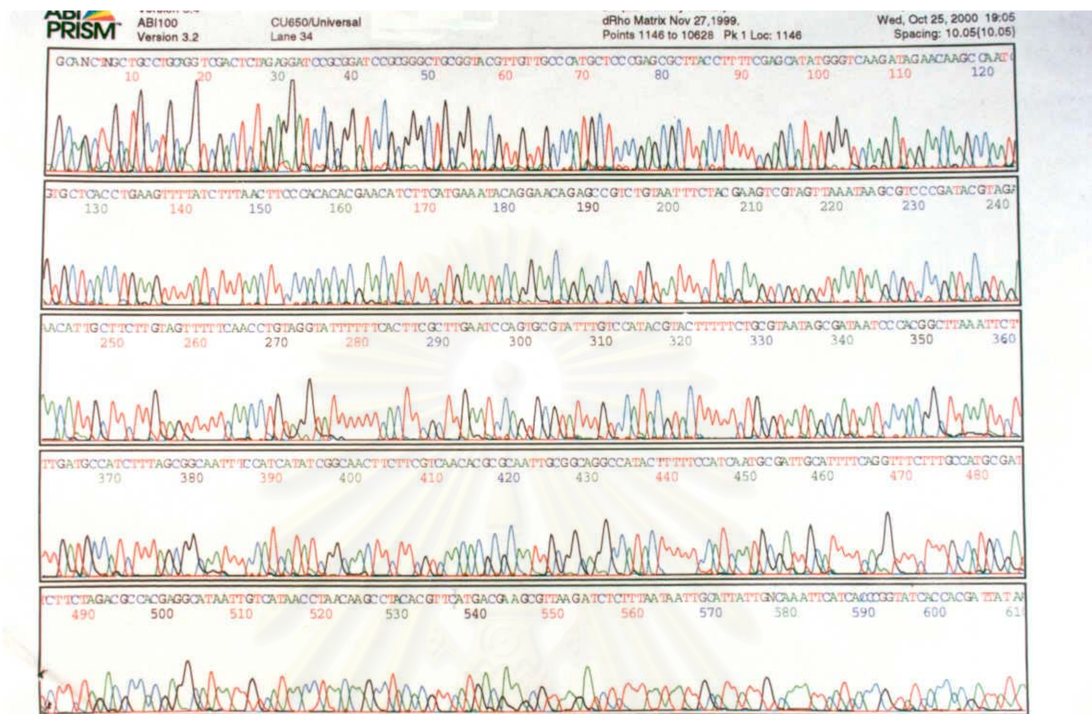
จุฬาลงกรณ์มหาวิทยาลัย



Clone B

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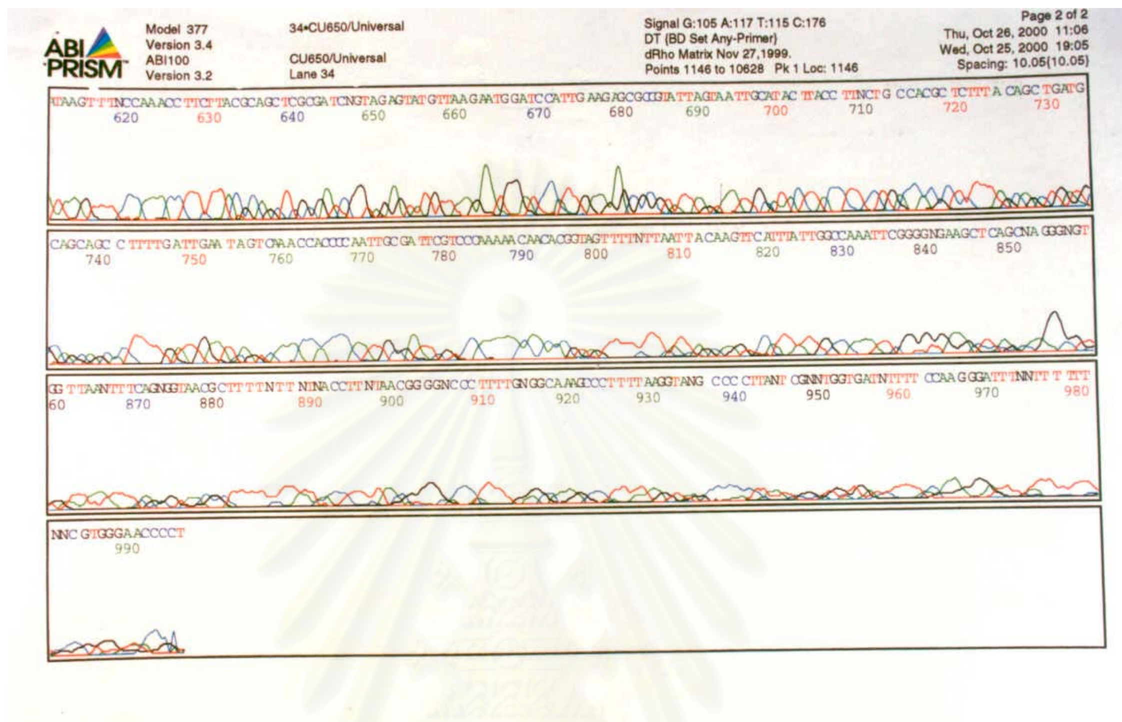
จุฬาลงกรณ์มหาวิทยาลัย



Clone B(continued)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Clone C

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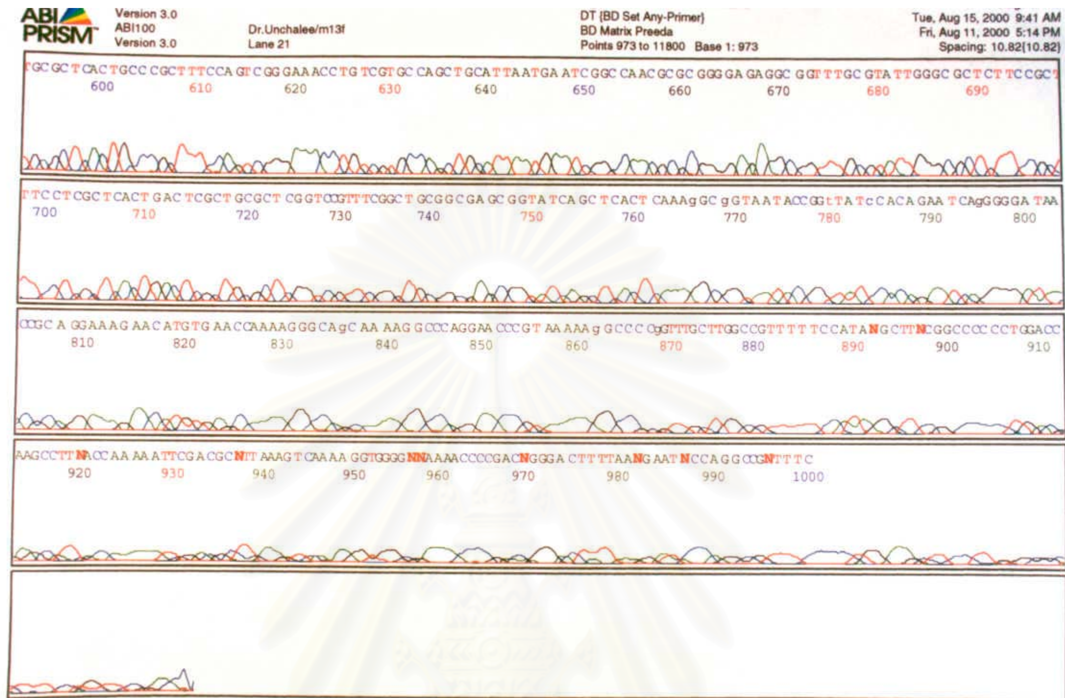

Clone C (continued)

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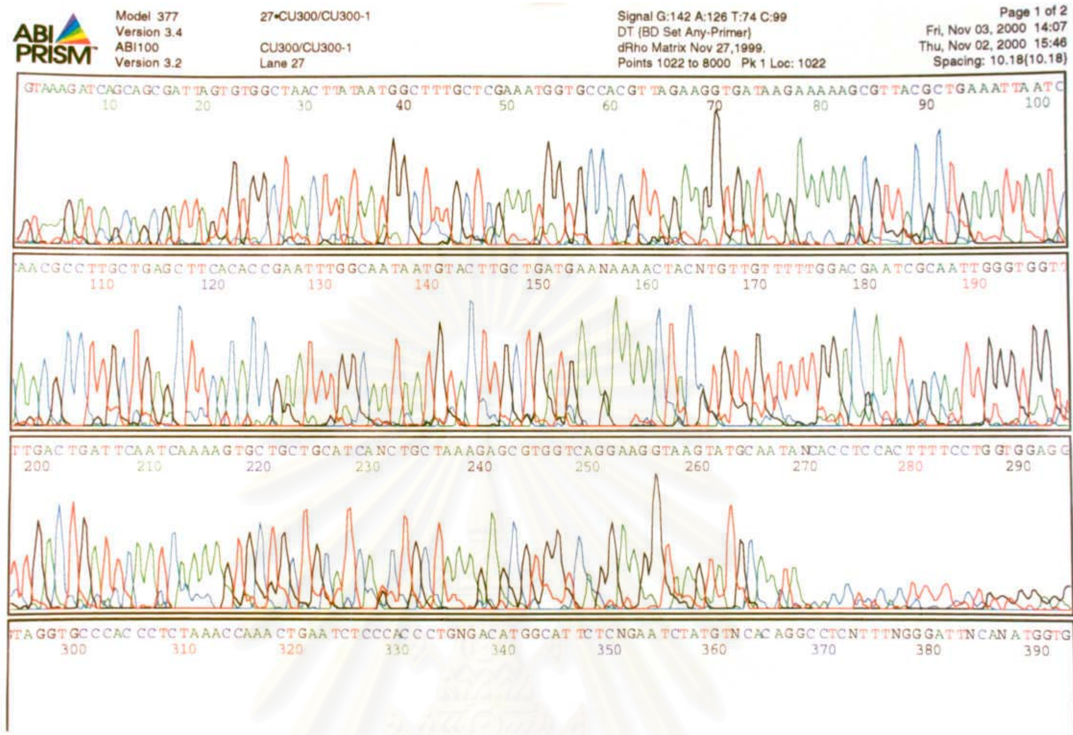

The 300 bp PCR product

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BIOGRAPHY

Miss Tithima Nuanboon was born on September 26, 1973 in Petchaboon. She graduated with the degree of Bachelor of Science from the department of Medical Technology at Mahidol University in 1995. In 1997, she has studies in Master degree of Science at the department of Biotechnology, Chulalongkorn University.

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