GENETIC DIVERSITY OF TROPICAL ABALONE IN THAILAND USING RAPD-PCR

Ms. Aporn Popongviwat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Marine Science
Department of Marine Science
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ความหลากหลายทางพันธุกรรมของหอยเป๋าฮื้อเขตร้อนในประเทศไทย โดย RAPD-PCR

นางสาวอาภรณ์ โพธิ์พงศ์วิวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางทะเล ภาควิชาวิทยาศาสตร์ทางทะเล
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KEY WORD: TROPICAL ABALONE / *HALIOTIS ASININA* / *H. OVINA* / *H. VARIA* / RAPD-PCR / GENETIC DIVERSITY / POPULATION STRUCTURE

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Randomly amplified polymorphic DNA (RAPD) analysis was used to identify species-specific genetic markers of three abalone species in Thailand; *Haliotis asinina*, *H. ovina*, *H. varia*. Five decanucleotide primers (UBC101, OPB11, UBC195, UBC197 and UBC271) were selected for genetic analysis of abalone in Thailand.

Seventy-two reproducible and polymorphic RAPD fragments (320-2300 bp in length) were generated using primers UBC101 and OPB11. One hundred percent of polymorphic bands were found for both primers. High genetic diversity levels between *H. asinina*, *H. ovina* and *H. varia* was observed

Genetic diversity of *H. asinina* were examined using 5 primers (UBC101, OPB11, UBC195, UBC197 and UBC271). One hundred and thirteen reproducible and polymorphic fragments (250-2300 bp in length) were generated. All primers provided high polymorphic levels in this abalone.

The percentages of polymorphic bands of *H. asinina*, *H. ovina* and *H. varia* were 84.91, 94.74 and 91.23, respectively. The average similarity index across overall samples resulted from primer UBC101, OPB11, UBC195, UBC197 and UBC271 were 0.7715, 0.6830, 0.8002, 0.8444 and 0.8396, respectively. The average similarity index within samples of *H. asinina*, *H. ovina* and *H. varia* were 0.7927-0.8496, 0.6010-0.7032 and 0.5259-0.6102, respectively. Average genetic differences within species were 0.2995, 0.4328 and 0.4295 for *H. asinina*, *H. ovina* and *H. varia*, respectively. The average genetic distance of *H. asinina* in the Gulf of Thailand were 0.0243 implying genetically close relationship compared to Talibong Island from the Andaman Sea and the Philippines samples.

A neighbor-joining tree constructed from the average genetic distance between paired geographic samples indicated phylogenetically clear separation between investigated abalone species (using two primers) and geographic samples of *H. asinina* (using five primers). Phylogeography was obviously observed between *H. ovina* originating from the Andaman Sea and the Gulf of Thailand.

The primer UBC101 yielded a species-specific markers in *H. asinina* (1700 bp) and the Philippines sample (380 bp). The primer UBC195 showed species-specific nature in *H. asinina* (1030 and 650 bp). Primer UBC197 provided RAPD markers in *H. asinina* (1450 and 750 bp). Additional species-specific RAPD markers in *H. asinina* (680 bp) and *H. asinina* from Talibong Island (880 bp) were identified by the primer UBC271. No species-specific RAPD markers were observed in *H. ovina* and *H. varia*.

Department	Marine Science	Student's signature	
Field of Study	Marine Science	Advisor's signature	
Academic year	2001	Co-advisor's signature	

อาภรณ์ โพธิ์พงศ์วิวัฒน์ ความหลากหลายทางพันธุกรรมของหอยเป๋าฮื้อเขตร้อนในประเทศไทยโดย RAPD-PCR (GENETIC DIVERSITY OF TROPICAL ABALONE IN THAILAND USING RAPD-PCR) อาจารย์ที่ปรึกษา : รศ. ดร. เผดิมศักดิ์ จารยะพันธุ์, อาจารย์ที่ปรึกษาร่วม : ดร. ศิราวุธ กลิ่นบุหงา, 149 หน้า. ISBN 974-17-0038-5.

ในการค้นหาเครื่องหมายทางพันธุกรรมที่จำเพาะต่อหอยเป๋าฮื้อ 3 ชนิดที่พบในประเทศไทย ได้แก่ *Haliotis asinina*. *H. ovina* และ *H. varia* ด้วยเทคนิคอาร์เอพีดี โดยการคัดเลือกไพรเมอร์ขนาด 10 นิวคลีโอไทด์ จำนวน 5 ไพรเมอร์ ได้แก่ UBC101, OPB11, UBC195, UBC197 และ UBC271 นำมาใช้ในการวิเคราะห์ลักษณะทางพันธุกรรมของหอยเป๋าฮื้อในประเทศไทย

จากการศึกษาหอยเป๋าฮื้อทั้งสามชนิดได้แก่ *H. asinina*, *H. ovina* และ *H. varia* ด้วยไพรเมอร์ UBC101, OPB11 พบว่าสามารถให้รูปแบบของแถบดีเอ็นเอเหมือนเดิมเมื่อทำซ้ำและให้ความหลากหลายของแถบดีเอ็นเอ จำนวนทั้งสิ้น 72 แถบ ซึ่งมีขนาดอยู่ในช่วง 320-2300 คู่เบส และพบเปอร์เซ็นต์ความหลากหลายของแถบดีเอ็นเอเท่ากับ 100% ในทั้งสองไพรเมอร์ ระดับความหลากหลายทางพันธุกรรมระหว่างทั้งสามชนิดมีค่าสูง

ความหลากหลายทางพันธุกรรมของ *H. asinina* โดยเทคนิคอาร์เอพีดีด้วยไพรเมอร์ UBC101, OPB11, UBC195, UBC197 และ UBC271 พบแถบดีเอ็นเอจำนวน 113 แถบ ซึ่งมีขนาดอยู่ในช่วง 250-2300 คู่เบส ทุกไพรเมอร์ให้ระดับความหลากหลายทางพันธุกรรมสูงในหอยเป๋าฮื้อชนิดนี้

เมื่อคำนวณเปอร์เซ็นต์ความหลากหลายของแถบดีเอ็นเอใน *H. asinina*, *H. ovina* และ *H. varia* พบว่ามีค่าเท่ากับ 84.91%, 94.74% และ 91.23% ตามลำดับ ค่าดัชนีความเหมือนเฉลี่ยของตัวอย่างทั้งหมดจาก 5 ไพรเมอร์มีค่า 0.7715, 0.6830, 0.8002, 0.8444 และ 0.8396 ในไพรเมอร์ OPB11, UBC101, UBC195, UBC197 และ UBC271 ตามลำดับ ค่าดัชนีความเหมือนระหว่างชนิดมีค่าอยู่ในช่วง 0.7927-0.8496, 0.6010-0.7032 และ0.5259-0.6102 ในชนิด *H. asinina*, *H. ovina* และ *H. varia* ตามลำดับ ค่าความแตกต่างทางพันธุกรรมเฉลี่ยระหว่างหอยเป๋าฮื้อ 3 ชนิดมีค่าเท่ากับ 0.2995, 0.4328 และ 0.4295 ในชนิด *H. asinina*, *H. ovina* และ *H. varia* ตามลำดับ ค่าความแตกต่างทางพันธุกรรมเฉลี่ยของ *H. asinina* ในอ่าวไทยมีค่าเท่ากับ 0.0243 ซึ่งแสดงถึงความคล้ายคลึงกันภายในกลุ่มประชากร *H. asinina* ในอ่าวไทยสูงเมื่อเทียบกับกลุ่มประชากร *H. asinina* เกาะตะลิบงจากฝั่งอันดามันและจากประเทศฟิลิปปินส์

แผนภูมิความสัมพันธ์เชิงวิวัฒนาการที่ได้จากค่าความแตกต่างทางพันธุกรรมเฉลี่ยระหว่างกลุ่มประชากรต่าง ๆ พบความแตกต่างอย่างชัดเจนระหว่างหอยเป๋าฮื้อทั้งสามชนิด (ข้อมูลจาก 2 ไพรเมอร์) และระหว่างกลุ่มประชากรของหอยเป๋าฮื้อชนิด *H. asinina* (ข้อมูลจาก 5 ไพรเมอร์) ส่วน *H. ovina* พบความแตกต่างอย่างชัดเจนระหว่างกลุ่มประชากรจากฝั่งทะเลอ่าวไทยและทะเลอันดามัน

พบเครื่องหมายทางพันธุกรรมที่ได้จากไพรเมอร์ UBC101 ให้แถบดีเอ็นเอที่จำเพาะต่อชนิด *H. asinina* (1700 คู่เบส) และจำเพาะต่อกลุ่มประชากรประเทศฟิลิปปินส์ (380 คู่เบส) ไพรเมอร์ UBC195 ให้แถบดีเอ็นเอที่จำเพาะต่อชนิด *H. asinina* (1030 และ 650 คู่เบส) ไพรเมอร์ UBC197 ให้แถบดีเอ็นเอที่จำเพาะต่อชนิด *H. asinina* (1450 และ 750 คู่เบส) นอกจากนี้ไพรเมอร์ UBC271 ให้แถบดีเอ็นเอที่จำเพาะต่อชนิด *H. asinina* (680 คู่เบส) และกลุ่มประชากรจากเกาะลิบง (880 คู่เบส) โดยการศึกษาครั้งนี้ไม่พบแถบดีเอ็นเอที่จำเพาะต่อ *H. ovina* และ *H. varia*

ภาควิชา	วิทยาศาสตร์ทางทะเล	ลายมือชื่อนิสิต	
สาขาวิชา	วิทยาศาสตร์ทางทะเล	ลายมือชื่ออาจารย์ที่ปรึกษา	
ปีการศึกษา	2544	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม	

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LIST OF ABBREVIATIONS

A, C, G, T	=	nucleotide containing the base adenine, cytosine, guanine and thymine, respectively
bp	=	base pair
°C	=	degree Celsius
cm	=	centimetre
dATP	=	deoxyadenosine triphosphate
dCTP	=	deoxycytosine triphosphate
dGTP	=	deoxyguanosine triphosphate
dTTP	=	deoxythymidine triphosphate
DNA	=	deoxyribonucleic acid
EDTA	=	ethylene diamine tetracetic acid (disodium salt)
EtBr	=	ethydium bromide
HCl	=	hydrochloric acid
Kb	=	kilobase
KCl	=	potassium chloride
L	=	length
$MgCl_2$	=	magnesium chloride
mg	=	milligram
ml	=	millilitre
M	=	molar
MtDNA	=	mitochondrial DNA
ng	=	nonogram
OD	=	optical density
PCR	=	polymerase chain reaction
RAPD	=	randomly amplified polymorphic DNA
RFLP	=	restriction fragment length polymorphism
RNase A	=	ribonuclease A
SDS	=	sodium dodecyl sulfate
Tris	=	tris (hydroxy methyl) aminomethane
µg	=	microgram
µl	=	mocrolitre
µM	=	micromolar
UV	=	ultraviolet
W/V	=	weight by volume

CHAPTER I

INTRODUCTION

1.1 Introduction

Abalones are economically important marine gastropods, commonly consumed as food and utilized decorative ornaments. They can be found throughout the tropical and the temperate zones. A total of 75-100 existing species is allocated within a single family; Haliotidae (Hahn, 1989; Uki, 1989 cited in Jarayabhand and Paphavasit, 1996). The classification of abalone had been determined through external characteristics. The characters for classification of these taxa are based on the ratio of shell to body size, shell sculpture, epipodial structures and biology of tremata (Linberg, 1992 cited in Kaenmanee, 1996). Recently, Geiger (1998) have reported that all existing abalone belong to 17 genera.

The total world production of abalone was estimated to be approximately 13,000 metric tons in 1999 from both fisheries and aquaculture sectors. Of which, 7,165 metric tons accounting for 55.1 % of the total production were from farming. A total of 5,500 metric tons accounting for 75% of the farming production were from Asia, mainly China and Taiwan (Gordon, 2000). There are at least 15 species of abalone, which are being farmed and commercially important (Jarayabhand and Paphavasit, 1996) (Table 1.1). Abalone products are usually in fresh (with shell), frozen, canned, and dried forms.

Table 1.1 Commercially important abalone species (Jarayabhand and Paphavasit, 1996)

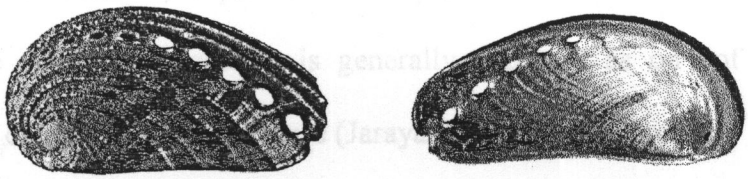
Scientific name	Common name	Shell length (mm)
H. rufescens	Red	> 275
H. fulgens	Green, Southern Green or Blue	125-200
H. corrugata	Pink or Corrugated	150-175
H. sorenseni	White or Sorensen	125-200
H. assimilis	Threaded	<100
H. cracherodii	Black	75-125
H. walallensis	Flat or Northern Green	75-125
H. kamtschatkana	Pinto	100
H. discus hannai	Ezo Awabi	180-200
H. discus	Kuro Awabi, Oni or Onigai	200
*H. diversicolor supertexta**	Tokobushi	50
H. gigantea	Madaka	250
H. sieboldii	Megae	170
*H. asinina**	Mimigai, Donkey's ear	70-100
H. rubra	Black lip	120-140
H. laevigata	Green lip	130-140
H. roei	Roe's	70-80
H. iris	Paua or Black	170
H. australis	Silver or Queen Paua	125
H. virginea	Virgin	70
H. tuberculata	Ormer	120
H. midae	Perlemon	90

* Tropical species

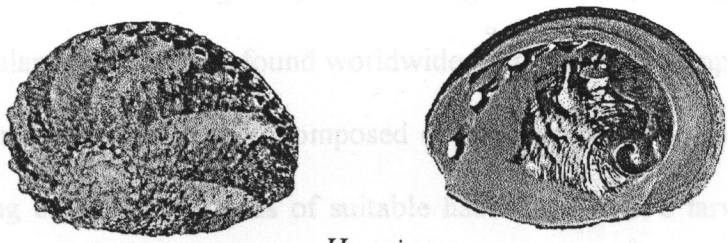
The first fisheries of abalone originated in China and Japan more than 1,500 years ago. However, it is only within the last 30 years that abalone fisheries have spreaded worldwide and become economically important in several countries (Shepherd *et al.*, 1992). Farming of abalone began between the late 1950's and early 1960's in China and Japan and was rapidly developed in the 1990's. It is now widespread in many countries including USA, Mexico, South Africa, Australia, Japan, China, Taiwan, Ireland, Iceland, etc. The largest producer of cultured abalone is China with a total annual production of approximately 3,500 metric tons. The world abalone production from fisheries have consistently decreased by 3% annually since the last 10 years (abalone fisheries was 12,995 metric tons in 1989 and estimated to be 10,150 metric tons in 1999) while the world culture abalone production has increased over 60% each year (689 metric tons in 1989 and 7,775 metric tons in 1999) (Gordon, 2000).

In many Asian countries (China, Japan, Taiwan), large abalone is very popular, but Chen (1989) reported that small abalone are preferred than large species in Taiwan owing to their delicate flavor, appropriate size for banquets and price. The majority of production of the small Taiwanese abalone, *H. diversicolor supertexta*, is from China.

In Thailand, only three species have been reported, *H. asinina* Linnaeus, 1758; *H. ovina* Gemlin, 1791; and *H. varia* Linnaeus, 1758 (Nateewathana and Hylleberg, 1986) (Figure 1.1).



H. asinina



H. ovina

H. varia

Figure 1.1 Three species of abalone found in Thailand; *H. asinina, H. ovina* and *H. varia* (Geiger, 2000).

Some basic biological characteristics of temperate abalone species (growth rate and spawning season) have hindered attempts to cultivated abalone, commercially. In contrast, tropical abalone species lack such disadvantages. Among Thai abalone species, *H. asinina* is generally accepted as one of the promising candidate for commercial scale culture (Jarayabhand *et al.*, in press).

1.2 Biology and Life history of Abalone

Abalones are primitive gastropods featuring a low spire, enlarged body whorl and large muscular foot. They are found worldwide in tropical and temperate oceans.

Abalone stocks are usually composed of discrete local populations, patchily distributed along coastlines in areas of suitable habitats. Abalone larvae have short planktonic larval phase and do not disperse widely from their spawning grounds. Because of their limited dispersal range, genetically distinct populations may occur within few kilometers of each other (Shepherd and Brown, 1993).

1.2.1 Classification

Abalones belong to the phylum Mollusca. They have soft body surrounded by the mantle, an anterior head and a large muscular foot. Mollusks are best known for their beautiful forms and colored shells secreted by the mantle. The abalone joins other snails, whelks and sea slugs as members of the class Gastropoda (one shell). The spiral structure, so common in snail shells, is flattened in abalone. All abalone are members of the family Haliotidae and the genus *Haliotis*, which means "sea ear", referring to the flattened shape of the shell.

The taxonomic definitions of Thai abalone are as follows (Nateewathana and Hylleberg, 1986):

Phylum: Mollusca

Class: Gastropoda

Subclass: Prosobranchia

Order: Archeogastropoda

Suborder: Zygobranchia

Superfamily: Pleurotomariacea

Family: Haliotidae

Genus: *Haliotis*

Scientific name of Thai abalone:

Haliotis asinina (Linnaeus, 1758)

Haliotis ovina (Gmelin, 1791)

Haliotis varia (Linnaeus, 1758)

1.2.2 Morphology and Anatomy

The most noticeable part of abalone is the shell with the row of respiratory pores. The shells are valued because of their inner iridescent layers. The muscular foot has a strong suction power consenting the abalone to clamp tightly to rocky surfaces. The mantle circles the foot as does the epipodium, a sensory structure and extension of foot which bears tentacles. The epipodium projects beyond the shell edge in the living animal. It is the most reliable structure for identifying abalone species, morphologically. The internal organs are arranged around the foot and under the shell. The most conspicuous organ, the gonad, is gray or green in females and cream colored in males. It extends around the side opposite the pores and to the rear of

abalone. Abalone has a pair of eyes, a mouth and an enlarged pair of tentacles. Inside the mouth is a long, file-like tongue called radula, which scrapes algal matter to a size that can be ingested. The gill chamber is next to the mouth and under the respiratory pores. Water is drawn in under the edge of the shell, and then flows over the gills and out the pores. Wastes and reproductive products are carried out in the flow of water. Since it has no obvious brain structure, the abalone is considered to be a primitive animal. However, it does have a heart on its left side and blood flows through the arteries, sinuses and veins, assisted by the surrounding tissues and muscles.

1.2.3 Reproduction

Tropical abalone reaches sexual maturity at a small size. Fertility is high and increase exponentially with size. Abalones are dioecious with external fertilization. Spawning may be controlled by the water temperature. The presence of eggs and sperm in the water mass stimulate other abalones to spawn simultaneously, thus increasing the chance of fertilization. The fertilized eggs then hatch as microscopic, free living trochophore and subsequently veliger larvae. Afterwards, the abalone larvae set to the bottom of the sea, shed their cilia and begin developing the adult shell form. If suitable habitat is located it may grow to maturity. The life cycle of abalone is illustrated by Figure 1.2.

In Thailand, Bussarawit, *et al.*, (1990) studied reproductive biology of *H. varia* at Bon island, Phuket. Mature gonads, with ripe eggs, were found in January, March-April, June and August-October. Spawning, as indicated by gonad index and examination of ovaries, occurred in January-February, April-May, June-July, November-December. The smallest specimen with mature eggs measured was 17.3 mm in shell length. Maximum fecundity was about 3.5 million eggs from a specimen

with the shell length of 41.8 mm. The sex ratio was not significantly different from 1:1 with respect to months and size classes.

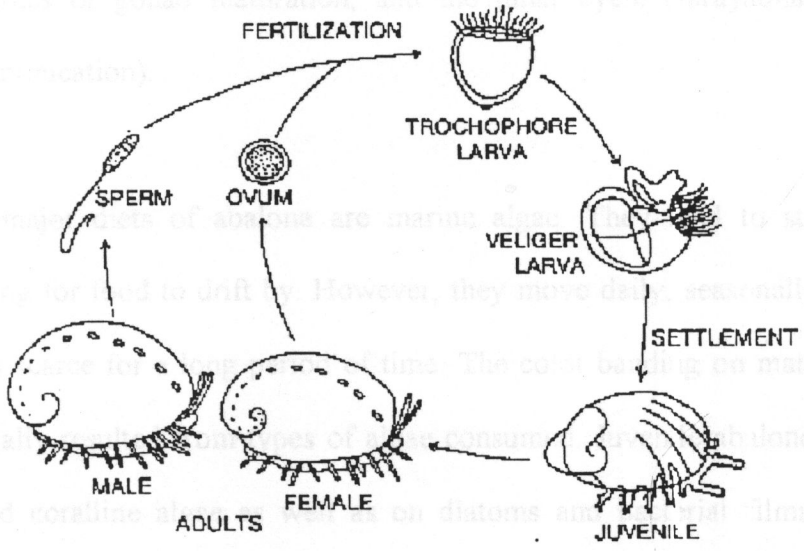


Figure 1.2 Life cycle of abalone (www.abalone.net).

Jarayabhand *et al.* (1992) reported that *H. ovina* are dioecious with the external fertilization, as other species. So far, there has been no evidence of *hermaphrodite. During the spawning season, males can easily be distinguished from* females visually by the color of reproductive tissues lining the surface of the conical appendage, which can be observed by folding back the mantle edge at the rear part of a live specimen. The colors of testes range from creamy white to orange whereas the colors of ovaries are dark green to black. There are two peaks of spawning seasons, June and from November- January.

Recently, Jarayabhand and Paphavasit (1996) revealed that all natural abalones in Thailand spawn all year round. Additionally, year-round spawning was also observed in *H. asinina* cultured in the semi-closed recirculated land-based system and fed the artificial diet. Spawning potential depended on the rearing conditions, nutrition, degrees of gonad maturation, and the lunar cycle (Jarayabhand, 2001; personal communication).

1.2.4 Food

The major diets of abalone are marine algae. They tend to stay in one location waiting for food to drift by. However, they move daily, seasonally or when food becomes scarce for a long period of time. The color banding on many abalone shells are usually resulted from types of algae consumed. Juvenile abalone graze on rock-encrusted coralline algae as well as on diatoms and bacterial films. As they grow, they increasing rely on drifted algae.

1.2.5 Age and growth

Abalone growth rates are highly variable and depend on the availability of food. Determining the age of an individual abalone is difficult. Unlike the hard parts of some animals, abalone shells have no marks or bands suitable for assigning ages. However, juvenile abalone in aquaria grow an inch or more per year for the first two years. Tagging studies have provided estimation larger abalone age in the wild. Red abalones are mature at 1.5 to 2.2 inches when the growth rate begins to slow with increasing age. For instance, a seven-inch red abalone may be 7-10 years old, while one which is only 3/4 of an inch longer may be 15 years old. *H. asinina* in the semi-closed recirculated land-based system have been observed to grow from the shell length of 2-3 cm to 5-6 cm within 8-12 months with artificial diet (Jarayabhand, 2001;

personal communication). This makes *H. asinina* a good candidate for investigating molecular and cellular bases of growth and reproduction.

1.2.6 Habitats

Abalones are usually found on rocky inter-tidal and sub-tidal areas. Each abalone species prefers a particular habitat, which is possibly related to the local sea temperature. Small abalones seek cover in crevices, under rocks or in the spines of sea urchins. This behavior protects them from many predators. Though small abalone hide during the day, they are active at night.

Thai abalones show a very clear nocturnal behavior both naturally and in the hatchery. During the day they seek shelter under rocks or in crevices, dead coral heads or under provided shelters in the case of the hatchery. *H. ovina* prefers the habitat of 1.5-4 meters in depth, and rock crevices (larger than 0.1 meters^2) rather than the coral substrates (Jarayabhand and Paphavasit, 1996). In the Gulf of Thailand, Kakhai and Petjamrat (1992) collected the broodstock of abalone in coral reef and rocky shore areas in Chon Buri Province. They sympatrically found two species of abalone collected at the depths of 2.0 - 8.0 meters composing of *H. asinina* with the shell length of 37.77 - 83.08 mm and *H. ovina* with the shell length of 35.29 - 64.19 mm, respectively. Furthermore, Jarayabhand *et al.*, (1992) studied distributions of abalone around Khang Khao Island and revealed that *H. ovina* was the dominant abalone species. They could be found at the depth of approximately 3.5 meters along the rocky shores around the island. *H. ovina* were usually found attached to the undersides the rocks and some coral heads (*Porites sp.*), often in the same areas where sea urchins (*Diadema serosum*) and some gastropods such as the top shell (*Trochus sp.*) were

found. They attached firmly to rocks along the exposed side of the island where strong water current created well - oxygenated seawater.

1.2.7 Distributions

Members of Haliotidae are globally distributed in modern oceans, especially in the tropical Western Pacific, Australia, Japan, South Africa and along the coast of Northeastern Pacific margins. They can be found in the intertidal areas to the depth of approximately 400 meters (Kaenmanee, 1996).

Geiger (2000) concluded the distributions and biogeography of the Haliotidae worldwide. Three models for the origin of the family Haliotidae are shown in Figure 1.3. The first model; Pacific Rim, was reported by Talmadge (1963) who has identified an arc spanning from Japan to northeastern Australia (Figure 1.3A). The second model; Indo-Pacific, was proposed by Lindberg (1992) who indicated that the highest diversity of the family at the present is found in the Indo-Malayan area. Although correlations of high present day diversity with the origin of the group in question is highly problematic, it provides one possible center of radiation for the abalone family (Lindberg, 1992; Briggs; 1999) (Figure 1.3B). The final model; Tethys, considered that Haliotidae has an origin in the Tethys Sea, based on the data from the numbers of chromosome (Geiger and Groves, 1999). Since other Vetigastropoda and basal gastropods have a relatively low chromosome number (2n = 18-20): (Patterson, 1967; Haszprunar, 1998) as compared to those found in the Haliotidae (2n = 28-36): (Geiger and Groves, 1999), a progression from low to high values of chromosome numbers was considered (Figure 1.3C). In addition, Bieler (1992) also concluded that the low chromosome number of patellogastropods is the plesiomorphic conditions within the archaeogastropod grade. It was suggested that a

radiation starting in the Tethys today, represented by the Mediterranean species *Haliotis tuberculata* Linnaeus, 1758, with 2n = 28, moving eastwards to the Indo Pacific (2n = 32), and finally reaching the North Pacific (2n = 36) (Geiger, 2000).

H. asinina Linnaeus, 1758 and *H. ovina* Gmelin, 1791 were found in more than 100 localities whereas *H. varia* Linnaeus, 1758 had the highest occurrence, 317 data points. Figure 1.4 shows the worldwide distribution of *H. asinina*, *H. ovina* and *H. varia* (Geiger, 2000). Distribution of *H. asinina* are found only in South east Asia, Japan and Australia regions but *H. ovina* and *H. varia* are wider spread including Indian Ocean and East Coast of Africa.

Among ASEAN countries, information regarding the biology and checklists have been reported from the Philippines (Fuze, 1981), Indonesia (Robert *et al.*, 1982), Malaysia and Singapore (Purchon and Purchon, 1981). Some studies have been undertaken in Thailand. The first survey on abundance and distribution of *Haliotis* spp along the coast of the Andaman Sea, Thailand, was carried out between 1985-1986 (Nateewathana and Bussarawit, 1988). In addition, Nateewathana and Hylleberg (1986) reported a survey on Thai abalone around Phuket Island and feasibility study of abalone culture in Thailand. A survey on species and distribution of *Haliotis* spp. in Surat Thani, Nakhon Si Thammarat and Songkla in the Gulf of Thailand were reported in the same year (Tookvinas *et al.*, 1986).

Basically, *H. asinina* and *H. ovina* are found around the islands along the eastern coasts of upper Gulf of Thailand, and all three species occur in the Andaman Sea.

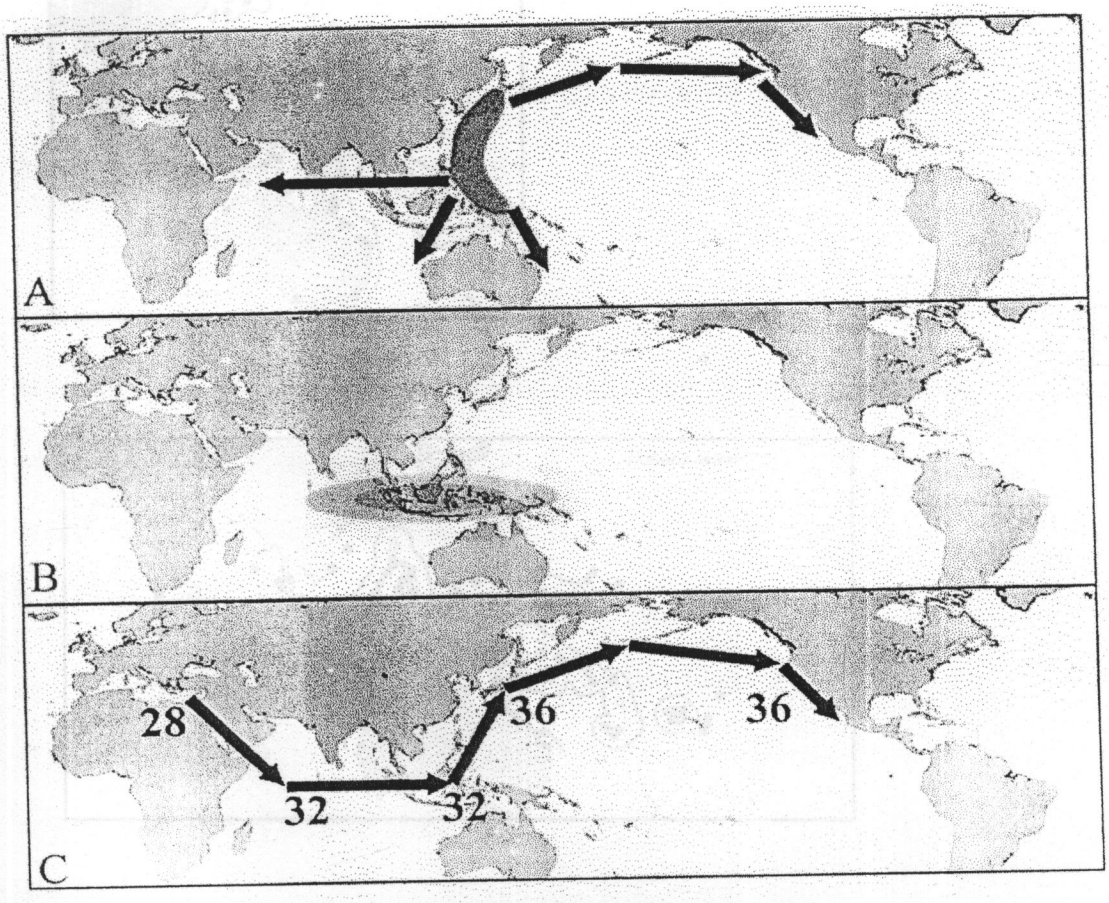
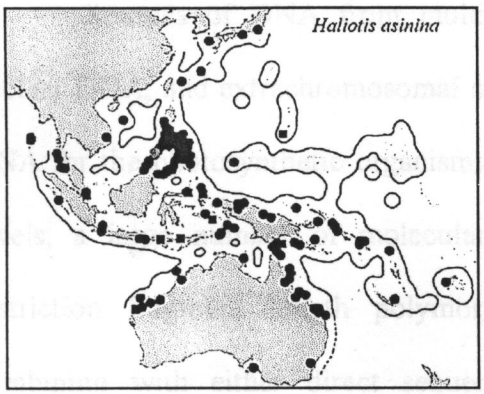


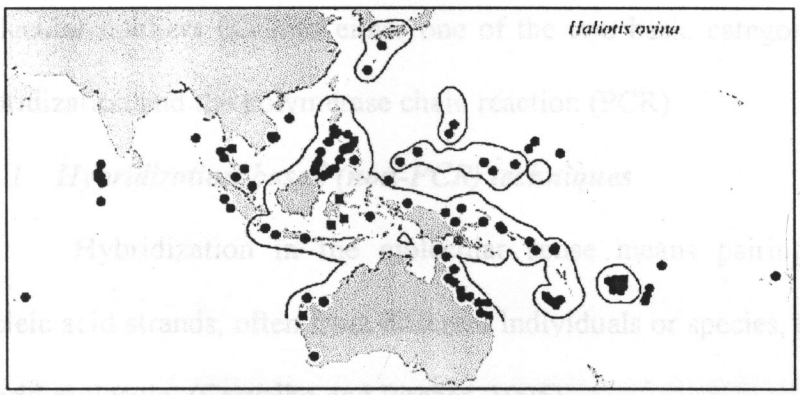
Figure 1.3 Three bio-geographical models proposing the origin of the *Haliotidae* (Geiger, 2000).

A) Pacific Rim origin (Talmadge, 1963)

B) Indo-malayan origin (Lindberg, 1992)

C) Tethys origin (Geiger and Groves, 1999)





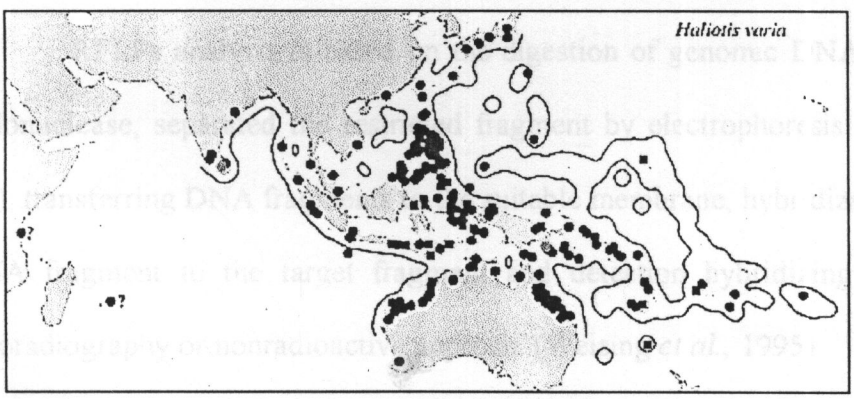


Figure 1.4 Worldwide distributions of abalone, *Haliotis asinina, H. ovina* and *H. varia* (Geiger, 2000).

1.3 DNA Markers

Sources of DNA from multi-cellular living organisms are composed of nuclear DNA, and extrachromosomal mitochondrial DNA (and additional chloroplast DNA for the photosynthetic organisms). To detect the genetic variation at the DNA levels, a large number of molecular techniques has been developed, including restriction fragment length polymorphism (RFLP), polymerase chain reaction combining with either direct sequencing or restriction endonuclease digestion, randomly amplified polymorphic DNA (RAPD) and recently microsatellite loci. Most molecular markers fall into either one of the two basic categories of technique, i.e. hybridization and the polymerase chain reaction (PCR).

1.3.1 Hybridization-based (non-PCR) techniques

Hybridization in the molecular sense means pairing of complementary nucleic acid strands, often from different individuals or species, to from a DNA-DNA hybrid molecule. (Carvalho and Pitcher, 1995).

1.3.1.1 Restriction fragment length polymorphisms (RFLPs)

RFLPs analysis is based on the digestion of genomic DNA by a restriction endonuclease, separated the restricted fragment by electrophoresis (usually agarose gel), transferring DNA fragments to the suitable membrane, hybridization of a labeled DNA fragment to the target fragment and detection hybridizing fragments with autoradiography or nonradioactive approch. (Weising *et al.*, 1995)

The limitations of this method are laborious and expensive. The use of radioactive isotopes for labeling the hybridization probes are also hazardous and required many safety precautions. However, non-radioactive methods have been developed to be an alternative method (Karp *et al.*, 1998).

1.3.1.2 DNA fingerprinting with VNTR sequences

Variable number of tandem repeats (VNTR loci) are the variable number of repeat core sequences at specific loci in the genome. Variation in the length of the alleles patterned from the repeats provided the basis fo. detected the polymorphism. Tandemly repetitive sequences are classified into three major groups. First, satellites DNA are very high repetitive with repeat lengths of one to several thousand based pairs. Second, minisatellites DNA are moderately repetitive, tandemly repeated of a basic motif, about 9-100 bp. Finally, microsatellites are tandem repeats of very short motif, mostly 1-6 bp. Copy numbers are characteristically variable within a population. Satellite DNAs show exceptional variability among individuals, especially with regard to the number of repeats at a given locus.

1.3.1.2.1 Multilocus DNA fingerprints

Multilocus DNA fingerprinting was first described by Jeffrey *et al.* in 1985. The procedure comprises general experimental procedure as conventional RFLP analysis but the minisatellite probes is used. The probe detects many loci simultaneously. The final product of this procedure is a pattern of bands resembling a bar code. This pattern is usually specific to an individual. In this case, it is usually not possible to identify alleles of the same loci or estimate levels of heterozygosity.

1.3.1.2.2 Single-locus minisatellite

Minisatellite is tandemly arranged of two to several hundred copies of a short *(9-100 bp)* sequences of repetitive DNA, usually interspersed but often clustered in telomeric regions of the chromosome. Numbers of copies on different chromosomes are variable, when cut by restriction enzymes produces DNA fragments of different lengths.

Single-locus minisatellites use a similar protocol with that of multilocus DNA fingerprint. In this method, a single locus probe is employed using flanking sequences as a part of the probe to identify allelic products at a single locus. The banding patterns typically obtain from single-locus minisatellites consist of either one for homozygote or two for heterozygote DNA fragments. Although single locus DNA fingerprinting needs a lot of efforts due to isolations of appropriate probe, this technique is a powerful tool in parentage and population genetic studies.

Hybridization - based techniques is requires the high quality and quantity of DNA (clean and high molecular weight), which are the limitations of their applications (Karp *et al.*, 1998).

1.3.2 PCR based techniques

The polymerase chain reaction (PCR) has had a profound impact on molecular biology and has great potential as a tool for detecting genetic polymorphism (Saiki *et al.*, 1998). This method was invented by Kary Mullis (Mullis *et al.*, 1986) The PCR is an *in vitro* method for the enzymatic synthesis of specific DNA segments, using two small oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. The amplification takes place in a thermocycler and is mediated by a thermostable DNA polymerase. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers results in the exponential accumulation of a specific DNA fragments (Erlich, 1992). Only a small amount of DNA template is required by this approach and the polymorphism can be easily scored on EtBr-stained gels (Karp *et al.*, 1998).

1.3.2.1 PCR amplification of specific sequence and microsatellite primers

Microsatellite are short core (1-6 bp) tandem repeat sequences (200 up to 500 bp long) distributed along the genome. Polymorphism due to length variation and base changes in these repeats occur at a higher rate than in the coding region. Microsatellite regions are screened by the insertion of up to 500 bp genomic fragments into plasmids, subsequently amplified using competent cells. Following the detection of suitable colonies, inserts are sequenced and the conserved flanking regions, identified in this method, are used to design/locus specific microsatellite primers. The primers are used in the PCR reaction, involves the incorporation of a radioisotope into the resultant PCR fragment. The samples are run on a polyacrylamide sequencing gel and visualized by autoradiography (Queller *et al.*, 1993). The utility of a PCR approach allow the possible processing of a large number of samples generally required for population surveys and large breeding programs (Brooker *et al.*, 1994).

1.3.2.2 PCR - RFLP

The concept of the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) technique is amplification of DNA fragments from known primers, digested the product with restriction enzymes (sometimes need purifiation of the fragment from the other contamination by agarose gel electrophoresis). It's requiring only agarose gels and ethidium bromide staining. This provided the chance of finding polymorphism within the specific DNA fragment (Karp *et al.*, 1998). The advantage of this technique is that a little DNA template is required.

1.3.2.3 DNA sequencing

Polymorphisms at the DNA level can be studies by several methods but the most direct approach is determination of nucleotide sequences of a defined region. DNA sequencing provides a highly reproducible and informative analysis of data (Weising *et al.*, 1995). DNA sequencing has two major approaches, PCR based and conventional dideoxy DNA sequencing. Both approaches require a DNA template and a specific primer for the DNA polymerization reaction.

Chain elongation of the polymerization process in PCR goes to fulfillment the DNA template, whereas it is interrupted by the dideoxy derivatives of the natural dNTPs in DNA sequencing. When the synthesis was terminated on the addition of a modified ddNTPs, a mixture of single-stranded fragments is generated from the amplification process and fractionated by polyacrylamide gel or sequencing gel electrophoresis. The sizes of the termination fragments can be transferred to the sequence of DNA template (Karp *et al.*, 1998).

The development of cycle sequencing allows rapid analysis of DNA sequences. Cycle sequencing is a combination of PCR and sequencing reaction where the annealing and extending steps are performed repeatedly using the same template. This enables sequencing from a much smaller amount of template than with the standard protocol (Carvalho and Pitcher, 1995). However, DNA sequencing is tedious, time consuming and expensive compared with other molecular genetic techniques.

1.3.2.4 Randomly Amplified Polymorphic DNA (RAPD)

RAPD analysis is amplification of genomic DNA by PCR with single short oligonucleotides of arbitrary sequence acting as both a forward and reverse primers at

low stringency (Welsh and McClelland, 1990 and Williams *et al.*, 1990). Under these conditions, a number of PCR products are generated from random locations within the genome (Dear, 1997). The technique detects genetic variation without requiring any prior DNA sequence information. Oligonucleotide primers are deranged follows simple criteria: at least ten bases in length; G+C content of 50-80%; no palindromic motifs of six or more nucleotides.

The PCR reactions are carried out at low stringency, as a result, their products can vary with only minor changes in reaction conditions; this can lead to inconsistencies between laboratories. A more serious problem is that RAPD markers are typically dominant rather than co-dominant. Many sequence polymorphism are simply reflected as the presence or absence of a given RAPD marker. This means that it is not possible to distinguish a homozygote from a heterozygote with one 'null' allele (Karp *et al.*, 1998).

RAPD can be used for the study on genetic diversity among conspecific populations of several species (Hardys *et al.*, 1992). It is a simple and rapid method and non-radioactive technique that requires only a tiny amount of DNA template. Therefore this technique has been increasingly used for population genetic studies in various species.

1.4 Genetic study in abalone

Geiger and Groves (1999) gathered documents concerning chromosomal numbers of 14 abalone species (*H. tuberculata, H. lamellosa, H. aquatilis, H. diversicolor aquatilis, H. diversicolor, H. exigua, H. planata, H. asinina, H. ovina, H. varia, H. cracherodii, H. discus discus, H. discus hannai,* and *H. madaka).* These organisms can be divided into 3 groups according to geographical regions, namely

European-Mediterranean (2n=28): *H. tuberculata, H. lamellosa*, Indo-Pacific (2n=32): *H. divericolor aquatilis, H. aquatilis, H. diversicolor, H. exigua, H. planata, H. varia, H. asinina* and *H. ovina* and North Pacific (2n=36): *H. cracherodii, H. discus discus* and *H. madaka*. They suggested that *H. tuberculata* was a relic species from the ancient Tethys Sea that abalone dispersed eastward.

Jarayabhand *et al.* (1998) karyotyped three abalone species found in Thai waters. The karyotype of *H. asinina* had ten pairs of metacentric chromosomes and six pairs of submetacentric chromosomes while *H. ovina* had nine pairs of metacentric chromosomes, six pairs of submetacentric chromosomes, and one pair of telocentric chromosomes. *H. varia* had eight pairs of both metacentric chromosomes and submetacentric chromosomes. Three types of chromosomes (metacentric, submetacentric and telocentric) were found only in *H. ovina*. They suggested that *H. asinina* and *H. varia* were more closely related than *H. ovina*.

There have been few publications concerning molecular genetic studies of abalone. Lee and Vacquier (1995) studied the phylogeny species identification of the genus *Haliotis* using molecular data. They compared complementary DNA (cDNA) sequences of sperm lysin of 27 abalone species from California, Japan, Australia, New Zealand, Taiwan, Borneo, Madagascar, South Africa, Greece, France, Italy and the Azores. Results allocated investigated species into three groups, all Californian species and three Japanese species (*H. gigantea, H. discus hannai*, and *H. madaka*), one New Zealand species (*H. iris*) and finally, one Japanese species (*H. diversicolor aquatilis*), Indo-West Pacific species and European species.

Naganuma *et al.* (1998) distinguished two closely related abalones, *H. discus discus* and *H. discus hannai*, with the 18S rDNA sequencs. The adults of *H. discus*

discus, *H. discus hannai*, were collected from central Japan, northeastern Japan whereas *H. madaka* and *H. gigantea*, were collected from western Japan, respectively. The hatchery larvae of *H. discus hannai* were also collected from abalone farms (original parents were from Kesennuma) and at Numazu, Shizuoka Prefecture, respectively. The primer sequences for amplification of 18S rDNA were 5'-AAC CTG GTT GAT CCT GCC AGT- 3' (forward, 21-mer) and 5' -TGA TCC TTC TGC AGG TTC A- 3' (reverse, 19-mer) (Medlin *et al.*, 1998 cited in Naganuma *et al.*, 1998). PCR products (1800-1900 bp) was cloned and multiple-aligned. Two or three clones from two or three individuals of *H. discus discus* and *H. discus hannai* were sequenced with two other abalones (*H. madaka* and *H. gigantea*) and a land gastropod.

Two minisatellite loci of the blacklip abalone (*H. rubra*) were isolated. One contained a 33 bp repeated units (5'-CCC AAG GTC CCC CAA GGT CAG GGA GGC GGA GGC-3') located in the 3' untranslated region of a putative growth hormone (GH) gene and the other contained a 18 bp repeat units (5'-ACC CGG CGC TTA TTA GAG-3') located in the 3' untranslated region of a putative molluscan insulin-related peptide (MIP) gene. The preliminary study on 100 individuals of *H. rubra* indicated that these minisatellite DNA are useful for genetic studies in this abalone including paternity testing, confirmation of triploidy, population genetic structure and gene flow (Huang *et al.*, 1997).

Huang and Hanna (1998) developed three microsatellite loci for the blacklip abalone (*H. rubra* Leach) using random amplified polymorphic DNA products and a genomic DNA library. There were RUBGT1, $(GT)_n$ repeats, RUBCA1, $(CA)_n$ repeats, and RUBGACA1, $(GACA)_n$ repeats. All of these microsatellites were polymorphic in

100 blacklip abalone samples collected from the Victorian coast and Eden, New South Wales. The number of alleles observed was 41, 30 and 8 alleles for RUBGT1, RUBCA1 and RUBGACA1, respectively. Three microsatellites and two minisatellites (a growth hormone gene repeat, GHR and a molluscan insulin-related peptide gene repeat, MIPR) were used for cross-species amplification of 14 abalone species from the United States, South Africa, South Korea, and Australia. No amplifications occurred for the overseas abalone species, with the exception of the South Korean species *H. gigantea* and *H. sieboldi* at the RUBGT1 locus. The minisatellite MIPR was a species-specific locus for blacklip abalone.

The first polymorphic microsatellite locus, Hruf200 (a GT repeat) of the red abalone (*H. rufescens*) was characterized by establishment size-selecting genomic libraries and screening for all combination of dinucleotide and trinucleotide repeats. Genomic libraries were constructed from the northern, central and southern California DNA samples. Twenty-one microsatellites were found from initial sequencing of positive clones identified. A total of 21 alleles ranging from 97 to 149 base pairs in length were detected at the locus Hruf200 (Kirby and Powers, 1998).

A tandemly repeated satellite DNA of 290 bp from the red abalone (*H. rufescens*) was directly sequenced (Muchmore *et al.*, 1998). A tandemly repeated satellite DNA of 290 bp was identified by *Sal*I digestion of genomic DNA of five species of Eastern Pacific abalone including *H. rufescens* (Red abalone), *H. kamtschatkana* (Pinto abalone), *H. corrugata* (Pink abalone), *H. sorenseni* (White abalone), and *H. walallensis* (Flat abalone). The 290-291 bp *Sal*I satellite is potential molecular marker for identifications of abalone species because of its high copy number, the consensus sequence can be obtained with direct cloning (satellite DNA

was ligated into pBluescript, and transformed into XL1-blue *Escherichia coli)*. This satellite DNA presented approximately 0.5% of total *H. rufescens* DNA corresponding to 28,000 copies per haploid genome and can be determined from alcohol-fix or dried tissue.

Sweijd *et al.* (1998) developed a PCR technique targeting a portion of the lysin gene of several abalone species to distinguish *H. midae* and *H. spadicea*. The PCR primers specifically amplify approximately 1,300 bp of genomic DNA from dried, cooked, and fresh abalone tissue. A smaller fragment of 146 bp is used for canned abalone. Restrictions fragment length polymorphism (RFLP) showed interspecific polymorphisms that discriminate these two species unambiguously.

Huang and Hanna (2000) analyzed genetic structure of the blacklip abalone, *H. rubra* from nine sites along the Victorian coast and from one at Eden, New South Wales, by RAPD, minisatellite and microsatellite markers. DNA markers included 84 RAPD bands from six primers, two minisatellites, GHP and MIPR, and three microsatellite, RUBGT1, RUBCA1 and RUBGACA1. All DNA markers revealed significant subdivision in the *H. rubra* populations. Results of microsatellites indicated excessive homozygotes across all populations at all three microsatellite loci.

1.5 Objectives

The objectives of this study are to identify molecular genetic markers showing species-specific nature of Thai abalone and to determine whether population differentiation is existent in *H. asinina*.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

- Autoclave: HEV-50 (Hirayama, Japan)
- Automatic micropipettes P10, P20, P100, P200, and P1000 (Gilson S.A., France)
- Camera: K1000 (Pentax, Japan)
- -20 °C Freezer (Songserm Intercool, Thailand)
- -30 °C Freezer (Sanyo, Japan)
- -80 °C Freezer (Sanyo, Japan)
- Microcentrifuge: MicroCen 13D (Herolab, Germany)
- Microcentrifuge tube 0.5 and 1.5 ml (Bio-RAD Laboratories, USA)
- Microwave (Hitachi, Japan)
- pH meter (Orion, USA)
- PCR Thermal cycle: Omnigene-E (Hybaid Limited, England)
- PCR Thermal cycle: Sprint (Hybaid Limited, England)
- Pipette tips 0.2, 10, 200, 1000 µl (Bio-RAD Laboratories, USA)
- Power supply: Power Pac 300 (Bio-RAD Laboratories, USA)
- Refrigerated centrifuge: 3K18 (Sigma, Germany)
- Scuba gear
- Shaker bath: SBS30 (STUART Scientific, UK)
- Spectrophotometer: Spectronic GENESYS5 (MiltonRoy, USA)

- Sterilize syringe 1 ml
- Underwater camera
- UV transilluminator: UVP (USA)

2.1.2 Chemicals

- Absolute ethanol (Merck, Germany)
- Boric acid (Merck, Germany)
- Bromphenol blue (Merck, Germany)
- Chloroform (Merck, Germany)
- 100 mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medison, Wisconsin)
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Sigma Chemical Co., USA)
- Ficoll Type400 (Sigma Chemical Co., USA)
- GeneAmp PCR core reagents (Perkin Elmer Cetus, USA)

  : 10x PCR buffer (100mM Tris-HCl pH8.3, 500 mM KCl)

  : 25 mM $MgCl_2$
- Isoamyl alcohol (Merck, Germany)
- Mineral oil (Sigma Chemical Co., USA)
- Oligonucleotide primers: 10-mers (BSU, National Center for Genetic Engineering and Biotechnology)
- Phenol, redistilled (Aldrich Chemical Co., USA)
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)

- Tris (hydroxy - methyl) amino-methane (Sigma Chemical Co., USA and Pharmacia)
- Ultrapure agarose (SeakemGTG, FMC)

2.1.3 Enzymes

- Ampli *Taq* DNA polymerase (Perkin Elmer Cetus, USA)
- DyNAzyme^{TM} II DNA Polymerase (Finnzymes, Finland)
- Proteinase K (Gibco BRL life technologies, Inc., USA)
- RNase A (Sigma Chemical Co., USA)

2.2 Methods

2.2.1 Choices for sample collection sites

The primary data for sources of abalone in Thailand was obtained by interviewing researchers, fishermen and seafarmers. The secondary data, about habitats and distributions of *H. asinina*, *H. ovina* and *H. varia* in Thai waters, was obtained through previous publications between 1986 to 1998 (Nateewathana and Hylleberg, 1986; Tookwinas *et al.*, 1986; Nateewathana and Bussarawit, 1988; Jarayabhand *et al.*, 1991; Kakhai and Petjamrat, 1992; Singhagriwan, 1992; Tanawansombat, 1992; Ngow and Jarayabhand, 1993; Jarayabhand and Paphavasit, 1996 and Jarayabhand *et al.*, 1998). Coral reef maps for the Gulf of Thailand and the Andaman Sea were used in order to choose suitable sampling sites of abalone (Department of Fisheries, 1999). Geographically different locations of *H. asinina* and *H. ovina* were selected covering both sides of peninsular Thailand. Additionally, *H. varia*, which has not been reported in the Gulf of Thailand, was collected only from the Andaman Sea.

2.2.2 Sources of specimens

2.2.2.1 Natural habitat

Abalone were collected by snorkeling and SCUBA diving covering their geographic distributions in Thailand (the upper Gulf of Thailand and the Andaman Sea). Live specimens collected from various locations in the Gulf of Thailand were brought back to the hatchery at the Angsila Marine Biological Research Station located at Chon Buri Province. Dead specimens were transported on ice to the laboratory at the Marine Biotechnology Research Unit (MBRU), Chulalongkorn University, as soon as possible.

To collect specimens from the Andaman Sea, a one-week trip was usually required due mainly to the long traveling distance. The collected specimens were sent back to the laboratory by plane as soon as possible. Alternatively, specimens collected from the first few days of the trip were kept alive in the sea prior to final transportation to the laboratory.

Abalone specimens of each species were collected from different locations depending on their habitats, for example, *H. asinina* and *H. ovina* were collected from the subtidal zones (*H. asinina* live under dead corals or in the crevices of the branching corals whereas *H. ovina* was collected from the crevices of the living corals). *H. varia* were collected from the intertidal areas as well as from the rock crevices in the subtidal zones.

Information regarding abalone habitats from some publications appeared to be out of date. Specimens were not present in several locations that were previously reported.

2.2.2.2 Abalone populations

Apart from wild specimens, *H. asinina* were also obtained from three hatchery locations. They are from the Angsila Marine Biological Research Station, Phuket Abalone Farm and SEAFDEC (the Philippines) for the hachery *H. asinina* 's stock. No hatchery stocks were available for *H. ovina* and *H. varia*, therefore only broodstock was collected.

A total number of collected specimens was three hundred and thirty-one individuals, comprising of *H. asinina* (*N*=163), *H. ovina* (*N*=135) and *H. varia* (*N*=33). Geopraphic locations of sampling sites were illustrated by Fig 2.1 and Table 2.1. The whole specimens were collected at -30°C while dissected foot tissue was kept at -80°C.

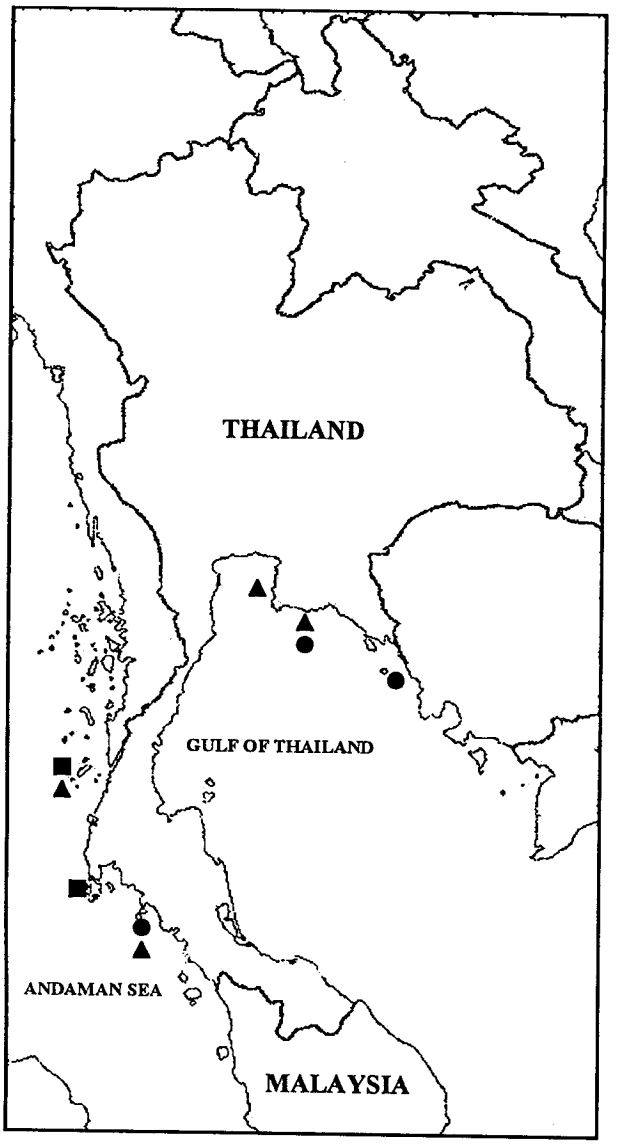


Figure 2.1 Map of Thailand indicating collection sites of abalone in the Gulf of Thailand and Andaman Sea used in this study.
(*Haliotis asinina* = ● , *H. ovina* = ▲ and *H. varia* =■).

Table 2.1 Sample collection sites, code of populations and number of collected individuals (and number of individuals used in this study) for three species of abalone in Thailand.

Collection site	Code of populations	No. of individuals (No. used in this study)
H. asinina		
Samet Island, Rayong	HASM	19 (14)
Talibong Island, Trang	HALB	28 (19)
Cambodia	HACB	23 (19)
Hatchery stock, P_o (Broodstock from Samet Island, Rayong)	HASH	28 (14)
Hatchery stock, P_o (Broodstock from Cambodia)	HACH	15 (13)
Hatchery stock, F_1 (Broodstock from Philippines)	HAPH	30 (20)
H. ovina		
Khang Kao Island, Chon Buri	HOSC	29 (29)
Samet Island, Rayong	HOSM	43 (27)
Chuak Island, Trang	HOTR	47 (23)
Similan Island, Phang-nga	HOPG	16 (16)
H. varia		
Aeo Island, Phuket	HVPK	29 (28)
Similan Island, Phang-nga	HVPG	4 (4)
Total		**311 (206)**

2.2.3 DNA extraction

Genomic DNA was extracted from the frozen foot tissue or haemolymp of each abalone using a phenol-chloroform proteinase K method (Klinbuga *et al.*, 1999). A piece of foot tissue was dissected out, placed in a 1.5 ml microcentrifuge tube containing 360 µl of extraction buffer (200 mM Tris-HCl, 100 mM Na_2EDTA and 250 mM NaCl; pH 8.0) and 80 µl of 10% SDS, and homogenized with a micropestle. RNA was digested by adding 8 µl of RNaseA (10 mg/ml). Additional 360 µl of extraction buffer was added. The homogenate was further homogenized for a few strokes and incubated at 37°C for 1 hr. A proteinase K solution (10 mg/ml) was added to make a final concentration of 200 mg/ml. The mixture was further incubated at 55° C for 4 hours. An equal volume of buffer equilibrated phenol was added and gently mixed for 20 min. The sample was centrifuged at 12,000 rpm for 10 min at room temperature. The upper aqueous phase was removed without disturbing the organic/aqueous interface. Phenol extraction was repeated twice. An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and mixed gently. The upper aqueous phase was transferred to a new microcentrifuge tube. This step was repeated twice. The upper phase from the final chloroform:isoamyl alcohol extraction was removed. One - half volume of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA pH 8.0) was added followed by one-tenth volume of 3M sodium acetate, pH 5.2 and 2 final volumes of ice - cold absolute ethanol. The mixture was thoroughly mixed by invertion of the tube. If the DNA pellet is not observed at this stage, the sample was kept in a - 20°C freezer for at least 1 hours. DNA was recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature. The DNA pellet

was washed twice, at least 30 minutes each, with 70 % ethanol. The pellet was air dried and redissolved with appropriate volume of a TE solution (usually 50-100 µl). The DNA solution was incubated at 37°C for 1-2 hours and kept at 4°C until further analysis.

2.2.4 Measurement of DNA concentrations

2.2.4.1 Spectrophotometry

The amount of DNA was estimated by determination of the optical density at 260 nm. The OD value at 260 allows calculation of total nucleic acids whereas the value reading at 280 nm determine the amount of protein in the sample.

An OD at 260 nm corresponds to approximately 50 µg/ml for double stranded DNA. (Maniatis *et al.*, 1982). The ratio between OD 260/280 provides a roughly estimate for the purity of extracted DNA. A pure preparation of DNA has a 260/280 ratio of 1.8 - 2.0 (Kirby, 1992). To determine DNA concentration, 5 µl of extracted DNA was transferred to an Eppendorf tube containing 995 µl of TE buffer. The tube was shaken vigorously. The diluted DNA solution was transferred to a semimicro-UV cuvette where a cuvette containing 1 ml of TE was served as the reagent blank. DNA concentration is estimated in µg/ml using the following equation;

$$[\text{DNA}] = OD_{260} \times \text{Dilution factors} \times 50 \qquad (2.1)$$

2.2.4.2 Mini-gel method

DNA concentration can be roughly estimated by comparing the amount of fluorescence of the DNA after electrophoresis through mini agarose gels. One or two

microlitres of the extracted DNA was diluted to a 10 µl final volume. Two microlitres of loading buffer (0.25% Bromophenol blue, 25% Ficoll) was added and mixed thoroughly. A series of undigested DNA at different amount (25, 50, 100, 150, 200 ng) was prepared and included as the quantitative standards.

DNA was electrophoresed through 0.8 % agarose gels in the presence of 0.5 µg/ml EtBr at 5 - 7.5 Volt/cm for approximately 30 minutes to 1 hr. The approximate amount of DNA concentration were obtained by comparing the fluorescent level of an investigated band with those of a λ- *Hind* III marker and undigested λ DNA. A portion of the DNA solution was then diluted to 25 µg/ml final concentration for using in RAPD-PCR analysis.

2.2.5 RAPD Analysis

One hundred and seventeen decatanucleotide primers, six microsatellites and seven minisatellites primers (Table 3.1) were screened for the successful amplification of 3 abalone species in Thai waters. An individual of each abalone were used for screening of primers.

RAPD - PCR was carried out in a final volume of 25 µl containing 0.2 µM of an appropriate primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, dTTP, 1 unit of Ampli*Taq* DNA polymerase and approximately 25-50 ng of genomic DNA. The reagent mix without the DNA template was included as the negative control.

The amplification reaction was operated for 1 cycles at 94 ^{o}C for 3 min followed by 40 cycles at 94^{o}C for 30 sec (denaturation), 36^{o}C for 60 sec for all specimen except the primer UBC271 for which 40 ^{o}C was used (annealing) and 90 sec at 72^{o}C (extension) and finally, 7 min at 72^{o}C. After amplification, 5-10 µl of the resulting PCR mixture was electrophoretically analysed as soon as possible. The remaining reaction mixture was kept at - 20°C for long storage.

Five primers (UBC101, OPB11, UBC195, UBC197 and UBC271) producing reliable and reproducible results were chosen. Primers UBC101 and OPB11 were used for determination of genetic diversity and identification of species - specific markers among three abalone, whereas all 5 primers were used for determination of intraspecific genetic diversity in the economically promising species, *H. asinina*.

2.2.6 Agarose gel electrophoresis

An appropriate amount of ultrapure agarose was weighed out and mixed with 1X TBE buffer (8.9 mM Tris - HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.3) to make 1.6% and 0.8% of agarose gels for analysis of the resulting RAPD products and for detection of quality of extracted genomic DNA, respectively. The suspension was heated until complete solubilization in a microwave. The melted agarose were left at room temperature to cool to approximately 50°C and poured into a gel mould. An appropriate comb was then inserted. The gel was left at room temperature for at least 30 minutes to completely solidify. The gel was placed in the electrophoretic chamber containing 1X TBE buffer covering the gel for 1-2 mm in depth. The comb was gently removed. Air bubbles trapped within the well were carefully removed.

One-quarter volume of the gel loading dye (0.25% Bromphenol blue and 25% Ficoll) was added and mixed. After briefly centrifugation, the sample was slowly loaded into the wells using an adjustable automatic pipette.

A 100 bp DNA ladder (BioLabs and SibEnzyme) and the λ - *Hind* III DNA were used as DNA markers. Electrophoresis was carried out at 5 V/cm until bromophenol blue migrated about three-quater of the gels. The gel was stained with ethidium bromide (0.5 μg/ml) for 10 min and destained in distilled water for 15 min to leach out unbound ethidium bromide from the gel. Migration distance of the DNA marker was recorded. The gel was photographed using a K1000 camera (Pentax, Japan).

2.2.7 Statistical analysis

Standard DNA markers (100 bp ladder and λ-*Hin*dIII) were used to assign the size of each RAPD fragment. Only fragments that could be accurately scored (250 bp - 2300 bp) were chosen. Each RAPD fragment was assigned a molecular length and recorded in a binary matrix for each individual as presence (1) or absence (0) of a given band.

The percentages of polymorphic and monomorphic bands were evaluated base on the assumption that bands that are present in less than 95% of investigated individuals are polymorphic. The RAPD patterns of individuals between different populations of three species of Thai abalone were compare using primer UBC101 and OPB11. Intraspecific genetic diversity of *H. asinina* was estimated using primers UBC101, OPB11, UBC195, UBC197 and UBC271.

Similarity index

A similarity index between individuals within geographic samples was calculated from band sharing of each pair of individuals using the formula:

$$S_{xy} = 2\ N_{xy} / N_x + N_y \qquad (2.2)$$

Where N_x and N_y represent the total number of band observed for compared individuals, and N_{xy} is the number of band which are present in both individual x and y (Lynch, 1990).

For overall species, similarity index within a geographic sample (S) and between samples (S_{ij}) were calculated as the average of S_{xy} across all possible comparisons between individuals within a geographic sample and between individuals from different geographic samples, respectively.

Between sample similarity, corrected for within sample similarity effect (S_{aij}) was used to estimate the similarity index among different samples of *H. asinina*, and can be calculated using the formula:

$$S_{aij} = 1 + S_{ij} - (S_i + S_j)/2 \qquad (2.3)$$

where S_i and S_j are the values of S for sample i and j, respectively, and S_{aij} is the average similarity between randomly paired individuals from samples i and j (Lynch, 1990)

Genetic distance

A genetic distance between pairs of genotypes, sample or species is a quantitative estimate of genetic divergence between two compared operational taxonomic unit (Avise, 1994). For RAPD, S_{aij} or S_{ij} is converted to the genetic distance (D_{aij} or D_{ij}) using the formula:

$D = 1 - S$ (Lynch, 1990) (2.4)

Phylogenetic reconstruction

Phylogenetic relationships between investigated sample of abalone were constructed based on a neighbor-joining/UPGMA method using Neighbor in PHYLIP (Felsenstein, 1993) version 3.562c. The neighbor-joining trees were illustrated using Treeview.

CHAPTER III

RESULTS

3.1 DNA extraction

Genomic DNA was extracted from either the foot tissue or haemolymp of each abalone using a proteinase K-phenol-chloroform extraction method. The quality of extracted genomic DNA was electrophoretically determined in a 0.8% agarose (w/v) gel while the concentration of DNA was estimated spectrophotometrically and electrophoretically. As can be seen from Figure 3.1, the migration distance of the extracted DNA was as the same as a 23.1 kb band of a λ-*Hin*d III marker indicating that high molecular weight DNA was consistently obtained. The intensity of the extracted DNA were compare with series of undigested λ DNA (10, 25, 50, 75, 100, 250 ng) and DNA concentrations were determined by measuring the optical density at 260 nm (1 OD_{260} unit was equivalent to 50 μg DNA/ml). The ratio of OD_{260}/OD_{280} was 1.8-2.0 indicating a possible contamination of RNA in the extracted DNA samples. Some DNA samples contained RNA contamination as visualized by the smear at the bottom of gel. Approximately 25-50 μg DNA were usually obtained from each specimen.

3.2 Primer screening

DNA samples of *H. asinina*, *H. ovina* and *H. varia* were tested for the amplification success against one hundred and thirty primers (Table 3.1). Thirty-four RAPD primers yielded successful amplification and were further tested against a larger number of individuals of three abalone species. In this study, two primers (UBC101 and

OPB11) were used to study population genetics of three abalone species whereas intraspecific genetic diversity was examined for *H. asinina* by those two primers together with additional three primers (UBC195, UBC197 and UBC271).

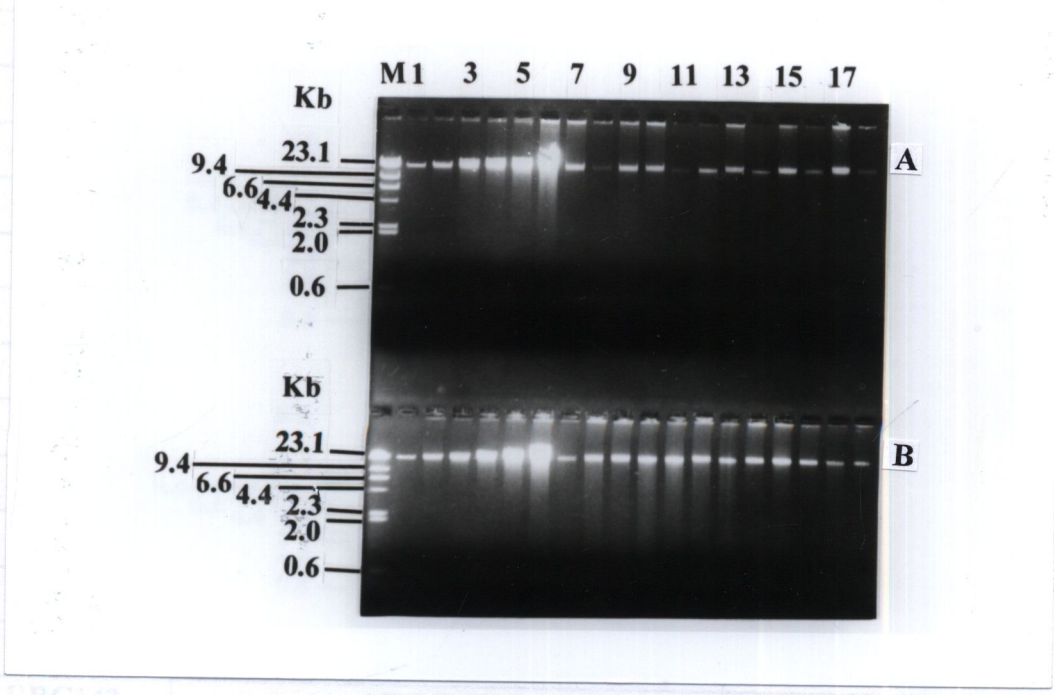


Figure 3.1 A 0.8% ethidium bromide stained-agarose gel showing the quality of total DNA extracted from the foot tissue of abalone.

lane M = λ-*Hin*d III

lanes 1-6 = undigested lamda DNA 10, 25, 50, 75, 100, 250 ng

lanes 7-18 (A and B) = Total DNA extracted from individuals of abalone (*H. asinina*)

Table 3.1 The amplification success of RAPD primers and their sequences initially screened by this study

Primer	Sequence	Amplification Strength
UBC101	GCG CCT GGA G	+++
UBC111	AGT AGA CGG G	-
UBC114	TGA CCG AGA C	-
UBC115	TTC CGC GGG C	-
UBC117	TTA GCG GTC T	-
UBC118	CCC GTT TTG T	-
UBC119	ATT GGG CGA T	++
UBC120	GAA TTT CCC C	-
UBC121	ATA CAG GGA G	-
UBC122	GTA GAC GAG C	-
UBC128	GCA TAT TCC G	-
UBC132	AGG GAT CTC C	-
UBC135	AAG CTG CGA G	-
UBC138	GCT TCC CCT T	-
UBC139	CCC AAT CTT C	-
UBC140	GTC GCA TTT C	-
UBC142	ATC TGT TCG G	-
UBC143	TCG CAG AAC G	-
UBC144	AGA GGG TTC T	-
UBC146	ATG TGT TGC G	-
UBC148	TGT CCA CCA G	-
UBC153	GAG TCA CGA G	-
UBC158	TAG CCG TGG C	-
UBC159	GAG CCC GTA G	-
UBC160	CGA TTC AGA G	++
UBC161	CGT TAT CTC G	-
UBC163	CCC CCC AGA T	-

Table 3.1 (continued)

Primer	Sequence	Amplification Strength
UBC164	CCA AGA TGC T	-
UBC166	ACT CCT ACA G	-
UBC167	CCA ATT CAC G	-
UBC168	CTA GAT GTG C	++
UBC169	ACG ACG TAG G	-
UBC170	ATC TCT CCT G	-
UBC171	TGA CCC CTC C	-
UBC174	AAC GGG CAG C	++
UBC175	TGG TGC TGA T	-
UBC187	AAC GGG GGA G	-
UBC189	TGC TAG CCT C	-
UBC191	CGA TGG CTT T	-
UBC193	TGC TGG CTT T	+++
UBC195	GAT CTC AGC G	+++
UBC197	TCC CCG TTC C	+++
UBC200	TCG CGA TAT G	++
UBC210	GCA CCG AGA G	++
UBC217	ACA GGT AGA C	-
UBC220	GTC GAT GTC G	++
UBC222	AAG CCT CCC C	-
UBC228	GCT GGG CCG A	-
UBC233	CTA TGC GCG C	-
UBC235	CTG AGG CAA A	-
UBC237	CGA CCA GAG C	-
UBC255	TTC CTC CGG A	-
UBC259	GGT ACG TAC T	-
UBC262	CGC CCC CAG T	-
UBC263	TTA GAG ACG G	-

Table 3.1 (continued)

Primer	Sequence	Amplification Strength
UBC264	TCC ACC GAG C	++
UBC267	CCA TCT TGT G	++
UBC268	AGG CCG CTT A	-
UBC270	TGC GCG CGG G	-
UBC271	GCC ATC AAG A	+++
UBC272	AGC GGG CCA A	++
UBC273	AAT GTC GCC A	-
UBC277	AGG AAG GTG C	-
UBC281	GAG AGT GGA A	-
UBC282	GGG AAA GCA G	-
UBC286	CGG AGC CGG C	-
UBC293	TCG TGT TGC T	-
UBC295	CGC GTT CCT G	-
UBC297	GCG CATT TAG A	-
UBC298	CCG TAC GGA C	-
UBC299	TGT CAG CGG T	-
UBC428	GGC TGC GGT A	-
UBC456	GCG GAG GTC C	++
UBC457	CGA CGC CCT G	++
UBC459	GCG TCG AGG G	++
OPA1	CAG GCC CTT C	++
OPA2	TGC CGA GCT G	++
OPA3	AGT CAG CCA C	-
OPA4	AAT CGG GCT G	-
OPA5	AGG GGT CCT G	-
OPA6	GGT CCC TGA C	-
OPA7	GAA ACG GGT G	-
OPA8	GTG ACG TAG G	-

Table 3.1 (continued)

Primer	Sequence	Amplification Strength
OPA9	GGG TAA CGC C	-
OPA10	GTG ATC GCA G	++
OPA11	CAA TCG CCG T	-
OPA12	TCG GCG ATA G	-
OPA13	CAG CAC CCA C	-
OPA14	TCT GTG CTG G	-
OPA15	TTC CGA ACC C	++
OPA16	AGC CAG CGA A	-
OPA17	GAC CGC TTG T	-
OPA18	AGG TGA CCG T	-
OPA19	CAA ACG TCG G	++
OPA20	GTT GCG ATC C	++
OPB1	GTT TCG CTC C	-
OPB2	TGA TCC CTG G	-
OPB3	CAT CCC CCT G	-
OPB4	GGA CTG GAG T	-
OPB5	TGC GCC CTT C	-
OPB6	TGC TCT GCC C	-
OPB7	GGT GAC GCA G	-
OPB8	GTC CAC ACG G	-
OPB9	TGG GGG ACT C	-
OPB10	CTG CTG GGA C	-
OPB11	GTA GAC CCG T	+++
OPB12	CCT TGA CGG A	-
OPB13	TTC CCC CGC T	-
OPB14	TCC GCT CTG G	-
OPB15	GGA GGG TGT T	-
OPB16	TTT GCC CGG A	++

Table 3.1 (continued)

Primer	Sequence	Amplification Strength
OPB17	AGG GAA CGA G	++
OPB18	CCA CAG CAG T	-
OPB19	ACC CCC GAA G	-
OPB20	GGA CCC TTA C	-
OPM9		-
OPZ9	-	-
Microsatellites	$(CA)_8$	-
Microsatellites	$(CT)_8$	-
Microsatellites	$(CAC)_5$	+
Microsatellites	$(GTG)_5$	++
Microsatellites	$(GATA)_4$	+
Microsatellites	$(GACA)_4$	+
HRU33	CCC AAG GTC CCC AAG GTC AGG GAG GCG AAG GCT	-
HRU18	ACC CGG CGC TTA TTA GAG	-
PER I	GAC NGG NAC NGG	-
INS	ACA GGG GTG TGG GG	+++
M13	GAG GGT GGN GGN TCT	++
YNZ22	CTC TGG GTG TCG TGC	-
YN73	CCC GTG GGG CCG CCG	+++

+, ++ and +++ indicate that the strength of amplification success.
- indicates that the amplification was not successful.

The RAPD-PCR amplification was performed in a DNA thermal Cycle (Hybaid Model Omnigene-E for primer UBC271 and Sprint PCR for the other primers). The annealing temperature for UBC271 was carried out at 40°C. This high amplification temperature RAPD (HAT-RAPD) provided more reproducible results than the standard low amplification temperature RAPD (LAT-RAPD).

3.3 Genetic diversity of tropical abalone using RAPD

3.3.1 Between species diversity

High genetic diversity levels between *H. asinina, H. ovina and H. varia* was observed based on RAPD analysis. Two decanucleotide primers, UBC101 and OPB11, were used for genetic analysis of all three Thai abalone species. Seventy-two RAPD fragments ranged from 320 bp to 2300 bp in length were generated. The size of RAPD bands ranged from 320 bp to 1850 bp for primer UBC101 and 390 bp to 2300 bp for primer OPB11. The number of reproducible bands across all investigated samples were 37 and 35 bands for respective primers. One hundred percent of polymorphic bands (bands found in less than 95% of overall investigated individuals within a particular species) were found for both primers (Table 3.2A).

The percentage of polymorphic bands of *H. asinina*, *H. ovina* and *H. varia* using UBC101 and OPB11 primers were 84.91%, 94.74% and 91.23% respectively. *H. ovina* exhibited the greatest levels of polymorphic bands followed by *H. varia* and *H. asinina* (Table 3.3). The total number of bands, percentage of polymorphic and monomorphic bands within each abalone species are shown in Table 3.3.

RAPD amplification patterns generated by primer UBC101 and primer OPB11 are shown by Figure 3.2 - 3.6. RAPD patterns of all specimens examined in this study are shown in the appendices B1 and B2.

3.3.2 Genetic diversity of H. asinina

High genetic diversity levels of *H. asinina* was also observed based on RAPD analysis using 5 decanucleotide primers. The number of reproducible bands across all investigated samples were 32, 21, 25, 23 and 12 bands for primers UBC101, OPB11, UBC195, UBC197 and UBC271, respectively. One hundred and thirteen RAPD fragments ranged from 250 bp to 2300 bp were generated. All primer provided high polymorphic levels in this abalone species. The percent polymorphic bands of UBC101, OPB11, UBC195, UBC197 and UBC271 were 81.25, 90.48, 84.00, 86.96 and 83.33, respectively. Size-range of RAPD bands in each primer are shown in Table 3.2B. RAPD amplification patterns generated by those primers are shown in Figures 3.2-3.10. RAPD patterns of all tested specimens in this study are shown in an appendix B.

Table 3.2 Sequence of RAPD primers, size-range, number of amplified bands, and the percentage of polymorphic and monomorphic bands resulted from RAPD analysis of three species of abalone; *H. asinina*, *H. ovina* and *H. varia* (A) and *H. asinina* (B)

A.

Primer	Sequence	Size-range (bp)	No. of RAPD bands	Polymorphic bands (%)	Monomorphic bands (%)
UBC101	GCG CCT GGA G	320-1850	37	100	0
OPB11	GTA GAC CCG T	390-2300	35	100	0
Total		320-2300	72	100	0

B.

Primer	Sequence	Size-range (bp)	No. of RAPD bands	Polymorphic bands (%)	Monomorphic bands (%)
UBC101	GCG CCT GGA G	320-1800	32	81.25	18.75
OPB11	GTA GAC CCG T	390-2300	21	90.48	9.52
UBC195	GAT CTC AGC G	520-1480	25	84.00	16.00
UBC197	TCC CCG TTC C	500-1480	23	86.96	13.04
UBC271	GCC ATC AAG A	250-1020	12	83.33	16.67
Total		250-2300	113	85.20	14.80

Table 3.3 Total number of bands, percentage of polymorphic and monomorphic bands within each abalone revealed by RAPD analysis

Primer No.	*H. asinina*			*H. ovina*			*H. varia*		
	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
UBC101	32	26	6	26	23	3	24	20	4
OPB11	21	19	2	31	31	0	33	32	1
UBC195	25	21	4						
UBC197	23	20	3						
UBC271	12	10	2						
Total	113	96 (84.96%) 45 (84.91%)*	17 (15.32%)	57	54 (94.74%)	3 (5.26%)	57	52 (91.23%)	5 (8.77%)

* When consider only UBC101 and OPB11

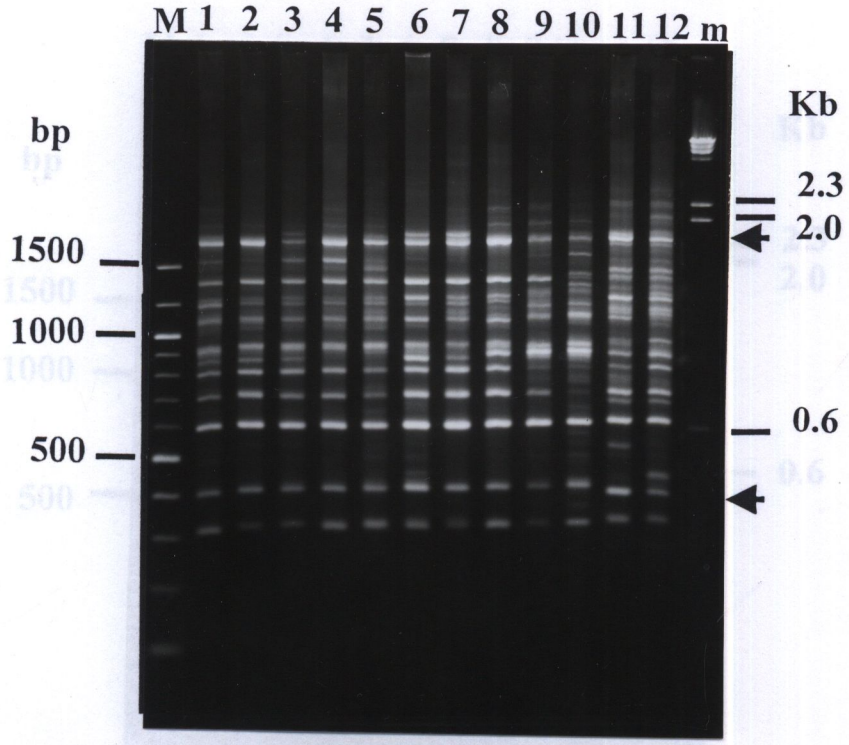


Figure 3.2 RAPD patterns resulted from analysis of *H. asinina* from Samet Island, Rayong (lanes 1-4), Cambodia (lanes 5-8), Talibong Island, Trang (lanes 9-10), and the Philippines (lanes 11-12) with the primer UBC101. Lanes M and m are a 100 bp ladder and λ-*Hin*d III, respectively.

Arrows indicated species-specific markers for *H. asinina* (1700 bp) and a marker specifically observed in *H. asinina* from the Philippines (380 bp).

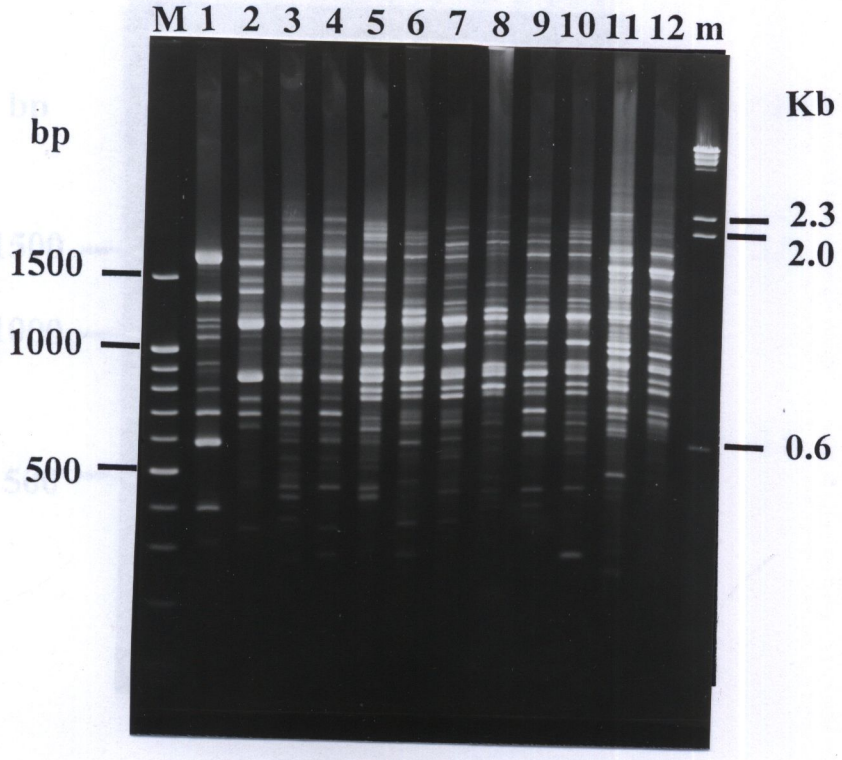


Figure 3.3 RAPD patterns resulted from analysis of *H. asinina* from Samet Island, Rayong (lanes 1), *H. ovina* from Khang Kao Island, Chon Buri (lanes 2-3), Samet Island, Rayong(lanes 4-5), Chuak Island, Trang (lanes 6-7), Similan Island, Phang-nga (lane 8), *H. varia* from Aeo Island, Phuket, (lanes 9-10), Similan Island, Phang-nga (lane 11-12) with the primer UBC101. Lanes M and m are a 100 bp ladder and λ-*Hin*d III, respectively.

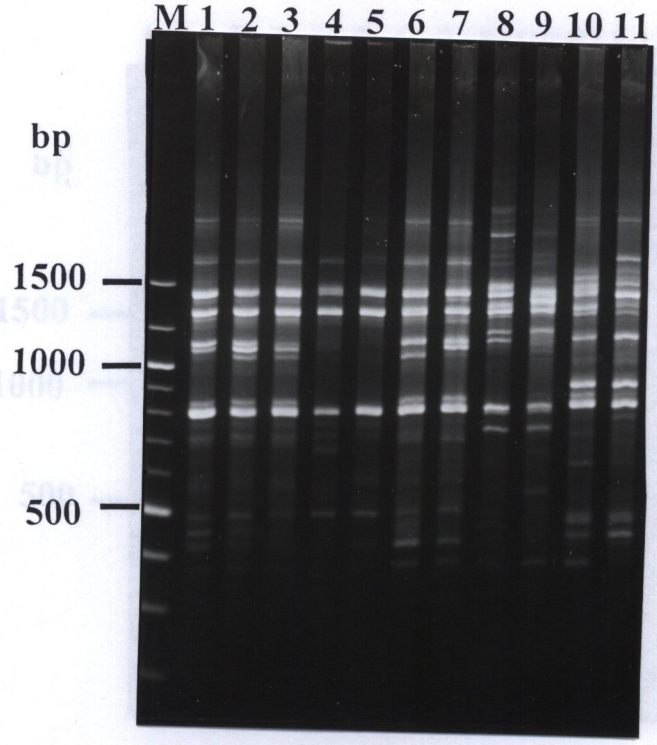


Figure 3.4 RAPD patterns resulted from analysis of *H. asinina* from Samet Island, Rayong (lanes 1-3), Cambodia (lanes 4-7), Talibong Island, Trang (lanes 8-9), and the Philippines (lanes 10-11) with the primer OPB11. Lane M is a 100 bp ladder.

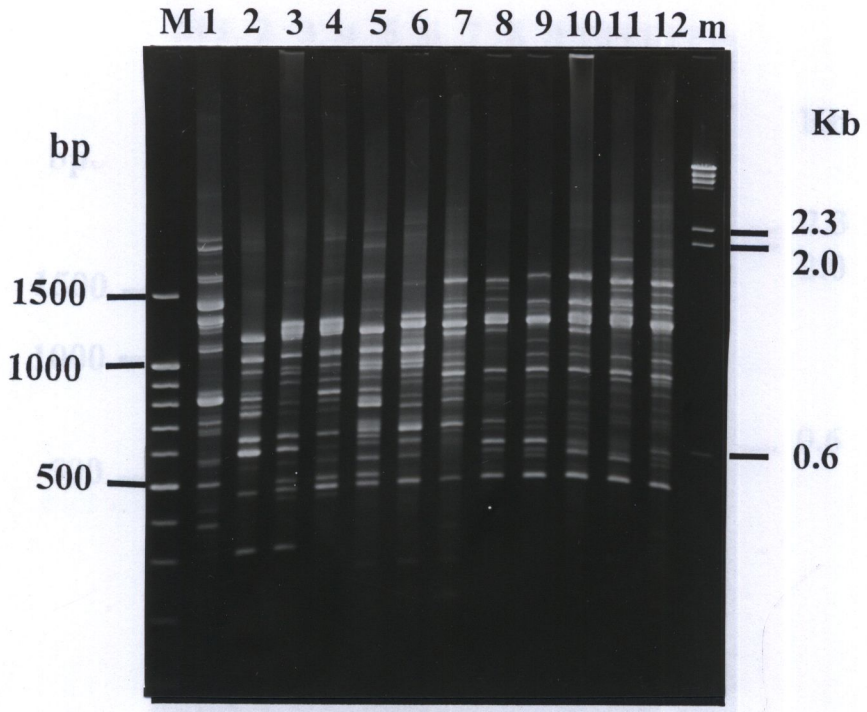


Figure 3.5 RAPD patterns resulted from analysis of *H. asinina* from Samet Island, Rayong (lanes 1), *H. ovina* from Khang Kao Island, Chon Buri (lanes 2-4), Samet Island, Rayong(lanes 5-6), Similan Island, Phang-nga (lane 7-9), Chuak Island, Trang (lanes 10-12) with the primer OPB11. Lanes M and m are a 100 bp ladder and λ-*Hin*d III, respectively.

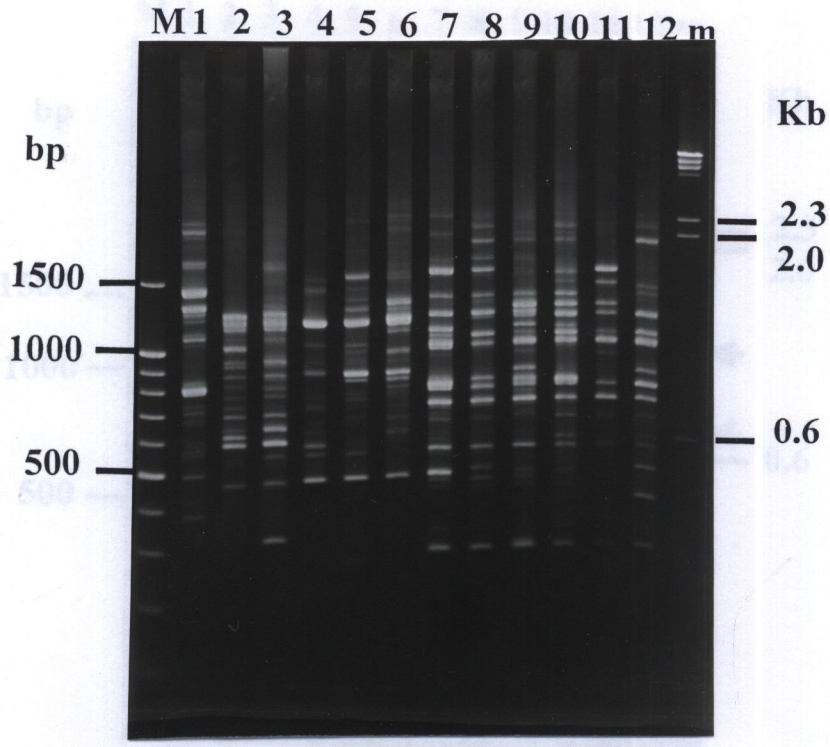


Figure 3.6 RAPD patterns resulted from analysis of *H. asinina* from Samet Island, Rayong (lanes 1), *H. ovina* from Samet Island, Rayong(lanes 2-3), Similan Island, Phang-nga (lane 4), Chuak Island, Trang (lanes 5-6), *H. varia* from Aeo Island, Phuket, (lanes 7-10), Similan Island, Phang-nga (lane 11-12) with the primer OPB11. Lanes M and m are a 100 bp ladder and λ-*Hind* III, respectively.

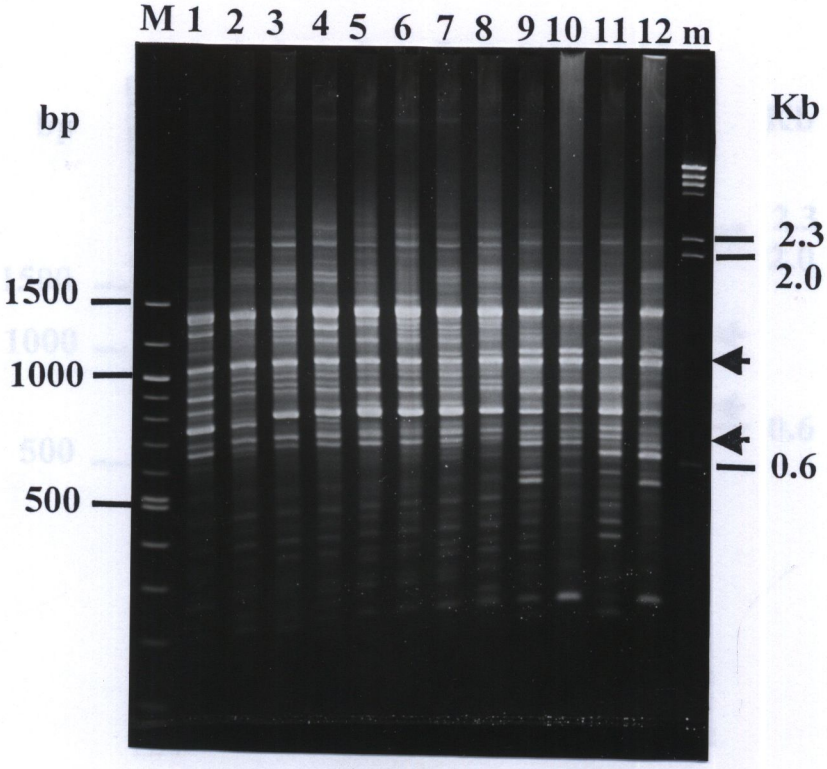


Figure 3.7 RAPD patterns resulted from analysis of *H. asinina* from Samet Island, Rayong (lanes 1-4), Cambodia (lanes 5-8), and The Philippines (lanes 9-12) with the primer UBC195. Lanes M and m are a 100 bp ladder and λ-*Hind* III, respectively. Arrows indicate species-specific markers for *H. asinina* (1030 bp and 650 bp) found in this study.

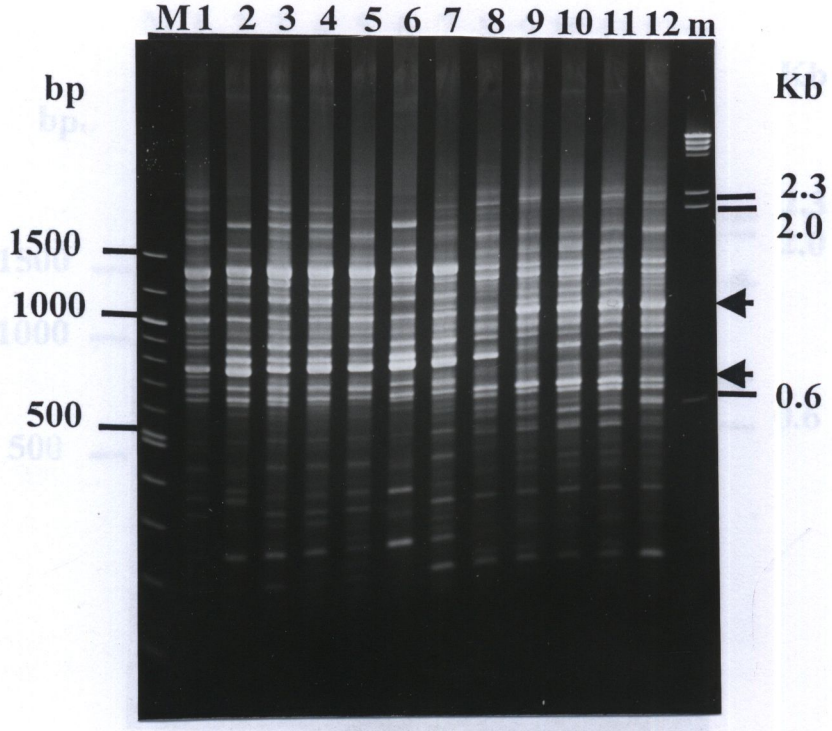


Figure 3.8 RAPD patterns resulted from analysis of *H. asinina* from Samet Island, Rayong (lanes 1), Cambodia (lanes 2-7), and Talibong Island, Trang (lanes 8-12) with the primer UBC195. Lanes M and m are a 100 bp ladder and λ-*Hin*d III, respectively. Arrows indicate species-specific markers for *H. asinina* (1030 bp and 650 bp) found in this study.

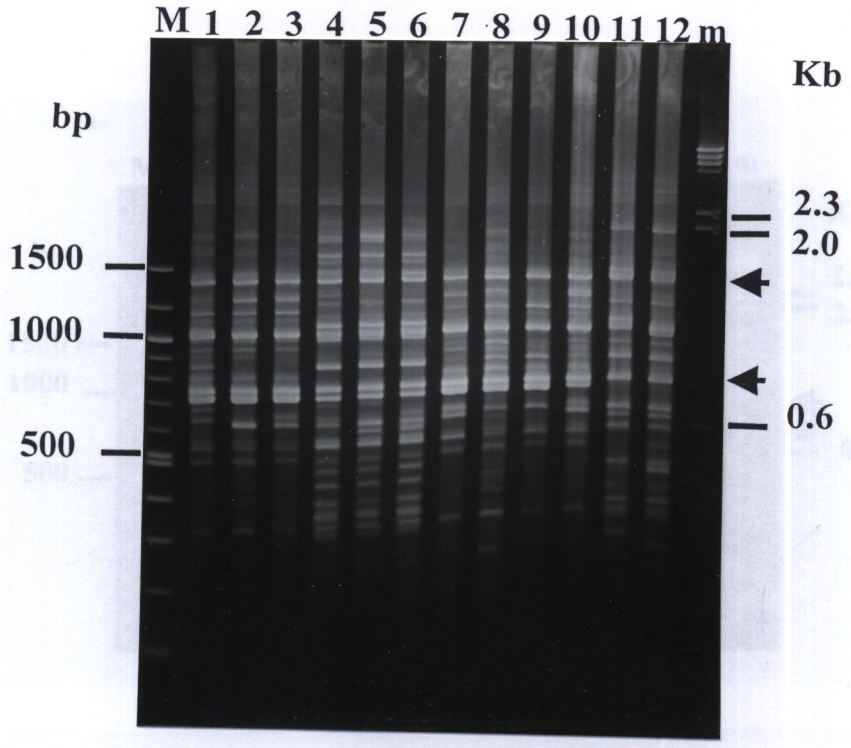


Figure 3.9 RAPD patterns resulted from analysis of *H. asinina* from Samet Island, Rayong (lanes 1-3), Talibong Island, Trang (lanes 4-6), Cambodia (lanes 7-10), and The Philippines (lanes 11-12) with the primer UBC197. Lanes M and m are a 100 bp ladder and λ-*Hin*d III, respectively.

Arrows indicate species-specific fragments (1450 bp and 750 bp) for *H. asinina* found in this study.

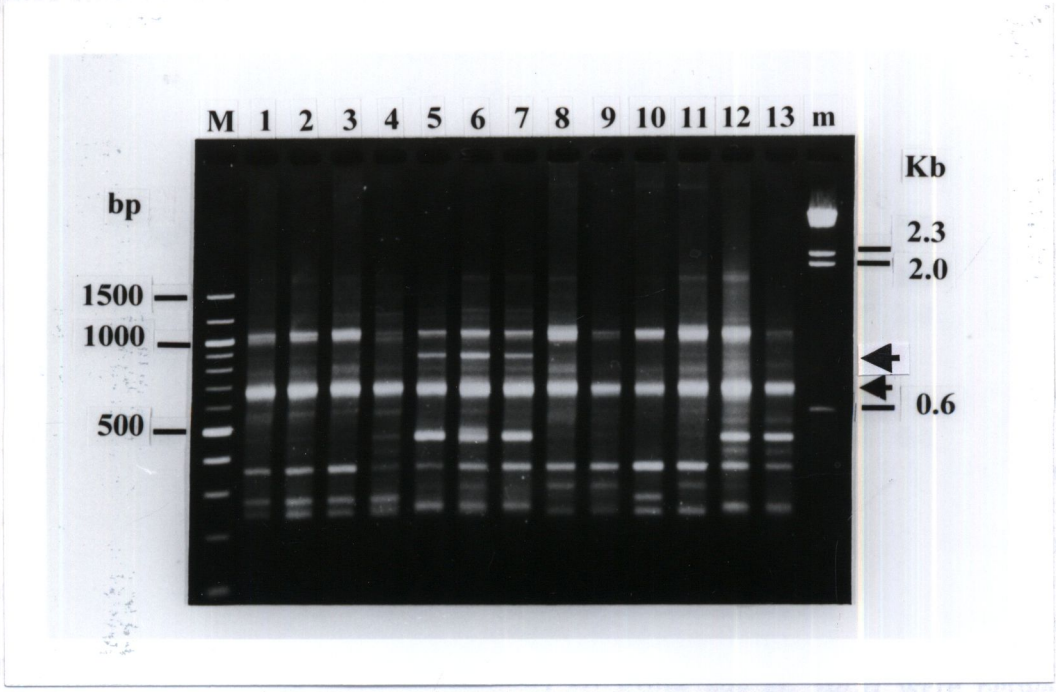


Figure 3.10 RAPD patterns resulted from analysis of *H. asinina* from Samet Island, Rayong (lanes 1-4), Talibong Island, Trang (lanes 5-7), Cambodia (lanes 8-11), and the Philippines (lanes 12-13) with the primer UBC271. Lanes M and m are a 100 bp ladder and λ-*Hind* III, respectively.

Arrows indicated a species-specific marker (680 bp) for *H. asinina* and a population-specific marker for *H. asinina* originating from Talibong Island (880 bp), respectively.

3.4 Genetic relationships of tropical abalone

3.4.1 Similarity index and Genetic distances

The similarity indices between and within species of 12 groups of samples (Table 2.1) analyzed by 5 primers (UBC101, OPB11, UBC195, UBC197 and UBC271) are illustrated by Table 3.4.

The average similarity index across overall samples resulted from primer UBC101, OPB11, UBC195, UBC197 and UBC271 were 0.7715, 0.6830, 0.8002, 0.8444 and 0.8396, respectively (Table 3.4). The mean similarity index within each geographic sample overall primers ranged from 0.5259 (HVPG) - 0.8496 (HALB). Basically, *H. asinina* exhibited the highest level of the similarity index within a species (0.8297) and ranged from 0.7927 (HASM) - 0.8496 (HALB) whereas *H. varia* showed the lowest similarity level (0.5681) within species. These results suggested that *H. asinina* has genetically closer related within species than that of *H. ovina* and *H. varia.* The average similarity index within samples of *H. asinina, H. ovina* and *H. varia* were between 0.7927-0.8496, 0.6010-0.7032 and 0.5259-0.6102, respectively.

Using primers OPB11 and UBC101, the similarity index between samples (S_{ij}) of each primer were calculated. Genetic distances (D_{ij}) were calculated from the similarity index between samples by the equation $D_{ij} = 1 - S_{ij}$. The average genetic distance between geographic samples within species across primers of *H. asinina, H. ovina* and *H. varia* ranged from 0.1578 - 0.4208, 0.3259 - 0.4827 and 0.4295, respectively (Table 3.5). Average genetic differences within 3 species were 0.2995, 0.4328 and 0.4295 for *H. asinina, H. ovina* and *H. varia,* respectively. The results from each primer are shown in an Appendix D. Notably, *H. ovina* showed the highest genetic diversity within species compared to *H. varia* and *H. asinina.*

Table 3.4 Estimated similarity indices (S) within geographic samples of abalone in Thailand using 5 selected RAPD primers. Detail information and abbreviations of sample sites are shown in Appendix A.

Sample	Primer					Average similarity within a sample across all primers
	UBC101	OPB11	UBC195	UBC197	UBC271	
HASH	0.8812	0.8112	0.8652	0.8666	0.8011	0.8451±0.0362
HASM	0.8571	0.7831	0.7066	0.8192	0.7974	0.7927±0.0556
HACH	0.8686	0.7973	0.8048	0.8941	0.8432	0.8416±0.0412
HACB	0.8640	0.8411	0.7749	0.8667	0.8317	0.8357±0.0371
HALB	0.8146	0.8133	0.8674	0.8385	0.9141	0.8496±0.0423
HAPH	0.8392	0.8143	0.7825	0.7811	0.8503	0.8135±0.0317
HOSC	0.7027	0.6431	-	-	-	0.6729±0.0421
HOSM	0.7092	0.6971	-	-	-	0.7032±0.0086
HOPG	0.7371	0.4648	-	-	-	0.6010±0.1925
HOTR	0.7053	0.5382	-	-	-	0.6218±0.1182
HVPK	0.7313	0.4891	-	-	-	0.6102±0.1713
HVPG	0.5482	0.5036	-	-	-	0.5259±0.0315
Average similarity of each primer across all samples	0.7715 ± 0.0997	0.6830 ± 0.1474	0.8002 ± 0.0608	0.8444 ± 0.0403	0.8396 ± 0.0424	

Table 3.5 The average genetic distance (D_{ij}, below diagonal) and similarity indices (S_{ij}, above diagonal) between and within species of 3 species of tropical abalone

	HASH	HASM	HACH	HACB	HALB	HAPH	HOSC	HOSM	HOTR	HOPG	HVPK	HVPG
HASH	-	**0.8068**	**0.8346**	**0.8423**	**0.6304**	**0.6268**	0.2528	0.2359	0.2556	0.2703	0.2957	0.2436
HASM	**0.1933**	-	**0.7985**	**0.8235**	**0.6122**	**0.6282**	0.2682	0.2493	0.2626	0.2872	0.2917	0.2464
HACH	**0.1655**	**0.2015**	-	**0.8302**	**0.6172**	**0.6093**	0.2706	0.2535	0.2660	0.2865	0.2825	0.2253
HACB	**0.1578**	**0.1765**	**0.1698**	-	**0.6182**	**0.6508**	0.2726	0.2553	0.2620	0.2907	0.3081	0.2451
HALB	**0.3697**	**0.3878**	**0.3828**	**0.3818**	-	**0.5793**	0.2969	0.2830	0.3384	0.3491	0.3508	0.3011
HAPH	**0.3732**	**0.3718**	**0.3907**	**0.3492**	**0.4208**	-	0.3038	0.5873	0.3489	0.3737	0.3182	0.2945
HOSC	0.7473	0.7319	0.7295	0.7275	0.7032	0.6962	-	**0.6741**	**0.5173**	**0.5201**	0.4388	0.3981
HOSM	0.7641	0.7508	0.7466	0.7447	0.7170	0.4127	**0.3259**	-	**0.5518**	**0.5464**	0.4209	0.4101
HOTR	0.7445	0.7374	0.7340	0.7381	0.6616	0.6512	**0.4827**	**0.4483**	-	**0.5933**	0.3761	0.3684
HOPG	0.7298	0.7128	0.7135	0.7093	0.6509	0.6264	**0.4799**	**0.4536**	**0.4067**	-	0.3803	0.3558
HVPK	0.7043	0.7084	0.7175	0.6919	0.6493	0.6818	0.5612	0.5791	0.6240	0.6197	-	**0.5706**
HVPG	0.7565	0.7536	0.7747	0.7550	0.6990	0.7055	0.6020	0.5899	0.6316	0.6443	**0.4295**	-

The similarity index between populations of *H. asinina* were calculated by the same procedure but concerning the similarity index within geographic sample. The genetic distances (D_{aij}) between pairs of geographic samples were corrected for effects of similarity within samples. The average genetic distance between geographic samples within species across all primers of *H. asinina* ranged from 0.0156-0.2381 (Table 3.6). The average genetic distance of *H. asinina* in the Gulf of Thailand (HASH, HASM, HACH and HACB) was 0.0243 indicating that they are mostly resembling compared to the Andaman (HALB) and The Philippines (HAPH).

Table 3.6 The average genetic distance (D_{aij}, below diagonal) and similarity indices (S_{aij}, above diagonal) within species of *H. asinina*

	HASH	HASM	HACH	HACB	HALB	HAPH
HASH	-	0.9730	0.9737	0.9844	0.7619	0.7781
HASM	0.0270	-	0.9683	0.9781	0.7686	0.7871
HACH	0.0263	0.0317	-	0.9766	0.7904	0.7764
HACB	0.0156	0.0219	0.0234	-	0.7691	0.7933
HALB	0.2381	0.2314	0.2096	0.2309	-	0.8203
HAPH	0.2219	0.2129	0.2236	0.2067	0.1797	-

3.4.2 Phylogeography

A neighbor-joining tree constructed from the average genetic distance between paired geographic samples indicated phylogenetically clear separation between investigated abalone species (using two primers, Figure 3.11) and geographic samples (using five primers, Figure 3.12). The neighbor-joining trees from each RAPD primer are shown in the Appendix E and F, respectively.

Genetic relationships from six geographic samples of *H. asinina* illustrated by Figure 3.12, demonstrated clearly differences between the Andaman (HALB) and The Philippines (HAPH) from the Gulf of Thailand populations (HASH, HASM, HACH and HACB). Likewise, phylogeographic differentiation from different coastal areas were observed in *H. ovina*. The phylogenetic relationships clearly divided in *H. ovina* to 2 different groups, HOSC and HOSM from the Gulf of Thailand and HOPG and HOTR from the Andaman Sea. For *H. varia*, due to small sample size from Similan Island, Phang-nga (HVPG), such a conclusion could not be made because population size were too small to be concluded accurately.

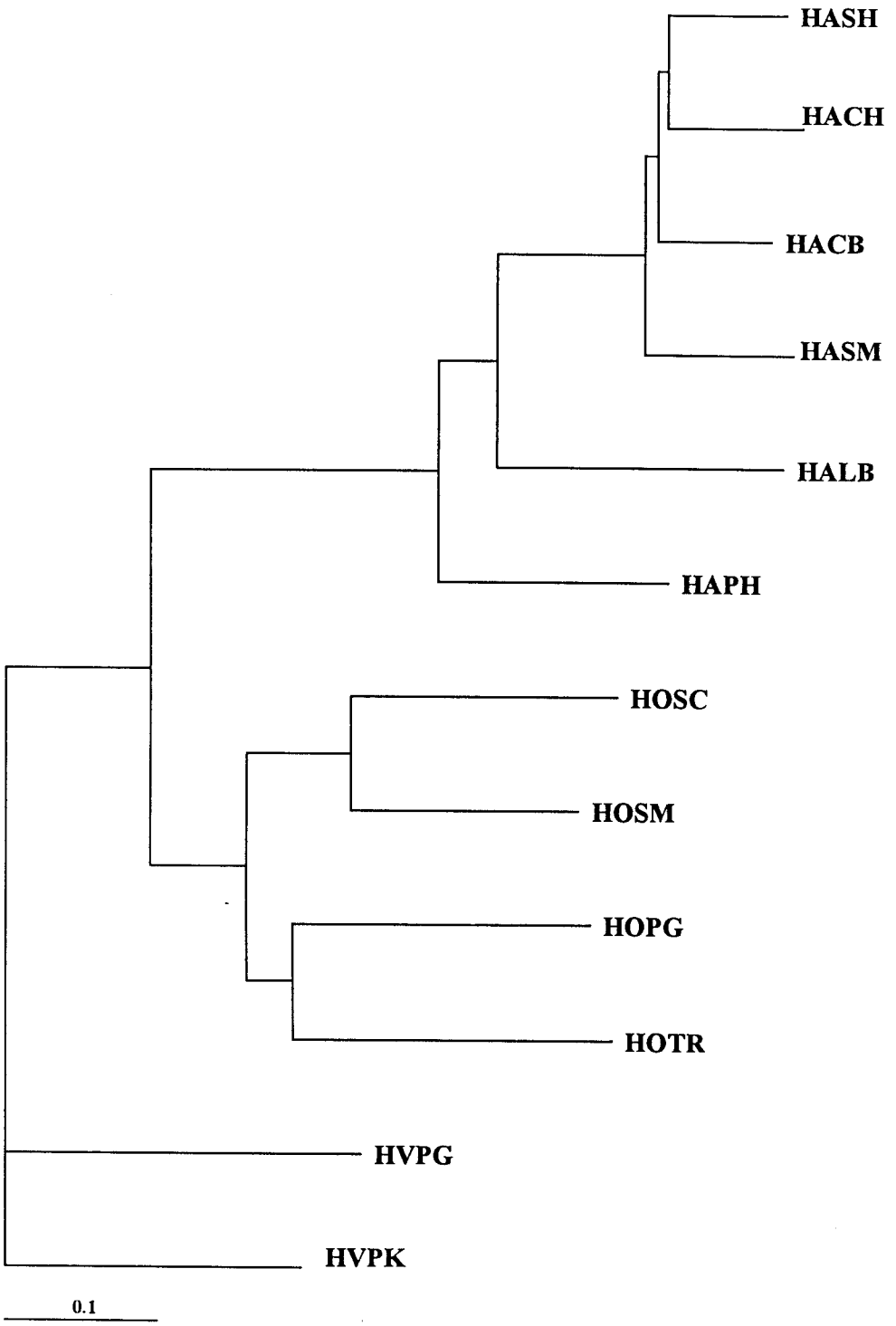


Figure 3.11 A neighbor-joining tree illustrating genetic relationships of Thai abalone in Thailand based on genetic distances resulted from RAPD analysis using two primers (UBC101 and OPB11). Detailed information and abbreviations of sample sites are shown in the Appendix A.

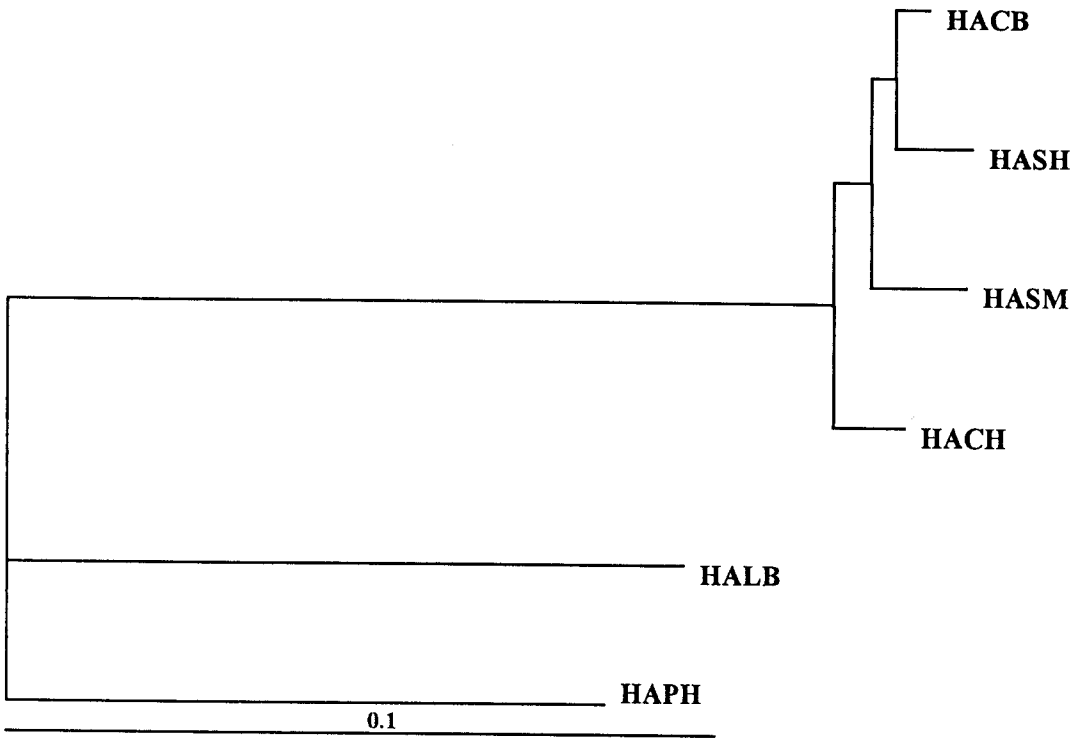


Figure 3.12 A neighbor-joining tree illustrating genetic relationships of *H. asinina* based on genetic distances resulted from RAPD analysis using five primers (UBC101, OPB11, UBC195, UBC197 and UBC271). Detailed information and abbreviations of sample sites are shown in the Appendix A.

3.5 Specific genetic markers of Tropical abalone found in Thailand

Four primers (UBC101, UBC195, UBC197 and UBC271) generated RAPD fragment exhibiting at least 95% of overall investigated specimens. The primer UBC101 yielded a species-specific marker in *H. asinina* (1700 bp) and the the Philippines samples (380 bp). The primer UBC195 showed species-specific nature in *H. asinina* (1030 and 650 bp) while the primer UBC197 provided RAPD markers in *H. asinina* (1450 and 750 bp). Additional species-specific RAPD markers in *H. asinina* (680 bp) and *H. asinina* from Talibong Island (880bp) were identified by the primer UBC271. No species-specific RAPD fragments in *H. ovina* and *H. varia* were found (Table 3.7).

Table 3.7 Specific RAPD markers of tropical abalone in Thailand revealed by RAPD analysis

Species markers	Primer	RAPD marker
Haliotis asinina	**UBC101**	**1700**
	UBC195	**1030, 650**
	UBC197	**1450, 750**
	UBC271	**680**
Population markers		
H. asinina **Talibong Island, Trang**	**UBC271**	**880**
H. asinina **The Philippines**	**UBC101**	**380**

CHAPTER IV

DISCUSSION

Population genetic studies in abalone provided important information for both breeding and conservation in these taxa. Nevertheless, there have been no reports on this and related topics in Thai abalone (*H. asinina*, *H. ovina* and *H. varia*). One of the most difficult problems for genetic studies of abalone is collection of appropriate specimens to be studied by effective techniques. The sample collection sites in this study were essentially different from data previously reported by Geiger (2000) who did not report that *H. asinina* is existent in the Gulf of Thailand and *H. varia* is commonly found in 317 points of the world. Practically, *H. asinina* is available in the Gulf of Thailand but it was not possible to collect *H. asinina*. The latter species was only found as a rare species in the west of peninsular Thailand (Andaman Sea).

The reason to explain contradiction between data of Geiger (2000) and those of the present study is that most publication of species and distribution of abalone in Thai waters are in Thai whereas database from Geiger (2000) were accumulated from the past finding published in English. Among three Thai abalone, *H. varia* has been regarded as the most abundant species in the west of peninsular Thailand. It could commonly be found in Phuket and neighboring provinces. Overexploitation and destruction of the habitats are probably resulted in decreasing of *H. varia* rapidly.

DNA extracted from hemolymp, live and frozen specimens and those preserved in absolute ethanol provided comparable DNA quality to be used for RAPD analysis if DNA extraction was carried out as soon as possible. Specimens died during the collection period provided severe degraded genomic DNA suggesting a low quality for RAPD analysis.

Molecular markers are powerful tools for analysis of genetic relationships and diversity at different taxonomic levels. Hadrys *et al.*, (1992) illustrated advantages of RAPD analysis in various applications in molecular genetics and ecology including its suitability to examine genome which is not well studied using only limited quantity of DNA in a rapid time period. As a result, RAPD is one of the potential techniques for genetic investigation of various organisms particularly when dealing with a large number of specimens.

In the present study, five primers (OPB11, UBC101, UBC195, UBC197 and UBC271) were selected for RAPD analysis. However, the last three primers provided difficulties for amplification of *H. ovina* and *H. varia* DNA. They were then used for determination of intraspecific genetic diversity of *H. asinina* while OPB11 and UBC101 were used for analysis of diversity in three abalone species. Notably, the annealing temperature for RAPD-PCR of the primer UBC271 was 40° C (high temperature amplification, HAT-RAPD) which gave more reproducible patterns than the typical annealing temperature at 36° C (low temperature amplification, LAT-RAPD)

RAPD analysis revealed high genetic diversity of abalone in Thailand. The percentage of polymorphic bands in *H. asinina*, *H. ovina* and *H. varia* was 84.91%, 94.74% and 91.23%, respectively. The polymorphic band levels in abalone is greater than

47.92% - 77.59% in mud crabs (*Scylla serrata, S. oceanica* and *S. transquebarica*) from eastern Thailand (Klinbunga *et al.*, 2000), 24.2%-47.8% in the black tiger shrimp (*P. monodon*) from different geographic locations in Thailand (Tassanakajon *et al.*, 1997) and 53.23%-77.67% of Crassostrea oysters but as high as Saccostrea and Striostrea oysters in Thailand (Klinbunga *et al.*, 2001).

Nevertheless, the percentage of polymorphic bands in RAPD analysis is largely dependent on taxonomic levels and geographic scales of taxa under investigation therefore this parameter should be considered with similarity indices and genetic distances.

Using primers OPB11 and UBC101, the similarity index within geographic samples indicated closer genetic relations between *H. asinina* sample (0.7927-0.8496) than *H. ovina* (0.6010-0.7032) and *H. varia* (0.5259-0.6102) samples. Genetic distances of abalone at the interspecific level (0.1578-0.4827) were greater than those at intraspecific levels (0.4127-0.7550). These differences were as similar as genetic distances in mud crabs (0.425-0.751; Klinbunga et al., 2000) but much less than those in the freshwater bryozoan, *Cristatella muceda* (0.9633-0.9977; Okamura *et al.*, 1993).

A lower level of genetic differences within *H. asinina* than *H. ovina* and *H. varia* can be explained by the potential mobility of *H. asinina* than other two species. This may have homogenized differentiation between geographically different samples of *H. asinina*. In contrast, genetic differentiation between the Gulf of Thailand and west of peninsular Thailand samples of *H. ovina* is observed owing to lower genetic differences within regions than between regions.

Although high genetic diversity of *H. varia* was found, results from this species should be considered with caution as the number of samples and sample sizes are limited

(only 2 sample sites with N = 28 and 4 for specimens from Phuket and Phangnga, respectively). Nevertheless, the percent of polymorphic bands, similarity index and genetic distance within a Phuket sample was higher than any sample of *H. asinina* suggesting the high genetic diversity in this rare species.

Shepherd and Brown (1993) predicted that population differentiation within each abalone species should be occurred due to short planktonic larval stages and their limited dispersal ranges. Genetically differentiated populations may be found within the scale of a few kilometers. Nevertheless, results from this study revealed clear genetic diversity within *H. ovina* from the west (Andaman) and the east (Gulf) coasts of peninsular Thailand, partial differentiation between *H. asinina* from the Gulf of Thailand and the Andaman and the Philippines but lack of differentiation within the same coastal regions in *H. asinina* and *H. varia* and possibly in *H. varia*. Therefore, factors relevant with population differentiation is not simply explained by the period of planktonic larval stages and dispersing ability of adults and should be further examined in details.

To consider genetic diversity within *H. asinina*, the average parameters from 5 primers would provide more accurate data than data from only OPB11 and UBC101. The average genetic distance of *H. asinina* within the Gulf of Thailand was 0.0243 (0.0156-0.0317) whereas the distance between each of the Gulf samples and *H. asinina* from Talibong and Philippines were 0.2096-0.2381. Results suggested partial differentiation between *H. asinina* from the Gulf of Thailand and the further east sample (Philippines) and the different coastal sample (Talibong).

The genetic diversity of three hatchery stocks (P_0 for HASH and HACH and F_1 for HAPH) exhibited similar levels of genetic diversity as that of natural *H. asinina*. This may

be resulted from the large number of male and female broodstock used for mass spawning breeding scheme in this species.

Tassanakajon *et al.* (1997) examined genetic diversity in 3 geographic samples of *P. monodon* (Trat and Angsila located in the Gulf of Thailand and Satun-Trang, located in the west of peninsular Thailand) using RAPD analysis of 5 primers and found that genetic distance within samples was 0.032-0.070. Conversely, higher diversity (0.171-0.199) was observed within each *S. scyllata*, *S. oceanuca* and *S. tranquebarica* (Klinbunga *et al.*, 2000).

It should be noted that the Indonesian specimen (hemolymp was collected from broodstock) was eliminated from the analysis because reproducible patterns and degree of success of these specimens could not be obtained. The parallel studies with this thesis based on PCR-RFLP of 18S and 16S rDNA and three microsatellite loci (Has2, Has3 and Has8) indicated the failure to amplify those target regions in abalone from Indonesia. Based on the fact that PCR-RFLP and a few microsatellite loci showed cross amplification in *H. ovina* and *H. varia*, the suspected question is arisen. *Why amplification could be carried out successfully in different species but not in a different geographic sample of the same species? Is the Indonesian sample actually H. asinina?* These questions are very interesting and should be studied further.

Phylogenetic analysis of Thai abalone was carried out using a neighbor-joining approach. A neighbor-joining tree of three abalone indicated clear genetic separation between different species. All branches within a species revealed mononphyletic status in Thai abalone when analyzed with primers OPB11 and UBC101. Phylogeography was

obvious in *H. ovina* (the Andaman and Gulf of Thailand samples) and partially observed *H. asinina* (Talibong and the Gulf samples) but not in *H. varia*.

The phylogenetic status of abalone in Thai waters was similar with that of cupped oysters in Thailand analyzed by 5 RAPD primers (OPA09, OPB01, OPB08, UBC210 and UBC220) where phylogeography was found in the white scar oyster (*C. belcheri*) and the black scar oyster (*C. iredalei*) but not in other species (*Saccostrea cucullata, S. forskali* and *Striostrea mytiloides*). However, clear differentiation of each species was found phylogenetically from RAPD analysis of mud crabs (*S. serrata, S. oceanica* and *S. tranquebarica*) in eastern Thailand. An intraspecific neighbor-joining tree of *H. asinina* based on 5 RAPD primers also revealed differentiation of this species.

The topology of a neighbor-joining tree between geographically different samples of three abalones in Thailand also indicated that *H. asinina* and *H. ovina* are more closely related one another than *H. varia*. This was contradictory to results from karyotyping of these abalone (Jarayabhand *et al.*, 1998) where *H. asinina* and *H. varia* were regarded as sister species. The parallel studies of this thesis based on PCR-RFLP of 16S and 18S rDNA and sequencing analysis of 16S rDNA using the same sample set (Pripue, 2001) confirms interspecific genetic relationships of Thai abalone determined by RAPD analysis.

Basically, three species of Thai abalone can be distinguished easily using external characteristics at the adult stage but taxonomic difficulties is found at the larval stages where they cannot be differentiated from each other (and possibly from other mollusc larvae). Therefore, identification of species-specific markers of abalone in Thailand is necessary for such application.

Using RAPD analysis, six *H. asinina*-specific RAPD markers were found from this study. In addition, RAPD markers specifically found in *H. asinina* originating from Talibong and the Philippines were also observed. Nevertheless, species-and/or population-specific markers were not found in *H. ovina* and *H.varia*. The species-specific markers found in this study may be used for quality control of the commercially traded seed. An inability to identify species-specific markers in those two species should be related to a limited number of RAPD primers used for genetic characterization of *H. ovina* and *H. varia*. Alternatively, the nature of genetic diversity in each abalone may have reflected the possibility to identify specific markers within species. In oysters, low genetic diversity (percentage of polymorphic bands and genetic distances) were found in three commercially cultured oysters; *C. belcheri*, *C. iredalei* and *S. cucullata* but extremely high diversity was observed in *S. forskali*, *S. mytiloides* and other morphologically unclear oysters. Several species-specific RAPD markers were determined in the first group of oysters but not in the other group even though PCR-RFLP of three genes (16S, 18S and COI-COII) was additionally used. Comparing with oysters, *H. asinina* showed the pattern and the level of genetic diversity as similar as three commercially cultured oysters whereas *H. ovina* and *H. varia* exhibited similar genetic diversity and patterns as the second group of oysters. Specific markers could easily be identified from the former but not from the latter.

Nonetheless, RAPD markers may provided false negative results due to susceptibility of this technique on various components of PCR and quality of DNA examined. Therefore, these markers should be cloned and sequenced. Species-specific PCR can then be developed using newly designed forward and reverse primers. This

Sequence Characterized Amplification Regions (SCAR) markers would eliminated problems arisen from inconsistency of RAPD markers.

More recently, molecular taxonomic key based on RAPD markers was constructed in three commercially important oysters; *C. belcheri*, *C. iredalei* and *S. cuculalata* (Klinbunga *et al.*, 2001). Three specie-specific SCAR markers were further developed by cloning and sequencing of *C. belcheri*-specific RAPD markers. A primer set was designed from clones representing each markers. Specificity and sensitivity tests indicated their species-specific nature that can detect DNA of the target species at approximately 30 pg.

RAPD-PCR has been successfully used to examine genetic diversity and population differentiation in several economically important marine species in Thailand including *P. monodon* (Tassanakajon *et al.*, 1997 and 1998), mud crabs (Klinbunga *et al.*, 2000) and oysters (Klinbunga *et al.*, 2001) and to develop SCAR markers in *C. belcheri* (Klinbunga *et al.*, 2000). In the thesis, it has been used to identify species-specific markers in *H. asinina* and phylogeography of abalone in Thailand. Population differentiation of *H. asinina* and *H. varia* in Thailand suggests the existence of different stocks within a particular species which should be treated as separate management units. In the conservation point of view, transferring of different stocks to other habitat should be limited. Restocking stocking programs, if carried out, should be restricted to the local stock. For aquaculture application, heritability for economically important phenotypes (growth, mortality and disease-resistance) between different coastal regions of *H. asinina* (and possibly *H. ovina* if can be cultured) should be carried out. Consistent quality control for seed production and canned products of *H. asinina* can be performed after SCAR markers are further developed from RAPD markers found in this study.

CHAPTER V

CONCLUSIONS

1. High genetic diversity levels of *H. asinina, H. ovina* and *H. varia* was observed based on RAPD analysis.
2. Clear genetic differentiation was observed between *H. ovina* originating from the Gulf of Thailand and the Andaman Sea.
3. Within *H. asinina,* partial genetic differentiation between the Gulf of Thailand, the Andaman Sea and the Philippines samples were found from RAPD analysis with primers UBC101, OPB11, UBC195, UBC197 and UBC271).
4. Phylogenetically clear separation between investigated abalone species (using two primers) and geographic samples of *H. asinina* (using five primers) were observed.
5. Six species-specific markers were found in *H. asinina.* Population-specific markers were found in the Philippines (UBC101, 380 bp) and Talibong (UBC271, 880 bp) samples. No species-specific RAPD markers were observed in *H. ovina* and *H. varia.*

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APPENDICES

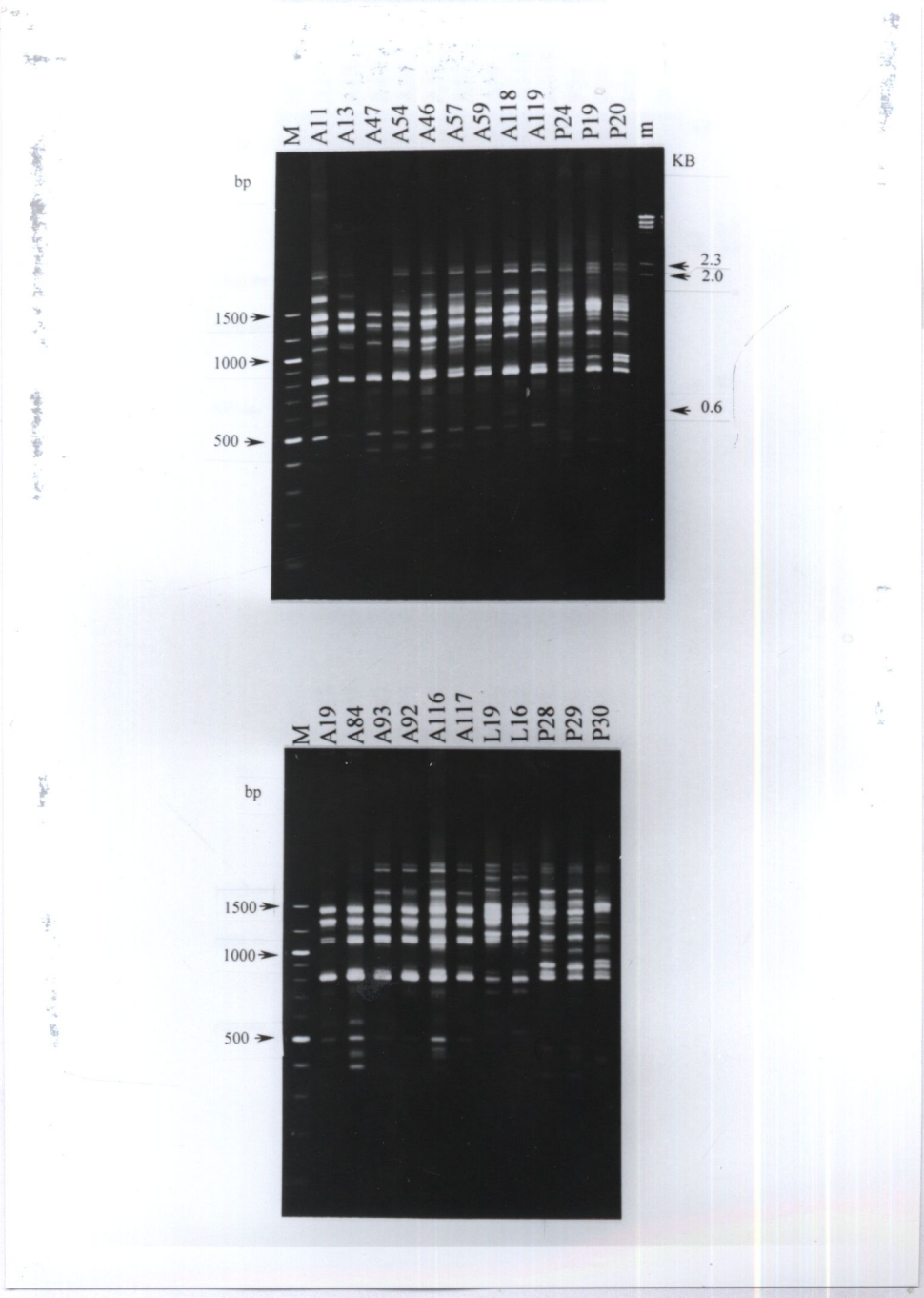
A. Sampling sites, date of collection and code of abalone used in this study

Species	Location	Code of populations	Code of samples	Remark
H. asinina	Angsila Marine Biological Research Station, Angsila, Chon Buri	HASH	A01-A30	Hatchery stock, P_o (Brood stock from Samet Island, Rayong)
	Samet Island, Rayong	HASM	A41-A60	Haemolymp was collected from each specimens
	Phuket Abalone Farm	HACH	A81-A95	Hatchery stock, P_o (Brood stock from Cambodia)
	Cambodia	HACB	A100-A122	
	Talibong Island, Trang	HALB	L01-L28	
	The Philippines	HAPH	P01-P30	Hatchery stock, F_1 (Brood stock from The Philippines)
	Indonesia	-	A61-A80	
H. ovina	Khang Kao Island, Chon Buri	HOSC	O1-O30	
	Samet Island, Rayong	HOSM	O41-O80	
	Chuak Island, Trang	HOTR	T01-T47	
	Similan Island, Phang-nga	HOPG	O81-O96	
H. varia	Aeo Island, Phuket	HVPK	V01-V30	
	Similan Island, Phang-nga	HVPG	V31-V34	

Appendix B

RAPD patterns of all individuals of *Haliotis asinina* (n=99), *H. ovina* (n=95) and *H. varia* (n=33) analyzed by primers OPB11 (B.1), UBC101 (B.2), and *H. asinina* analyzed with UBC195 (B.3), UBC197 (B.4), and UBC271 (B.5). The DNA markers were a 100 bp (Lane M) and λ *Hin*dIII (Lane m), respectively.

B. 1 Primer OPB11



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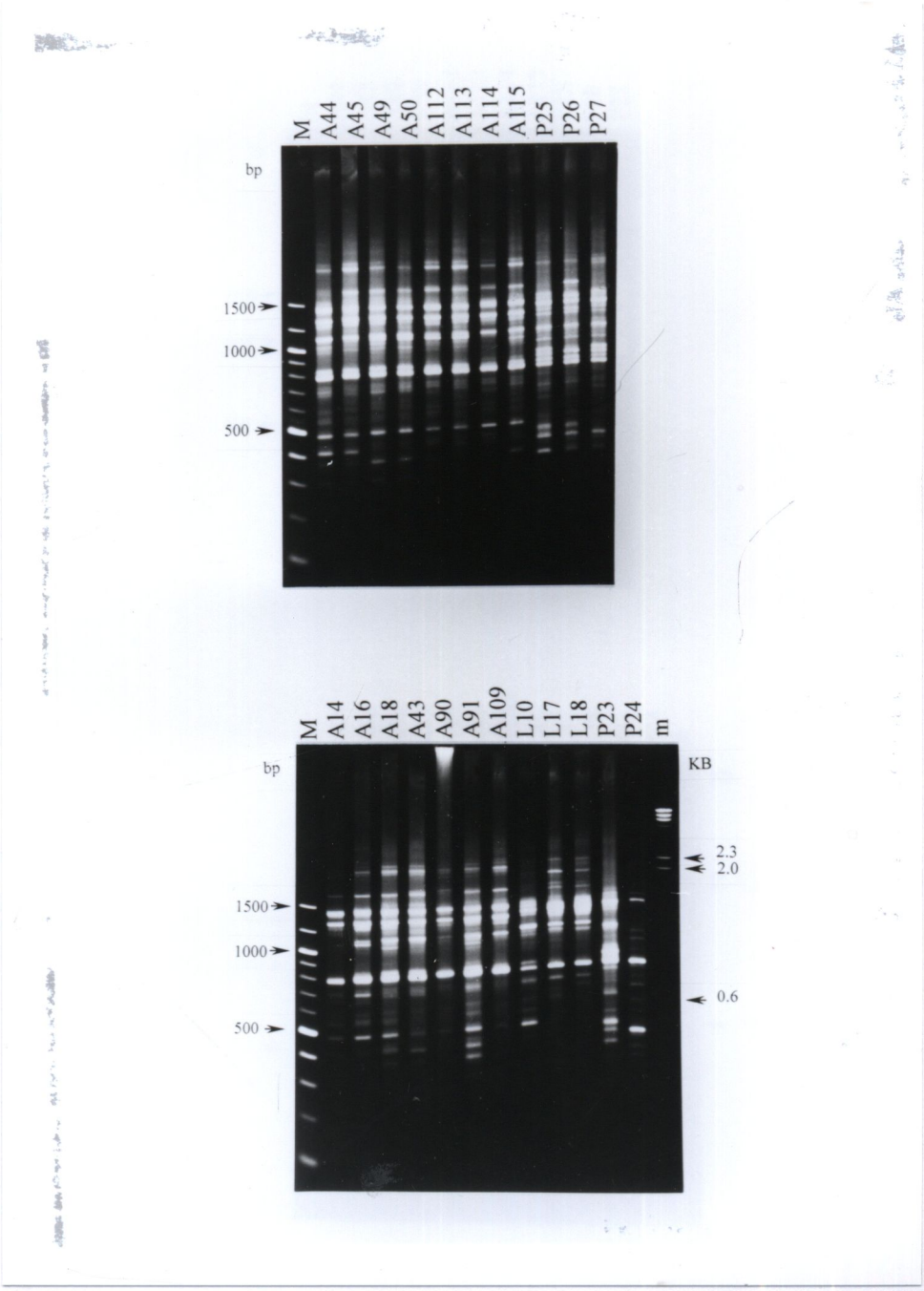

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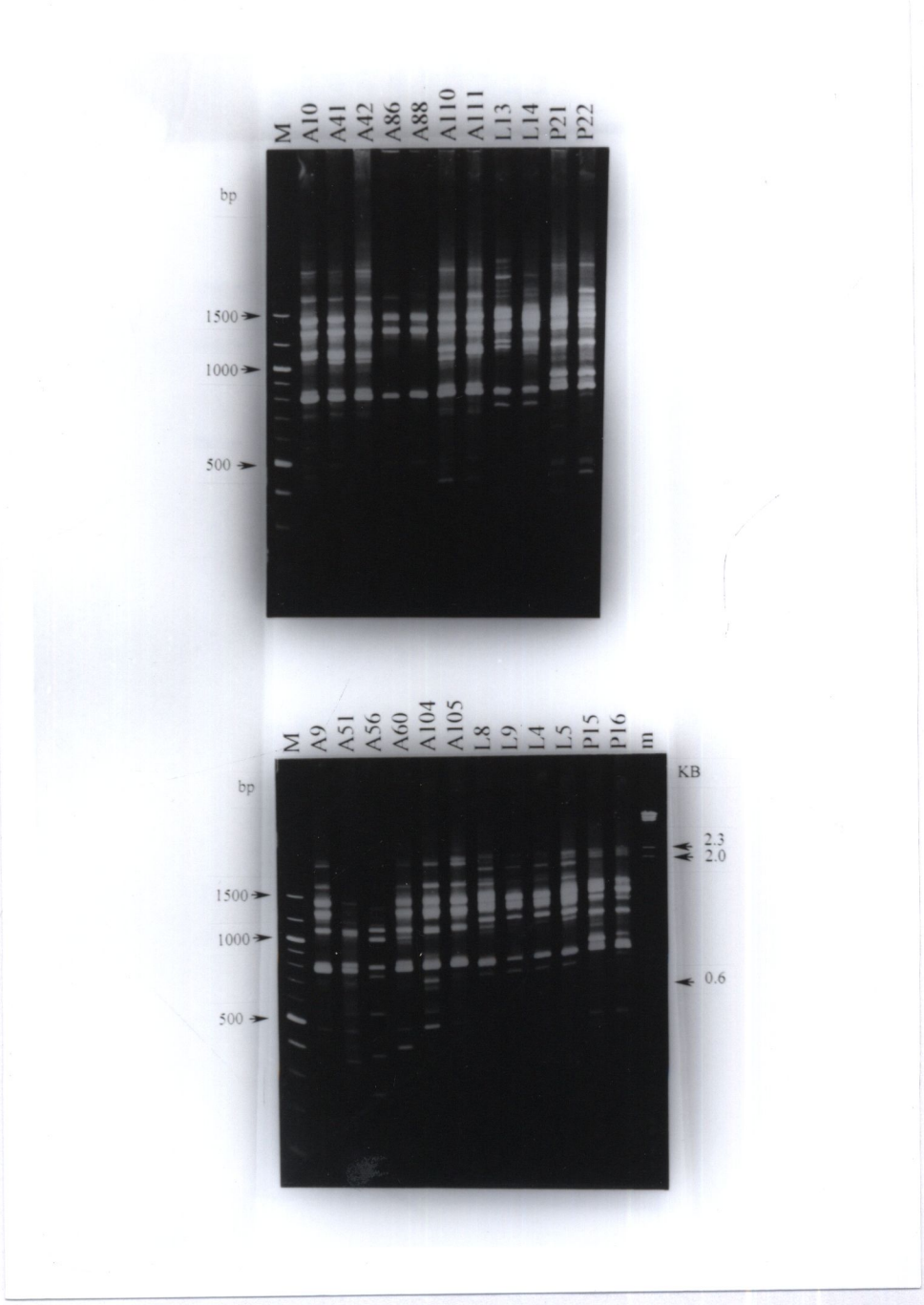

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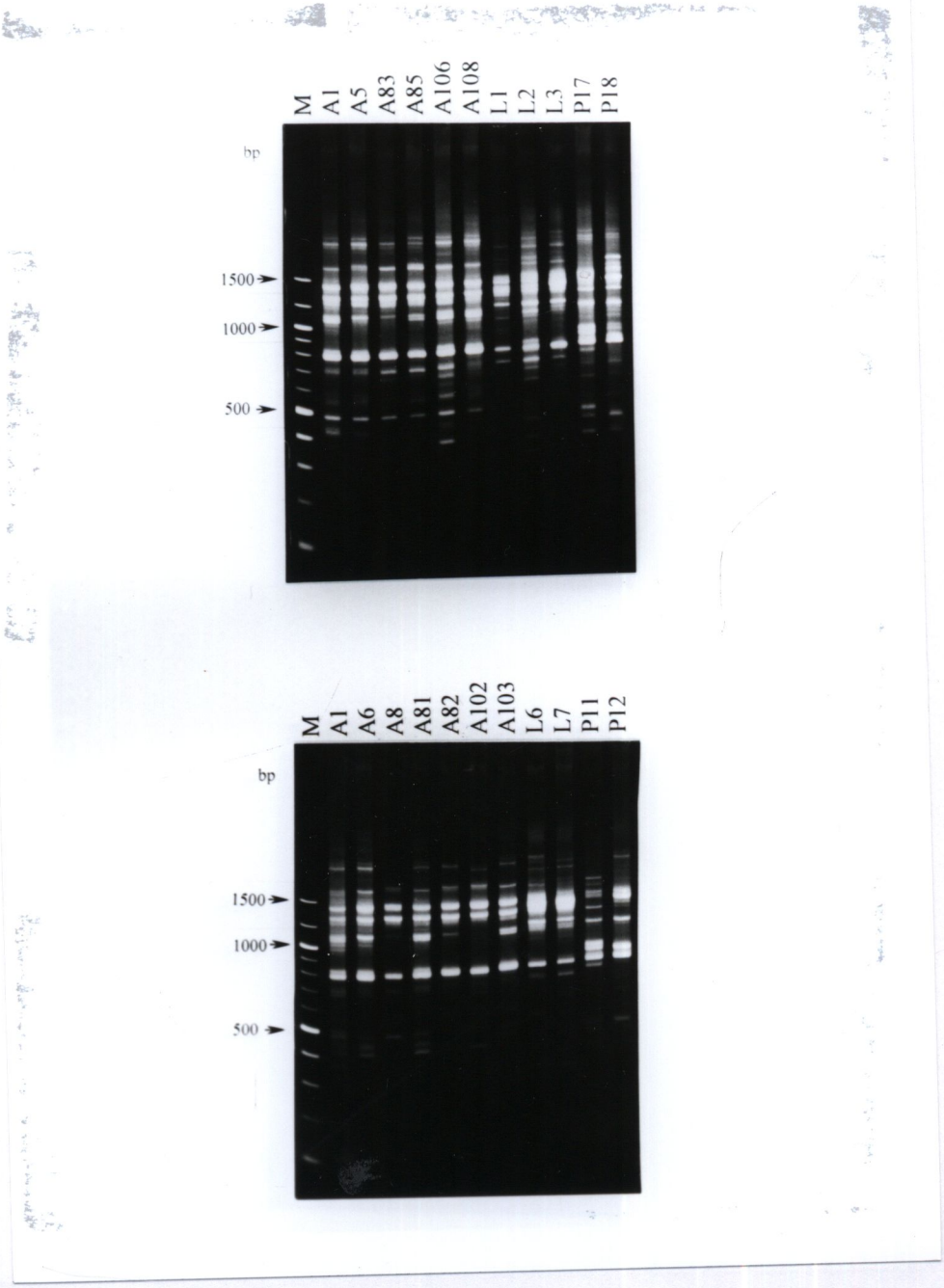

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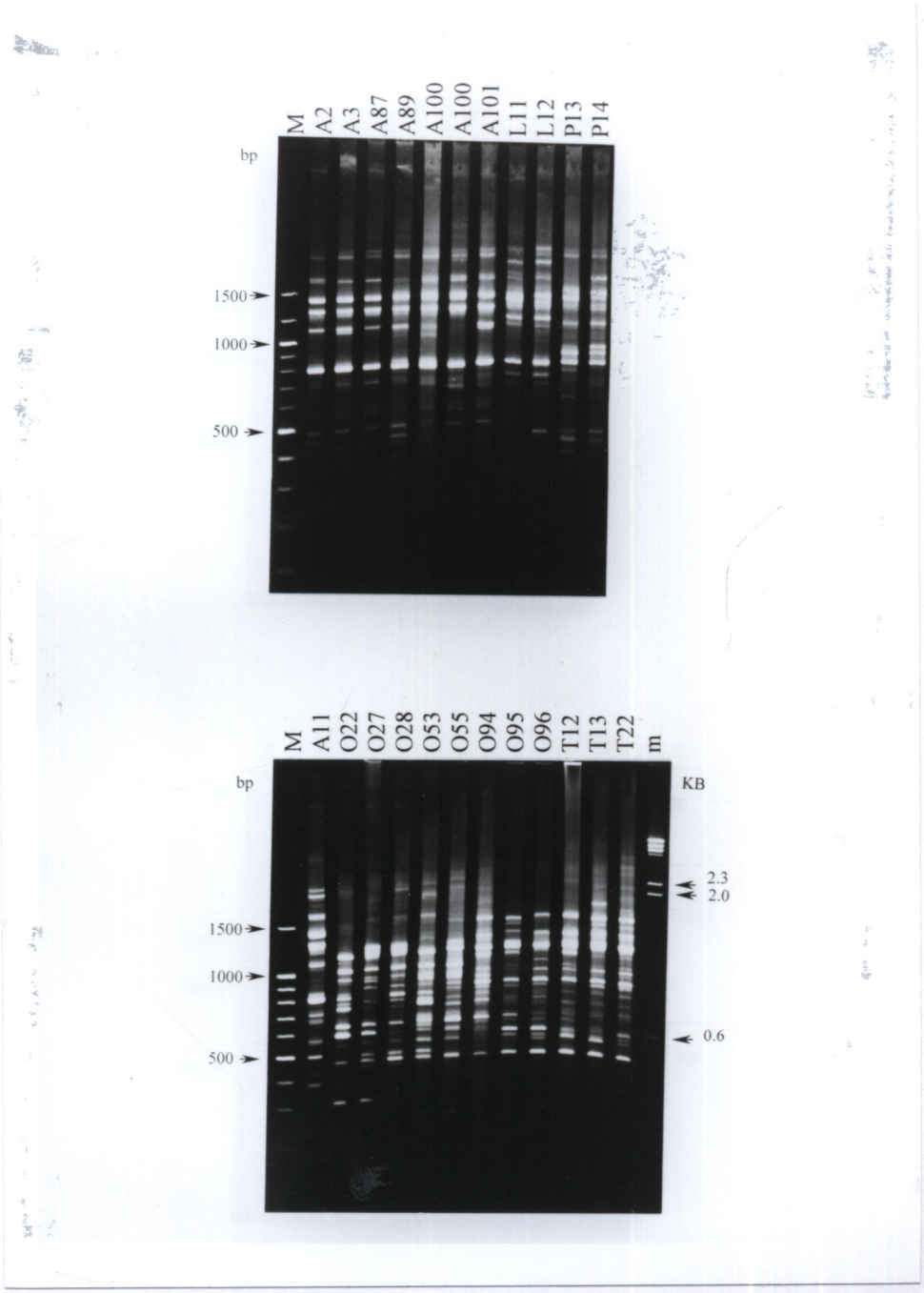

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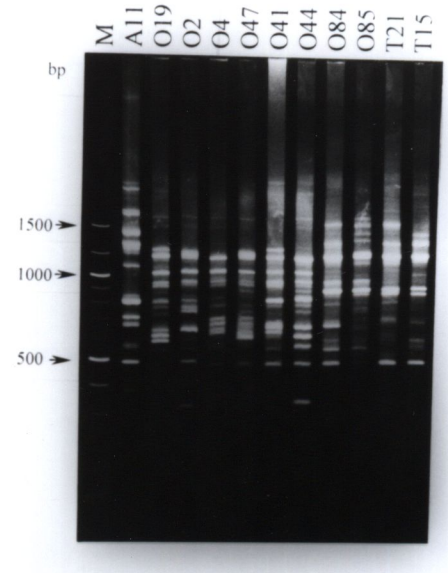

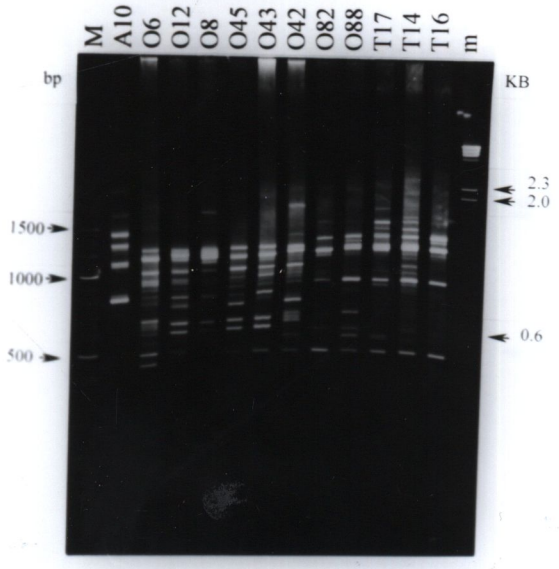

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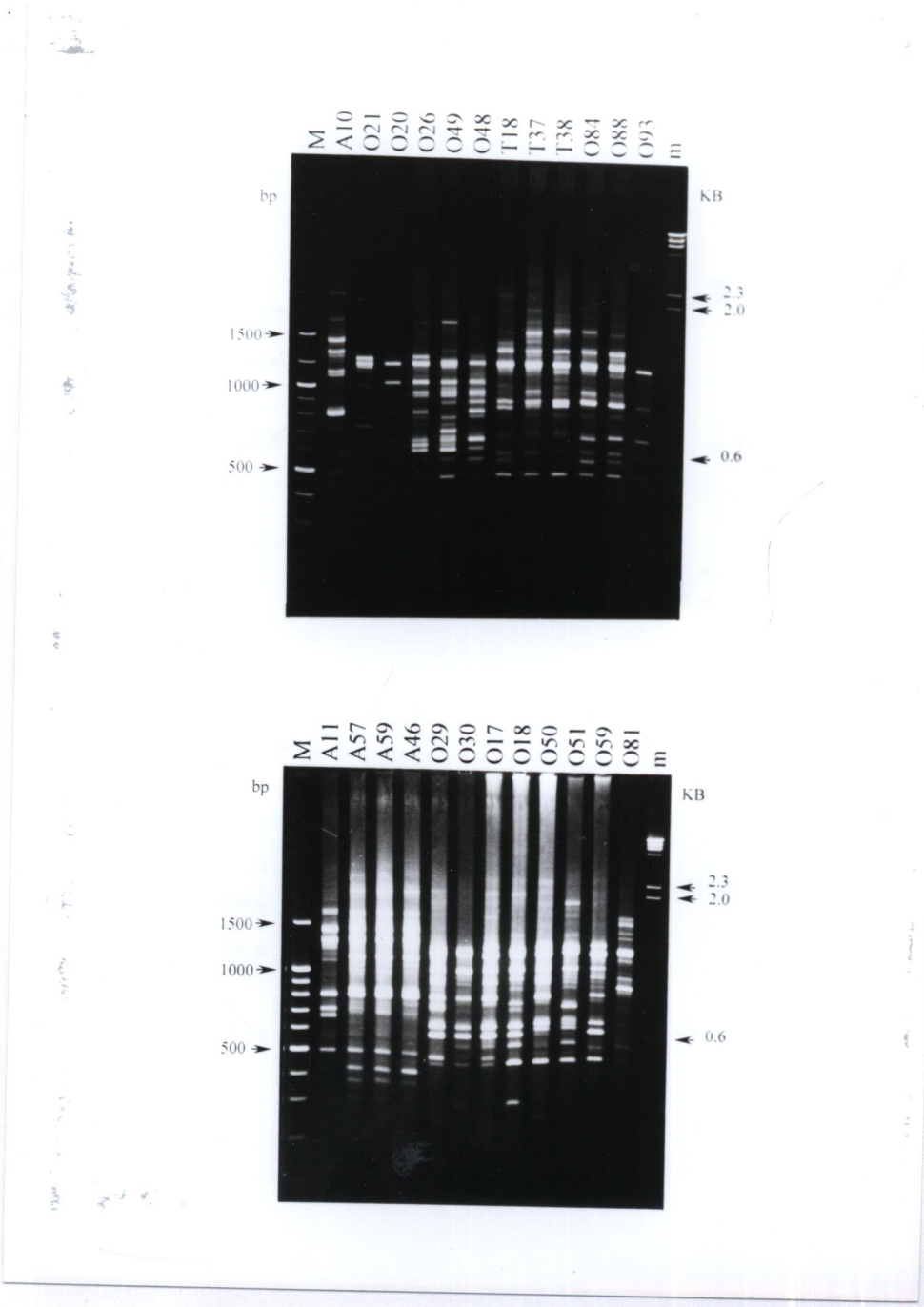

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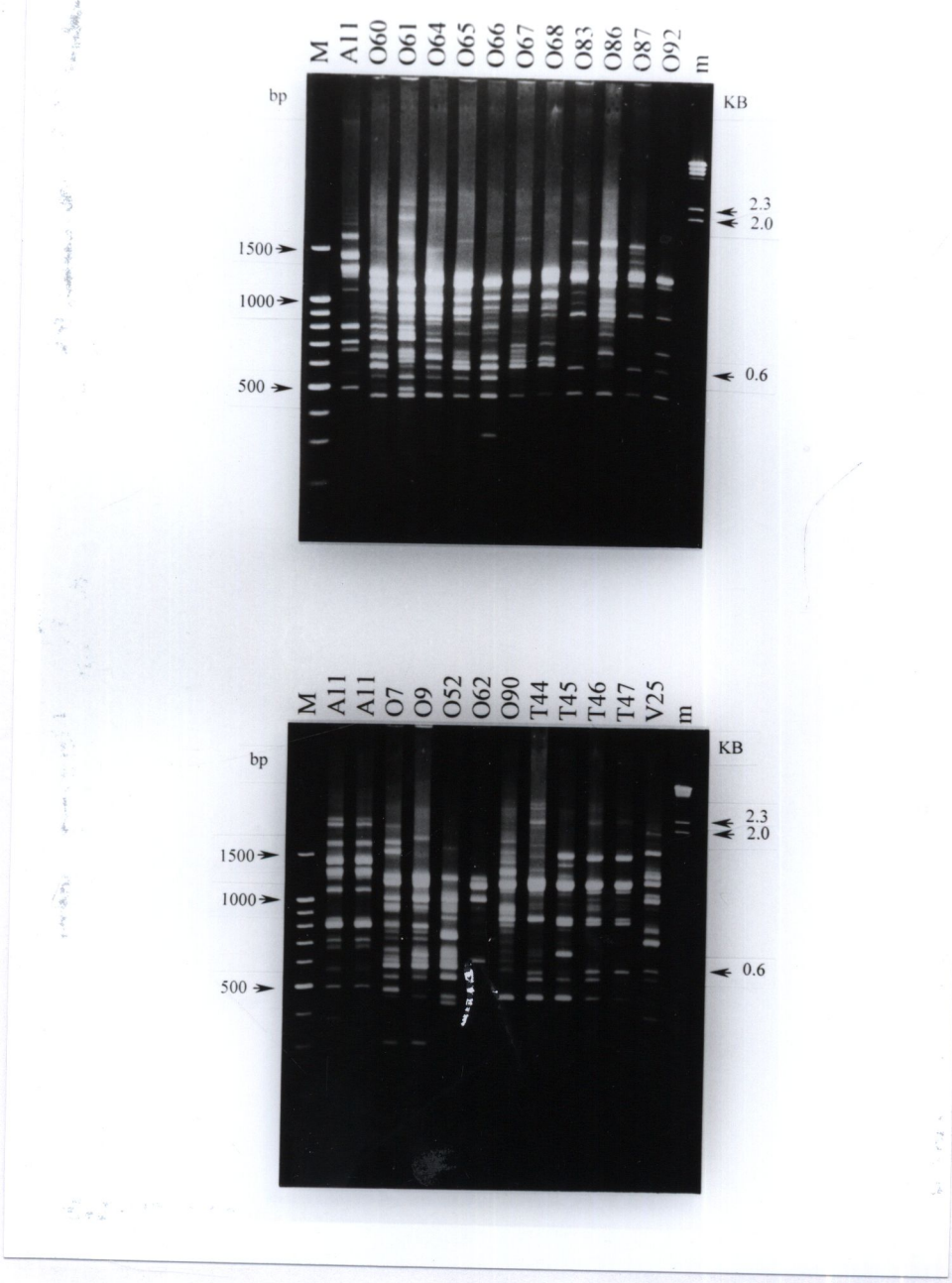

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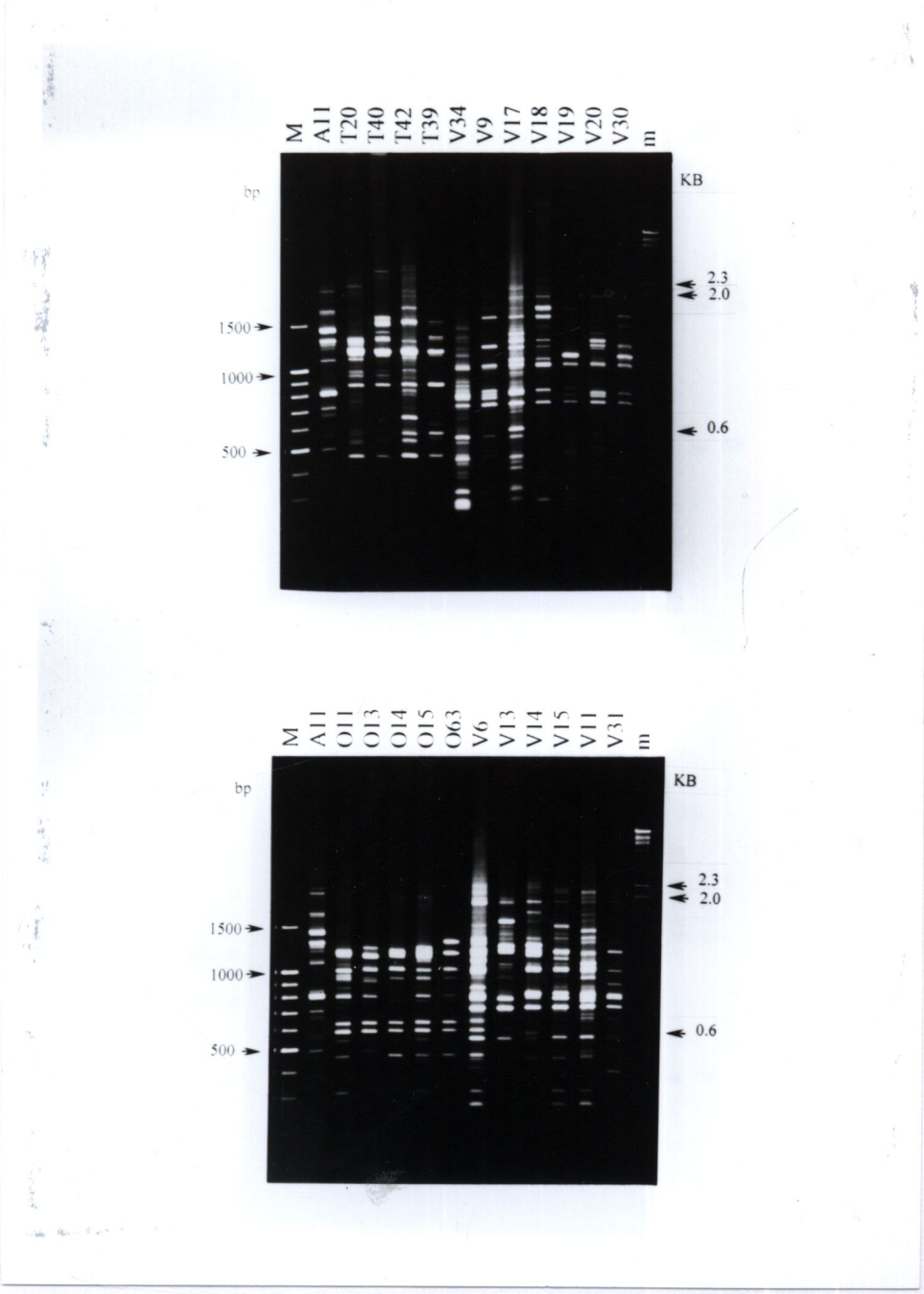

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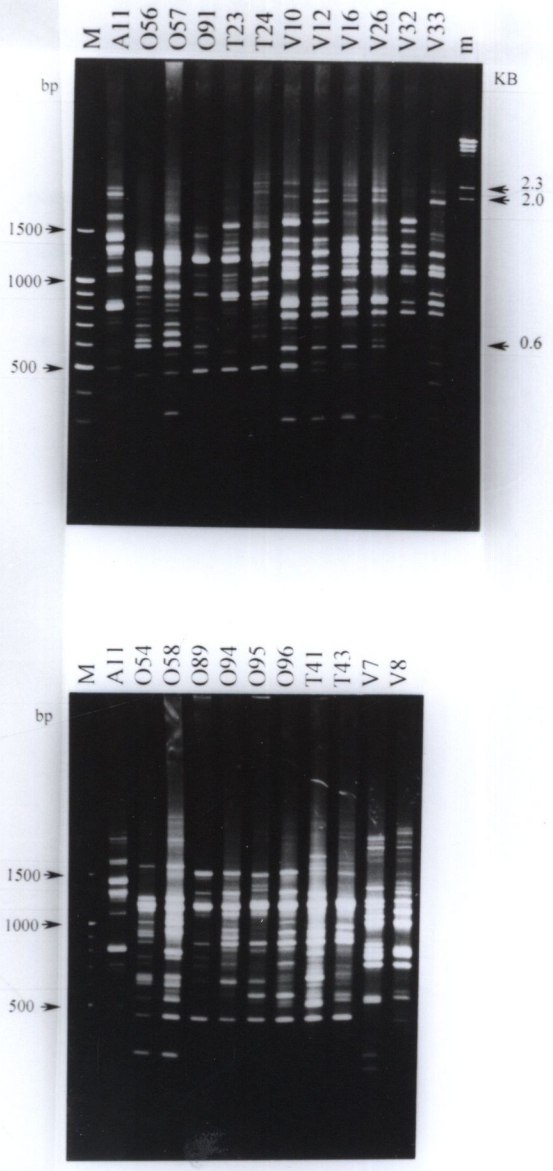

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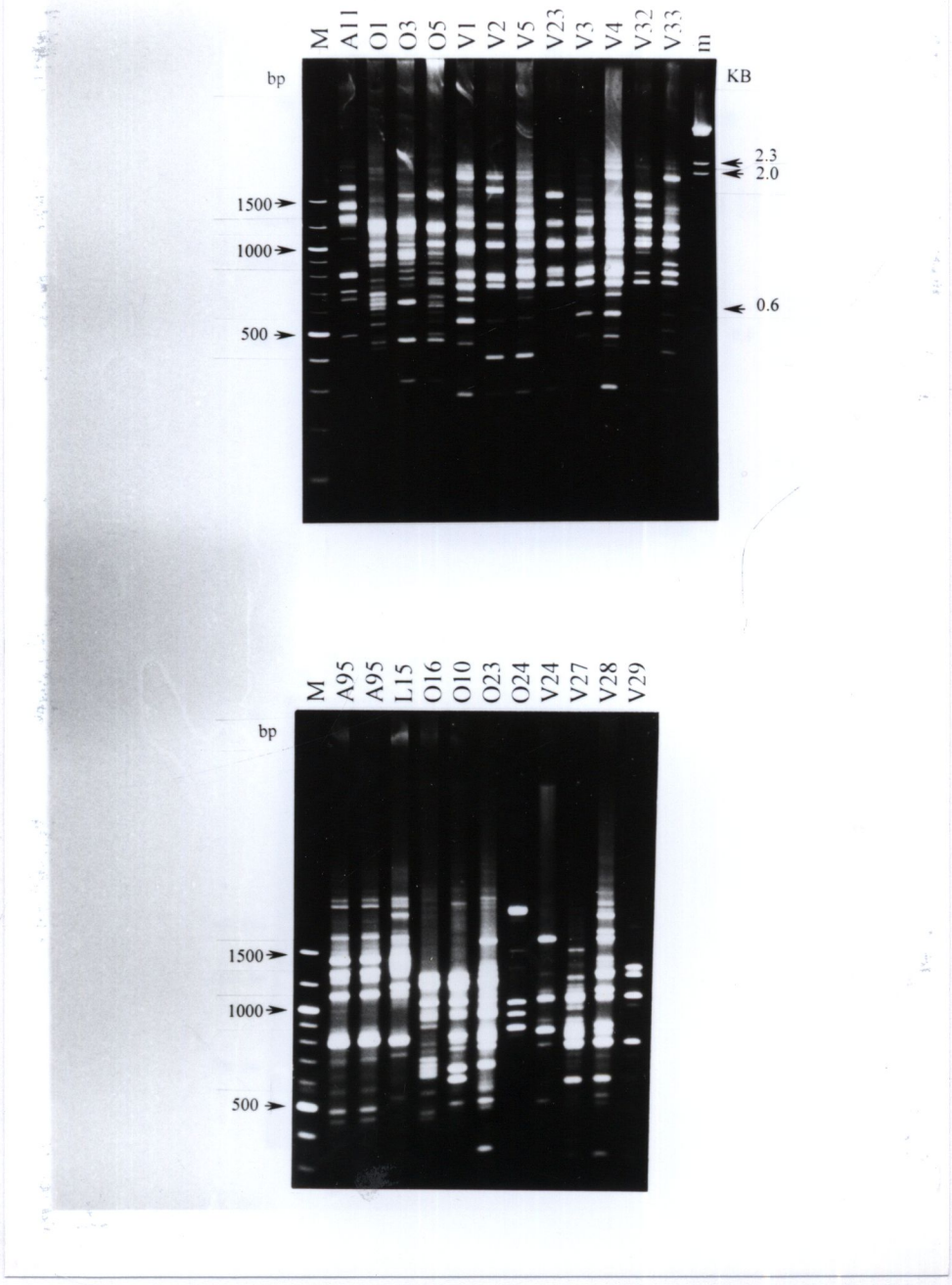
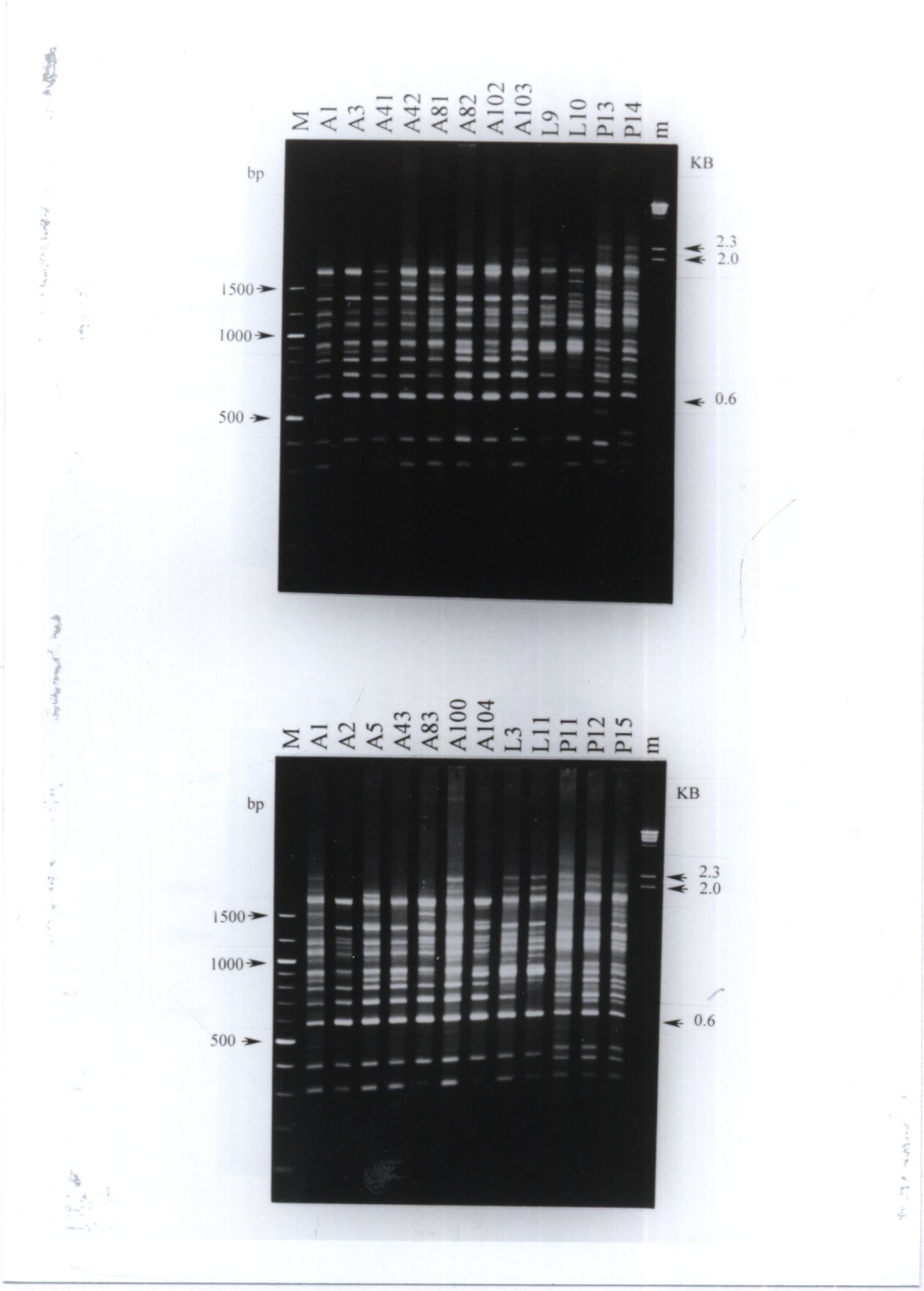


B. 2 Primer UBC101



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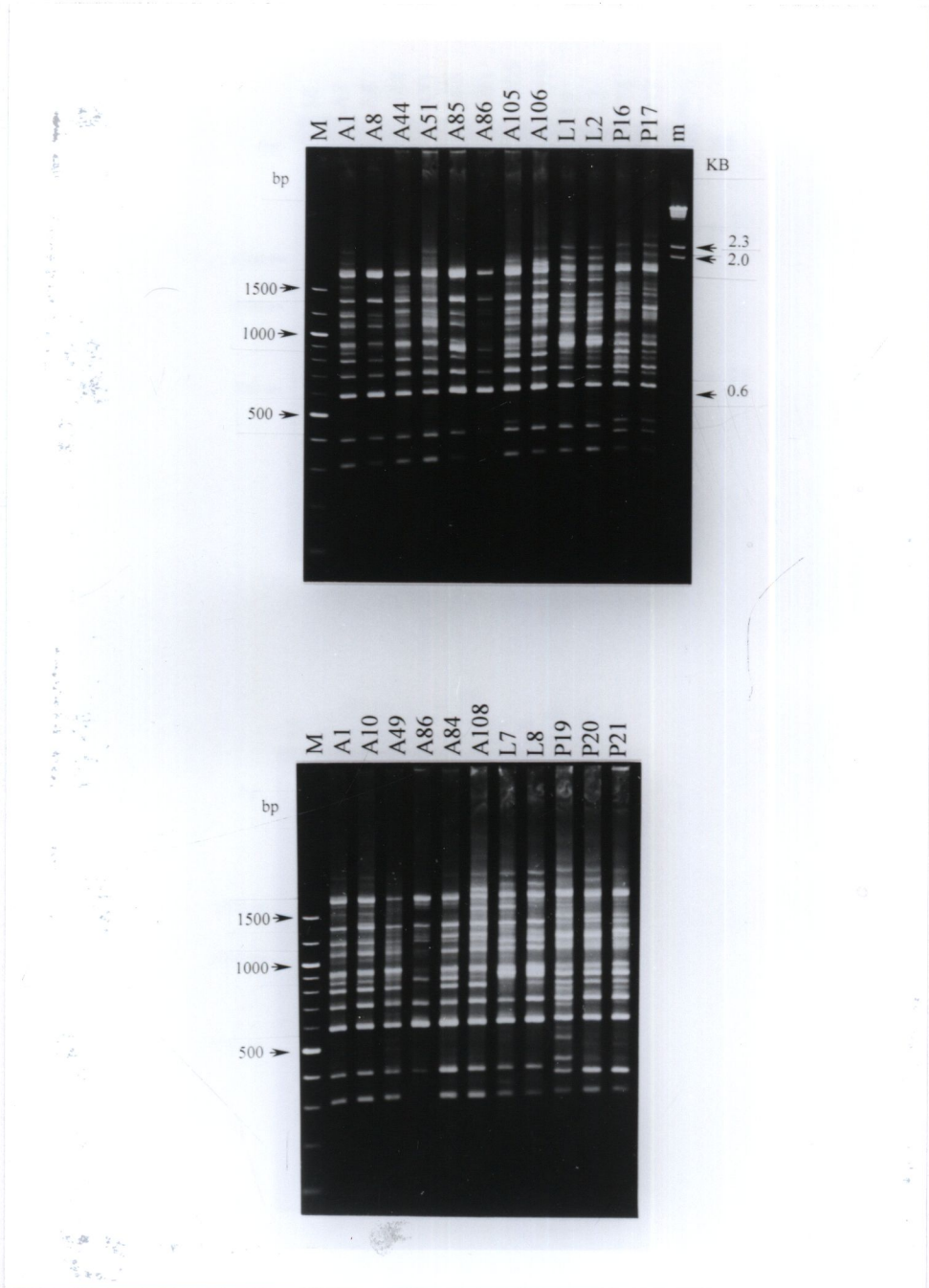

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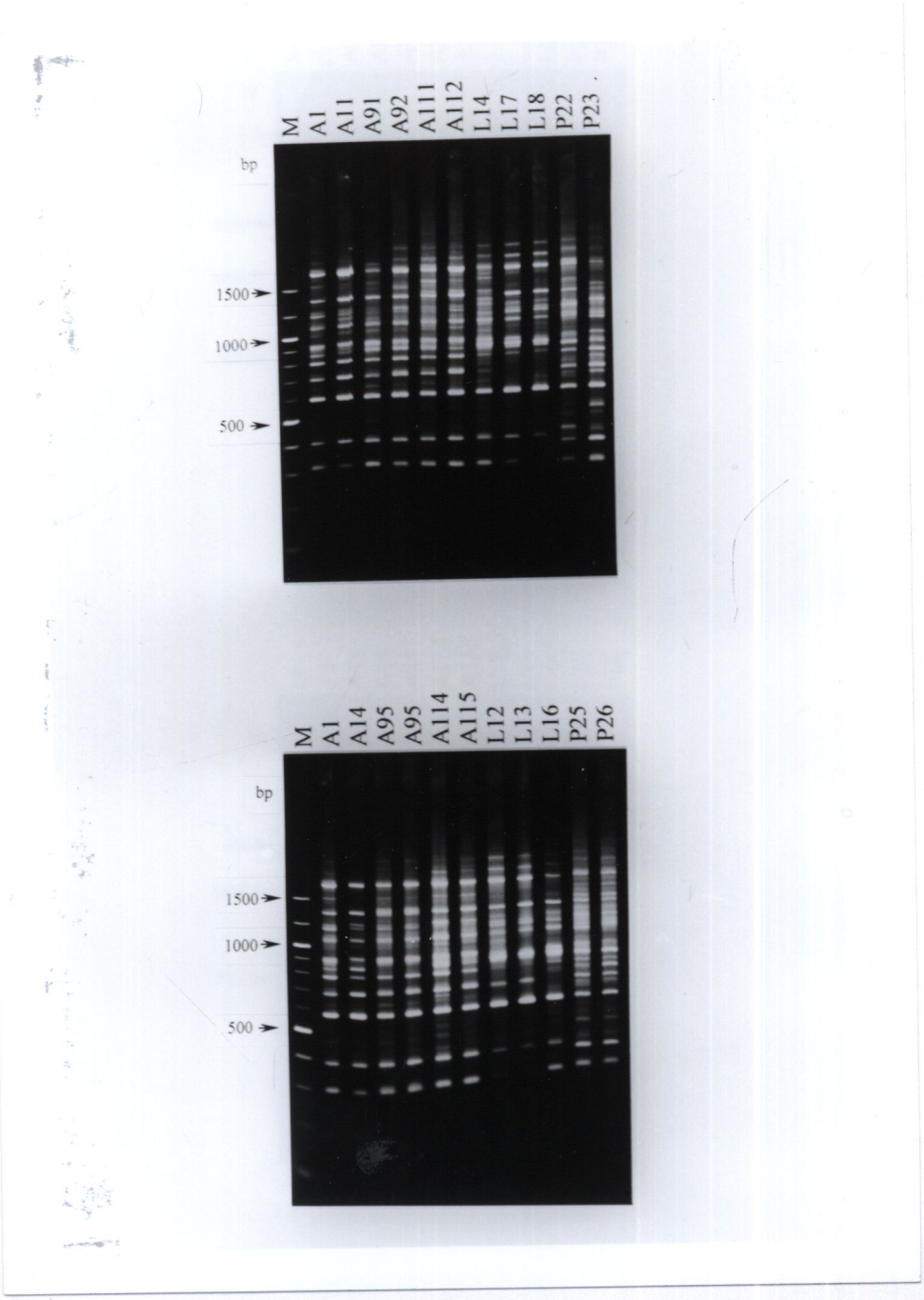

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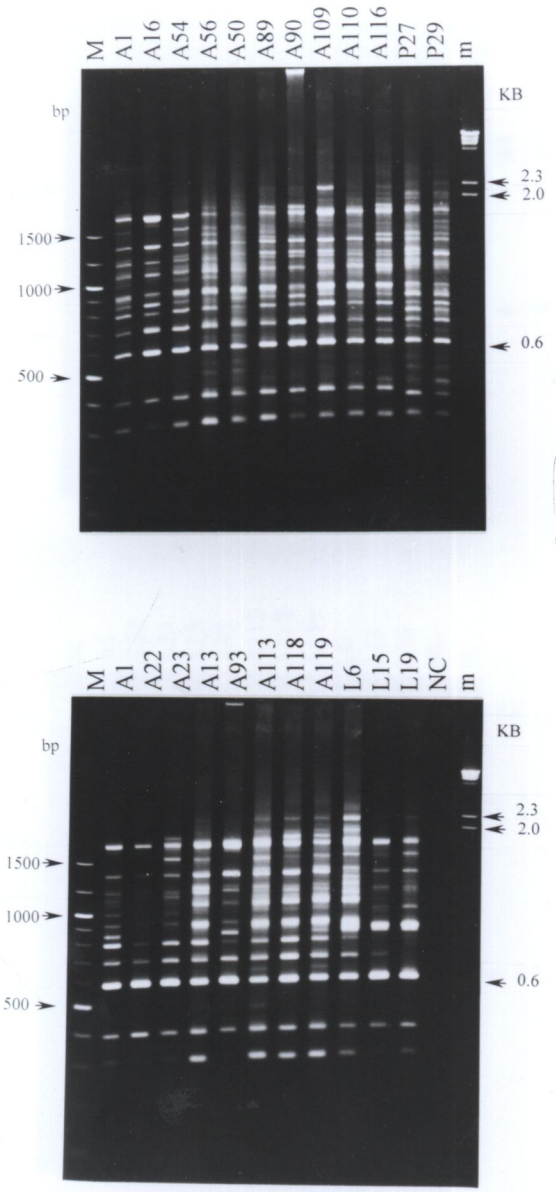
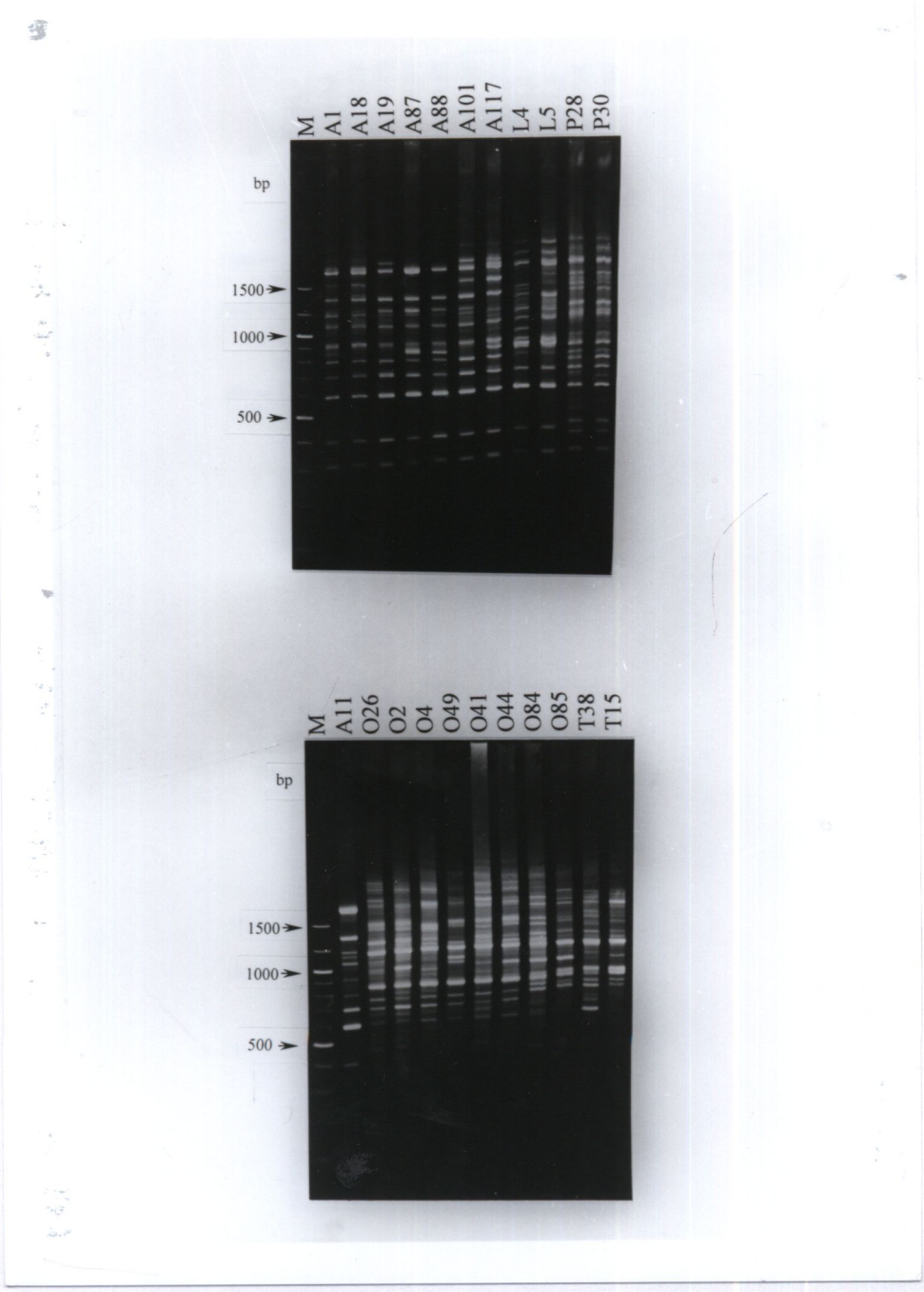
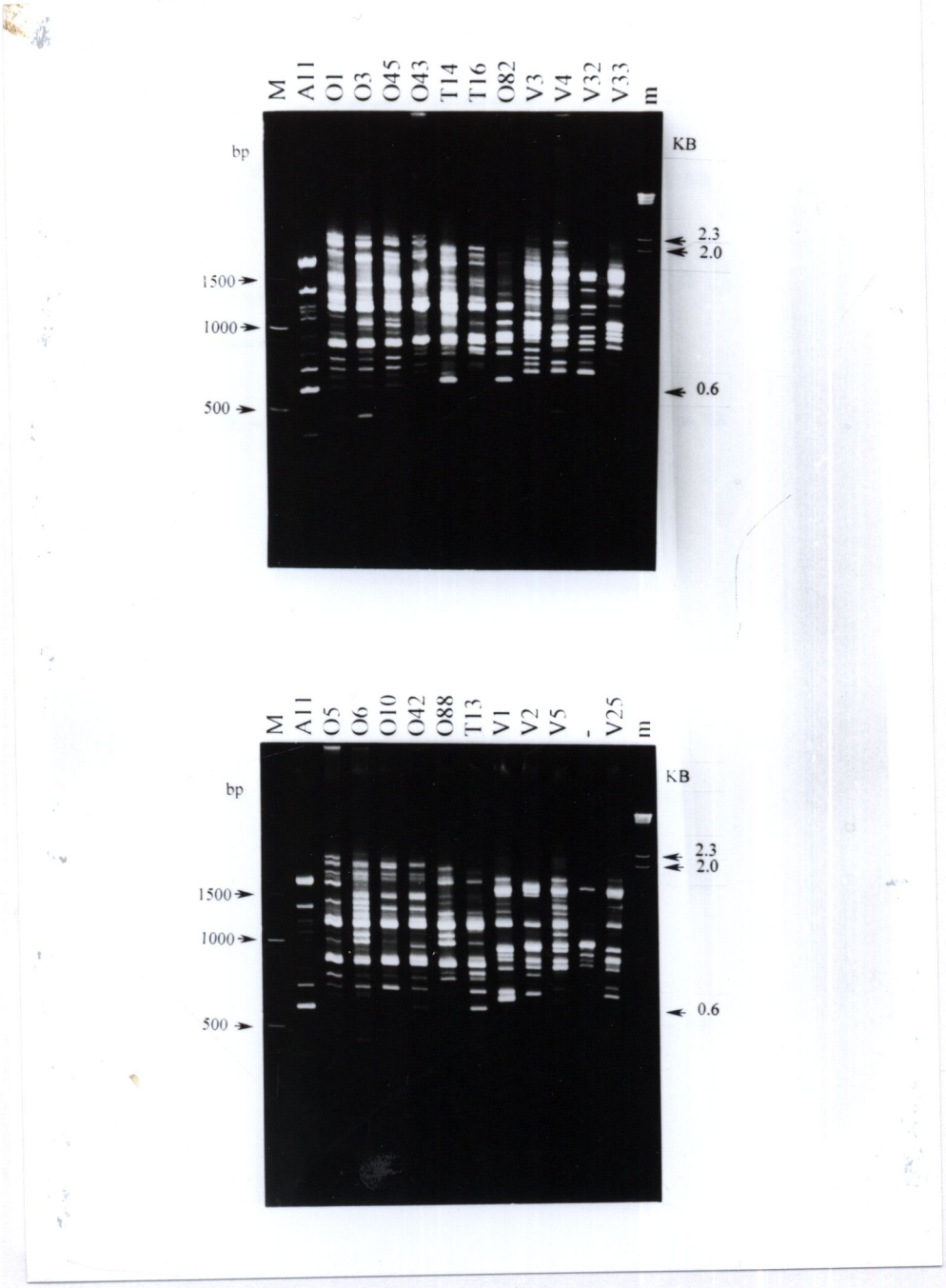


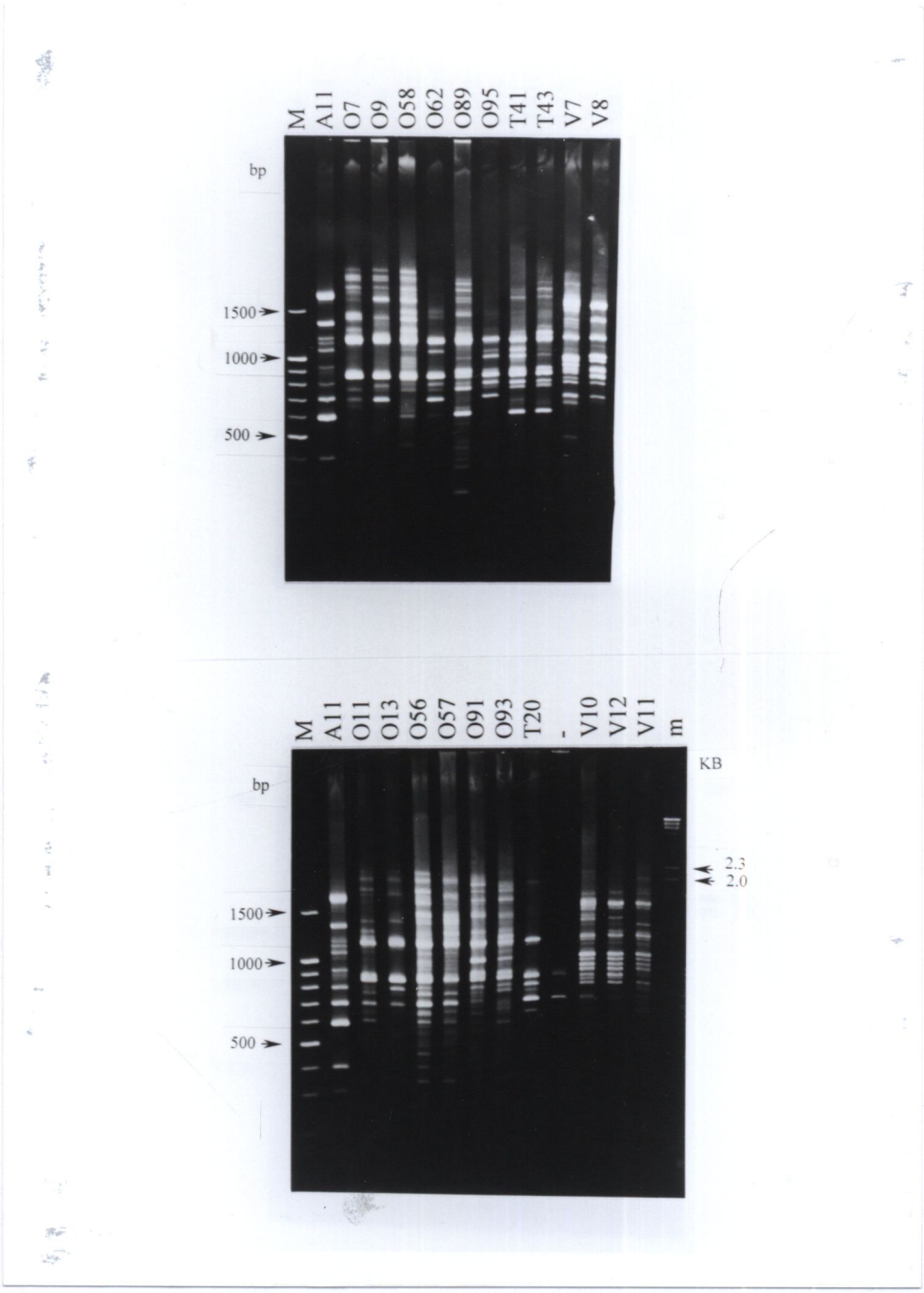
B. 2 Primer UBC101 (continued)



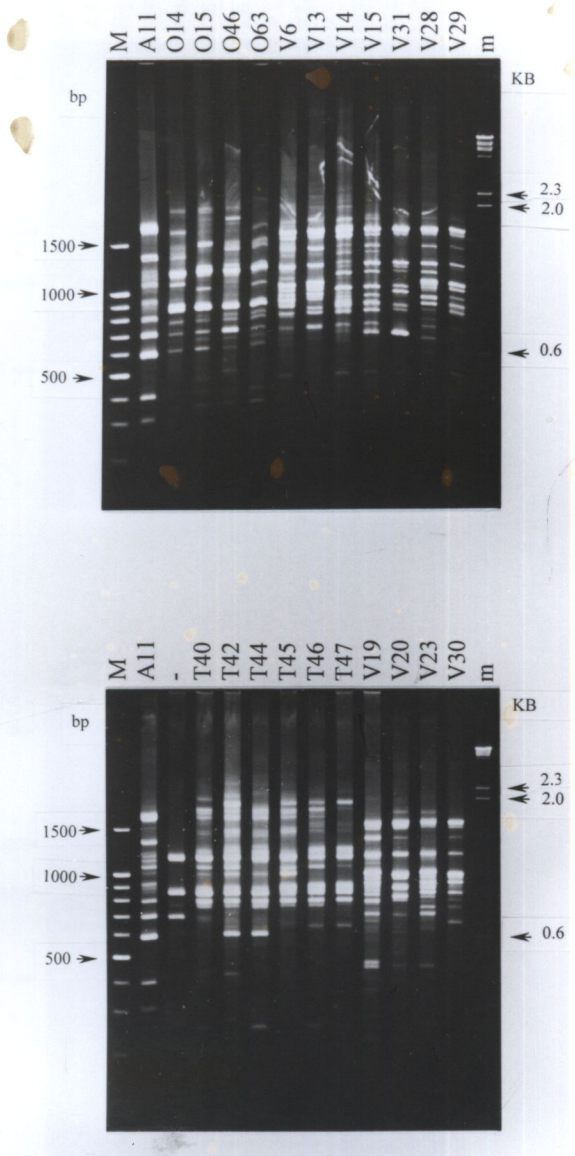
B. 2 Primer UBC101 (continued)



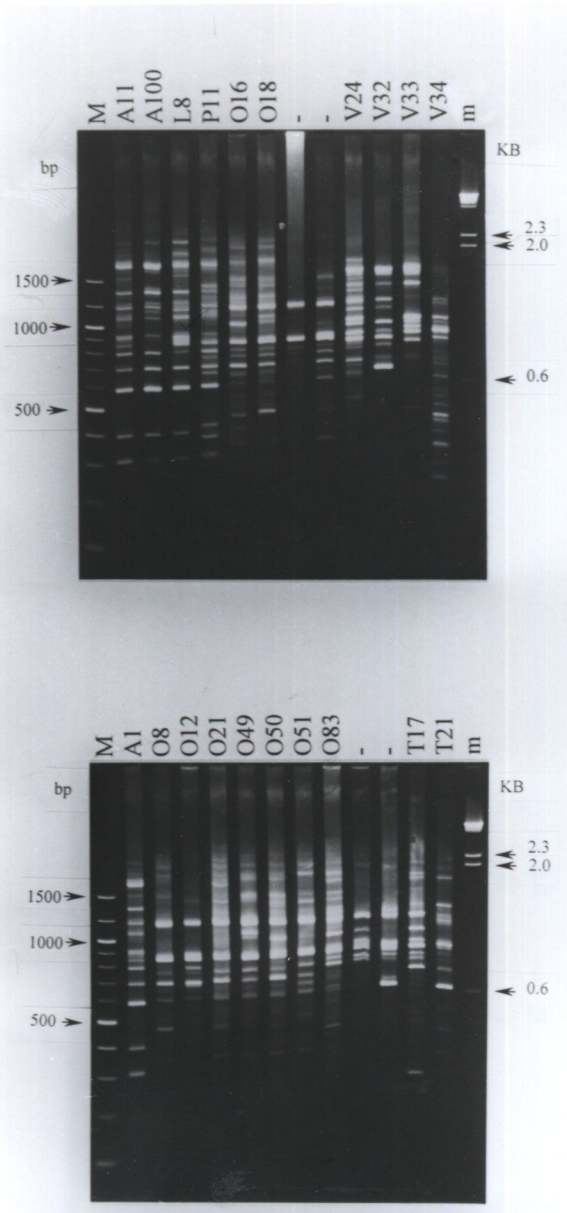
B. 2 Primer UBC101 (continued)



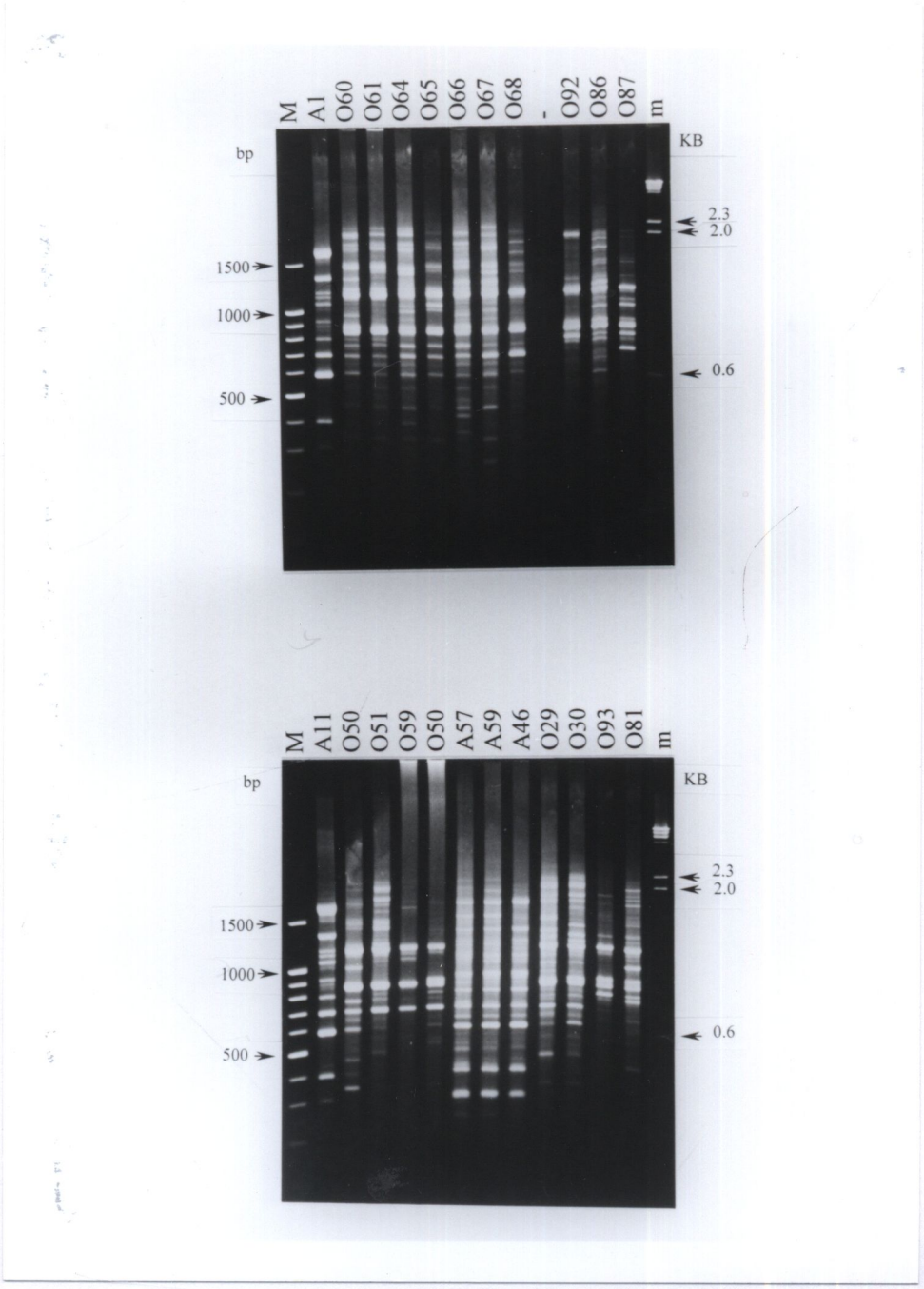
B. 2 Primer UBC101 (continued)



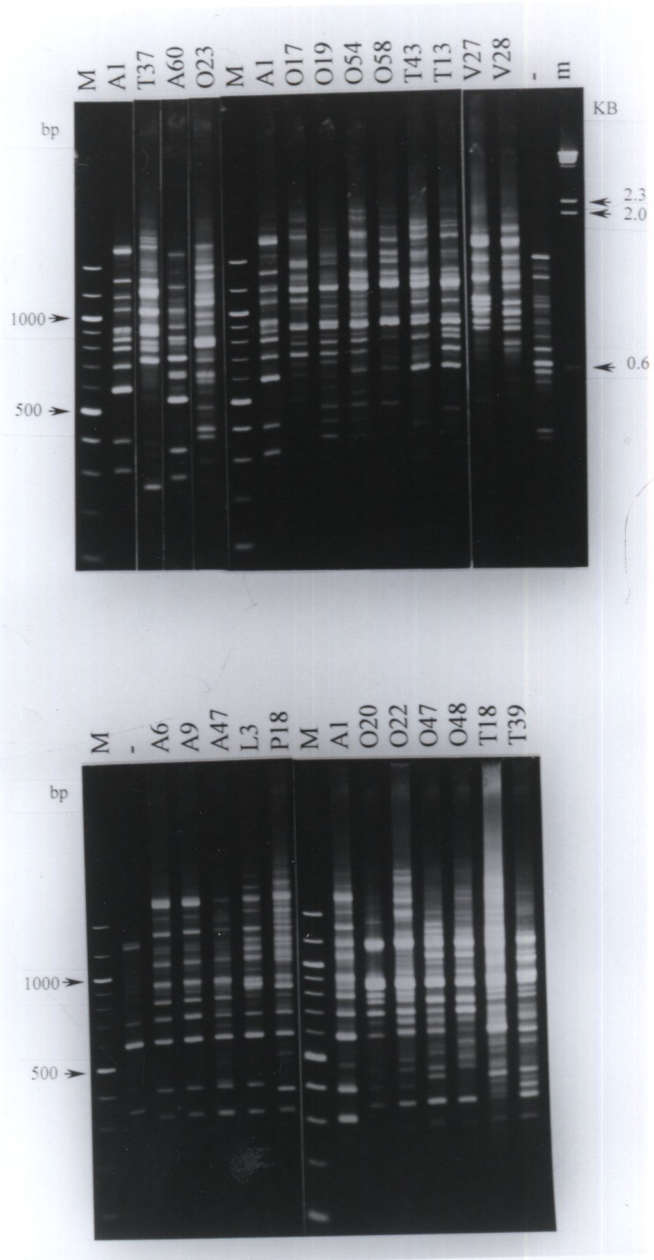
B. 2 Primer UBC101 (continued)



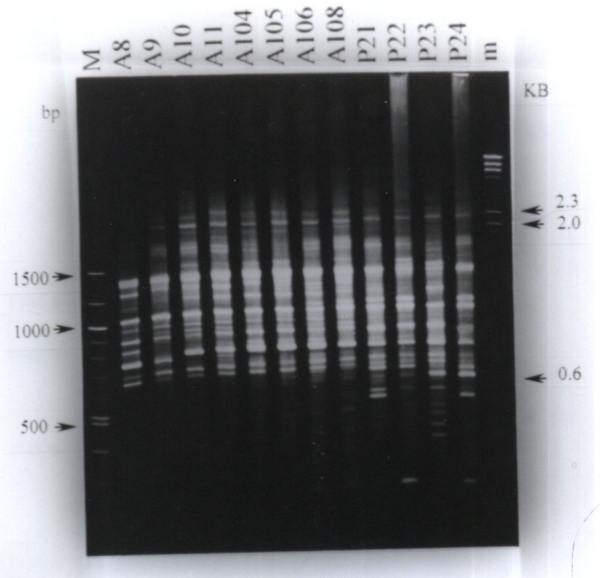
B. 2 Primer UBC101 (continued)

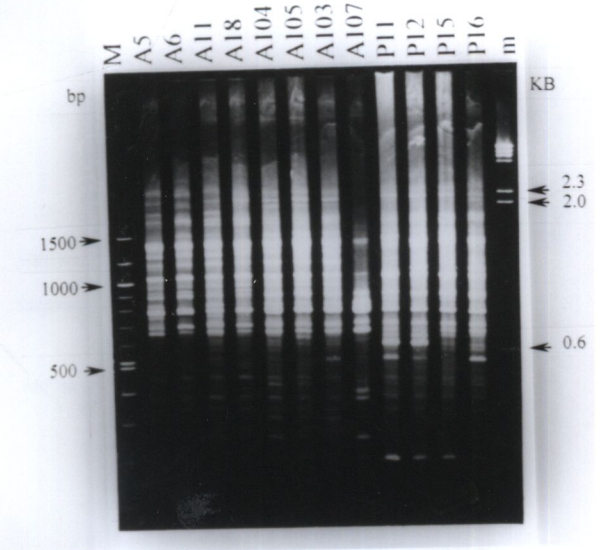


B. 2 Primer UBC101 (continued)

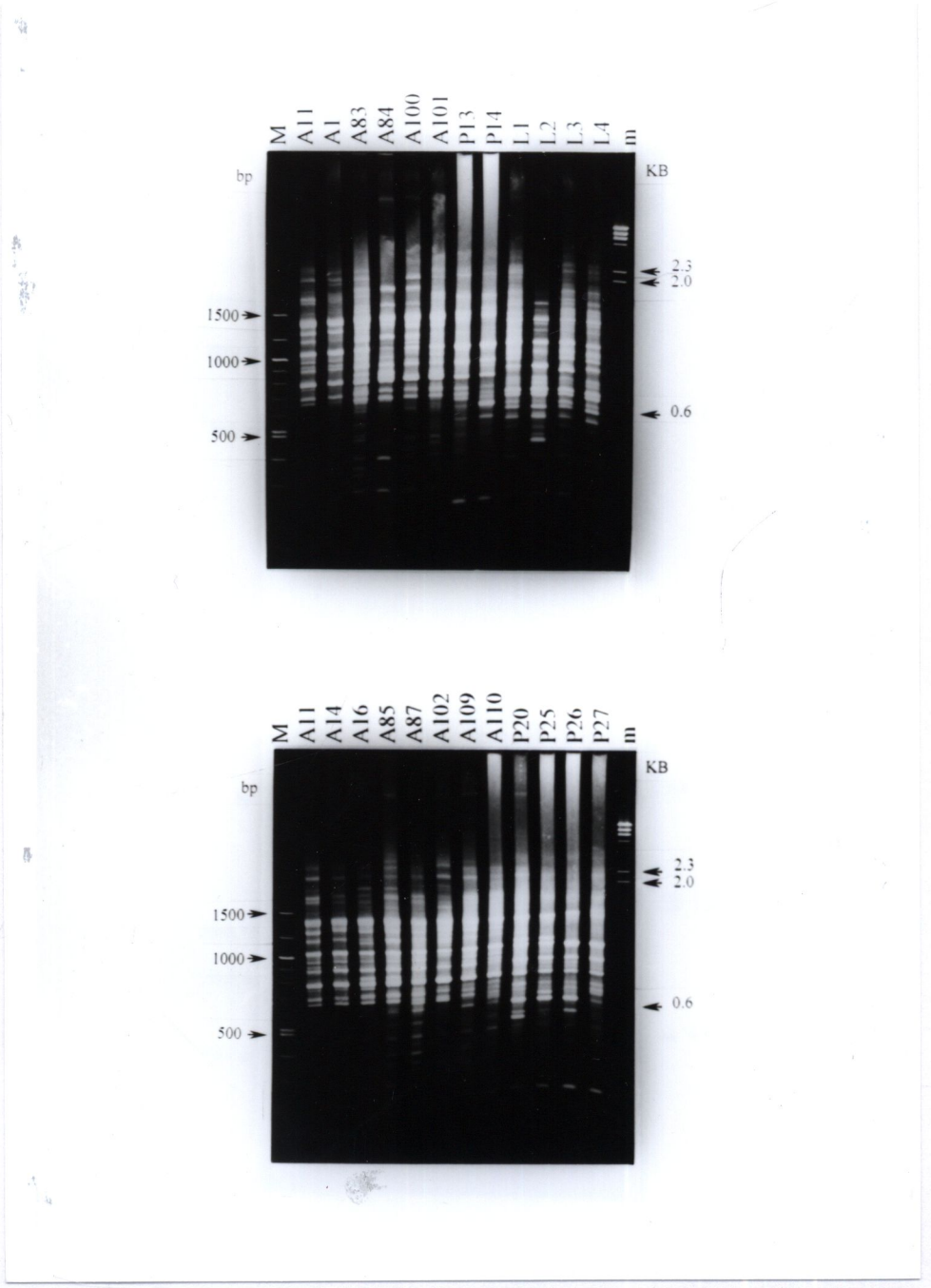


B.3 Primer UBC195 (continued)

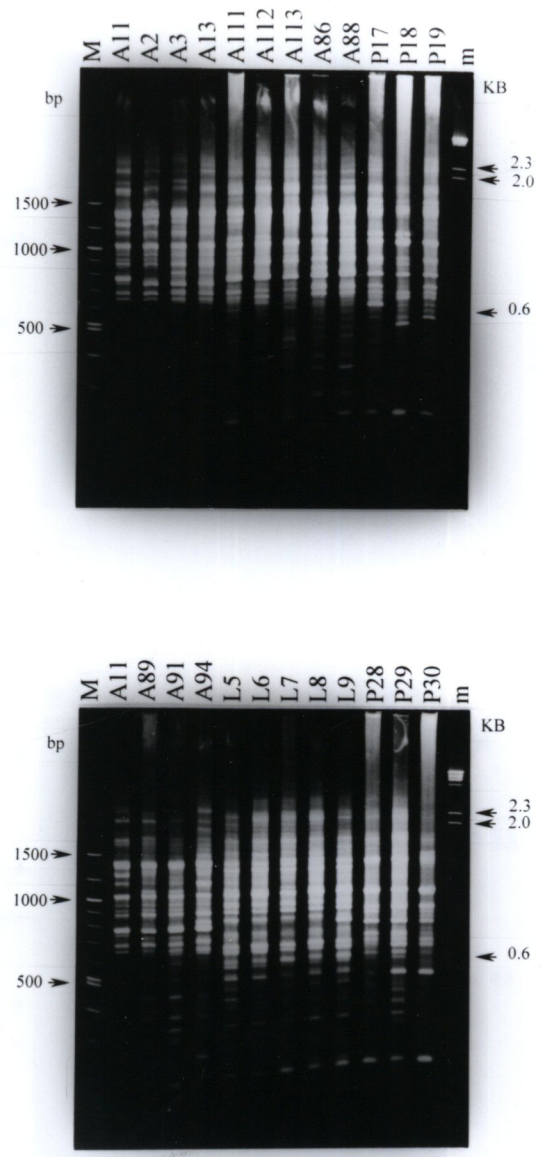




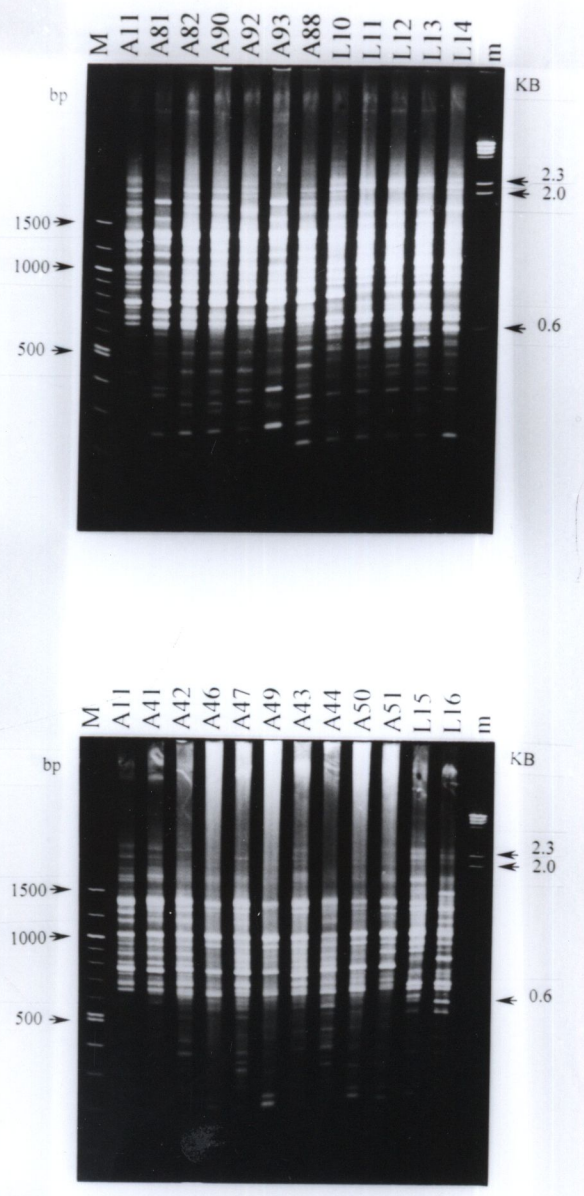
B.3 Primer UBC195 (continued)



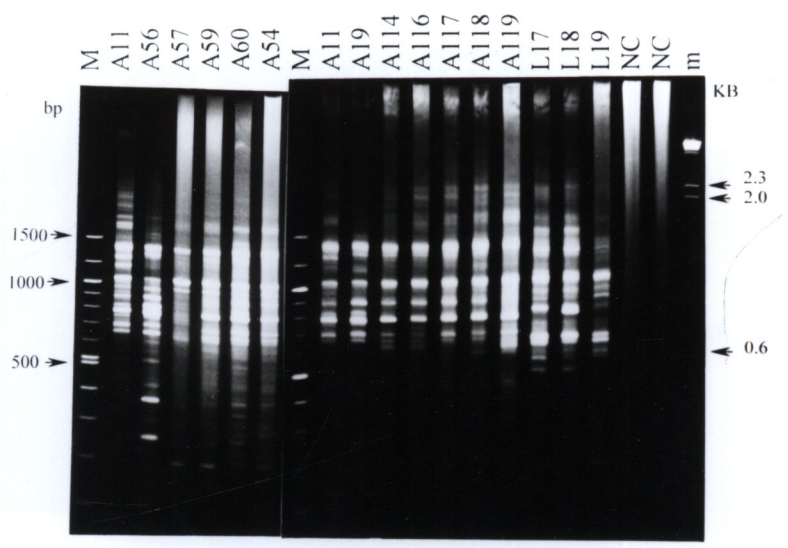
B.3 Primer UBC195 (continued)



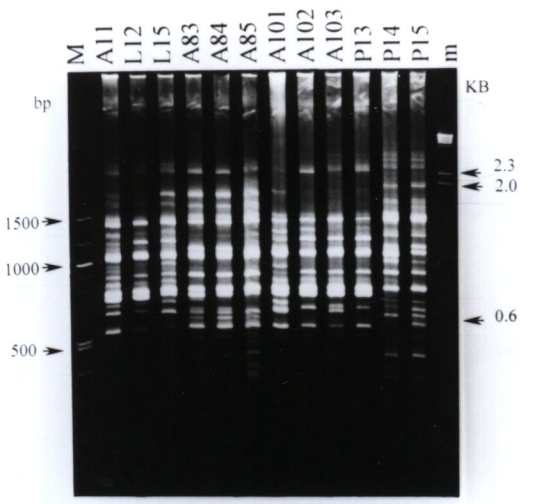
B.3 Primer UBC195 (continued)

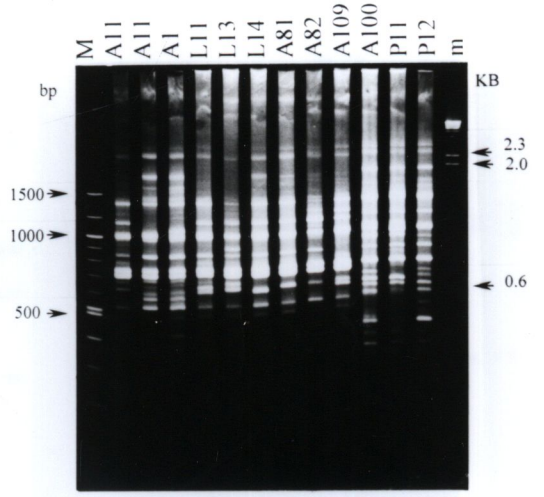


Primer UBC195 (continued)

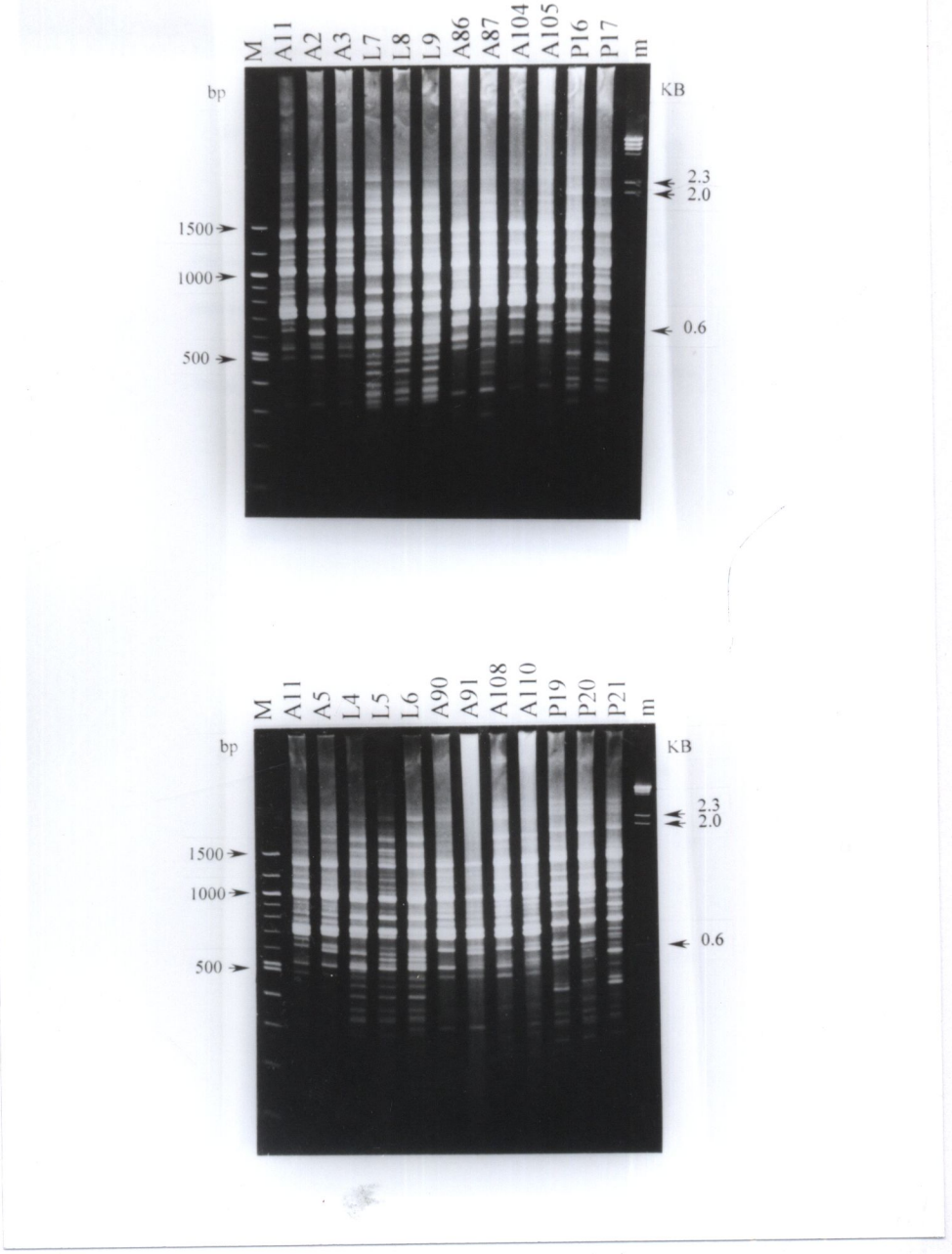


B.4 Primer UBC197

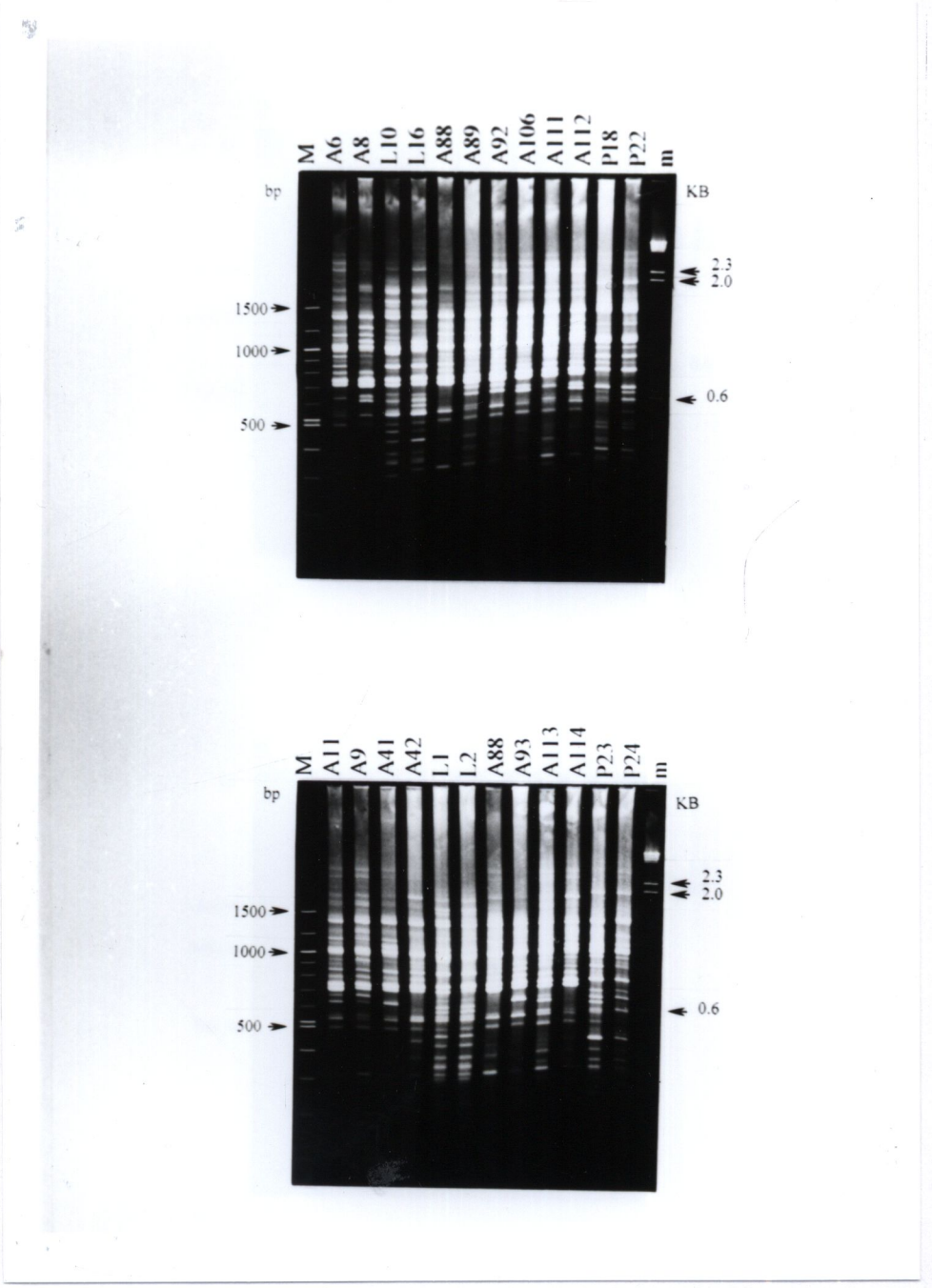




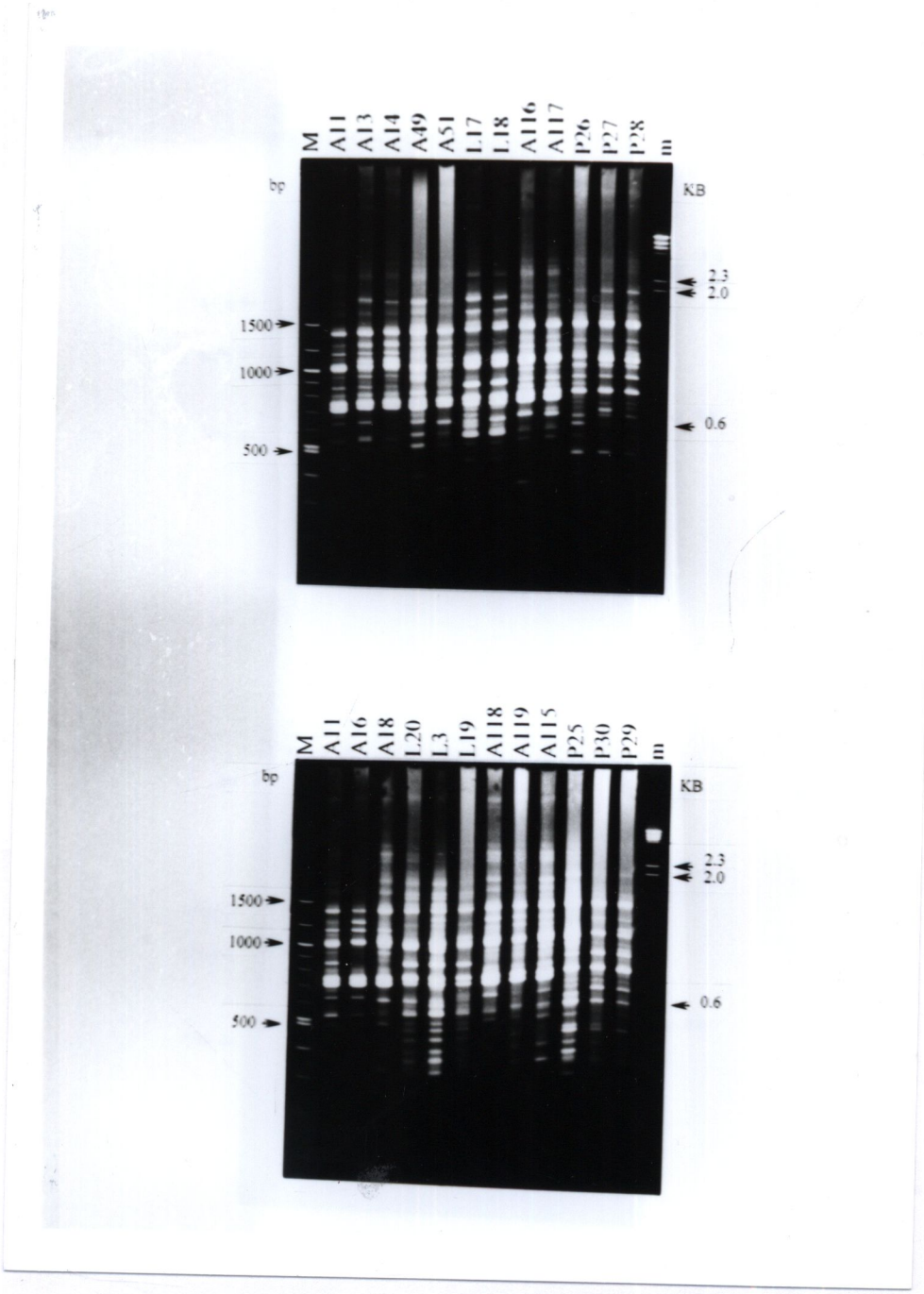
B.4 Primer UBC197 (continued)



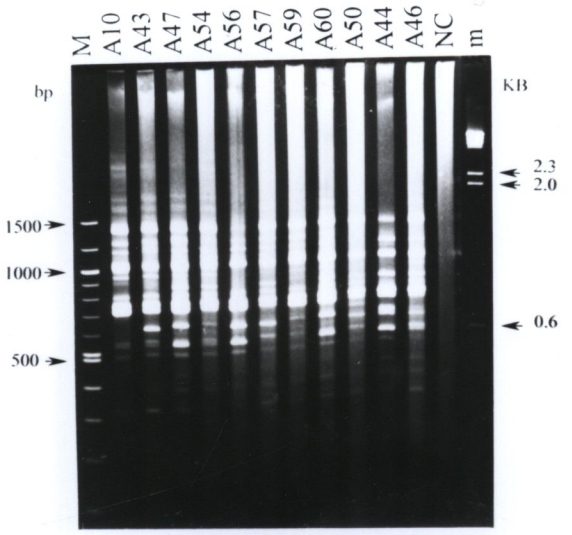
B.4 Primer UBC197 (continued)



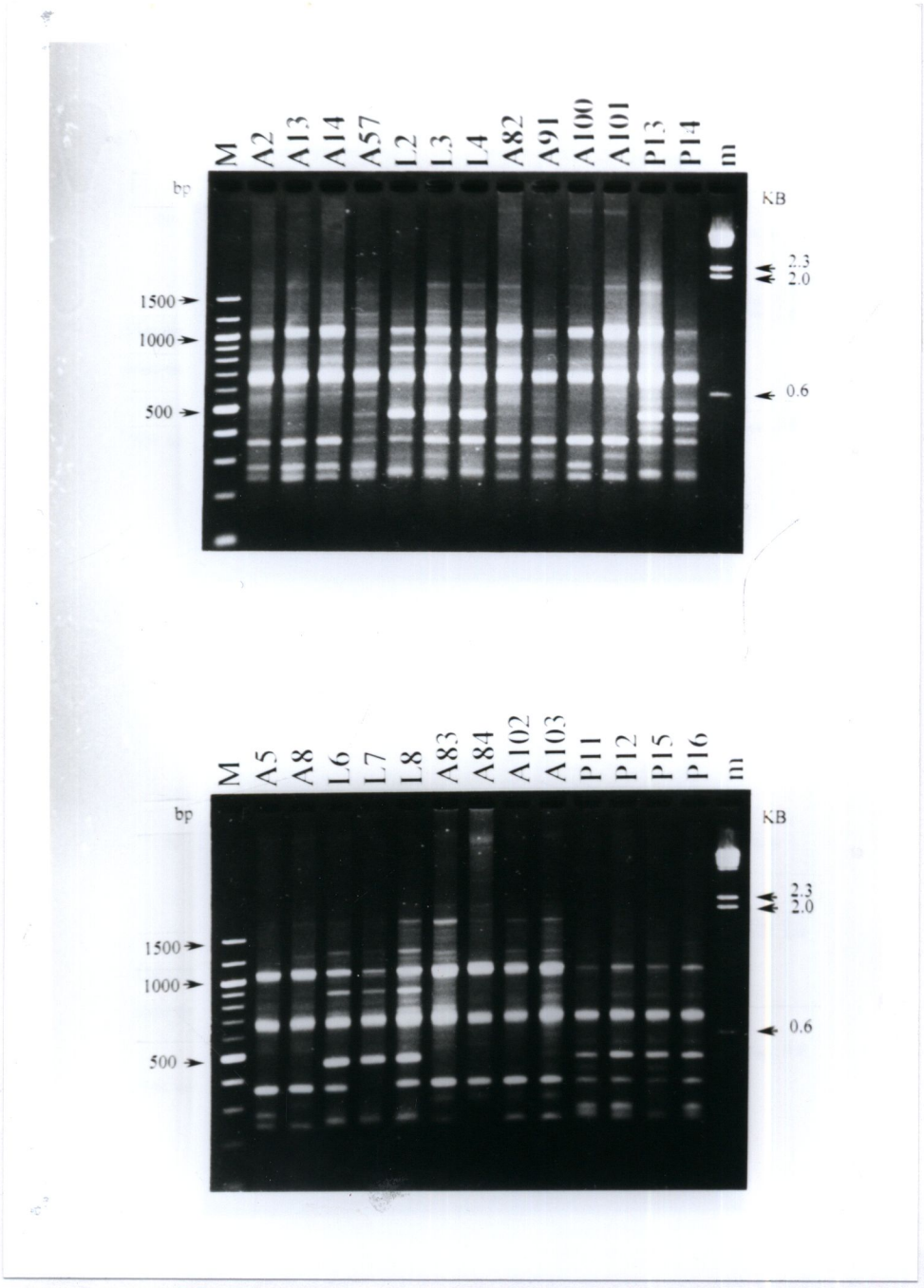
B.4 Primer UBC197 (continued)



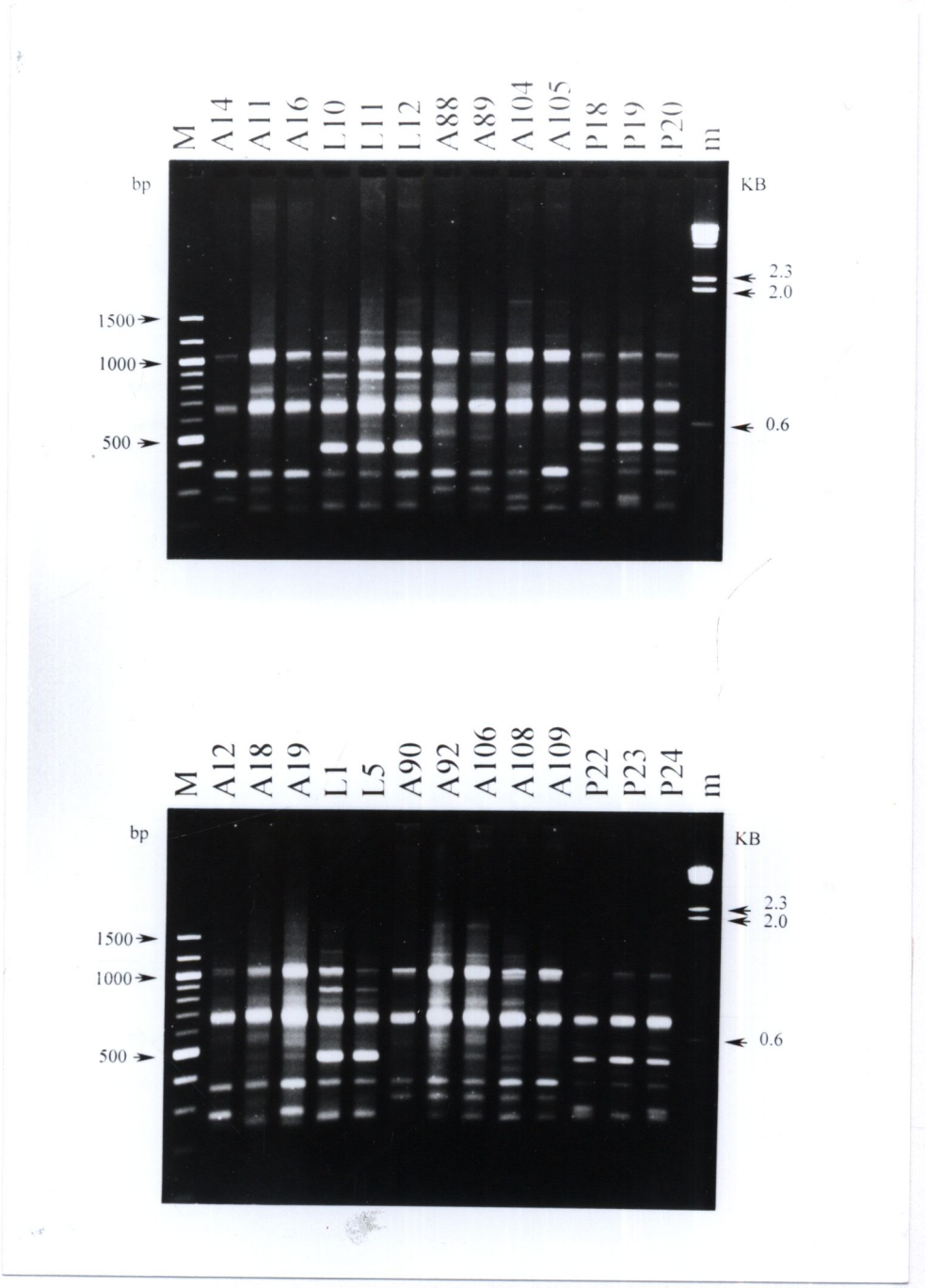
B.4 Primer UBC197 (continued)



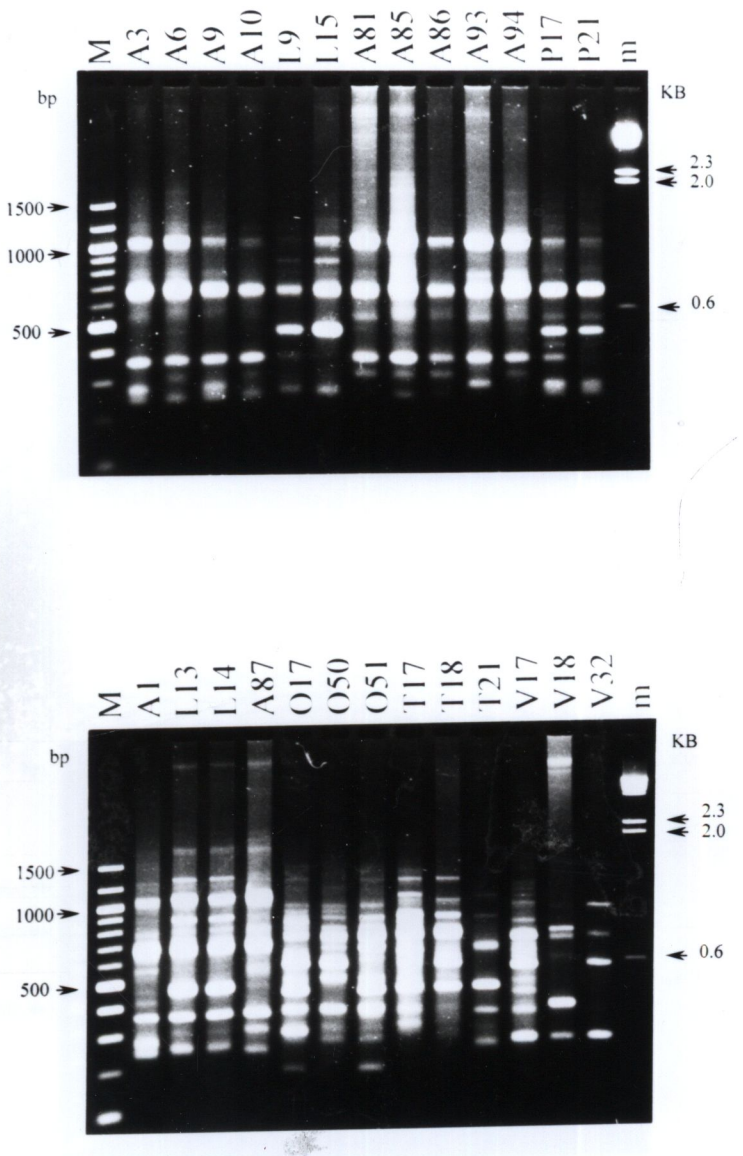
B.5 Primer UBC271



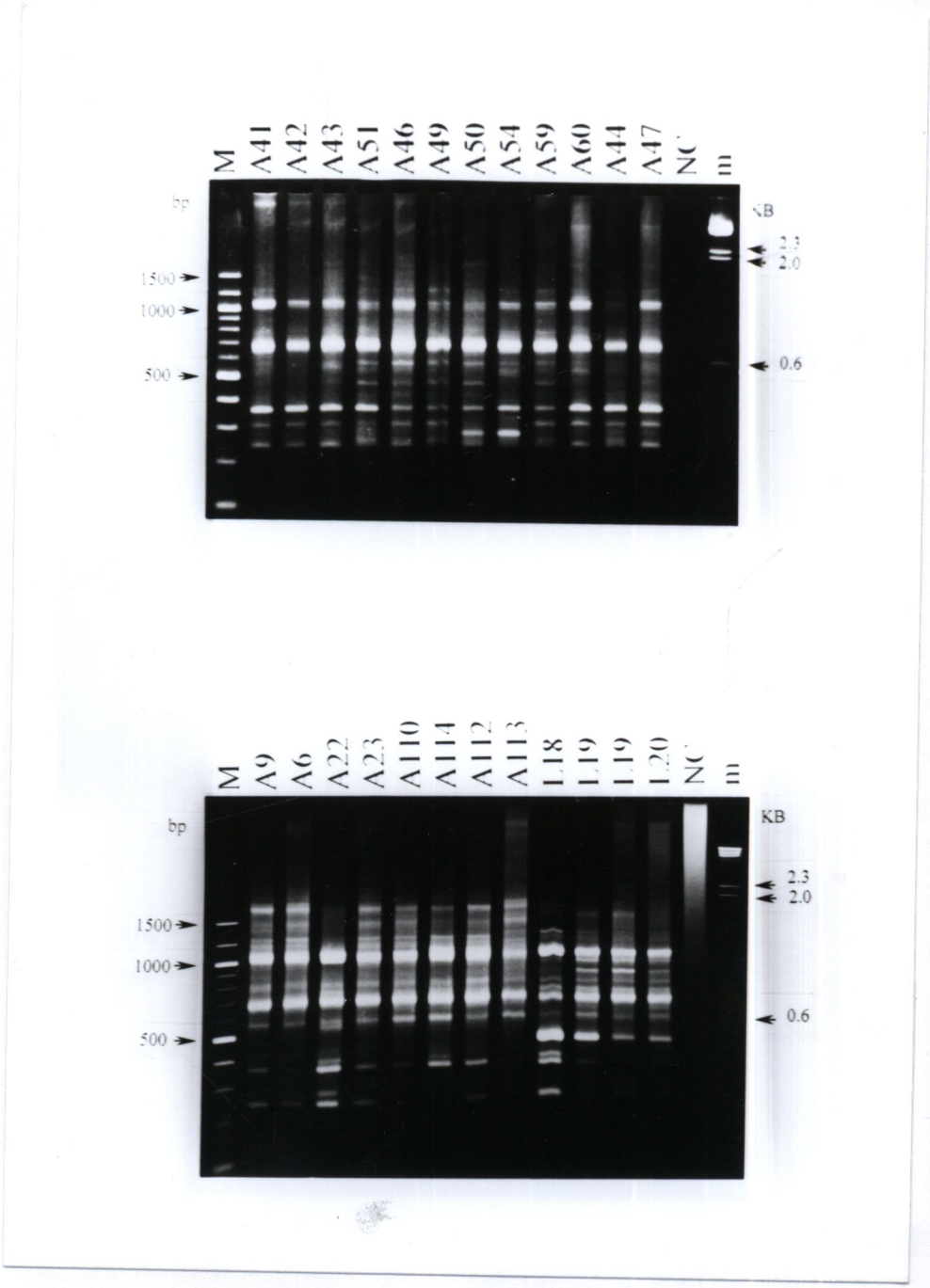
B.5 Primer UBC271 (continued)



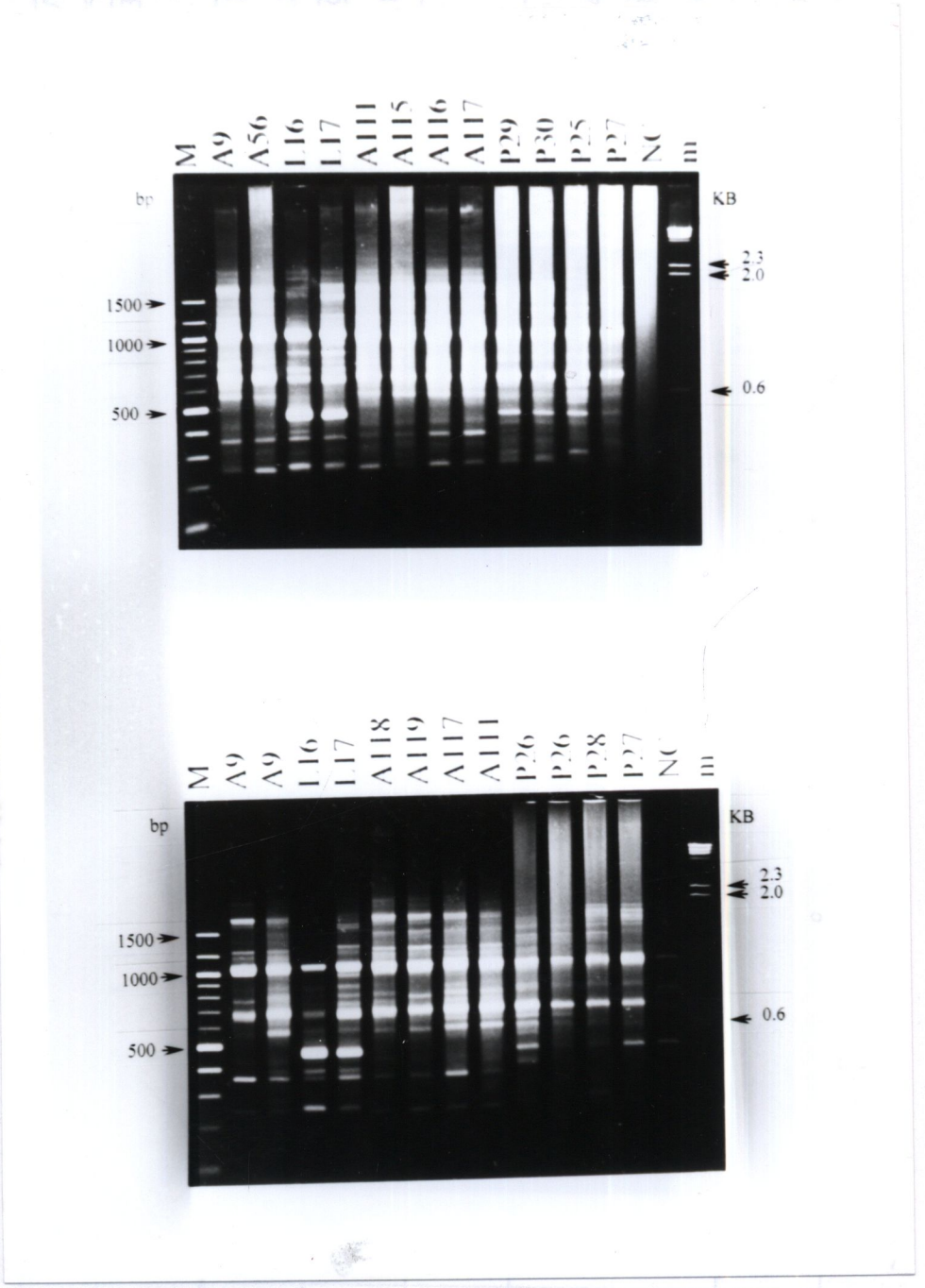
B.5 Primer UBC271 (continued)



B.5 Primer UBC271 (continued)



B.5 Primer UBC271 (continued)



Appendix C

C. 1 Frequencies of each amplified RAPD band within each investigated samples of *H. asinina, H. ovina* and *H. varia* generated from primer UBC101

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)	HOSC (N=29)	HOSM (N=27)	HOPG (N=16)	HOTR (N=23)	HVPK (N=28)	HVPG (N=4)
1850	-	-	-	-	-	-	17	17	12	11	3	-
%	-	-	-	-	-	-	58.62	62.96	75.00	47.83	10.71	-
1800	1	3	4	6	-	-	18	15	4	11	11	-
%	7.14	21.43	30.77	31.58	-	-	62.07	55.56	25.00	47.83	39.29	-
1750	6	1	4	10	4	20	9	8	14	18	19	2
%	42.86	7.14	30.77	52.63	21.05	100.00	31.03	29.63	87.50	78.26	67.86	50.00
1700	14	14	13	19	19	19	-	-	-	-	-	-
%	100.00	100.00	100.00	100.00	100.00	95.00	-	-	-	-	-	-
1650	-	-	-	-	-	1	10	7	4	10	6	1
%	-	-	-	-	-	5.00	34.48	25.93	25.00	43.48	21.43	25.00
1600	-	-	-	-	-	-	-	-	-	2	28	3
%	-	-	-	-	-	-	-	-	-	8.70	100.00	75.00
1540	11	10	5	15	10	10	-	-	-	-	24	2
%	78.57	71.43	38.46	78.95	52.63	50.00	-	-	-	-	85.71	50.00

C. 1 (Continued)

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)	HOSC (N=29)	HOSM (N=27)	HOPG (N=16)	HOTR (N=23)	HVPK (N=28)	HVPG (N=4)
1490	-	-	-	-	-	1	14	21	10	10	-	-
%	-	-	-	-	-	5.00	48.28	77.78	62.50	43.48	-	-
1480	-	3	4	1	-	10	9	13	5	13	-	-
%	-	21.43	30.77	5.26	-	50.00	31.03	48.15	31.25	56.52	-	-
1450	1	-	1	7	4	18	23	17	6	10	26	2
%	7.14	-	7.69	36.84	21.05	90.00	79.31	62.96	37.50	43.48	92.86	50.00
1350	14	14	13	19	18	20	3	1	-	3	15	-
%	100.00	100.00	100.00	100.00	94.74	100.00	10.34	3.70	-	13.04	53.57	-
1300	-	-	-	-	4	2	-	-	-	-	-	-
%	-	-	-	-	21.05	10.00	-	-	-	-	-	-
1260	-	-	-	-	14	4	19	14	13	17	-	-
%	-	-	-	-	73.68	20.00	65.52	51.85	81.25	73.91	-	-
1220	10	12	12	19	2	17	18	21	11	21	-	-
%	71.43	85.71	92.31	100.00	10.53	85.00	62.07	77.78	68.75	91.30	-	-
1190	14	13	13	19	17	15	-	-	-	-	-	-
%	100.00	92.86	100.00	100.00	89.47	75.00	-	-	-	-	-	-

C. 1 (Continued)

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)	HOSC (N=29)	HOSM (N=27)	HOPG (N=16)	HOTR (N=23)	HVPK (N=28)	HVPG (N=4)
1170	1	3	-	-	17	-	29	27	16	23	27	4
%	7.14	21.43	-	-	89.47	-	100.00	100.00	100.00	100.00	96.43	100.00
1150	3	8	5	11	-	9	-	-	-	-	-	-
%	21.43	57.14	38.46	57.89	-	45.00	-	-	-	-	-	-
1100	14	14	13	19	17	19	16	17	12	8	11	2
%	100.00	100.00	100.00	100.00	89.47	95.00	55.17	62.96	75.00	34.78	39.29	50.00
1040	-	-	-	-	-	1	11	7	1	8	14	3
%	-	-	-	-	-	5.00	37.93	25.93	6.25	34.78	50.00	75.00
1000	-	-	-	-	-	-	19	19	15	19	18	2
%	-	-	-	-	-	-	65.52	70.37	93.75	82.61	64.29	50.00
980	-	-	-	-	-	-	-	-	-	-	28	4
%	-	-	-	-	-	-	-	-	-	-	100.00	100.00
920	14	14	13	19	6	19	-	-	-	-	-	-
%	100.00	100.00	100.00	100.00	31.58	95.00	-	-	-	-	-	-
900	2	5	-	3	18	-	-	-	-	-	18	4
%	14.29	35.71	-	15.79	94.74	-	-	-	-	-	64.29	100.00

C. 1 (Continued)

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)	HOSC (N=29)	HOSM (N=27)	HOPG (N=16)	HOTT (N=23)	HVPK (N=28)	HVPG (N=4)
870	-	-	-	-	-	-	20	15	15	23	4	-
%	-	-	-	-	-	-	68.97	55.56	93.75	100.00	14.29	-
850	9	10	11	12	19	18	29	27	16	23	22	2
%	64.29	71.43	84.62	63.16	100.00	90.00	100.00	100.00	100.00	100.00	78.57	50.00
820	-	-	-	-	1	-	-	-	-	-	21	1
%	-	-	-	-	5.26	-	-	-	-	-	75.00	25.00
800	14	14	13	19	16	20	21	15	16	23	23	2
%	100.00	100.00	100.00	100.00	84.21	100.00	72.41	55.56	100.00	100.00	82.14	50.00
750	-	1	-	-	4	18	29	26	16	22	17	2
%	-	7.14	-	-	21.05	90.00	100.00	96.30	100.00	95.65	60.71	50.00
700	14	14	13	19	13	20	29	26	6	11	27	3
%	100.00	100.00	100.00	100.00	68.42	100.00	100.00	96.30	37.50	47.83	96.43	75.00
680	-	-	-	1	1	17	-	-	-	-	-	-
%	-	-	-	5.26	5.26	85.00	-	-	-	-	-	-
650	2	10	3	10	5	5	28	23	9	14	17	2
%	14.29	71.43	23.08	52.63	26.32	25.00	96.55	85.19	56.25	60.87	60.71	50.00

C. 1 (Continued)

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)	HOSC (N=29)	HOSM (N=27)	HOPG (N=16)	HOTR (N=23)	HVPK (N=28)	HVPG (N=4)
600	14	14	13	19	19	20	22	26	10	13	-	-
%	100.00	100.00	100.00	100.00	100.00	100.00	75.86	96.30	62.50	56.52	-	-
440	-	-	1	3	-	14	11	15	3	7	7	2
%	-	-	7.69	15.79	-	70.00	37.93	55.56	18.75	30.43	25.00	50.00
400	14	14	13	19	19	3	5	5	3	3	-	1
%	100.00	100.00	100.00	100.00	100.00	15.00	17.24	18.52	18.75	13.04	-	25.00
380	-	-	-	-	-	19	-	-	-	-	-	-
%	-	-	-	-	-	95.00	-	-	-	-	-	-
350	-	-	-	-	-	2	11	18	3	1	1	1
%	-	-	-	-	-	10.00	37.93	66.67	18.75	4.35	3.57	25.00
320	14	14	13	19	19	19	-	-	-	1	-	-
%	100.00	100.00	100.00	100.00	100.00	95.00	-	-	-	4.35	-	-

C. 2 Frequencies of each amplified RAPD band within each investigated samples of *H. asinina, H. ovina* and *H. varia* generated from primer OPB11

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)	HOSC (N=29)	HOSM (N=27)	HOPG (N=16)	HOTR (N=23)	HVPK (N=28)	HVPG (N=4)
2300	9	12	7	17	13	15	-	4	-	6	6	-
%	64.29	85.71	53.85	89.47	68.42	75.00	-	14.81	-	26.09	21.43	-
2150	12	10	11	19	12	17	4	2	1	7	12	-
%	85.71	71.43	84.62	100.00	63.16	85.00	13.79	7.41	6.25	30.43	42.86	-
1700	14	10	13	19	18	7	3	5	1	5	8	-
%	100.00	71.43	100.00	100.00	94.74	35.00	10.34	18.52	6.25	21.74	28.57	-
1480	-	-	-	-	12	18	-	-	1	4	2	-
%	-	-	-	-	63.16	90.00	-	-	6.25	17.39	7.14	-
1450	14	13	13	19	-	-	-	-	2	4	6	2
%	100.00	92.86	100.00	100.00	-	-	-	-	12.50	17.39	21.43	50.00
1420	-	-	-	-	19	19	-	-	3	4	7	2
%	-	-	-	-	100	95	-	-	18.75	17.39	25.00	50.00
1350	-	-	-	-	19	-	-	2	6	8	12	1
%	-	-	-	-	100.00	-	-	7.41	37.50	34.78	42.86	25.00
1300	14	12	13	19	19	19	1	1	7	14	11	1
%	100.00	85.71	100.00	100.00	100.00	95.00	3.45	3.70	43.75	60.87	39.29	25.00

C. 2 (Continued)

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)	HOSC (N=29)	HOSM (N=27)	HOPG (N=16)	HOTR (N=23)	HVPK (N=28)	HVPG (N=4)
1220	6	2	4	8	1	11	18	18	1	6	12	2
%	42.86	14.29	30.77	42.11	5.26	55.00	62.07	66.67	6.25	26.09	42.86	50.00
1190	-	-	-	-	-	-	13	11	9	9	13	1
%	-	-	-	-	-	-	44.83	40.74	56.25	39.13	46.43	25.00
1180	-	-	-	-	19	-	25	20	9	20	13	1
%	-	-	-	-	100.00	-	86.21	74.07	56.25	86.96	46.43	25.00
1100	13	14	11	17	15	20	1	2	10	9	10	1
%	92.86	100.00	84.62	89.47	78.95	100.00	3.45	7.41	62.50	39.13	35.71	25.00
1080	6	6	6	10	7	-	-	-	-	-	-	-
%	42.86	42.86	46.15	52.63	36.84	-	-	-	-	-	-	-
1050	-	-	-	-	-	-	1	2	-	-	18	3
%	-	-	-	-	-	-	3.45	7.41	-	-	64.29	75.00
1000	-	-	-	-	-	-	28	26	3	1	27	4
%	-	-	-	-	-	-	96.55	96.30	18.75	4.35	96.43	100.00
950	-	-	-	-	-	-	23	24	8	13	4	-
%	-	-	-	-	-	-	79.31	88.89	50.00	56.52	14.29	-

C. 2 (Continued)

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)	HOSC (N=29)	HOSM (N=27)	HOPG (N=16)	HOTR (N=23)	HVPK (N=28)	HVPG (N=4)
920	-	-	-	-	-	-	22	24	2	10	6	-
%	-	-	-	-	-	-	75.86	88.89	12.50	43.48	21.43	-
900	-	-	-	-	-	9	-	-	-	-	-	-
%	-	-	-	-	-	45.00	-	-	-	-	-	-
880	-	-	-	-	-	19	10	23	15	23	2	3
%	-	-	-	-	-	95.00	34.48	85.19	93.75	100.00	7.14	75.00
840	4	12	5	8	4	14	6	3	3	13	6	1
%	28.57	85.71	38.46	42.11	21.05	70.00	20.69	11.11	18.75	56.52	21.43	25.00
800	14	14	13	19	19	20	19	15	4	2	18	3
%	100.00	100.00	100.00	100.00	100.00	100.00	65.52	55.56	25.00	8.70	64.29	75.00
760	-	-	-	-	-	-	23	20	3	5	17	2
%	-	-	-	-	-	-	79.31	74.07	18.75	21.74	60.71	50.00
740	-	-	-	-	-	-	19	16	4	1	5	1
%	-	-	-	-	-	-	65.52	59.26	25.00	4.35	17.86	25.00
700	-	-	-	-	-	-	-	3	3	-	16	4
%	-	-	-	-	-	-	-	11.11	18.75	-	57.14	100.00

C. 2 (Continued)

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)	HOSC (N=29)	HOSM (N=27)	HOPG (N=16)	HOTR (N=23)	HVPK (N=28)	HVPG (N=4)
660	-	-	-	-	-	-	4	8	4	10	3	1
%	-	-	-	-	-	-	13.79	29.63	25.00	43.48	10.71	25.00
650	-	-	-	-	-	-	27	24	3	6	6	1
%	-	-	-	-	-	-	93.10	88.89	18.75	26.09	21.43	25.00
620	-	-	-	-	-	-	9	16	5	3	1	-
%	-	-	-	-	-	-	31.03	59.26	31.25	13.04	3.57	-
590	-	-	-	-	-	-	22	23	5	4	2	-
%	-	-	-	-	-	-	75.86	85.19	31.25	17.39	7.14	-
570	3	2	1	4	-	-	1	2	4	13	3	-
%	21.43	14.29	7.69	21.05	-	-	3.45	7.41	25.00	56.52	10.71	-
550	-	-	-	-	-	-	20	19	7	15	20	3
%	-	-	-	-	-	-	68.97	70.37	43.75	65.22	71.43	75.00
540	-	-	-	-	1	-	2	2	2	9	4	-
%	-	-	-	-	5.26	-	6.90	7.41	12.50	39.13	14.29	-
500	14	13	13	19	3	5	14	2	-	-	4	-
%	100.00	92.86	100.00	100.00	15.79	25.00	48.28	7.41	-	-	14.29	-

C. 2 (Continued)

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)	HOSC (N=29)	HOSM (N=27)	HOPG (N=16)	HOTR (N=23)	HVPK (N=28)	HVPG (N=4)
470	-	-	-	-	-	-	18	26	16	23	15	3
%	-	-	-	-	-	-	62.07	96.30	100.00	100.00	53.57	75.00
440	10	9	3	12	2	20	-	-	-	-	2	1
%	71.43	64.29	23.08	63.16	10.53	100.00	-	-	-	-	7.14	25.00
390	3	11	4	11	2	11	-	-	-	-	1	-
%	21.43	78.57	30.77	57.89	10.53	55.00	-	-	-	-	3.57	-

C. 3 Frequencies of each amplified RAPD band within each conspecific population of *H. asinina* generated from primer UBC195

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)
1480	-	-	1	-	-	9
%	-	-	7.69	-	-	45.00
1450	14	8	12	19	11	16
%	100.00	57.14	92.31	100.00	57.89	80.00
1300	14	14	13	18	18	18
%	100.00	100.00	100.00	94.74	94.74	90.00
1280	8	5	11	9	15	-
%	57.14	35.71	84.62	47.37	78.95	-
1250	13	8	6	14	18	-
%	92.86	57.14	46.15	73.68	94.74	-
1200	2	-	6	5	6	7
%	14.29	-	46.15	26.32	31.58	35
1160	13	5	13	14	16	9
%	92.86	35.71	100.00	73.68	84.21	45.00
1150	5	1	2	3	-	-
%	35.71	7.14	15.38	15.79	-	-
1080	-	-	6	5	13	9
%	-	-	46.15	26.32	68.42	45.00
1030	14	14	13	19	19	20
%	100.00	100.00	100.00	100.00	100.00	100.00
1000	-	-	1	4	-	-
%	-	-	7.69	21.05	-	-
970	13	14	13	14	19	10
%	92.86	100.00	100.00	73.68	100.00	50.00
910	9	9	6	15	-	1
%	64.29	64.29	46.15	78.95	-	5.00
890	14	10	13	19	18	20
%	100.00	71.43	100.00	100.00	94.74	100.00

C. 3 (Continued)

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)
850	-	-	-	-	-	12
%	-	-	-	-	-	60.00
810	5	7	11	7	17	12
%	35.71	50.00	84.62	36.84	89.47	60.00
790	14	13	13	19	4	20
%	100.00	92.86	100.00	100.00	21.05	100.00
750	4	5	-	1	-	-
%	28.57	35.71	-	5.26	-	-
720	-	5	5	5	19	-
%	-	35.71	38.46	26.32	100.00	-
690	14	11	13	19	7	18
%	100.00	78.57	100.00	100.00	36.84	90.00
650	14	14	13	19	19	20
%	100.00	100.00	100.00	100.00	100.00	100.00
620	-	-	5	7	19	17
%	-	-	38.46	36.84	100.00	85.00
600	-	-	4	10	18	19
%	-	-	30.77	52.63	94.74	95.00
570	-	4	3	-	15	16
%	-	28.57	23.08	-	78.95	80.00
520	1	6	9	1	18	9
%	7.14	42.86	69.23	5.26	94.74	45.00

C. 4 Frequencies of each amplified RAPD band within each conspecific population of *H. asinina* generated from primer UBC197

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)
1480	12	6	12	18	19	18
%	85.71	42.86	92.31	94.74	100.00	90.00
1450	14	14	13	19	19	20
%	100.00	100.00	100.00	100.00	100.00	100.00
1250	13	11	12	17	5	3
%	92.86	78.57	92.31	89.47	26.32	15.00
1210	-	-	-	-	-	4
%	-	-	-	-	-	20.00
1200	-	-	-	-	18	19
%	-	-	-	-	94.74	95.00
1180	14	12	13	19	-	-
%	100	85.71	100	100	-	-
1170	2	1	-	-	9	-
%	14.29	7.14	-	-	47.37	-
1150	11	7	1	5	15	13
%	78.57	50.00	7.69	26.32	78.95	65.00
1050	12	14	13	19	19	20
%	85.71	100.00	100.00	100.00	100.00	100.00
980	-	-	-	-	10	-
%	-	-	-	-	52.63	-
920	13	9	7	14	-	19
%	92.86	64.29	53.85	73.68	-	95.00
900	11	2	10	12	14	6
%	78.57	14.29	76.92	63.16	73.68	30.00
850	8	11	13	13	17	20
%	57.14	78.57	100.00	68.42	89.47	100.00
800	14	13	13	18	1	6
%	100.00	92.86	100.00	94.74	5.26	30.00

C. 4 (Continued)

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)
750	14	14	13	19	19	20
%	100.00	100.00	100.00	100.00	100.00	100.00
700	14	14	13	19	19	0
%	100.00	100.00	100.00	100.00	100.00	0.00
680	4	1	4	-	4	13
%	28.57	7.14	30.77	-	21.05	65.00
650	0	3	1	8	7	4
%	0.00	21.43	7.69	42.11	36.84	20.00
620	10	13	10	15	7	17
%	71.43	92.86	76.92	78.95	36.84	85.00
600	5	-	5	-	17	13
%	35.71	-	38.46	-	89.47	65.00
570	-	1	-	1	14	11
%	-	7.14	-	5.26	73.68	55.00
520	14	13	13	19	19	13
%	100.00	92.86	100.00	100.00	100.00	65.00
500	14	12	13	18	19	12
%	100.00	85.71	100.00	94.74	100.00	60.00

C. 5 Frequencies of each amplified RAPD band within each conspecific population of *H. asinina* generated from primer UBC271

Size (bp) %	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)
1020	14	13	13	19	19	20
%	100.00	92.86	100.00	100.00	100.00	100.00
1000	3	5	5	13	3	1
%	21.43	35.71	38.46	68.42	15.79	5.00
880	-	-	-	-	19	-
%	-	-	-	-	100.00	-
680	14	14	13	19	19	20
%	100.00	100.00	100.00	100.00	100.00	100.00
480	-	-	-	-	19	20
%	-	-	-	-	100.00	100.00
475	6	6	7	5	-	-
%	42.86	42.86	53.85	26.32	-	-
470	3	5	-	5	2	14
%	21.43	35.71	-	26.32	10.53	70.00
370	14	14	13	19	17	14
%	100.00	100.00	100.00	100.00	89.47	70.00
320	5	10	12	7	6	1
%	35.71	71.43	92.31	36.84	31.58	5.00
280	7	3	1	2	-	11
%	50.00	21.43	7.69	10.53	-	55.00
270	-	-	-	-	19	19
%	-	-	-	-	100.00	95.00
250	14	14	10	19	-	-
%	100.00	100.00	76.92	100.00	-	-

Appendix D

D. 1 Pairwise comparisons of inter - and intraspecific similarity indices (above diagonal) and genetic distances (below diagonal) of *H. asinina, H. ovina* and *H. varia* samples using primers UBC101

Dij/Sij	HASH (*N*=14)	HASM (*N*=14)	HACH (*N*=13)	HACB (*N*=19)	HALB (*N*=19)	HAPH (*N*=20)	HOSC (*N*=29)	HOSM (*N*=27)	HOPG (*N*=16)	HOTR (*N*=23)	HVPK (*N*=28)	HVPG (*N*=4)
HASH	-	0.8531	0.8668	0.8613	0.7354	0.6953	0.3448	0.3501	0.3380	0.3287	0.3284	0.2603
HASM	0.1469	-	0.8494	0.8511	0.7277	0.6749	0.3892	0.3927	0.3528	0.3547	0.3395	0.2801
HACH	0.1332	0.1506	-	0.8540	0.7068	0.7027	0.3802	0.3900	0.3615	0.3657	0.3045	0.2326
HACB	0.1387	0.1489	0.1460	-	0.6998	0.7177	0.3860	0.3876	0.3576	0.3613	0.3509	0.2728
HALB	0.2646	0.2723	0.2932	0.3002	-	0.5886	0.4133	0.4030	0.4113	0.3866	0.3940	0.3663
HAPH	0.3047	0.3251	0.2973	0.2823	0.4114	-	0.4520	0.4552	0.4358	0.4420	0.3892	0.2987
HOSC	0.6552	0.6108	0.6198	0.6140	0.5867	0.5480	-	0.6989	0.6685	0.6657	0.4863	0.4173
HOSM	0.6499	0.6073	0.6100	0.6124	0.5970	0.5448	0.3011	-	0.6627	0.6491	0.4450	0.3973
HOPG	0.6620	0.6472	0.6385	0.6424	0.5887	0.5642	0.3315	0.3373	-	0.7115	0.4448	0.3806
HOTR	0.6713	0.6453	0.6343	0.6387	0.6134	0.5580	0.3343	0.3509	0.2885	-	0.4588	0.3862
HVPU	0.6716	0.6605	0.6955	0.6491	0.6060	0.6108	0.5137	0.5550	0.5552	0.5412	-	0.6377
HVPG	0.7397	0.7199	0.7674	0.7272	0.6337	0.7013	0.5827	0.6027	0.6194	0.6138	0.3623	-

D. 2 Pairwise comparisons of inter - and intraspecific similarity indices (above diagonal) and genetic distances (below diagonal) of *H. asinina, H. ovina* and *H. varia* samples using primers OPB11

Dij/Sij	HASH (*N*=14)	HASM (*N*=14)	HACH (*N*=13)	HACB (*N*=19)	HALB (*N*=19)	HAPH (*N*=20)	HOSC (*N*=29)	HOSM (*N*=27)	HOPG (*N*=16)	HOTR (*N*=23)	HVPK (*N*=28)	HVPG (*N*=4)
HASH	-	0.7604	0.8023	0.8232	0.5253	0.5583	0.1607	0.1217	0.1731	0.2118	0.2630	0.2268
HASM	0.2396	-	0.7476	0.7959	0.4967	0.5815	0.1471	0.1058	0.1724	0.2197	0.2438	0.2127
HACH	0.1977	0.2524	-	0.8064	0.5276	0.5159	0.1609	0.1169	0.1705	0.2073	0.2605	0.2180
HACB	0.1768	0.2041	0.1936	-	0.5366	0.5839	0.1591	0.1230	0.1663	0.2201	0.2653	0.2173
HALB	0.4747	0.5033	0.4724	0.4634	-	0.5699	0.1804	0.1630	0.2655	0.3116	0.3075	0.2358
HAPH	0.4417	0.4185	0.4841	0.4161	0.4301	-	0.1556	0.7194	0.2619	0.3053	0.2472	0.2903
HOSC	0.8393	0.8529	0.8391	0.8409	0.8196	0.8444	-	0.6493	0.3661	0.3745	0.3913	0.3788
HOSM	0.8783	0.8942	0.8831	0.8770	0.8370	0.2806	0.3507	-	0.4408	0.4437	0.3968	0.4229
HOPG	0.8269	0.8276	0.8295	0.8337	0.7345	0.7381	0.6339	0.5592	-	0.4751	0.3073	0.3562
HOTR	0.7882	0.7803	0.7927	0.7799	0.6884	0.6947	0.6255	0.5563	0.5249	-	0.3018	0.3253
HVPU	0.7370	0.7562	0.7395	0.7347	0.6925	0.7528	0.6087	0.6032	0.6927	0.6982	-	0.5034
HVPG	0.7732	0.7873	0.7820	0.7827	0.7642	0.7097	0.6212	0.5771	0.6438	0.6747	0.4966	-

D. 3 Pairwise comparisons of intraspecific similarity indices (above diagonal) and genetic distances (below diagonal) of *H. asinina* using primer UBC101

Daij /Saij	HASH (*N*=14)	HASM (*N*=14)	HACH (*N*=13)	HACB (*N*=19)	HALB (*N*=19)	HAPH (*N*=20)
HASH	-	0.9840	0.9919	0.9887	0.8875	0.8351
HASM	0.0160	-	0.9865	0.9905	0.8919	0.8268
HACH	0.0081	0.0135	-	0.9877	0.8652	0.8488
HACB	0.0113	0.0095	0.0123	-	0.8605	0.8661
HALB	0.1125	0.1081	0.1348	0.1395	-	0.7617
HAPH	0.1649	0.1732	0.1512	0.1339	0.2383	-

D. 4 Pairwise comparisons of intraspecific similarity indices (above diagonal) and genetic distances (below diagonal) of *H. asinina* using primer OPB11

Daij/Saij	HASH (*N*=14)	HASM (*N*=14)	HACH (*N*=13)	HACB (*N*=19)	HALB (*N*=19)	HAPH (*N*=20)
HASH	-	0.9632	0.998	0.997	0.7131	0.7455
HASM	0.0368	-	0.9574	0.9838	0.6985	0.7828
HACH	0.0020	0.0426	-	0.9872	0.7223	0.7101
HACB	0.0030	0.0162	0.0128	-	0.7094	0.7562
HALB	0.2869	0.3015	0.2777	0.2906	-	0.7561
HAPH	0.2545	0.2172	0.2899	0.2438	0.2439	-

D. 5 Pairwise comparisons of intraspecific similarity indices (above diagonal) and genetic distances (below diagonal) of *H. asinina* using primer UBC195

Daij/Saij	HASH (*N*=14)	HASM (*N*=14)	HACH (*N*=13)	HACB (*N*=19)	HALB (*N*=19)	HAPH (*N*=20)
HASH	-	0.9565	0.9417	0.9766	0.7548	0.7926
HASM	0.0435	-	0.9405	0.9494	0.8152	0.8373
HACH	0.0583	0.0595	-	0.9652	0.9003	0.8881
HACB	0.0234	0.0506	0.0348	-	0.8289	0.8782
HALB	0.2452	0.1848	0.0997	0.1711	-	0.839
HAPH	0.2074	0.1627	0.1119	0.1218	0.1610	-

D. 6 Pairwise comparisons of intraspecific similarity indices (above diagonal) and genetic distances (below diagonal) of *H. asinina* using primer UBC197

Daij/Saij	HASH (*N*=14)	HASM (*N*=14)	HACH (*N*=13)	HACB (*N*=19)	HALB (*N*=19)	HAPH (*N*=20)
HASH	-	0.9678	0.9767	0.9834	0.8084	0.8098
HASM	0.0322	-	0.9658	0.982	0.7898	0.8102
HACH	0.0233	0.0342	-	0.9908	0.8092	0.8008
HACB	0.0166	0.0180	0.0092	-	0.8028	0.7976
HALB	0.1916	0.2102	0.1908	0.1972	-	0.8785
HAPH	0.1902	0.1898	0.1992	0.2024	0.1215	-

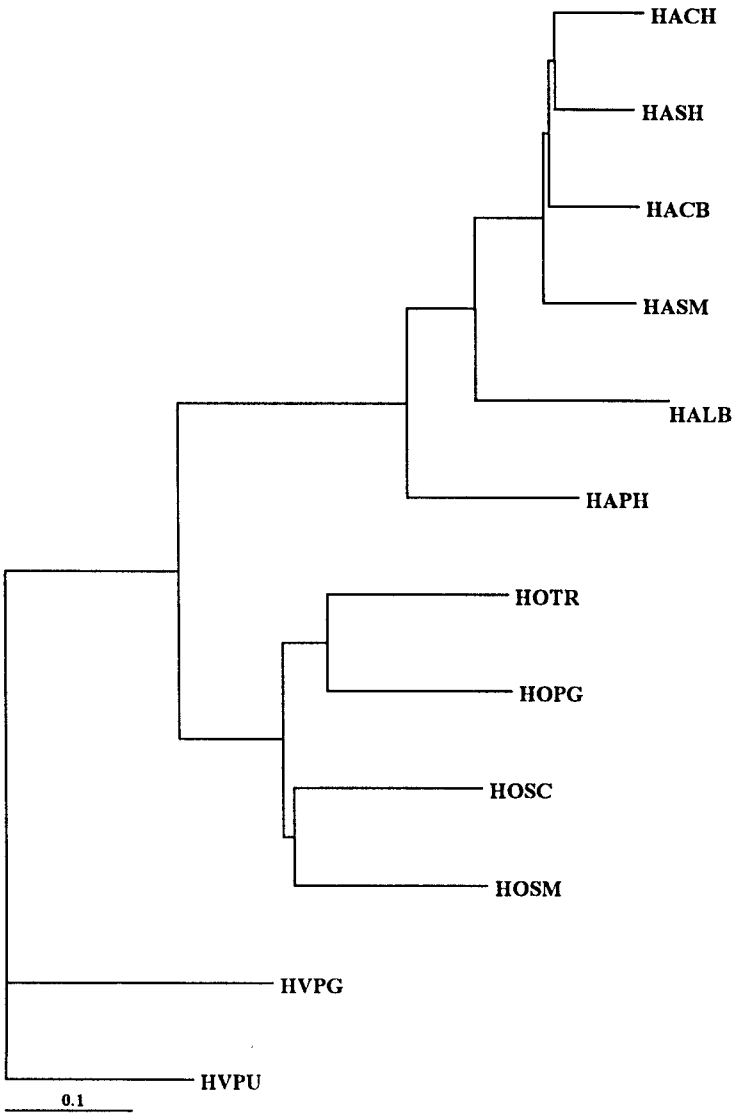
D. 7 Pairwise comparisons of intraspecific similarity indices (above diagonal) and genetic distances (below diagonal) of *H. asinina* using primer UBC271

Daij/Saij	HASH (N=14)	HASM (N=14)	HACH (N=13)	HACB (N=19)	HALB (N=19)	HAPH (N=20)
HASH	-	0.9935	0.9604	0.9763	0.6458	0.7073
HASM	0.0065	-	0.9915	0.9847	0.6476	0.6784
HACH	0.0396	0.0085	-	0.9523	0.6551	0.6344
HACB	0.0237	0.0153	0.0477	-	0.6437	0.6683
HALB	0.3542	0.3524	0.3449	0.3563	-	0.8661
HAPH	0.2927	0.3216	0.3656	0.3317	0.1339	-

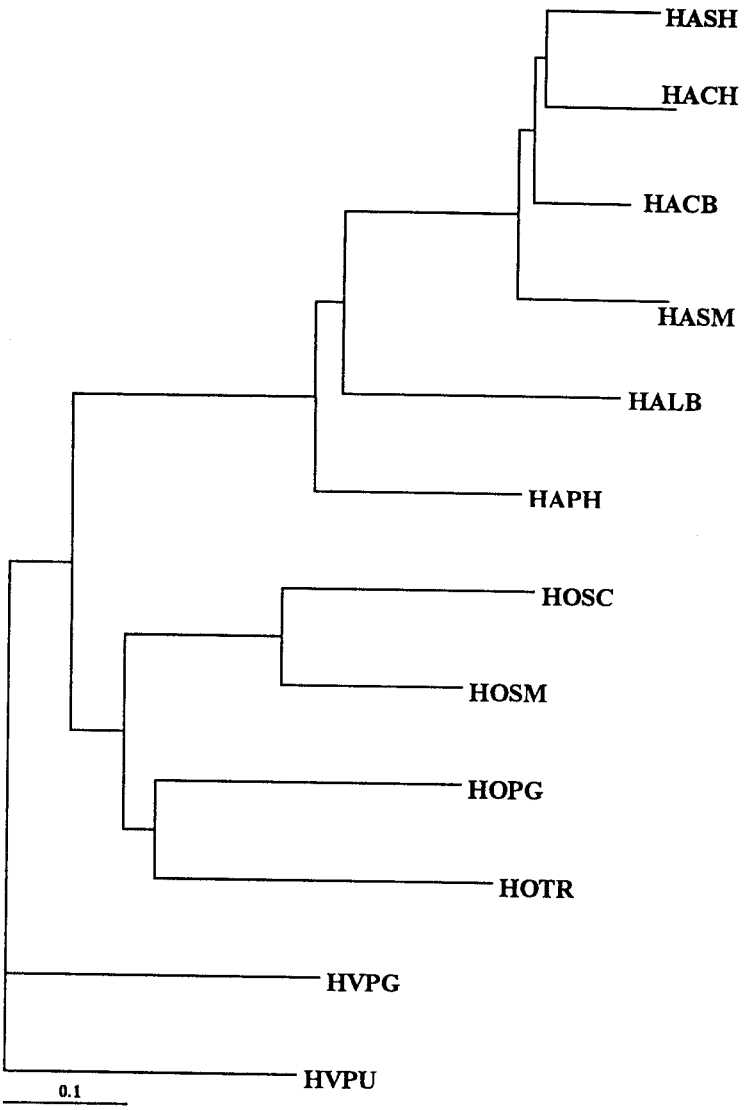
Appendix E

Neighbor - joining trees illustrating genetic relationships of 3 tropical abalone found in Thailand, *Haliotis asinina*, *H. ovina*, and *H. varia*, base on genetic distances resulted from RAPD analysis using primer UBC101 (E. 1), OPB11 (E. 2). Detailed information and abbreviations of sample sites are shown in Appendix A.

E. 1 UBC101



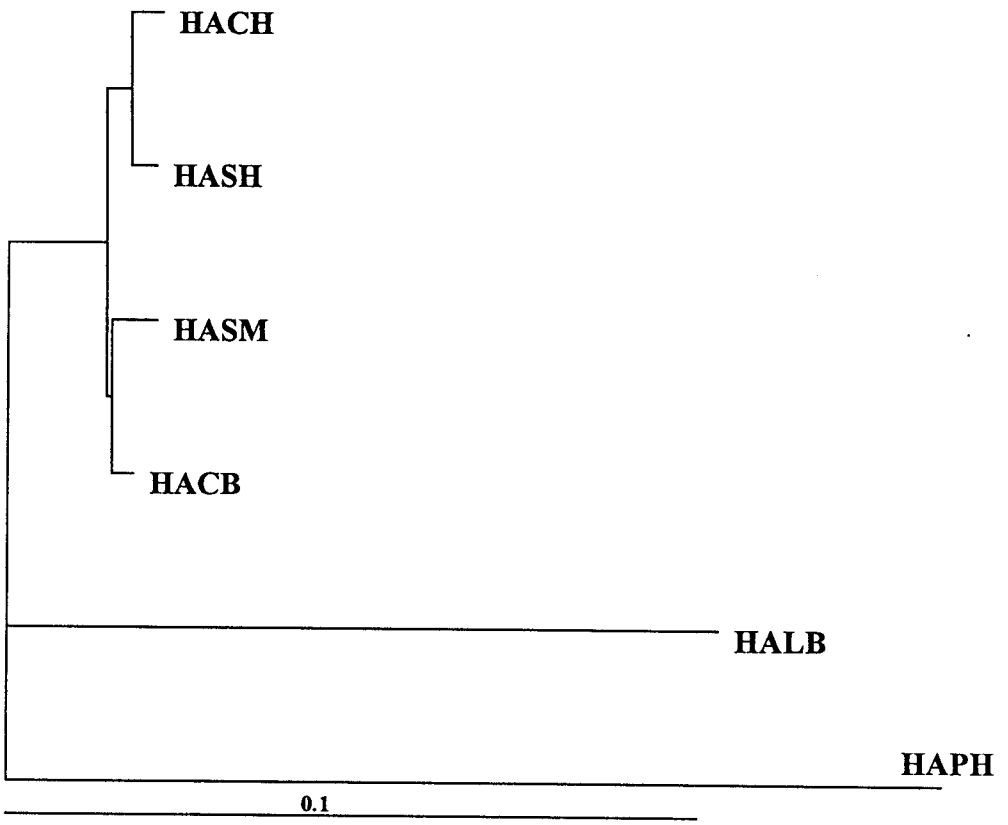
E. 2 OPB11


HASH
HACH
HACB
HASM
HALB
HAPH
HOSC
HOSM
HOPG
HOTR
HVPG
HVPU
0.1

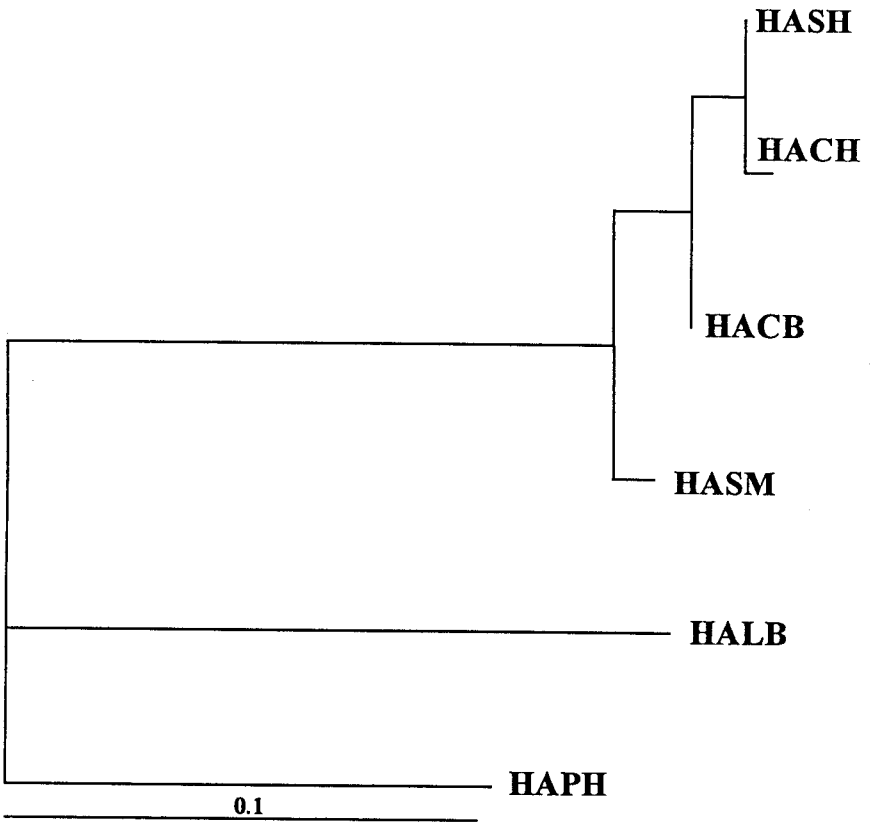
Appendix F

Neighbor - joining trees illustrating genetic relationships of 6 populations of *Haliotis asinina*, base on genetic distances resulted from RAPD analysis using primer UBC101 (F. 1), OPB11 (F. 2), UBC195 (F. 3), UBC197 (F. 4), and UBC271 (F. 5). Detailed information and abbreviations of sample sites are shown in Appendix A.

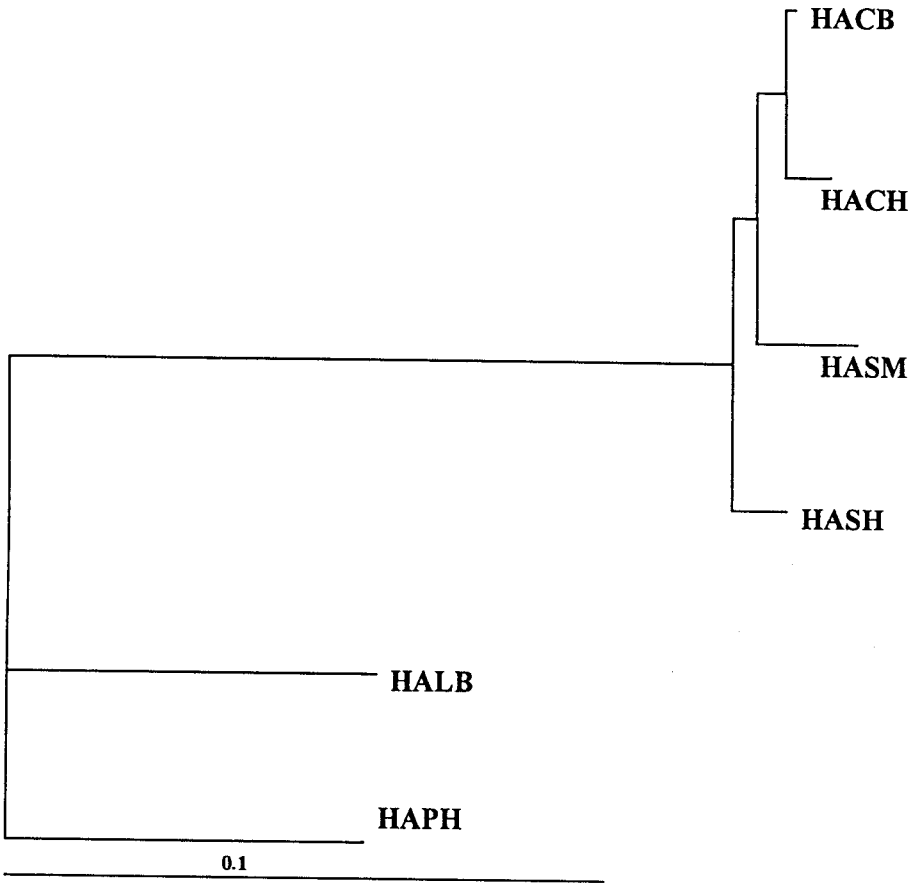
F. 1 UBC101



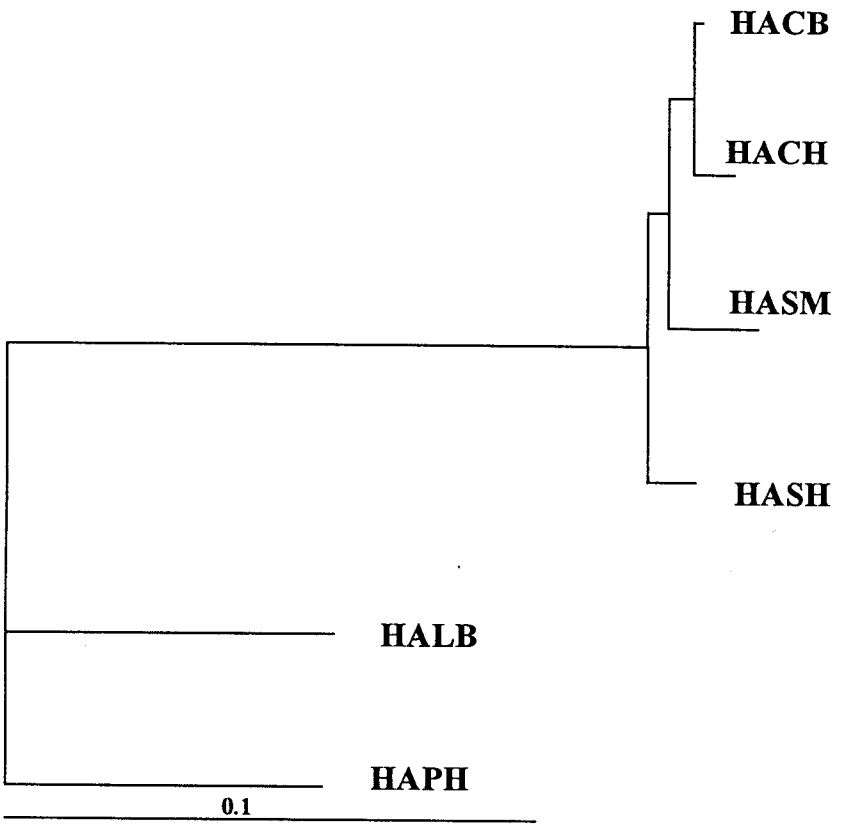
F. 2 OPB11



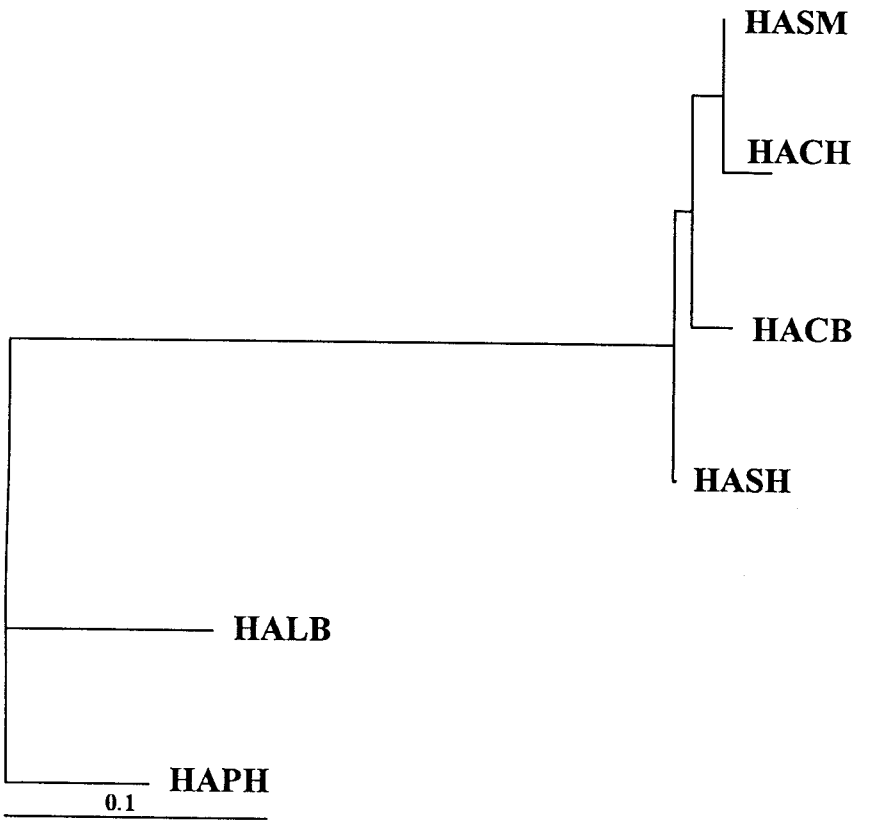
F. 3 UBC195



F. 4 UBC197



F. 5 UBC271



BIOGRAPHY

Miss Aporn Popongviwat was born on June 16, 1974 in Kanchanaburi, Thailand. She graduated with the Bachelor of Science degree in Marine Science from Department of Marine Science, Faculty of Science, Chulalongkorn University in 1995.