DETECTION OF GENETIC VARIATION IN POPULATIONS OF BLACK TIGER PRAWN *Penaeus monodon* BY DNA FINGERPRINTING

MISS SIRIPRORN PONGSOMBOON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY
GRADUATE SCHOOL
CHULALONGKORN UNIVERSITY
ACADEMIC YEAR 1996

ISBN 974-636-763-3

Ah 47

โครงการพัฒนาองค์ความรู้และศึกษานโยบายการจัดการทรัพยากรชีวภาพในประเทศ
c/o ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
อาคารสำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ
73/1 ถนนพระรามที่ 6 เขตราชเทวี
กรุงเทพฯ 10400

การตรวจหาความแปรผันทางพันธุกรรมในประชากรกุ้งกุลาดำ *Penaeus monodon* โดยการตรวจลายพิมพ์ดีเอ็นเอ

นางสาว สิริพร พงษ์สมบูรณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
หลักสูตรเทคโนโลยีทางชีวภาพ
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2539

ISBN 974-636-763-3

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science

Department of Biotechnology

Graduate School

Chulalongkorn University

Academic Year 1996

ISBN 974-636-763-3

Thesis Title	Detection of Genetic Variation in Populations of Black Tiger Prawn *Penaeus monodon* by DNA Fingerprinting
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พิมพ์ต้นฉบับบทคัดย่อวิทยานิพนธ์ภายในกรอบสีเขียวนี้เพียงแผ่นเดียว BRT 5 390248

สิริพร พงษ์สมบูรณ์ : การตรวจหาความแปรผันทางพันธุกรรมในประชากรกุ้งกุลาดำ *Penaeus monodon* โดยการตรวจลายพิมพ์ดีเอ็นเอ (DETECTION OF GENETIC VARIATION IN POPULATIONS OF BLACK TIGER PRAWN *Penaeus monodon* BY DNA FINGERPRINTING) อ. ที่ปรึกษา : รศ.ดร. อัญชลี ทัศนาขจร, อ. ที่ปรึกษาร่วม : ผศ.ดร. วิเชียร ริมพณิชยกิจ, 128 หน้า. ISBN 974-636-763-3

ในการตรวจสอบความแปรผันทางพันธุกรรม ของกลุ่มตัวอย่างกุ้งกุลาดำในแหล่งน้ำธรรมชาติ ด้วยเทคนิค RAPD-PCR ได้ทำการตรวจสอบระหว่างกลุ่มตัวอย่างจากทะเลอันดามันและอ่าวไทย กลุ่มตัวอย่างจากทะเลอันดามัน ทำการเก็บจากจังหวัด สตูล, ตรัง และ เมดาน (ประเทศอินโดนีเซีย) กลุ่มตัวอย่างจากอ่าวไทยทำการเก็บจากจังหวัดชลบุรี (ต. อ่างศิลา) และ ตราด

จากการคัดเลือกไพรเมอร์ซึ่งมีขนาด 10 นิวคลีโอไทด์ จำนวน 300 ไพรเมอร์ ได้คัดเลือกไพรเมอร์ 7 ตัว นำไปใช้ในการตรวจสอบความแปรผันของดีเอ็นเอ จากตัวอย่างกุ้งกุลาดำทั้งสิ้น 75 ตัว แบ่งเป็นกุ้งจากสตูล-ตรัง 17 ตัว, ตราด 28 ตัว, อ่างศิลา 15 ตัว และ เมดาน 15 ตัว พบว่ามีจำนวนแถบดีเอ็นเอที่เกิดจากไพรเมอร์ ทั้ง 7 ตัว รวม 80 แถบ ซึ่งมีขนาดอยู่ในช่วง 200-2000 คู่เบส เมื่อคำนวณเปอร์เซ็นต์ความหลากหลายของแถบดีเอ็นเอ พบว่าเป็น 57.7, 52.2, 45.6 และ 53.4% ตามลำดับ นอกจากนี้ได้ทำการวิเคราะห์ข้อมูลโดยการคำนวณค่าทางสถิติ ได้แก่ similarity index ภายในและระหว่างกลุ่มประชากร, genetic distance และ การทดสอบไคสแคว์ นอกจากนี้นำค่า genetic distance มาสร้าง dendrogram จากค่า similarity index ภายในกลุ่มประชากร และ เปอร์เซ็นต์ความหลากหลายของแถบดีเอ็นเอ แสดงให้เห็นว่า จากกลุ่มตัวอย่างทั้ง 4 กลุ่ม กลุ่มตัวอย่างจากอ่างศิลา มีความแปรผันทางพันธุกรรมน้อยสุด ค่า similarity index ระหว่างกลุ่มประชากร และ dendrogram แสดงให้เห็นว่า ตัวอย่างกุ้งกุลาดำจากเมดาน มีลักษณะทางพันธุกรรมแตกต่างจากตัวอย่างกุ้งจากประเทศไทย ทั้ง 3 กลุ่ม นอกจากนี้ยังพบว่า ไพรเมอร์ 428 ให้แถบดีเอ็นเอที่มีขนาดประมาณ 950 คู่เบส ที่พบเฉพาะในกุ้งที่มาจากทะเลอันดามัน คือ จาก สตูล-ตรัง และเมดาน ซึ่งแถบดีเอ็นเอนี้น่าจะนำมาใช้เป็น marker ในการจำแนกกลุ่มประชากรจากฝั่งทะเลอันดามันและอ่าวไทยได้

ลายพิมพ์ดีเอ็นเอจากตัวอย่างทั้ง 4 กลุ่ม มีทั้งสิ้น 214 แบบ ซึ่งพบลายพิมพ์ดีเอ็นเอที่มีความจำเพาะกับกลุ่มตัวอย่าง มีจำนวน 160 แบบ มีความจำเพาะกับแหล่งน้ำ จำนวน 10 แบบ จากลายพิมพ์ดีเอ็นเอที่มีความจำเพาะกับกลุ่มตัวอย่าง พบว่า 97 แบบ ปรากฏในตัวอย่างกุ้งเพียง 1 ตัว เท่านั้น เมื่อนำลายพิมพ์ ดีเอ็นเอ มาวิเคราะห์ความแตกต่างโดยใช้ไคสแคว์ พบว่าเมื่อทำการเปรียบเทียบระหว่างกลุ่มตัวอย่างจาก เมดานกับกลุ่มตัวอย่าง 3 กลุ่มของประเทศไทย ลายพิมพ์ดีเอ็นเอที่เพิ่มขยายด้วยทุกๆไพรเมอร์ มีความแตกต่างกันอย่างมีนัยสำคัญ ($P<0.0001$) เมื่อทำการเปรียบเทียบระหว่างกลุ่มตัวอย่างของประเทศไทยด้วยกัน พบว่าลายพิมพ์ดีเอ็นเอที่เพิ่มขยายด้วยไพรเมอร์ 101, 268, 428, 457, และ 459 มีความแตกต่างระหว่างกลุ่มตัวอย่างจากทะเลอันดามันกับอ่าวไทย ($P=0.0049$, $P<0.0001$, $P<0.0001$, $P=0.0014$, และ $p=0.0156$) เมื่อนำลายพิมพ์ดีเอ็นเอมาวิเคราะห์ความแตกต่างโดยใช้ไคสแคว์ สามารถแยกกลุ่มตัวอย่างจากสตูล-ตรัง ออกจาก ตราด และ อ่างศิลาได้ แสดงถึงกลุ่มตัวอย่างในประเทศไทยมีความแตกต่างกัน

ภาควิชา -

สาขาวิชา หลักสูตรเทคโนโลยีทางชีวภาพ

ปีการศึกษา 2539

ลายมือชื่อนิสิต สิริพร พงษ์สมบูรณ์

ลายมือชื่ออาจารย์ที่ปรึกษา .....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

C 727043 : MAJOR BIOTECHNOLOGY
KEY WORD: *Penaeus monodon* / RAPD / GENETIC VARIATION

SIRIPORN PONGSOMBOON : DETECTION OF GENETIC VARIATION IN POPULATIONS OF BLACK TIGER PRAWN *Penaeus monodon* BY DNA FINGERPRINTING. THESIS ADVISOR : ASSOC. PROF. ANCHALEE TASSANAKAJON, Ph.D. THESIS CO-ADVISOR : ASST. PROF. VICHIEN RIMPHANITCHAYAKIT, Ph.D. 128 pp. ISBN 974-636-763-3.

Randomly amplified polymorphic DNA analysis (RAPD) was used to examine genetic variation of four geographically separated wild populations of *Penaeus monodon* from two regions: the Andaman Sea and the Gulf of Thailand. Samples from the Andaman Sea were collected from Satun-Trang and Medan (Indonesia) and samples from the Gulf of Thailand were collected from Chonburi (Angsila district) and Trad.

A total of 75 individuals were tested, which included 17, 28, 15 and 15 from Satun-Trang, Trad, Angsila and Medan respectively. A screen of 300 random decaoligonucleotide primers identified 7 primers, which were selected for the analysis of black tiger prawn DNA. A total of 80 reproducible RAPD fragments ranging in size from 200-2000 bp were scored and 62 fragments (77.5%) were polymorphic. The percentages of polymorphic bands were 57.7, 52.2, 45.6 and 53.4% for samples from Satun-Trang, Trad, Angsila and Medan respectively. The index of similarity within and between populations and genetic distances were performed based on the RAPD data. The values of genetic distance were used to construct dendrograms using arithmetic mean (UPGMA). The results of similarity index within population illustrated that the Angsila sample was the most similar among themselves. The results of similarity index between populations and UPGMA dendrograms showed that the Medan sample was significantly different from the 3 samples of Thai *P. monodon*. The results show that primer 428 detected a RAPD marker that was found only in samples from the Andaman Sea; Satun-Trang and Medan suggesting the use of this marker as a region-specific marker.

RAPD patterns among the 4 samples gave 214 genotypes. One hundred and sixty of these were population-specific genotypes and 10 were region-specific genotypes. Ninety-seven of these genotypes were represented by a single individual. In chi-square analysis of the genotypes show that Thai and Indonesian *P.monodon* were significant different for all primers ($P<0.0001$) and Thai *P.monodon* from the Andaman Sea and the Gulf of Thailand were also different for primers 101, 268, 428, 457 and 459 ($P = 0.0049$, <0.0001, <0.0001, 0.0014 and 0.0156 respectively).

ภาควิชา – ลายมือชื่อนิสิต สิริพร พงษ์สมบูรณ์

สาขาวิชา หลักสูตรเทคโนโลยีทางชีวภาพ ลายมือชื่ออาจารย์ที่ปรึกษา

ปีการศึกษา 2539 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

Acknowledgement

I would like to express my deepest gratitude to my advisor, Associate Professor Dr. Anchalee Tassanakajon for her guidance, supervision, encouragement and supports throughout my study.

I am very grateful to Assistance Professor Dr. Vichien Rimphanitchayakit for his great helps, guidances, and suggestions in laboratory techniques. The special thanks are also extended to Dr. Sirawut Klinbunga for his help in data computerized analysis. Thanks are also expressed to all my friends of the Biochemistry and Biotechnology department especially in R707 and R708 for their helps in the laboratory and friendships that help me enjoy and happy through out my study. Collaboration and kindness that help me enjoy and happy throughout my study. And to Miss Somjai Wongtripop for her kind assistance in collecting shrimp samples. I wish to acknowledge to contributions of the Thailand Research Fund. for financial support for all laboratory equipments and chemicals, and the Programme for Biodiversity Research and training in Thailand for my finance.

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

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bp	=	base pair
°C	=	degree Celcius
dATP	=	deoxyadenosine triphosphate
dCTP	=	deoxycytosine triphosphate
dGTP	=	deoxyguanosine triphosphate
dTTP	=	deoxythymidine triphosphate
DNA	=	deoxyribonucleic acid
EtBr	=	ethidium bromide
Fig	=	figure
$\bar{H}$	=	mean heterozygosities
M	=	molar
ml	=	millilitre
mM	=	millimolar
min	=	minute
MT	=	metric ton
$MgCl_2$	=	magnesium chloride
mg	=	milligram
nm	=	nanometre
ng	=	nanogram

O.D.	=	optical density
PCR	=	polymerase chain reaction
RAPD	=	randomly amplified polymorphic DNA
RFLP	=	restriction fragment length polymorphism
sec	=	second
µg	=	microgram
µl	=	microlitre
µM	=	micromolar

Chapter I

Introduction

The term "shrimp" is commonly used by FAO to refer to species in the marine Penaeidae, while prawn is more commonly used to call freshwater Palaemonidae. Shrimps are the most valuable fisheries, particularly in terms of value of foreign exchange earning. This is because of a strong market demand, with the highest prices coming in the international markets. United State of America and Japan are the two major consuming markets that have caused a rapid growth of this industry. In 1982 about 4.84 % of the total world harvest shrimps was from culture whereas the other 95.16% was from normal fisheries. In 1993, cultured shrimp production was increased to 22.50% of the total world shrimp production (Table 1.1) (Shrimp culture newsletter group, 1995a). With all of the tropical shrimp fisheries are overexploited, any increases in supply to the market place will be from increased of the cultured production. There are at least 60 species of commercially important penaeid shrimps. In 1994 the world cultured shrimp production accounted for 61% of *P. monodon,* 15% of *P.vannamei* and 24% of the others. In Thailand, cultured shrimp production (mainly, *P.monodon*) accounted for 1.34% of total production in 1983 and dramatically increased to 67% of the total production in 1991. The intensive

farming system has been widely used for *P.monodon* farming actively resulting in the consistent increase in the outcome production. Among all members of Penaeidae, *P.monodon* is the most economically cultured species in the Southeast Asia (Anderson 1993; Shrimp culture newsletter group, 1995a).

The total world cultured shrimp production had increased rapidly from 298,573 MT in 1986 to 762,000 MT in 1995. Thailand is the leader in cultured shrimp for five consecutive years, followed by Ecuador, Indonesia and India. (Table 1.2) (Anderson, 1993; Asian shrimp culture council, 1995). Thailand exports a huge quantity of cultured shrimps which provide a substantial income to the country. (Table 1.3) (Shrimp culture newsletter group, 1995b). For example in 1993, approximately 50% of the value of marine animals exported came from cultured shrimp production. As a result, cultured shrimp production has economically important value of Thai marine animal production and the most economically important cultured species is the black tiger prawn (*Penaeus monodon*, Fabricius) (Shrimp culture newsletter group, 1994).

Table 1.1 World total shrimp production: 1982-1993.

Production (MT)			
Year	Cultured shrimp production	Captured shrimp production	Total production
1982	84,000	1,652,000	1,736,000
1983	143,000	1,683,000	1,826,000
1984	174,000	1,733,000	1,907,000
1985	213,000	1,908,000	2,121,000
1986	309,000	1,909,000	2,218,000
1987	551,000	1,733,000	2,284,000
1988	604,000	1,914,000	2,518,000
1989	611,000	1,832,000	2,443,000
1990	633,000	1,968,000	2,601,000
1991	690,000	2,118,000	2,808,000
1992	721,000	2,191,000	2,912,000
1993	610,000	2,100,000	2,710,000

Source : Shrimp culture newsletter 1995.

Table 1.2 World cultured shrimp production: 1993-1995.

Country	Head-on Production (MT)			Variance' 94/95	
	1993	1994	1995	MT	%
Thailand	209,000	250,000*	270,000*	+20,000	+8
Ecuador	76,000	100,000	100,000	-	-
Indonesia	100,000	100,000	80,000	-20,000	-20
China	30,000	35,000	70,000	+35,000	+100
India	55,000	70,000	60,000	-10,000	-14
Vietnam	40,000	50,000	50,000	-	-
Bangladesh	30,000	35,000	30,000	-5,000	-14
Taiwan	20,000	25,000	30,000	+5,000	+20
Philippines	25,000	30,000	20,000	-10,000	-33
Mexico	9,000	12,000	12,000	-	-
other	45,000	51,000	40,000	-11,000	-17
Total	639,000	758,000	762,000	+4,000	+1

Source: Asian shrimp news, 1995.

Table 1.3 Exports of Thailand shrimp: 1986-1995.

Year	Quantity		Value	
	MT	Difference	Billion baths	Difference
1986	28,063	-	4,391	-
1987	33,909	+21	5,749	+31
1988	49,829	+47	9,701	+69
1989	74,294	+49	16,057	+66
1990	84,724	+14	20,454	+27
1991	121,240	+43	26,681	+30
1992	140,432	+16	31,696	+19
1993	148,889	+6	37,843	+19
1994	190,650	+28	48,109	+27
1995	202,000	-	50,600	-

Source: Shrimp culture newsletter, 1995.

Taxonomy

The taxonomic definition of black tiger prawn is as follow:

Phylum Arthropoda

Class Crustacea

Subclass Malacostraca

Order Decapoda

Suborder Natantia

Infraorder Penaeid

Family Penaeidae Rafinesque,1815

Genus *Penaeus* Fabricius, 1798

Subgenus *Penaeus*

Species *monodon*

Scientific name: *Penaeus (Penaeus) monodon* Fabricius, 1798.

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

FAO name : giant tiger shrimp (or prawn)

Morphology

Externally the shrimp can be basically divided into the thorax and abdomen (Fig.1.1). The thorax (or head) is covered by a single, immobile carapace which protects the internal organs and supports muscle origin. The rostrum, extending beyond the tip of the antennular peduncle, is sigmoidal in

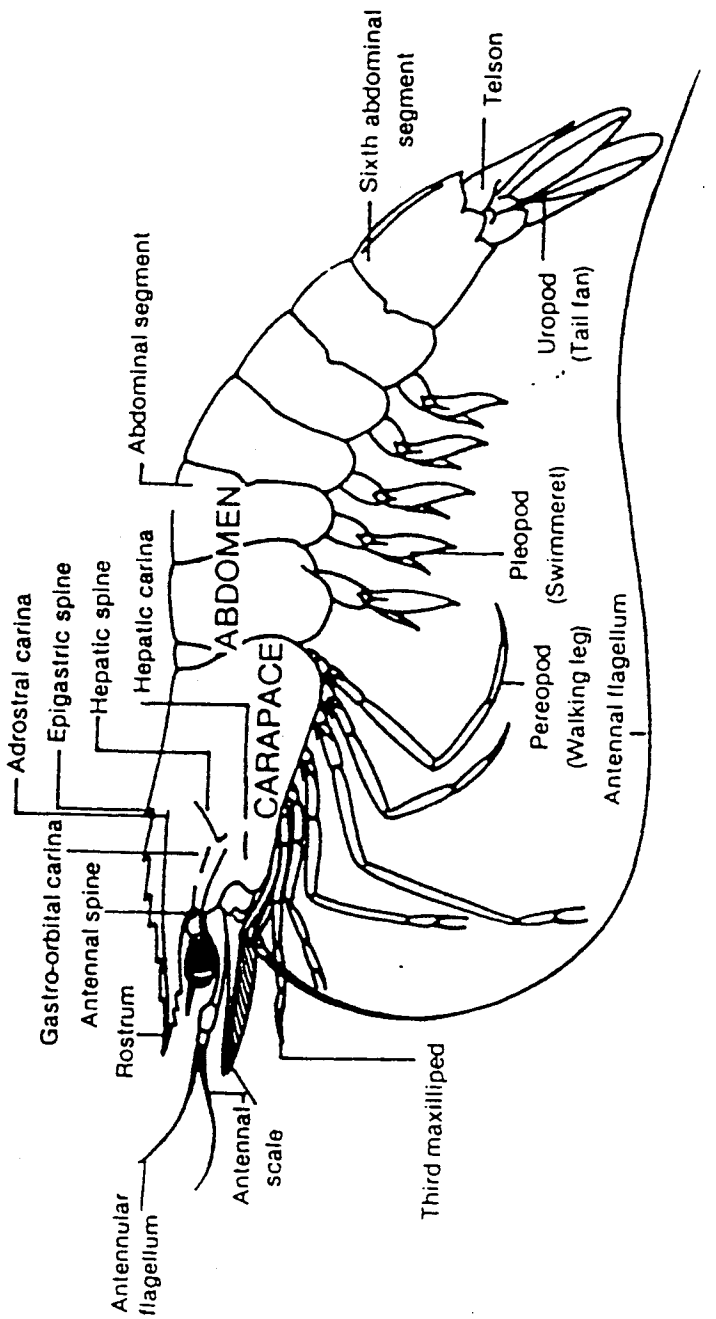


Figure 1.1 Lateral view of *P. monodon* showing important parts.

shape, and has 6-8 dorsal and 2-4 ventral teeth, mostly 7 and 3, respectively. The carapace is carinate with the adrostral carina , almost reaching to or not as far as the epigastric or first tooth. The gastro-orbital carina occupies the posterior one-third to one half distance between the post-orbital marine of the carapace and the hepatic spine. The hepatic carina is prominent and slightly curved, extending behind the antennal spine. The antennular flagellum is sub-equal or slightly longer than the peduncle. The walking legs or pereiopods are the thoracic appendages. Exopods are present on the 1^{st} and 4^{th} but absent on the 5^{th} pereiopods. Gills are formed from sac-like out growths of the base of the walking legs and sit in branchial chambers on either side of the thorax. The carapace extends laterally to cover the gills completely. The abdomen has the obvious segmentation of invertebrates. A pair of swimming legs or pleopods arise appendages of each of the six abdominal segments. A tail fan comprise of a telson, which bears the anus and two uropods, attached to the last (6^{th}) abdominal segment. The telson has deep median groove, without dorso-lateral spines. A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of shrimp (Anderson,1993).

Distribution

P.monodon is the largest penaeid shrimp. It can tolerate a wide range of fluctuating environments (e.g., water temperature, salinity) in tropical areas and performing well in ponds at high density. Most of the adults inhabit deeper

water down to 162 m. The fry, juvenile and adolescent stages occupy shore areas and mangrove estuaries (Panya Suwansamut, 1991; Anderson, 1993). *P.monodon* is naturally distributed in the Indo-West Pacific region; South Africa, Tanzania, Kenya, Somalia, Madagascar, Saudi Arabia, Oman, Pakistan, India, Bangladesh, Sri Lanka, Indonesia, Thailand, Malaysia, Singapore, Philippines, Hong kong, Taiwan, Korea, Japan, Australia and Papua New Guinea (Motoh, 1985). In the Gulf of Thailand, this species is widely distributed. The abundance of it is found at the depth of 26-30 m., closed to the coastal areas, the mouths of the rivers and the islands (Atchara Vibhasiri, 1989).

The farming areas of penaeid are found throughout the world (Table 1.4). However, South-East Asia is the center of the most farming areas of *P.monodon* in the world (Anderson,1993).

Life cycle

During the spawning season, adult shrimps undertake spawning migration to various depths on the continental where copulation occurs. The number of spawned eggs depend on the size of spawners. The fecundity of spawners at 70-150 g. in size is approximately 1,000,000 -1,200,000 eggs. In *P.monodon* the thelycum modified for internal storage of sperm ("close thelycum"), mating occurs just after the female moult. At this time the male inserts the spermatophore (encapsulated spermatozoa) through the soft cuticle of

Table 1.4 Some of the prawns and shrimps farmed throughout the world.

Scientific name*	Common name	Natural distribution	Cultured in
P.monodon	Black (black) tiger prawn	Indo-West Pacific	Taiwan, SE Asia+, China, India, Vietnam
P.chinensis (*P. orientalis*)	Chinese white shrimp or Oriental shrimp	West Pacific	China, Korea
P. vannamei	Western white shrimp	Eastern Pacific	Ecuador, Central and South America
P.merguiensis	Banana prawn	Indo-West Pacific	SE-Asia, India, China
P.indicus	Indian white prawn	Indo-West Pacific	SE-Asia, India
M.ensis	Greasy back shrimp	Indo-West Pacific	Taiwan
P. stylirostris	Blue shrimp	Eastern Pacific	Central and South America
P. japonicus	Kuruma prawn	Indo-West Pacific	Japan, Korea, Taiwan, Italy
P. aztecus	Northern brown shrimp	Western Atlantic	USA
P. duorarum	Northern pink shrimp	Western Atlantic	USA
P.setiferus	White shrimp	Western Atlantic	USA
P. penicillatus	Red-tailed prawn	Indo-West Pacific	China, Taiwan
P.semisulcatus	Green tiger prawn	Indo-West Pacific and Eastern Mediterranean	Taiwan
P.esculentus	Brown tiger prawn	Indo-West Pacific	Australia
P. schmitti	Southern White shrimp	Western Atlantic	Central and South America
P. californienis	Yellow-leg shrimp	Eastern Pacific	USA
M.dobsoni	Kadal shrimp	Indo-West Pacific	India

*P = *Penaeus*, M = *Metapenaeus*.

+SE Asia = Indonesia, Philippines, Thailand, Brunei, Singapore and Malaysia

the thelycum, a special seminal receptacle for the storage of spermatozoa. During eggs are extruded from the paired genital pore located at the base of 3rd pereiopods. Meanwhile , spermatozoa are also extruded from the thelycum for fertilization. The time required for each spawning is approximately 3-5 min. Fertilized eggs gradually sink to the bottom. Hatching of the eggs, occurs about 12 hours later. The larval stages consist of three to six nauplius, three protozoea and three mysis substages. This larval development period is between 10-14 days depended on temperature and feeding level. Mysis III larvae metamorphose into post-larvae (PL) which have all the appendages and organs as in adult shrimps.

Postlarvae continue moulting as they grow. They migrate shoreward and settle in nursery areas close to shore or in estuaries, where they grow quickly to juvenile and subsequently to sub-adult, they highly tolerate variable physico-chemical factor in their living environment. Sub-adults migrate back to the sea where they are finally mature and able to mate and spawn. The life span of penaeid spawns are rarely longer than two years (Panya Suwansamut, 1991; Anderson, 1993).

Why employ molecular genetic markers in *Penaeus monodon*?

Black tiger prawn, *Penaeus monodon* is an economically important species. The culture of this species has rapidly developed in the last decade.

However, the culture of this species depends on wild caught spawners that can finally result in a serious bottleneck effect. At present this exploitation has resulted in increasing pressure on wild populations. As the industry grow, some of the most heavily harvest stocks are showing signs of over-exploitation. If, however, broodstock availability declines, the cost of gravid females will be increased imposing and increasing burden on the financial viability of hatchery operations. Some efforts have been made to enhance wild populations but genetic effects of the release of large numbers of hatchery reared seed with unknown origin may harmful to local population. Reliance on wild seed and/or broodstock can limit availability of postlarvae either seasonally or geographically in area without abundant indigenous stocks. Demand for seed has also encouraged transfer of live shrimps between geographically distinct areas resulting in the spreading of certain pathogens. Distribution of seed stock heavily infected with significant pathogens can have important negative effects for growers. For all these reasons, the breeding program is required to manage this species. The used of captive broodstocks will offer many advantages including disease control, maintenance of genetic diversity and selection to improve commercially important traits (Browdy, 1996).

Basic knowledge on population genetics is essential for improving a selective breeding program. These data can provide information on rare alleles, decrease heterozygosities, an increased level of inbreeding within a breeding

program. Population genetic analysis provided genetic markers which may be utilized for making hatchery broodstock in order to maintain line purity, and for constructing sophisticated breeding efforts. Using molecular data, scientists can developed breeding programs aimed at specific needs (Garcia et al., 1994.).

Molecular markers for population genetic analysis

Traditional methods such as comparative anatomy, morphology and physiology had been used to evaluated genetic variability in the past but these methods are not adequate for studying intraspecific genetic variation of several species. During the past decade, traditional methods have been increasingly complemented by molecular techniques, the development of so-called "molecular markers" which are based on polymorphisms found in protein or DNA molecules.

Morphological characters have long been used for identification and classification of organisms at different levels (genera, families and species). Levels of variability can be estimated from morphological characters and their responses to selection and their genetic background can be determined. Morphological characters are, often, influenced by the environment. Molecular methods, on the other hand, are not effected by the environment and, provide almost unlimited numbers of potential markers. Theoretically, the general properties of the desirable molecular marker are (1) highly polymorphic

behavior (2) codominant inheritance (allows to discriminate homo- and heterozygotic states in diploid organisms) (3) frequent occurrence in the genome (4) everly distribution throughout the genome (5) selectively neutral behavior (i.e., no pleiotropic effects) (6) easy access (7) easy and fast assay (e.g. by proceduces amenable to automation and highly reproducible). The data from the analysis of these markers are acceptable among laboratories.

No molecular markers that fulfill all of these criteria are available yet. However, according to the systems of study, a suitable molecular marker can already be chosen from a variety of marker systems, each of which combines at least some of the above-mentioned properties (Weising et al., 1995).

A. Protein markers

For a long period of time, the first molecular approach employed widely in population genetic studies was protein electrophoresis of various enzymes (allozyme and isozyme systems). Generally, only allozyme data is used in either systematic study or phylogenetic analysis. Allozyme methods were introduced in the mid-1960s had been dominated in molecular systematics until the late 1980s where other DNA approaches have increasingly used instead. The use of allozyme and other DNA techniques opened a new approach to understand population and the evolution of organisms.

The protein markers are based on protein polymorphisms. This method

involves the separation of native proteins under electric field. The proteins are identified by histochemical staining to reveal the enzymes. The majority of protein markers are represent by allozymes.

Allozyme electrophoresis has been successfully applied to many organisms from bacteria to numerous animal and plant species. The studies have encompassed various fields (e.g., physiology, biochemistry, genetic breeding) and purposes (e.g., population structure analysis, mating system planning) (Avise, 1994).

Allozyme analysis has been used as the standard molecular technique in population genetic research. This technique has been enormously successful for three primary reasons. First, it is inexpensive particularly in comparison to other molecular genetic methods. Second the method allows for quick processing time. A laboratory can assay hundreds of samples per day for many different loci and this translates to low labor costs as well. Finally, allozyme markers are codominant therefore both alleles in a diploid organism are usually clearly identifiable. Accordingly heterozygotes can be discriminated from homozygotes, which is a prerequisite for estimation of allele frequencies in population genetic studies.

However, the resolution of protein electrophoresis is not always adequate for detecting differences between populations or individuals.

(Carvoalho and Pitcher, 1995). There are certain limitations to the allozyme studies. A new allele will only be detected if nucleotide substitution resultes in an amino acid substitution that affects the electrophoretic mobility of the protein. Since sixteen out of 20 common amino acids are electrostatically neutral, mutations do not usually alter the total charge of the protein. Only 30% of all nucleotide substitutions result in polymorphic fragment patterns. Therefore, allozyme analysis underestimates the genetic variability (Selander and Whittam, 1983 ; Weising et al., 1995).

B. DNA markers

Although traditional allozyme analysis serves as the basis for many studies, the limitation of polymorphic markers in the investigated populations have frequently limited the power of analysis. The DNA of techniques have been developed. They uncover a great number of DNA markers. Because each individual DNA sequence is unique, this sequence information can be exploited for any studies as genetic markers. (Hallerman and Beckman, 1988).

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 developmental influences on the phenotype are not a concern. Second, since different regions of DNA evolve at different rates, appropriate regions may be chosen for a given study. Third, DNA markers are not restricted to coding regions. Therefore, an almost

unlimited number of detectable polymorphisms exist. 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).

1. DNA-level variation

Variation at the DNA level can be generalized into two categories, namely, base substitution and insertion/deletion. The simplest form of variation is a single nucleotide substitution or point mutation. Insertion or deletion of one or more nucleotides can occur within a DNA sequence. The actual insertion/deletion can be single nucleotide or hundreds to thousands of nucleotides long. A common type of insertion/deletion mutation is copy number variation. The variation results from a difference in the number of copies of a basic unit, or core sequence. The sequence on either side of the variant region is the same in different individuals. Another type of insertion variation is found in some multi-copy elements, such as Alu sequence. Some of these elements display a high degree of polymorphism at specific insertion sites.

The proportion of DNA sites that are polymorphic will depends on certain species-specific population parameters, population size and mutation rate in particular. In general, the level of polymorphism is expected to be inversely correlated with the functional constraints of the DNA. Coding regions of the genes, for example, tend to have little polymorphism compared to introns

and noncoding regions. By this account of variation, it is widely thought that the highest level of polymorphism will be found in regions of DNA having no function. The most likely place to find polymorphism, therefore, is in noncoding regions of DNA. Introns, for example, are generally relatively polymorphic (Kreitman, 1990; Carvalho and Pitcher, 1995).

2. DNA techniques used in population genetic

2.1 Restriction fragment length polymorphisms

Restriction fragment length polymorphisms (RFLPs) are based on differences in fragment lengths obtained by digesting the DNA samples with restriction endonucleases (Yu, Deynze and Pauls, 1993). Digestion of a particular DNA fragment with enzymes result in a reproducible set of fragments of well defined lengths. Point mutations as well as insertions or deletions within the restriction enzyme recognition sequences result in an altered pattern of restriction fragments (Weising et al., 1995).

In eukaryote, the genome is so large that digestion with a typical restriction enzyme produces hundreds of thousands of fragments. The DNA appears as a continuous smear on the gel upon EtBr staining. It is therefore necessary to visualize only particular fragments. The specific fragments are made visible by hybridization with specific labeled probes (Hallerman and Beckman, 1988; Avise, 1994; Carvolho and Pitcher, 1995). RFLPs have been used extensively to develop genomic maps, establish linkages to traits and

develop phylogenetic trees. (Yu et al., 1993).

2.2 Hybridization-based fingerprinting

In 1985 Jeffreys et al. introduced multilocus DNA fingerprinting techniques which reveals enormous variability in a wide range of organisms. Multilocus DNA fingerprinting detects genetic variation at hypervariable regions (HVR). The HVR consists of tandem repeats of short sequence. Hypervariability at these minisatellites or microsatellites result from change in the number of repeats, presumably driven either by unequal recombination or by slippage at replication forks leading to the gain or loss of repeat units. The resulting length variability can be high, and the frequency of heterozygotes can sometimes approach 100% (Jeffreys, 1987; Jeffreys and Pena, 1993). The technique of classical DNA fingerprinting (hybridization-based fingerprinting) is methodologically derived from RFLP analysis.

Two categories of multilocus probes are mainly used. The first category comprises of cloned DNA fragment called " minisattellite". Its tandem repeat is about 10 to 60 bp long. The second category is examplified by "simple sequences" or "microsatellites" whose tandem repeats is 1 to 5 bp long (Ali, Muller, and Epplen, 1986; Weising et al., 1995).

The resulting DNA fingerprints allow the identification of individuals in populations. Thus frequently permit assignments of paternity and thereby

promote analysis systems on a fine scale. They provide a potential source of markers for population studies where genetic variation is extremely limited. They should be especially valuable in systems where other markers are lacking (Turner, Elder and Laughlin, 1991). RFLPs and hybridization-based fingerprinting markers have advantages that they are phenotypically neutral, codominantly inherited, non specific to growth stages. However, RFLPs and hybridization-based fingerprinting require fairly large amounts of DNA and are time-consuming and expensive.

2.3 PCR based fingerprinting, randomly amplified polymorphic DNA analysis

Randomly amplified polymorphic DNA analysis (RAPD) has been as a method of detecting polymorphisms since 1990 (Williams et al., 1990). This technique is based on the polymerase chain reaction (PCR). With standard PCR, it is first necessary to determine the sequence of the DNA to be analyzed. Then two specific primers complementary to sequences flanking the target segment are synthesized and used to prime the DNA amplification reaction. The RAPD method, however uses a single arbitrary primer and lower annealing temperature than the average PCR reaction. The arbitrary primers used for the procedure are usually at 10 bp in size. They have GC content of 50 to 80% and do not contain palindromic sequences. The number and size of fragment(s) that are amplified are dependent on the primer and the genomic

DNA used. For a shorter primer, the probability that two priming sites occur in the genome close to each other in an inverted orientation increases. A subset of elongation products in the first cycle can serve as templates for the following cycles because it may have primer annealing sites within the templates suitable for amplification. Priming sites are randomly distributed throughout the genome. The various of DNA fragments of different lengths amplified in this fashion are inherited as classical Mendelian traits and, thus, can be used for genetic analysis (Paran, Kessili, and Michelmore, 1991; Yu, et al., 1993). A single nucleotide substitution in a primer can result in a complete change in the RAPD pattern, this is an indication of the sensitivity of the system. The RAPD products are detected as DNA fragment length polymorphism in multiple loci by the presence or absence of bands at various positions in agarose gel after electrophoresis (Mullis, Ferre, and Gibbs, 1994). DNA polymorphism revealed by this method results from either chromosome rearrangements within the amplified sequence, deletion of priming sites, insertion or deletion of the sequence between of the priming sites and base substitution in priming sites (Michelmore, Paran, and Kessili, 1991; Paran et al., 1991). Insertion of a large piece of DNA between the two annealing sites may render the original fragment too large to be amplified, resulting in its absence in the gel. The deletion of a DNA fragment carrying one of the two primer annealing sites also results in loss of a fragment. A nucleotide substitution may affect the annealing of one of the two primers at a given site. Insertion or deletion of a small pieces of DNA can

lead to change in size of amplified fragment.

The amplification products can be separated by agarose gelelectropheresis or nondenaturing polyacrylamide gel electrophoresis. The choice of system depends on the size of the DNA fragments to be separated. Agarose gel electrophoresis is relatively simple to perform and separates DNA fragments from 0.5 to 25 kb in size. Polyacrylamide gel electrophoresis is the method of choice to separate DNA fragments less than 1 kb. The DNA in the gel can be visualized by staining with ethidium bromide or by silver staining which gives better resolution, especially of minimal length-difference fragments in polyacrylamide gel electrophoresis (Yu et al., 1993).

The advantages of using RAPD markers are the followings. First, RAPD analysis is simpler, faster, and demands less technical expertise. Second, RAPD-PCR requires small amount of DNA. Third, the same single short primers usually can be used for the analyses of widely different taxonomic group. Forth, prior sequence data of the target DNA is not necessary. Fifth, the availability of a large number of random primers makes RAPD-PCR a powerful tool for detecting numerous polymorphism, which are useful for the studies of population genetics. Finally, RAPD-PCR does not require probes, DNA libraries, and radioactive chemicals (Williams et al., 1993; Narang et al., 1994).

There are some disadvantages in using the RAPD-PCR approach for

population genetics, genetic mapping, and taxonomic studies. First, many fragments (especially those arising from mispairing of a primer with the genomic DNA) may not be reproducible among different laboratories because amplifications are sensitive to slightly change in temperature cycle.

Second, in contrast to RFLP (on autoradiographs), which are usually codominant, most of the polymorphisms detected by RAPD-PCR are inherited as dominant markers. Therefore, homozygotes and heterozygotes can not be differentiated. In RAPD-PCR, both genotypes will show one band because the size difference is more likely to appear as the present or absent of a band.

Third, RAPD bands of the same size may not actually have the same or similar sequence. Comigrating RAPD bands may not be allelic (Narang et al., 1994).

Genetic Studies in Crustaceans

Dating back to the earliest years of modern genetics, the crustacean genetic literature had never been summarized, except for more specific reviews such as those on sex determination, chromosomal evolution in copepods or the genetics of genera such as *Artemia* or *Daphnia*. In the 1940s, the synthetic or neo-Darwinian theory of evolution and the "new systematics" cast a spot light on polychromatisms as visible examples of polyallelic systems, though to be the medium of genetic change. Polychromatism is difference in colour pattern. The

first comprehensive genetic studies of visible polymorphisms in marine organisms began. There soon appeared treatments of polychromatisms of the copepod *Tisbe reticulata*, the flabelliferan isopod *Sphaeroma serratum*, the asellote isopod *Jaera albifrons* and a few decapods.

By the mid-1960s, electrophoretic techniques for protein separation followed by histochemical localization of specific enzymes allowed geneticists, for the first time, to survey natural populations for the amount of variation in a random sample of primary gene products. As investigators turned to biochemical techniques. The interest in externally visible polymorphisms of crustacean declined. The enzyme polymorphisms posses significant advantages, including codominant expression and much greater frequency of occurrence (only a very small percentage of crustacean species exhibit well-marked polychromatism).

Protein polymorphism data from 97 crustacean species had been surveyed for variation at 12 to 43 loci ($\bar{x}$ =23). 30.5% of the loci in the average population are polymorphic and 7.3% of the loci in an average individual are heterozygous ($\bar{H}$, Mean heterozygosities = 0.073). Nevo, et al.(1984) had been reported mean heterozygosities of 0.082 for crustaceans (Hedgecock et al., 1982).

Decapods are known to display relatively low level of enzyme

polymorphisms. Mean heterozygosities of 0.048 were reported for decapods. Geographic variation among conspecific decapod populations had been tested by allozyme variation. The grass shrimp, *Palaemonetes pugio* has a much more fragmented distribution than does the penaeid shrimp, being sometimes isolates in small ponds. These pond populations tend to have less allozyme variation compared with open bay populations, and Nei's statistics show significant genetic subdivision among samples from the Galveston, Texas, area (Fuller and Lester, 1980). Likewise pandalid shrimp, although, not subject to obvious gene flow barriers, exhibits some slight allozyme frequency variation, at least among wider spread localities (Hedgecock et al., 1982).

The identification of the genetic diversity in Penaeid Shrimp is through the examination of allozyme variability indicates relatively few allozyme polymorphisms. Low levels of genetic variation and little geographic differentiation within wild penaeid shrimp species have been reported (Garcia et al., 1994). Using three penaeid shrimps from the Gulf of Mexico, *Penaeus aztecus*, *P.setiferus* and *P. duorarum*, Lester (1979), could not demonstrate the significant differences among locality differentiation of allozyme frequencies using Nei's (1973) procedures for partitioning gene diversity. However, Benzie et al. (1992) found significant allozyme frequency differences among Australian populations of *P. monodon*. However mtDNA analysis indicated higher levels of variation among the Australian populations of *P.monodon*, suggesting that

DNA analysis would provide a better source of markers for penaeid prawns. RFLPs have been used to examine the degree of genetic diversity in the mitochondrial and nuclear DNA of *P.vannamei* (Garcia and Benzie, 1995). The large differences in mtDNA between species of *Penaeus* have been reported by direct mtDNA sequencing and RFLP analysis (Garcia et al., 1994). RAPD technique has also been developed for monitoring genetic diversity. The results from the RAPD technique are found to be consistent with those of other techniques for the identification of individuals at the population and subspecies levels and for the investigation of the genetic relatedness of population (Garcia et al., 1994).

By using RAPD technique, D'Amato and Corach (1996) reported a high level diversity detected in *Macrobrachium borellii* from Arroyo Pescodo stream and Canteras de Berisso, an artificial pond with a surface area of 4 ha. Polymorphic RAPD bands/individuals range from 33.33-50.00% in both populations. The average percentage difference (APD), as derived by Gilbert et al. (1990) and Yuhki and O'Brient (1990), was calculated for the two populations. The APD for Pescodo and Berisso (close population) are more similar than that of the Pescado (open population). The measure of genetic distance [Nei's genetic (D) = 0.001] indicates high genetic similarity among populations. Nevertheless, population-specific marker was found.

Garcia et al. (1994) had evaluated the genetic diversity of *P. vannamei*

by using 3 different techniques, namely, restriction fragment length polymorphisms (RFLPs), random amplification of polymorphic DNA (RAPD) and allozyme variation. The RAPD technique revealed a high level of genetic variation which polymorphic bands/individuals range from 39-77%. A population and family-specific marker was found in this technique. For RFLPs technique, variability was found in cytochrome oxidase subunit I (CO I) of mtDNA, digested with *Hha* I or *Cfo*I enzymes, among the populations and families. Allozyme variation was examined on thirty loci. Seven loci were polymorphic. The percentages of polymorphic loci range from 3.33-16.67%. The levels of heterozygosity were low and consistent with reports by other researchers. Garcia et al.(1994) concluded that the low levels of allozyme variability in the populations were not high enough for the establishment of a breeding program based on genetic variation. Therefore, mtDNA polymorphisms can be used together with nuclear DNA polymorphisms to establish a breeding program (Garcia et al., 1994).

Garcia and Benzie (1995) had also investigated the RAPD patterns in six families of *P. monodon*. The percentage of polymorphic bands was 6.2%. The ratios of segregation for each family were consistent with Mendelian inheritance. They suggested 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 (Garcia and Benzie, 1995).

In conclusion, although RAPD technique has some disadvantages, it is superior to other methods in uncovering genetic variability for distinguishing of difficult taxa. Therefore, this research described herein possibility of using the RAPD technique to evaluate genetic variability in wide populations of Thai *P.monodon.*

Objectives

1. To find a suitable system (optimal conditions and primers) for identification of DNA-markers in wild populations of *P.monodon* by PCR-based fingerprinting method.
2. To assess the genetic variation among wild populations of *P.monodon* from the Andaman Sea and the Gulf of Thailand.
3. To evaluate the evolutionary relationship among wide populations of *P.monodon* based on the RAPD data.

Chapter II

Materials and Methods

Materials and chemicals

-Absolute ethanol (Merck, Germany.)

-Agarose gel type 1-A Low (Sigma Chemical Co., U.S.A.)

-Ammonium acetate (Merck, Germany.)

-Boric acid (Merck, Germany.)

-Chloroform (Merck, Germany.)

-100mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medison, Wisconsin.)

-Ethylene diaminetetraacetic acid disodium salt dihydrate (Fluka Chemika-Bio Chemika, Switzerland.)

-Isoamyl alcohol (Merck, Germany.)

-25 mM $MgCl_2$ (Perkin-elmer Cetus, Norwalk, Connecticus.)

-10 X PCR buffer ; 10 mM Tris-HCl, pH 8.3, 50 mM KCl (Perkin-elmer Cetus, Norwalk, Connecticus.)

- Phenol crystal (Fluka Chemika-Bio Chemika, Switzerland.)

- Potassium acetate (Merck, Germany.)

- Proteinase-K (Gibco BRL life Technologies, Inc., U.S.A.)
- RNase (Sigma Chemical Co., U.S.A.)
- Sodium chloride (Merck, Germany.)
- Sodium dodecyl sulfate (Sigma Chemical Co., U.S.A.)
- Sucrose (Sigma Chemical Co., U.S.A.)
- Tris-(hydroxy methyl)-amino methane (Fluka Chemika-Bio Chemika, Switzerland.)

Enzyme

AmpliTaq DNA polymerase (Perkin-elmer Cetus, Norwalk, Connecticus.)

DNA primers

Oligonucleotide primers (University of British Columbia)

Primer sequences were shown in table 3.1.

Shrimp samples

The wild populations of *Penaeous monodon* were obtained from different location sites on the Andaman Sea and the Gulf of Thailand during March 1995 to August 1996. The shrimps of the Andaman Sea were collected from Satun, Trang provinces (Thailand) and Medan (Indonesia) whereas the shrimps of the Gulf of Thailand were collected from Chonburi (Angsila district)

Figure 2.1 Map of collection sites for *P.monodon* samples.

and Trad provinces. The pleopods were dissected out from the live shrimps, kept on ice or absolute ethanol during transportation and stored individually at -80°C.

DNA extraction

The shrimp DNA were prepared from the pleopods by using proteinase-K / phenol-chloroform extraction. This method has been shown to be effective removing protein from nucleic acid and become a standard method for DNA extraction (Kirby, 1992).

A pleopod of individual specimen was homogenized with a micropestel in a 1.5 ml microcentrifuge tube, on ice, containing 400 µl of extraction buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 200 mM sucrose and 50 mM Na_2EDTA, pH 8.0). The nucleated cell was lysed by incubating with 0.4 % sodium doducyl sulfate for 1 hour at 65°C. Subsequently protein and RNA were destroyed by incubating with proteinase-K (500 µg/ml) and RNase solution (100 µg/ml), respectively, at 65°C for 3 hours. Protein was precipitated from nucleic acid by adding 5 M potassium acetate to the final concentration of 1 M. The sample was chilled on ice for 30 min and centrifuged at 12,000 rpm for 10 min. The supernatant was added with an equal volume of redistilled phenol and mixed very gently. The sample was then spun in a microfuge for 5 min at 7,000 rpm, the upper aqueous phase was removed without disturbing the

organic/aqueous interface and added with and equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1, by volume) and gently mixed. After centrifugation, the lower organic phase was discarded, the upper aqueous phase was added with an equal volume of chloroform/isoamyl alcohol (24:1, by volume) and mixed gently. After centrifugation, the upper aqueous was removed, added with 1/10 volume of 3M sodium acetate, pH 5.5, mixed gently and added with two volume of prechilled -20°C absolute ethanol. The mixture was kept at -20°C overnight. The DNA pellet was collected by hooking out and washed twice with 70% ethanol, air-dried and dissolved in 300 μl of TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8.0). The DNA was stored over night at 4 °C for complete solubilization.

Spectrophotometric measuring of DNA concentration

DNA concentration is estimated by measuring the OD_{260}. An absorbance optical density (O.D.) of 1.0 corresponds to 50 μg 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 the O.D. at 260 and 280 nm. For a pure preparation of DNA, $OD_{260/280}$ should be ≥ 1.8 (Kirby, 1992).

RAPD analysis

RAPD-PCR conditions for amplification reactions were 35 cycles of 5 sec at 94°C (denaturation), 45 sec at 36°C (annealing) and 90 sec at 72°C (extension). 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 µM each dATP, dCTP, dGTP, dTTP, 0.2 µM primer, 50 ng genomic DNA and 0.2 unit of AmpliTaq DNA polymerase. Reactions were performed in a Perkin-Elmer Cycler model 2400.

Agarose gel electrophoresis

DNA was analyzed by convection sub-marine gel electrophoresis. Agarose was mixed with Tris-Borate-EDTA (TBE) buffer (89 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 amplification products and 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 a comb. After the gel hardened, the comb was carefully withdrawn and the seal was removed from the ends of the plateform. Sufficient 1XTBE buffer was added to cover the gel for approximately 0.5 cm. An appropriate 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 Lambda *Hind* III fragments were used as standard DNA markers. The electrophoresis was carried out in 1XTBE buffer from cathode to anode at 100 volts until the bromphenol blue marker dye migrated almost out of the gel. After finishing, the gel was stained in 2.5μg/ml ethidium bromide (EtBr) solution for 5 min and destained (to remove excessive EtBr) by submerged in an excessive amount of distilled water for 15 min. The nucleic acid bands were visualized as fluorescent bands under UV transilluminator and photographed through a red filter using Kodak Tri-X-Pan 400 film. The exposure time was usually about 10-15 seconds.

Statistical procedures

The photographs of RAPD patterns were evaluated directly by eye with the help of a transparent ruler. Only unambigous fragments that were 200 bp-2 Kbp and bands that could be accurately scored throughout all lanes were chosen to score. Standard DNA markers were used to assign the size of each RAPD fragment, and were markers for the accurate comparison of fragments among lanes, derived from the same or different gels. Each RAPD fragment was assigned a molecular length and recorded in a binary matrix for each individual as present (1) or absent (0) of a given band. The percentages of polymorphic bands and monomorphic bands were evaluated. The RAPD patterns of

individuals were compared within and between the 4 geographic samples.

1. Similarity index

The index of similarity between individuals was calculated using the formula: $S_{xy}=2n_{xy}/n_x+n_y$, where n_{xy} is the number of fragments shared by individuals x and y, and n_x and n_y are the number of fragments scored for each individual (Lynch, 1990). Within population similarity (S) is calculated as the average of S_{xy} across all possible comparisons between individuals within a population. Between population similarity, corrected for within population similarity, is: $S_{ij} = 1+S'_{ij}-0.5\ (S_i+S_j)$, where S_i and S_j are the values of S for population i and j, respectively, and S'_{ij} is the average similarity between randomly paired individuals from populations i and j (Lynch, 1990).

2. Genetic distance

S'_{ij} is also converted to a measure of genetic distance (D_{ij}) using the equation: $D_{ij} = -\ln[S'_{ij} /\sqrt{(S_iS_j)}]$, (Lynch, 1991).

3. Dendrograms

The distance values were subjected to cluster analysis using the UPGMA method of Phylip version 3.57 c.

4. Chi-square analysis

A RAPD pattern is referred to as genotype which is generated from randomly amplified polymorphic DNA. Chi-square (χ^2) test, a Monte Carlo

simulation, was also performed on genotypes to ascertain the difference between the 4 geographic samples for the number and size of RAPD fragments.

Chapter III

Results

DNA Extraction

The shrimp DNAs were prepared from the pleopods by using proteinase-K / phenol-chloroform extraction. The quality and quantity of DNA from individual shrimp were determined by electrophoresis on a 0.7% mini-gel and measuring absorbance at 260 and 260/280 nm.

The extracted DNA from one pleopod showed O.D. at 260 between 0.20-0.32 and O.D. 260/280 ratio between 1.8-2.0. Since $O.D._{260}$ of 1.0 corresponds to 50 μg double-stranded DNA per ml, thus, the recovery yield was about 250-400 μg/ml = 75-100 μg/one pleopod. The O.D. 260/280 ratio also indicated the purity of the extracted DNA. High MW. DNA (>23 kb) was observed after electrophoresed through a 0.7% agarose mini-gel (Fig 3.1).

Optimization of RAPD-PCR

Experiments were performed to optimize PCR program parameters for reproducible amplification of discrete *P.monodon* DNA.. Inconsistent amplification was minimized by including three quantities of template DNA

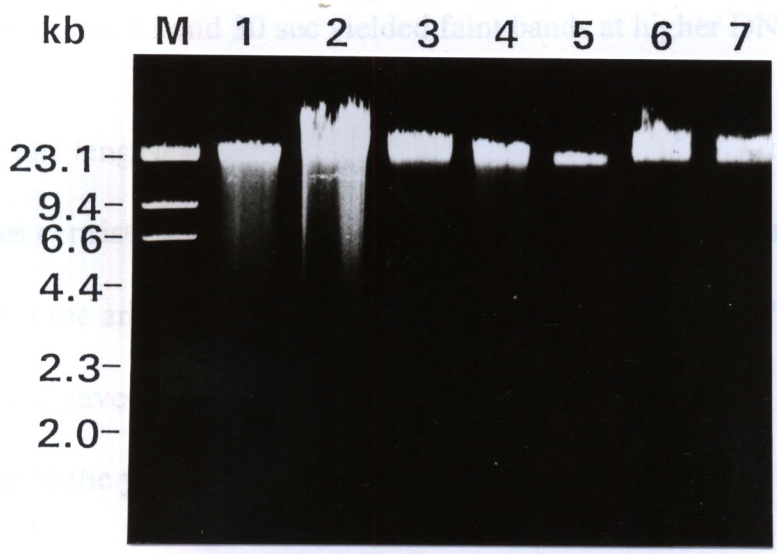


Figure 3.1. Ethidium bromide staining of 0.7% agarose gel showing DNA extracted from pleopods of *P.monodon*.

Lane M = λ DNAs / *Hind* III size marker.

Lanes 1-7 = genomic DNAs of 7 individuals of *P.monodon*.

(25, 50 and 100 ng) in the program evaluation and selecting optimal program parameters based upon consistent amplification at all DNA quantities. The first assay was attempted with the following profiles: 35 cycles consisting of denaturation at 94°C for 5 sec, 15 sec, and 30 sec, annealing at 36°C for 45 sec and extension at 72°C for 90 sec. In this assay, denaturation for 5 sec yielded consistent amplifications at all three DNA concentrations (Fig 3.2 A), whereas denaturation for 15 and 30 sec yielded faint bands at higher DNA concentration.

The length of annealing step was attempted with the following profiles: 35 cycles consisting of denaturation at 94°C for 5 sec, annealing at 36°C for 30, 45 and 60 sec and extension at 72°C for 90 sec. The results of annealing for 45 and 60 sec gave bands of acceptable intensity (Fig 3.2 B) but annealing for 30 sec reduced the yield of PCR products. Annealing length of 45 sec was chosen.

The length of extension step was attempted with the following profiles: 35 cycles consisting of denaturation at 94°C for 5 sec, annealing at 36°C for 45 sec and extension at 72°C for 75, 90 and 105 sec. It was found that short extension for 75 sec resulted in faint bands, whereas too long extension for 105 sec resulted in smearing an agarose gel. Therefore, the optimized extension time was 90 sec (Fig 3.2 C).

The last assay was attempted by varying the number of cycles with the following profiles: 30, 35 and 40 cycles consisting of denaturation at 94°C for 5

sec, annealing at 36°C for 45 sec and extension at 72°C for 90 sec. The 30 cycles of amplification produced lesser amounts of PCR products. Increasing the number of cycles from 35 to 40 cycles did not significantly increase the PCR products (Fig 3.2 D).

Therefore the optimal program parameters identified for the RAPD analysis of *P. monodon* were 35 cycles of 5 sec at 94°C, 45 sec at 36°C and 90 sec at 72°C.

Primer screening

A total of 300 ten-base primers were screened for their ability to prime PCR amplifications of the black tiger prawn DNA. Only 138 RAPD primers (46%) yielded amplification products while the rest of the primers did not amplified the DNA template or resulted in smear patterns (Table 3.1). Examples of primer screening are shown in Fig 3.3. The primers which yielded intense or consistent bands or both were selected for further analysis. In this study, seven positive primers 101, 174, 268, 428, 456, 457 and 459 were selected for the detection of genetic variation in wild populations of *P.monodon.*

Figure 3.2 Optimization of the PCR parameters for RAPD analysis of *P.monodon* using primer 174.

A . Variation of the length of denaturation step at 94 °C.

Lane M = 100 bp DNA ladder.

Lanes 1-3 = denaturation for 5 sec with 25, 50 and 100 ng template DNA, respectively.

Lanes 4-6 = denaturation for 15 sec with 25, 50 and 100 ng template DNA, respectively.

Lanes 7-9 = denaturation for 30 sec with 25, 50 and 100 ng template DNA, respectively.

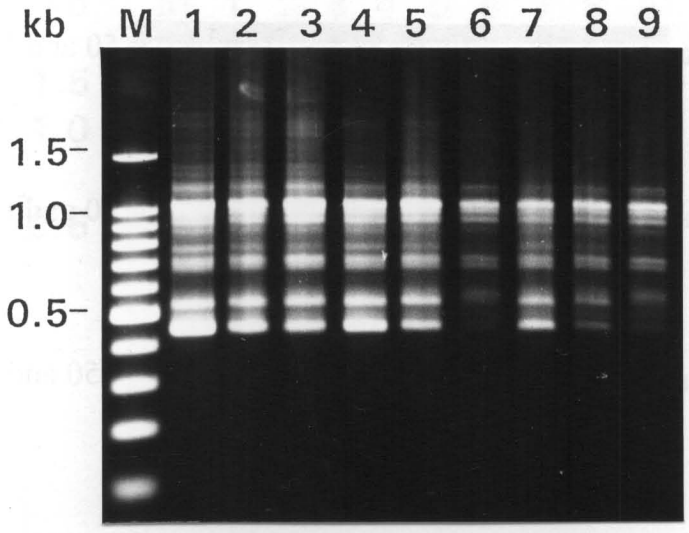
B. Variation of the length of annealing step at 36 °C.

Lane M = 100 bp DNA ladder.

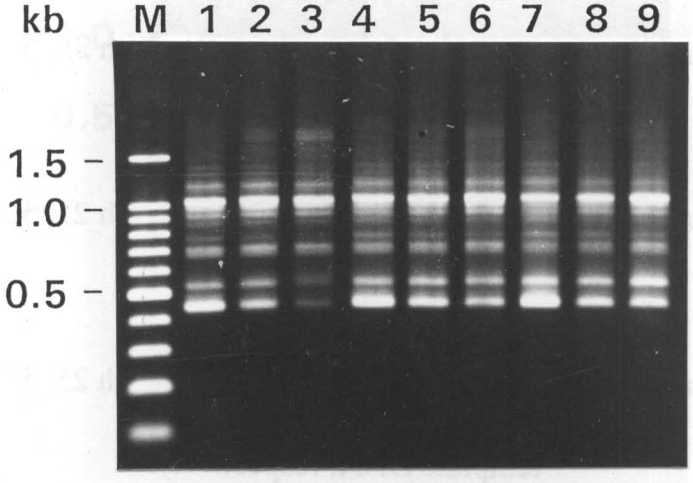
Lanes 1-3 = annealing for 30 sec with 25, 50 and 100 ng template DNA, respectively.

Lanes 4-6 = annealing for 45 sec with 25, 50 and 100 ng template DNA, respectively.

Lanes 7-9 = annealing for 60 sec with 25, 50 and 100 ng template DNA, respectively.



A



B

C. Variation of the length of extension step at 72°C.

Lane M = 100 bp DNA ladder.

Lanes 1-3 = extension for 75 sec with 25, 50 and 100 ng template DNA, respectively.

Lanes 4-6 = extension for 90 sec with 25, 50 and 100 ng template DNA, respectively.

Lanes 7-9 = extension for 105 sec with 25, 50 and 100 ng template DNA, respectively.

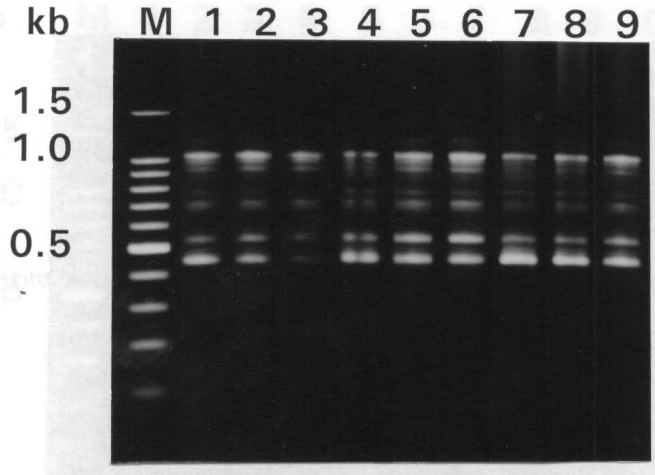
D) Variation of the number of cycles.

Lane M = 100 bp DNA ladder.

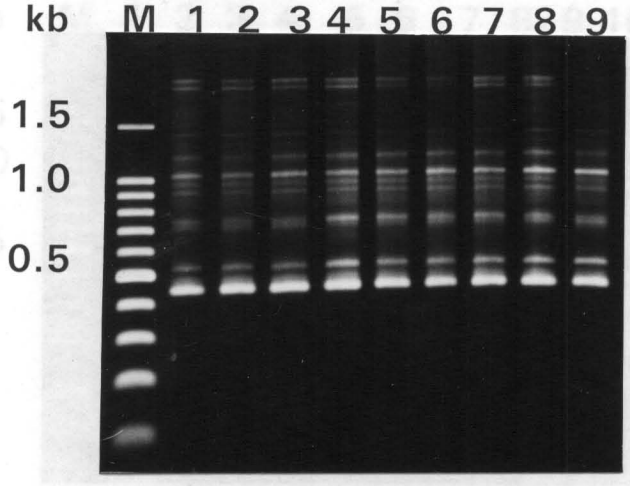
Lanes 1-3 = amplification for 30 cycles with 25, 50 and 100 ng template DNA, respectively.

Lanes 4-6 = amplification for 35 cycles with 25, 50 and 100 ng template DNA, respectively.

Lanes 7-9 = amplification for 40 cycles with 25, 50 and 100 ng template DNA, respectively.



C



D

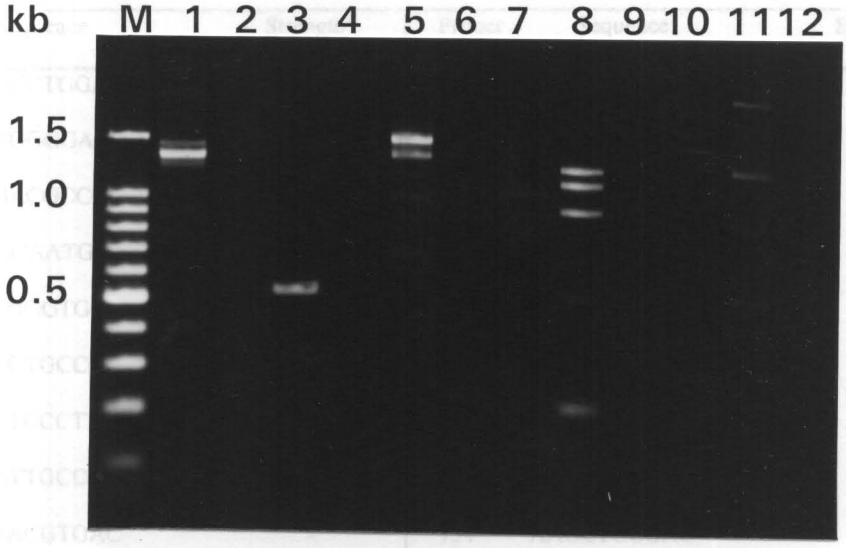
Figure 3.3. RAPD patterns obtained from amplification of *P.monodon* DNA with various primers.

A. Lane M = 100 bp DNA ladder.

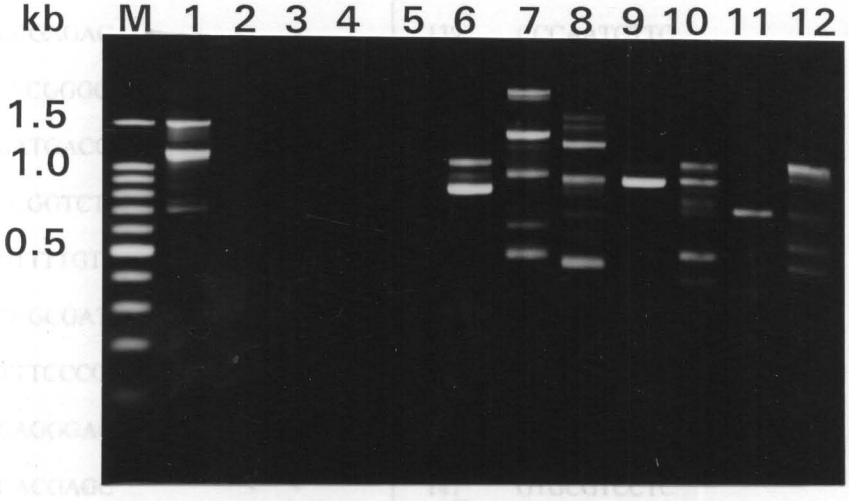
Lanes 1-12 = amplifications with primers 421- 432, respectively.

B. Lane M = 100 bp DNA ladder.

Lanes 1-12 = amplifications with primers 450- 460 and 174, respectively.



A



B

Table 3.1 The sequences of random primers included in the screening and amplification strength values for *P.monodon*.

Primer	Sequence	Strength*	Primer	Sequence	Strength*
101	GCGCCTGGAG	+++	126	CTTTCGTGCT	-
102	GGTGGGGACT	-	127	ATCTGGGAGC	-
103	GTGACGCCGC	+	128	GCATATTCCG	-
104	GGGCAATGAT	-	129	GCGGTATAGT	-
105	CTCGGGTGGG	-	130	GGTTATCCTC	-
106	CGTCTGCCCG	-	131	GAAACAGCGT	+
107	CTGTCCCTTT	-	132	AGGGATCTCC	++
108	GTATTGCCCT	-	133	GGAAACCTCT	++
109	TGTACGTGAC	-	134	AAGCTGCGAG	-
110	TAGCCCGCTT	-	135	AAGCTGCGAG	++
111	AGTAGACGGG	-	136	TACGTCTTGG	-
112	GCTTGTGAAC	-	137	GGTCTCTCCC	+
113	ATCCCAAGAG	-	138	GCTTCCCCTT	+
114	TGACCGAGAC	-	139	CCCAATCTTC	-
115	TTCCGCGGGC	-	140	GTCGCATTTC	-
116	TACGATGACG	-	141	ATCCTGTTCG	-
117	TTAGCGGTCT	-	142	ATCTGTTCGG	-
118	CCCGTTTTGT	-	143	TCGCAGAACG	-
119	ATTGGGCGAT	-	144	AGAGGGTTCT	-
120	GAATTTCCCC	-	145	TGTCGGTTGC	-
121	ATACAGGGAG	-	146	ATGTGTTGCG	-
122	GTAGACGAGC	+	147	GTGCGTCCTC	-
123	GTCTTTCAGG	-	148	TGTCCACCAG	+
124	ACTCGAAGTC	-	149	AGCAGCGTGG	-
125	GCGGTGAGAGG	-	150	GAAGGCTCTG	-

Table 3.1 Continued

Primer	Sequence	Strength*	Primer	Sequence	Strength*
151	GCTGTAGTGT	+++	176	CAAGGGAGGT	-
152	CGCACCGCAC	+++	177	TCAGGCAGTC	+++
153	GAGTCACGAG	++	178	CCGTCATTGG	-
154	TCCATGCCGT	-	179	TCACTGTACG	-
155	CTGGCGGCTG	++	180	GGCCACGCT	+++
156	GCCTGGTTGC	++	181	ATGACGACGG	++
157	CGTGGGCAGG	-	182	GTTCTCGTGT	-
158	TAGCCGTGGC	-	183	CGTGATTGCT	-
159	GAGCCCGTAG	-	184	CAAACGGCAC	+++
160	CGATTCAGAG	-	185	GTGTCTTCAG	+
161	CGTTATCTCG	-	186	GTGCGTCGCT	++
162	AACCTACCGC	-	187	AACGGGGGAG	-
163	CCCCCCAGAT	-	188	GCTGGACATC	++
164	CCAAGATGCT	+	189	TGCTAGCCTC	-
165	GAAGGCACGT	++	190	AGAATCCGCC	+++
166	ACTCCTACAG	-	191	CGATGGCTTT	-
167	CCAATTCACG	-	192	GCAAGTCACT	++
168	CTAGATGTGC	-	193	TGCTGGCTTT	-
169	ACGACGTAGG	-	194	AGGACGTGCC	-
170	ATCTCTCCTG	-	195	GATCTCAGCG	-
171	TGACCCCTCC	+++	196	CTCCTCCCCC	++
172	ACCGTCGTAG	-	197	TCCCCGTTCC	-
173	CAGGCGGCGT	+++	198	GCAGGACTGC	++
174	AACGGGCAGC	+++	199	GCTCCCCCAC	-
175	TGGTGCTGAT	++	200	TCGGGATATG	-

Table 3.1 Continued

Primer	Sequence	Strength*	Primer	Sequence	Strength*
201	CTGGGGATTT	-	226	GGGCCTCTAT	+ + +
202	GAGCACTTAC	-	227	CTAGAGGTCC	+ + +
203	CACGGCGAGT	+	228	GCTGGGCCGA	+ +
204	TTCGGGCCGT	+	229	CCACCCAGAG	+ +
205	CGGTTTGGAA	+ +	230	CGTCGCCCAT	+ + +
206	GAGGACGTCC	+	231	AGGGAGTTCC	+ + +
207	CATATCAGGG	-	232	CGGTGACATC	+ + +
208	ACGGCCGACC	-	233	CTATGCGCGC	-
209	TGCACTGGAG	+ +	234	TCCACGGACG	+ + +
21O	GCACCGAGAG	+	235	CTGAGGCAAA	+ +
211	GAAGCGCGAT	+ + +	236	ATCGTACGTG	+
212	GCTGCGTGAC	+ + +	237	CGACCAGAGC	+ + +
213	CAGCGAACTA	+ + +	238	CTGTCCAGCA	+ + +
214	CATGTGCTTG	+	239	CTGAAGCGGA	+ + +
215	TCACACTTGC	+ +	240	ATGTTCCAGG	+ +
216	CATAGACTCC	+ +	241	GCCCGACGCG	+ + +
217	ACAGGTAGAC	+ +	242	CACTCTTTGC	-
218	CTCAGCCCAG	+ +	243	GGGTGAACCG	+ + +
219	GTGACCTCAG	+ + +	244	CAGCCAACCG	+ + +
220	GTCGATGTCG	+ + +	245	CGCGTGCCAG	+ + +
221	CCCGTCAATA	+ + +	246	TATGGTCCGG	+
222	AAGCCTCCCC	+ + +	247	TACCGACGGA	-
223	GATCCATTGC	+ +	248	GAGTAAGCGG	+ + +
224	TCTCCGGTAT	-	249	GCATCTACCG	+ + +
225	CGACTCACAG	+ + +	250	CGACAGTCCC	+ + +

Table 3.1 Continued

Primer	Sequence	Strength*	Primer	Sequence	Strength*
251	CTTGACGGGG	++	276	AGGATCAAGC	+++
252	CTGGTGATGT	+++	277	AGGAAGGTGC	++
253	CCGTGCAGTA	+++	278	GGTTCCAGCT	+++
254	CGCCCCCATT	+++	279	AGACATTAGA	-
255	TTCCTCCGGA	-	280	CTGGGAGTGG	+++
256	TGCAGTCGAA	+++	281	GAGAGTGGAA	+++
257	CGTCACCGTT	+++	282	GGGAAAGCAG	++
258	CAGGATACCA	++	283	CGGCCACCGT	+++
259	GGTACGTACT	-	284	CAGGCGCACA	++
260	TCTCAGCTAC	+	285	GGGCGCCTAG	+++
261	CTGGCGTGAC	+++	286	CGGAGCCGGC	-
262	CGCCCCCAGT	+++	287	CGAACGGCGG	+++
263	TTAGAGACGG	-	288	CCTCCTTGAC	+
264	TCCACCGAGC	+++	289	ATCAAGCTGC	+++
265	CAGCTGTTCA	+++	290	CCGCGAGCAC	+++
266	CCACTCACCG	+++	291	AGCTGAAGAG	++
267	CCATCTTGTG	-	292	AAACAGCCCG	+++
268	AGGCCGCTTA	+++	293	TCGTGTTGCT	-
269	CCAGTTCGCC	+++	294	TGATTGGCCA	+++
270	TGCGCGCGGG	++	295	CGCGTTCCTG	+++
271	GCCATCAAGA	+++	296	CCGCTGGGAG	+++
272	AGCGGGCCAA	++	297	GCGCATTAGA	+
273	AATGTCGCCA	+++	298	CCGTACGGAC	++
274	GTTCCCGAGT	-	299	TGTCAGCGGT	++
275	CCGGGCAAGC	++	300	GGCTAGGGCG	+++

Table 3.1 Continued

Primer	Sequence	Strength*	Primer	Sequence	Strength*
401	TAGAACAGTC	-	426	CTCCCGGTG	-
402	CCCGCCGTTG	-	427	GTAATCGACG	-
403	GGAAGGCTGT	+++	428	GGCTGCGGTA	+++
404	TCTCTACGAC	-	429	AAACCTGGAC	-
405	CTCTCGTGCG	++	430	AGTCGGCACC	+
406	GCCACCTCCT	+++	431	CTGCGGGTCA	+
407	TGGTCCTGGC	+++	432	AGCGTCGACT	-
408	CCGTCTCTTT	-	433	TCACGTGCCT	+++
409	TAGGCGGCGG	+++	434	TCGCTAGTCC	-
410	CGTCACAGAG	+++	435	CTAGTAGGGG	-
411	GAGGCCCGTT	+	436	GAGGGGGCCA	-
412	TGCGCCGGTG	++	437	AGTCCGCTGC	+++
413	GAGGCGGCGA	+	438	AGACGGCCGG	+++
414	AAGGCACCAG	+	439	GCCCCTTGAC	+++
415	GTTCCAGCAG	-	440	CTGTCGAACC	++
416	GTGTTTCCGG	+++	441	CTGCGTTCTT	-
417	GACAGGCCAA	++	442	CTACTCGGTT	-
418	GAGGAAGCTT	+	443	TGATTGCTCG	-
419	TACGTGTTTG	-	444	GCAGCCCCAT	+++
420	GCAGGGTTCG	++	445	TAGCAGCTTG	+++
421	ACGGCCCACC	+++	446	GCCAGCGTTC	++
422	CACCTGCGGG	-	447	CAGGCTCTAG	++
423	GGGTCTCGAA	++	448	GTTGTGCCTG	+
424	ACGGAGGTTC	-	449	GAGGTTCAAC	+
425	CGTCGGGCCT	+++	450	CGGAGAGCCC	+++

Table 3.1 Continued

Primer	Sequence	Strength*	Primer	Sequence	Strength*
451	CTAATCTCGC	-	476	TTGAGGCCCT	+
452	CTAATCACGG	-	477	TGTTGTGCCC	-
453	AGTACAAGGG	-	478	CAGGCTGGTC	++
454	GCTTACGGCA	-	479	CTCATACGCG	-
455	AGCAAGCCGG	+++	480	GGAGGGGGGA	++
456	GCGGAGGTCC	+++	481	GTAAGGGCGC	-
457	CGACGCCCTG	+++	482	CTATAGGCCG	-
458	CTCACATGCC	+++	483	GCACTAAGAC	-
459	GCGTCGAGGG	++	484	CTGGCAAGGA	-
460	ACTGACCGGC	++	485	AGAATAGGGC	-
461	CCCGTATGTC	-	486	CCAGCATCAG	++
462	CATAGCGGCA	++	487	GTGGCTAGGT	++
463	AGGCGGAAGC	-	488	TTCGCTTCTC	-
464	CACAAGCCTG	+++	489	CGCACGCACA	-
465	GGTCAGGGCT	-	490	AGTCGACCTT	+++
466	TTCTTAGCGG	-	491	TCCTGTCCAG	-
467	AGCACGGGCA	++	492	GTGACTGCTC	+
468	ACGGAAGCGC	+++	493	CCGAATCACT	-
469	CTCCAGCAAA	-	494	TGATGCTGTC	-
470	AGGAGCTGGG	++	495	CTTTCCTTCC	-
471	CCGACCGGAA	-	496	CCTTTCAAGG	-
472	AGGCGTGCAA	++	497	GCATAGTGCG	-
473	ATCCCCAAGA	-	498	GACAGTCCTG	-
474	AGGCGGGAAC	+++	499	GGCCGATGAT	++
475	CCAGCGTATT	-	500	TTGCGTCATG	+

* Primer amplification strength was scored as -, +, ++, +++, where - indicates no amplification, + indicates weak amplification, ++ indicates moderately amplification, and +++ indicates strong amplification.

Detection of genetic variation in wild populations of *P.monodon*

Four geographically separated wild populations of *P. monodon* were collected from the Andaman Sea and the Gulf of Thailand. Samples from the Andaman Sea were from Satun-Trang and Medan (Indonesia) provinces. Samples from the Gulf of Thailand were from Chonburi (Angsila district) and Trad provinces.

The RAPD analysis of 2 geographically separated populations using the 7 selected primers produced a total of 80 scorable bands ranging in size from 200 to 2,200 bp. Eighteen of these bands (22.5%) were monomorphic (present in at least approximately 95% of all individuals) and 62 bands (77.5%) were polymorphic (present in some individuals, absent in others) (Table 3.2). The percentages of polymorphic bands varied between primers. The percentages of polymorphic bands generated by primers 101, 174, 268, 428, 456, 457 and 459 were 84.6, 70.0, 88.9, 100.0, 66.7, 69.2 and 72.7% respectively. Each primer generated between 9-15 scorable bands. The complexity of the banding patterns varied between the primers. Primer 456 gave the highest number of amplified bands (15 bands) but yielded least percentage of polymorphic bands (66.7%). Primers 428 yielded the highest percentage of polymorphic bands which was 100 %.

Individuals of the shrimps from Satun-Trang (17), Trad (28), Angsila

(15) and Medan (15) produced 71, 69, 68 and 73 scorable bands respectively (Table 3.3). The percentages of polymorphic bands from individual shrimps of the Satun-Trang, Trad, Angsila and Medan were 57.7, 52.2, 45.6 and 53.4%, respectively. The results suggested that the shrimps collected from Satun-Trang were the highest polymorphic, while the shrimps collected from Angsila were the least polymorphic among the 4 geographic samples.

The RAPD patterns of all samples tested are shown in Appendix A. Examples of RAPD amplification patterns and the bands scored generated by the selected primers are shown in Fig 3.4-3.10. Only reproducible bands were scored for their presence or absence. Difference in staining intensity of RAPD fragments between profiles was not scored as real variation. Primer 428 appeared to identify a more variable region among the samples of Thai *P.monodon* from the Andaman Sea and the Gulf of Thailand, whereas the remaining primers showed less variable RAPD patterns. When comparing RAPD patterns among wild populations of *P.monodon* from Indonesia (Medan) and Thailand, all of the seven primers gave different RAPD patterns.

RAPD analysis by primer 101

When genomic DNAs of the 4 geographically separated *P.monodon* were amplified by primer 101, bands at about 900 and 520 were found in all individuals of the 4 geographic samples (Table 3.4A). A band at about 1550 bp, were found in 66.7 and 100% of the Angsila and Medan samples, respectively,

Table 3.2 Nucleotide sequences of seven selected primers, number of amplified bands and range of sizes of amplified fragments (bp) shown in the RAPD analysis in *P.monodon*.

Primer No.	Sequences	Size-rang	No. of amplified bands	No. of polymorphic bands	No. of monomorphic* bands
101	GCGCCTGGAG	1800-420	13	11(84.6%)	2(15.4%)
174	AACGGGCAGG	1500-450	10	7(70.0%)	3(30.0%)
268	AGGCCGCTTA	1300-400	9	8(88.9%)	1(11.1%)
428	GGCTGCGGTA	1800-200	9	9(100.0%)	0(0.0%)
456	GCGGAGGTCC	2200-260	15	10(66.7%)	5(33.3%)
457	CGACGCCCTG	2000-350	13	9(69.2%)	4(30.8%)
459	GCGTCGAGGG	1550-430	11	8(72.7%)	3(27.3%)
		Total	80	62(77.5%)	18(22.5%)

*These bands are present in at least approximately 95% of total investigated individuals.

Table 3.3 Total number of bands, percentage of polymorphic and monomorphic bands found in the 4 geographic samples of *P.monodon*.

	Satun-Trang			Trad			Angsila			Medan		
Primer No.	No. of bands	No.of polymor-phic bands	No.of monomor-phic bands	No. of bands	No. of polymor-phic bands	No. of monomor-phic bands	No. of bands	No. of polymor-phic bands	No. of monomor-phic bands	No. of bands	No. of polymor-phic bands	No. of monomor-phic bands
101	11	5	6	11	4	7	12	6	6	10	4	6
174	10	5	5	10	6	4	10	5	5	10	6	4
268	9	7	2	9	6	3	9	6	3	8	5	3
428	8	6	2	7	5	2	5	2	3	6	2	4
456	13	7	6	12	4	8	12	3	9	15	9	6
457	12	7	5	12	8	4	12	5	7	13	7	6
459	8	4	4	8	3	5	8	4	4	11	6	5
Total	71	41 (57.7%)	30 (42.3%)	69	36 (52.2%)	33 (47.8%)	68	31 (45.6%)	37 (54.4%)	73	39 (53.4%)	34 (46.6%)

whereas in the Satun-Trang and Trad samples, this band was rare. Bands at about 690 and 790 bp were absent in the Medan sample but showed approximately 64-100% in Thai *P.monodon*. By contrast, bands at about 1800 and 810 bp were found in 93.3 and 100% of the Medan sample, respectively but approximately 0-7% were found in Thai *P.monodon*. The bands at 1800 and 810 bp make it possible to distinguish between Thai and Indonesian *P. monodon* (Fig 3.4).

RAPD analysis by primer 174

By using primer 174, bands at about 1050 and 700 were found in all individuals of the 4 geographic samples (Table 3.4B). When comparing RAPD patterns among the samples of Thai *P.monodon*, they were quite similar. The RAPD patterns compared between Thai and Medan (Indonesia) *P.monodon* were observed to contain a few differences. A band at about 520 bp was found in 100% and 53.3% of Thai *P.monodon* and Medan respectively, and bands at about 1450 and 1350 bp were found more frequently (>33%) in Thai *P.monodon* than in Medan (6.7%) (Fig 3.5).

RAPD analysis by primer 268

When using primer 268 for the amplification, a common band at about 690 bp was shown in all individuals of the 4 samples (Table 3.4C). This primer showed some variable RAPD patterns between Thai and Indonesian *P.monodon*. A band with size about 890 bp was found in approximately 88.2%

of Satun-Trang, whereas this band was found in approximately 3.6 and 40% of Trad and Angsila respectively. In Medan, this band was absent. A band about 400 bp was found in 100% of Thai *P.monodon* but only 42.9% was found in Medan (Fig 3.6).

RAPD analysis by primer 428

For amplification by primer 428, some RAPD fragments were monomorphic in particular samples. Several distinctive scorable bands were detected between Thai and Indonesian *P.monodon.* Bands at about 590 and 200 bp were found in approximately 73-89 % and 100% of Thai *P.monodon* respectively but were not found in Medan (Table 3.4D). In contrast, a band at about 1800 bp was absent in all Thai *P.monodon* but was found in 100% of Medan. The results suggested that these bands were possible to distinguish between Thai *P.monodon*; Satun-Trang, Trad, and Angsila and Indonesian *P.monodon*; Medan. A band with size about 950 bp was present in 100% of Satun-Trang and Medan, but absent in samples from Trad and Angsila. This band can be used as a region-specific marker (Fig 3.7).

RAPD analysis by primer 456

Bands at size range about 2000, 480 and 290 were monomorphic in the 4 geographic samples (Table 3.4E). When compared between Thai and Indonesian *P.monodon*, slightly different patterns were found but no specific fragment for either population was observed. A band at 1,300 bp was a

monomorphic band in *P.monodon* from the Gulf of Thailand but found only in 30.8% of Indonesian *P.monodon*. Bands at 2,100 and 1,400 bp were absent in Thai *P.monodon* but were present in 53.8 and 76.9% of Medan, respectively. Bands at 1,250 and 700 bp were found in 100 and 76.9% of Medan but were found approximately in 0-18% of Thai *P.monodon* (Fig 3.8).

RAPD analysis by primer 457

Bands at size range about 1250, 550 and 420 were monomorphic in all 4 geographic samples (Table 3.4F). A band at 950 bp was found at 70.6, 78.6 and 57.1% in Satun-Trang, Trad and Medan, respectively but was found only in 40.0% of Angsila. A band at about 900 bp was found in 52.9% and 32.1% of Satun-Trang and Trad but was found in 86.7% of Angsila. Moreover, it was found only in 7.1% of Medan. Bands at about 730 and 600 bp were found approximately in 18-46% of Satun-Trang, Trad and Medan but were found in 100% of Angsila. A band at 500 bp was found in 88.2, 80 and 100% of Satun-Trang, Angsila and Medan, respectively, but was found only in 28.6% of Trad. Although the majority of bands appeared in all 4 geographic samples, there was a specific band for Medan. A band at 2,200 bp was found in 100% of Medan but absent in all Thai *P.monodon* samples (Fig 3.9).

RAPD analysis by primer 459

Bands at 480 and 430 bp were found in all individuals of the 4 different geographic samples. A band at 900 bp found in 96.4% of Trad was monomorphic in the remaining geographic samples (Table 3.4G). A band at 620 bp was rare in all 4 samples. A band specific for Thai *P.monodon* was not found. In contrast, a band at 730 bp was found in 100% of Medan but absent in Thai *P.monodon*. Moreover, it was shown that the frequency of several bands differed between Thai and Indonesian *P.monodon*, such as the bands at 1,550, 1,450 and 790 bp. Bands at 1,550 and 1,450 were absent in Thai *P.monodon* but were found approximately in 66-73% of Medan. A band at 790 bp was found approximately in 60-100% of Thai *P.monodon* but was found only in 20% of Medan (Fig 3.10).

Table 3.4 The number of each amplified band found in each of the 4 geographic sample of *P. monodon*

A. Primer 101

Size-range	Satun-Trang (17 individuals)	Trad (28 individuals)	Angsila (15 individuals)	Medan (15 individuals)
1800	0(0.0.0%)	0(0.0%)	0(0.0%)	14(93.3%)
1550	2(11.7%)	0(0.0%)	10(66.7%)	15(100.0%)
1350	2(11.7%)	7(25.0%)	2(13.3%)	15(100.0%)
1050	13(76.5%)	7(25.0%)	9(60.0%)	15(100.0%)
900	17(100.0%)	28(100.0%)	15(100.0%)	15(100.0%)
810	0(0.0%)	1(3.6%)	1(6.7%)	15(100.0%)
790	17(100.0%)	27(96.4%)	14(93.3%)	0(0.0%)
710	14(82.3%)	27(96.4%)	13(86.4)	14(93.3%)
690	11(64.7%)	20(71.4%)	15(100.0%)	0(0.0%)
590	17(100.0%)	27(96.4%)	15(100.0%)	12(80.0%)
520	17(100.0%)	28(100.0%)	15(100.0%)	15(100.0%)
470	17(100.0%)	28(100.0%)	15(100.0%)	5(33.3%)
420	17(100.0%)	28(100.0%)	15(100.0%)	0(0.0%)

B. Primer 174

Size-range	Satun-Trang (17 individuals)	Trad (28 individuals)	Angsila (15 individuals)	Medan (15 individuals)
1500	8(47.1%)	7(25.0%)	7(46.7%)	5(33.3%)
1450	16(94.1%)	17(60.7%)	5(33.3%)	1(6.7%)
1350	7(41.2%)	9(32.1%)	11(73.3%)	1(6.7%)
1200	17(100.0%)	21(75.0%)	15(100.0%)	15(100.0%)
1050	17(100.0%)	28(100.0%)	15(100.0%)	15(100.0%)
720	10(58.8%)	19(67.9%)	10(66.7%)	15(100.0%)
700	17(100.0%)	28(100.0%)	15(100.0%)	15(100.0%)
600	8(47.1%)	6(21.4%)	4(26.7%)	1(6.7%)
520	17(100.0%)	28(100.0%)	15(100.0%)	8(53.3%)
420	17(100.0%)	28(100.0%)	15(100.0%)	14(93.3%)

C. Primer 268

Size-range	Satun-Trang (17 individuals)	Trad (28 individuals)	Angsila (15 individuals)	Medan (14 individuals)
1250	3(17.6%)	13(46.4%)	4(26.3%)	14(100.0%)
1150	13(76.5%)	9(32.2%)	15(100.0%)	4(28.6%)
980	7(41.2%)	5(17.9%)	3(20.0%)	14(100.0%)
890	15(88.2%)	1(3.6%)	6(40.0%)	0(0.0%)
790	14(82.3%)	28(100.0%)	14(93.3%)	9(64.3%)
710	4(23.5%)	9(32.1%)	8(53.3%)	9(64.3%)
690	17(100.0%)	28(100.0%)	15(100.0%)	14(100.0%)
490	14(82.3%)	26(92.9%)	11(73.3%)	4(28.6%)
400	17(100.0%)	28(100.0%)	15(100.0%)	6(42.9%)

D. Primer 428

Size-range	Satun-Trang (17 individuals)	Trad (28 individuals)	Angsila (15 individuals)	Medan (15 individuals)
1800	0(0.0%)	0(0.0%)	0(0.0%)	15(100.0%)
1400	6(35.3%)	2(7.1%)	0(0.0%)	15(100.0%)
1250	15(88.2%)	27(96.4%)	15(100.0%)	15(100.0%)
1150	9(52.9%)	20(71.4%)	12(80.0%)	6(40.0%)
950	17(100.0%)	0(0.0%)	0(0.0%)	15(100.0%)
900	15(82.3%)	17(60.7%)	15(100.0%)	5(33.3%)
880	1(5.9%)	10(35.7%)	0(0.0%)	0(0.0%)
590	15(88.2%)	25(89.3%)	11(73.3%)	0(0.0%)
200	17(100.0%)	28(100.0%)	15(100.0%)	0(0.0%)

E.Primer456

Size-range	Satun-Trang (17 individuals)	Trad (28 individuals)	Angsila (15 individuals)	Medan (13 individuals)
2100	0(0.0%)	0(0.0%)	0(0.0%)	7(53.8%)
2000	17(100.0%)	28(100.0%)	15(100.0%)	13(100.0%)
1400	0(0.0%)	0(0.0%)	0(0.0%)	10(76.9%)
1300	16(94.1%)	28(100.0%)	15(100.0%)	4(30.8%)
1250	3(17.6%)	5(17.9%)	0(0.0%)	13(100.0%)
1200	14(82.3%)	25(89.3%)	15(100.0%)	13(100.0%)
1100	11(64.7%)	17(60.8%)	14(93.3%)	2(15.4%)
920	17(100.0%)	28(100.0%)	15(100.0%)	8(61.5%)
750	16(94.1%)	24(85.7%)	14(93.3%)	9(69.2%)
700	3(17.6%)	0(0.0%)	1(6.7%)	10(76.9%)
650	16(94.1%)	27(96.4%)	15(100.0%)	13(100.0%)
600	17(100.0%)	28(100.0%)	15(100.0%)	12(92.3%)
520	17(100.0%)	28(100.0%)	15(100.0%)	11(84.6%)
480	17(100.0%)	28(100.0%)	15(100.0%)	13(100.0%)
290	17(100.0%)	28(100.0%)	15(100.0%)	13(100.0%)

F. Primer 457

Size-range	Satun-Trang (17 individuals)	Trad (28 individuals)	Angsila (15 individuals)	Medan (14 individuals)
2200	0(0.0%)	0(0.0%)	0(0.0%)	14(100.0%)
1250	17(100.0%)	28(100.0%)	15(100.0%)	14(100.0%)
950	12(70.6%)	22(78.6%)	6(40.0%)	8(57.1%)
900	9(52.9%)	9(32.1%)	13(86.7%)	1(7.1%)
850	13(76.5%)	25(89.3%)	10(66.7%)	8(57.1%)
800	17(100.0%)	27(96.4%)	14(93.3%)	14(100.0%)
730	5(29.4%)	13(46.4%)	15(100.0%)	3(21.4%)
650	17(100.0%)	26(92.9%)	15(100.0%)	5(35.7%)
600	4(23.5%)	5(17.9%)	15(100.0%)	5(35.7%)
550	17(100.0%)	28(100.0%)	15(100.0%)	14(100.0%)
500	15(88.2%)	8(28.6%)	12(80.0%)	14(100.0%)
420	17(100.0%)	28(100.0%)	15(100.0%)	14(100.0%)
350	11(64.7%)	20(71.4%)	15(100.0%)	5(35.7%)

G. Primer 459

Size-range	Satun-Trang (17 individuals)	Trad (28 individuals)	Angsila (15 individuals)	Medan (15 individuals)
1550	0(0.0%)	0(0.0%)	0(0.0%)	11(73.3%)
1450	0(0.0%)	0(0.0%)	0(0.0%)	10(66.7%)
900	17(100.0%)	27(96.4%)	15(100.0%)	15(100.0%)
800	17(100.0%)	25(89.3%)	15(100.0%)	15(100.0%)
790	11(64.7%)	28(100.0%)	11(73.3%)	3(20.0%)
730	0(0.0%)	0(0.0%)	0(0.0%)	15(100.0%)
700	11(64.7%)	28(100.0%)	11(73.3%)	7(47.7%)
690	15(88.2%)	26(92.9%)	14(93.3%)	9(60.0%)
620	1(5.9%)	5(17.9%)	2(13.3%)	1(6.7%)
480	17(100.0%)	28(100.0%)	15(100.0%)	15(100.0%)
430	17(100.0%)	28(100.0%)	15(100.0%)	15(100.0%)

Figure 3.4 RAPD patterns using primer 101.

Lane M = 100 bp DNA ladder.

Lanes 1-4 = individuals collected from Medan.

Lanes 5-8 = individuals collected from Satun-Trang.

Lanes 9-12 = individuals collected from Trad.

Lanes 13-15 = individuals collected from Angsila.

— = indicate bands that were scored.

Figure 3.5 RAPD patterns using primer 174.

Lane M = 100 bp DNA ladder.

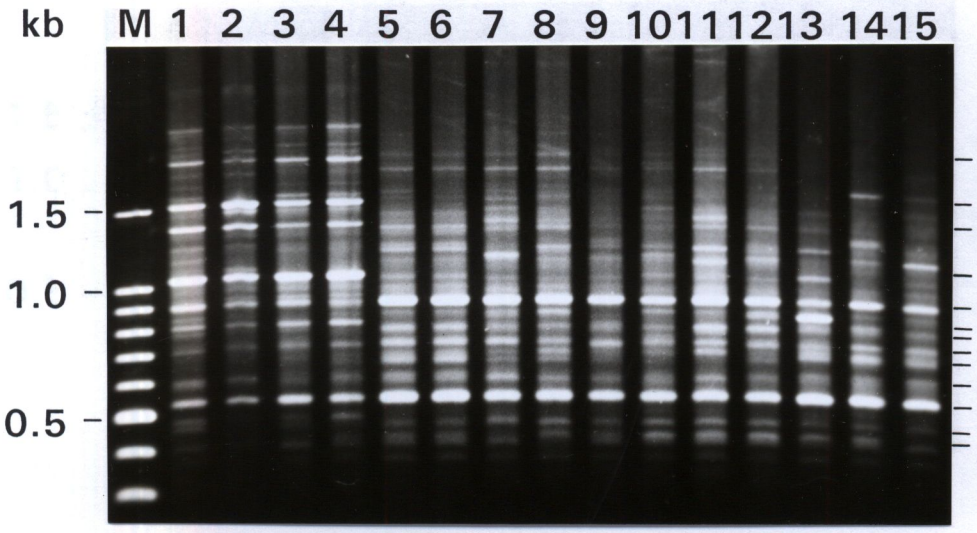
Lanes 1-4 = individuals collected from Medan.

Lanes 5-8 = individuals collected from Satun-Trang.

Lanes 9-12 = individuals collected from Trad.

Lanes 13-15 = individuals collected from Angsila.

— = indicate bands that were scored.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1.5
1.0
0.5

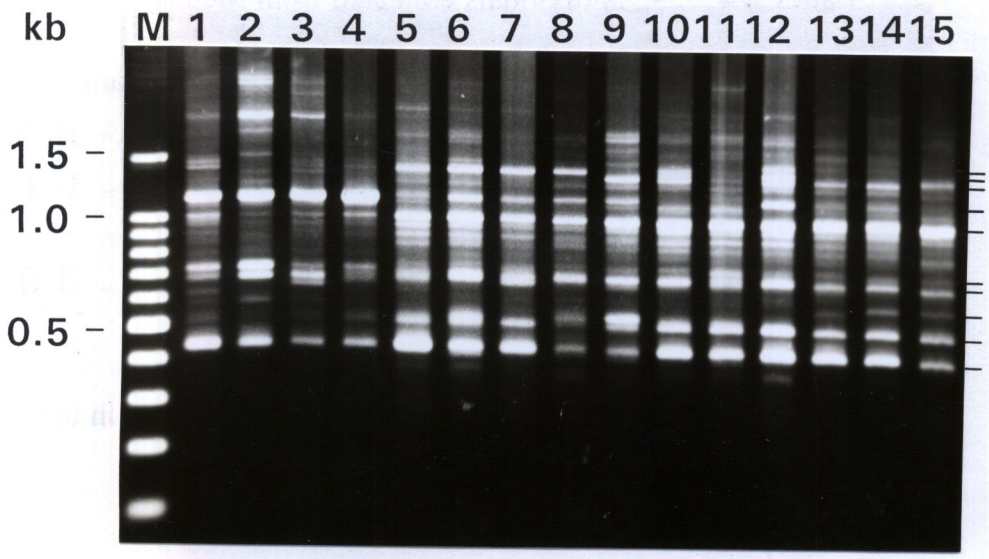

kb
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1.5
1.0
0.5

Figure 3.6 RAPD patterns using primer 268.

Lane M = 100 bp DNA ladder.

Lanes1-4 = individuals collected from Medan.

Lanes 5-8 = individuals collected from Satun-Trang.

Lanes 9-12 = individuals collected from Trad.

Lanes 13-15 = individuals collected from Angsila.

— = indicate bands that were scored.

Figure 3.7 RAPD patterns using primer 428.

Lane M = 100 bp DNA ladder.

Lanes 1-4 = individuals collected from Medan.

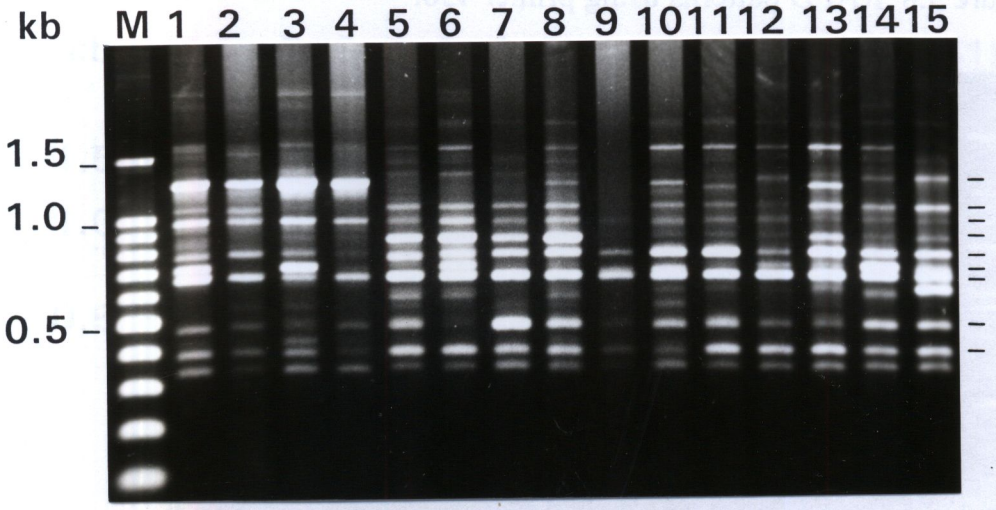
Lanes 5-8 = individuals collected from Satun-Trang.

Lanes 9-12 = individuals collected from Trad.

Lanes 13-15 = individuals collected from Angsila.

— = indicate bands that were scored.

← = indicate a band that was found only in the Andaman Sea.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1.5
1.0
0.5

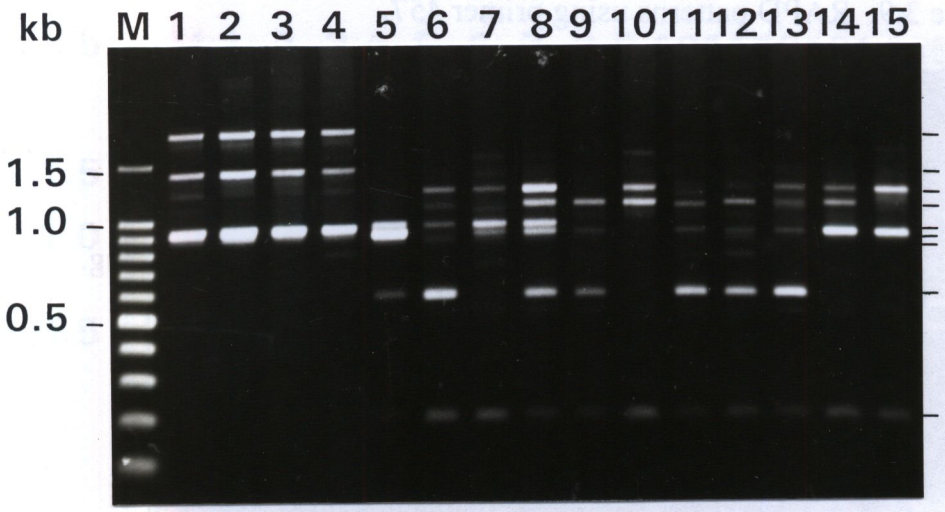

kb
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1.5
1.0
0.5

Figure 3.8 RAPD patterns using primer 456.

Lane M = 100 bp DNA ladder.

Lanes 1-5 = individuals collected from Medan.

Lanes 6-9 = individuals collected from Satun-Trang.

Lanes 10-12 = individuals collected from Trad.

Lanes 13-15 = individuals collected from Angsila.

— = indicate bands that were scored.

Figure 3.9. RAPD patterns using primer 457.

Lane M = 100 bp DNA ladder.

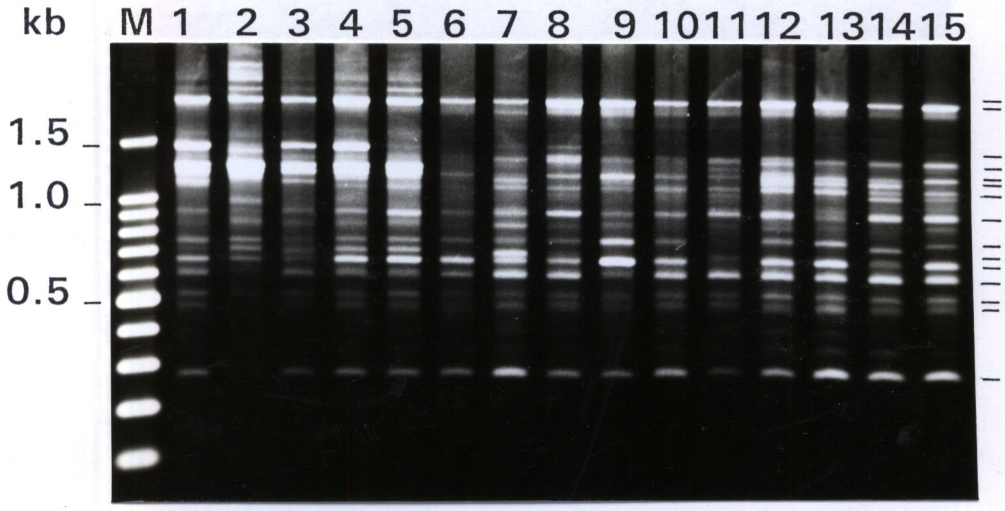
Lanes 1-4 = individuals collected from Medan.

Lanes 5-8 = individuals collected from Satun-trang.

Lanes 9-12 = individuals collected from Trad.

Lanes 13-15 = individuals collected from Angsila.

— = indicate bands that were scored.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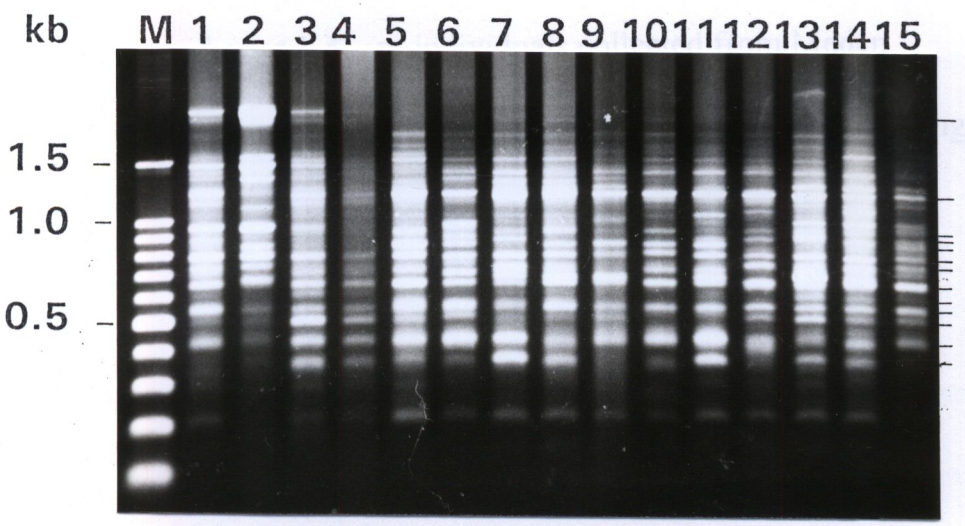
1.5
1.0
0.5


kb
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1.5
1.0
0.5

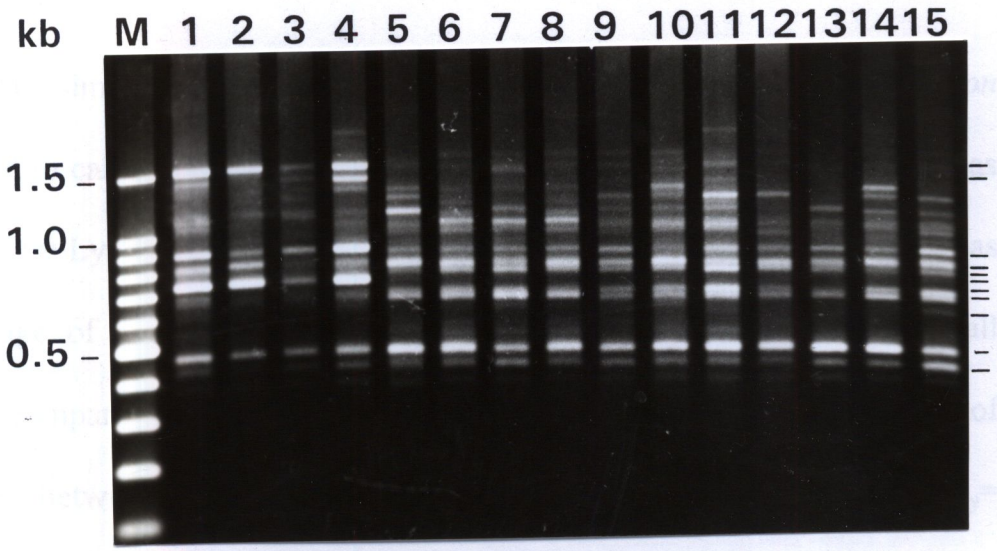


Figure 3.10 RAPD patterns using primer 459.

Lane M = 100 bp DNA ladder.

Lanes 1-4 = individuals collected from Medan.

Lanes 5-8 = individuals collected from Satun-Trang.

Lanes 9-12 = individuals collected from Trad.

Lanes 13-15= individuals collected from Angsila.

— = indicate bands that were scored.

Data analysis

The similarities in the RAPD patterns of all 75 individuals *P.monodon* were used to calculated the similarity index within and between populations as described by Lynch (1991). Within population, similarity (S) was calculated as the average of the index of similarity between individuals (S_{xy}) across all possible comparisons between individuals within a population. The index of similarity between individuals was calculated using the formula: $S_{xy}= 2n_{xy}/n_x+n_y$, where n_{xy} is the number of fragments shared by individuals x and y and n_x and n_y are the number of fragments scored for each individual. Between population, similarity (S_{ij}) is calculated using: $S_{ij} = 1+S'_{ij}-0.5\ (S_i+S_j)$, where S_i and S_j are the values of S for populations i and j, respectively and S'_{ij} is the average similarity between randomly paired individuals from populations i and j. Table 3.5 and 3.6 show the similarity within (S) and between (S_{ij}) populations for the 4 geographic samples based on RAPD patterns with 7 primers. The average similarity within populations (S) across all the primers ranged from 0.8626 to 0.9047. Samples from Angsila showed the highest similarity index (0.9047) where that of Satun-Trang, Trad and Medan was 0.8655, 0.8646 and 0.8626, respectively. The calculation suggested that Angsila was the least polymorphic. The average of similarity between populations (S_{ij}) across all the primers ranged from 0.7548 to 0.9578. The comparison of Satun-Trang with Angsila gave highest similarity index (S_{ij} = 0.9578), while comparison of Trad

and Medan showed the least similarity index (S_{ij} = 0.7548). The average of similarity between populations across all the primers of Thai *P.monodon*; the comparisons of Satun-Trang with Trad, Satun-Trang with Angsila and Trad with Angsila were 0.9478, 0.9578 and 0.9463, respectively. The results indicated that the 3 geographic samples of Thai *P.monodon* were similar. When compared between Thai and Indonesian *P.monodon*, the index of similarity between populations indicated significant difference between the two populations. The comparisons of Satun-Trang, Trad and Angsila with Medan showed similarity index of 0.7882, 0.7548 and 0.7563, respectively. The results indicated that Satun-Trang was genetically more similar to Medan than Trad and Angsila. S'_{ij} was converted to measure the genetic distance (D_{ij}) using the equation: $D_{ij} = -\ln[S'_{ij}/\sqrt{(S_iS_j)}]$. The results of the calculations are shown in Table 3.7. The average of genetic distance across all the primers yielded the values ranging from 0.0487 to 0.334. The comparisons of the Satun-Trang with Trad, Angsila and Medan; Trad with Angsila and Medan; and Angsila with Medan were 0.0622, 0.0487, 0.2812, 0.0624, 0.3340 and 0.3225, respectively. The comparison of Satun-Trang with Angsila showed the least genetic distance (D_{ij} = 0.0487), while comparison of Trad with Medan showed the highest (D_{ij} = 0.334). Distance values were used to construct dendrograms using the unweighted pair-group method of analysis (UPGMA). The resultant phenograms based on UPGMA are shown in Fig 3.11. The dendrogram across all the primers presented clusters that the 4 geographic samples of *P.monodon*

clearly divided into 2 distinct groups. Group 1 contained the 3 georaphic samples of Satun-Trang, Trad and Angsila. Group 2 contained the 1 geographic sample of Medan. For group 1, Satun-Trang and Angsila were more closely linked than Trad. To assess the significance of this grouping, a compatibility analysis was carried out by constructing separate dendrograms for each of the 7 primers. All primers clearly divided the isolates into 2 distinct groups which were Satun-Trang, Trad and Angsila as 1 group and the other was Medan. Samples of Thai *P.monodon*, Satun-Trang and Angsila were more closely linked than Trad for dendrograms of primers 101, 174, 268, 456 and 459. For dendrogram of primer 428, Trad and Angsila were more closely linked than Satun-Trang, while dendrogram of primers 457 Trad and Satun-Trang were more closely linked than Angsila.

Although the samples of Satun-Trang are separated from Trad and Angsila by geographic distribution, the genetic distances showed slight difference among the 3 geographic samples of Thai *P.monodon*. When compared RAPD patterns for each primer, the results showed that only primer 428 gave a specific marker for the samples of Satun-Trang (Fig 3.7). Thus, the samples of Thai *P.monodon* appeared to evolve from a single ancestral gene pool.

Table 3.5 Estimated similarity (S) for each primer within the 4 geographic samples of *P. monodon*

Primer	Satun-Trang	Trad	Angsila	Medan
101	0.9149	0.9099	0.9146	0.9605
174	0.8619	0.8216	0.8412	0.8651
268	0.8044	0.8239	0.8283	0.7640
428	0.8208	0.8086	0.9151	0.9420
456	0.9149	0.9284	0.9937	0.8016
457	0.8512	0.8279	0.9269	0.8648
459	0.8904	0.9316	0.9120	0.8403
Mean	0.8655	0.8646	0.9047	0.8626
SD	0.0437	0.0557	0.0556	0.0705

Table 3.6 Estimated similarlity (S_{ij}) for each primer between the 4 geographic samples of *P. monodon*

Primer	S-T/T*	S-T/A*	S-T/M*	T/A*	T/M*	A/M*
101	0.9614	0.9704	0.6518	0.9303	0.6448	0.7011
174	0.9442	0.9813	0.9144	0.9552	0.8762	0.8902
268	0.9512	0.9743	0.6886	0.8787	0.7186	0.7000
428	0.8801	0.8621	0.6344	0.9863	0.5174	0.5151
456	0.9820	0.9868	0.8712	0.9717	0.8696	0.8369
457	0.9641	0.9318	0.9133	0.9219	0.8831	0.8117
459	0.9516	0.9978	0.8435	0.9799	0.7739	0.8389
Mean	0.9478	0.9578	0.7882	0.9463	0.7548	0.7563
SD	0.0323	0.047	0.125	0.0385	0.1382	0.1283

*S-T = Satun-Trang, T = Trad, A = Angsila and M = Medan

Table 3.7 Estimated of genetic distance for each primer between the 4 geographic samples of *P. monodon*

A. All primers

	S-T*	T*	A*	M*
S-T	-			
T	0.0622	-		
A	0.0487	0.0624	-	
M	0.2812	0.3340	0.3225	-

B. Primer 101

	S-T	T	A	M
S-T	-			
T	0.0432	-		
A	0.0330	0.0795	-	
M	0.4638	0.4774	0.3837	-

C . Primer 174

	S-T	T	A	M
S-T	-			
T	0.0684	-		
A	0.0222	0.0553	-	
M	0.1044	0.1585	0.1378	-

D. Primer 268

	S-T	T	A	M
S-T	-			
T	0.0618	-		
A	0.032	0.1588	-	
M	0.5056	0.4370	0.4722	-

E. Primer 428

	S-T	T	A	M
S-T	-			
T	0.1592	-		
A	0.1716	0.0142	-	
M	0.5334	0.7987	0.7386	-

F. Primer 456

	S-T	T	A	M
S-T	-			
T	0.0197	-		
A	0.0131	0.0294	-	
M	0.1605	0.1607	0.1948	-

G. Primer 457

	S-T	T	A	M
S-T	-			
T	0.0437	-		
A	0.0790	0.0916	-	
M	0.1066	0.1484	0.2354	-

H. Primer 459

	S-T	T	A	M
S-T	-			
T	0.0544	-		
A	0.0023	0.0220	-	
M	0.1991	0.2934	0.2023	-

*S-T = Satun-Trang, T = Trad, A = Angsila and M = Medan

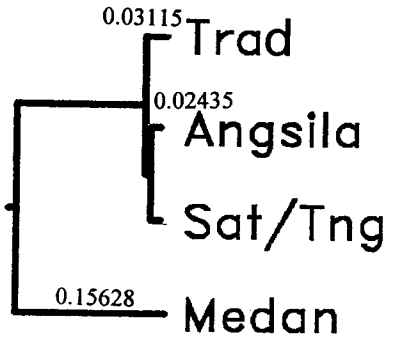
Figure 3.11 Phenograms showing the relationships among the 4 geographic samples of *P.monodon*, generated according to UPGMA method of cluster analysis based on distance matrix in Table 3.7.

A. For all primers.

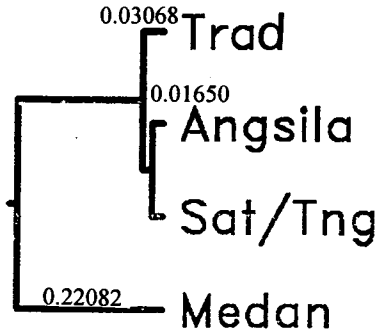
B. For primer 101.

C. For primer174.

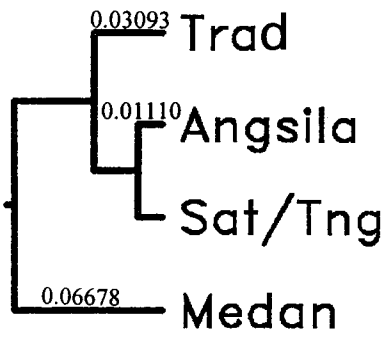
D. For primer 268.


0.03115
Trad
0.02435
Angsila
Sat/Tng
0.15628
Medan

A


0.03068
Trad
0.01650
Angsila
Sat/Tng
0.22082
Medan

B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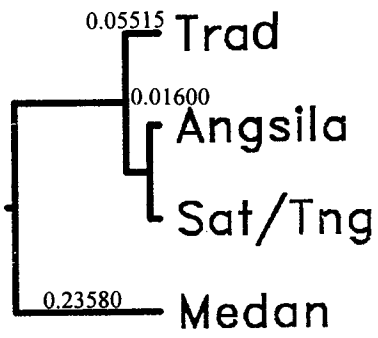
Trad
0.01110
Angsila
Sat/Tng
0.06678
Medan

C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Trad
0.01600
Angsila
Sat/Tng
0.23580
Medan

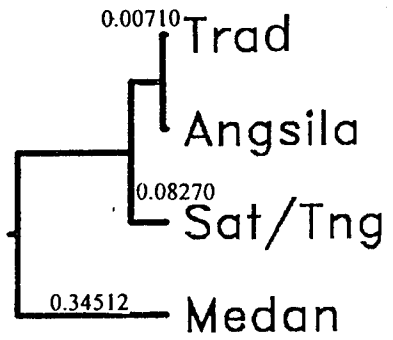
D

E . For primer 428

F . For primer 456

G . For primer 457

H . For primer 459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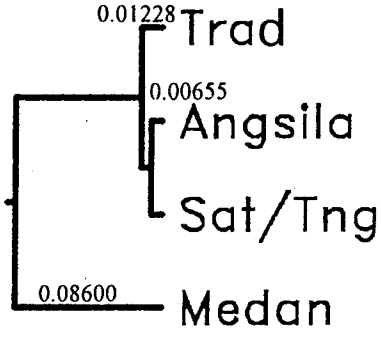
Trad
Angsila
0.08270
Sat/Tng
0.34512
Medan

E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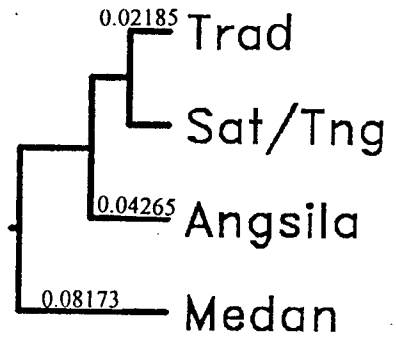
Trad
0.00655
Angsila
Sat/Tng
0.08600
Medan

F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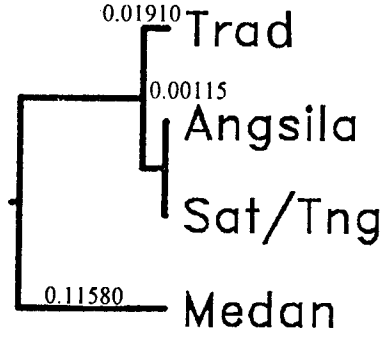
Trad
Sat/Tng
0.04265
Angsila
0.08173
Medan

G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Trad
0.00115
Angsila
Sat/Tng
0.11580
Medan

H

Geographic heterogeneity in genotype frequency distributions

A RAPD pattern is referred to as a genotype which is generated from randomly amplified polymorphic DNA. In this study using 7 RAPD primers, 214 genotypes were observed from the 4 geographic samples; 29 individuals from Satun-Trang, 28 individuals from Trad, 15 individuals from Angsila and 15 individuals from Medan (for details of each genotype see Appendix B). One hundred and sixty of these were population-specific genotypes and 10 were region-specific genotypes for the sample from the Gulf of Thailand (Table 3.8). Ninety-seven of these genotypes were only represented by single individual. Primers 101, 174, 268, 428, 456, 457 and 459 yielded 24, 34, 35, 30, 22, 45 and 24 genotypes, respectively. Population-specific genotypes of primers 101, 174, 268, 428, 456, 457 and 459 were 18, 20, 27, 27, 15, 35 and 18, respectively. Only primers 174, 268, 428 and 457 gave region-specific genotypes which were 4, 2, 2 and 2, respectively. Genotypes from the 4 geographic samples of *P.monodon* which were generated by each primer, are shown in Table 3.8.

A chi-square (χ^2) analysis, a Monte Carlo simulation was used to analyze heterogeneity of the frequencies of 213 genotypes among the 4 geographic samples of *P.monodon* (Table 3.9). For overall samples, significant differences were observed for every primers ($P<0.0001$). Therefore, it could be concluded that the heterogeneity among samples existed. Highly significant differences were also observed between Thai and Indonesian *P.monodon*.

Heterogeneity for all Thai samples was not significantly different when primer 174 and 456 were employed (P = 0.1582 and P = 0.5600, respectively). When comparing between Thai *P.monodon* from the Gulf of Thailand and the Andaman Sea, there was no significant difference when using primers 174 and 456 (P = 0.0599 and P = 0.6663, respectively). Significant differences were observed for primers 101, 268, 428, 457 and 459 (P = 0.0049, <0.0001, <0.0001, 0.0014 and 0.0156, respectively). Therefore, the chi-square analysis showed the existent of region heterogeneity among Thai *P.monodon*.

Table 3.8 Geographic heterogeneity in genotype frequency distributions generated from randomly amplified polymorphic DNA patterns of *P. monodon*

A. primer 101

Population	genotype																							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
Satun-Trang	3	0	4	3	1	4	5	0	0	2	0	2	1	0	1	1	1	0	0	0	0	0	0	1
Trad	13	0	2	5	0	0	0	3	0	1	3	0	0	0	0	0	0	1	0	0	0	0	0	0
Angsila	0	0	3	0	6	0	0	0	0	0	0	0	1	2	0	0	0	0	1	1	1	0	0	0
Medan	0	10	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0

B. primer 174

Population	genotype																																		
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH
Satun-Trang	6	5	0	0	0	1	0	1	3	0	0	1	2	1	1	0	0	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Trad	3	3	0	0	3	1	0	2	0	1	1	1	1	1	1	1	1	1	2	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0
Angsila	0	1	0	0	1	1	4	0	0	2	2	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Medan	0	0	5	5	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1

C. primer 268

Population	genotype																																		
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI
Satun-Trang	7	0	5	4	2	0	0	0	0	0	0	2	2	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Trad	0	7	0	0	1	0	1	4	3	3	3	0	0	2	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Angsila	1	0	1	2	2	0	3	0	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
Medan	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1

D. primer 428

Population	genotype																													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD
Satun-Trang	0	0	1	6	0	0	5	4	0	2	2	2	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0
Trad	7	0	4	0	5	0	0	0	3	0	0	0	2	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	0	0
Angsila	9	0	2	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Medan	0	9	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

E. primer 456

Population	genotype																						
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
Satun-Trang	13	5	3	2	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0
Trad	13	3	4	2	0	1	0	1	1	2	0	0	1	0	0	0	0	0	0	0	0	0
Angsila	14	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Medan	0	0	0	0	4	0	2	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0

F. primer 457

Population	genotype																																														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR	AS
Satun-Trang	4	0	3	0	0	0	0	2	0	0	2	2	2	2	2	0	0	0	0	0	1	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Trad	1	0	1	3	3	0	0	1	2	2	0	0	0	0	0	2	2	0	0	0	1	0	1	1	1	1	0	0	0	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0
Angsila	0	7	0	0	0	3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
Medan	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2	2	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1

G. primer 459

Population	genotype																							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
Satun-Trang	11	1	3	3	3	3	0	2	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
Trad	17	6	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0
Angsila	8	1	2	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Medan	0	0	0	0	0	0	3	0	0	2	2	2	2	0	0	0	0	0	0	0	1	1	1	1

Table 3.9 Analysis of geographic heterogeneity in genotype frequency distributions generated from randomly amplified polymorphic DNA of *P.monodon* using a Monte Carlo simulation

	Primer						
	101	174	268	428	456	457	459
Satun-Trang v Gulf of Thailand	P=0.0049	P=0.0599	P<0.0001	P<0.0001	P=0.6663	P=0.0014	P=0.0156
Andaman v Medan	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001
Gulf of Thailand v Medan	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001
All Thai samples	P<0.0001	P=0.1582	P<0.0001	P<0.0001	P=0.5600	P<0.0001	P=0.0006
Over all samples	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001

Chapter IV

Discussion

Genetic variation has the potential to play an important role in shrimp selective breeding programs. So far, however, only allozyme variability has been extensively used in penaeid shrimp and the few DNA studies that have been performed have shown that more genetic variation was detected using the DNA techniques (Garcia et al., 1994). In this study, RAPD technique was used to evaluate the extent of genetic variability in wild populations of *P. monodon* collected from four different geographic locales. These samples covered the main supplying sources of *P.monodon* broodstock used for hatchery purpose in Thailand.

The RAPD technique has been used to detect polymorphism in many organisms. It has uncovered cryptic genetic variability in organisms that distinguishing closely related taxa (Narang et al., 1994). Theoretically, the RAPD technique is expected to scan the whole target genome more randomly than other conventional methods. Since primers are constructed at random, both coding and non coding regions including VNTR loci should be targeted for PCR amplification. As a result, an unbiased used of DNA markers from significantly

different evolutionary rate is, at least theoretically, compromised.

Any parts of the PCR program can change RAPD-PCR patterns (Yu et al., 1993). Therefore, it is useful to spend some time testing various PCR when developing a new RAPD application (Weising et al., 1995). In this study, the reaction buffer components were similar to those described in several protocols but temperature profile, shorter time interval at each step was used. Too long a time at each step unnecessarily prolongs the procedure and may decrease the effectiveness of Taq polymerase. The length of time also depends on the thermocycler's construction and thermal control; therefore the length of time could differ from one brand to another (Yu et al., 1993). The optimized PCR temperature profile for RAPD analysis of the *P.monodon* DNA was denaturation at 94°C for 5 sec; annealing at 36°C for 45 sec and extension at 72°C for 90 sec. During optimization of PCR program parameters, only denaturation for 5 sec yielded consistent amplifications at all three DNA concentrations because shorter periods at 94°C, of course, prolonged the life time of the polymerase. Moreover, short denaturation for 5 sec is indicated that shrimp DNAs consisted of AT rich sequences. Hedgecock et al.(1982) had reported that within the Crustacea, satellite DNAs isolated from species of Cancer consisted of 90-97% alternating A-T. Too much DNA resulted in a smear presumably because DNA might contain some inhibitors (Yu et al., 1993). Annealing at 36°C for 30 sec reduced the yield of PCR products

indicated that insufficient primer annealing may occur, particularly when primer concentrations decline in the later cycles of a long PCR run. Extension for 75 sec and 105 sec resulted in faint bands and smearing respectively. In general, the length of extension step longer than might be expected to get a good yield of PCR products (Rosalind and Stamps, 1993). At the optimal temperature, rate of nucleotide incorporation for Taq polymerase is up to 150 nucleotides per second (Geland, 1989). Benter et al. (1995) had found that 60 sec was enough for extension step for human DNA (about 400-3,000 bp amplification products) but more than 240 sec increased smearing. The 30 cycles of amplifications were not sufficient to gave intense bands. Corresponding to several reports, it was suggested that above 35 cycles were enough for sufficient amplifications (Benter et al., 1995). In this study, the RAPD conditions were optimized and samples were amplified on the same machine. Thus, any differences in banding patterns among individuals should not be attributable to differences in PCR; only polymorphic loci that gave high intensity, easily scorable were used for data collection.

Three hundred RAPD primers (primer 101-300 and 401-500) were screened for their ability to prime PCR amplifications. On the basis of a preliminary results, primers 101, 174, 268, 428, 456, 457 and 459 were chosen. These primers produced strongly reproducible amplification products and gave several fragments. All seven ten-base oligonucleotide primers used in this study

have 60-80% GC content. Generally, primer with ten nucleotides and GC content of at least 50% are generally used (Weising et al., 1995). William et al.(1990) found that the minimum of a primer length to detect amplification in ethidium bromide-stained agarose-gel was 9 bases.

To assess the usefulness of RAPD markers in the determination of genetic diversity of *P.monodon*, samples from the Andaman sea were compared with those from the Gulf of Thailand. The samples from the Andaman Sea were collected from Satun-Trang and Medan (Indonesia), whereas those of the Gulf of Thailand were from Chonburi (Angsila district) and Trad. In Thailand, the Satun-Trang and Trad samples are the most commonly used as spawners in shrimp farming. However, broodstock from the Satun-Trang sample tends to have much bigger size and exhibit different color than those from Trad. Moreover, although get controversial, the shrimp farmers claim that the Andaman Sea broodstock produces more eggs and better quality seeds making the price of the Andaman Sea broodstock higher than that of the Gulf of Thailand.

Decapods are known to display relatively low level of enzyme polymorphism. Mean heterozygosities of 0.048 were reported for decapods, 0.082 for crustaceans (D'Amato and Corach, 1996). A high level of diversity was detected in *P. vannamai* Boone by RAPD analysis, being higher than the level of polymorphism with allozyme markers (Garcia et al., 1994). The

percentages of polymorphic bands range from 39-77%.

In this study, the percentages of polymorphic bands in *P.monodon* varied from 45.6-57.7%. The percentages of polymorphic bands were comparable in the Satun-Trang, Trad, and Medan samples, suggesting similar level of polymorphisms among these three geographic samples. The Angsila sample was the least polymorphic. These correspond to the fact that broodstock from Satun-Trang, Trad and Medan is commonly used as spawners in shrimp farming but broodstock from Angsila has never been used for shrimp industry. Moreover, there was no *P.monodon* found in Angsila until recently when farming of the species is common in such an area and a few were caught by fishermen (Menasveta, personal communication) suggesting that they might be the escapees from the farm. Thus, low level of polymorphism was expected for the Angsila samples. Recently the RAPD analysis of the six crossing families of *P. monodon* had shown a much low level of polymorphism (6.2%) (Garcia and Benzie, 1995). This may be due to the different in the total number of scoring bands, evolutionary rate of the primer's target per se, and the fact that the individuals from wild-caught populations would exhibit higher level of genetic differences than those of the breeding families.

Various RAPD primers used in this study make the possibility to resolve genetic variability between samples of Thai and Indonesian *P.monodon.* Some of RAPD markers identified here are monomorphic in *P.monodon* from

particular geographic samples. For example, primer 459 produced a major band that appeared to be monomorphic and fixed in all individuals from Medan. Other RAPD primers, primer 101, 428, and 457 also produced specific markers. Although Thai *P.monodon* from Trad and Angsila are geographically separated from Satun-Trang, the primers which gave geographically-specific fragments were rare. Only primer 428 gave a specific band which was found only in Satun-Trang. Band with size about 950 bp which was amplified by primer 428, was present in 28 out of 29 individuals from Satun-Trang. This band was also found in all individuals from Medan suggesting that this band should be specific for the populations of the Andaman Sea. Far more samples would need to be screened to establish whether a band at 950 bp would provide the real region-specific marker. After that, this marker can be cloned and sequenced to design a specific set of primers to be used as a region-specific marker or alternatively, the 950 bp can be reamplified by a "touch down" PCR and used as a DNA probe through Southern analysis for the same purpose. The lack of a band at 950 bp in samples from the Gulf of Thailand can also be explained by the lack of primer binding site within the window of size ranges.

For data analysis, the RAPD patterns were used to score for the presence or absence of the amplified DNA fragments. These allow comparisons of genetic similarity among individuals or populations. The similarity index and genetic distance which are described by Lynch (1991), were used in analysis of

the RAPD data. The S and S_{ij} values are estimators of genetic variation within and between populations respectively. They indicate the level of similarity between the individuals or populations being compared. Thus, if S or S_{ij} is high, the individuals or populations share a high level of sequence similarity. In the present study, intrapopulation RAPD variation was detected with all primers. It is noteworthy that samples from Angsila showed the highest S value, indicated that these individuals were more similar among themselves than those in other populations. This value corresponded to the lower percentage of polymorphic bands observed in this sample. It is not surprising that the Angsila sample has the lowest genetic diversity within population in comparison to others. This may be due to *P.monodon* fry which escaped from farms, were recaptured for the analysis in this study as discussed above. The values of S_{ij} and genetic distance indicated a high genetic similarity between samples of Thai *P.monodon*, but indicated a lower genetic similarity between Thai and Indonesian *P.monodon*. Genetic distances were used to cluster the data using the UPGMA algorithum. Most notable, in this presentation, dendrograms implied that the Thai *P.monodon* (Satun-Trang, Trad, and Angsila) shared an ancestral gene pool. More importantly, the dendrograms illustrate a clear "genetic brake" between the Thai and Indonesian *P. monodon* at the Strait of Malacca.

The Satun-Trang and Medan samples are located on the Andaman Sea, so, it was expected to form a cluster. For this reason, the samples from Satun-

Trang, which were kindly provided by Banchong Farm, were suspected for the real locality. New samples, (12 individuals) were then collected from fishermen in Satun-Trang to further tested. The new Satun-Trang sample showed similar level of genetic variation as from the previous sample (see Appendix C). The similarity index within population was 0.8791 which was comparable to that of the previous one (S = 0.8655). The index of similarity between populations, genetic distances and UPGMA dendrograms also indicated that they were the similar samples (see Appendix C). This suggested that the previous Satun-Trang specimens were collected from the real locale.

The similarity index is assumed that any comigration of non allelic markers can be resolved either by differences in band intensity or from other information (Lynch, 1990). The RAPD bands of the same size may not actually have the same or similar sequence (Narang et al., 1994). This may be leading to lose of genetic informations.

A RAPD pattern is referred to as a genotype. A genotype, which specifies for particular population or geography, can be developed a genetic brand. Such as in this study, a genotype B of primer 101 was a genetic brand of *P. monodon* from Medan. This genetic brand can be used to detect the Medan sample. Thus, the advantages of genetic brands are the same as the specific-markers. Consequently, it is not possible to assign individuals taken at random to any specific population. Although the presence of private genotype appears to

be a characteristic of the population or region, this observation requires a larger sampling to confirm.

A chi-square (χ^2) analysis, a Monte Carlo simulation, was also used to suggest genetic differentiation. Chi-square enabled discrimination with highly significant among Thai and Indonesian *P.monodon* as dendrograms. Moreover, chi-square can provided the geographic heterogeneity among Thai *P.monodon*, the Andaman Sea and the Gulf of Thailand. Using a chi-square analysis, several primers can discriminate among groups of Thai *P.monodon*. Although the UPGMA dendrograms clustered Satun-Trang with Angsila and Trad, the chi-square enabled the separation of Satun-Trang samples from those of the Gulf of Thailand. These results suggested the existence of differentiation between 2 geographically seperated population of Thai *P.monodon*. The UPGMA dendrograms and values of chi-square suggested clearly the genetic differences between Thai and Indonesian *P.monodon*.

The genetic population structure of *P.monodon* had also been analyzed by using mtDNA RFLP (Sirawut Klinbunga, 1995). Geographic heterogeneity based on chi-square test and UPGMA dendrogram showed that the Satun was clustered with Aceh (North of Sumatra island) and these two samples were isolated from Trad. Since Aceh locates nearly to Medan, on the Andaman Sea, the result of mtDNA RFLP was different from that of the RAPD analysis. However, there are many factors that effect the analyses of geographic

heterogeneity in this 2 studies. The collection of samples is one factor. The samples used in mtDNA RFLP analysis were collected in 1993, while in this study the samples were collected between 1995-1996. The population structure may be different in this 2 periods of time. Because, in Thailand, most female broodstock were transferred from the Andaman Sea to other parts of the country. *P.monodon* fries of these female broodstock were released to the Gulf of Thailand leading to stock mixing. The weak differentiation found among Thai *P.monodon* by the RAPD analysis may be the consequent of the mixing of stocks of Thai *P.monodon.* Therefore, determination of genetic polymorphism in Thailand should employ more than one technique to the same samples (Wilkerson et al., 1995).

To increase the reliability of the RAPD technique, a more vigorously search of polymorphism would most likely have to be performed. This may involve, more RAPD primers and more samples. A large number of primers must specific be tested with the expectation that at least some of the primers may amplify population-specific DNA fragments. Better resolution and more sensitive detection of RAPD fragments can be achieved by using polyacrylamide gel electrophoresis followed by silver staining. Polyacrylamide gel electrophoresis is known to increase the resolution of band separation whereas silver staining is more sensitive than ethidium bromide staining (Stothard and Rollinson, 1995).

RAPD markers inherited in a dominant fashion, because the presence of a given RAPD band does not distinguish whether its respective locus is homozygous or heterozygous. On the one hand less information is obtained from dominant markers (RAPD) than from codominant markers, caused loss of basic estimators for population genetic studies (Williams et al., 1993). On the other hand, the great advantage of RAPD technology is the ability to obtain DNA polymorphisms without having sequence or otherwise characterize genetic DNA of interest. The possibility of carrying out compatibility analysis with unlimited numbers of primers, each detecting variation of several regions in the genome, provides an advantage over other techniques. Even if some primers amplify identical regions of the genome or if the technique itself is noisy, it should be possible to build up a quickly consensus from patterns of interpopulation variation (Bardakcl and Skibinski, 1994; Wilkerson et al., 1995). This is contrast to other DNA based methods (e.g., southern blotting, DNA sequencing) which may take several days for completion and often require elaborate steps such as cloning. RAPD does not require the use of hazardous-radioactive chemicals. RAPD also allows for the processing of many samples simultaneously. The number of samples that can be processed is a function of the number of tubes that a thermalcycler will hold and the number of thermalcyclers that are available. Whereas the time required to obtain results from RAPD and allozyme analyses is comparable, allozyme analyses has certain limitations (Kambhampati, Black IV, and Rai, 1992).

Chapter IV

Conclusions

1. A Randomly Amplified Polymorphic DNA analysis (RAPD) can be used to assess the genetic variation among populations of *P.monodon*.
2. Optimized PCR temperature profile for amplification of *P.monodon* DNA is 35 cycles; 94°C, 5 sec; 36°C, 45 sec and 72°C, 90 sec.
3. The percentages of polymorphic bands and the values of similarity index within population showed that samples from Angsila were more similar among themselves than samples from Satun-Trang, Trad and Medan, respectively.
4. The values of similarity index between population and genetic distances indicated a high genetic similarity between samples of Thai *P.monodon*, but indicated a lower genetic similarity between Thai and Indonesian *P.monodon*.
5. The UPGMA dendrograms could divide samples of *P.monodon* into two clearly distinct gene pools which were composed of Satun-Trang, Trad and Angsila as Group 1, and Medan as Group 2.
6. A monte Carlo simulation could divide samples of *P.monodon* into three distinct groups which were composed of Satun-Trang as group 1, Trad and Angsila as Group 2, and Medan as group 3.

7. Amplification by primer 428 gave region-specific marker for samples from the Andaman Sea.

Bibliography

ภาษาไทย

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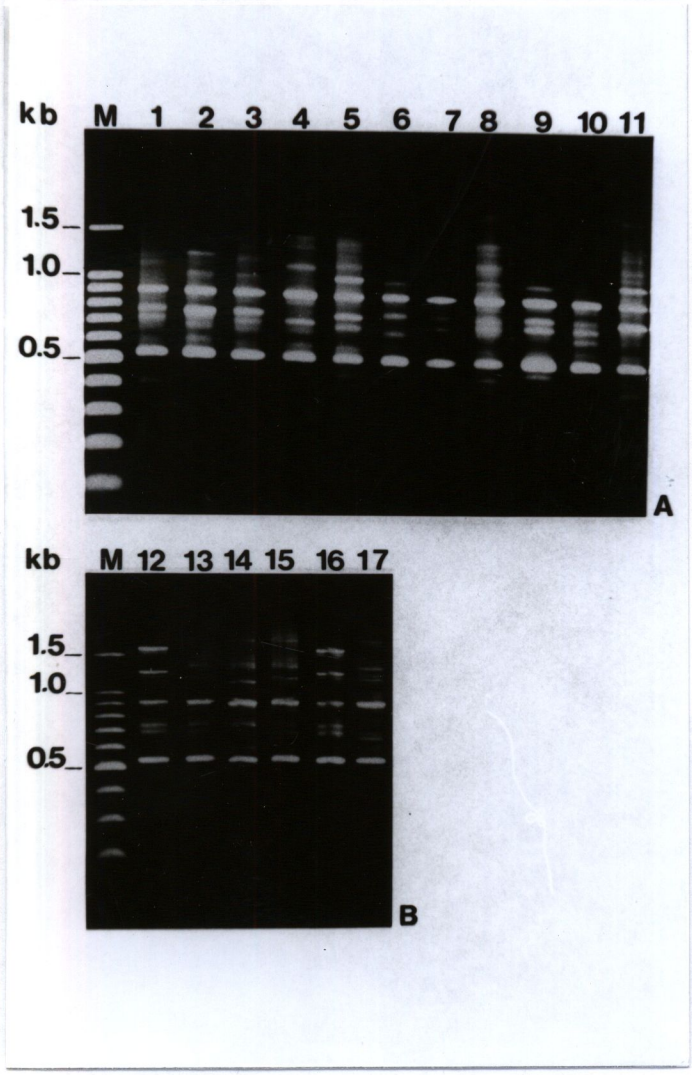
APPENDIX A

RAPD patterns of the 4 geographic samples of *P.monodon*

RAPD patterns of 17 individuals of the Satun-Trang sample



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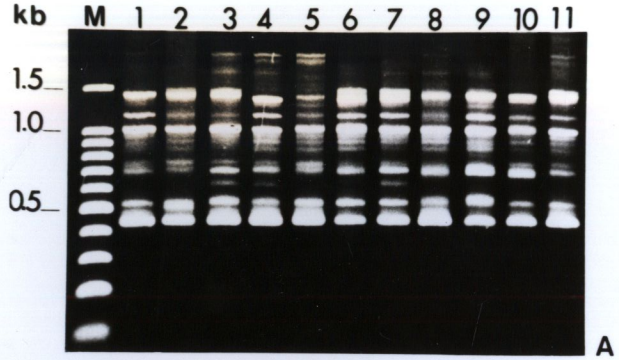

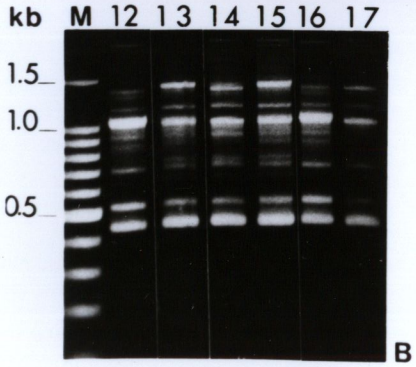

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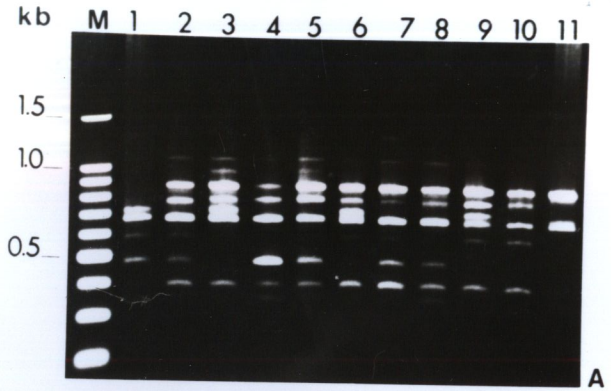

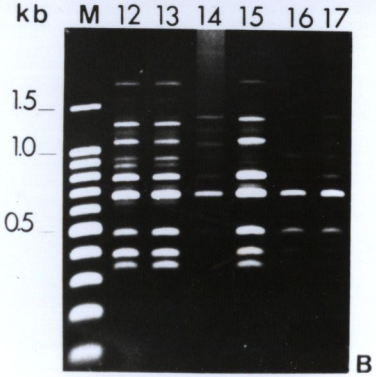

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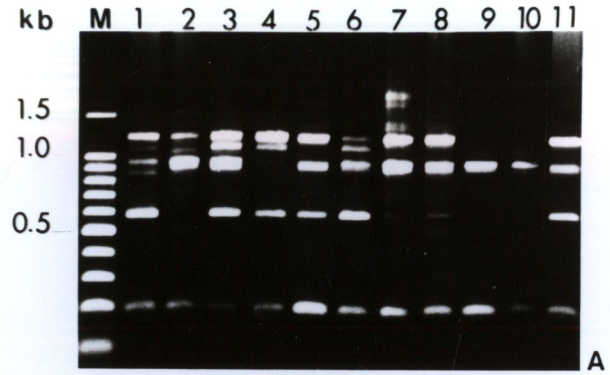

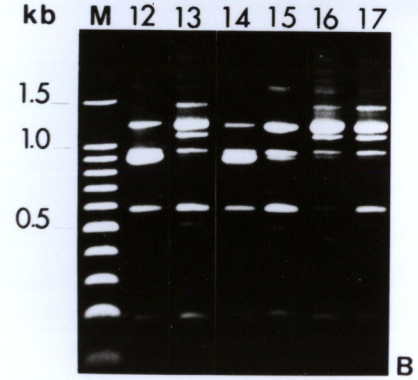

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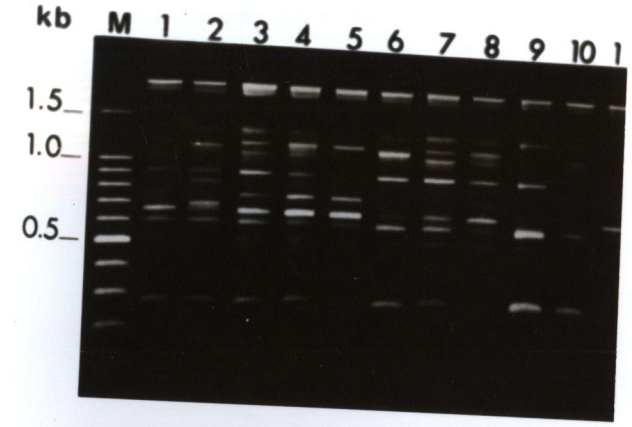

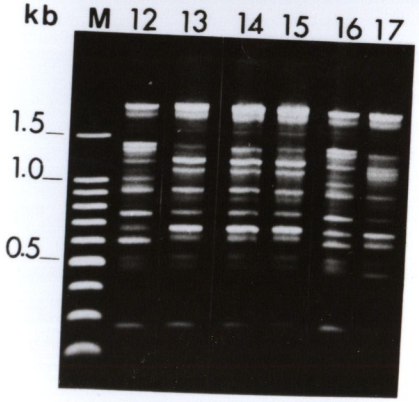

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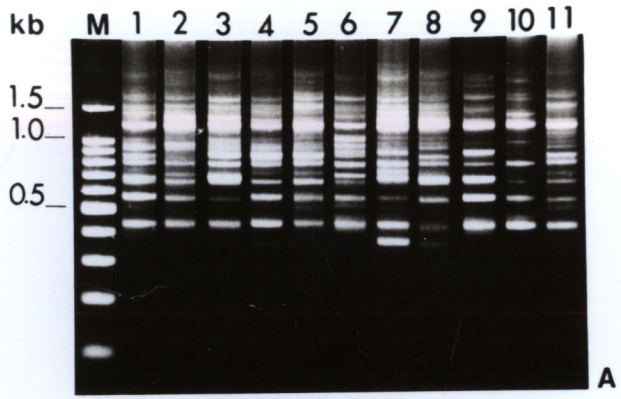

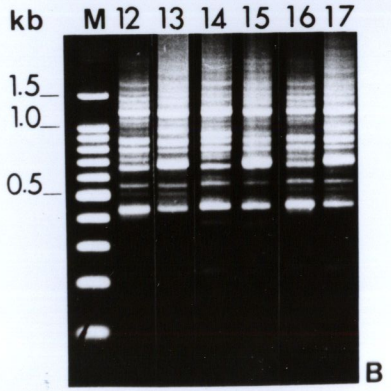

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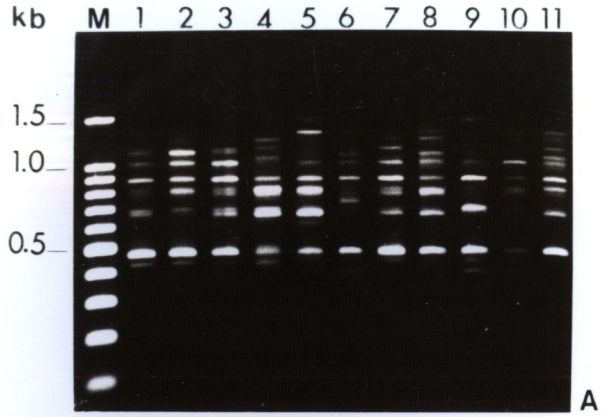
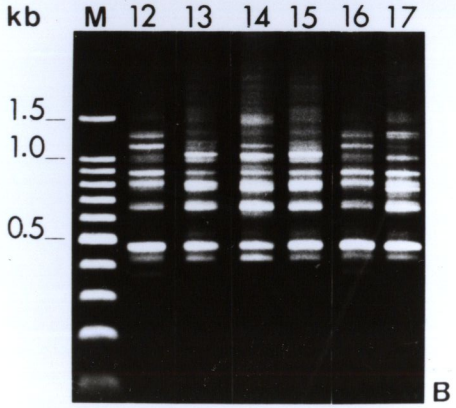
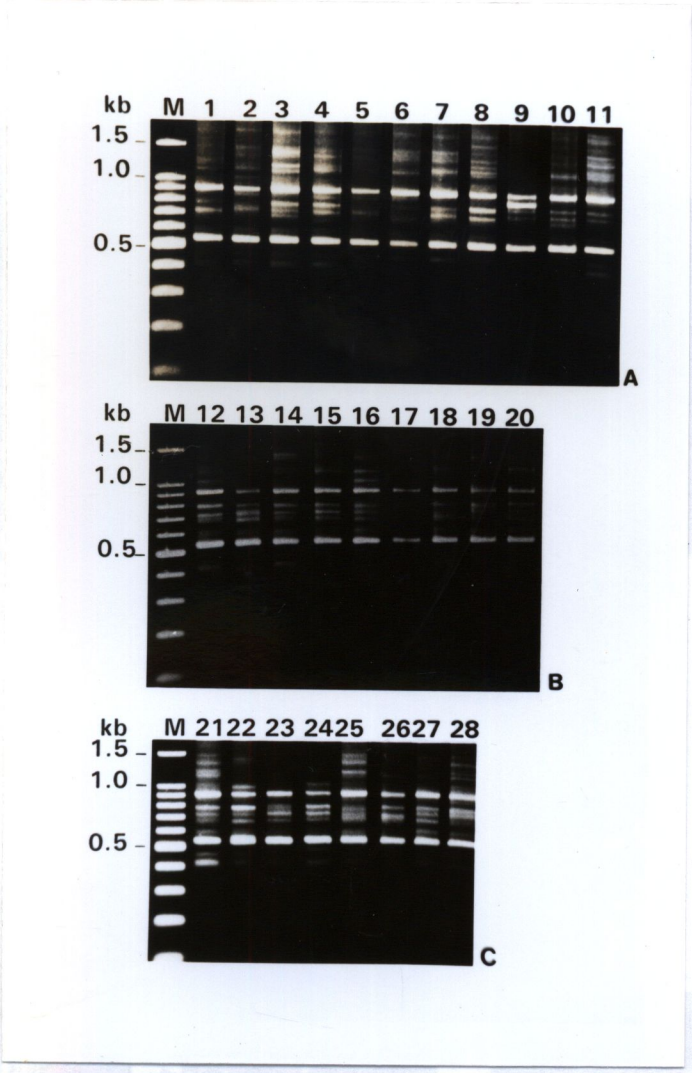




Primer 459

RAPD patterns of 28 individuals of the Trad sample



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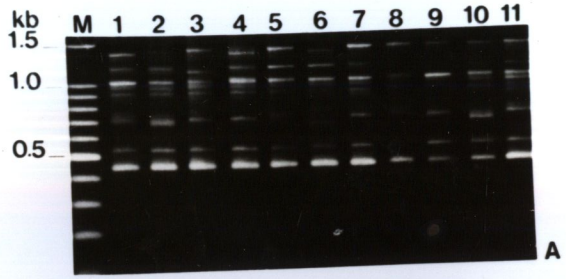

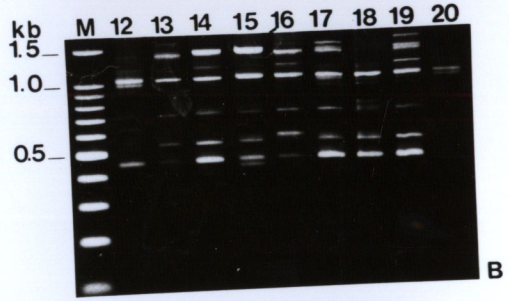

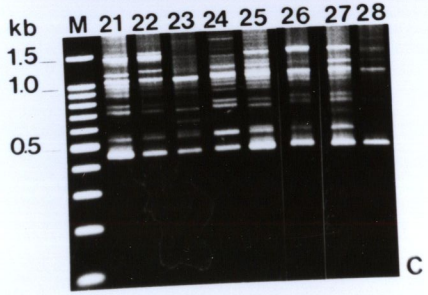

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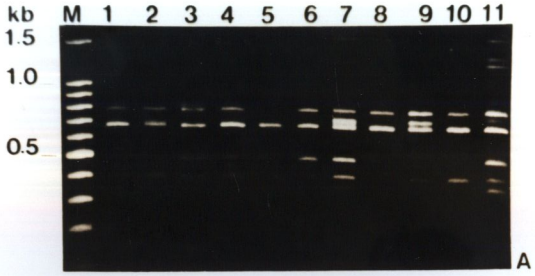

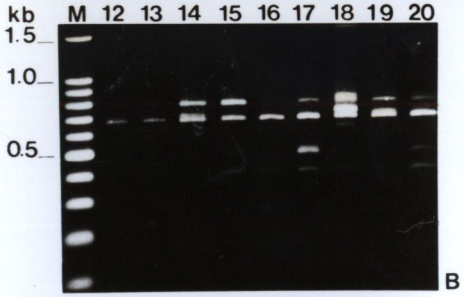

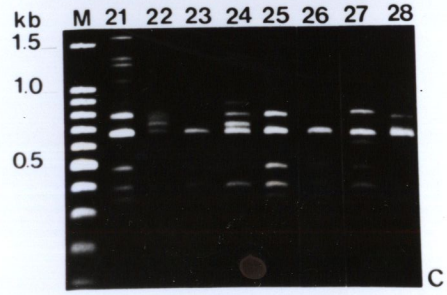

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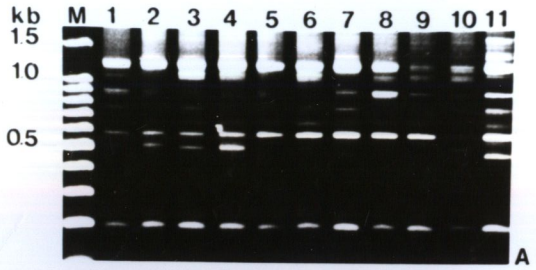

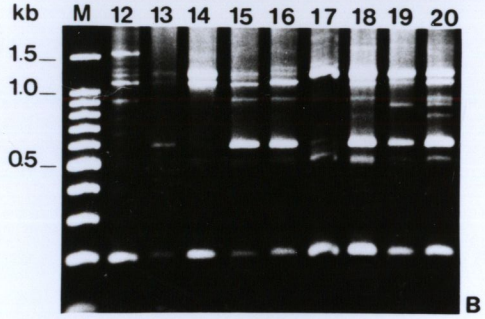

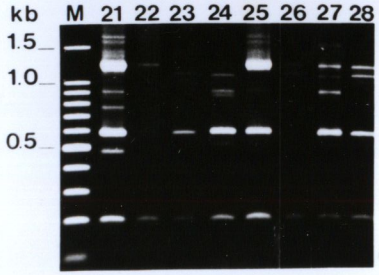

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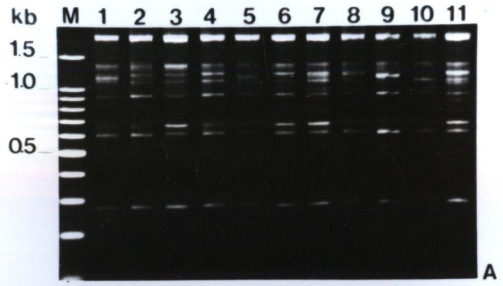

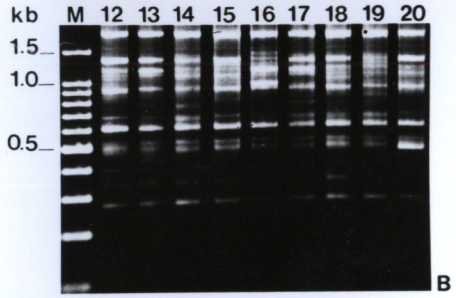

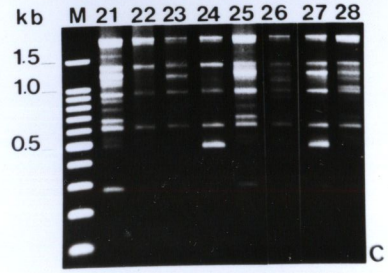

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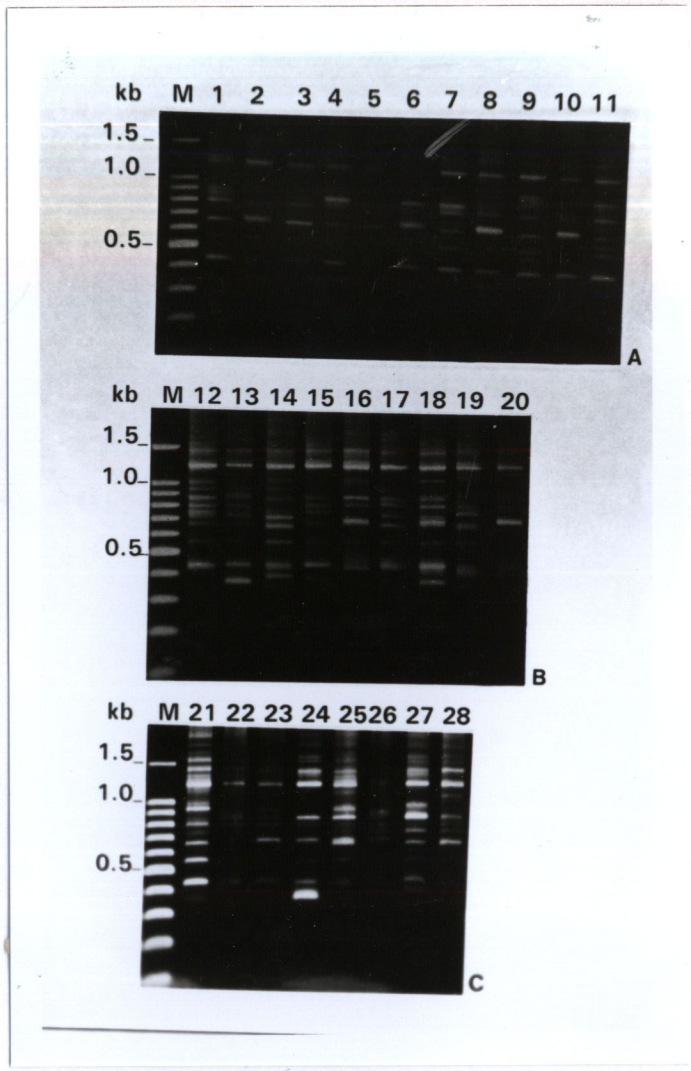

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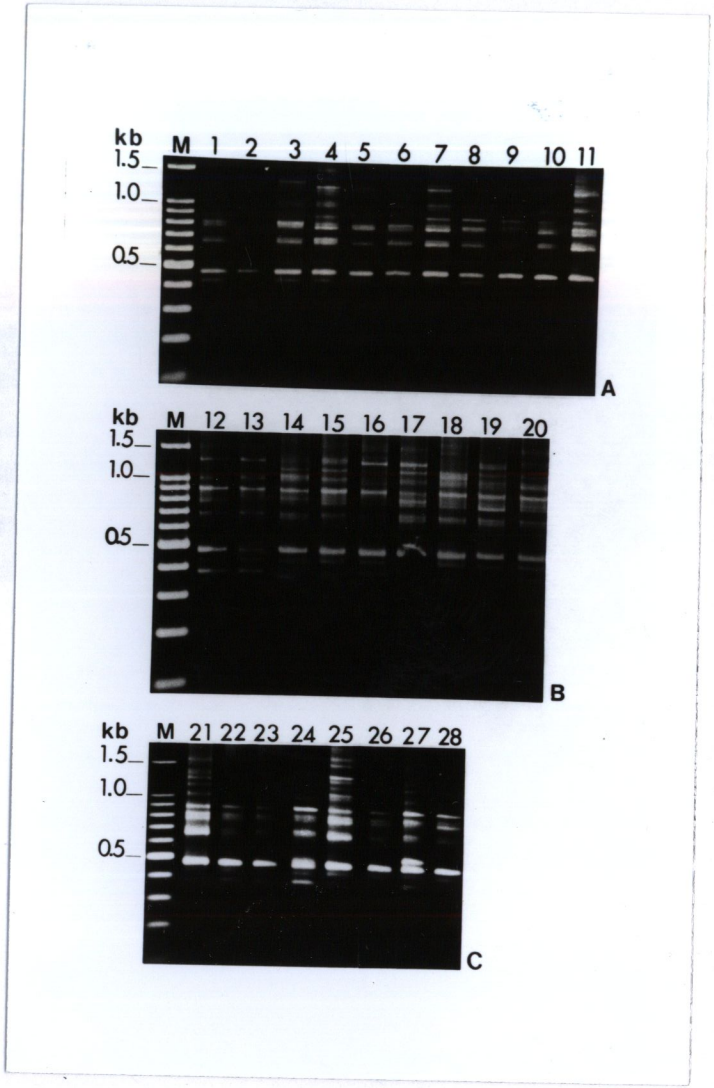
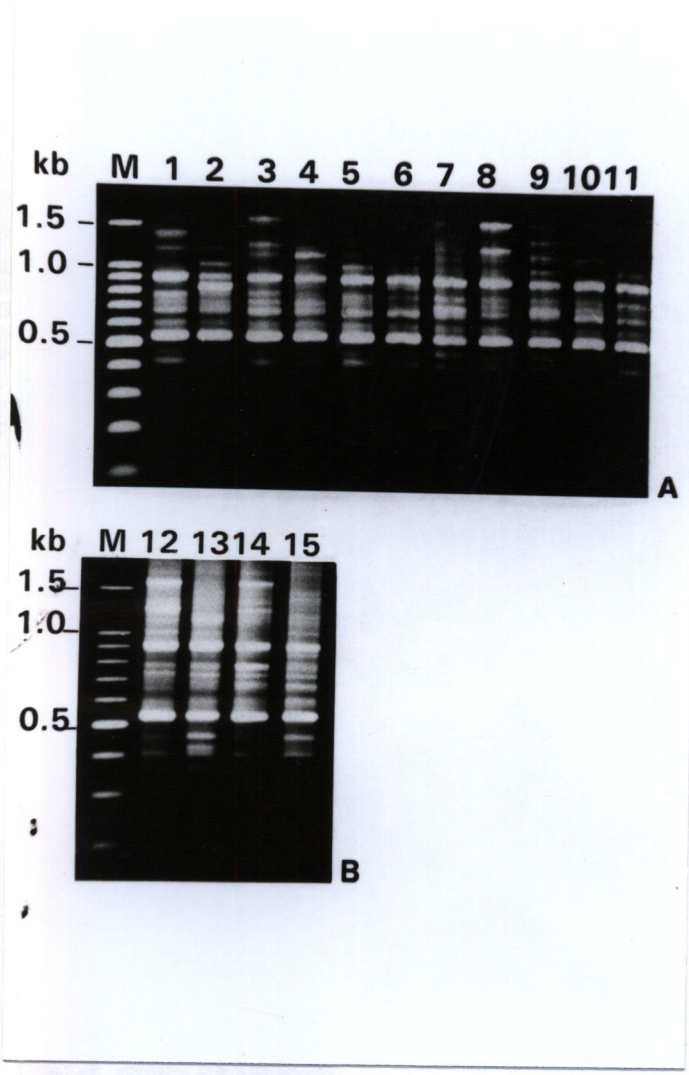


Primer 459

RAPD patterns of 15 individuals of the Angsila sample



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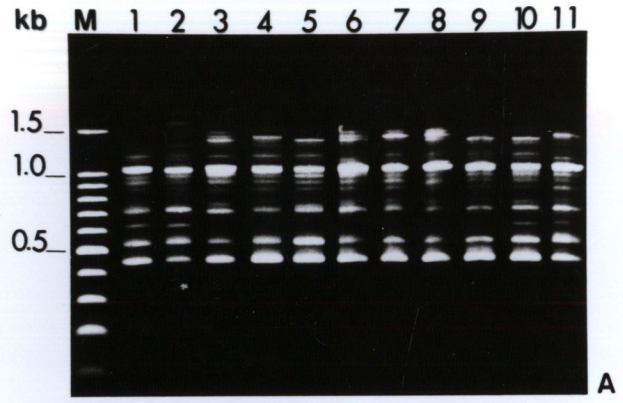

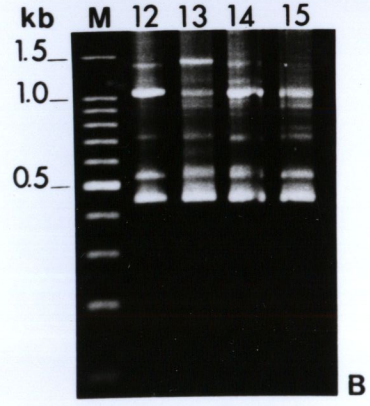

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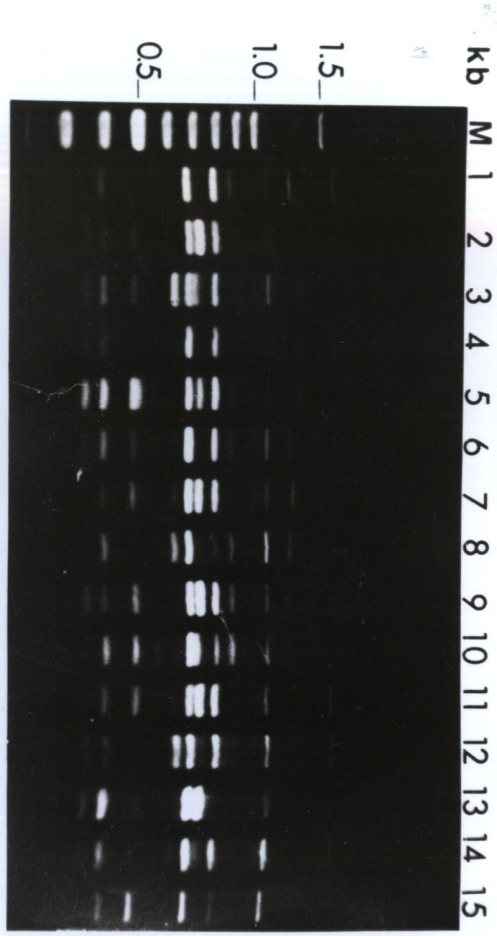

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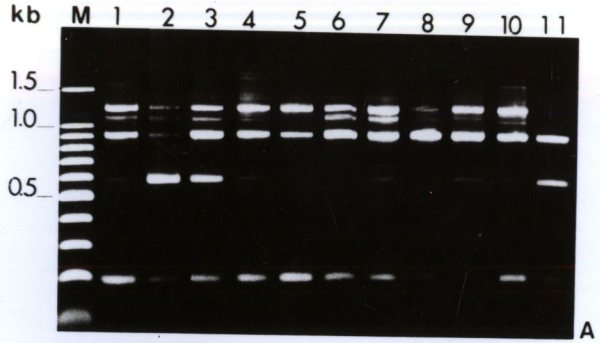

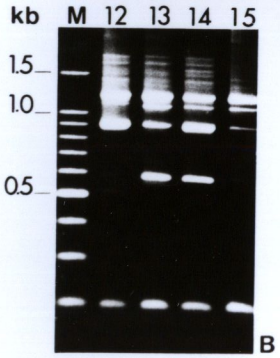

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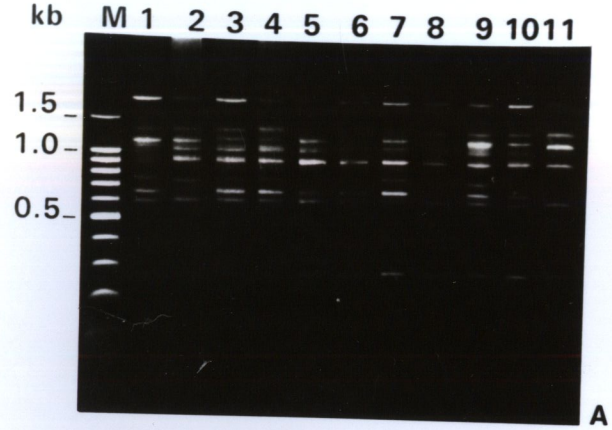

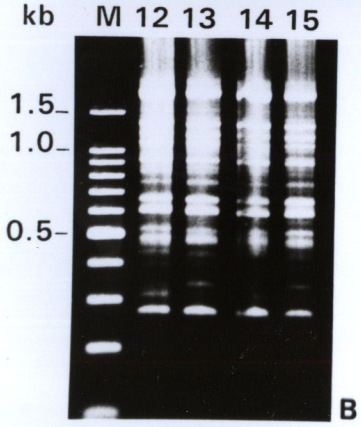

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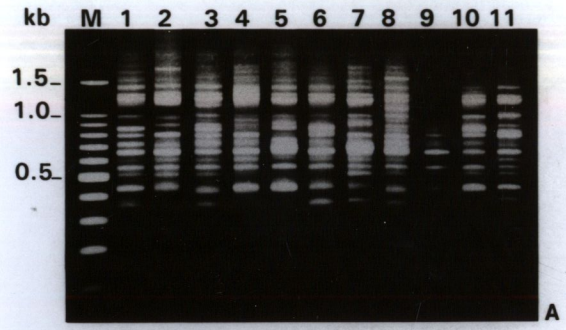

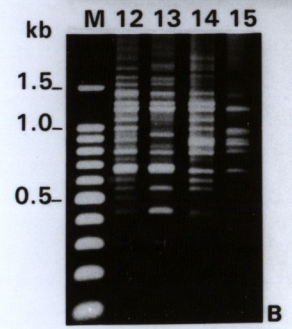

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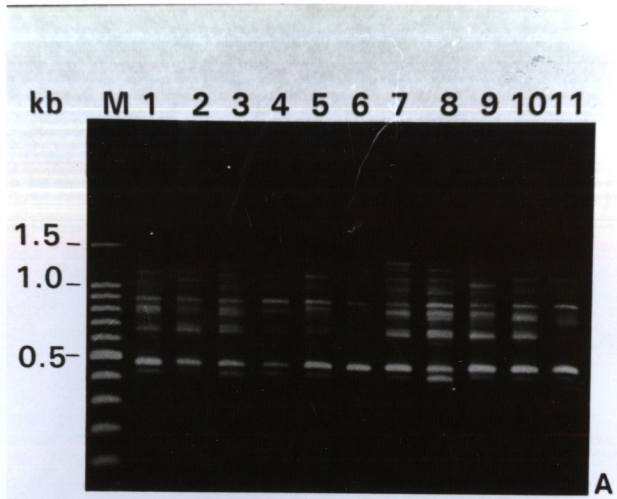
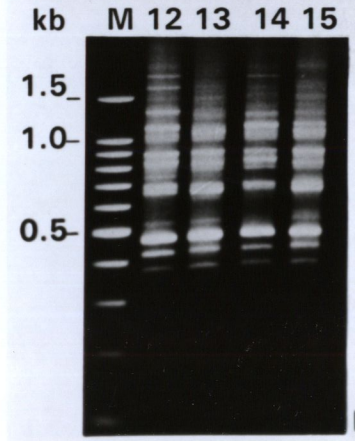
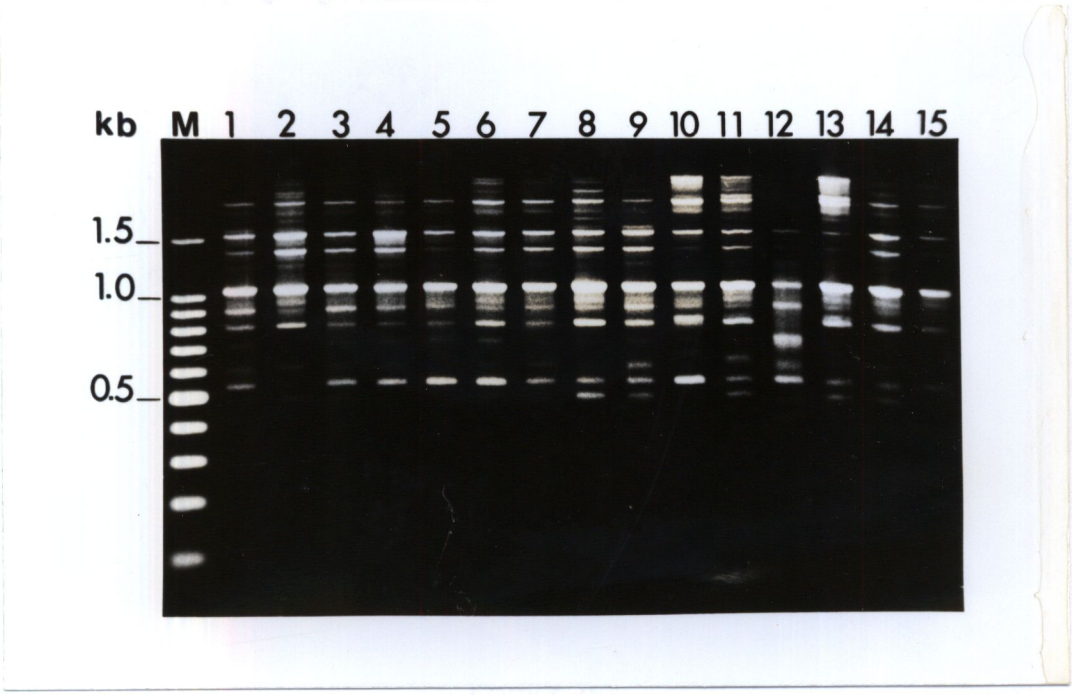


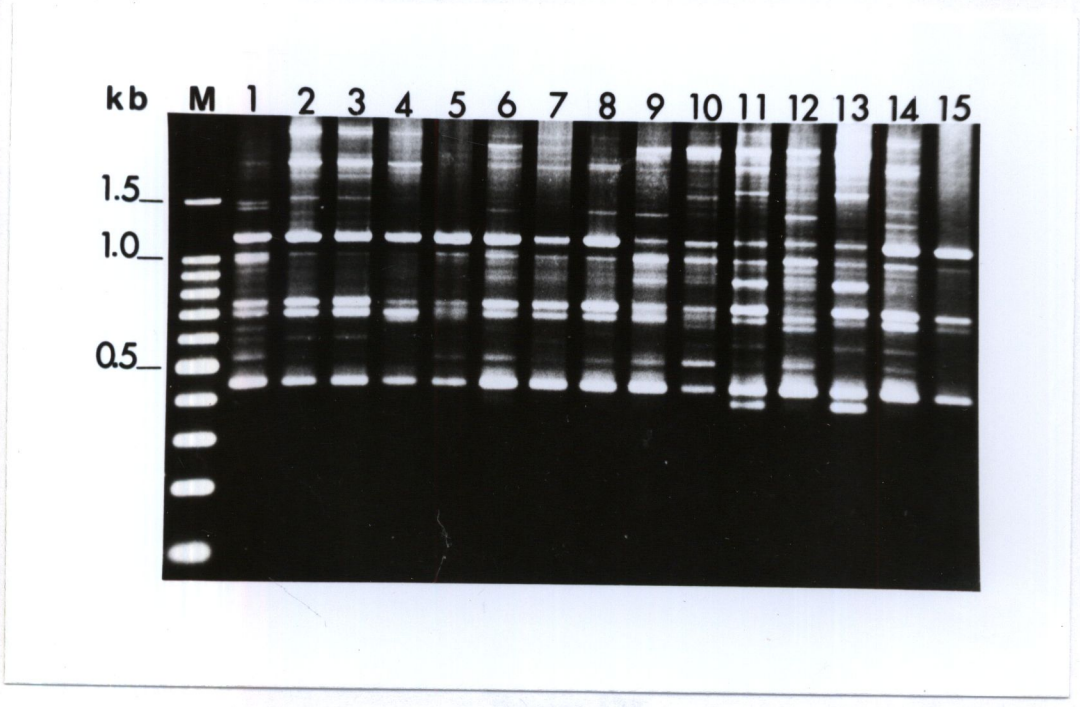


Primer 459

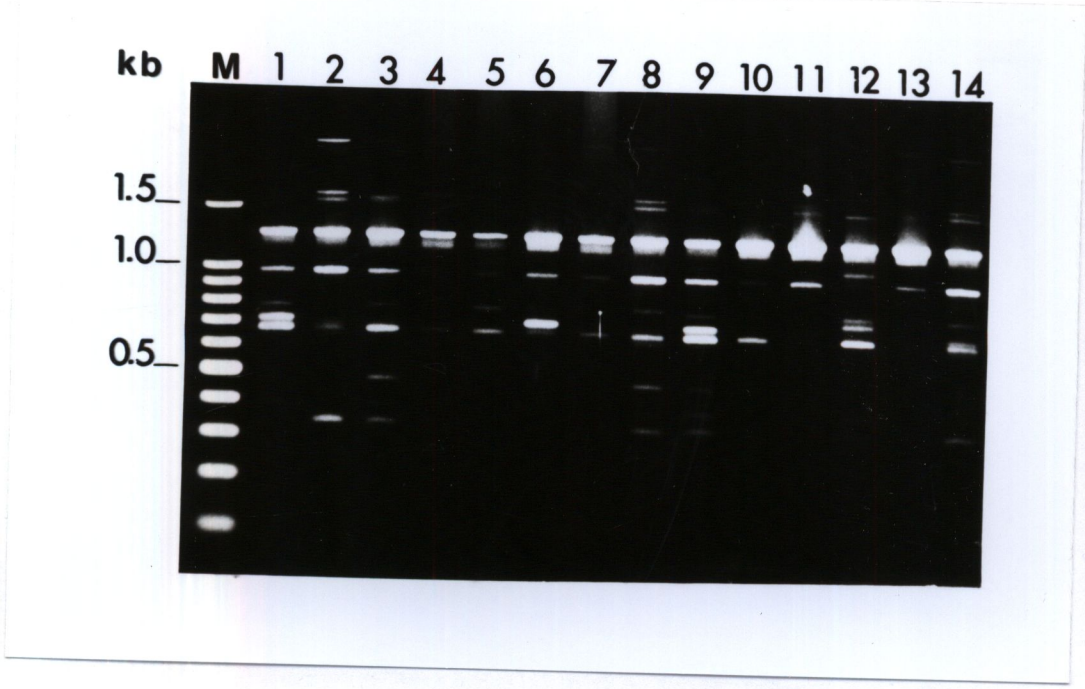
RAPD patterns of 15 individuals of the Medan sample



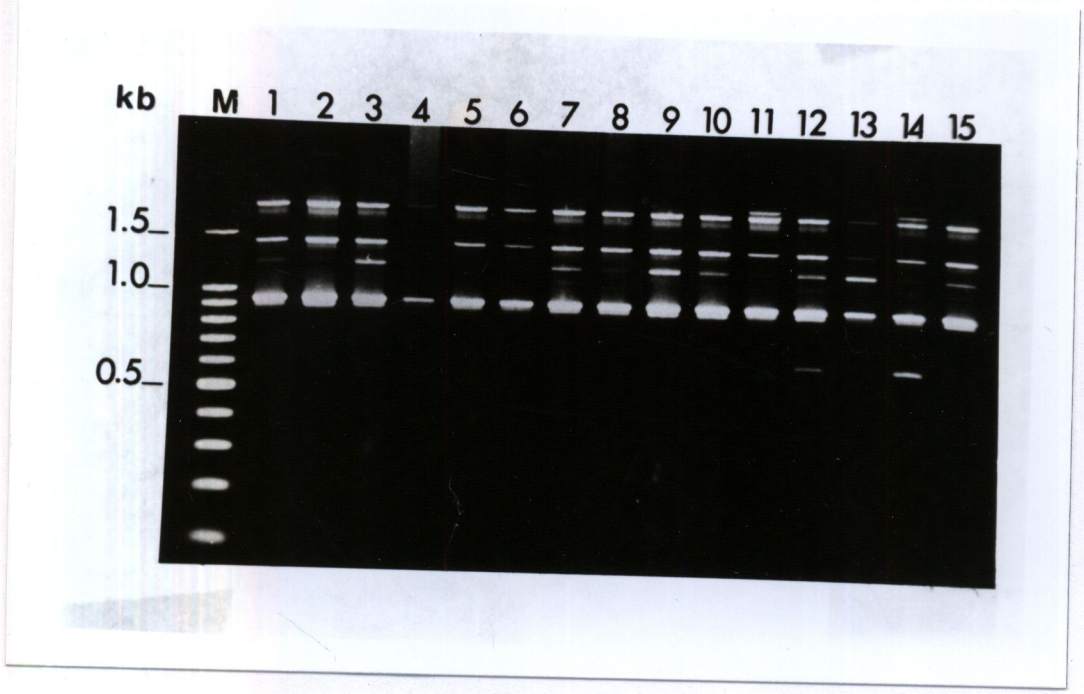
Primer 101



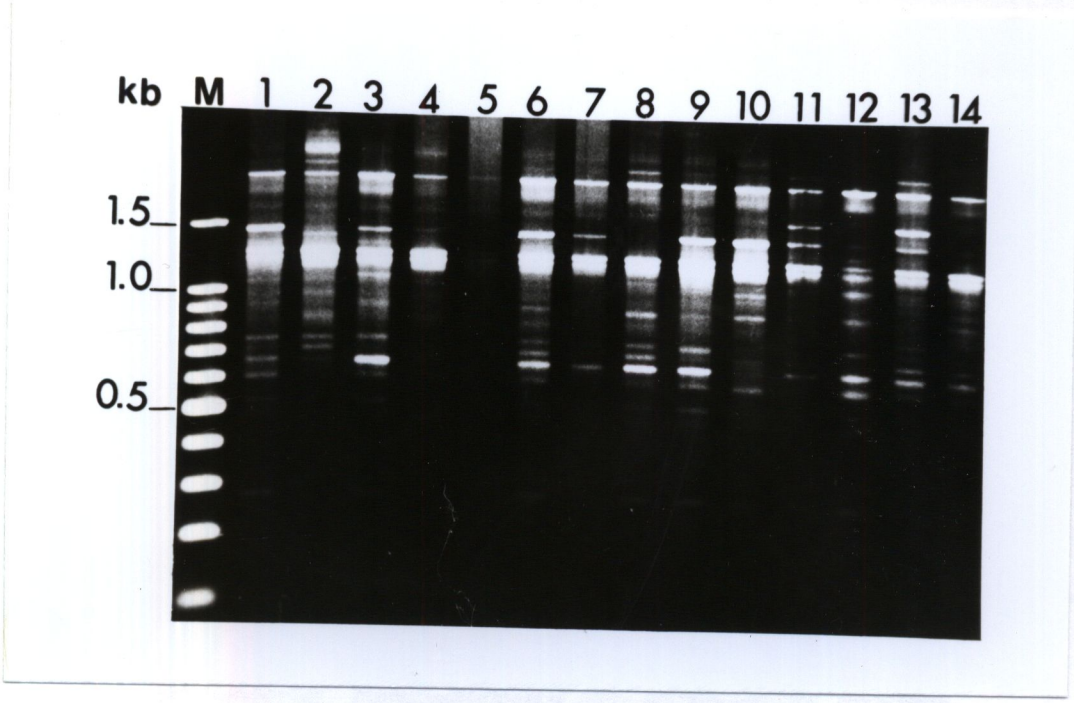
Primer 174



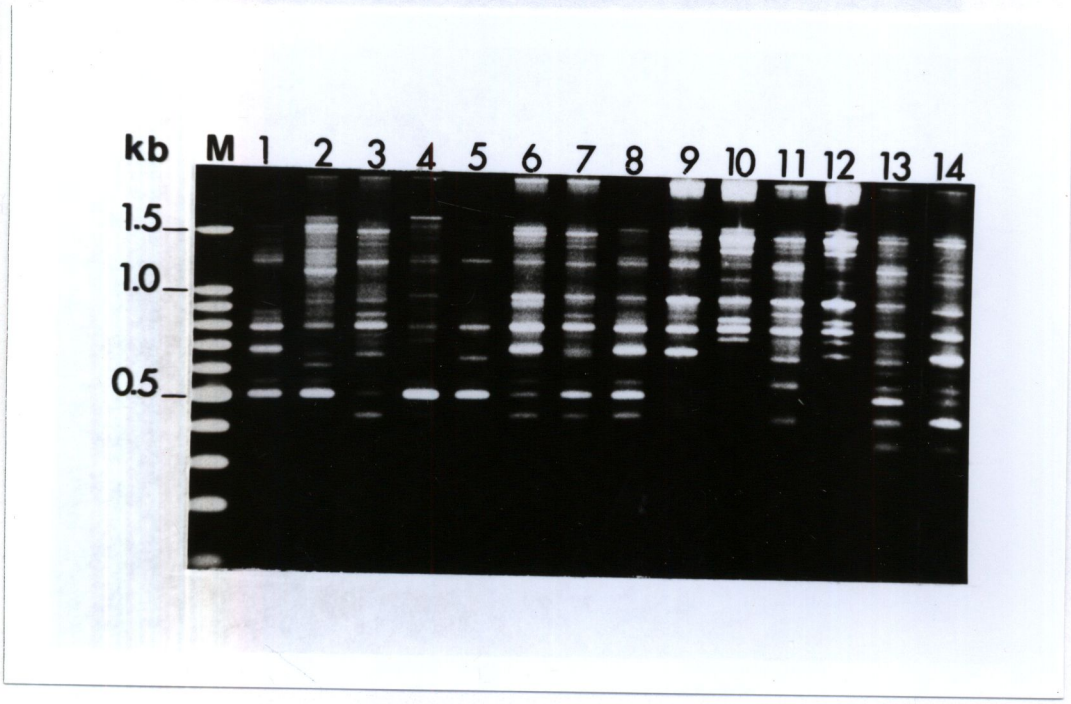
Primer 268



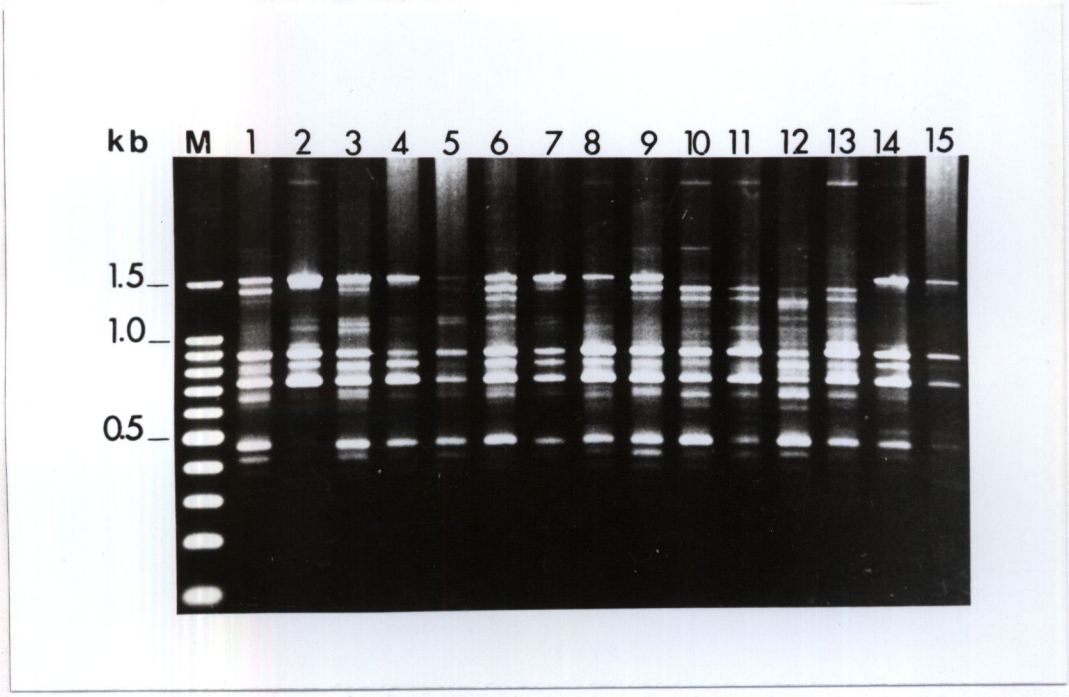
Primer 428



Primer 456



Primer 457



Primer 459

APPENDIX B

Genotypes for each primer in the 4 geographic samples of *P. monodon*

A. primer 101

size (bp)	genotype																							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
1800	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
1550	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	1	0	0	1	1	1	1	1	0
1350	0	1	0	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	1
1050	0	1	1	0	1	1	0	1	1	1	0	0	0	0	1	1	1	1	0	0	0	1	1	1
900	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
810	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	1	1	1
790	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	1	0	0	0	0
710	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	0	1
690	1	0	1	0	1	0	0	0	0	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0
590	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1
520	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
470	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
420	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0

B. primer 174

size (bp)	genotype																																					
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH				
1500	0	0	0	1	0	0	1	1	1	1	0	1	0	0	1	0	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0				
1450	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0	1	0				
1350	0	0	0	0	1	0	1	0	1	1	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	0	0	0	0	1	0	0	0				
1200	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1	1	0	0	1	0	1	1	1	1				
1050	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
720	1	0	1	1	1	1	1	1	1	0	1	1	1	0	0	0	1	0	1	0	1	0	0	0	1	0	1	1	0	1	1	0	1	1				
700	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
600	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	1	1	0	0	1	0	1	1	1	1	1	0	0	0	0	1	1	0				
520	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0				
420	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0				

C. primer 268

size (bp)	genotype																																					
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI			
1250	0	0	0	1	0	1	0	1	1	1	0	0	1	0	0	1	1	1	1	1	0	1	0	0	0	0	0	1	0	1	1	1	1	1	1			
1150	1	0	1	1	1	0	1	0	1	0	0	0	1	0	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	1	1			
980	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1			
890	1	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0			
790	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	0	1	1			
710	0	0	1	1	0	0	1	0	0	1	1	1	0	0	0	1	1	1	1	0	1	0	1	0	0	0	0	0	1	0	1	1	1	1	1			
690	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
490	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0			
400	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0			

D. primer 428

size (bp)	genotype																													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD
1800	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1400	0	1	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0	1
1250	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1
1150	1	0	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1	0	0	0	0	1	0	1	1	0	0	1	1	1
950	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	0	0	1
900	1	0	1	1	0	1	0	1	1	1	1	0	0	1	1	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0
880	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	0	0
590	1	0	1	1	1	0	1	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	0	1	0	0	0
200	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0

E. primer 456

size (bp)	genotype																					
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
2100	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0
2000	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1400	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	1	1	1	0	0	0
1300	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	1
1250	0	1	0	0	1	0	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	0
1200	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
1100	1	1	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
920	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	1	1
750	1	1	1	1	1	0	1	1	0	0	1	0	1	1	1	1	0	0	0	0	0	1
700	0	0	0	0	1	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1
650	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1
600	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1
520	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1
480	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
290	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

F. primer 457

size (bp)	genotype																																												
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR	AS
2200	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
1250	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
950	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	1	1	0	1	1	1	1	0	0	0	1	0	1	1	1	0	0	0	0	1	0	1	0	0
900	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	1	1	0	1	0	1	0	0	1	1	0	0	0	0	0
850	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0	0	0	1	1	0	0	1	0	0	0	1
800	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
730	1	1	0	0	1	1	0	1	1	1	0	0	1	1	1	0	0	0	0	1	0	1	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1	0
650	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	0	0	0
600	0	1	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0	1	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0
550	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
500	0	1	1	0	0	1	1	1	0	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	0	0	1	1	1	0	1	0	1	0	1	1	0	0	1	1	1	1	1	1	1
420	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
350	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	0	0	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	0	0	0

G. primer 459

size (bp)	genotype																							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
1550	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	1	1	0	0
1450	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	1	1	1
900	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1
800	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
790	1	1	0	0	1	1	0	1	1	0	0	0	0	1	1	1	1	1	1	0	1	0	1	1
730	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1
700	1	1	0	1	0	0	0	1	1	1	1	0	1	0	0	1	1	1	1	0	0	0	1	0
690	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1
620	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	1	0	0	0	1	0
480	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
430	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

APPENDIX C

Data analysis of new Satun-Trang sample

Estimated similarity (S) for each primer within the 5 samples of *P. monodon*

Primer	Satun-Trang1*	Satun-Trang2*	Angsila	Trad	Medan
101	0.9149	0.8818	0.9146	0.9099	0.9605
174	0.8619	0.8056	0.8412	0.8216	0.8651
268	0.8044	0.8874	0.8283	0.8239	0.7640
428	0.8208	0.8488	0.9151	0.8086	0.9420
456	0.9149	0.9598	0.9937	0.9284	0.8016
457	0.8512	0.9243	0.9269	0.8279	0.8648
459	0.8904	0.8457	0.9120	0.9316	0.8403
Mean	0.8655	0.8791	0.9047	0.8646	0.8626
SD	0.0437	0.0518	0.0556	0.0557	0.0705

*Satun-Trang1 = previous Satun-Trang, Satun-Trang2 = new Satun-Trang

Estimated similarlity (S_{ij}) for each primer between the 5 samples of *P. monodon*

Primer	S-T1/T*	S-T1/A*	S-T1/M*	S-T1/S-T2*	S-T2/T*	S-T2/A*	S-T2/M*	T/A*	T/M*	A/M*
101	0.9614	0.9704	0.6518	0.9860	0.9688	0.9539	0.6467	0.9303	0.6448	0.7011
174	0.9442	0.9813	0.9144	1.0300	1.0438	1.0158	0.8958	0.9552	0.8762	0.8902
268	0.9512	0.9743	0.6886	0.9501	0.9407	0.9733	0.6726	0.8787	0.7186	0.7000
428	0.8801	0.8621	0.6344	0.9555	0.8745	0.8586	0.6662	0.9863	0.5174	0.5151
456	0.9820	0.9868	0.8712	0.9968	1.0019	0.9960	0.8084	0.9717	0.8696	0.8369
457	0.9641	0.9318	0.9133	0.9217	0.9562	0.8572	0.8483	0.9219	0.8831	0.8117
459	0.9516	0.9978	0.8435	0.9628	0.9482	1.0291	0.7990	0.9799	0.7739	0.8389
Mean	0.9478	0.9578	0.7882	0.9718	0.9620	0.9548	0.7624	0.9463	0.7548	0.7563
SD	0.0323	0.047	0.125	0.0354	0.0527	0.0708	0.0994	0.0385	0.1382	0.1283

*S-T1= previous Satun-Trang, S-T2 = new Satun-Trang, T = Trad, A = Angsila and M = Medan

Estimated of genetic distance for each primer between the 5 samples of *P. monodon*

A. All primers

	S-T1*	S-T2*	T*	A*	M*
S-T1	-				
S-T2	0.0328	-			
T	0.0622	0.0466	-		
A	0.0487	0.0519	0.0624	-	
M	0.2812	0.3186	0.3340	0.3225	-

B. Primer 101

	S-T1*	S-T2*	T*	A*	M*
S-T1	-				
S-T2	0.0155	-			
T	0.0432	0.0353	-		
A	0.0330	0.0526	0.0795	-	
M	0.4638	0.4829	0.4774	0.3837	-

C. Primer 174

	S-T1*	S-T2*	T*	A*	M*
S-T1	-				
S-T2	- 0.0359	-			
T	0.0684	- 0.0525	-		
A	0.0222	- 0.0192	0.0553	-	
M	0.1044	0.1326	0.1585	0.1378	-

D. Primer 268

	S-T1*	S-T2*	T*	A*	M*
S-T1	-				
S-T2	0.0596	-			
T	0.0618	0.0711	-		
A	0.032	0.0310	0.1588	-	
M	0.5056	0.5022	0.4370	0.4722	-

E. Primer 428

	S-T1*	S-T2*	T*	A*	M*
S-T1	-				
S-T2	0.05466	-			
T	0.1592	0.1639	-		
A	0.1716	0.1740	0.0142	-	
M	0.5334	0.4651	0.7987	0.7386	-

F. Primer 456

	S-T1*	S-T2*	T*	A*	M*
S-T1	-				
S-T2	0.0032	-			
T	0.0197	-0.0022	-		
A	0.0131	0.0040	0.0294	-	
M	0.1605	0.2413	0.1607	0.1948	-

G. Primer 457

	S-T1*	S-T2*	T*	A*	M*
S-T1	-				
S-T2	0.0915	-			
T	0.0437	0.0498	-		
A	0.0790	0.1675	0.0916	-	
M	0.1066	0.1853	0.1484	0.2354	-

H. Primer 459

	S-T1*	S-T2*	T*	A*	M*
S-T1	-				
S-T2	0.0435	-			
T	0.0544	0.0589	-		
A	0.0023	- 0.0333	0.0220	-	
M	0.1991	0.2725	0.2934	0.2023	-

*S-T1 = previous Satun-Trang, S-T2 = new Satun-Trang, T = Trad, A = Angsila and M = Medan

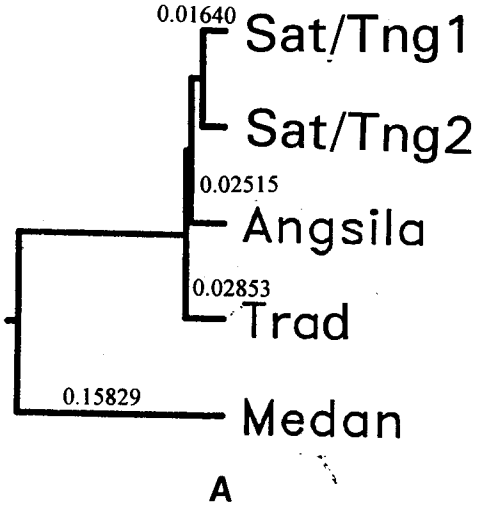
Phenograms showing the relationships among the 5 samples of *P.monodon*, generated according to UPGMA method of cluster analysis based on distance matrix.

A. For all primers.

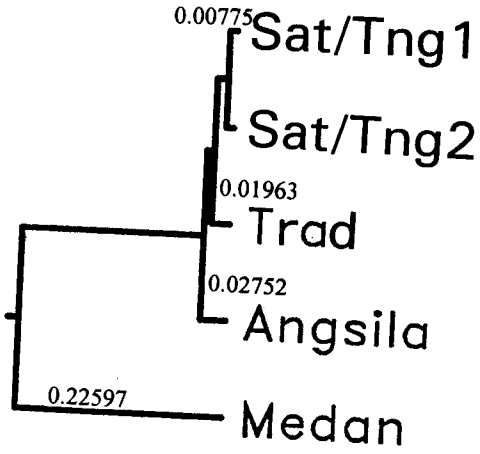
B. For primer 101.

C. For primer174.

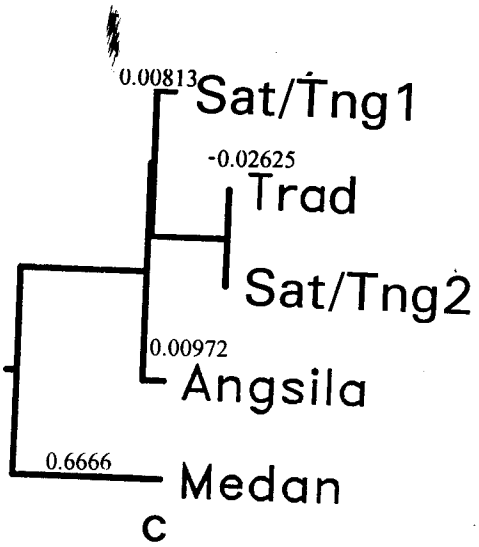
D. For primer 268.


0.01640
Sat/Tng1
Sat/Tng2
0.02515
Angsila
0.02853
Trad
0.15829
Medan

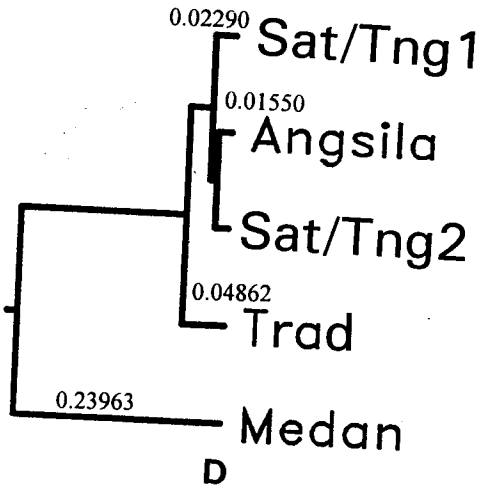
A


0.00775
Sat/Tng1
Sat/Tng2
0.01963
Trad
0.02752
Angsila
0.22597
Medan

B


0.00813
Sat/Tng1
-0.02625
Trad
Sat/Tng2
0.00972
Angsila
0.6666
Medan

C


0.02290
Sat/Tng1
0.01550
Angsila
Sat/Tng2
0.04862
Trad
0.23963
Medan

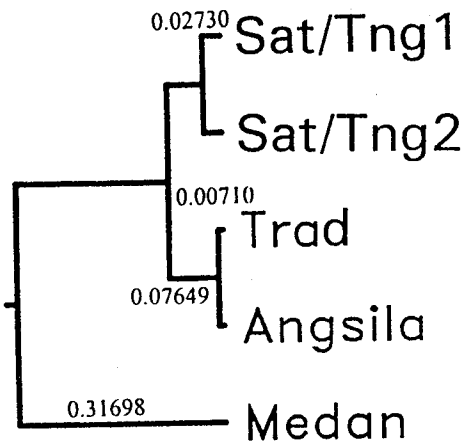
D

E . For primer 428

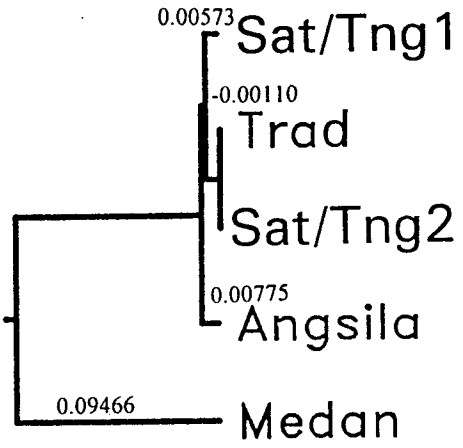
F . For primer 456

G . For primer 457

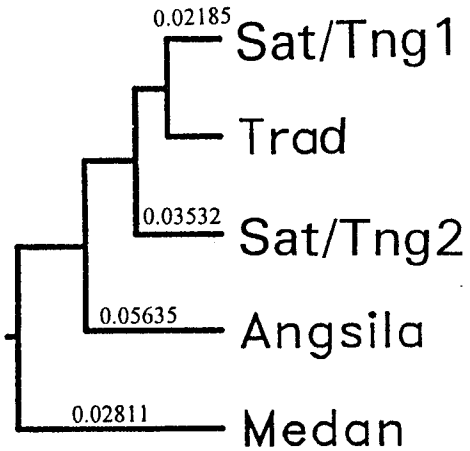
H . For primer 459


0.02730
Sat/Tng1
Sat/Tng2
0.00710
Trad
0.07649
Angsila
0.31698
Medan

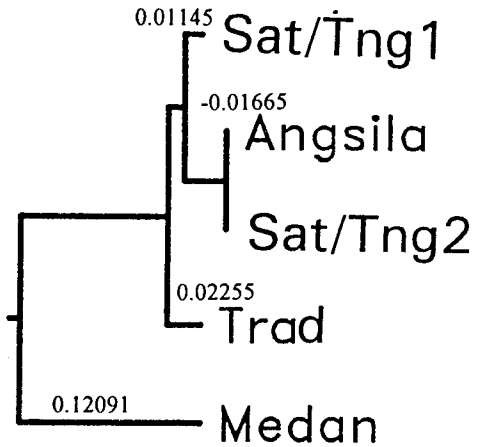
E


0.00573
Sat/Tng1
-0.00110
Trad
Sat/Tng2
0.00775
Angsila
0.09466
Medan

F


0.02185
Sat/Tng1
Trad
0.03532
Sat/Tng2
0.05635
Angsila
0.02811
Medan

G


0.01145
Sat/Tng1
-0.01665
Angsila
Sat/Tng2
0.02255
Trad
0.12091
Medan

H

Biography

Miss Siriporn Pongsomboon was born on January 22, 1971 in Chonburi. She graduated with the degree of Bachelor of Science from the Department of Medical Technology at Mahidol University in 1993. In 1994, she has studied in Master degree of Science at the department of Biotechnology, Chulalongkorn University.