การพัฒนาเครื่องหมายทางพันธุกรรมของหอยเป๋าฮื้อเขตร้อนในประเทศไทย

นางสาวปาริฉัตร พรายภู่

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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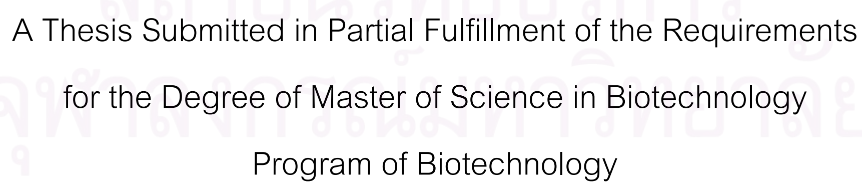
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

DEVELOPMENT OF GENETIC MARKERS OF TROPICAL ABALONE

IN THAILAND

Miss Parichart Praipue



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ปาริฉัตร พรายภู่ : การพัฒนาเครื่องหมายทางพันธุกรรมของหอยเป๋าฮื้อเขตร้อนในประเทศไทย (DEVELOPMENT OF GENETIC MARKERS OF TROPICAL ABALONE IN THAILAND) อ.ที่ปรึกษา : รศ.ดร. เผดิมศักดิ์ จารยะพันธุ์, อ.ที่ปรึกษาร่วม : ดร. ศิราวุธ กลิ่นบุหงา 173 หน้า. ISBN 974-17-0037-7

ในการวิเคราะห์ความหลากหลายทางพันธุกรรมของหอยเป๋าฮื้อในประเทศไทย 3 ชนิดคือ *Haliotis asinina*, *H. ovina*, และ *H. varia* โดยเทคนิค RAPD-PCR และ PCR-RFLP ของ 18S และ 16S rDNAs พบชิ้น RAPD ที่จำเพาะต่อชนิดหอยเป๋าฮื้อจากการใช้ไพรเมอร์ INS, YN73 และ M13 การวิเคราะห์โดยการตัดของ 18S rDNA (nuclear marker) ด้วย *Alu* I, *Taq* I และ *Hae* III และ 16S rDNA (mitochondrial marker) ด้วย *Bam*H I, *Eco*R I, *Hae* III และ *Alu* I ให้ 12 และ 13 แบบการตัดสำหรับ 18S rDNA และ 16S rDNA ตามลำดับ พบ composite haplotype ทั้งหมดจำนวน 49 haplotypes โดยค่าระยะห่างทางพันธุกรรมระหว่างคู่ composite haplotypes ภายในสปีชีส์ต่ำกว่าระหว่าง สปีชีส์

จากการสร้างแผนภูมิความสัมพันธ์ในเชิงวิวัฒนาการโดยใช้วิธี UPGMA ที่สร้างจากค่า divegence ระหว่าง composite haplotypes ระหว่างกลุ่มตัวอย่างและระหว่างชนิดแสดงให้เห็นความแตกต่างของ gene pools ของหอยเป๋าฮื้อแต่ละชนิด โดย *H. asinina* มีความสัมพันธ์ทางพันธุกรรมใกล้เคียงกับ *H. ovina* มากกว่า *H. varia* การวิเคราะห์ geographic heterogeneity และการประมาณค่า F_{ST} ชี้ให้เห็นถึงโครงสร้างประชากรในหอยเป๋าฮื้อแต่ละชนิด และพบโครงสร้างประชากร (population structure) ที่ชัดเจนระหว่าง *H. ovina* ที่มาจากทะเลอันดามันและอ่าวไทย (P<0.0001) ในขณะที่พบความแตกต่างระหว่าง *H. asinina* จาก Philippines กับกลุ่มตัวอย่างที่เหลือทั้งหมด (P<0.0021)

ทำการโคลนชิ้น 16S rDNA จากตัวแทนหอยเป๋าฮื้อที่มี composite haplotype ทั้ง 10 haplotypes นำมาหาลำดับเบส แผนภูมิความสัมพันธ์ทางวิวัฒนาการที่สร้างจาก divergence ของลำดับเบสของ 16S rDNA แสดงความสัมพันธ์ที่ใกล้เคียงกันภายในแต่ละชนิดของหอยเป๋าฮื้อ นอกจากนั้นการหาลำดับนิวคลีโอไทด์ยังแสดงถึงความเป็นไปได้ที่จะพัฒนา PCR ที่จำเพาะต่อชนิดของหอยเป๋าฮื้ออีกด้วย

หลักสูตร ....เทคโนโลยีชีวภาพ.........ลายมือชื่อนิสิต ....

สาขาวิชา ....เทคโนโลยีชีวภาพ.........ลายมือชื่ออาจารย์ที่ปรึกษา ....

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Genetic diversity of three species of abalone in Thailand; *Haliotis asinina*, *H. ovina*, and *H. varia* were analyzed by RAPD-PCR and PCR-RFLP of 18S and 16S rDNAs. Several species-specific RAPD fragment was found using primers INS, YN73, and M13. Restriction analysis of 18S rDNA (nuclear marker) with *Alu* I, *Taq* I and *Hae* III and 16S rDNA (mitochondrial marker) with *Bam*H I, *Eco*R I, *Hae* III and *Alu* I gave 12 and 13 digestion patterns for 18S rDNA and 16S rDNA, respectively. A total of 49 composite haplotype were observed.

Genetic distances between pairs of composite haplotypes within species were lower than those between species. A UPGMA dendogram constructed from divergence between composite haplotypes, samples and species revealed separate gene pools of these abalones. The *H. asinina* alone showed closer genetic relationships with *H. ovina* than *H. varia.* Geographic heterogeneity analysis and F_{ST} estimate indicated the existence of population structure of each abalone. Disregarding *H. varia* due to small sample sizes, strong genetic differentiation was observed in *H. ovina* whereas partial differentiation was observed between the Philippines and the remaining sample (P<0.0021)

The 16S rDNA of individual representing 10 composite haplotypes of three abalone species were cloned. Comparing and sequenced. The aligned sequences indicated the possibility to develop species-specific PCR from these sequences. A neighbor-joining tree constructed from sequence divergence of these sequences allocated relationships of sequences according to species origins of abalone.

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CONTENTS

Page

THAI ABSTRACT……………………………………………………………...iv

ENGLISH ABSTRACT………………………………………..………..v

ACKNOWLEDGMENTS……..…………………………………………vi

CONTENTS……………………………………………………………vii

LIST OF TABLES……………………………………………….……x

LIST OF FIGURES…………………………………………………xiii

LIST OF ABBREVIATIONS……………………………………...xv

CHAPTER I INTRODUCTION…………………………………...…1

1.1 Taxonomy of abalone……………………………………………...6

1.2 Biology and morphology…………………………………………..6

1.3 Life cycle……………………………………………………………12

1.4 Distributions of abalone…………………………………………….14

1.5 Molecular genetic markers………………………………………….15

1.6 Genetic studies in abalones…………………………………………21

1.7 Objective……………………………………………………………29

CHAPTER II MATERIALS AND METHODS

2.1 Materials……………………………………………………………30

2.2 Sampling……………………………………………………………33

2.3 DNA extraction……………………………………………………..34

CONTENTS (cont.)

Page

2.4 Measuring concentrations of extracted DNA using spectrophotometry and electrophoresis…....……………………………..35

2.5 Screening of primers for population genetic studies in Thai abalone…...38

2.6 PCR analysis……………………………………………………………41

2.7 Agarose gel electrophoresis…...……………………………………...42

2.8 Restriction endonuclease digestion…………………………………...…45

2.9 Data analysis……………………………………………………………47

2.10 Cloning of 16S rDNA fragments………………………………………49

CHAPTER III RESULTS

3.1 DNA extraction…………………………………………………………55

3.2 Analysis of genetic diversity of *H. asinina*, *H. ovina*, and *H. varia* using RAPD analysis…………………………………………………………..55

3.3 Analysis of genetic diversity of *H. asinina*, *H. ovina*, and *H. varia* using PCR-RFLP analysis ……………………………………………………56

3.4 Genetic distance between composite haplotypes of abalone and their phylogenetic relationships……………………………………………86

3.5 Haplotype and nucleotide diversity within species, nucleotide divergence between sample and phylogenetic relationships at the sample and species levels………………………………………………………………..…87

3.6 Geographic heterogeneity analysis between geographic samples of abalone……………………………………………………………………..99

3.7 Cloning and sequencing of 16S rDNA…………………………………100

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CONTENTS (cont.)

Page

CHAPTER IV DISCUSSIONS…………………………………………..108

CHAPTER V CONCLUSIONS……………………………………………116

REFERENCES…………………………………………………………….117

APPENDICES……………………………………………………………..133

Appendix A……………………………………………………………………134

Appendix B……………………………………………………………………144

Appendix C……………………………………………………………………152

Appendix D……………………………………………………………………155

Appendix E……………………………………………………………………156

Appendix F……………………………………………………………………162

Appendix G……………………………………………………………………163

Appendix H……………………………………………………………………167

Appendix I……………………………………………………………………..168

BIOGRAPHY……………………………………………………………..173

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

Tables **Page**

Table 1.1 Diversity of members of the genus *Haliotis* from different geographic locations and climatic zones……………………………………………….…...4

Table 1.2 Commercially important abalone species……………..…………………..5

Table 1.3 Morphological characteristics, habitat, distribution and abundance of abalone found in Thailand……………………………………………….11

Table 1.4 Chromosome number in *Haliotis* from various geographic areas……….28

Table 2.1 Sample collection sites and sample sizes of abalone specimens used in this study……………………………………………………………………37

Table 2.2 Primers and primer sequences screened for genetic studyies of Thai abalone...……………………………………………………………....39

Table 2.3 Primers and primer sequences screened for population genetic studies of Thai abalone………………………………………………..40

Table 2.4 The amplification conditions of RAPD-PCR analysis abalone………….43

Table 2.5 The amplification condition of 16S rDNA and 18S rDNA of abalone….43

Table 2.6 The optimal concentration of Seakem LE and MetaPhor agarose prepared in 1xTBE buffer for separating double straned DNA…………44

Table 2.7 Restriction endonucleases and reconizing site used for screening of informative enzymes……………………………………………….…46

Table 3.1 Restriction fragment patterns observed from digestion of the 18S rDNA of three abalone species (*H. asinina*, *H. ovina*, and *H. varia*) with restriction endonucleases used in this study…………..…………………74

LIST OF TABLES (cont.)

Page

Table 3.2 Restriction fragment patterns observed from digestion of the 16S rDNA of three abalone species (*H. asinina*, *H. ovina*, and *H. varia*) with restriction endonucleases used in this study…………………..………75

Table 3.3 Frequency distribution patterns of 18S rDNA in each geographic sample analyzed by *Alu* I, *Taq* I, and *Hae* III….……………………………...76

Table 3.4 Frequency distribution patterns of 16S rDNA in each geographic sample analyzed by *Bam*H I, *Eco*R I, *Hae* III, and *Alu* I………………………...78

Table 3.5 Frequency distribution of composite haplotypes within each abalone sample resulted from restriction analysis of 16S rDNA…………………80

Table 3.6 Frequency distribution of composite haplotypes within each abalone sample resulted from restriction analysis of 18S and 16S rDNAs…….…81

Table 3.7 Haplotype and nucleotide diversity within samples of Thai abalone examined by restricton analysis of 16S rDNA …….……………………92

Table 3.8 Haplotype and nucleotide diversity within samples of Thai abalone examined by restricton analysis of 18S rDNA and 16S rDNA ……..…...93

Table 3.9 Nucleotide diversity (above diagonal) and divergence (below) between geographic samples of abalone resulted from restriction analysis of 16S rDNA ……………………………………………………..…..……..95

Table 3.10 Nucleotide diversity (above diagonal) and divergence (below) among geographic samples of abalone resulted from restriction analysis of 18S rDNA and 16S rDNAs ……………………………………………...96

Table 3.11 Geographic heterogeneity analysis in distribution frequency of 16S composite haplotypes using a Monte Carlo simulation………………...101

LIST OF TABLES (cont.)

Page

Table 3.12 Geographic heterogeneity analysis in distribution frequency of 18S rDNA and 16S rDNA composite haplotypes using a Monte Carlo simulation..102

Table 3.13 Estimated gene flow (above diagonal) and F_{ST} (and P-value, below diagonal) between pairs of geographic samples based on restriction analysis of 16S rDNA……………………………………………………..103

Table 3.14 Estimated gene flow (above diagonal) and F_{ST} (and P-value, below diagonal) between pairs of geographic samples based on restriction analysis of 18S rDNA……………………………………………………..104

LIST OF FIGURES

Figures **Page**

Figure 1.1 Shell morphology of three abalone species (*H. asinina*, *H. ovina*, and *H. varia)* Thailand…………………………………………………………………...9

Figure 1.2 Top view of abalone…………………………………...……………10

Figure 1.3 Internal organs of abalone……………………………………………...10

Figure 1.4 Life cycle of abalone…………………………………………………...13

Figure 2.1 Map of Thailand indicating sample sites of abalone used in this study…..36

Figure 3.1 Genomic DNA extracted from adductor muscle of abalone……………...58

Figure 3.2 RAPD patterns resulted from amplification of DNA of three abalone species with primer INS……..…………………………………………….59

Figure 3.3 RAPD patterns resulted from amplification of DNA of three abalone species with primer YN73…………………………………………...60

Figure 3.4 RAPD patterns resulted from amplification of DNA of three abalone species with primer M13……………………………………………….61

Figure 3.5 The amplification product of 18S rDNA (A), and 16S rDNA (B) of abalone…………………………………………………………………...62

Figure 3.6 An example of restriction patterns of 18S rDNA digested with *Alu* I……66

Figure 3.7 An example of restriction patterns of 18S rDNA digested with *Taq* I…...67

Figure 3.8 An example of restriction patterns of 18S rDNA digested with *Hae* III…68

Figure 3.9 An example of restriction patterns of 16S rDNA digested with *Bam*H I...69

Figure 3.10 An example of restriction patterns of 16S rDNA digested with *Eco*R I...70

Figure 3.11 An example of restriction patterns of 16S rDNA digested with *Hae* III..71

Figure 3.12 An example of restriction patterns of 16S rDNA digested with *Alu* I ….72

Figure 3.13 An example of restriction patterns of 16S rDNA digested with *Alu* I…..73

LIST OF FIGURES (cont.)

Page

Figure 3.14 A UPGMA dendogram illustrating genetic relationships of ten composite haplotypes in the three abalone species resulted from PCR-RFLP of 16S rDNA……………………………………………………………………88

Figure 3.15 A UPGMA dendogram illustration genetic relationships between samples of three abalone species based on PCR-RFLP of 16S rDNA…………...89

Figure 3.16 A UPGMA dendogram indicating genetic relationships between composite haplotypes of *H. asinina*, *H. ovina*, and *H. varia* based on PCR-RFLP of 18S and 16S rDNAs……………………………………90

Figure 3.17 A UPGMA dendogram illustration genetic relationships between geographic samples of three abalone species based on PCR-RFLP of 18S and 16S rDNAs…………………………………………………91

Figure 3.18 A UPGMA dendogram illustrating genetic relationships between *H. asinina*, *H. ovina*, and *H. varia* based on PCR-RFLP of 16S rDNA……………………………………………………………97

Figure 3.19 A UPGMA dendogram illustrating genetic relationships between *H. asinina*, *H. ovina*, and *H. varia* based on PCR-RFLP of 18S and 16S rDNA……………………………………………………………..98

Figure 3.20 Sequence aligment of abalone individuals exhibiting different composite haplotype of 16S DNA found in Thai abalone………………………...106

Figure 3.21 A neighbor-joining tree indication relationships between abalone possessing different 16S rDNA composite haplotypes based on DNA sequences……………………………………………………………107

LIST OF ABBREVIATIONS

A, C, G, T	=	nucleotides containing the bases adenine, cytosine, guanine and thymine, respectively
ATP	=	adenosine triphosphate
bp	=	base pair
BSA	=	bovine serum albumin
^{o}C	=	degree celcius
cm	=	centimetre
dATP	=	deoxyadenosine triphosphate
dCTP	=	deoxycytosine triphosphate
dGTP	=	deoxyguanosine triphosphate
dTTP	=	deoxythymidine triphosphate
DNA	=	deoxyribonucleic acid
EDTA	=	ethylene diamine tetra acetic acid
Fig.	=	figure
HCl	=	hydrochloric acid
IPTG	=	isopropyl-thiogalactoside
kb	=	kilobase
KCl	=	potassium chloride
M	=	molar
min	=	minute
$MgCl_2$	=	magnesium chloride
$MgSO_4$	=	magnesium sulfate

สถาบันวิทยบริการ
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mg	=	milligram
ml	=	millilitre
mM	=	millimolar
mtDNA	=	mitochondrial DNA
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
ng	=	nanogram
OD	=	optical density
PCR	=	polymerase chain reaction
pg	=	picogram
RAPD	=	randomly amplified polymorphic DNA
rDNA	=	ribosomal DNA
RFLP	=	restriction fragment length polymorphism
RNase A	=	ribonuclease A
rpm	=	revolution per minute
sec	=	second
SDS	=	sodium dodecyl sulfate
Tris	=	tris (hydroxy methyl) aminomethane
μg	=	microgram
μl	=	microlitre
μM	=	micromolar
U	=	unit
UV	=	ultraviolet
V	=	volt
W/V	=	weight / volume

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

Abalone are marine gastropod which are ecologically and commercially important occurring in most tropical and temperate areas, particularly in the subtidal zones (Geiger, 1998). There are over 100 species of abalone distributed worldwide and all of them are allocated into the genus *Haliotis*. Diversity of *Haliotis* species from different geographic locations and climatic zones is shown by Table 1.1.

The first information mentioned about abalone was made in the forth century B.C. by Aristotle. In the first century A.D., *otia* (little ear) was used by Pliny. The root of *Haliotis* were from the two Greek words, *halios* (the sea) and *ous*, *otis* (the ear) (Geiger, 1998). Shell morphological characters clearly separate abalone from other families of fossil as well as extent gastropods. Abalone shells are easily recognized by their flat, limpetlike shape and row of tremata toward the left periphery (Geiger and Groves, 1999).

At least 20 species of abalone are commercially important (Table 1.2.). Most of which are large size species (except *Haliotis diversicolor supertexta*) harvested from natural stocks (Jarayabhand and Paphavasit, 1996). Farming of abalone has been carried out commercially for the last few decades in various parts of the world (Hahn, 1989; Shepherd *et al*., 1992). The knowledge about abalone biology is thus increased substantially. In Australia, abalone culture was established about 16 years ago in South Australia and Tasmania. In Japan, abalone aquaculture has been carried out

over 50 years. Overexploitation of natural abalone resulted in the impetus for developing abalone aquaculture in several Asian countries, and others including USA, Mexico and South Africa. Japan, Australia, New Zealand, the United States, Mexico, and South Africa which are major producer of abalone at present (Uki, 1989).

In Australia, three species of interest showing the importance of the commercial fishery; the green lip abalone (*H. laevigata*), the brown lip (*H. conicopora*) and the Roe's abalone (*H. roei*). The brown lip may be regarded as the synonym of the black lip abalone (*H. rubra*) from the south-eastern Australia (Sherpherd, 1975; Brown, 1991b; Brown and Murray, 1992a).

In North America, nine species of abalone was found; the red abalone (*H. rufescens*), the green abalone (*H. fulgens*), the pink abalone (*H. corrugata*), the black abalone (*H. cracherodii*), the flat abalone (*H. walallensis*), the pinto abalone (*H. kamtschatkana*), the threaded abalone (*H. assimilis*), the white abalone (*H. sorenseni*) and the Western Atlantic abalone (*H. pourtalesii*).

Tantanasiriwong (1978) reported the existence of four tropical species of abalone, i.e., *H. asinina* (Linnaeus, 1758), *H. ovina* (Gmelin, 1791), *H. varia* (Linnaeus, 1758) and *H. planata* (Sowerby, 1882) in Thailand, but subsequently only three; *H. asinina, H. ovina,* and *H. varia* were truely found (Nateewathana and Hyleberg, 1986; Nateewathana and Bussarawit, 1988; Singhagraiwan and Doi, 1993; Jarayabhand *et al.*, 1995). There have been limited information about tropical abalone in Thailand. The researches related to establishment of abalone culture in *H. asinina*

and *H. ovina* began in 1986 and 1989, respectively. However, there have been no researched about culturing of *H. varia* (Jarayabhand and Paphavasit, 1996).

Among these species, *H. asinina* has the highest percentage of a ratio between the meat weight and the total weight (85%) compared to that of 40% and 30% for *H. ovina* and *H. varia*, respectively (Singhagraiwan and Doi, 1993). Therefore, *H. asinina* has a high value for 'cocktail-sized' (40-70mm) market, as happening with *H. diversicolor supertexta* in Taiwan (Jarayabhand and Paphavasit, 1996). It is currently promoted for culture activity in Thailand.

Abalones show a very clear nocturnal behaviour as other *Haliotis* species. During the day, they are usually found clinging to undersides of rocks and dead coral plates or within crevices of the rocks or dead coral heads. They are nocturnally active and usually venture out of hiding to feed only after dark (Wood and Buxton, 1996).

Relatively little knowledge about the genetic diversity and population structure of Thai abalone is available. This information is essential for the construction of appropriate breeding programs and for broodstock selection and management scheme leading to sustainable culturing actively of these taxa. Population genetic studies of abalone can be applied in several aspects, including determination of stocks, analysis of gene flow, and phylogenetic relationships. Additionally, species-specific can be used for quality control to prevent supplying incorrect abalone larvae for commercial culture, and incorrect canned species.

Table 1.1 Diversity of members of the genus *Haliotis* from different geographic locations and climatic zones

Region[1]	No. of species	Mean size (mm)	Climatic zone
Europe/Africa	3	57	Warm temperate/ subtropical
Southern Africa	5		
	3	103	Warm temperate
	2	51	Subtropical
Indian Ocean	3	50	Subtropical/ tropical
Australia	15-17		
	5	58	Tropical
	7	124	Warm temperate
	4	84	Temperate
New Zealand	6	65	Warm temperate/ temperate
West Pacific	10-13	47	Tropical
Japan	11		
	6	43	Warm temperate/ subtropical
	5	125	Temperate
North Amerca	7	142	Temperate
Panamic Caribbean	3	21	Tropical

[1] regions that include several distinct climatic zones, the total number of species is shown followed by their distributions in each respective climatic zone (Brown and Murray, 1992a).

Table 1.2 Commercially important abalone species

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 of northern green	75-125
H. kamtschatkana	Pinto	100
H. discus hannai	Ezo awabi	180-200
H. discus	Kuro awabi, oni or onigal	200
H. diversicolor supertexta[a]	Tokobushi	50
H. gigantea	Madaka	250
H. sieboldii	Megae	170
H. asinina[a]	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 balck	170
H. australis	Silver or queen paua	125
H. virginea	Virgin	70
H. tuberculata	Ormer	120
H. midae	Perlemon	90

After Hahn (1989) and Fallu (1991). [a] Tropical species

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

1.1 Taxonomy of abalone

Taxonomic definition of Thai abalone is as followed (Cox, 1960);

Phylum Mollusca

Class Gastropoda

Subclass Prosobranchia

Order Archaeogastropoda

Suborder Pleurotomariina

Superfamily Pleurotomariacea

Family Haliotidae

Haliotis asinina (Linnaeus, 1758)

-Ass's Ear Shell (Eng.), Mimigai (Jap.)

Haliotis ovina (Gmelin, 1791)

-Sheep Ear Shell (Eng.), Maanago (Jap.)

Haliotis varia (Linnaeus, 1758)

-Varied Ear Shell (Eng.), Iboanago (Jap.)

1.2 Biology and Morphology

Taxonomic identification of Thai abalone is based primarily on shell morphology (Fig 1.1). The shell characters generally use to differentiate abalone species: a size, shape, respiratory pores, epipodia and radula. Morphological characteristics, habitat, species distribution and abundance of Thai abalone are shown by Table 1.3.

1.2.1 Shell

The shell is on the top and covers most of the body part of the abalone (Fig 1.2). It generally has an oval shape with the long anterior-posterior axis, though some species are more elongated. The abalone shell is in a spiral form. The head of the abalone is the anterior and the apex of the shell spiral is the posterior. The outside of the shell is usually rough often with other molluscs, sponges, algae or hard red coralline encrusting algae grow on it. The inside of the shell is smooth, generally pearly and in some species is iridescent. A row of holes is found in the left-hand side of the shell. The anterior holes are the biggest and those toward the back are usually blocked. The holes assist with respiration and removal wastes. The abalone shell grows as a spiral by the addition of new material on the anterior.

1.2.2 Foot

Abalone holds onto the sea-bed with its foot. In most species, the foot is largely hidden by the shell but it is clearly visible when abalone is turned upside-down. A series of tentacles is found around the outside of the foot, presumably for detection predators and food by touch and taste. Internal organs of an abalone are shown by Fig 1.3.

1.2.3 Head

The head is located in front of the foot. The mouth is at the base of the head and is underneath the lips. The mouth is a tongue-like organ covered with teeth called the radula, which is used to rasp food.

1.2.4 Gills

Paired gills are located in a chamber called the mantle cavity, which is located under series of holes in the shell. The sea water is drawn into the anterior of the mantle cavity and passed over the gills. Oxygen is taken up and waste gases are given off. The used water is passed out through the holes of the shell.

1.2.5 Gut

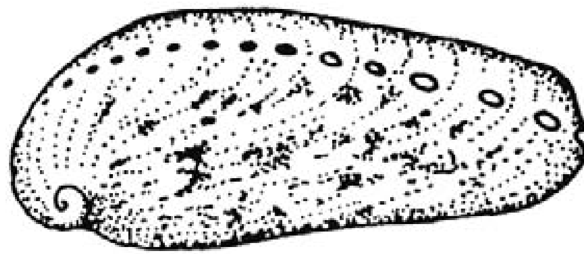
Gut is hidden above the foot. The shell muscle, or stalk of the foot, extends up and attached to the middle of the shell. Gut coils around the space between the stalk of the foot and the rim of the shell.

1.2.6 Reproductive glands

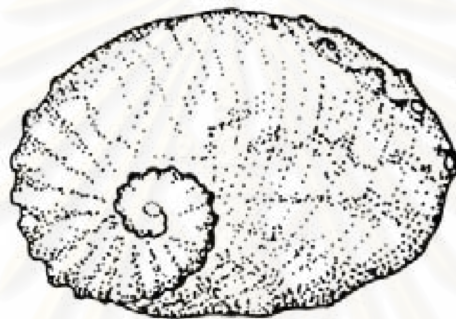
The reproductive glands (or gonads) envelop the tubes of the gut. They form a large cone-shaped appendage between the shell and the foot. Gonad are located on the same side of the shell and extends up into coiled apex of the shell.

1.2.7 Circulatory system

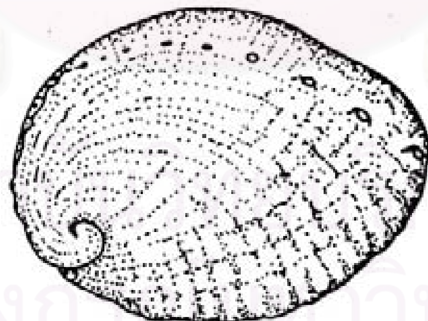
Abalones blood contains a copper-based respiratory pigment, haemocyanin, which is blue when highly oxygenated but colorless when no oxygen is present. The abalone's heart pumps oxygenated blood from gills into the foot along two central vessels which branch into smaller tubes. From the small tubes, blood and oxygen infiltrate into tissues. The blood then drains into another system of small tubes and moves back to a larger central cavity in the foot which carries it to gills to be oxygenated again.



H. asinina



H. ovina



H. varia

Fig. 1.1 Shell Morphology of three abalone species (*H. asinina*, *H. ovina*, and *H. varia*) in Thailand (Nateewathana and Bussarawit, 1988).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

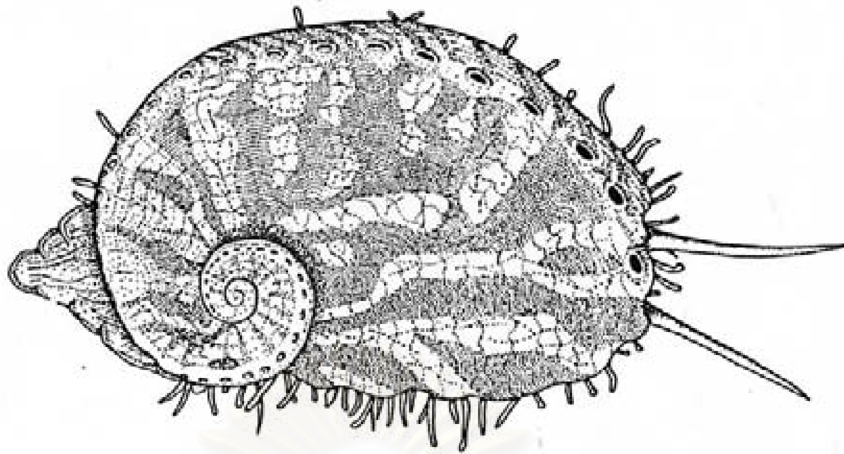


Fig. 1.2 Top view of abalone. Most abalone conform a general pattern and have a similar appearance (Fallu, 1991).

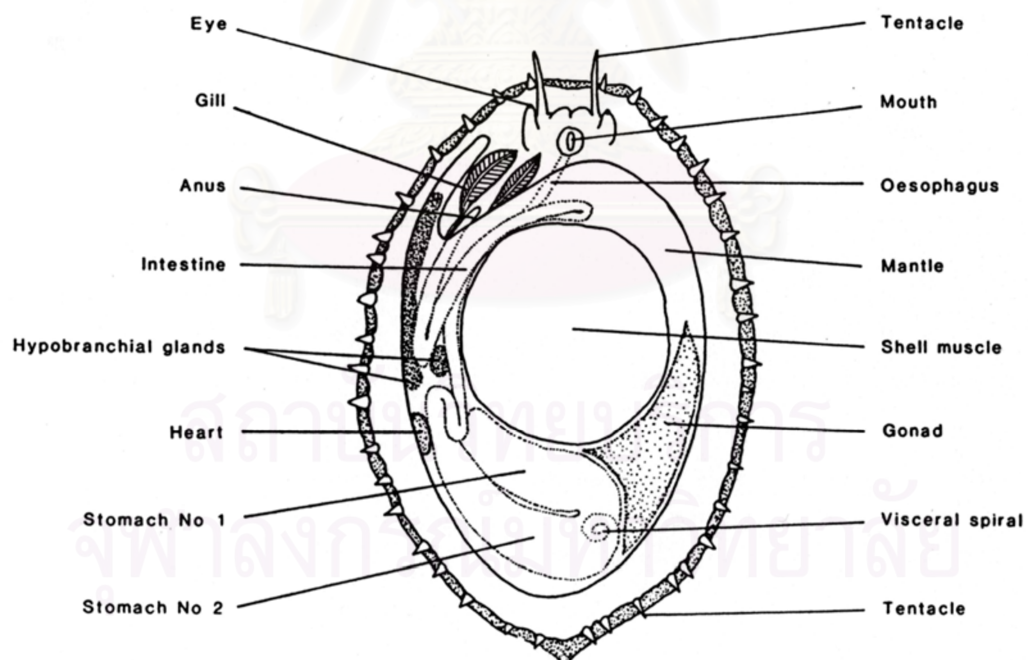


Fig. 1.3 Internal organs of abalone. The abalone's internal organs are hidden between the shell and the foot (Fallu, 1991).

Table 1.3 Morphological characteristics, habitat, distribution and abundance of abalone found in Thailand

Description	*H. asinina*	*H. ovina*	*H. varia*
Morphology			
Shell shape	Elongate	Oval	Oval
Color	Greenish brown	Olive green	Dark brown
Sculpture	Spiral	Prominent spiral	Spiral
Thickness	Thin	Thick	Moderate
Tremata	6-7, smooth	4-6, highly elevated	3-5, slightly elevated
Foot color	Grey	Cream to orange	Cream to orange
Regression coefficient			
Length and weight	3.225	3.034	2.705
Length and width	0.527	0.716	0.687
Maximum size			
Shell length (mm)	100	80	60
Total weight (g)	250-280	75	25
Habitat			
Zone	Inter- to subtidal	Subtidal	Inter- to subtidal
Substrate	Rock, dead coral	Rock crevice	Rock crevice
Depth (m)	1-7	1-7	1-7
Distribution			
Gulf of Thailand	Chon Buri, Rayong, Trad	Chon Buri, Rayong, Trad	No occurrence
Andaman Sea	Puket	Ranong to Satul	Ranong to Satul
Abundance			
Gulf of Thailand	Rare	Dominant	No record
Andaman Sea	Rare	Moderate	Dominant

After Jarayabhand and Paphavasit (1996).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

1.3 Life cycle

Abalones are dioecious broadcast spawners and usually have a seasonal reproductive cycle consisting of an annual spawning preceded by gametogenesis. The periodicity and duration of spawning vary both intra- and interspecifically (Shepherd & law, 1974; McShane *et al*., 1988; Tutschulte & Connell, 1981). The eggs of abalone are buoyant and generally hatch within 24 hours after fertilization (Mottet, 1978).

Abalone eggs are external fertilized. Adjacent female and male abalone shed eggs and sperm (spawning). These are mixed in the sea water. When gametes are fused, the fertilized egg divides repeatedly and forms a larva. At the beginning abalone larvae are tiny and have no shell. The life cycle of abalone is shown by Fig 1.4. Initially, an upward swimming (trochophore) larvae are produced, probably as an adaptation to avoid predation by benthic filter feeders (Thorson, 1964; Mileikovsky, 1971; Crisp, 1974). The larvae go through a series of changes in the body form, to veliger stages. After about one week, the larvae sink to take up residence on the sea-bed. This process is called settlement and the developmental stage of abalone is termed spat. The abalone's body transforms into a miniature copy of adults. The minimum duration of larval stages is about 4 days (for temperate abalone species) (Leighton, 1972, 1974) but larval life duration would be expected to be much longer for most exploited abalone inhabiting cooler temperature waters (Cox, 1962; Mottet, 1978). Almost abalone become mature between the first and third year. The life span of abalone is longer than a decade.

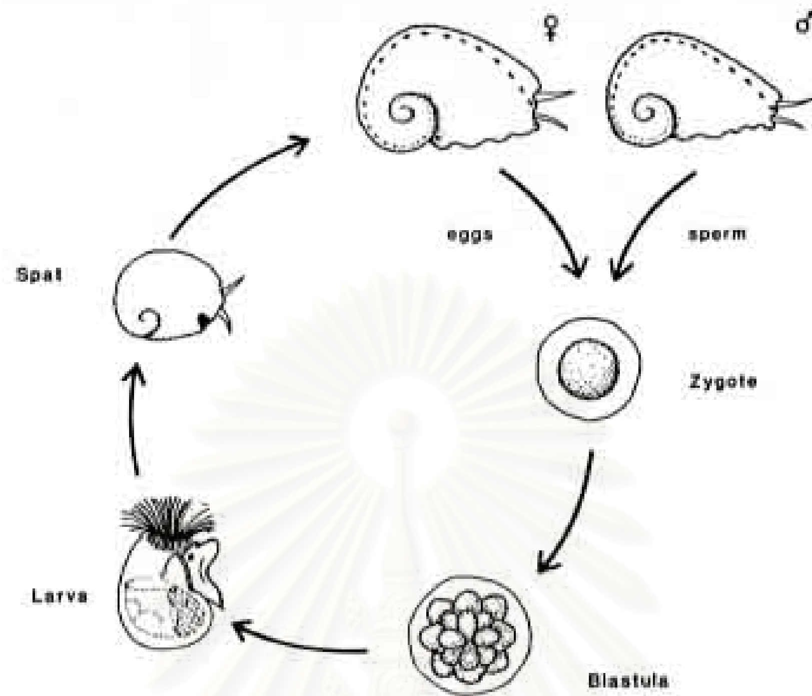


Fig. 1.4 Life cycle of abalone (Fallu, 1991).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

1.4 Distributions of abalone

Abalones occur in all of the major oceans of the world but they are more abundantly distributed in the temperate zones. Generally, abalones prefer shallow, turbulent waters with high levels of dissolved oxygen and hard surfaces for settlement. Abalones are thus commonly found at rocky headlands in the cool seas.

In Thailand, *H. asinina* and *H. ovina* are found on islands along the eastern coasts of the upper Gulf of Thailand, and all three species occur in the Andaman Sea (Tookwinas *et al*., 1986; Nateewathana and Bussarawit, 1988; Jarayabhand *et al*., 1991; Kakhai and Petjamart, 1992; Ngow and Jarayabhand, 1993). *H. ovina* has been reported to be more common than *H. asinina* along the upper eastern Gulf of Thailand, only small numbers of *H. ovina* have been found along the lower eastern coast (Kakhai and Petjamart, 1992). Along the Andaman coastline, the relative abundance of each species was 81% for *H. varia*, 17.3% for *H. ovina* and 1.7% for *H. asinina* (Jarayabhand and Paphavasit, 1996).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

1.5 Molecular genetic markers

Analysis of genetic diversity and relatedness between different species, populations, and individuals is a central task for many biological disciplines. During the past decade, classical strategies of evaluating genetic variability such as comparative anatomy, morphology, embryology, and physiology have been increasingly complemented by molecular techniques. These techniques have proved most powerful in making identifications when morphological differentiate are ambigious (Burton, 1996). The development of species-specific molecular markers (protein or DNA) has greatly facilitated researches in a variety of disciplines such taxonomy, phylogeny, ecology, genetics, and breeding. Allozymes have been molecular markers of choice initially. In recent years, however, attention has increasingly focused on polymorphism of DNA molecule as a source of information. Because each individual's DNA sequence is unique, this sequence information can be exploited for any study of genetic diversity and relatedness between organisms. The use of the PCR to amplify DNA *in vitro* increases sensitivity of the detection assay since it only requires very few DNA template molecules. This property makes PCR based approaches particularly suitable for application in early developmental stages of organisms or in small organisms for which a limited amount of biological material is available.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

1.5.1 Protein markers

For generation of molecular markers based on protein polymorphism, the most frequently used technique is the electrophoretic separation of proteins, followed by specific staining of a distinct protein subclass. The majority of protein markers are represented by allozymes. Allozyme electrophoresis has been successfully applied for

genetic studies of organisms from bacteria to animal and plant species since the 1960s (May, 1992). Allozyme analysis is relatively straightforward and easy to carry out. A tissue extract is prepared and electrophoresed on a starch or polyacrylamide gel. The proteins of this extract are thereby separated according to net charges and sizes. After electrophoresis, the gel is stained for a particular enzyme by adding a substrate and a dye under appropriate reactions, resulting in a band at the position to where the enzyme has migrated. Depending on the number of loci, their states of homo- or heterozygosity, and the enzyme molecule configuration, from one to several bands are visualized. The positions of these bands can be polymorphic and thus informative.

Allozyme studies (Brown and Murray, 1992; Hara and Fujio, 1992; Brown, 1993) revealed information on relationships among several abalone species. However, there are problems with this method; (1) all isozymes may not sepatate completely on the gel (Lewontin, 1991), (2) distantly related species cannot be compared due to the interference from convergence (Hills and Moritz, 1990; Brown, 1993), (3) phylogenetic trees inferred from isozyme polymorphism is sometimes in disagreement with trees based on nuclear or mitochondrial DNA (Karl and Avise, 1992). Advances in DNA technique have lead to the rapid development of analysis identification methods. The DNA molecule offers a number of advantages when compared to proteins including stability of DNA at high temperatures, its presence in all tissue types and greater variation owing to degeneracy of the genetic code.

1.5.2 DNA markers

1.5.2.1 MtDNA

The animal mitochondrial DNA (mtDNA) molecule is small, and its gene order is very conservative within the phylum. The rate of sequence evolution is higher than that in nuclear DNA. Its high sequence variability, small genome size, and relatively easy isolation make mtDNA a valuable marker for population studies, especially for the analysis of maternal lineages and population history.

Since mtDNA is haploid, and transmitted maternally, the effective population size estimated from mtDNA is generally smaller than that estimated from nuclear markers such as allozymes and single copy nuclear DNA (Birky *et al*., 1989). This increases its sensitivity to inbreeding and bottleneck effects compared to nuclear DNA markers (O'Connell *et al.*, 1998). The mtDNA genetic polymorphism information allows inferring relationships between closely related species groups for a wide range of taxa and the population history of a species and also used for genetic stock structure analysis (Avise, 1994).

1.5.2.2 Nuclear ribosomal DNA

The rDNA genes in most eukaryote nuclear genomes are highly repeated on specific chromosomes, and contain regions that evolved at distinct rates. Genes coding the 18S, 5.8S, and 28S (or their equivalents) are separated by internal spacer sequences (ITS) to form a single repeat unit, which is arranged head-to-tail in multiple copies (Ibrahim *et al*., 1994). While the rDNA genes are highly conserved, the intergenic spacer (IGS) and internal transcribed spacer sequences (ITS) are

polymorphic, and provide useful tools for taxonomy and phylogenetic studies at higher taxonomic levels (Henrion *et al*., 1994).

1.5.2.3 Repetitive DNA sequences

Satellite DNA, a type of tandemly arranged highly repetitive sequence, has been found widely exist in animals and plants (Beridze 1986). The repeated size within 7-70 base pairs (bp) is known as minisatellites, while the repeat unit size between 1-6 bp is known as microsatellites or short tandem repeats (STR) (Budowle et al., 1991; Kimpton et al., 1993). Minisatellites are usually located in introns (Griffiths et al., 1993), or 3' end nontranslated regions of the genes (Budowle et al., 1991; Huang et al., 1997). In contrast, microsatellites are located abundantly in both genic and extragenic regions of the eukaryotic cell genome (Kimpton et al., 1993). The abundance of repetitive sequences particularly microsatellites and their polymorphic nature and amenability to amplification by polymerase chain reaction (PCR) have made microsatellites (and some minisatellites loci) to be ideal markers for genetic analysis, including population genetic structure, and evolutionary studies (Bosch et al., 1993; Jeffreys et al., 1991; Budowle et al., 1991; Deka et al., 1995; Primer et al., 1996).

1.5.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) created a revolution in molecular biology research and its applications. PCR is an *in vitro* method that enzymatically amplifies specific DNA sequences using oligonucleotide primers that flank the region of interest in the target DNA. The principal involves a repetitive series of cycles each of which consist of template denaturation, primer annealing and extension of the

สถาบันวิทยบริการ จุฬาลงกรณ์มหาวิทยาลัย

annealed primers by a DNA polymerase resulting in the exponential accumulation of a specific fragment.

1.5.4 Randomly Amplified Polymorphic DNA (RAPD)

RAPD analysis (Williams *et al*., 1990) is conceptually simple. Nanogram amounts of total genomic DNA are subjected to PCR using short synthetic oligonucleotide of random sequences. The amplification protocol differs from the standard PCR conditions (Erlich, 1989) in that only a single random oligonucleotide primer is employed and no prior knowledge of the genome subjected to analysis is required. When the primer is short (e.g. 10-mer), there is a high probability that the genome contains several priming sites close to one another that are in an inverted orientation. The technique essentially scans a genome for these small inverted repeats and amplifies intervening DNA segments of variable length. The profile of amplification products depends on the template-primer combination. The amplification products are resolved on agarose gels and polymorphism is treated as dominant genetic markers, which are inherited in a Mendelian fashion (Williams *et al*., 1990; Carlson *et al*., 1991; Martin *et al.*, 1991; Welsh *et al.*, 1991). Amplification of non-nuclear RAPD (e.g. mtDNA) markers is negligible because of the relatively small non-nuclear genome sizes.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

1.5.5 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) analysis is used for indirect evaluation of genetic variation at the nucleotide (DNA) lavel. Variation in restriction enzyme cleavage sites (conventionally detected by Southern blot hybridization)

generates size differences of the resulting fragments. Therefore, RFLP representing the polymorphism of restricted DNA fragments.

For the conventional RFLP, genomic DNA is cut using one or more restriction endonucleases generally tetra- or hexanucleases that recognise sites on the DNA template. The digested DNA fragments was electrophoresed through the agarose gel and transferred to a supporting membrane (nylon or nitrocellulose) before hybridized with a specific radiolabeled DNA probe. Results from restriction analysis can be visualised by autoradiography.

RFLP has been widely used for population genetic studies in various species, but conventional technique have some limitation. RFLP technique requires fairly large amounts of genomic DNA, cloned probes that should be specific to an organism, tedious, and time consuming particularly when dealing with a large number of specimens. Therefore, it has been replaced by polymerase chain reaction (PCR)-based methods including PCR-based DNA fingerprinting, PCR-RFLP, Amplified fragment length polymorphism (AFLP) and microsatellites.

PCR-RFLP is a technique for *in vitro* amplification of specific DNA sequences by the stimultaneous primer extension of complementary strands of DNA. The followed by restriction analysis of the amplification product with informative restriction endonucleases. Large number of copies of a particular target DNA fragment are produced from a very low amount of starting template DNA within a short period of time. As a result, simple detection method by ethidium bromide staining is sensitive enough to determine polymorphism.

Restriction analysis can be carried out considering restriction site (cleavage map) or fragment length polymorphism. This technique is simpler, and requires shorter time consuming than the conventional RFLP where hybridization of labeled DNA probes to the target restricted DNA is not necessary.

1.6 Genetic studies in abalones

There have been few publications concerning molecular genetics studies in abalone. Among these, a tandemly repeated satellite DNA of 290-291 bp was identified by *Sal* I digestion of genomic DNA of five species of the Eastern Pacific (California) abalone including the Red abalone (*H. rufescens*), the White abalone (*H. sorenseni*), the Flat abalone (*H. walallensis*), the Pinto abalone (*H. kamtschatkana*), and the Pink abalone (*H. corrugata*). The fragment was cloned into pBluescript (Stratagene), transformed into XL1-Blue *Escherichia coli* and sequenced using T3 and T7 primers. Satellite-specific primers were synthesized from a repeat unit of *H. rufescens.* The consensus sequences of satellite DNA were determined in all five species by directly sequencing of genomic DNA using the satellite-specific primers. In *H. rufescens*, the 290 bp *Sal* I satellite represents approximately 0.5% of total DNA, equivalent to approximately 28,000 copies per haploid genome. (Muchmore *et al*., 1998)

Huang and Hanna (1998) identified microsatellite DNA in abalone and obtained three microsatellite from screening randomly amplified polymorphic DNA (RAPD) products and a genomic DNA library of the blacklip abalone (*H. rubra* Leach). RAPD products generated from primers UBC-101 (5’-GCG GCT GGA G-3’), UBC-135 (5’-AAG CTG CGA G-3’), and M13 (5’-GAG GGT GGC GGT TCT-

3'), were transferred to membranes and hybridized with 3' end fluorescein-labeled oligonucleotides probes; $(CA)_{10}$, $(GA)_{10}$, $(CT)_{10}$, $(GT)_{10}$, $(GGT)_7$, $(GATA)_5$, $(GACA)_5$, $(GGGT)_5$, and $(AACT)_5$, to detect bands containing microsatellite sequences. Positive bands were excised from agarose gels. DNA fragments were eluted and cloned into a pCR-Script vector for sequencing. DNA inserts were sequenced for both directions with T3 and T7 primers. Three loci of microsatellites were found. They were RUB*GT1* (forward: 5'-AGG GTG GCG GTT CTG GTC CTA AAT C-3', reverse: 5'-GGC AGT GAT GAT ATA GCG TTG TTC G-3'), RUB*CA1* (forward: 5'-CCA ATT TTA CTT GAA GAC TTG TGA TGC-3', reverse: 5'-ATG TGT ACG CGT TGG TGG ATG G-3'), and RUB*GACA1* (forward: 5'-CGC CGT TTT ATT CGT CAC CAA TC-3', reverse: 5'-CCA CAT ATA CAA ATA AAT ATA TC-3'), which contain $(GT)_n$, $(CA)_n$, and $(GACA)_n$ repeats, respectively. All three microsatellite loci were polymorphic when tested with 100 blacklip abalone originating from nine geographic sites along the Victorian coast and one site from Eden, New South Wales. The number of alleles for RUB*GT1*, RUB*CA1*, and RUB*GACA1* were 41 alleles, 30 alleles, and 8 alleles, respectively.

The first microsatellite locus in the red abalone (*H. rufescens*) from California was obtained by creating size-selected genomic libraries and screened for all combination of dinucleotide and trinucleotide repeats. Genomic libraries were created from 12 adults from northern California (Punta Gorda Reserve), two adults from central California (Morro Bay), and five adults from southern California (Santa Babara). Genomic DNA was digested with *Sau3*AI and size-fractionated on the agarose gel. The 200-700 bp size-ranges were excised and purified by Centricon spin columns, ligated to KSpUC18 vector and inserted into *Escherichia coli* XL1-BLUE.

Colonies were lifted onto nylon membranes for hybridization. Dinucleotide and trinucleotide repeats were end-labeled with P^{32}-dATP and used as the probe. The plasmid DNA was sequenced. Primers were then designed. The most common microsatellite repeats were GT/TG and AC/CA. Locus specific primers were designed for the microsatellite locus Hruf200. A total of 21 alleles ranging from 97 to 149 base pairs in size were observed (Kirby *et al*., 1998).

Huang *et al*. (1997) cloned and sequenced growth-promoting genes of the blacklip abalone (*H. rubra* Leach, 1814). Two minisatellites loci were identified in the cDNA libraries. One contained a 33 bp repeat unit (5'-CCC AAG GTC CCC AAG GTC AGG GAG GCG AAG GCT-3') located in the 3' untranslated region of a putative growth hormone (GH) gene, and the repeat was designated as GHR. The other contained a 18 bp repeat unit (5'- ACC CGG CGC TTA TTA GAG-3') located in the 3' untranslated region of a putative molluscan insulin-related peptides (MIP) gene, and was designated as MIPR. The preliminary population study on 100 blacklip abalone from the Victorian coastline indicated a highly polymorphic level implying that these DNA minisatellites can be used in molecular genetic studies of abalone, including paternity testing, triploid testing, population genetic structure, and gene flow.

Huang *et al*. (2000) analyzed genetic structure of one hundred blacklip abalone, *H. rubra* (Leach) of nine sites along the Victorian coast and from one at Eden, New South Wales, Australia using three PCR-based DNA fingerprinting. Six random primers; UBC-101 (5'-GCG GCT GGA G-3'), UBC-135 (5'-AAG CTG CGA G-3'), UBC-149 (5'-AGC AGC GTG G-3'), UBC-159 (5'-GAG CCC GTA G-

3'), UBC-169 (5'-ACG ACG TAG G-3'), and M13 (GAG GGT GGC GGT TCT-3'), two minisatellites (GHR: putative growth hormone gene repeat, and MIPR: putative mollusca insulin-like peptide gene repeat), and three microsatellites (RUB*GT1*, RUB*CA1*, and RUB*GACA1*) were used. All types of DNA markers revealed significant subdivision of the *H. rubra.* Genotypes of three microsatellite loci indicated excessive homozygotes across all populations, in contrast to those observed in two minisatellite loci which conformed Hardy-Weinberg equilibrium.

Okamura *et al*. (1999) studies chromosome morphology in a member of Haliotidae. Karyotyping of *H. discus hannai* larvae obtained at 15-20 hours after fertilization were analyzed using a scanning electron microscopy (SEM), banding analysis, and nucleolus organizer region (NOR) analysis. Standard values of the relative length and arm ratio of each pair of chromosome in *H. discus hannai* were determined by SEM measurements of the chromosome arm length. This abalone species possessed 11 pairs of metacentric and 7 pairs of submetacentric chromosomes (2n = 36).

Karyotyping of three species of Thai abalone (2n = 32); *H. asinina* and *H. ovina* samples collected from the east coast and the upper Gulf of Thailand, respectively, and *H. varia* collected from the Andaman sea were carried out. The gill was dissected out, and fixed. Chromosomes were prepared and observed under a light microscope. Two types of chromosomes, metacentric and submetacentric chromosome were found in *H. asinina* and *H. varia* whereas additional telocentric was found in *H. ovina*. The numbers of metacenteric and submetacentric were 20 and 12 in *H. asinina*, and were both 16 in *H. varia*. There were 18, 12, and 2 for

metacentric, submetacentric and telocentric chromosomes in *H. ovina*, respectively (Jarayabhand *et al*., 1998). The chromosome number in extant *Haliotis* spp respected to geographic areas are shown by Table 1.4 (Geiger and Groves, 1999). Generally, diploid (2n) are 28 chromosomes in the Europe-Mediterranean, 32 chromosomes in Indo-Pacific, and 36 chromosomes in the North Pacific abalone.

Lee and Vacquire (1995) compared complementary DNA (cDNA) sequences of the sperm lysin from 27 species of abalone from California, Japan, Australia, New Zealand, Taiwan, Borneo, Madagascar, South Africa, Greece, France, Italy, and the Azores. The sampling localities in that study represended most of the abalone. Total RNA was isolated and mRNA was purified using the poly A/T tract system. One micrograms of mRNA was hybridized with 1mg of Dynabeads oligo $(dT)_{25.}$ The cDNA was synthesized on the beads, denatured mRNA and cDNA hybrids and then purified. The lysin cDNA sequences revealed that 22 of 27 investigated taxa are clearly distinguishable by at least 20 nucleotide differences. For the remaining taxa, *H. coccinea* may be a subspecies of *H. tuberculata* and other four taxa are most probably the same species as one of the 22 taxa. The lysin sequences are almost identical between *H. makada* and *H. discus hannai*, *H. conicopora* and *H. rubra*, *H. diversicolor supertexta* and *H. diversicolor aquatilis*, and *H. tuberculata lamellosa* and *H. tuberculata tuberculata.* The phylogeny of lysin cDNA suggests that there are three groups among the 27 species-group taxa: (1) all California species (*H. rufescens*, *H. sorenseni*, *H. kamtschatkana*, *H. walallensis*, *H. cracherodii*, *H. corrugata*, *H. fulgens*) and 3 Japanese species (*H. gigantea*, *H. discus hannai*, *and H. madaka*); (2) The New Zealand species (*H. iris*); and (3) 1 Japanese species (*H. diversicolor aquatilis*), Indo-West Pacific species (*H. roei*, *H. scalaris*, *H. laevigata*,

H. cyclobates, *H. rubra*, *H. ovina*, *H. conicopora* from Australia; *H. australis* from New Zealand; *H. diversicolor supertexta* from Taiwan; *H. varia* from Borneo), and European species (*H. pustulata* from Madagascar; *H. midae* from South Africa; *H. tuberculata lamellosa* from Greece; *H. tuberculata tuberculata* from France; *H. coccinea* from Azores).

Naganuma *et al*. (1998) compared partial 18S rDNA sequences of closely related abalones, *H. discus discus* Reeve (from Izu Peninsula, Shizuoka Prefecture, central Japan) and *H. discus hannai* Ino (from Kesennuma, Iwate Prefecture, northeastern Japan). The PCR product of 18S rDNA (forward 5'-AAC CTG GTT GAT CCT GCC AGT-3' and reverse 5'-TGA TCC TCC TGC AGG TTC A-3') were directly sequenced. The sequences were multiple-aligned with those from other abalones (*H. madaka* Haba and *H. gigantea* Gmelin, both from Naruto, Tokushima Prefecture, western Japan). A land gastropod (*Limicolaria kambeul*) was also included as an outgroup. The inferred 18S rDNA phylogenies indicated that the *H. discus discus* and *H. discus hannai* are closely related but distinguishable presumably at the subspecies level.

Hamm and Burton (2000) determined allelic frequencies of 400 individuals of the black abalone (*H. cracherodii*) from seven geographic sites of southern and central California (Vandenberg Marine Ecological Reserve, Cambria, San Simeon, Big Creek Marine Ecological Reserve, Carmel Point, Point Pinos, and Scotts Creek) at three polymorphic enzyme-encoding loci (GPI: glucose-6-phosphate isomerase, AAT-1: aspartate aminotransferase, and PGM: phosphoglucomutase). Sample was used for protein electrophoresis and as a source of the template for PCR

amplification. Protein electrophoresis was performed on polyacrylamide gels. DNA from the mitochondrial gene cytochrome oxidase subunit I was amplified by PCR using primers designed for abalone COI, ABCOI F (forward): 5'-TGA TCC GGC TTA GTC GGA CTG C-3' and ABCOI R (reverse): 3'-GAT GTC TTG AAA TTA CGG TCG GT-5'. The resulting 580-bp fragment was sequenced, aligned and analyzed. Significant allelic frequency differences among sites was observed at all three loci. Genetic distance was found to be independent from geographic distance over the approximately 300 km sampling range. In addition, a limited number of DNA sequences (*N*=51) were obtained for the mitochondrial cytochrome oxidase subunit I gene (COI) from five of the populations. The observed level of population differentiation using allozyme of *H. cracherodii* was three-fold higher than that observed in the California red abalone, *H. refescens.*

For identification of species origins of abalone tissue from South Africa (False Bay near Cape Town), PCR-RFLP was carried out and using a portion of the lysin cDNA sequences of several abalone species to distinguish *H. midae* (only commercially exploited abalone in South Africa) from *H. spadicea* (a sympatrically congeneric species). DNA was extracted using the CTAB extraction method. The lysin gene was amplified and directly sequenced. Species-specific PCR primers were designed. The PCR primers successfully specifically amplify approximately 1,300 bp of genomic DNA from dried, cooked, and fresh *H. midae* tissue. A smaller fragment of 146 bp product was used for identification of canned *H. midae*. RFLP analysis revealed interspecific polymorphism that discriminate between these two species (Sweijd *et al*., 1998).

Table 1.4 Chromosome number in *Haliotis* from various geographical areas

Taxon	Haploid No.	Diploid No.	Geographic Occurrence	Reference
H. tuberculata	14	28	EM	Colombera and Tagliaferri, 1983
		28	EM	Arai and Wilkins, 1986
H. lamellosa[1]	14		EM	Colombera and Tagliaferri, 1983
H. aquatilis[3]	17	34	IP	Nakamura, 1985
H. diversicolor aquatilis[2]	16	32	IP	Nakamura, 1985, 1986
H. diversicolor		32	IP	Arai *et al.*, 1988; Yang *et al.*, 1998
H. exiqua[4]		32	IP	Arai *et al.*, 1988
H. planata[5]		32	IP	Arai *et al.*, 1988
H. asinina		32	IP	Jarayabhand *et al.*, 1998
H. ovina		32	IP	Jarayabhand *et al.*, 1998
H. varia		32	IP	Jarayabhand *et al.*, 1998
	16	32	IP	Nakamura, 1986
H. cracherodii		36	NP	Minkler, 1977
H. discus discus		36	NP	Arai *et al.*, 1982
H. discus hannai		36	NP	Arai *et al.*, 1982
H madaka[6]		36	NP	Nakamura, 1986

Geographic occurrence; EM, European-Mediterranean; IP, Indo-Pacific; NP, North Pacific.

[1] *H. lamellosa* has been shown to be a synonym/ecomoroph of *H. tuberculata* (Lee and Vacquire, 1995).

[2] a synonym of *H. japonica.*

[3] a synonym of *H. diversicolor aquatilis* in Nakamura (1985), but as *H. aquatilis* in Nakamura (1986).

[4] a synonym of *H. planata.*

[5] a synonym of *H. varia.*

[6] a synonym of *H. gigantea.*

1.7 Objective

The objectives of this thesis are to examine an intraspecific genetic variation and differentiation of geographic samples of *H. asinina* and to determine molecular genetic markers showing species-specific nature to *H. asinina*, *H. ovina* and *H. varia* in Thailand by PCR-RFLP analysis. The basic information can be applied for the construction of effective breeding programs and conservation of abalone in Thailand.

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CHAPTER II

Materials and Methods

2.1 Materials

2.1.1 Equipment

- Autoclave : HVE-50 (Hirayama, Japan)
- Automatic micropipettes P10, P20, P100, P200 and P1000 (Gilson Medical Electronic S.A., France)
- Camera : K1000 (Pentax, Japan)
- Electrophoresis apparatus (Bio-RAD Laboratories, USA)
- -20°C Freezer (Songserm Intercool, Thailand)
- -80°C Freezer (SANYO, Japan)
- Gene pulser (Bio-RAD Laboratories, USA)
- Incubator : BM-600 (Memmert GambH, Germany)
- Laminar Flow Cabinet : NU-440-300E (Nuaire)
- Magnetic stirrer : M21/1 (Franz Morat KG GambH, Germany)
- Microcentrifuge : Microcen 13D (Herolab, Germany)
- Microwave oven : Power Boost 900 (Hitachi, Japan)
- PCR Thermal Cycler

    : Omnigene-E (Hybaid Limited, England)

    : PCR Sprint (Hybaid Limited, England)
- Power supply (Bio-RAD Laboratories, USA)

    : Power Pac 300

    : Power Pac Junior

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

: Model 200/2.0

- Refrigerated microcentrifuge : 3K18 (Sigma Osterode and Harz, Germany)
- Refrigerated microcentrifuge : Kubota 1300 (Kubota, Japan)
- Shaking waterbath : SBS 30 (Stuart Scientific, UK)
- Spectrophotometer : Spectronic GeneSys 5 (MiltonRoy, USA)
- Standard film : FORMAPAN DX100
- UV transilluminator : M-26 (UVP, USA)

2.1.2 Chemicals

- Absolute ethanol (BDH, England)
- Agarose gel (FMC Bioproducts, USA)
  : MetaPhor Agarose
  : Seakem LE agarose
- Bacto-agar (Oxoid, England)
- Bacto-tryptone (Oxoid, England)
- Bacto-yeast extract (Oxoid, England)
- Boric acid (Merck, Germany)
- Bromophenol blue (Merck, Germany)
- Chloroform (Merck, Germany)
- λ-DNA (Promega Corporation Medison, Wiscconsin, USA)
- 100 base-pair DNA ladder (New England Biolabs, USA)
- 100 mM dATP, dCTP, dGTP and dTTP (New England Biolabs, USA)
- Ethidium bromide (Sigma Chemical Co., USA)

- Ethidium diamine tetraacetic acid, disodium salt dihydrate (Fluka Chemika, Switzerland)
- Ficoll, type 400 (Sigma Chemical Co., USA)
- D-Glucose (Sigma Chemical Co., USA)
- Hydrochloric acid (Merck, Germany)
- 8-Hydroxy Quinoline (Sigma Chemical Co., USA)
- Isoamyl alcohol (Sigma Chemical Co., USA)
- 2-Mercaptoethanol (Sigma Chemical Co., USA)
- 25 mM $MgCl_2$ (Perkin-Elmer Cetus, USA)
- Mineral oil (Sigma Chemical Co., USA)
- 10X PCR Buffer : 100 mM Tris-HCl, pH8.3, 500 mM KCl (Perkin-Elmer Cetus, USA)
- Phenol, redistilled (Aldrich Chemical Co., USA)
- Prep-A-Gene^R DNA Purification Kit (Bio-RAD Laboratories, USA)
- Potassium chloride (Merck, Germany)
- Sodium acetate (Merck, Germany)
- Sodium chloride (APS Chemicals Limited CAN.)
- Sodium dodecyl sulfate : SDS (Sigma Chemical Co., USA)
- Sodium hydroxide pellets (Merck, Germany)
- Spermidine trihydrochloride (Sigma Chemical Co., USA)
- Tris (USB, Amersham Life Science, England)

2.1.3 Enzymes

- Ampli*Taq* DNA Polymerase (Perkin-Elmer Cetus, USA)
- DyNAzyme^{TM} II DNA Polymerase (Finnzymes, Finland)

- Proteinase K (Promega Corporation Medison, Wiscconsin, USA)
- Ribonuclease A (Sigma Chemical Co., USA)
- T4 DNA ligase (Pharmacia, USA)
- Restriction endonucleases

  : *Acs* I (Boehringer Mannheim, Germany)

  : *Alu* I, *Bam*H I, *Bfr* I, *Dde* I, *Dra* I, *Eco*R I, *Hae* III, *Hin*d III, *Hin*f I, *Mbo* I, *Nde* I, *Rsa* I, *Swa* I, *Taq* I (Promega Corporation Medison, Wisconsin, USA)

  : *Bgl* II, *Bst*E II, *Cla* I, *Kpn* I, *Pst* I, *Sal* I, *Sma* I, *Ssp* I, *Vsp* I (New England Biolabs, USA)

2.1.4 Bacterial strain

- *Escherichia coli* : strain XL1 Blue

  (F′:: Tn$10proA^{+}B^{+}lac^{q}$ $\Delta(lacZ)M15/recA1$ *endA1* $gyrA96(Nal^{r})$ $thihsdR17(r_{k}^{-}\ m_{k}^{+})$ *supE44 relA1lac)*

2.1.5 Cloning vector

- pGEM^{R}-T easy vertor (Promega Corporation Medison, Wisconsin, USA)

2.2 Sampling

Two abalone species, *H. asinina* (*N*=59) and *H. ovina* (*N*=71) were collected from the Gulf of Thailand and the Andaman Sea. *H. asinina* from Cambodia (*N*=36), and the Philippines (*N*=20) was also collected and included in this study. In addition, *H. varia* abalone (*N*=23) was collected from the Andaman Sea. Geographic locations,

abbreviations and sample sizes of each abalone are shown by Fig. 2.1 and Table 2.1, respectively.

All abalones were collected and maintained on ice and transported back to Marine Biotechnology Research Unit, Chulalongkorn University except *H. asinina* from Rayong, which the blood was collected and *H. asinina* from the Philippines, where the whole experimental abalones were collected in absolute ethanol. Each abalone was kept in a –30°C freezer until required. Alternatively, the adductor muscle was dissected out individually and kept in a –80°C freezer until used for DNA extraction.

2.3 DNA Extraction

Total DNA was extracted from the adductor muscle of each abalone using a phenol-chloroform-proteinase K method. A piece of adductor muscle was dissected from each specimen, homogenized with a micropestle in a prechilled 1.5 ml. microcentrifuge tube containing 600 µl of TEN buffer (200 mM Tris-HCl, 100 mM EDTA and 250 mM NaCl, pH 8.0). A 10% SDS solution was added to a final concentration of 1.0% (w/v). RNA was removed by an addition of a RNase A solution (10 mg/ml) to a final concentration of 0.1 mg/ml and incubated at 37°C for 1 hour. A proteinase K solution (10 mg/ml) was then added to a final concentration of 0.2 mg/ml and further incubated at 55°C for 3-4 hours. Unless indicated, subsequent steps were carried out at the room temperature. An equal volume of equilibrated phenol was added and gently mixed for 15 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new sterile microcentrifuge tube. The phenol extraction was repeated and further extracted once

with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and twice with chloroform-isoamyl alcohol (24:1). DNA was precipitated by adding one-tenth volume of 3 M sodium acetate, pH 5.2 and two volumes of chilled absolute ethanol, gently mixed and incubated at -20°C for 1 hour. DNA was recovered by centrifugation at 12,000 rpm for 10 minutes and briefly washed twice with 70% ethanol, for 30 minutes each. The DNA pellet was air-dried and resuspended in 50 µl of TE buffer (10 mM Tris-HCl, and 0.1 mM EDTA, pH 8.0) The DNA solution was incubated at 37°C for 1-2 hours for complete solubilization and kept at 4°C until further used.

2.4 Measuring concentrations of extracted DNA using spectrophotometry and electrophoresis

2.4.1 Spectrophotometry

The concentration of extracted DNA can be estimated by measure the optical density at 260 nanometre (OD_{260}). The value at OD_{260} allows calculation of total nucleic acid whereas the value at OD_{280} determines the amount of proteins in the DNA solution. The ratio between OD_{260}/OD_{280} provides an estimate on the purity of extracted DNA. A pure preparation of DNA has an OD_{260}/OD_{280} ratio of 1.8-2.0. The ratio that much lower than 1.8 indicated contamination of residual proteins or phenol (Kirby, 1992). An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml of double stranded DNA, therefore the DNA concentration is estimated in µg/ml by the following equation;

$$[DNA] = OD_{260} \times \text{Dilution factors} \times 50$$

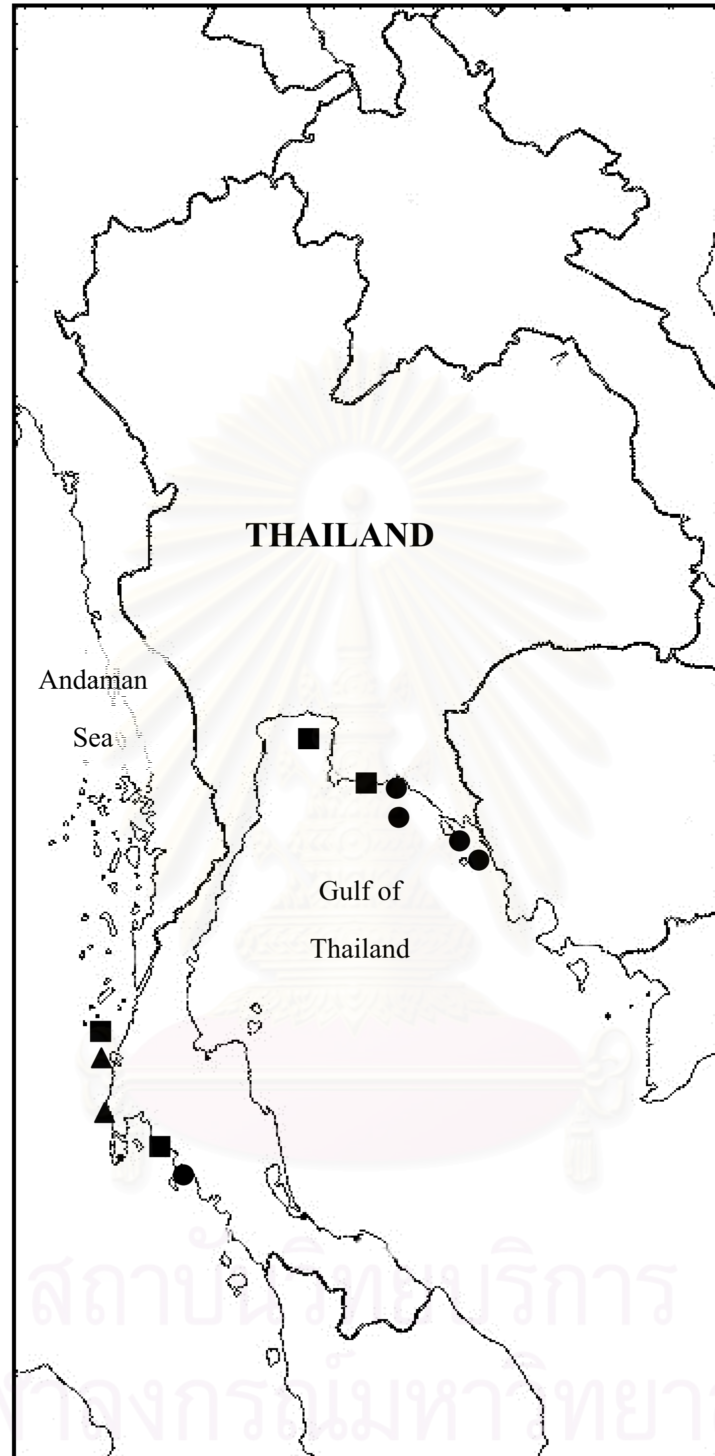

Fig. 2.1 Map of Thailand indicating sample sites of abalone used in this study;

● = *H. asinina*,■ = *H. ovina* and▲ = *H. varia* (detailed information and abbreviations of sample sites are shown in Table 2.1).

Table 2.1 Sample collection sites and sample sizes of abalone specimens used in this study

Sample	Abbreviation	Sample size (*N*)
H. asinina		
Hatchery (P_0), Angsila, Chon Buri originated from Rayong (Gulf of Thailand), HA 001-019	HAHt	19
Samet Island, Rayong (Gulf of Thailand), HA 041-052	HASt	12
Talibong Island, Trang (Andaman Sea), HL 001-028	HALb	28
Hatchery (P_0), Cambodia, HA 081-095	HACbh	15
Cambodia, HA 101-121	HACb	21
The Philippines (F_1), HP 001-020	HAPhi	20
H. ovina		
Sichang Island, Chon Buri (Gulf of Thailand), HO 001-024	HOSi	24
Samet Island, Rayong (Gulf of Thailand), HO 041-058	HOSt	18
Churk Island, Trang (Andaman Sea), HT 001-018	HOTg	18
Similan Island, Phangnga (Andaman Sea), HO 081-091	HOSl	11
H. varia		
L-Island, Phuket (Andaman Sea), HV 001-021	HVPhu	21
Similan Island, Phangnga (Andaman Sea), HV 031-032	HVSl	2
Total (*N*)		209

2.4.2 Mini-gel electrophoresis

DNA concentration can also be estimated on the basis of its direct relationship between the amount of DNA and the level of fluorescence after ethidium bromide staining. DNA was electrophoresed through 0.8% agarose gel prepared in 1XTBE buffer (89 mM Tris-HCl, 8.91 mM boric acid, and 2.5 mM EDTA, pH 8.0) at 100 V. After electrophoresis, the gel was stained with ethidium bromide. DNA concentration was estimated by comparing the fluorescent intensity of interested band with that of λ/*Hin*d III and undigested λ-DNA.

2.5 Screening of primers for population genetic studies of Thai abalone

2.5.1 RAPD primers

Thirteen primers, composing of 7 minisatellite primers; HRU18, HRU33, INS, M13, PERI, YN73 and YNZ22, and 6 microsatellite primers; $(CA)_8$, $(CAC)_5$, $(CT)_8$, $(GTG)_5$, $(GACA)_4$ and $(GATA)_4$, were screened for the amplification success in 2-3 representative individuals of each species. Three primers (INS, M13, and YN73) were selected for analysis using larger sample sizes (Table 2.2).

2.5.2 RFLP primers

Five primers (COI, COI-COII, 12S rDNA, 16S rDNA, and 18S rDNA) were screened. For amplification of 16S rDNA, 3 reverse primers of 16S rDNA were tested with a universal forward primer (Table 2.3).

Table 2.2 Primers and primer sequences screened for genetic studies of Thai abalones

Primer	Sequence	$MgCl_2$ (mM)	Reference
Minisatellite primers			
HRU18	5’ ACC CGG CGC TTA TTA GAG 3’	2	Huang *et al.*, 1997
HRU33	5’ CCC AAG GTC CCC AAG GTC AGG GAG GCG AAG GCT 3’	2	Huang *et al.*, 1997
INS	5’ ACA GGG GTG TGG GG 3’	4	Heath *et al.*, 1993
M13	5’ GAG GGT GGN GGN TCT 3’	4	Heath *et al.*, 1993
PERI	5’ GAC NGG NAC NGG 3’	3	Heath *et al.*, 1993
YN73	5’ CCC GTG GGG CCG CCG 3’	3	Heath *et al.*, 1993
YNZ22	5’ CTC TGG GTG TCG TGC 3’	3	Heath *et al.*, 1993
Microsatellite primers			
$(CA)_8$	5’ CAC ACA CAC ACA CAC A 3’	3	Weising *et al.*, 1994
$(CAC)_5$	5’ CAC CAC CAC CAC CAC 3’	3	Weising *et al.*, 1994
$(CT)_8$	5’ CTC TCT CTC TCT CTC T 3’	3	Weising *et al.*, 1994
$(GTG)_5$	5’ GTG GTG GTG GTG GTG 3’	2	Weising *et al.*, 1994
$(GACA)_4$	5’ GAC AGA CAG ACA GAC A 3’	4	Weising *et al.*, 1994
$(GATA)_4$	5’ GAT AGA TAG ATA GAT A 3’	3	Weising *et al.*, 1994

Table 2.3 Primers and primer sequences screened for population genetic studies of Thai abalone

Primer	Sequence	Reference
COI	F : 5’ GGT CAA CAA ATC ATA AAG ATA TTG G 3’ R : 5’ TAA ACT TCA GGG TGA CCA AAA AAT CA 3’	Folmer *et al.*, 1994
COI-COII	F : 5’ TTG ATT TTT TGG TCA TCC AGA AGT 3’ R : 5’ CCA CAA ATT TCT GAA CAT TGA CC 3’	Sihanunthavong *et al.*, 1999
12S rDNA	F : 5’ AAA CTA GGA TTA TAT ACC CTA TTA 3’ R : 5’ AAG AGG GAC GGG CGA TTT GT 3’	Crozier and Crozier, 1993
$16S_{F1R1}$ rDNA	F_1 : 5’ CGC CTG TTT AAC AAA AAC AT 3’ R_1 : 5’ CCG GTC TGA ACT CAG ATC ATG T 3’	Palumbi *et al.*, 1991
$16S_{F1R2}$ rDNA	F_1 : 5’ CGC CTG TTT AAC AAA AAC AT 3’ R_2 : 5’ GGT CTG AAC TCA GAT CAG ATC ACG T 3’	Small and Chapman, 1997
$16S_{F1R3}$ rDNA	F_1 : 5’ CGC CTG TTT AAC AAA AAC AT 3’ R_3 : 5’ CCG GTC TGA ACT CAG ATC AGA TCA CGT 3’	Small and Chapman, 1997
18S rDNA	F : 5’ TGG ATC CGG GCA AGT CTG GTG CC 3’ R : 5’ TGA AGT CAA GGG CAT CAC AGA CC 3’	Aoki, T (personal communication)

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2.6 PCR analysis

2.6.1 RAPD-PCR

Each PCR component and the amplification conditions of RAPD-PCR was optimized until clear intensity of the PCR products and reproducible results were obtained. RAPD-PCR was performed in a 25 µl reaction volume containing 1xPCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), an appropriate amount of $MgCl_2$ (Table 2.2), 100 µM of each of dNTPs (dATP, dCTP, dGTP and dTTP), 0.2 µM (INS and YN73) or 0.4 µM (M13) of a primer, 1 unit of Ampli*Taq* DNA Polymerase (Perkin-Elmer Cetus) and 25 ng of DNA template. The reaction mixture was overlaid by the mineral oil to prevent evaporation during amplification. PCR was performed in a thermal cycler (Omnigene-E, Hybaid Limited). The amplification conditions using primer INS, M13 and YN73 are shown by Table 2.4. After amplification, PCR products were electrophoretically analyzed as soon as possible.

2.6.2 PCR-RFLP of 18S and 16S rDNA

Two different regions; 16S rDNA (mitochondrial gene) and 18S rDNA (nuclear gene) were analyzed by PCR-RFLP. The 16S rDNA was amplified using primers $16S_{F1}$ and $16S_{R1}$, specimens which are not successfully amplified with those primers were then amplified with $16S_{F1}$ and $16S_{R2}$ primers (Table 2.3). After optimized, the amplification reaction was performed in a 50 µl containing 1xPCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), appropriate $MgCl_2$; 1.5 mM (18S rDNA) or 2 mM (16S rDNA) $MgCl_2$, 100 µM (18S rDNA) or 200 µM (16S rDNA) of each dNTP (dATP, dCTP, dGTP and dTTP), 0.5 µM of each primer, 1 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes) and 50 ng of DNA template. PCR

was performed in a thermal cycler (PCR Sprint, Hybaid Limited). The amplification conditions of primer 16S rDNA and 18S rDNA are shown in Table 2.5.

Fives microlitres of the amplified product were electrophoresed through 1% agarose gel to determine whether the reaction was successfully amplified. Samples showing positive results were subjected to restriction enzyme analysis.

2.7 Agarose gel electrophoresis

The amplification products were separated by agarose gel electrophoresis on the basis of molecular sizes (the optimal concentration of agarose and range of fragment size to be separated are shown by Table 2.6). RAPD products were analyzed by electrophoresed through 1.6% agarose gels while 16S and 18S PCR products were analyzed through 1%. The digested products of those gene segments were analyzed through 2% agarose gels except *Alu* I-digested 16S rDNA which was analyzed by 3% Metaphor agarose gels. The appropriate amount of agarose was weighed out and mixed with 1xTBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). The solution was boiled in a microwave to complete solubilization and left at room temperature to approximately 60°C before poured into a gel mould. The gel was left at room temperature for 30-45 minutes to completely solidified. In case of metaphor agarose, the gel was placed at 4°C for at least 30 minutes to achieve sieving ability. When needed, the gel was placed in the electophoretic chamber containing an enough amount of 1xTBE buffer covering the gel for approximately 0.5 cm and the comb was gently removed.

Table 2.4 The optimal amplification conditions of RAPD-PCR analysis of abalone

	INS	M13	YN73
Denaturation Annealing Extension	40 cycles: 92°C, 30 sec. 50°C, 60 sec. 72°C, 90 sec.	40 cycles: 92°C, 30 sec. 60°C, 60 sec. 72°C, 90 sec.	40 cycles: 92°C, 30 sec. 50°C, 60 sec. 72°C, 90 sec.
Final extension	1 cycle: 72°C, 10 min.	1 cycle: 72°C, 10 min.	1 cycle: 72°C, 10 min.

Table 2.5 The optimal amplification conditions of 16S rDNA and 18S rDNA of abalone

	16S rDNA	18S rDNA
Predenaturation	1 cycle: 94°C, 3 min.	1 cycle: 94°C, 3 min.
Denaturation Annealing Extension	5 cycles: 94°C, 1 min. 48°C, 1 min. 72°C, 1 min.	10 cycles: 94°C, 1 min. 48°C, 1 min. 72°C, 1 min.
Denaturation Annealing Extension	40 cycles: 94°C, 1 min. 58°C, 1 min. 72°C, 1 min.	35 cycles: 94°C, 1 min. 53°C, 1 min. 72°C, 1 min.
Final extension	1 cycle: 72°C, 7 min.	1 cycle: 72°C, 7 min.

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Table 2.6 The optimal concentration of Seakem LE and Metaphor agarose prepared in 1xTBE buffer for separating double stranded DNA

Gel percentage	Range of fragment size to be separated (bp)	
	Seakem LE agarose	Metaphor agarose
0.50	1,000-23,000	-
0.70	800-10,000	-
0.85	400-8,000	-
1.00	300-7,000	-
1.25	200-4,000	-
1.75	100-3,000	-
2.00	-	100-600
3.00	-	50-250
4.00	-	20-130
5.00	-	<80

Reference : FMC BioProducts, Denmark.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

The products were mixed with one-fifth volume of a loading dye solution (0.25% bromophenol blue and 25% ficoll) and then loaded into the well. The 100 bp DNA ladder and λ/*Hin*d III were used as standard DNA markers. Electrophoresis was operated at 4 volts/cm until bromophenol blue moved to approximately 1 cm from the bottom of the gel. The agarose gel was stained with 0.5 mg/ml ethidium bromide solution for 15 minutes and destained in distilled water twice for 15 minutes each to removed unbound ethidium bromide from the gel. The DNA was visualized under a UV transilluminator and photographed through a red filter using a typical camera (K100, Pentex).

2.8 Restriction endonuclease digestion

The 16S rDNA and 18S rDNA amplification products were screened against 24 restriction endonucleases (restriction endonucleases and their recognized site shown by Table 2.7). The digestion reaction was performed in 15 µl containing 1.5 µl of 10x restriction enzyme buffer, 1.5 µl of 40 mM spermidine trihydrochloride, 1.5 µg BSA, 2.5-3 units of each restriction endonuclease, 10 µl of amplification products, and appropriate amount of sterile deionized water. The reaction mixture was incubated for 10-12 hours at 37^oC except *Taq* I where the reaction was incubated at 65^oC. The reaction was electrophoretically through 2% agarose gel or 3% Metaphor (for 16S rDNA digested with *Alu* I).

Table 2.7 Restriction endonucleases and reconizing site used for screening of informative enzymes

Restriction endonuclease	Recognizing site	Restriction endonuclease	Recognizing site
Acs I	G(A)AATTT(C)	*Hin*f I	G/ANTC
Alu I	AG/CT	*Kpn* I	GGTAC/C
*Bam*H I	G/GATCC	*Mbo* I	/GATC
Bfr I	CTTAAG	*Nde* I	CA/TATG
Bgl II	A/GATCT	*Pst* I	CTGCA/G
*Bst*E II	G/GTNACC	*Rsa* I	GT/AC
Cla I	AT/CGAT	*Sal* I	G/TCGAC
Dde I	C/TNAG	*Sma* I	CCC/GGG
Dra I	TTT/AAA	*Ssp* I	AAT/ATT
*Eco*R I	G/AATTC	*Swa* I	ATTT/AAAT
Hae III	GG/CC	*Taq* I	T/CGA
*Hin*d III	A/AGCTT	*Vsp* I	AT/TAAT

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จุฬาลงกรณ์มหาวิทยาลัย

2.9 Data analysis

2.9.1 Restriction pattern analysis

The restriction pattern generated from each restriction endonuclease was given letter designations according to their frequencies. Haplotype A refers to the most common digestion pattern in investigated specimens. The remaining alphabetical profile names (B, C, etc.) indicate digestion patterns reflecting their frequencies in order. The fragment sizes of restriction profiles were compared and estimated with a 100 bp DNA ladder. The composite haplotypes were constructed from combination of all restriction patterns of 18S rDNA and 16S rDNA respectively. The binary matrix was recorded the presence (1) and absence (0) of restriction patterns for statistical analysis using Restriction Enzyme Analysis Package (REAP), version 4.0 (McElroy, 1991).

2.9.2 Genetic distance

The relationships of composite haplotype were observed by genetic distance values (d) that could be calculated by the equation :

$$d = - (2 / r) \ln G$$

where r is the number of recognized sequences at the restriction site and G is $[F (3 - 2G_1)]^{1/4}$ and repeat calculated until $G = G_1$. The $G_1 = F^{1/4}$ is recommended to initial trial value.

F is the similarity between haplotype patterns and calculated by the equation :

$$F = 2m_{xy} / (m_x + m_y)$$

where m_x and m_y are the numbers of restriction fragments in the xth and y^{th} haplotypes, respectively, and m_{xy} is the number of shared fragments between two haplotypes (Nei and Li, 1979).

2.9.3 Haplotype and nucleotide diversity with geographic samples

Genetic diversity within geographic samples was estimated from haplotype and nucleotide diversity, the haplotype diversity was calculated by the equation :

$$h = n\,(1 - \Sigma x_i^2) / (n - 1)$$

where n is the number of individuals investigated and x_i is the frequency of the ith haplotype (Nei and Tajima, 1981).

The nucleotide diversity within sample is the average number of nucleotide substitution within a sample was calculated by the equation :

$$d_x = [n_x / (n_x - 1)]\ \Sigma_{ij}\, x_i\, x_j\, d_{ij}$$

where n_x is the number of sequences sampled and d_{ij} is the number of nucleotide substitutions per site between the ith and j^{th} haplotype. The x_i and x_j values are the sample frequencies of the ith and j^{th} haplotypes in geographic sample X (Nei, 1987).

2.9.4 Nucleotide divergence

Nucleotide diversity between two samples is the average number of nucleotide substitutions between DNA haplotypes from samples X and Y was calculated by the equation :

$$d_{xy} = \Sigma_{ij} x_i y_j d_{ij}$$

where d_{ij} is the nucleotide substitutions between the ith and j^{th} haplotype from geographic sample X and Y, respectively. Nucloetide divergence between two samples is the average number of nucleotide substitution per site where the effect of within geographic sample polymorphism has been substracted was calculated by the equation :

$$d_A = d_{xy} - (d_x + d_y) / 2$$

2.9.5 Phylogenetic reconstruction

Phylogenetic relationship between investigated samples of Thai abalones were constructed based on a UPGMA method (Saitou and Nei, 1987) using Neighbor implemented in PHYLIP, version 3.56c (Felsenstein, 1993).

2.10 Cloning of 16S rDNA fragments

2.10.1 Preparation of RFLP products for cloning

2.10.1.1 Amplification of 16S rDNA fragments

The 16S rDNA segments were amplified from an individual representing each composite haplotype, using conditions described in section 2.6.2. Three 16S rDNA

exhibiting composite haplotypes AAAA, AAAE, and ABBB were amplified and cloned by the auther of this thesis whereas cloning the remaining 7 haplotypes were performed by Ms. Neerawan Khamnamtong.

2.10.1.2 Recovery of 16S rDNA fragment from the agarose gels

After the amplified 16S rDNA gene segment was fractionated through 1.5% agarose gels, an approximately 580 bp DNA fragment was excised from the gel using a scalpel and placed in a preweighed 1.5 ml microcentrifuge tube individually.

The gel volume was calculated. DNA was eluted from agarose gels using Prep-A-Gene DNA purification kit (Bio-Rad Laboratories). Three volumes of Prep-A-Gene binding buffer (sodium perchlorate) was added. The mixture was incubated at 50°C for 5-10 minutes until gel slice was completely dissolved. The Prep-A-Gene matrix (7.5 μl) was added, mixed by vortexing and left at room temperature for 10 minutes to allow binding of DNA and matrix. The mixture was mixed by inversion of the tube every 2 minutes during this period. At the end of incubation time, the mixture was centrifuged at 7,000xg for 30 seconds at room temperature. The supernatant was removed. The Prep-A-Gene buffer was added (25 volume of the matrix). The mixture was vortexed and centrifuged as described previously. The supernatant was removed. The pellet was washed twice with Prep-A-Gene wash buffer (25 volume of the matrix). Trace amount of the supernatant was carefully removed from the final wash. Prep-A-Gene elution buffer (15 μl) was added, gently mixed and incubated in a 37°C waterbath for 5 minutes followed by centrifugation. The eluted DNA was removed to a new microcentrifuge tube. The elution step was repeated using additional 10 μl of the elution buffer. The eluted DNA solution was adjusted to 100 μl using a TE

solution. The proteinase K solution was added to eluted DNA to 50 μg/ml final concentration in presence of 0.5% SDS. The mixture was incubated at 65°C for 1 hour. After cooling at room temperature, the mixture was extracted once with phenol-chloroform-isoamylalcohol (25:24:1 v/v) and once with chloroform-isoamylalcohol (24:1 v/v). DNA was recovered by ethanol precipitation. Appropriate amount of TE buffer was added. DNA was kept at 4°C until further used.

2.10.2 Ligation of 16S rDNA to T-vector

Taq I polymerase have a terminal transferase activity which results in the non-template addition of a single nucleotide to the 3' end of PCR products for which deoxyadenosine is almost preferentially added. This allow cloning of PCR-amplified fragments to the modified vector containing a single 3'- overhang thymine residue (T-A cloning method).

The gel-eluted 16S rDNA (25 ng) was ligated to pGEM^{R}-T easy vector (Promega Corporation Medison) in a ligation reaction (10 μl) containing 5 μl of 2x rapid ligation buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol; MW 8,000), 3 weiss units of T4 DNA ligase and 25 ng of pGEM^{R}-T easy vector. The reaction mixture was incubated at 4°C overnight before electrotransformed into *E. coli* XL1-BLUE.

2.10.3 Transformation of ligated products to *E. coli* host cells by electroporation (Dower *et al*., 1988)

2.10.3.1 Preparation of host cells

A single colony of *E. coli* XL1-BLUE was inoculated in 15 ml of LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) supplemented with tetracycline and vigorous shaking overnight at 37°C. The starting culture was inoculated to 1 liter of LB medium and continued culture at 37°C with vigorous shaking to the OD_{600} of 0.5 to 0.8. The cells were chilled briefly on ice for 15 to 30 minutes and transferred to a centrifuge bottle and centrifuged in a prechilled rotor at 4,000xg at 4°C for 15 minutes. The pellets were resuspended and centrifuged as above using 1 liter and 0.5 liter of prechilled sterile water, respectively. The pellets were resuspended in 20 ml of 10% glycerol and recentrifuged. Finally, the pellets were resuspended in 2-3 ml of 10% glycerol and devided to 45 μl aliquots. These cells could be used immediately or stored at -70°C until used.

2.10.3.2 Electrotransformation

The competent cells were thawed on ice for 5 minutes. Approximately 1-2 μl of ligation product were added, mixed by pipetting and left on ice for 1 minutes. The mixture was electroporated in a prechilled 0.2 cm cuvette using a Gene pulser (Bio-Rad Laboratories) with the setting parameters of 25 μF, 200 Ω and 2.5 KV. After electroporation, the mixture was transferred to a new tube containing 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). The cell suspension was shaking incubated at 37°C for 1-2 hours. The cell suspension (10-30 μl) was spread on the LB

agar plate containing 50 μg/ml of ampicillin, 25 μg/ml of IPTG and 20 μg/ml of X-Gal and incubated overnight at 37°C (Sambrook *et al*., 1989).

2.10.4 Isolation of recombinant plasmid DNA

Plasmid DNA was isolated using a modification of alkaline lysis minipreparation method (Li *et al*., 1997). A single white colony was inoculated into a sterile tube containing 3 ml of LB medium supplemented with 50 μg/ml of ampicillin and incubated with vigorous shaking overnight at 37°C. The culture was transferred into a new 1.5 ml microcentrifuge tube and centrifuged at 10,000xg for 30 seconds. The supernatant was carefully removed. 100 μl of solution I (50 mM glucose, 10 mM EDTA, pH 8.0, 25 mM Tris-HCl, pH 8.0) were added to the cell pellet and vigorously vortexed following by an addition of 200 μl of freshly prepared solution II (0.2 N NaOH and 1% SDS). The mixture was gently mixed by inverse the tube for 10-15 times before 150 μl of solution III (3 M sodium acetate, pH 4.8) was added, mixed by inversion and flicking the tube. The mixture was centrifuged at 10,000xg for 30 seconds to pellet the cell debris. The supernatant was transferred to a new microcentrifuge tube and extracted with a phenol-chloroform solution. An equal volume of cold absolute ethanol was added, mixed by inversion and centrifuged at 10,000xg for 10 minutes. The pellet was washed twice with 70% cold ethanol. The pellet was dried *in vacuo* for 5 minutes and dissolved in 50 μl of TE buffer. The solution was then incubated for 10 minutes at 65°C for inactive residual DNase activity. The solution was centrifuged at 10,000 for 5 minutes. The supernatant was transferred to a new tube and RNase A was added to final concentration of 200 μg/ml to eleminate contaminating RNA. The solution was incubated at 37°C for 30 minutes before kept at -20°C.

2.10.5 Detection of an inserted DNA

The presence of a 580 bp insert was examined by digestion the recombinant plasmids with *Eco*R I in 20 µl reaction mixture at 37°C overnight. The digestion products were electrophoretically through 1% agarose gel. The size of DNA insert was compared with that of a 100 bp DNA ladder.

2.10.6 DNA sequencing

The recombinant clones of all composite haplotype were unidirectional sequenced at the Bio Service Unit (BSU), National Science and Technology Development Agency (NSTDA) and further confirmed by sequencing for both direction using an Licor 4100 automated DNA sequence (Licor).

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CHAPTER III

RESULTS

3.1 DNA extraction

Total genomic DNA extracted from the adductor muscle of each abalone showed acceptable quality for further used on population genetic analysis of abalone. High molecular weight DNA at approximately 23.1 kb along with slightly sheared DNA was observed (Fig 3.1). The ratio between the optical density at 260 and 280 nm was 1.5-2.4, indicated that extracted DNA was contaminated with protein or phenol (samples having OD260/280 <1.8) or with RNA (samples having OD260/280 >2.0). DNA samples showed possible contamination with residual proteins or RNA was re-extracted once with phenol/chloroform followed by ethanol precipitation before used.

3.2 Analysis of genetic diversity of *H. asinina*, *H. ovina* and *H. varia* using RAPD analysis

Simple repeated primers used in this study did not yield positive amplification success in abalone. Three minisatellite primers (INS, YN73, and M13) provided amplification bands fixed in representative individuals of *H. asinina* and were chosen for analysis of larger sample sizes in *H. asinina* (N = 24), *H. ovina* (N = 24) and *H. varia* (N = 24)

Using the primer INS, complex band patterns (170-1600 bp) were observed in all abalone species but RAPD patterns of *H. asinina* revealed genetically closed relationships between individuals of this species. Three RAPD bands (1450 bp, 1000 bp,

and 780 bp) were specifically found in *H. asinina*. No species-specific bands were observed in *H. ovina* and *H. varia* (Fig. 3.2).

The primer YN73 was probably the best primer for RAPD analysis of abalone in this study. The patterns of this primer were not complex in abalone (400-2200 bp in size). Although high similarity was found in all species, the *H. asinina* patterns was closely related than *H. ovina* and *H. varia*. Based on limited sample sizes of each species, species-specific markers were found in all species (1190 bp, 980 bp, 710 bp, and 500 bp in *H. asinina*, 2100 bp and 420 bp in *H. ovina* and 820 bp in *H. varia*; Fig. 3.3).

Complex band patterns were observed when *H. asinina*, *H. ovina* and *H. varia* were genetically analyzed using the primer M13. The generated RAPD fragment ranged from 180-2200 bp. Species-specific fragments were not possible to deduce from this primer owing to inconsistent band patterns against large sample sizes (Fig 3.4).

Due to difficulties arisen from reproducible of RAPD patterns of these primers when analyzed with larger sample sizes, RAPD analysis was not carried out further.

3.3 Analysis of genetic diversity of *H. asinina*, *H. ovina* and *H. varia* using PCR-RFLP analysis

Two pairs of primers for amplification of nuclear (18S rDNA) and mitochondrial (16S rDNA) DNAs were successfully amplified in abalone (*H. asinina*, *H. ovina* and *H. varia*) used in this study (Figs. 3.5A and 3.5B). Almost all of the investigated specimens were successfully amplified by primers $18S_{F1}$+$18S_{R1}$ and $16S_{F1}$+$16S_{R1}$. A few specimens which were not successfully amplified at the 16S rDNA region were then amplified with $16S_{F1}$ and $16S_{R2}$ primers. The product of amplified 16S rDNA was approximately 580

bp. A 18S rDNA product (900 bp) were usually amplified accompanying with non-specific fragments particularly in *H. varia*. Therefore, the gel eluted product of each individual rather than direct amplification product was used for restriction analysis.

The amplified 18S and 16S rDNAs of *H. asinina*, *H. ovina* and *H. varia* were screened with 24 restriction endonucleases. Three informative enzymes (*Alu* I, *Taq* I, and *Hae* III) were found in 18S rDNA whereas four polymorphic enzymes (*Bam*H I, *Eco*R I, *Hae* III, and *Alu* I) were found in 16S rDNA.

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Fig. 3.1 Genomic DNA extracted from adductor muscle of abalone. The extracted DNA was electrophoresed through 1.0 % agarose gel and stained with ethidium bromide.

Lane M = A λ/*Hin*d III DNA marker

Lanes 1-6 = undigested λ DNA (25, 50, 75, 100, 200, and 500 ng, respectively)

Lanes 7-19 = DNA extracted from abalone individuals

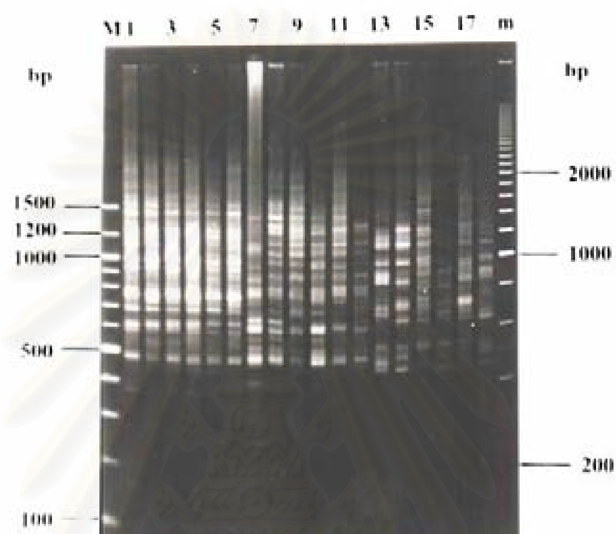

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Fig 3.2 RAPD patterns resulted from amplification of DNA of three abalone species with the primer INS.

Lane M = A 100 bp DNA ladder

Lanes 1-6 = *H. asinina* (HAHt)

Lanes 7-12 = *H. ovina* (HOSi)

Lanes 13-18 = *H. varia* (HVPhu)

Lane m = A 200 bp DNA ladder

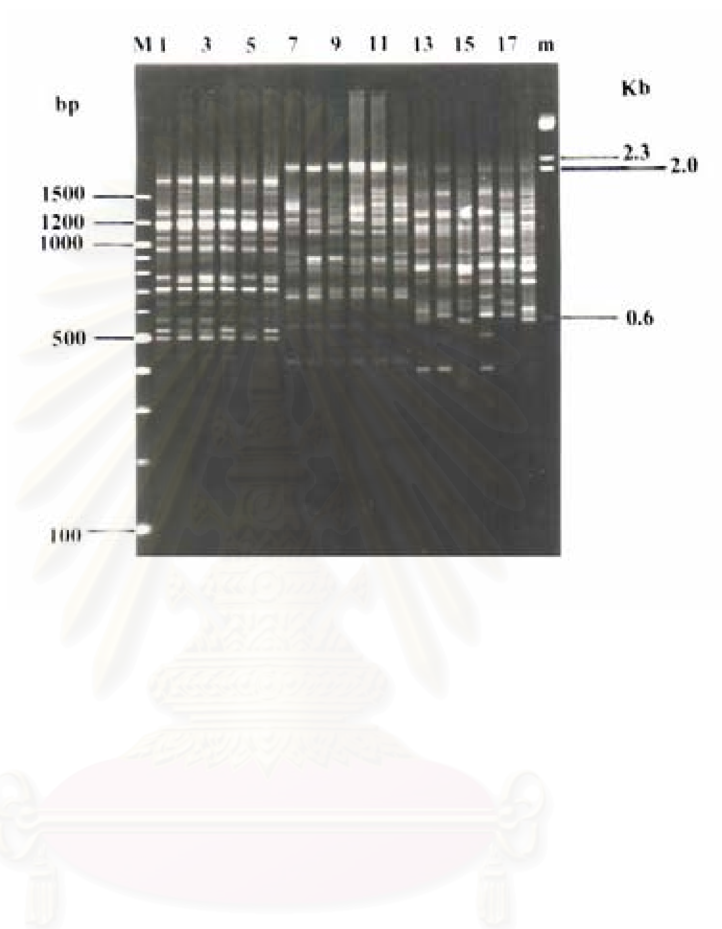


Fig 3.3 RAPD patterns resulted from amplification DNA of three abalone species with the primer YN73.

Lane M = A 100 bp DNA ladder

Lanes 1-6 = *H. asinina* (HAHt)

Lanes 7-12 = *H. ovina* (HOSi)

Lanes 13-18 = *H. varia* (HVPhu)

Lane m = A λ/*Hin*d III DNA marker

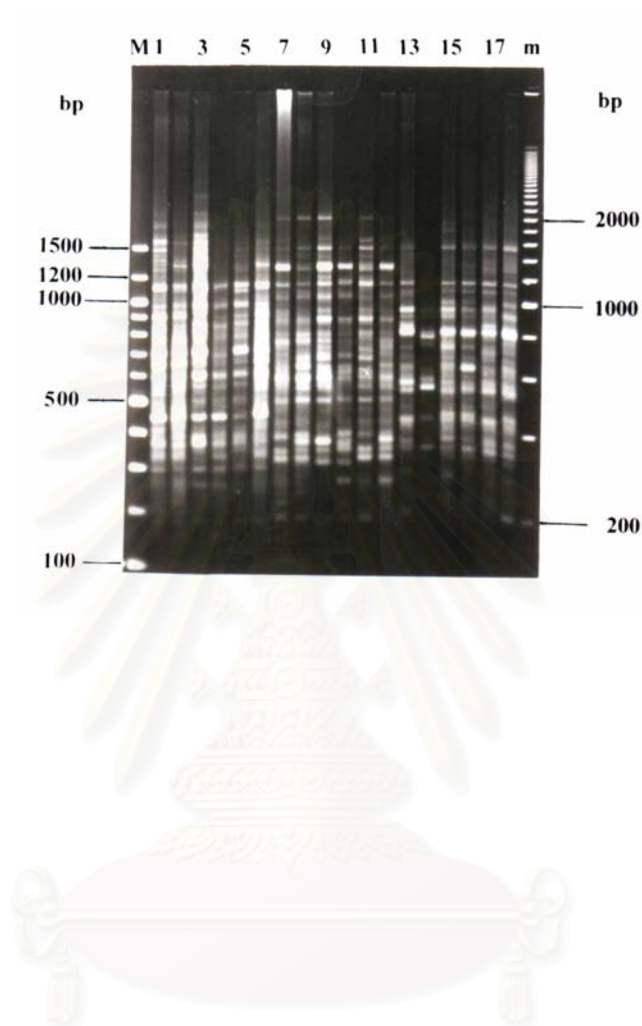


Fig 3.4 RAPD patterns resulted from amplification of DNA of three abalone species with the primer M13.

Lane M = A 100 bp DNA ladder

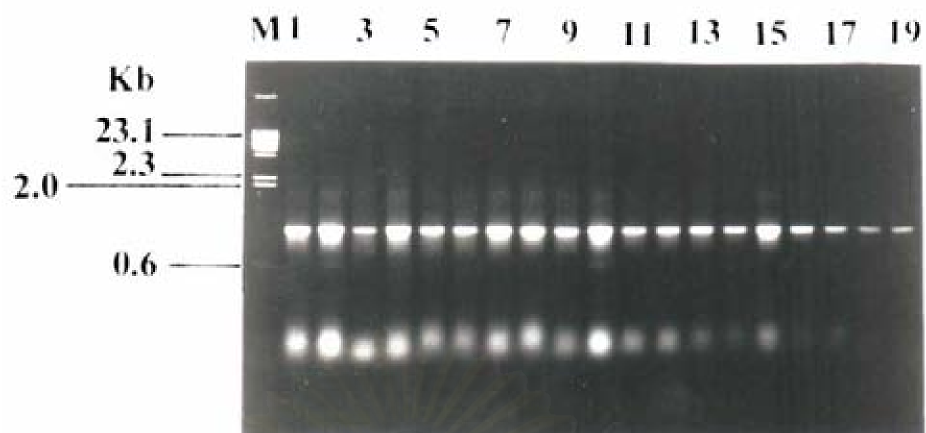
Lanes 1-6 = *H. asinina* (hatchery, P_0)

Lanes 7-12 = *H. ovina* (Sichang Island)

Lanes 13-18 = *H. varia* (Phuket Island)

Lane m = A 200 bp DNA ladder

(A)



(B)

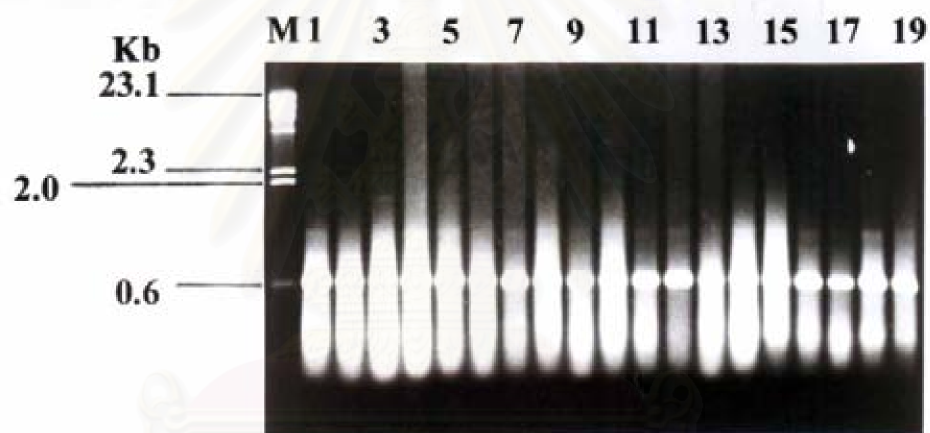


Fig 3.5 The amplification product of 18S rDNA (A), and 16S rDNA (B) of abalone.

Lane M = A λ/*Hin*d III DNA marker

Lanes 1-7 = *H. asinina*

Lanes 8-13 = *H. ovina*

Lanes 14-19 = *H. varia*

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Twenty-five of restriction patterns was found from analysis of 209 individuals of abalone with those restriction endonucleases (Fig. 3.6-3.13 and Table 3.1-3.2). Digestion of 18S rDNA with *Alu* I, *Taq* I, and *Hae* III generated a total of 12 patterns and 13 patterns were resulted from digestion of 16S rDNA with *Bam*H I, *Eco*R I, *Hae* III, and *Alu* I.

Distributions of restriction patterns of 18S and 16S rDNA with each restriction endonuclease across geographic sample of each abalone species were shown by Tables 3.3 and 3.4, respectively.

Complex patterns of digested bands were observed in 18S rDNA (Table 3.3). Digestion of this region with *Alu* I yielded 6 restriction patterns (A, B, C, D, E, and F). The most common restriction profile in *H. asinina* was the B pattern (66.96%), while that of *H. ovina* and *H. varia* was the pattern A (83.09% and 43.48%, respectively). When 18S rDNA was analyzed with *Taq*, I, 4 patterns (A, B, C, and D) were obtained and the common pattern in *H. asinina* was the pattern A (75.65%), Patterns B and C was common in *H ovina* originating from the Andaman sea (23.94%) and the Gulf of Thailand (36.62%), respectively. The B pattern was also the most common pattern in *H. varia* (82.61%). Two patterns were found from *Hae* III digestion, the pattern A was predominate in *H. asinina* and *H. varia* (94.78% and 100%, respectively) but the B pattern was predominate in *H. ovina* (73.24%).

Restriction digestion of 16S rDNA with *Bam*H I generated patterns A (580 bp) and B (380, 200 bp) (Fig 3.9). Two restriction profiles; A (580 bp) and B (300, 280 bp) were obtained from digested of 16S rDNA with *Eco*R I (Fig 3.10) and *Hae* III; A (380,

120, and 80 bp) and B (500, 80 bp) (Fig. 3.11), respectively. Restriction analysis of 16S rDNA with *Alu* I, provided 7 haplotypes (A, B, C, D, E, F, and G, Fig. 3.12-3.13).

Frequency of *BamH* I-digested 16S rDNA patterns was fixed in *H. asinina* and *H. ovina* (Table 3.4) Both A and B were found in *H. varia* but the pattern B was predominate (95.65%). Restriction analysis of 16S rDNA with *EcoR* I indicated that all specimens of abalone except *H. ovina* from the Gulf of Thailand possessed the pattern A. Therefore, the origin of *H. ovina* from the east and west coast of peninsular Thailand could be simply determined by *Eco*R I digestion. The pattern A of *Hae* III-digested 16S rDNA was predominate in *H. asinina* (100%) but showed slightly less frequent in *H. ovina* (39.44%) where the pattern B was predominate (60.56%) in *H. ovina* and 100% in *H. varia*. Digestion of 16S rDNA with *Alu* I, *H. asinina* provided two haplotypes; haplotype A (95.65%) and haplotype E (4.35%) while all *H. ovina* exhibited the B pattern (100%). Four restriction patterns were observed in *H. varia* (C, D, F, and G). Using this single enzyme digestion, obtained profiles were not overlapped and could be used for simple differentiation between different species of abalone in this study (Table 3.4).

Considering only 16S rDNA, ten composite haplotypes were generated. (Table 3.5), *H. asinina* exhibited two composite haplotypes (I, AAAA, and II, AAAE) but the haplotype I was predominate (95.65%). Only haplotypes III, ABBB was observed in *H. ovina* originating from Gulf of Thailand (59.15%), while *H. ovina* from Andaman Sea most possessed haplotypes IV, AAAB (39.44%) and V, AABB (1.41%). Five composite haplotypes (VI BABD, VII BABC, VIII BABG, IX BABF, and X AABC) were found in *H. varia* but the VII BABC was existent in most individual (60.87%).

Forty-nine composite haplotypes were generated from combining each restriction digestion pattern (18S rDNA digested with *Alu* I, *Taq* I, and *Hae* III folowed by 16S rDNA digested with *Bam*H I, *Eco*R I, *Hae* III, and *Alu* I). Fifteen composite haplotypes were generated in *H. asinina* (I BBAAAAA, II ACAAAAA, III BCAAAAA, IV BBAAAAE, V BAAAAAA, VI BAAAAAE, VII CAAAAAA, VIII AAAAAAA, IX ABAAAAA, X CABAAAA, XI EAAAAAA, XII BABAAAA, XIII AABAAAA, XIV EBAAAAA, and XV DBAAAAA) while nine composite haplotypes (XVI BBAABBB, XVII ACAABBB, XVIII ACBABBB, XIX ADBABBB, XX DBBABBB, XXI ABBABBB, XXI DCBABBB, XXIII ADAABBB, and XXIV ABAABBB) were found in *H. ovina* from Gulf of Thailand, thirteen additional composite haplotypes (XXV ABBAAAB, XXVIF BAAAAB, XXVII ACBAAAB, XXVIII FBBAAAB, XXIX DDBAAAB, XXX DCBAAAB, XXXI CBAAAAB, XXXII BBAAABB, XXXIII ABAAAAB, XXXIV ADBAAAB, XXXV DBAAAAB, XXXVI ACAAAAB, and XXXVII BAAAAAB) were found in *H. ovina* from Andaman Sea. A total of twelve composite haplotypes (XXXVIII BBABABD, XXXIX BBABABC, XL BBABABG, XLI ABABABC, XLII DBABABC, XLIII EAABABC, XLIV EAABABF, XLV ABAAABC, XLVI ACABABC, XLVII CBABABC, XLVIII DBABABD, and XLIX ABABABD) were observed in *H. varia* (Table 3.6).

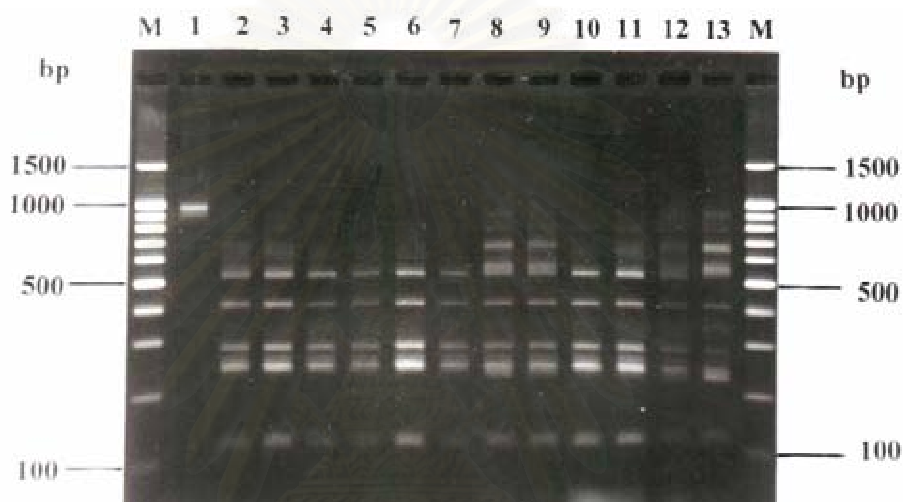

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Fig 3.6 An example of restriction patterns of 18S rDNA digested with *Alu* I in *H. asinina* (D and B, lanes 2-3 and 4-5, respectively), *H. ovina* (B and A, lanes 6-7 and 8-9, respectively), and *H. varia* (B, C and A, lanes 10, 11 and 12-13, respectively). Lanes M and 1 are a 100 bp DNA ladder and undigested 18S rDNA (approximately 900 bp in length), respectively.

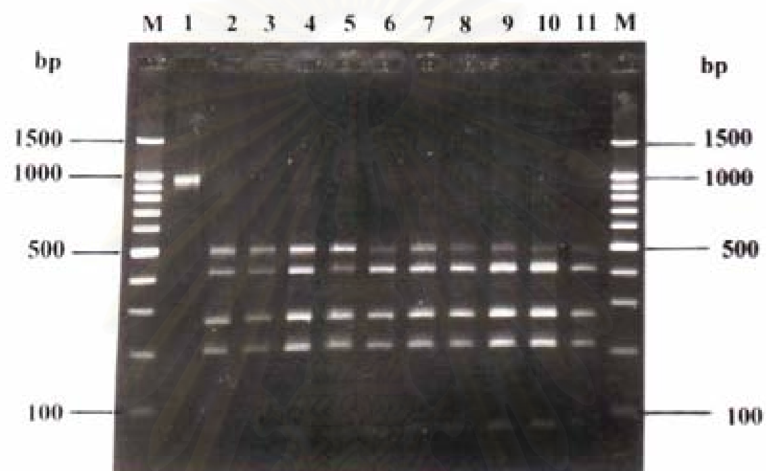

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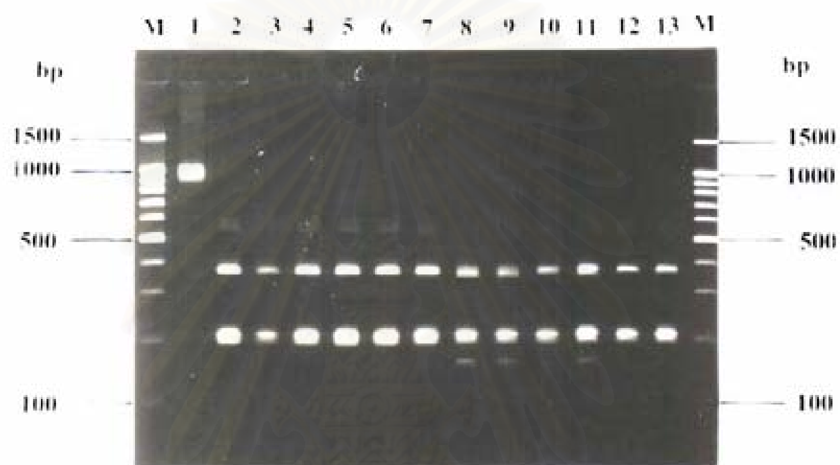
Fig 3.7 An example of restriction patterns of 18S rDNA digested with *Taq* I in *H. asinina* (C, lanes 2-4), *H. ovina* (D, A and B, lanes 5, 6 and 8, and 7, respectively) and *H. varia* (B, and A, lanes 9-10 and 11, respectively). Lanes M and 1 are a 100 bp DNA ladder and undigested 18S rDNA (approximately 900 bp in length), respectively.



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จุฬาลงกรณ์มหาวิทยาลัย

Fig 3.8 An example of restriction patterns of 18S rDNA digested with *Hae* III in *H. asinina* (A, lanes 2-5), *H. ovina* (A and B, lanes 6-7 and 8-9, respectively) and *H. varia* (A and B, lanes 10, 12 and 13, and 11, respectively). Lanes M and 1 are a 100 bp DNA ladder and undigested 18S rDNA (approximately 900 bp in length), respectively.

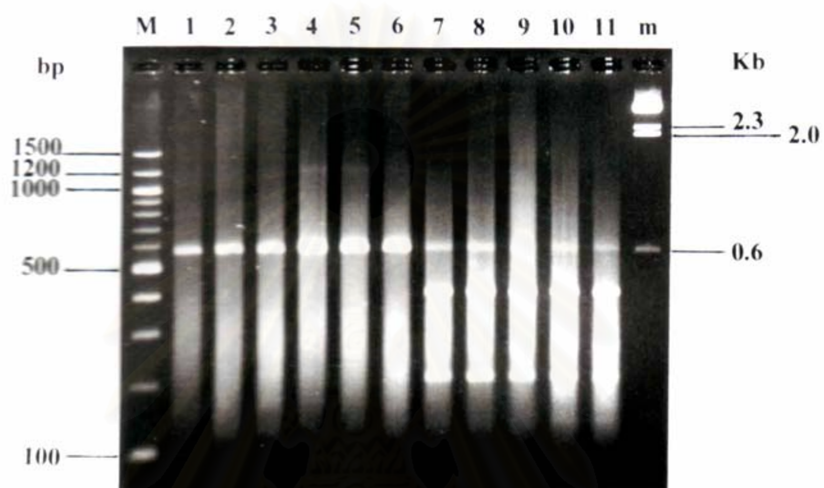

Fig 3.9 An example of restriction patterns of 16S rDNA digested with *Bam* H I.

Lane M = A 100 bp DNA ladder

Lane 1 = An undigested 16S rDNA products (580 bp in length)

Lanes 2-4 = *H. asinina* (pattern A)

Lanes 5-6 = *H. ovina* (pattern A)

Lanes 7-11 = *H. varia* (pattern B)

Lane m = A λ/*Hin*d III DNA marker

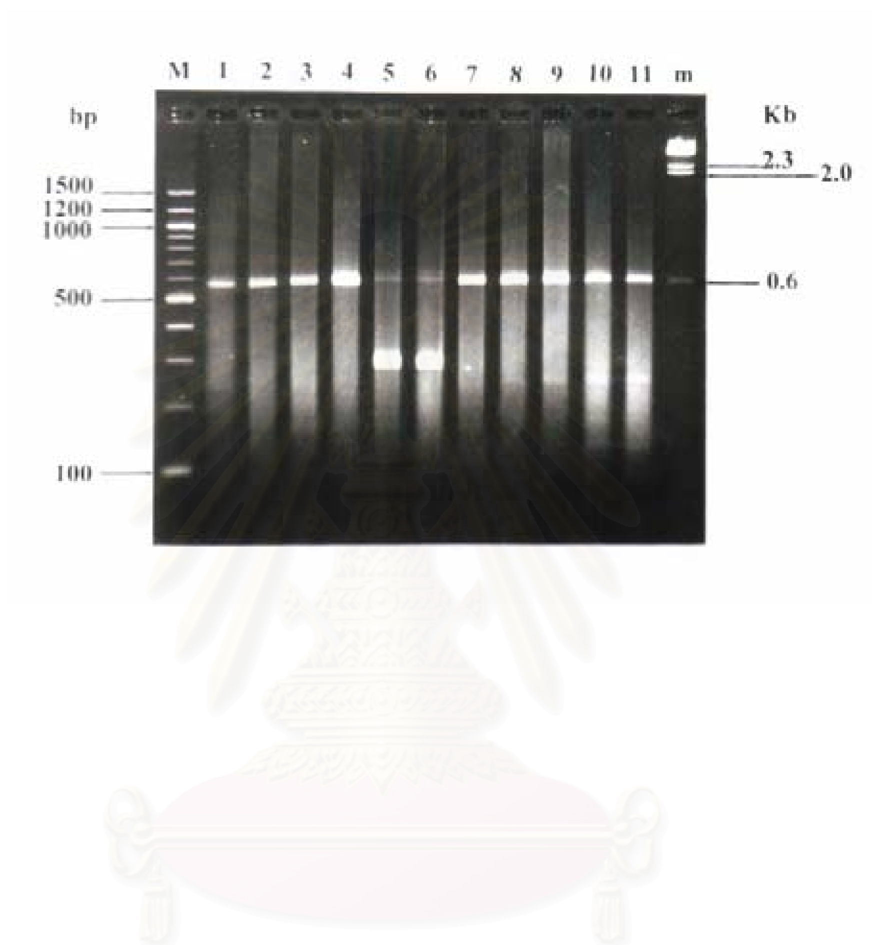

Fig 3.10 An example of restriction patterns of 16S rDNA digested with *Eco*R I.

Lane M = A 100 bp DNA ladder

Lane 1 = An undigested 16S rDNA products (580 bp in length)

Lanes 2-4 = *H. asinina* (pattern A)

Lanes 5-6 = *H. ovina* (pattern B)

Lanes 7-11 = *H. varia* (pattern A)

Lane m = A λ/*Hin*d III DNA marker

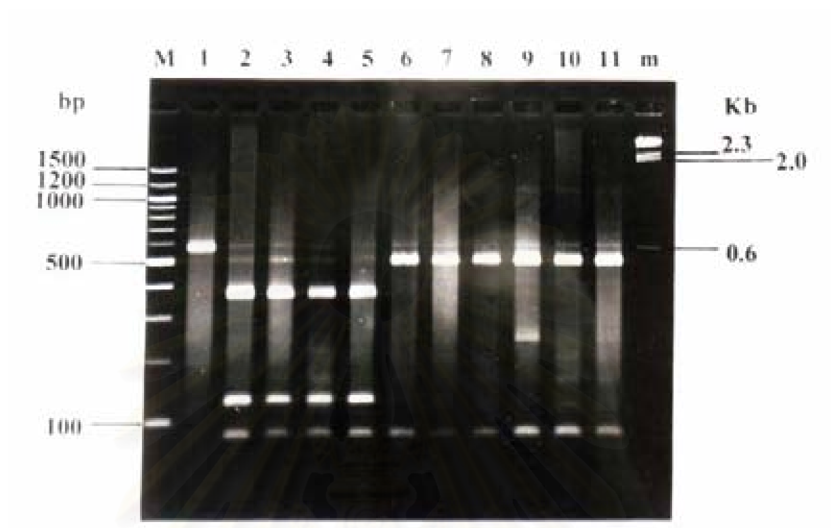

Fig 3.11 An example of restriction patterns of 16S rDNA digested with *Hae* III.

Lane M = A 100 bp DNA ladder

Lane 1 = An undigested 16S rDNA products (580 bp in length)

Lanes 2-5 = *H. asinina* (pattern A)

Lanes 6-8 = *H. ovina* (pattern B)

Lanes 9-11 = *H. varia* (pattern B)

Lane m = A λ/*Hin*d III DNA marker

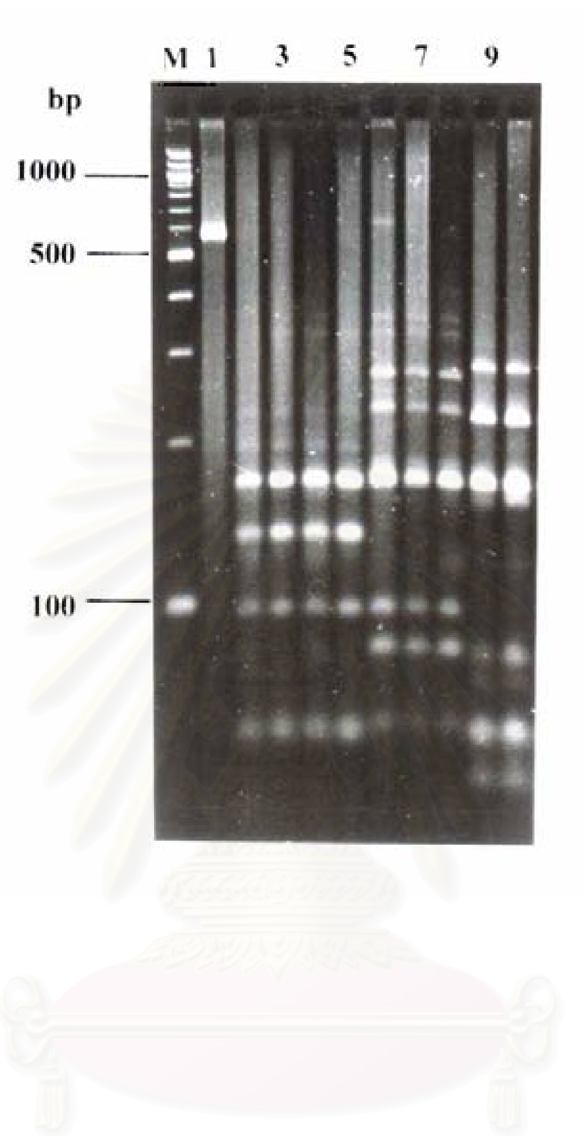

Fig 3.12 An example of restriction patterns of 16S rDNA digested with *Alu* I.

Lane M = A 100 bp DNA ladder

Lane 1 = An undigested 16S rDNA products (580 bp in length)

Lanes 2-5 = *H. asinina* (pattern A)

Lanes 6-8 = *H. ovina* (pattern B)

Lanes 9-10 = *H. varia* (pattern C)

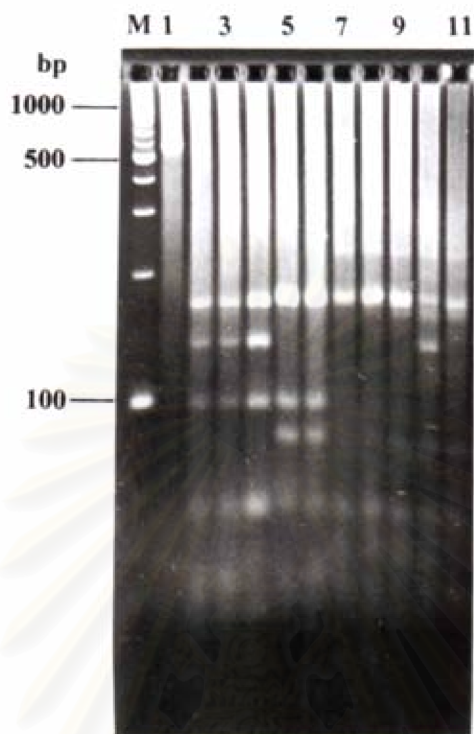

Fig 3.13 An example of restriction patterns of 16S rDNA digested with *Alu* I.

Lane M = A 100 bp DNA ladder

Lane 1 = An undigested 16S rDNA products (580 bp in length)

Lanes 2-4 = *H. asinina* (pattern A)

Lanes 5-6 = *H. ovina* (pattern B)

Lanes 7-11 = *H. varia* (pattern D, D, C, G, and C, respectively)

Table 3.1 Restriction fragment patterns observed from digestion of 18S rDNA of three abalone species (*H. asinina*, *H. ovina* and *H. varia*) with restriction endonucleases used in this study

Restriction pattern of 18S rDNA											
Alu I						*Taq* I				*Hae* III	
A	B	C	D	E	F	A	B	C	D	A	B
680	-	680	-	-	-	-	500	500	500	390	390
-	-	-	650	-	-	-	-	-	500	210	210
520	520	520	520	520	520	410	410	410	410	200	200
410	410	410	410	410	410	-	410	-	-	-	180
350	-	-	350	350	350	270	270	270	270		
290	290	290	290	290	290	210	210	210	210		
250	250	250	250	250	250	80	80	80	80		
240	-	-	240	240	-						
110	110	110	110	110	110						
-	110	-	-	-	-						

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.2 Restriction fragment patterns observed from digestion of 16S rDNA of three abalone species (*H. asinina*, *H. ovina* and *H. varia*) with restriction endonucleases used in this study

Restriction pattern of 16S rDNA												
*Bam*H I (bp)		*Eco*R I (bp)		*Hae* III (bp)		*Alu* I (bp)						
A	B	A	B	A	B	A	B	C	D	E	F	G
580	-	580	-	-	500	-	-	220	-	-	220	-
-	380	-	300	380	-	175	175	175	175	175	-	175
-	200	-	280	120	-	-	175	-	175	-	-	-
				80	80	140	-	-	-	140	140	140
						95	95	-	-	95	-	-
						-	80	80	-	80	80	-
						50	50	50	50	50	50	50
						-	-	35	-	-	35	35

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.3 Frequency distribution patterns of 18S rDNA in each geographic sample analyzed by *Alu* I, *Hae* III and *Tag* I

Restriction pattern	*H. asinina*						*H. ovina*				*H. varia*	
	HAHt (*N*=19)	HASt (*N*=12)	HALb (*N*=28)	HACbh (*N*=15)	HACb (*N*=21)	HAPhi (*N*=20)	HOSi (*N*=24)	HOSt (*N*=18)	HOTg (*N*=18)	HOSl (*N*=11)	HVPhu (*N*=21)	HVSl (*N*=2)
18S rDNA-*Alu* I												
A	0.0530(1)	-	0.2140(6)	0.2670(4)	0.0480(1)	0.4000(8)	0.9160(22)	0.9440(17)	0.6660(12)	0.7270(8)	0.3810(8)	1(2)
B	0.8940(17)	1(12)	0.5710(16)	0.4660(7)	0.9040(19)	0.3000(6)	0.0420(1)	-	0.0560(1)	0.0910(1)	0.2860(6)	-
C	0.0530(1)	-	0.1430(4)	0.2670(4)	-	-	-	-	0.0560(1)	-	0.0950(2)	-
D	-	-	-	-	-	0.1500(3)	0.0420(1)	0.0560(1)	0.1110(2)	0.1820(2)	0.0950(2)	-
E	-	-	0.0710(2)	-	0.0480(1)	0.1500(3)	-	-	-	-	0.1430(3)	-
F	-	-	-	-	-	-	-	-	0.1110(2)	-	-	-

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.3 (cont.)

Restriction pattern	*H. asinina*						*H. ovina*				*H. varia*	
	HAHt (*N*=19)	HASt (*N*=12)	HALb (*N*=28)	HACbh (*N*=15)	HACb (*N*=21)	HAPhi (*N*=20)	HOSi (*N*=24)	HOSt (*N*=18)	HOTg (*N*=18)	HOSl (*N*=11)	HVPhu (*N*=21)	HVSl (*N*=2)
18S rDNA-*Taq* I												
A	0.5790(11)	1(12)	0.9640(27)	0.8670(13)	0.9050(19)	0.2500(5)	-	-	-	0.0910(1)	0.1430(3)	-
B	0.4210(8)	-	0.0360(1)	0.1330(2)	0.0950(2)	0.7500(15)	0.1250(3)	0.2220(4)	0.6110(11)	0.5450(6)	0.8090(17)	1(2)
C	-	-	-	-	-	-	0.7500(18)	0.4440(8)	0.3330(6)	0.2730(3)	0.0480(1)	-
D	-	-	-	-	-	-	0.1250(3)	0.3330(6)	0.0560(1)	0.0910(1)	-	-
18S rDNA-*Hae* III												
A	1(19)	1(12)	0.9640(27)	0.7330(11)	1(21)	0.9500(19)	0.2080(5)	0.1670(3)	0.2780(5)	0.5450(6)	1(21)	1(2)
B	-	-	0.0360(1)	0.2760(4)	-	0.0500(1)	0.7920(19)	0.8330(15)	0.7220(13)	0.4550(5)	-	-

HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.4 Frequency distribution patterns of 16S rDNA in each geographic sample analyzed by *Bam* HI, *Eco* RI, *Hae* III and *Alu* I

Restriction pattern	*H. asinina*						*H. ovina*				*H. varia*	
	HAHt (*N*=19)	HASt (*N*=12)	HALb (*N*=28)	HACbh (*N*=15)	HACb (*N*=21)	HAPhi (*N*=20)	HOSi (*N*=24)	HOSt (*N*=18)	HOTg (*N*=18)	HOSl (*N*=11)	HVPhu (*N*=21)	HVSl (*N*=2)
16S rDNA-*Bam*H I												
A	1(19)	1(12)	1(28)	1(15)	1(21)	1(20)	1(24)	1(18)	1(18)	1(11)	0.0480(1)	-
B	-	-	-	-	-	-	-	-	-	-	0.9520(20)	1(2)
16S rDNA-*Eco*R I												
A	1(19)	1(12)	1(28)	1(15)	1(21)	1(20)	-	-	1(18)	1(11)	1(21)	1(2)
B	-	-	-	-	-	-	1(24)	1(18)	-	-	-	-
16S rDNA-*Hae* III												
A	1(19)	1(12)	1(28)	1(15)	1(21)	1(20)	-	-	0.9440(17)	1(11)	-	-
B	-	-	-	-	-	-	1(24)	1(18)	0.0560(1)	-	1(21)	1(2)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.4 (cont.)

Restriction pattern	*H. asinina*						*H. ovina*				*H. varia*	
	HAHt (*N*=19)	HASt (*N*=12)	HALb (*N*=28)	HACbh (*N*=15)	HACb (*N*=21)	HAPhi (*N*=20)	HOSi (*N*=24)	HOSt (*N*=18)	HOTg (*N*=18)	HOSl (*N*=11)	HVPhu (*N*=21)	HVSl (*N*=2)
16S rDNA-*Alu* I												
A	0.8420(16)	1(12)	0.9290(26)	1(15)	1(21)	1(20)	-	-	-	-	-	-
B	-	-	-	-	-	-	1(24)	1(18)	1(18)	1(11)	-	-
C	-	-	-	-	-	-	-	-	-	-	0.6660(14)	0.5000(1)
D	-	-	-	-	-	-	-	-	-	-	0.2380(5)	0.5000(1)
E	0.1580(3)	-	0.0710(2)	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	0.0480(1)	-
G	-	-	-	-	-	-	-	-	-	-	0.0480(1)	-

HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.5 Frequency distribution of composite haplotypes within each abalone sample resulted from restriction analysis of 16S rDNA

Composite haplotype	*H. asinina*						*H. ovina*				*H. varia*	
	HAHt (*N*=19)	HASt (*N*=12)	HALb (*N*=28)	HACbh (*N*=15)	HACb (*N*=21)	HAPhi (*N*=20)	HOSi (*N*=24)	HOSt (*N*=18)	HOTg (*N*=18)	HOSl (*N*=11)	HVPhu (*N*=21)	HVSl (*N*=2)
I AAAA	0.8421(16)	1(12)	0.9286(26)	1(15)	1(21)	1(20)	-	-	-	-	-	-
II AAAE	0.1579(3)	-	0.0714(2)	-	-	-	-	-	-	-	-	-
III ABBB	-	-	-	-	-	-	1(24)	1(18)	-	-	-	-
IV AAAB	-	-	-	-	-	-	-	-	0.9444(17)	1(11)	-	-
V AABB	-	-	-	-	-	-	-	-	0.0556(1)	-	-	-
VI BABD	-	-	-	-	-	-	-	-	-	-	0.2381(5)	0.5000(1)
VII BABC	-	-	-	-	-	-	-	-	-	-	0.6191(13)	0.5000(1)
VIII BABG	-	-	-	-	-	-	-	-	-	-	0.0476(1)	-
IX BABF	-	-	-	-	-	-	-	-	-	-	0.0476(1)	-
X AABC	-	-	-	-	-	-	-	-	-	-	0.0476(1)	-

HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.6 Frequency distribution of composite haplotypes within each abalone sample resulted from restriction analysis of 18S and 16S rDNAs

Composite haplotype	*H. asinina*						*H. ovina*				*H. varia*	
	HAHt (*N*=19)	HASt (*N*=12)	HALb (*N*=28)	HACbh (*N*=15)	HACb (*N*=21)	HAPhi (*N*=20)	HOSi (*N*=24)	HOSt (*N*=18)	HOTg (*N*=18)	HOSl (*N*=11)	HVPhu (*N*=21)	HVSl (*N*=2)
I BBAAAAA	0.2632(5)	-	-	-	0.0953(2)	0.1500(3)	-	-	-	-	-	-
II ACAAAAA	0.0526(1)	-	-	-	-	-	-	-	-	-	-	-
III BCAAAAA	0.0526(1)	-	-	-	-	-	-	-	-	-	-	-
IV BBAAAAE	0.0526(1)	-	-	-	-	-	-	-	-	-	-	-
V BAAAAAA	0.4211(8)	1(12)	0.5000(14)	0.4000(6)	0.8095(17)	0.1500(3)	-	-	-	-	-	-
VI BAAAAAE	0.1053(2)	-	0.0714(2)	-	-	-	-	-	-	-	-	-
VII CAAAAAA	0.0526(1)	-	0.1072(3)	0.0667(1)	-	-	-	-	-	-	-	-
VIII AAAAAAA	-	-	0.1786(5)	0.1333(2)	0.0476(1)	0.0500(1)	-	-	-	-	-	-
IX ABAAAAA	-	-	0.0357(1)	0.1333(2)	-	0.3000(6)	-	-	-	-	-	-
X CABAAAA	-	-	0.0357(1)	0.2000(3)	-	-	-	-	-	-	-	-
XI EAAAAAA	-	-	0.0714(2)	-	0.0476(1)	-	-	-	-	-	-	-

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.6 (cont.)

Composite haplotype	*H. asinina*						*H. ovina*				*H. varia*	
	HAHt (*N*=19)	HASt (*N*=12)	HALb (*N*=28)	HACbh (*N*=15)	HACb (*N*=21)	HAPhi (*N*=20)	HOSi (*N*=24)	HOSt (*N*=18)	HOTg (*N*=18)	HOSl (*N*=11)	HVPhu (*N*=21)	HVSl (*N*=2)
XII BABAAAA	-	-	-	0.0667(1)	-	-	-	-	-	-	-	-
XIII AABAAAA	-	-	-	-	-	0.0500(1)	-	-	-	-	-	-
XIV EBAAAAA	-	-	-	-	-	0.1500(3)	-	-	-	-	-	-
XV DBAAAAA	-	-	-	-	-	0.1500(3)	-	-	-	-	-	-
XVI BBAABBB	-	-	-	-	-	-	0.0417(1)	-	-	-	-	-
XVII ACAABBB	-	-	-	-	-	-	0.1666(4)	0.0556(1)	-	-	-	-
XVIII ACBABBB	-	-	-	-	-	-	0.5833(14)	0.3333(6)	-	-	-	-
XIX ADBABBB	-	-	-	-	-	-	0.1250(3)	0.2777(5)	-	-	-	-
XX DBBABBB	-	-	-	-	-	-	0.0417(1)	-	-	-	-	-
XXI ABBABBB	-	-	-	-	-	-	0.0417(1)	0.1666(3)	-	-	-	-
XXII DCBABBB	-	-	-	-	-	-	-	0.0556(1)	-	-	-	-

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.6 (cont.)

Composite haplotype	*H. asinina*						*H. ovina*				*H. varia*	
	HAHt (*N*=19)	HASt (*N*=12)	HALb (*N*=28)	HACbh (*N*=15)	HACb (*N*=21)	HAPhi (*N*=20)	HOSi (*N*=24)	HOSt (*N*=18)	HOTg (*N*=18)	HOSl (*N*=11)	HVPhu (*N*=21)	HVSl (*N*=2)
XXIII ADAABBB	-	-	-	-	-	-	-	0.0556(1)	-	-	-	-
XXIV ABAABBB	-	-	-	-	-	-	-	0.0556(1)	-	-	-	-
XXV ABBAAAB	-	-	-	-	-	-	-	-	0.3335(6)	0.1818(2)	-	-
XXVI FBAAAAB	-	-	-	-	-	-	-	-	0.0555(1)	-	-	-
XXVII ACBAAAB	-	-	-	-	-	-	-	-	0.2223(4)	0.1818(2)	-	-
XXVIII FBBAAAB	-	-	-	-	-	-	-	-	0.0555(1)	-	-	-
XXIX DDBAAAB	-	-	-	-	-	-	-	-	0.0555(1)	-	-	-
XXX DCBAAAB	-	-		-	-	-	-	-	0.0555(1)	-	-	-
XXXI CBAAAAB	-	-	-	-	-	-	-	-	0.0555(1)	-	-	-
XXXII BBAAABB	-	-	-	-	-	-	-	-	0.0555(1)	-	-	-
XXXIII ABAAAAB	-	-	-	-	-	-	-	-	0.1112(2)	0.1818(2)	-	-

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.6 (cont.)

Composite haplotype	*H. asinina*						*H. ovina*				*H. varia*	
	HAHt (*N*=19)	HASt (*N*=12)	HALb (*N*=28)	HACbh (*N*=15)	HACb (*N*=21)	HAPhi (*N*=20)	HOSi (*N*=24)	HOSt (*N*=18)	HOTg (*N*=18)	HOSl (*N*=11)	HVPhu (*N*=21)	HVSl (*N*=2)
XXXIV ADBAAAB	-	-	-	-	-	-	-	-	-	0.0909(1)	-	-
XXXV DBAAAAB	-	-	-	-	-	-	-	-	-	0.1818(2)	-	-
XXXVI ACAAAAB	-	-	-	-	-	-	-	-	-	0.0909(1)	-	-
XXXVII BAAAAAB	-	-	-	-	-	-	-	-	-	0.0909(1)	-	-
XXXVIII BBABABD	-	-	-	-	-	-	-	-	-	-	0.1429(3)	-
XXXIX BBABABC	-	-	-	-	-	-	-	-	-	-	0.0952(2)	-
XL BBABABG	-	-	-	-	-	-	-	-	-	-	0.0476(1)	-
XLI ABABABC	-	-	-	-	-	-	-	-	-	-	0.2382(5)	0.5000(1)
XLII DBABABC	-	-	-	-	-	-	-	-	-	-	0.0476(1)	-
XLIII EAABABC	-	-	-	-	-	-	-	-	-	-	0.0952(2)	-
XLIV EAABABF	-	-	-	-	-	-	-	-	-	-	0.0476(1)	-

Table 3.6 (cont.)

Composite haplotype	*H. asinina*						*H. ovina*				*H. varia*	
	HAHt (*N*=19)	HASt (*N*=12)	HALb (*N*=28)	HACbh (*N*=15)	HACb (*N*=21)	HAPhi (*N*=20)	HOSi (*N*=24)	HOSt (*N*=18)	HOTg (*N*=18)	HOSl (*N*=11)	HVPhu (*N*=21)	HVSl (*N*=2)
XLV ABAAABC	-	-	-	-	-	-	-	-	-	-	0.0476(1)	-
XLVI ACABABC	-	-	-	-	-	-	-	-	-	-	0.0476(1)	-
XLVII CBABABC	-	-	-	-	-	-	-	-	-	-	0.0952(2)	-
XLVIII DBABABD	-	-	-	-	-	-	-	-	-	-	0.0476(1)	-
XLIX ABABABD	-	-	-	-	-	-	-	-	-	-	0.0476(1)	0.5000(1)

HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines;

Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket

Distribution frequencies of composite haplotypes within geographic samples are shown by Table 3.5 and 3.6. No overlapping haplotypes between different species of abalone were found neither from analysis of 16S rDNA alone nor from analysis of 16S and 18S rDNAs together. This illustrated that identification of species origin of *H. asinina*, *H. ovina*, and *H. varia* could be unambiguously carried out by PCR-RFLP of 16S rDNA alone and both 16S and 18S rDNAs.

3.4 Genetic distance between composite haplotypes of abalone and their phylogenetic relationships

Genetic distance between pairs of composite haplotypes of *H. asinina*, *H. ovina*, and *H. varia* (Appendix D for combined 16S rDNA and Appendix E for 18S and 16S rDNAs) based on polymorphism of 16S rDNAs alone (Fig. 3.14) and 18S+16S rDNA (Fig. 3.16) were used to construct a UPGMA phenogram.

Misclustering of composite haplotypes was observed from RFLP analysis using only 16S rDNA (Fig. 3.14). The UPGMA dendrogram allocated 10 composite haplotypes of this gene region to 2 major groups. However, the composite haplotypes AAAB and AABC found in *H. ovina* and *H. varia*, respectively were misallocated to be grouped with *H. asinina* and *H. ovina* haplotypes.

The latter dendrogram allocated all haplotypes into 3 groups (clusters I, II, and III). The cluster I contained fifteen composite haplotypes of all *H. asinina*, and eleven composite haplotypes of *H. ovina* from Andaman sea. Nevertheless, one composite haplotype (BAAAAAB) of *H. ovina* misclustered and was allocated into the *H. asinina* group. The cluster II contained nine composite haplotypes of *H. ovina* from

the Gulf of Thailand and one composite haplotype of *H. ovina* from the Andaman sea (BBAAABB). All composite haplotypes of *H. varia* were allocated into the cluster III.

3.5 Haplotype diversity and nucleotide diversity within samples, nucleotide divergence between sample and phylogenetic relationships at the sample and species levels

Haplotype and nucleotide diversity within sample populations of Thai abalone are shown by Table 3.7 (16S rDNA) and 3.8 (18S and 16S rDNA). The average haplotype diversity of 16S and 16S + 18S rDNA was 0.1458 (0.0000–0.6667) and 0.6762 (0.0000–0.9013), respectively. The nucleotide diversity within geographic samples were 0.2483% (0.0000–1.7440%) and 0.3716% (0.0000-0.8114%). It should be noted that only two specimens from Similan Island, Phangnga were analyzed in that geographic sample therefore, data resulted from a limited sample size of this sample must be interpreted with cautions.

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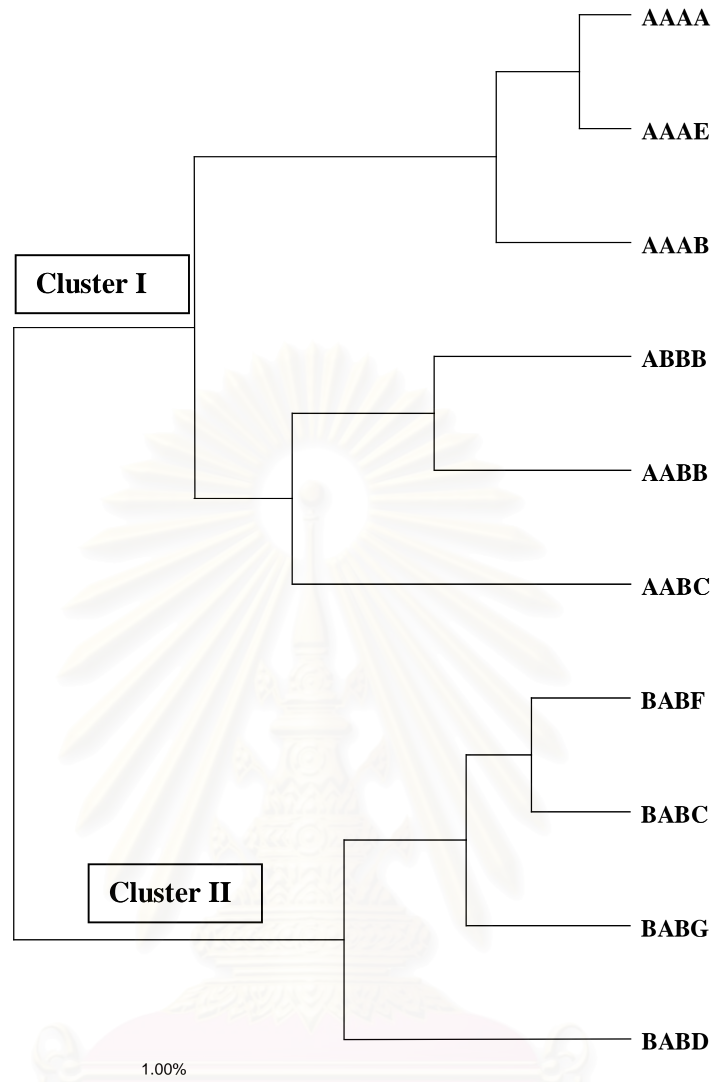

Fig 3.14 A UPGMA dendrogram illustrating genetic relationships of ten composite haplotypes in three abalone species resulted from PCR-RFLP of 16S rDNA.

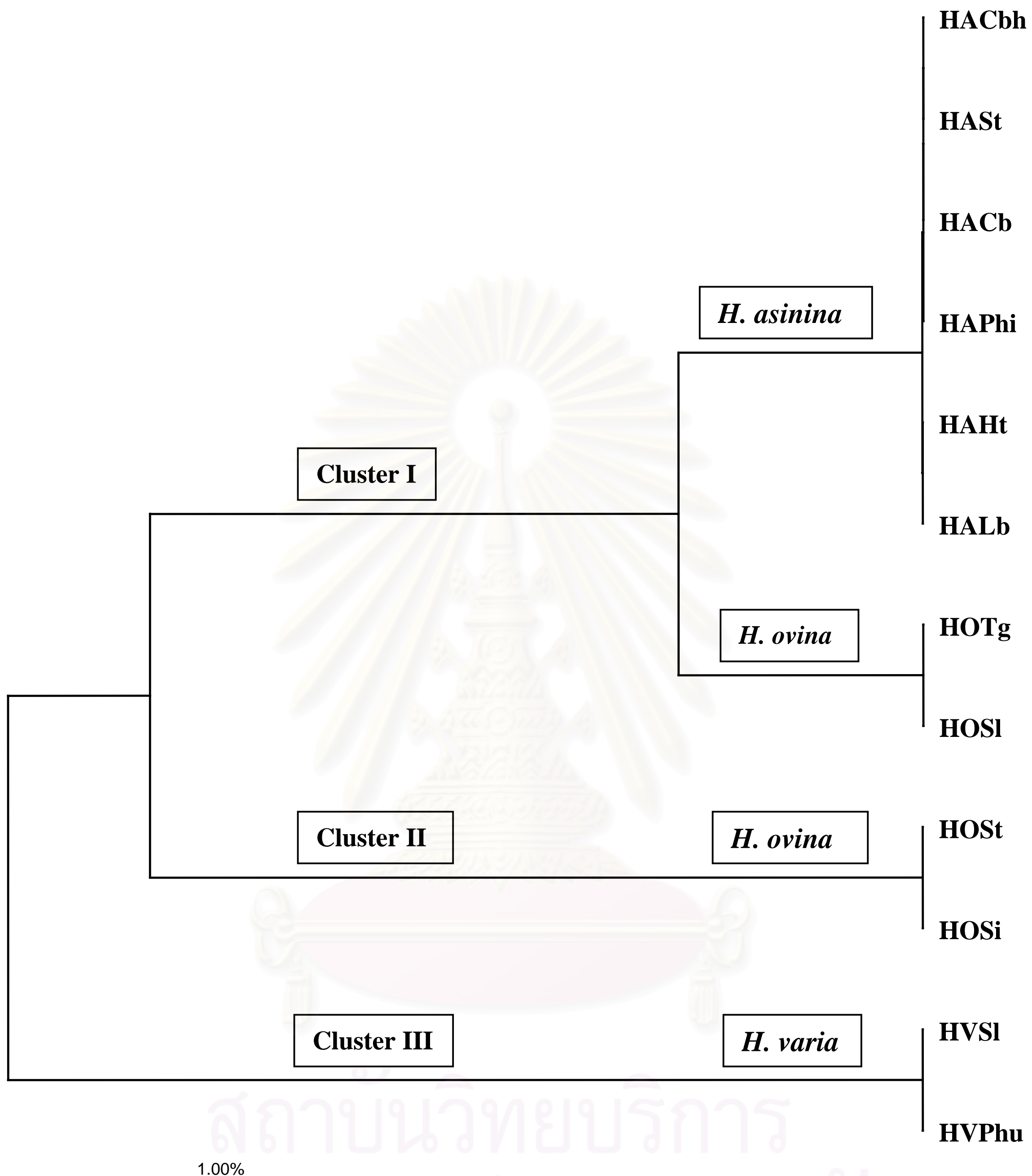

Fig. 3.15 A UPGMA dendrogram illustration genetic relationships between samples of three abalone species based on PCR-RFLP of 16S rDNA. HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket.

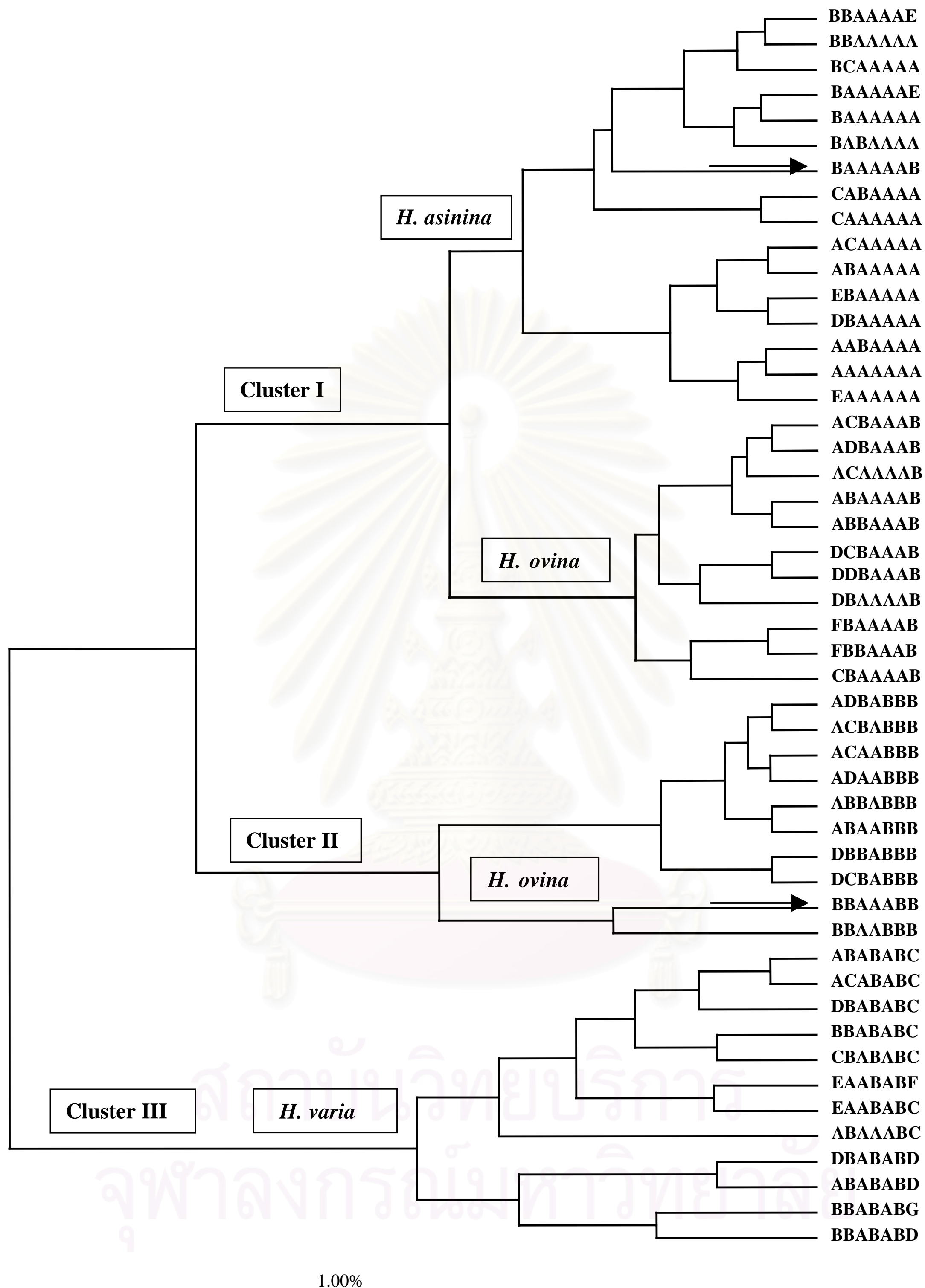

Fig. 3.16 A UPGMA dendrogram indicating genetic relationships between composite haplotypes of *H. asinina*, *H. ovina*, and *H. varia* based on PCR-RFLP of 18S and 16S rDNAs.

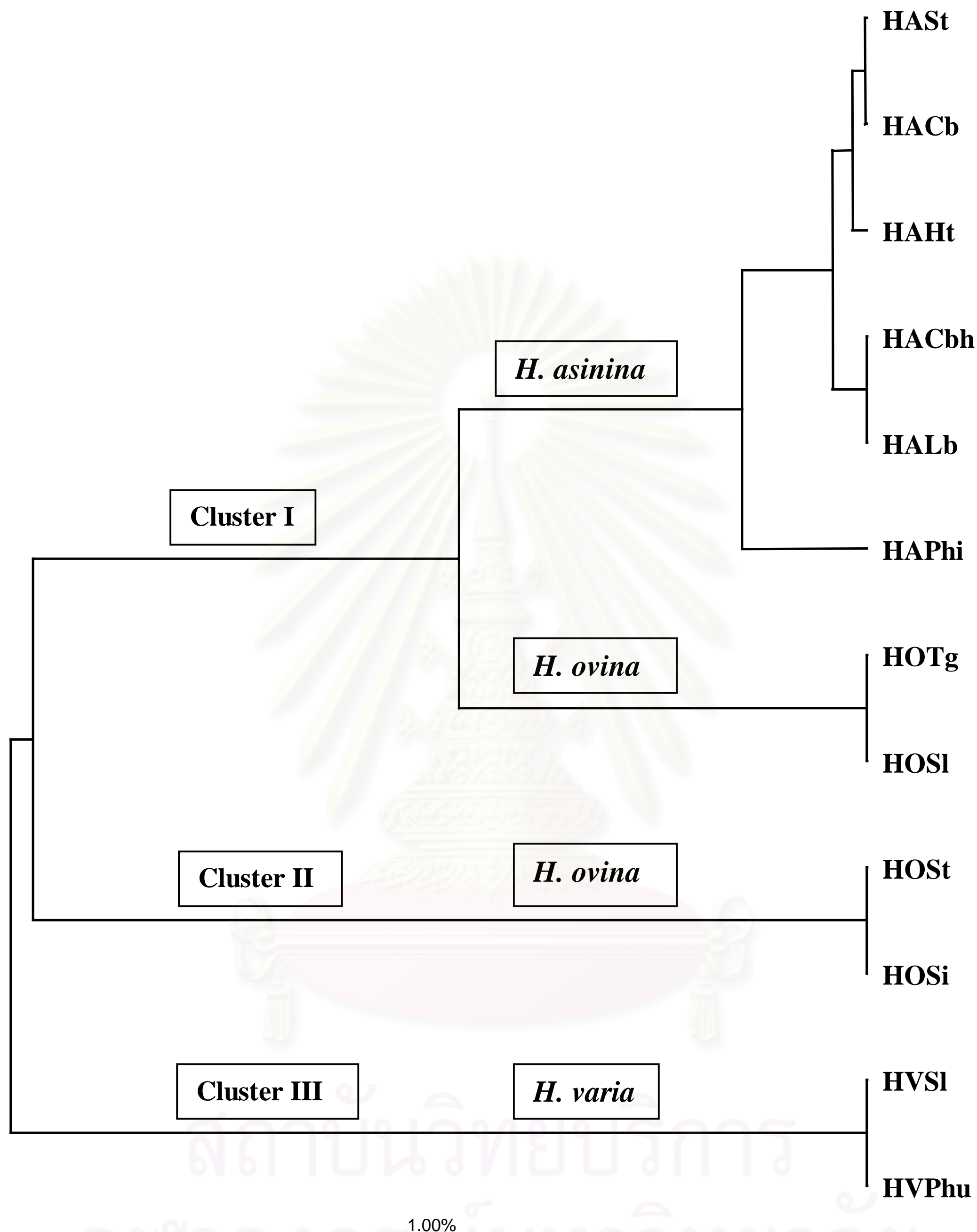

Fig. 3.17 A UPGMA dendrogram illustration genetic relationships between geographic samples of three abalone species based on PCR-RFLP of 18S and 16S rDNAs. HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket.

Table 3.7 Haplotype and nucleotide diversity within samples of Thai abalone examined by restriction analysis of 16S rDNA

Sample	Haplotype diversity ± SE	Nucleotide diversity (x100)
H. asinina		
Hatchery (P_0), Angsila, Chon Buri, originated from Rayong	0.2731±0.0816	0.1014
Samet Island, Rayong	0.0000±0.0000	0.0000
Talibong Island, Trang	0.1351±0.0591	0.0497
Hatchery (P_0), Cambodia	0.0000±0.0000	0.0000
Cambodia	0.0000±0.0000	0.0000
The Philippines	0.0000±0.0000	0.0000
H. ovina		
Sichang Island, Chon Buri	0.0000±0.0000	0.0000
Samet Island, Rayong	0.0000±0.0000	0.0000
Churk Island, Trang	0.1079±0.0680	0.1315
Similan Island, Phangnga	0.0000±0.0000	0.0000
H. varia		
L-Island, Phuket	0.5668±0.0712	0.9534
Similan Island, Phangnga	0.6667±0.2041	1.7440
Average	0.1458±0.0047	0.2483±0.0000

Table 3.8 Haplotype and nucleotide diversity within samples of Thai abalone examined by restriction analysis of 18S rDNA and 16S rDNA

Sample	Haplotype diversity ± SE	Nucleotide diversity (x100)
H. asinina		
Hatchery (P_0), Angsila, Chon Buri, originated from Rayong	0.7511±0.0508	0.3139
Samet Island, Rayong	0.0000±0.0000	0.0000
Talibong Island, Trang	0.7065±0.0536	0.3758
Hatchery (P_0), Cambodia	0.7816±0.0518	0.4809
Cambodia	0.3391±0.0898	0.1715
The Philippines	0.8359±0.0288	0.4910
H. ovina		
Sichang Island, Chon Buri	0.6241±0.0679	0.1897
Samet Island, Rayong	0.7937±0.0392	0.1903
Churk Island, Trang	0.8317±0.0417	0.4073
Similan Island, Phangnga	0.8831±0.0293	0.4222
H. varia		
L-Island, Phuket	0.9013±0.0248	0.8114
Similan Island, Phangnga	0.6667±0.2041	0.6057
Average	0.6762±0.0057	0.3716±0.0000

Nucleotide diversity and nucleotide divergence among samples are shown by Table 3.9 (16S rDNA) and 3.10 (18S+16S rDNAs). The average nucleotide diversity and nuclotide divergence from 16S rDNA alone were 0.0268 and 0.0243, respectively while the highest nucleotide divergence was 0.0485 (HVPhu-HOSi and HVPhu-HOSt). The average nucleotide diversity and nuclotide divergence were 0.0169 and 0.0132 (18S+16S rDNAs). The highest nucleotide diversity between geographic samples was 0.0312 (HASt-HVSl).

UPGMA dendrograms constructed from nucleotide divergence between geographic samples using 16S rDNA and combined 18S and 16S rDNAs (Fig. 3.15 and 3.17) could differentiate *H. asinina* into 3 groups largely according to species origin with the exception that *H. ovina* from the Andaman Sea was consistently regarded as the sister taxa of *H. asinina*.

UPGMA phenograms constructed from nucleotide divergence between abalone species revealed genetically closed relationships between *H. asinina* and *H. ovina* but distantly related to *H. varia* phylogenetically (Fig. 3.18 and 3.19).

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Table 3.9 Nucleotide diversity (above diagonal) and divergence (below) between geographic samples of abalone resulted from restriction analysis of 16S rDNA

	HAHt	HASt	HALb	HACbh	HACb	HAPhi	HOSi	HOSt	HOTg	HOSl	HVPhu	HVSl
HAHt	-	**0.0006**	**0.0008**	**0.0006**	**0.0006**	**0.0006**	0.0405	0.0405	0.0121	0.0111	0.0522	0.0527
HASt	**0.0000**	-	**0.0003**	**0.0000**	**0.0000**	**0.0000**	0.0417	0.0417	0.0128	0.0118	0.0532	0.0522
HALb	**-0.0000**	**0.0000**	-	**0.0003**	**0.0003**	**0.0003**	0.0412	0.0412	0.0125	0.0115	0.0527	0.0519
HACbh	**0.0001**	**0.0000**	**0.0000**	-	**0.0000**	**0.0000**	0.0417	0.0417	0.0128	0.0118	0.0532	0.0522
HACb	**0.0001**	**0.0000**	**0.0000**	**0.0000**	-	**0.0000**	0.0417	0.0417	0.0128	0.0118	0.0532	0.0522
HAPhi	**0.0001**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	-	0.0417	0.0417	0.0128	0.0118	0.0532	0.0522
HOSi	0.0399	0.0417	0.0409	0.0417	0.0417	0.0417	-	**0.0000**	**0.0239**	**0.0245**	0.0532	0.0523
HOSt	0.0399	0.0417	0.0409	0.0417	0.0417	0.0417	**0.0000**	-	**0.0239**	**0.0245**	0.0532	0.0523
HOTg	0.0109	0.0122	0.0116	0.0122	0.0123	0.0123	**0.0233**	**0.0233**	-	**0.0007**	0.0437	0.0409
HOSl	0.0106	0.0118	0.0113	0.0118	0.0118	0.0118	**0.0245**	**0.0245**	**0.0000**	-	0.0445	0.0417
HVPhu	0.0469	0.0484	0.0477	0.0484	0.0484	0.0484	0.0485	0.0485	0.0382	0.0398	-	**0.0101**
HVSl	0.0428	0.0435	0.0430	0.0435	0.0435	0.0435	0.0436	0.0436	0.0315	0.0329	**-0.0034**	-

HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket.

Average nucleotide diversity = 0.0268±0.0000

Average nucleotide divergence = 0.0243±0.0000

Table 3.10 Nucleotide diversity (above diagonal) and divergence (below) among geographic samples of abalone resulted from restriction analysis of 18S and 16S rDNAs

	HAHt	HASt	HALb	HACbh	HACb	HAPhi	HOSi	HOSt	HOTg	HOSl	HVPhu	HVSl
HAHt	-	**0.0021**	**0.0042**	**0.0049**	**0.0026**	**0.0066**	0.0262	0.0268	0.0139	0.0134	0.0263	0.0283
HASt	**0.0005**	-	**0.0027**	**0.0037**	**0.0009**	**0.0072**	0.0279	0.0288	0.0159	0.0151	0.0282	0.0312
HALb	**0.0007**	**0.0008**	-	**0.0043**	**0.0031**	**0.0067**	0.0249	0.0256	0.0138	0.0129	0.0270	0.0278
HACbh	**0.0009**	**0.0013**	**-0.0000**	-	**0.0040**	**0.0067**	0.0242	0.0247	0.0131	0.0126	0.0271	0.0272
HACb	**0.0002**	**0.0000**	**0.0004**	**0.0008**	-	**0.0068**	0.0271	0.0279	0.0151	0.0144	0.0275	0.0298
HAPhi	**0.0026**	**0.0047**	**0.0023**	**0.0019**	**0.0035**	-	0.0219	0.0221	0.0104	0.0101	0.0237	0.0218
HOSi	0.0236	0.0270	0.0221	0.0208	0.0253	0.0186	-	**0.0019**	**0.0132**	**0.0138**	0.0284	0.0248
HOSt	0.0243	0.0279	0.0227	0.0213	0.0261	0.0187	**-0.0000**	-	**0.0131**	**0.0137**	0.0285	0.0247
HOTg	0.0103	0.0139	0.0098	0.0087	0.0122	0.0059	**0.0102**	**0.0101**	-	**0.0040**	0.0217	0.0181
HOSl	0.0097	0.0130	0.0089	0.0081	0.0114	0.0054	**0.0107**	**0.0107**	**-0.0001**	-	0.0220	0.0183
HVPhu	0.0206	0.0241	0.0211	0.0206	0.0226	0.0171	0.0234	0.0235	0.0156	0.0159	-	**0.0069**
HVSl	0.0237	0.0281	0.0229	0.0218	0.0259	0.0163	0.0209	0.0207	0.0129	0.0132	**-0.0002**	-

HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket.

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Average nucleotide diversity = 0.0169±0.0000

Average nucleotide divergence = 0.0132±0.0000

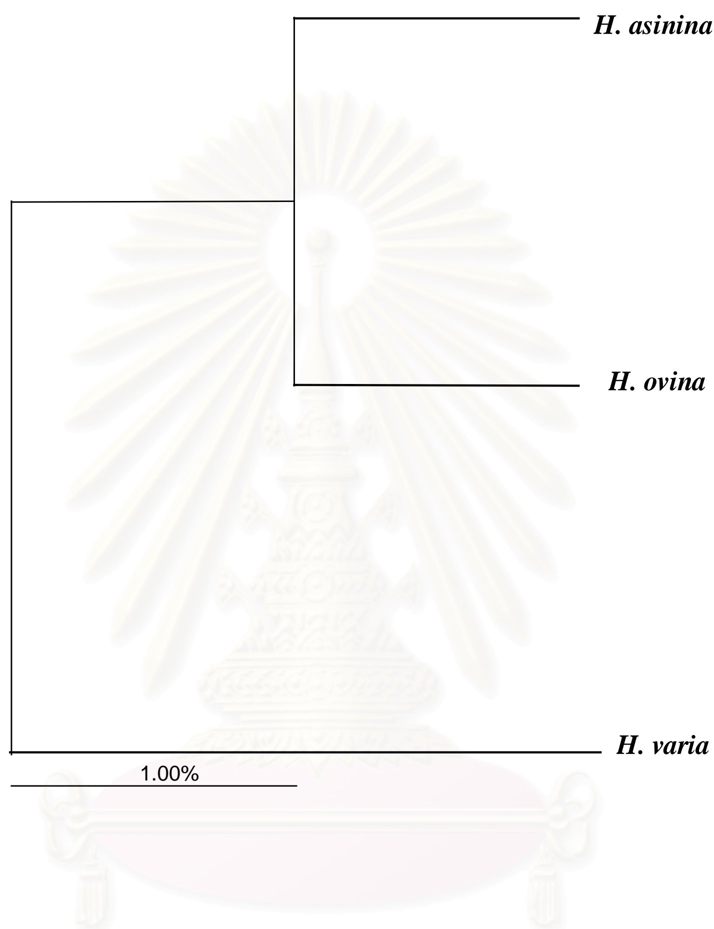

Fig. 3.18 A UPGMA dendrogram illustrating genetic relationships between *H. asinina*, *H. ovina*, and *H. varia* based on PCR-RFLP of 16S rDNA.

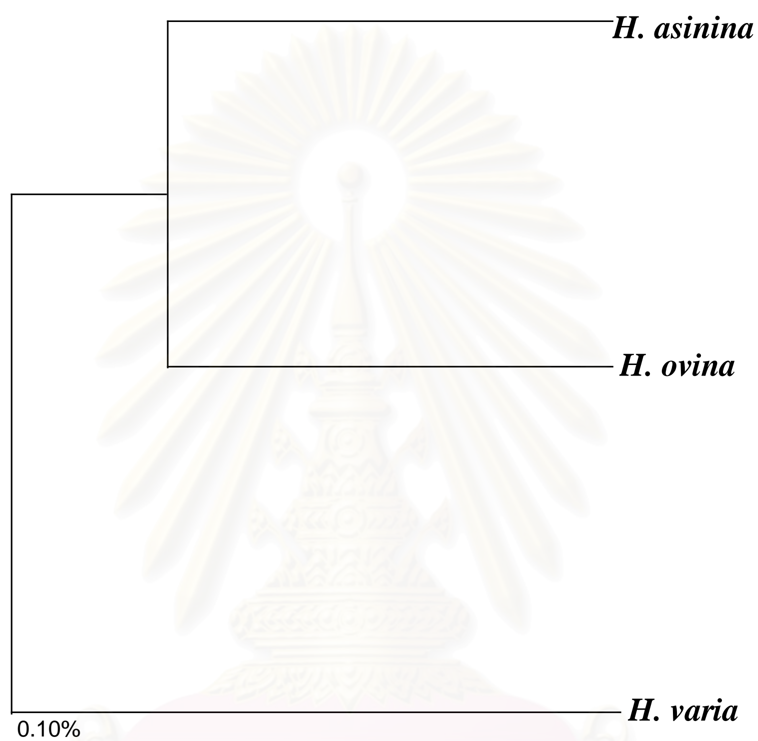

Fig. 3.19 A UPGMA dendrogram illustrating genetic relationships between *H. asinina*, *H. ovina*, and *H. varia* based on PCR-RFLP of 18S and 16S rDNAs.

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3.6 Geographic heterogeneity analysis between geographic samples of abalone

Results from geographic heterogeneity analysis across overall samples using both 18S+16S rDNAs and 16S rDNA were significant (P<0.0001) indicating the existence of genetic differentiation of abalone in this study. Overviews of genetic heterogeneity between different data set were identical (Tables 3.11 and 3.12).

Disregarding HVSl where only two specimens were examined, heterogeneity between sample of different abalone species were significantly different statistically for both 18S+16S and 16S rDNA data sets (P<0.0021 and P<0.0042, respectively). Within *H. asinina*, a panmictic gene pool was observed in this species when analyzed with only 16S rDNA. Including of 18S rDNA resulted in significant differences between the Philippines and the remaining samples except the HACbh sample (P<0.0021). Strong genetic differentiation between *H. ovina* originating from the Andaman Sea and the Gulf of Thailand were consistently observed for both data sets (P<0.0001) whereas a lack of heterogeneity was found in *H. vatia* (P=1.00 and 0.7710, see Table 3.11 and 3.12).

Genetic population differentiation within each species was also analyzed using F_{ST} estimate. Significant population structure was observed between *H. ovina* originating from different coastal regions (P<0.0001, Table 3.13) when analyzed with 16S rDNA. In addition, the 18S rDNA revealed the ability to differentiate between *H. asinina* from the Philippines from the remaining samples except the HACbh (P < 0.0008 for all cases, Table 3.14).

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Levels of gene flow between geographic samples within a species varied enormously due to different types of DNA markers. The 18S rDNA indicated that *H. asinina* experienced a low gene flow level whereas *H. ovina* and *H. varia* were moderate gene flow species. Considering female gene flow (analyzed from 16S rDNA), restricted female gene flow was observed in *H. ovina* whereas *H. asinina* and *H. varia* exhibited high female gene flow levels.

3.7 Cloning and sequencing of 16S rDNA

The amplified 16S rDNA of an individual representing each composite haplotype of 16S rDNA were cloned, sequenced and aligned (Fig. 3.20). Sequences were blasted against the GenBank using BlastN (www.ncbi.nih.nlm.gov). Resulted indicated significant matching of cloned fragments with 16S rDNA of other species previously deposited in the GenBank confirming that homologous DNA fragments were investigated for restriction analysis (Appendix G).

Large genetic distance between composite haplotypes from different abalone species was greater than that between species. A neighbor-joining tree of different sequences was constructed after sequence divergence was estimated using Kimura's (1980) two parameter procedure. The phylogenetic tree allocated composite haplotype sequences into three groups according to species origin of the abalone accurately composing of AAAA and AAAE (*H. asinina*), AAAB, AABB and ABBB (*H. ovina*) and AABC, BABC, BABD, BABF and BABG (*H. varia*) (Fig. 3.21).

Table 3.11 Geographic heterogeneity analysis in distribution frequency of 16S rDNA composite haplotypes using a Monte Carlo simulation

	HAHt	HASt	HALb	HACbh	HACb	HAPhi	HOSi	HOSt	HOTg	HOSl	HVPhu	HVSl
HAHt	-											
HASt	**0.2586**^{ns}	**-**										
HALb	**0.6413** ^{ns}	**0.5681** ^{ns}	**-**									
HACbh	**0.2368** ^{ns}	**1.0000** ^{ns}	**0.5341** ^{ns}	**-**								
HACb	**0.1007** ^{ns}	**1.0000** ^{ns}	**0.4989** ^{ns}	**1.0000** ^{ns}	**-**							
HAPhi	**0.1062** ^{ns}	**1.0000** ^{ns}	**0.4994** ^{ns}	**1.0000** ^{ns}	**1.0000** ^{ns}	-						
HOSi	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	-					
HOSt	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	**1.0000** ^{ns}	**-**				
HOTg	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	**<0.0001***	**<0.0001***	**-**			
HOSl	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	**<0.0001***	**<0.0001***	**1.0000** ^{ns}	**-**		
HVPhu	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	-	
HVSl	0.0049^{ns}	0.0113^{ns}	0.0053^{ns}	0.0078^{ns}	0.0048^{ns}	0.0041*	0.0031*	0.0037*	0.0179 ^{ns}	0.0134 ^{ns}	**1.0000** ^{ns}	-

HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket; ^{ns} not significant; * P<0.0042 following a sequential Bonferroni method (Rice, 1989).

Table 3.12 Geographic heterogeneity analysis in distribution frequency of 18S rDNA and 16S rDNA composite haplotypes using a Monte Carlo simulation

	HAHt	HASt	HALb	HACbh	HACb	HAPhi	HOSi	HOSt	HOTg	HOSl	HVPhu	HVSl
HAHt	-											
HASt	**0.0184**^{ns}	**-**										
HALb	**0.0087**^{ns}	**0.1371** ^{ns}	**-**									
HACbh	**0.0106** ^{ns}	**0.0086** ^{ns}	**0.2670** ^{ns}	**-**								
HACb	**0.0293** ^{ns}	**0.5741** ^{ns}	**0.0657** ^{ns}	**0.0035**^{ns}	**-**							
HAPhi	**0.0007***	**0.0003***	**<0.0000***	**0.0085** ^{ns}	**0.0001***	-						
HOSi	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	-					
HOSt	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	**0.1264** ^{ns}	**-**				
HOTg	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	**<0.0001***	**<0.0001***	**-**			
HOSl	<0.0001*	<0.0001*	<0.0001*	0.0001*	<0.0001*	<0.0001*	**<0.0001***	**<0.0001***	**0.3668** ^{ns}	**-**		
HVPhu	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	-	
HVSl	0.0639 ^{ns}	0.0102 ^{ns}	0.0172 ^{ns}	0.0572 ^{ns}	0.0114 ^{ns}	0.0100 ^{ns}	0.0328 ^{ns}	0.0784 ^{ns}	0.1565 ^{ns}	0.1249 ^{ns}	**0.7710** ^{ns}	-

HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket; ^{ns} not significant; * P<0.0021 following a sequential Bonferroni method (Rice, 1989).

Table 3.13 Estimated gene flow (above diagonal) and F_{ST} (and P-value, below diagonal) between pairs of geographic samples based on restriction analysis of 16S rDNA

	HAHt	HASt	HALb	HACbh	HACb	HAPhi	HOSi	HOSt	HOTg	HOSl	HVPhu	HVSl
HAHt	-	**6.6572**	**very large**	**5.0891**	**3.6398**	**3.8096**	ND	ND	ND	ND	ND	ND
HASt	**0.0699 (0.2656)^{ns}**	-	**very large**	**Not possible**	**Not possible**	**Not possible**	ND	ND	ND	ND	ND	ND
HALb	**-0.0058 (0.6350)^{ns}**	**-0.00738 (0.5692)^{ns}**	-	**97.5392**	**21.8414**	**24.6004**	ND	ND	ND	ND	ND	ND
HACbh	**0.0895 (0.2385)^{ns}**	**- Not possible**	**0.0051 (0.5347)^{ns}**	-	**Not possible**	**Not possible**	ND	ND	ND	ND	ND	ND
HACb	**0.1208 (0.0977)^{ns}**	**- Not possible**	**0.02238 (0.4999)^{ns}**	**- Not possible**	-	**Not possible**	ND	ND	ND	ND	ND	ND
HAPhi	**0.1160 (0.1059)^{ns}**	**- Not possible**	**0.0199 (0.5035)^{ns}**	**- Not possible**	**- Not possible**	-	ND	ND	ND	ND	ND	ND
HOSi	ND	ND	ND	ND	ND	ND	-	**Not possible**	**0.0249**	**x**	ND	ND
HOSt	ND	ND	ND	ND	ND	ND	**- Not possible**	-	**0.0294**	**x**	ND	ND
HOTg	ND	ND	ND	ND	ND	ND	**0.9524 (0.0000)***	**0.9444 (0.0000)***	-	**very large**	ND	ND
HOSl	ND	ND	ND	ND	ND	ND	**1.0000 (0.0000)***	**1.0000 (0.0000)***	**-0.0299 (1.0000)^{ns}**	-	ND	ND
HVPhu	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	**very large**
HVSl	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	**-0.2489 (1.0000)**	-

HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket; ND=not determine; ^{ns} not significant; * P<0.0042 following a sequential Bonferroni method (Rice, 1989).

Table 3.14 Estimated gene flow (above diagonal) and F_{ST} (and P-value, below diagonal) between pairs of geographic samples based on restriction analysis of 18S rDNAs

	HAHt	HASt	HALb	HACbh	HACb	HAPhi	HOSi	HOSt	HOTg	HOSl	HVPhu	HVSl
HAHt	-	**0.7256**	**3.4914**	**3.1111**	**2.65023**	**1.3217**	ND	ND	ND	ND	ND	ND
HASt	**0.2563 (0.0083)**^{ns}	-	**1.2734**	**0.6021**	**5.4656**	**0.3013**	ND	ND	ND	ND	ND	ND
HALb	**0.0668 (0.0388)**^{ns}	**0.1641 (0.0141)**^{ns}	-	**47.9196**	**4.7792**	**1.1381**	ND	ND	ND	ND	ND	ND
HACbh	**0.0744 (0.0483)**^{ns}	**0.2934 (0.0022)***	**0.0052 (0.3347)**^{ns}	-	**1.4036**	**4.2135**	ND	ND	ND	ND	ND	ND
HACb	**0.0862 (0.0686)**^{ns}	**0.04374 (0.2712)**^{ns}	**0.0497 (0.0747)**^{ns}	**0.1512 (0.0058)**^{ns}	-	**0.5070**	ND	ND	ND	ND	ND	ND
HAPhi	**0.1591 (0.0008)***	**0.4535 (0.0000)***	**0.1801 (0.0001)***	**0.0560 (0.0566)**^{ns}	**0.3302 (0.0000)***	-	ND	ND	ND	ND	ND	ND
HOSi	ND	ND	ND	ND	ND	ND	-	**6.2469**	**1.6709**	**2.1408**	ND	ND
HOSt	ND	ND	ND	ND	ND	ND	**0.0385 (0.1173)**^{ns}	-	**8.53426**	**very large**	ND	ND
HOTg	ND	ND	ND	ND	ND	ND	**0.1296 (0.0035)***	**0.0285 (0.1754)**^{ns}	-	**very large**	ND	ND
HOSl	ND	ND	ND	ND	ND	ND	**0.1046 (0.0226)**^{ns}	**-0.0017 (0.4295)**^{ns}	**-0.0134 (0.5747)**^{ns}	-	ND	ND
HVPhu	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	**2.6039**
HVSl	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	**0.0876 (0.3125)**^{ns}	-

HA=*H. asinina*; HO=*H. ovina*; HV=*H. varia*; Ht=hatchery(P_0); St=Samet Island; Lb=Talibong Island; Cbh=hatchery(P_0),Cambodia; Cb=Cambodia; Phi=the Philippines; Si=Sichang Island; Tg=Trang; Sl=Similan Island; Phu=Phuket; ND=not determine; ^{ns} not significant; * $P<0.0042$ following a sequential Bonferroni method (Rice, 1989).

$16S_{F1}$

```
AABB    CGCCTGTTTAACAAAAACATGGCTCCTTGGTTGTCTGA-GTGGATGAGGAGTCGGACCTG
AAAB    CGCCTGTTTAACAAAAACATGGCTCCTTGGTTGTCTGA-GTGGATGAGGAGTCGGACCTG
ABBB    CGCCTGTTTAACAAAAACATGGCTCCTCGGTTGTTTGA-GTGGATGGGGAGTCGGACCTG
BABC    CGCCTGTTTAACAAAAACATGGCTCCTCGGTCAATTAGTGTGGATGGGGAGTCGGACCTG
BABF    CGCCTGTTTAACAAAAACATGGCTCCTCGGTCAATTAGTGTGGATGGGGAGTCGGACCTG
BABD    CGCCTGTTTAACAAAAACATGGCTCCTCGGTCAATTAGTGTGGATGGGGAGTCGGACCTG
AABC    CGCCTGTTTAACAAAAACATGGCTCCTCGGTCAATTAGTGTGGATGGGGAGTCGGACCTG
BABG    CGCCTGTTTAACAAAAACATGGCTCCTCGGTCAGCTAGTGTGGATGGGGAGTCGGACCTG
AAAA    CGCCTGTTTAACAAAAACATGGCTCCTTG--TGTTTTAGGCGGATAAGGAGTCGGACCTG
AAAE    CGCCTGTTTAACAAAAACATGGCTCCTTG--TGTTTTAGGCGGATAAGGAGTCGGACCTG
        *************************** *      *   * ****  *************

AABB    CCCGGTGACTTACGGGTTAAACGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAATCA
AAAB    CCCGGTGACTTACGGGTTAAACGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAATCA
ABBB    CCCGGTGACTTACGGGTTAAACGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAATCA
BABC    CCCGGTGAC-TACGGGTTAAACGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAATCA
BABF    CCCGGTGACCTACGGGTTAAACGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAATCA
BABD    CCCGGTGAC-TACGGGTTAAACGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAATCA
AABC    CCCGGTGAC-TACGGGTTAAACGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAATCA
BABG    CCCGGTGAC-TACGGGTTAAACGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAATCA
AAAA    CCCGGTGAC-TACGGGTTAAACGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAATCA
AAAE    CCCGGTGACCTACGGGTTAAACGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAATCA
        ********* **************************************************

AABB    CTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTCTG
AAAB    CTTGCCTCTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTCTG
ABBB    CTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTCTG
BABC    CTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTTTG
BABF    CTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTTTG
BABD    CTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTTTG
AABC    CTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTTTG
BABG    CTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTTTG
AAAA    CTTGCCTCTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTTTG
AAAE    CTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTTTG
        ******* ************************************************* **

AABB    AAATATTTAAAAATTAACTTCTAGGTGAAAAGGCTTAGATTAGGCTGAGGGACGAGAAGA
AAAB    AAATATTTAAAAATTAACTTCTAGGTGAAAAGGCCTAGATTAGGCTGAGGGACGAGAAGA
ABBB    AAATATTTAAAAATTAACTTCTAGGTGAAAAGGCTTAGATTAGGCTGAGGGACGAGAAGA
BABC    AAATATTTAAAAATTAACTTCTAGGTGAAAAGGCTTAGATTAAGCTGAGGGACGAGAAGA
BABF    AAATATTTAAAAATTAACTTCTAGGTGAAAAGGCTTAGATTAAGCTGAGGGACGAGAAGA
BABD    AAATATTTAAAAATTAACTTCTAGGTGAAAAGGCTTAGATTAAGCTGAGGGACGAGAAGA
AABC    AAATATTTAAAAATTAACTTCTAGGTGAAAAGGCTTAGATTAAGCTGAGGGACGAGAAGA
BABG    AAATATTTAAAAATTAACTTCTAGGTGAAAAGGCTTAGATTAAGCTGAGGGACGAGAAGA
AAAA    GAATATTTAAAAATTAACTTCTAGGTGAAAAGGCCTAGATTGAGCTGAGGGACGAGAAGA
AAAE    GAATATTTAAAAATTAACTTCTAGGTGAAAAGGCCTAGATTGAGCTGAGGGACGAGAAGA
         ********************************* ******  *****************
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$16S_{RHV}$

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AABB    CCCTGTTGAGCTTTAGTGTGGAGTGAAGGTTC-TAATTTCTAGTTGTACTAGAGAATTTA
AAAB    CCCTGTTGAGCTTTAGTGTGGAGTGAAGGTTC-TAATTTCTAGTTGTACTAGAGAATTTA
ABBB    CCCTGTTGAGCTTTAGTGTGGAGTGAAGGTTTATAATTTCTAGTTGTACTAGAGAATTCA
BABC    CCCTGTTGAGCTTTAGTGTGGAATGAAGGGGTGTGCTCCTGAAATAAGTTAGAGAGCTCA
BABF    CCCTGTTGAGCTTTAGTGTGGAATGAAGGGGTGTGCTCCTGAAATAAGTTAGAGAGCTCA
BABD    CCCTGTTGAGCTTTAGTGTGGAATGAAGGGGTGTGCTCCTGAAATAAGTTAGAGAGCTCA
AABC    CCCTGTTGAGCTTTAGTGTGGAATGAAGGGGTGTGCTCCTGAAATAAGTTAGAGAGCTCA
BABG    CCCTGTTGAGCTTTAGTGTGGAATGAAGGGGTGTGCTCCTGAAATAAGTTAGAGAGCTCA
AAAA    CCCTGTTGAGCTTTAGTGTTGAATAAAGGTTTATATTTCCTAGTTGAATTTAGAGGTTTA
AAAE    CCCTGTTGAGCTTTAGTGTTGAATAAAGGTTTATATTTCCTAGTTGAATTTAGAGGTTTA
        ******************* ** * ****    *  *    *  *    *       * *
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$16S_{RHO}$

```
AABB    AATTCATTTTTACATCTTTAGTTGGGGTGACTGGGGAACATAGGTAGCTTCTCTGTTTTT
AAAB    AATTCATTTTTACATCTTTAGTTGGGGTGACTGGGGAACATAGGTAGCTTCTCTGTTTTT
ABBB    AATTCATTTTTACATCTTTAGTTGGGGTGACTGGGGAACATAGGTAGCTTCTCTGTTTTT
BABC    GGTTTGTTCTTACATCTTTAGTTGGGGTGACTGGGGAACATAAGAAGCTTCCCTGTTTAT
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BABF            GGTTTGTTCTTACATCTTTAGTTGGGGTGACTGGGGAACATAAGAAGCTTCCCTGTTTAT
BABD            GGTTTGTTTTTACATCTTTAGTTGGGGTGACTGGGGAACATAAGAAGCTTCCCTGTTTAT
AABC            GGTTTGTTTTTACATCTTTAGTTGGGGTGACTGGGGAACATAAGAAGCTTCCCTGTTTAT
BABG            GGTTTGTTCTTACATCTTTAGTTGGGGTGACTGGGGAACATAAGAAGCTTCCCTGTTTAT
AAAA            AGTTTATTCTTACATCTTTAGTTGGGGTGACTGGGGAACAAAAGTAGCTTCTCTACTTAT
AAAE            AGTTTATTCTTACATCTTTAGTTGGGGTGACTGGGGAACAAAAGTAGCTTCTCTACTTAT
                  **  ** ******************************* * * ****** **  ** *
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16S$_{RHA}$

```
AABB            AGTA-AATTAAATTTGGTCTGCTGACTGATGATCCGGCATTGTCGATTATCGGAAAAAGT
AAAB            AGTA-AATTAAATTTGGTCTGCTGACTGATGATCCGGCATTGTCGATTATCGGAAAAAGT
ABBB            AGTA-GATTGAATTTGGTCTGCCGACTGATGATCCGACATTGTCGATTATCGGAAAAAGT
BABC            AGTTTAATTATTTTCGGTTTTCTGACTAAGGATCCAGCATTGCTGATTGTCGGAAAAAGT
BABF            AGTTTAATTATTTTCGGTTTTCTGACTAAGGATCCAGCATTGCTGATTGTCGGAAAAAGT
BABD            AGTTTAATTATTTTCGGTTTTCTGACTAAGGATCCAGCATTGCTGATTGTCGGAAAAAGT
AABC            AGTTTAATTATTTTCGGTTTTCTGACTAAGGATCCAGCATTGCTGATTGTCGGAAAAAGT
BABG            AGTTTAATAATTTTTGGTTTTCTGACTAAGGATCCAGCATTGCTGATTGTCGGAAAAAGT
AAAA            -----TACTGGGTTTGGCTTGCTAGCTAATGATCCGGCATTGCTGATTATTGGAAAAAGT
AAAE            -----TACTGGGTTTGGCTTGCTAGCTAATGATCCGGCATTGCTGATTATTGGAAAAAGT
                      *     ** **  * *   ** * *****  *****  **** * *********

AABB            TACCACAGGGATAACAGCGTAATCTTTCTGGAGAGTTCACATTGAAAGAAGGGTTTGCGA
AAAB            TACCACAGGGATAACAGCGTAATCTTTCTGGAGAGTTCACATTGAAAGAAGGGTTTGCGA
ABBB            TACCACAGGGATAACAGCGTAATCTTTCTGGAGAGTTCACATTGAAAGAAGGGTTTGCGA
BABC            TACCACAGGGATAACAGCGTAATCTTTCTGGAGAGTTCACATTGAAAGAAGGGTTTGCGA
BABF            TACCACAGGGATAACAGCGTAATCTTTCTGGAGAGTTCACATTGAAAGAAGGGTTTGCGA
BABD            TACCACAGGGATAACAGCGTAATCTTTCTGGAGAGTTCACATTGAAAGAAGGGTTTGCGA
AABC            TACCACAGGGATAACAGCGTAATCTTTCTGGAGAGTTCACATTGAAAGAAGGGTTTGCGA
BABG            TACCACAGGGATAACAGCGTAATCTTTCTGGAGAGTTCACATTGAAAGAAGGGTTTGCGA
AAAA            TACCACAGGGATAACAGCGTAATCTTTTTGGAGAGTTCATATTGAAAAAAGGGTTTGCGA
AAAE            TACCACAGGGATAACAGCGTAATCTTTTTGGAGAGTTCATATTGAAAAAAGGGTTTGCGA
                *************************** *********** ******* ************

AABB            CCTCGATGTTGGATTAAGGTGTCCTGAGGGTGTAGCAGCTTTCGTTGGTTGGTCTGTTCG
AAAB            CCTCGATGTTGGATTAAGGTGTCCTGAGGGTGTAGCAGCTTTCGTTGGTTGGTCTGTTCG
ABBB            CCTCGATGTTGGATTAAGGTGTCCTGGGGGTGTAGCAGCTTTCGTTGGTTGGTCTGTTCG
BABC            CCTCGATGTTGGATTAAGGTGTCCTGAGGGTGTAGCAGCTTTCGTTGGTTGGTCTGTTCG
BABF            CCTCGATGTTGGATTAAGGTGTCCTGAGGGTGTAGCAGCTTTCGTTGGTTGGTCTGTTCG
BABD            CCTCGATGTTGGATTAAGGTGTCCTGAGGGTGTAGCAGCTTTCGTTGGTTGGTCTGTTCG
AABC            CCTCGATGTTGGATTAAGGTGTCCTGAGGGTGTAGCAGCTTTCGTTGGTTG-TCTGTTCG
BABG            CCTCGATGTTGGATTAAGGTGTCCTGAGGGTGTAACAGCTTTCGTTGGTTGGTCTGTTCG
AAAA            CCTCGATGTTGGATTAAGGTGTCCTAAGGGTGTAGCAGCTCTTGTTGGTTGGTCTGTTCG
AAAE            CCTCGATGTTGGATTAAGGTGTCCTAAGGGTGTAGCAGCTCTTGTTGGTTGGTCTGTTCG
                *************************  ******* ***** * ******** ********

AABB            ACCATTAAAACCTTACATGATCTGA-----GTTCAGACCGG
AAAB            ACCATTAAAACCTTACATGATCTGA-----GTTCAGACCGG
ABBB            ACCATTAAAACCTTACGTGATCTGATCTGAGTTCAGACCGG
BABC            ACCATTAAAACCTTACATGATCTGA-----GTTCAGACCGG
BABF            ACCATTAAAACCTTACATGATCTGA-----GTTCAGACCGG
BABD            ACCATTAAAACTTTACGTGATCTGATCTGAGTTCAGACCGG
AABC            ACCATTAAAACCTTACATGATCTGA-----GTTCAGACCGG
BABG            ACCATTAAAACCTTACATGATCTGA-----GTTCAGACCGG
AAAA            ACCATTAAATCCTTACATGATCTGA-----GTTCAGACCGG
AAAE            ACCATTAAAACCTTACATGATCTGA-----GTTCAGACCGG
                ********* ****** ********     ***********
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Fig. 3.20 Sequence aligment of abalone individuals exhibiting different composite haplotype of 16S rDNA found in Thai abalone. Asterisks indicate identical bases among compared sequences. Regions used to design primers for species-specific PCR are illustrated in boldface.

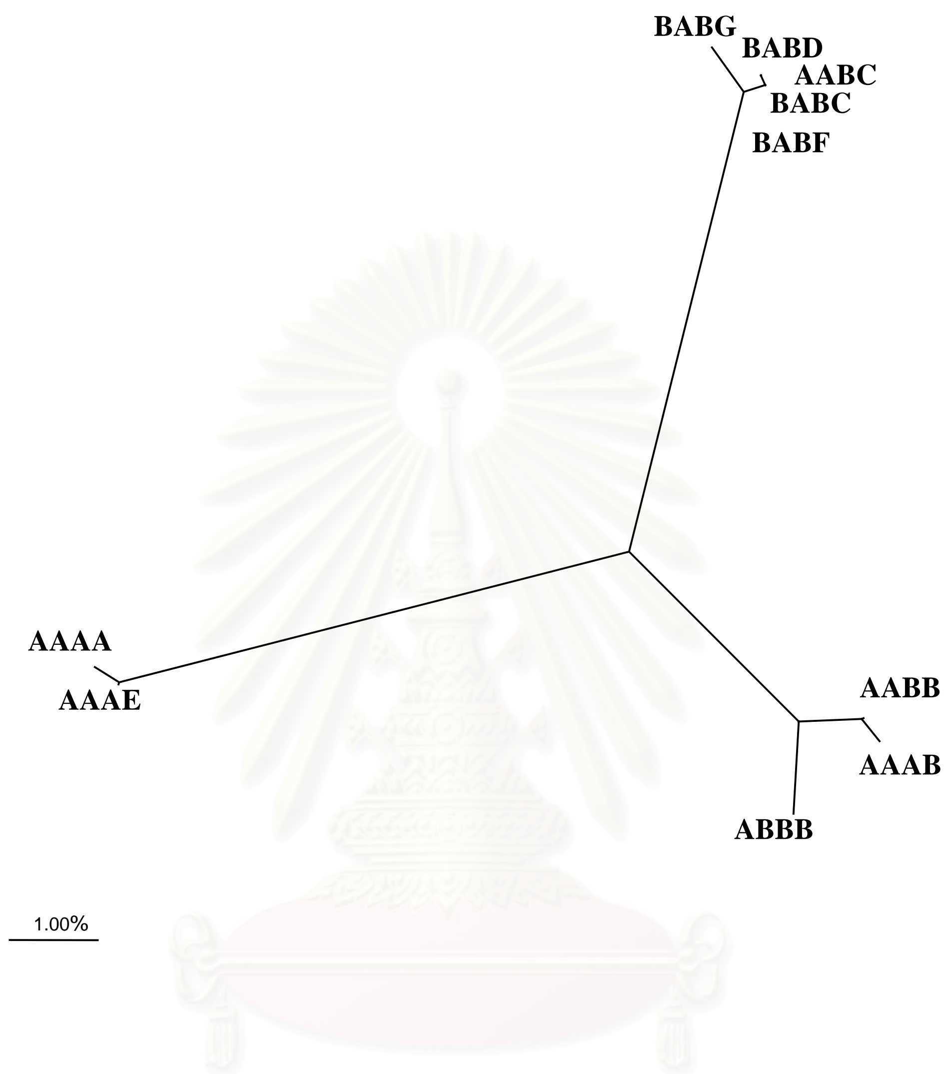

Fig 3.21 A neighbor-joining tree indicating relationships between abalone possessing different 16S rDNA composite haplotypes based on DNA sequences.

CHAPTER IV

DISCUSSION

Analysis of genetic diversity and differentiation at intra- and inter specific levels is essential for genetic researches (e.g. gene mapping, individuality and parentage, population genetics, phylogenetics, molecular taxonomy and systematics, and evolutionary studies) of various organisms.

Using RAPD analysis, a few promising genetic markers for differentiation of the commercially cultured species, *H. asinina* from *H. ovina* and *H. varia* were found. Results from RAPD analysis using INS, YN73 and M13 primers suggested that genetic diversity of *H. asinina* was lower than *H. ovina* and *H. varia*. Therefore, development of *H. asinina*-specific markers was possible due to the nature of its genetic diversity. Nonetheless, amplification of these microsatellite primers was not consistent. Therefore, RAPD-PCR was not carried out further.

PCR-RFLP of 18S and 16S rDNAs provided useful information on species identification, population differentiation and diversity of investigated abalone. Species-diagnostic markers were found both from single enzyme digestion (16S rDNA-*Alu* I) and composite haplotypes (16S rDNA and combined 18S and 16S rDNAs). In addition, a discrimination test on the species origin of suspected specimens could also be carried out by sequential digestion of 16S rDNA. For example, a digestion pattern of 16S rDNA with *Bam*H I and *Hae* III could differentiate all *H. varia* speciems from *H. asinina* and *H. ovina*.

Only 16S rDNA polymorphism was sufficient for species identification of *H. asinina*, *H. ovina*, and *H. varia* in this study. However, genetic patterns of 18S rDNA alone were not possible to distinguish different abalone species.

For quality control of exported abalone, species-specific markers for differentiation of the target species, *H. midae* and suspected *H. spadicea* were developed based on species-specific PCR and PCR-RFLP approaches. The PCR primers designed from the lysin gene sequences specifically amplified a 1300 bp fragment from genomic DNA of dried, cooked and fresh abalone tissues. A smaller fragment (146 bp) was used to verify that the canned abalone in question is *H. midae* (Sweijd *et al*., 1998).

Although composite haplotypes of 16S rDNA and 18S+16S rDNAs in *H. asinina*, *H. ovina*, and *H. varia* were not overlapping distributed among different abalone species, discrimination power was resulted from restriction patterns of 16S rDNA. This indicated well separate maternal lineages between *H. asinina*, *H. ovina*, and *H. varia*.

Limited genetic diversity within *H. asinina* and *H. varia* was observed when analyzed with mitochondrial DNA (16S rDNA). The haplotype diversity of investigated sample of each species was greater when 18S rDNA was included into the analysis. However, estimation of haplotype diversity depends solely on haplotype frequencies alone. Therefore, it is sensitive to the number of gene regions and restriction enzymes used in the experiment. As more region and/or enzymes are used,

more haplotypes can be detected, increasing the value of haplotype diversity (Nei, 1987, Graves and McDowell, 1994).

In contrast, nucleotide diversity within species provided more accurate estimation than haplotype diversity (average 0.2483% and 0.3716% overall samples). This parameter found in the present study was greater than that of oysters; *Crassostrea belcheri* (0.0945%) and *C. iredalei* (0.0912%) but comparable to that of the oyster (*Saccostrea cucullata*) and much less than that of *S. forskali* (2.8711%), *Striostrea* (*Parastriostrea*) *mytiloides* (3.2388%) and the giant tiger prawn, *Penaeus monodon* (3.328%).

The nucleotide divergence between geographic samples within each species was extremely low for both data sets. The results in this study was concordant with those of *H. cracherodii* along the central California coast where low genetic distance (0.001-0.057) resulted from allozyme (GPI, AAT-1, and PGM) and COI sequence analysis of specimens collected from geographic distance covering 300 km were found (Hamm and Burton, 2000).

Conversely, large nucleotide divergences were observed between abalone species. The percentage nucleotide divergence between paired sample from different species was 1.06% - 4.85%. Naganuma *et al*. (1998) examined divergence of COI between two morphological resamblant abalone, *H. discus discus* and *H. discus hannai* and found that the percentage sequence divergence between these taxa was 0.8%. Comparisons of those sequences with DNA sequences of the outgroups (*H. madaka* and *H. gigantea*) revealed the divergence about 5.0%.

Misclustering of composite haplotypes and allocation of *H. ovina* originating from the Andaman Sea with *H. asinina* should have resulted from the use of insufficient number of restriction endonucleases in this study. Although species identification was successfully developed, estimation of genetic diversity level should be reexamined using more gene regions and/or restriction endonucleases. RAPD analysis of the same sample set using RAPD (Popongviwat, personal communication) and microsatellite analyses (Tang, personal communication) illustrated obvious differentiation between *H. asinina*, *H. ovina*, and *H. varia* phylogenetically. The use of 16S rDNA sequences from individuals representing all composite haplotypes of investigated abalone showed identical tree topology with that from RAPD and microsatellite analysis.

Hybridization between two species of abalone (*H. rubra* and *H. laevigata*) and introgression of gene of each species into the gene pool of the other was reported using allozyme analysis (Brown, 1995). Interspecific hybrids of the California abalone could be produced by laboratory crosses. Moreover, morphological evidence also indicated the existence of interspecific hybridization of the commercial catch of the California abalone (Leighton and Lewis, 1982).

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It was expected that hybridization between different abalone in this study could be occurred but there have been no evidences on that phenomenon. PCR-RFLP analysis of 16S rDNA suggested a lack of bi-directional interspecific hybridization due to complete disassociation of mtDNA composite haplotypes between *H. asinina*, *H. ovina*, and *H. varia*. Nevertheless, the possibility of unidirectional hybridization between species (female of *H. asinina*, *H. ovina* or *H. varia* crossed with male of

different species) could not be completely eliminated because this circumstance cannot be examined by mtDNA markers.

Muchmore *et al*. (1998) identified tandemly repeated satellite DNA (290-291 bp in length) in five species of eastern Pacific abalone (*H. rufescens*, *H. kamtschatkana*, *H. corrugata*, *H. sorenseni*, and *H. walallensis*). Satellite specific primers were designed and used to determine the consensus sequences of five abalone species by direct sequencing of the PCR product. The specific sequence of this satellite could be used for identification of hybrid parentage, taxonomy, population identification and forensic studies.

Phylogenetic errors based on PCR-RFLP in this study are good example about the bridge between molecular diagnosis of sample/species and genetic diversity and phylogenetic studies. The former requires rapid and accurate method of the experiment. Therefore, species identification should be identified using the less number of restriction enzymes as possible. In contrast, accurate genetic diversity and phylogenic analysis should have been obtained if more number of restriction endonucleases are included in the analysis.

The topology of UPGMA dendrograms between composite haplotypes, geographically different samples, and species of abalone in this study indicated that *H. asinina* and *H. ovina* are genetically closely related whereas *H. varia* was more distantly related to those species. The results were not concordant with karyotyping of choromosomes of these abalone (Jarayabhand *et al*., 1998) where *H. asinina* and *H. varia* were regarded as more closely related species. Parallel studies of this thesis

based on RAPD analysis and sequencing analysis of 16S rDNA in this study confirms interspecific genetic relationships of Thai abalone reported by PCR-RFLP.

Large genetic discontinuity was found between *H. ovina* originating from the west (the Andaman Sea) and east (the Gulf of Thailand) of peninsular Thailand reflecting strong genetic differentiation of this species. This was also consistently supported by geographic heterogeneity and F_{ST} analyses ($P < 0.0001$). Genetic population structure within *H. asinina* were observed only when 18S rDNA was included into the analysis ($P < 0.0021$). A lack of population differentiation was found in small sample sizes of *H. varia*.

Huang *et al*. (2000) investigated populaton differentiation of the blacklip abalone (*H. rubra*) of Victoria, Australia using RAPD-PCR (UBC101, UBC135, UNC149, UBC159, UBC169, and RM13), minisatellites (GHR and MIPR) and microsatellites (RBUGT1, RUBCA1, and RUBGACA1). All types of DNA markers revealed intraspecific genetic differentiation in this species relating to the relatively short period and limited dispersion of abalone.

Moreover, evolution and systematics of 27 abalone species were examined using cDNA sequences of the lysin gene. The phylogeny of lysin cDNA suggested three phylogenetic groups composing of 1) all California species and three Japanese species (*H. gigantea*, *H. discus hannei*, and *H. madaka*), 2) *H. iris* from the New Zealand and 3) *H. diversicolor aquatilis* from Japan, Indo-West Pacific species and European species. Phylogenetic relationships indicated that *H. ovina* and *H. varia* were sister taxa. However, *H. asinina* was not included in their study.

Disregarding the Philippines sample, a panmictic gene pool was found in *H. asinina* investigated in this study. Therefore, establishment of the appropriate propagated stock for aquaculture does not require populations from several geographic locations. On the other hand, strong differentiation between *H. ovina* from different coastal regions suggested that these populations should be treated as separate management units. Transferring of different stocks of *H. ovina* to other habitat should be limited.

Assuming neutral mutation of molecular markers used in this study, biased gene flow between gender may have occurred. Based on the fact that, discriminatory power of 18S rDNA was lower than that of 16S rDNA, differentiation of *H. asinina* from the Philippines and the remaining geographic samples when the former was included into the analysis, restricted male gene flow may possible be existent in the Philippines sample.

Although species-specific markers was successfully developed based on PCR-RFLP approach, sequences of 16S rDNA in this study can be used to develop species-specific PCR which is more rapid and convenient and less expensive. Currently, that specific PCR is being developed. Species-specific PCR is completely successful in *H. varia*. Moreover, over 95% of *H. asinina* were also able to be specifically identified by PCR while *H. ovina* could not be determined specifically (N. Khamnumtong, personal communication).

Restriction analysis of mtDNA has been successfully used to estimate levels of genetic diversity and/or to identify population differentiation in several

commercially important marine species in Thailand including the cupped oysters; *C. belcheri, C. iredalei, S. cucullata, S. forskali*, *and S. mytiloides* (Khamnumtong, 2000) and the giant tiger prawn, *P. monodon* (Klinbunga *et al*., 1999 and 2001). The ability to identify species origins of Thai abalone in this study is crucial for the construction of broodstock management and conservation programmes in these taxa. Following which, these molecular markers can also be used for comparisons of growth performance among three abalone species in communal setting conditions.

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CHAPTER V

CONCLUSIONS

1. Species-specific markers were found in *H. asinina*, *H. ovina*, and *H. varia* using PCR-RFLP analysis of 18S and 16S rDNAs.

2. Forty-nine composite haplotypes were generated from digested 18S rDNA with *Alu* I, *Taq* I, and *Hae* III, and 16S rDNA with *Bam*H I, *Eco*R I, *Hae* III, and *Alu* I. No overlapping between composite haplotypes of different species was found.

3. Nucleotide divergence and phylogenetic studies revealed distant relationships between abalone species but closer relationships were observed within each species. *H. asinina* and *H. ovina* were closely related one another than *H. varia.*

4. Genetic population differentiation was clearly observed in *H. ovina*. Differentiation of *H. asinina* was observed when both 18S and 16S rDNA were used but not the 16S rDNA alone.

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDICES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix A

Code of samples, geographic location, and species of abalone used in this study.

Code of samples	Geographic location	Species
HA001	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA002	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA003	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA004	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA005	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA006	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA007	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA008	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA009	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA010	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA011	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA012	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA013	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA014	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA015	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA016	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA017	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA018	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*
HA019	Angsila, Chonburi (Hatchery, P_0)	*H. asinina*

Code of samples	Geographic location	Species
HA041	Samet Island, Rayong	*H. asinina*
HA042	Samet Island, Rayong	*H. asinina*
HA043	Samet Island, Rayong	*H. asinina*
HA044	Samet Island, Rayong	*H. asinina*
HA045	Samet Island, Rayong	*H. asinina*
HA046	Samet Island, Rayong	*H. asinina*
HA047	Samet Island, Rayong	*H. asinina*
HA048	Samet Island, Rayong	*H. asinina*
HA049	Samet Island, Rayong	*H. asinina*
HA050	Samet Island, Rayong	*H. asinina*
HA051	Samet Island, Rayong	*H. asinina*
HA052	Samet Island, Rayong	*H. asinina*
HL001	Libong Island, Trang	*H. asinina*
HL002	Libong Island, Trang	*H. asinina*
HL003	Libong Island, Trang	*H. asinina*
HL004	Libong Island, Trang	*H. asinina*
HL005	Libong Island, Trang	*H. asinina*
HL006	Libong Island, Trang	*H. asinina*
HL007	Libong Island, Trang	*H. asinina*
HL008	Libong Island, Trang	*H. asinina*
HL009	Libong Island, Trang	*H. asinina*
HL010	Libong Island, Trang	*H. asinina*
HL011	Libong Island, Trang	*H. asinina*

Code of samples	Geographic location	Species
HL012	Libong Island, Trang	*H. asinina*
HL013	Libong Island, Trang	*H. asinina*
HL014	Libong Island, Trang	*H. asinina*
HL015	Libong Island, Trang	*H. asinina*
HL016	Libong Island, Trang	*H. asinina*
HL017	Libong Island, Trang	*H. asinina*
HL018	Libong Island, Trang	*H. asinina*
HL019	Libong Island, Trang	*H. asinina*
HL020	Libong Island, Trang	*H. asinina*
HL021	Libong Island, Trang	*H. asinina*
HL022	Libong Island, Trang	*H. asinina*
HL023	Libong Island, Trang	*H. asinina*
HL024	Libong Island, Trang	*H. asinina*
HL025	Libong Island, Trang	*H. asinina*
HL026	Libong Island, Trang	*H. asinina*
HL027	Libong Island, Trang	*H. asinina*
HL028	Libong Island, Trang	*H. asinina*
HA081	Cambodia (Hatchery)	*H. asinina*
HA082	Cambodia (Hatchery)	*H. asinina*
HA083	Cambodia (Hatchery)	*H. asinina*
HA084	Cambodia (Hatchery)	*H. asinina*
HA085	Cambodia (Hatchery)	*H. asinina*
HA086	Cambodia (Hatchery)	*H. asinina*

Code of samples	Geographic location	Species
HA087	Cambodia (Hatchery)	*H. asinina*
HA088	Cambodia (Hatchery)	*H. asinina*
HA089	Cambodia (Hatchery)	*H. asinina*
HA090	Cambodia (Hatchery)	*H. asinina*
HA091	Cambodia (Hatchery)	*H. asinina*
HA092	Cambodia (Hatchery)	*H. asinina*
HA093	Cambodia (Hatchery)	*H. asinina*
HA094	Cambodia (Hatchery)	*H. asinina*
HA095	Cambodia (Hatchery)	*H. asinina*
HA101	Cambodia	*H. asinina*
HA102	Cambodia	*H. asinina*
HA103	Cambodia	*H. asinina*
HA104	Cambodia	*H. asinina*
HA105	Cambodia	*H. asinina*
HA106	Cambodia	*H. asinina*
HA107	Cambodia	*H. asinina*
HA108	Cambodia	*H. asinina*
HA109	Cambodia	*H. asinina*
HA110	Cambodia	*H. asinina*
HA111	Cambodia	*H. asinina*
HA112	Cambodia	*H. asinina*
HA113	Cambodia	*H. asinina*
HA114	Cambodia	*H. asinina*

Code of samples	Geographic location	Species
HA115	Cambodia	*H. asinina*
HA116	Cambodia	*H. asinina*
HA117	Cambodia	*H. asinina*
HA118	Cambodia	*H. asinina*
HA119	Cambodia	*H. asinina*
HA120	Cambodia	*H. asinina*
HA121	Cambodia	*H. asinina*
HP001	Philippines	*H. asinina*
HP002	Philippines	*H. asinina*
HP003	Philippines	*H. asinina*
HP004	Philippines	*H. asinina*
HP005	Philippines	*H. asinina*
HP006	Philippines	*H. asinina*
HP007	Philippines	*H. asinina*
HP008	Philippines	*H. asinina*
HP009	Philippines	*H. asinina*
HP010	Philippines	*H. asinina*
HP011	Philippines	*H. asinina*
HP012	Philippines	*H. asinina*
HP013	Philippines	*H. asinina*
HP014	Philippines	*H. asinina*
HP015	Philippines	*H. asinina*
HP016	Philippines	*H. asinina*

Code of samples	Geographic location	Species
HP017	Philippines	*H. asinina*
HP018	Philippines	*H. asinina*
HP019	Philippines	*H. asinina*
HP020	Philippines	*H. asinina*
HO001	Sichang Island, Chonburi	*H. ovina*
HO002	Sichang Island, Chonburi	*H. ovina*
HO003	Sichang Island, Chonburi	*H. ovina*
HO004	Sichang Island, Chonburi	*H. ovina*
HO005	Sichang Island, Chonburi	*H. ovina*
HO006	Sichang Island, Chonburi	*H. ovina*
HO007	Sichang Island, Chonburi	*H. ovina*
HO008	Sichang Island, Chonburi	*H. ovina*
HO009	Sichang Island, Chonburi	*H. ovina*
HO010	Sichang Island, Chonburi	*H. ovina*
HO011	Sichang Island, Chonburi	*H. ovina*
HO012	Sichang Island, Chonburi	*H. ovina*
HO013	Sichang Island, Chonburi	*H. ovina*
HO014	Sichang Island, Chonburi	*H. ovina*
HO015	Sichang Island, Chonburi	*H. ovina*
HO016	Sichang Island, Chonburi	*H. ovina*
HO017	Sichang Island, Chonburi	*H. ovina*
HO018	Sichang Island, Chonburi	*H. ovina*
HO019	Sichang Island, Chonburi	*H. ovina*

Code of samples	Geographic location	Species
HO020	Sichang Island, Chonburi	*H. ovina*
HO021	Sichang Island, Chonburi	*H. ovina*
HO022	Sichang Island, Chonburi	*H. ovina*
HO023	Sichang Island, Chonburi	*H. ovina*
HO024	Sichang Island, Chonburi	*H. ovina*
HO041	Samet Island, Rayong	*H. ovina*
HO042	Samet Island, Rayong	*H. ovina*
HO043	Samet Island, Rayong	*H. ovina*
HO044	Samet Island, Rayong	*H. ovina*
HO045	Samet Island, Rayong	*H. ovina*
HO046	Samet Island, Rayong	*H. ovina*
HO047	Samet Island, Rayong	*H. ovina*
HO048	Samet Island, Rayong	*H. ovina*
HO049	Samet Island, Rayong	*H. ovina*
HO050	Samet Island, Rayong	*H. ovina*
HO051	Samet Island, Rayong	*H. ovina*
HO052	Samet Island, Rayong	*H. ovina*
HO053	Samet Island, Rayong	*H. ovina*
HO054	Samet Island, Rayong	*H. ovina*
HO055	Samet Island, Rayong	*H. ovina*
HO056	Samet Island, Rayong	*H. ovina*
HO057	Samet Island, Rayong	*H. ovina*
HO058	Samet Island, Rayong	*H. ovina*

Code of samples	Geographic location	Species
HT001	Churk Island, Trang	*H. ovina*
HT002	Churk Island, Trang	*H. ovina*
HT003	Churk Island, Trang	*H. ovina*
HT004	Churk Island, Trang	*H. ovina*
HT005	Churk Island, Trang	*H. ovina*
HT006	Churk Island, Trang	*H. ovina*
HT007	Churk Island, Trang	*H. ovina*
HT008	Churk Island, Trang	*H. ovina*
HT009	Churk Island, Trang	*H. ovina*
HT010	Churk Island, Trang	*H. ovina*
HT011	Churk Island, Trang	*H. ovina*
HT012	Churk Island, Trang	*H. ovina*
HT013	Churk Island, Trang	*H. ovina*
HT014	Churk Island, Trang	*H. ovina*
HT015	Churk Island, Trang	*H. ovina*
HT016	Churk Island, Trang	*H. ovina*
HT017	Churk Island, Trang	*H. ovina*
HT018	Churk Island, Trang	*H. ovina*
HO081	Similan Island, Phangnga	*H. ovina*
HO082	Similan Island, Phangnga	*H. ovina*
HO083	Similan Island, Phangnga	*H. ovina*
HO084	Similan Island, Phangnga	*H. ovina*
HO085	Similan Island, Phangnga	*H. ovina*

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Code of samples	Geographic location	Species
HO086	Similan Island, Phangnga	*H. ovina*
HO087	Similan Island, Phangnga	*H. ovina*
HO088	Similan Island, Phangnga	*H. ovina*
HO089	Similan Island, Phangnga	*H. ovina*
HO090	Similan Island, Phangnga	*H. ovina*
HO091	Similan Island, Phangnga	*H. ovina*
HV001	L-Island, Phuket	*H. varia*
HV002	L-Island, Phuket	*H. varia*
HV003	L-Island, Phuket	*H. varia*
HV004	L-Island, Phuket	*H. varia*
HV005	L-Island, Phuket	*H. varia*
HV006	L-Island, Phuket	*H. varia*
HV007	L-Island, Phuket	*H. varia*
HV008	L-Island, Phuket	*H. varia*
HV009	L-Island, Phuket	*H. varia*
HV010	L-Island, Phuket	*H. varia*
HV011	L-Island, Phuket	*H. varia*
HV012	L-Island, Phuket	*H. varia*
HV013	L-Island, Phuket	*H. varia*
HV014	L-Island, Phuket	*H. varia*
HV015	L-Island, Phuket	*H. varia*
HV016	L-Island, Phuket	*H. varia*
HV017	L-Island, Phuket	*H. varia*

Code of samples	Geographic location	Species
HV018	L-Island, Phuket	*H. varia*
HV019	L-Island, Phuket	*H. varia*
HV020	L-Island, Phuket	*H. varia*
HV021	L-Island, Phuket	*H. varia*
HV031	Similan Island, Phangnga	*H. varia*
HV032	Similan Island, Phangnga	*H. varia*

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix B

Summary of restriction patterns of 18S rDNA and 16S rDNA of three abalone species digested with restriction endonucleases.

Sample	Restriction enzyme						
	18S rDNA			**16S rDNA**			
	***Alu* I**	***Taq* I**	***Hae* III**	***Bam*H I**	***Eco*R I**	***Hae* III**	***Alu* I**
HA001	B	B	A	A	A	A	A
HA002	A	C	A	A	A	A	A
HA003	B	C	A	A	A	A	A
HA004	B	B	A	A	A	A	E
HA005	B	B	A	A	A	A	A
HA006	B	B	A	A	A	A	A
HA007	B	B	A	A	A	A	A
HA008	B	B	A	A	A	A	A
HA009	B	A	A	A	A	A	A
HA010	B	A	A	A	A	A	A
HA011	B	A	A	A	A	A	E
HA012	B	A	A	A	A	A	E
HA013	B	A	A	A	A	A	A
HA014	B	A	A	A	A	A	A
HA015	B	A	A	A	A	A	A
HA016	B	A	A	A	A	A	A
HA017	C	A	A	A	A	A	A
HA018	B	A	A	A	A	A	A
HA019	B	A	A	A	A	A	A
HA041	B	A	A	A	A	A	A
HA042	B	A	A	A	A	A	A
HA043	B	A	A	A	A	A	A

Sample	**Restriction enzyme**						
	18S rDNA			**16S rDNA**			
	***Alu* I**	***Taq* I**	***Hae* III**	***Bam*H I**	***Eco*R I**	***Hae* III**	***Alu* I**
HA044	B	A	A	A	A	A	A
HA045	B	A	A	A	A	A	A
HA046	B	A	A	A	A	A	A
HA047	B	A	A	A	A	A	A
HA048	B	A	A	A	A	A	A
HA049	B	A	A	A	A	A	A
HA050	B	A	A	A	A	A	A
HA051	B	A	A	A	A	A	A
HA052	B	A	A	A	A	A	A
HL001	B	A	A	A	A	A	A
HL002	B	A	A	A	A	A	E
HL003	B	A	A	A	A	A	A
HL004	B	A	A	A	A	A	A
HL005	B	A	A	A	A	A	A
HL006	B	A	A	A	A	A	E
HL007	B	A	A	A	A	A	A
HL008	B	A	A	A	A	A	A
HL009	B	A	A	A	A	A	A
HL010	A	A	A	A	A	A	A
HL011	A	A	A	A	A	A	A
HL012	B	A	A	A	A	A	A
HL013	B	A	A	A	A	A	A
HL014	B	A	A	A	A	A	A
HL015	B	A	A	A	A	A	A
HL016	B	A	A	A	A	A	A
HL017	A	A	A	A	A	A	A
HL018	A	B	A	A	A	A	A

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Sample	Restriction enzyme						
	18S rDNA			16S rDNA			
	Alu I	*Taq* I	*Hae* III	*Bam*H I	*Eco*R I	*Hae* III	*Alu* I
HL019	A	A	A	A	A	A	A
HL020	A	A	A	A	A	A	A
HL021	B	A	A	A	A	A	A
HL022	B	A	A	A	A	A	A
HL023	C	A	B	A	A	A	A
HL024	C	A	A	A	A	A	A
HL025	C	A	A	A	A	A	A
HL026	C	A	A	A	A	A	A
HL027	E	A	A	A	A	A	A
HL028	E	A	A	A	A	A	A
HA081	B	A	A	A	A	A	A
HA082	B	A	A	A	A	A	A
HA083	B	A	A	A	A	A	A
HA084	C	A	B	A	A	A	A
HA085	B	A	A	A	A	A	A
HA086	B	A	A	A	A	A	A
HA087	A	A	A	A	A	A	A
HA088	C	A	A	A	A	A	A
HA089	A	A	A	A	A	A	A
HA090	A	B	A	A	A	A	A
HA091	A	B	A	A	A	A	A
HA092	B	A	A	A	A	A	A
HA093	C	A	B	A	A	A	A
HA094	B	A	B	A	A	A	A
HA095	C	A	B	A	A	A	A
HA101	B	A	A	A	A	A	A
HA102	B	A	A	A	A	A	A

Sample	Restriction enzyme						
	18S rDNA			16S rDNA			
	Alu I	*Taq* I	*Hae* III	*Bam*H I	*Eco*R I	*Hae* III	*Alu* I
HA103	B	A	A	A	A	A	A
HA104	B	A	A	A	A	A	A
HA105	B	A	A	A	A	A	A
HA106	B	A	A	A	A	A	A
HA107	A	A	A	A	A	A	A
HA108	B	A	A	A	A	A	A
HA109	B	A	A	A	A	A	A
HA110	B	A	A	A	A	A	A
HA111	B	A	A	A	A	A	A
HA112	B	A	A	A	A	A	A
HA113	B	A	A	A	A	A	A
HA114	B	A	A	A	A	A	A
HA115	B	A	A	A	A	A	A
HA116	B	A	A	A	A	A	A
HA117	B	A	A	A	A	A	A
HA118	B	A	A	A	A	A	A
HA119	E	A	A	A	A	A	A
HA120	B	B	A	A	A	A	A
HA121	B	B	A	A	A	A	A
HP001	A	B	A	A	A	A	A
HP002	A	B	A	A	A	A	A
HP003	A	B	A	A	A	A	A
HP004	A	B	A	A	A	A	A
HP005	A	B	A	A	A	A	A
HP006	A	A	A	A	A	A	A
HP007	B	A	A	A	A	A	A
HP008	B	A	A	A	A	A	A

Sample	Restriction enzyme						
	18S rDNA			16S rDNA			
	Alu I	*Taq* I	*Hae* III	*Bam*H I	*Eco*R I	*Hae* III	*Alu* I
HP009	B	B	A	A	A	A	A
HP010	B	A	A	A	A	A	A
HP011	B	B	A	A	A	A	A
HP012	B	B	A	A	A	A	A
HP013	A	B	A	A	A	A	A
HP014	A	A	B	A	A	A	A
HP015	E	B	A	A	A	A	A
HP016	E	B	A	A	A	A	A
HP017	E	B	A	A	A	A	A
HP018	D	B	A	A	A	A	A
HP019	D	B	A	A	A	A	A
HP020	D	B	A	A	A	A	A
HO001	B	B	A	A	B	B	B
HO002	A	C	A	A	B	B	B
HO003	A	C	B	A	B	B	B
HO004	A	C	B	A	B	B	B
HO005	A	C	B	A	B	B	B
HO006	A	C	B	A	B	B	B
HO007	A	C	B	A	B	B	B
HO008	A	C	B	A	B	B	B
HO009	A	D	B	A	B	B	B
HO010	D	B	B	A	B	B	B
HO011	A	C	B	A	B	B	B
HO012	A	C	A	A	B	B	B
HO013	A	D	B	A	B	B	B
HO014	A	C	B	A	B	B	B
HO015	A	D	B	A	B	B	B

Sample	Restriction enzyme						
	18S rDNA			**16S rDNA**			
	***Alu* I**	***Taq* I**	***Hae* III**	***Bam*H I**	***Eco*R I**	***Hae* III**	***Alu* I**
HO016	A	C	B	A	B	B	B
HO017	A	C	B	A	B	B	B
HO018	A	C	A	A	B	B	B
HO019	A	C	B	A	B	B	B
HO020	A	C	A	A	B	B	B
HO021	A	C	B	A	B	B	B
HO022	A	C	B	A	B	B	B
HO023	A	C	B	A	B	B	B
HO024	A	B	B	A	B	B	B
HO041	A	C	B	A	B	B	B
HO042	A	C	A	A	B	B	B
HO043	A	B	B	A	B	B	B
HO044	A	C	B	A	B	B	B
HO045	D	C	B	A	B	B	B
HO046	A	D	B	A	B	B	B
HO047	A	B	B	A	B	B	B
HO048	A	D	B	A	B	B	B
HO049	A	D	A	A	B	B	B
HO050	A	C	B	A	B	B	B
HO051	A	C	B	A	B	B	B
HO052	A	D	B	A	B	B	B
HO053	A	B	A	A	B	B	B
HO054	A	D	B	A	B	B	B
HO055	A	B	B	A	B	B	B
HO056	A	C	B	A	B	B	B
HO057	A	C	B	A	B	B	B
HO058	A	D	B	A	B	B	B

Sample	Restriction enzyme						
	18S rDNA			**16S rDNA**			
	***Alu* I**	***Taq* I**	***Hae* III**	***Bam*H I**	***Eco*R I**	***Hae* III**	***Alu* I**
HT001	A	B	B	A	A	A	B
HT002	F	B	A	A	A	A	B
HT003	A	C	B	A	A	A	B
HT004	F	B	B	A	A	A	B
HT005	A	B	B	A	A	A	B
HT006	D	D	B	A	A	A	B
HT007	A	B	B	A	A	A	B
HT008	D	C	B	A	A	A	B
HT009	C	B	A	A	A	A	B
HT010	B	B	A	A	A	B	B
HT011	A	B	A	A	A	A	B
HT012	A	C	B	A	A	A	B
HT013	A	C	B	A	A	A	B
HT014	A	B	B	A	A	A	B
HT015	A	B	B	A	A	A	B
HT016	A	B	A	A	A	A	B
HT017	A	C	B	A	A	A	B
HT018	A	C	B	A	A	A	B
HO081	A	C	B	A	A	A	B
HO082	A	B	B	A	A	A	B
HO083	A	C	B	A	A	A	B
HO084	A	B	A	A	A	A	B
HO085	A	D	B	A	A	A	B
HO086	D	B	A	A	A	A	B
HO087	D	B	A	A	A	A	B
HO088	A	C	A	A	A	A	B
HO089	B	A	A	A	A	A	B

Sample	Restriction enzyme						
	18S rDNA			**16S rDNA**			
	***Alu* I**	***Taq* I**	***Hae* III**	***Bam*H I**	***Eco*R I**	***Hae* III**	***Alu* I**
HO090	A	B	B	A	A	A	B
HO091	A	B	A	A	A	A	B
HV001	B	B	A	B	A	B	D
HV002	B	B	A	B	A	B	D
HV003	B	B	A	B	A	B	C
HV004	B	B	A	B	A	B	C
HV005	B	B	A	B	A	B	G
HV006	A	B	A	B	A	B	C
HV007	B	B	A	B	A	B	D
HV008	A	B	A	B	A	B	C
HV009	D	B	A	B	A	B	C
HV010	E	A	A	B	A	B	C
HV011	E	A	A	B	A	B	F
HV012	E	A	A	B	A	B	C
HV013	A	B	A	A	A	B	C
HV014	A	C	A	B	A	B	C
HV015	A	B	A	B	A	B	C
HV016	C	B	A	B	A	B	C
HV017	C	B	A	B	A	B	C
HV018	A	B	A	B	A	B	C
HV019	A	B	A	B	A	B	C
HV020	D	B	A	B	A	B	D
HV021	A	B	A	B	A	B	D
HV031	A	B	A	B	A	B	C
HV032	A	B	A	B	A	B	D

Appendix C

Size of fragments, the presence (1) and absence (0) of a particular fragment results from digestion of 18S rDNA and 16S rDNA genes with restriction enzymes.

18S rDNA / *Alu* I

Haplotype	Size of fragment (base pairs)									
	680	650	520	410	350	290	250	240	110	110
A	1	0	1	1	1	1	1	1	1	0
B	0	0	1	1	0	1	1	0	1	1
C	1	0	1	1	0	1	1	0	1	0
D	0	1	1	1	1	1	1	1	1	0
E	0	0	1	1	1	1	1	1	1	0
F	0	0	1	1	1	1	1	0	1	0

18S rDNA / *Taq* I

Haplotype	Size of fragment (base pairs)						
	500	500	410	410	270	210	80
A	0	0	1	0	1	1	1
B	1	0	1	1	1	1	1
C	1	0	1	0	1	1	1
D	1	1	1	0	1	1	1

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

18S rDNA / *Hae* III

Haplotype	Size of fragment (base pairs)			
	390	210	200	180
A	1	1	1	0
B	1	1	1	1

16S rDNA / *Bam*H I

Haplotype	Size of fragment (base pairs)		
	580	380	200
A	1	0	0
B	0	1	1

16S rDNA / *Eco*R I

Haplotype	Size of fragment (base pairs)		
	580	300	280
A	1	0	0
B	0	1	1

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

16S rDNA / *Hae* III

Haplotype	Size of fragment (base pairs)			
	500	380	120	80
A	0	1	1	1
B	1	0	0	1

16S rDNA / *Alu* I

Haplotype	Size of fragment (base pairs)							
	220	175	175	140	95	80	50	35
A	0	1	0	1	1	0	1	0
B	0	1	1	0	1	1	1	0
C	1	1	0	0	0	1	1	1
D	0	1	1	0	0	0	1	0
E	0	1	0	1	1	1	1	0
F	1	0	0	1	0	1	1	1
G	0	1	0	1	0	0	1	1

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix D

Pairwise genetic distances of 10 composite haplotypes generated from digestion of 16S rDNA with *Alu* I, *Taq* I, and *Hae* III.

I	*AAAA*	*0.00000000000*									
II	*AAAE*	*0.00361328922*	*0.00000000000*								
III	*ABBB*	*0.04172505968*	*0.03388005167*	*0.00000000000*							
IV	*AAAB*	*0.01183206496*	*0.00719893872*	*0.02449898874*	*0.00000000000*						
V	*AABB*	*0.02963083948*	*0.02179944432*	*0.01390835323*	*0.01183206496*	*0.00000000000*					
VI	*BABD*	*0.04759877800*	*0.05225102873*	*0.05003254731*	*0.03773920154*	*0.02336776823*	*0.00000000000*				
VII	*BABC*	*0.05684835786*	*0.04568262150*	*0.05457304079*	*0.04568262150*	*0.03036296776*	*0.01743955545*	*0.00000000000*			
VIII	*BABG*	*0.03773920154*	*0.04172505968*	*0.06247510346*	*0.05684835786*	*0.03773920154*	*0.01301654867*	*0.01160989367*	*0.00000000000*		
IX	*BABF*	*0.05684835786*	*0.04568262150*	*0.06527962740*	*0.06137527040*	*0.04172505968*	*0.03042574379*	*0.00700365718*	*0.01160989367*	*0.00000000000*	
X	*AABC*	*0.04574043972*	*0.03433967476*	*0.03036296776*	*0.03433967476*	*0.01752774157*	*0.03374684377*	*0.01390835323*	*0.02685114876*	*0.02134080574*	*0.00000000000*

Appendix E

Pairwise genetic distances of 49 composite haplotypes generated from digestion of 18S rDNA with *Alu* I, *Taq* I, and *Hae* III, and 16S rDNA with *Bam*H I, *Eco*R I, *Hae* III, and *Alu* I

49

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I      BBAAAAA     0.00000000000
II     ACAAAAA     0.00825759347 0.00000000000
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0.02909657314 0.03033609821 0.03157007019 0.03157007019

	0.02806220848 0.03403476242 0.03033609821 0.02685516421
	0.02210047202 0.02148942939 0.02447856387 0.02299132513
	0.03005489172 0.02855435304 0.01772069043 0.01623311063
	0.02065439636 0.02595117472 0.02447856387 0.02299132513
	0.02662946321 0.01011639370 0.00305886678 0.00813449181
	0.00294987007 0.00605675078 0.00813449181 0.01174321331
	0.00905924103 0.00456415480 0.00000000000
XLVIII DBABABD	*0.02395983237 0.02148942939 0.02662946321 0.02550687799*
	0.02952702519 0.03117570939 0.02952702519 0.02395983237
	0.01919446630 0.03117570939 0.02239722624 0.03117570939
	0.02550687799 0.01772069043 0.01562111812 0.02785197999
	0.02564335178 0.02685516421 0.02806220848 0.02161801161
	0.02475120305 0.02356987154 0.02685516421 0.02356987154
	0.01847581502 0.01772069043 0.02065439636 0.01919446630
	0.01847581502 0.01705502522 0.02148942939 0.01623311063
	0.01705502522 0.02210047202 0.01365764574 0.01919446630
	0.02662946321 0.00653949920 0.01335298570 0.01174321331
	0.00934204344 0.00605675078 0.01174321331 0.01559085364
	0.01562111812 0.01127620464 0.01335298570 0.00000000000
XLIX ABABABD	*0.02395983237 0.01772069043 0.02662946321 0.02550687799*
	0.02952702519 0.03117570939 0.02501708038 0.01997287804
	0.01562111812 0.02662946321 0.02239722624 0.03117570939
	0.02148942939 0.01772069043 0.01919446630 0.02785197999
	0.02238452330 0.02356987154 0.02475120305 0.02475120305
	0.02161801161 0.02685516421 0.02356987154 0.02046299084
	0.01505427449 0.01772069043 0.01705502522 0.01919446630
	0.02210047202 0.02065439636 0.01772069043 0.01623311063
	0.01365764574 0.01847581502 0.01705502522 0.01562111812
	0.02662946321 0.00653949920 0.01335298570 0.01174321331
	0.00605675078 0.00934204344 0.01174321331 0.01559085364
	0.01224860530 0.00782298328 0.00971339063 0.00305886678
	0.00000000000

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix F

F.1 a UPGMA treefile based on PCR-RFLP of 18S and 16S rDNA with restriction endonucleases (18S rDNA with *Alu* I, *Taq* I, and *Hae* III, 16S rDNA with *Bam*H I, *Eco*R I, *Hae* III, and *Alu* I)

((((((CABAAAA:0.00085,CAAAAAA:0.00085):0.00257,((((BBAAAAE:0.00078,
BBAAAAA:0.00078):0.00043,BCAAAAA:0.00121):0.00082,((BAAAAAE:0.00085,
BAAAAAA:0.00085):0.00042,BABAAAA:0.00126):0.00077):0.00110,
BAAAAAB:0.00314):0.00028):0.00109,(((AABAAAA:0.00078,AAAAAAA:0.00078):0.00043,
EAAAAAA:0.00121):0.00104,((ACAAAAA:0.00075,ABAAAAA:0.00075):0.00078,
(EBAAAAA:0.00075,DBAAAAA:0.00075):0.00078):0.00072):0.00226):0.00112,
((((ACAAAAB:0.00109,(ACBAAAB:0.00070,ADBAAAB:0.00070):0.00038):0.00023,
(ABAAAAB:0.00070,ABBAAAB:0.00070):0.00061):0.00112,(DBAAAAB:0.00180,
(DCBAAAB:0.00070,DDBAAAB:0.00070):0.00110):0.00063):0.00036,
((FBAAAAB:0.00075,FBBAAAB:0.00075):0.00118,CBAAAAB:0.00194):0.00085):0.00285):0.00389,
(((((ADBABBB:0.00069,ACBABBB:0.00069):0.00037,(ACAABBB:0.00071,
ADAABBB:0.00071):0.00035):0.00035,(ABBABBB:0.00069,ABAABBB:0.00069):0.00072):0.00098,
(DBBABBB:0.00069,DCBABBB:0.00069):0.00170):0.00340,(BBAAABB:0.00313,
BBAABBB:0.00313):0.00267):0.00373):0.00286,(((DBABABD:0.00153,
ABABABD:0.00153):0.00304,(BBABABG:0.00246,BBABABD:0.00246):0.00211):0.00156,
(((EAABABF:0.00159,EAABABC:0.00159):0.00211,((BBABABC:0.00153,
CBABABC:0.00153):0.00126,((ABABABC:0.00071,ACABABC:0.00071):0.00110,
DBABABC:0.00181):0.00098):0.00090):0.00118,ABAAABC:0.00488):0.00126):0.00625);

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

F.2 a UPGMA treefile based on PCR-RFLP of 16S rDNA with restriction endonucleases (*Bam*H I, *Eco*R I, *Hae* III, and *Alu* I)

(EAABABF:0.00007,((((BAAAAAE:0.00024,BBAAAAA:0.00326):0.05980,
((BBAAABB:0.00024,DBAAAAB:0.00326):0.00715,ADAABBB:0.01050):0.02726):0.05377,
BBABABG:0.00621):0.00256,(BBABABD:-0.00002,ABAAABC:0.00002):0.00119):0.00051,ABABABC:-
0.00007);

Appendix G

Results from sequence similarity search by comparing DNA sequences of individuals representing 16S rDNA composite haplotype shown in Table 3.5 with the GenBank using the Blast N programme.

Composite haplotype BABD (HV 007)

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|1272393|gb|U51989.1|HDU51989  Haliotis diversicolor 16S r...   363    1e-97
gi|10281749|gb|AF101007.2|AF101007  Cacozeliana lacertina tR...   137    1e-29
gi|15637191|gb|AF338145.1|AF338145  Dendropoma gregaria smal...   133    2e-28
gi|16554868|gb|AY010519.1|  Tarebia granifera tRNA-Thr, tRNA...   129    3e-27
gi|16554864|gb|AY010515.1|  Clypeomorus sp. tRNA-Thr, tRNA-G...   129    3e-27
gi|15637188|gb|AF338143.1|AF338143  Dendropoma sp. Chile sma...   129    3e-27
gi|16554863|gb|AY010514.1|  Cerithium coralium tRNA-Thr, tRN...   127    1e-26
gi|15637212|gb|AF338156.1|AF338156  Vasum muricatum small su...   127    1e-26
gi|16554906|gb|AY010316.1|  Cerithidea anticipata tRNA-Thr, ...   125    5e-26
gi|15637189|gb|AF338144.1|AF338144  Dendropoma corrodens sma...   125    5e-26
```

Composite haplotype BABC (HV 006)

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|1272393|gb|U51989.1|HDU51989  Haliotis diversicolor 16S r...   363    1e-97
gi|10281749|gb|AF101007.2|AF101007  Cacozeliana lacertina tR...   137    1e-29
gi|15637195|gb|AF338146.1|AF338146  Dendropoma maxima small ...   135    5e-29
gi|15637191|gb|AF338145.1|AF338145  Dendropoma gregaria smal...   133    2e-28
gi|16554868|gb|AY010519.1|  Tarebia granifera tRNA-Thr, tRNA...   129    3e-27
gi|16554864|gb|AY010515.1|  Clypeomorus sp. tRNA-Thr, tRNA-G...   129    3e-27
gi|15637188|gb|AF338143.1|AF338143  Dendropoma sp. Chile sma...   129    3e-27
gi|16554863|gb|AY010514.1|  Cerithium coralium tRNA-Thr, tRN...   127    1e-26
gi|15637212|gb|AF338156.1|AF338156  Vasum muricatum small su...   127    1e-26
gi|16554906|gb|AY010316.1|  Cerithidea anticipata tRNA-Thr, ...   125    5e-26
```

Composite haplotype BABG (HV 005)

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|1272393|gb|U51989.1|HDU51989  Haliotis diversicolor 16S r...   357    8e-96
gi|15637191|gb|AF338145.1|AF338145  Dendropoma gregaria smal...   155    6e-35
gi|15637195|gb|AF338146.1|AF338146  Dendropoma maxima small ...   153    2e-34
gi|10281749|gb|AF101007.2|AF101007  Cacozeliana lacertina tR...   137    1e-29
gi|16554868|gb|AY010519.1|  Tarebia granifera tRNA-Thr, tRNA...   129    3e-27
gi|16554864|gb|AY010515.1|  Clypeomorus sp. tRNA-Thr, tRNA-G...   129    3e-27
```

gi|15637188|gb|AF338143.1|AF338143 Dendropoma sp. Chile sma...	129	3e-27
gi|16554863|gb|AY010514.1| Cerithium coralium tRNA-Thr, tRN...	127	1e-26
gi|15637212|gb|AF338156.1|AF338156 Vasum muricatum small su...	127	1e-26
gi|16554906|gb|AY010316.1| Cerithidea anticipata tRNA-Thr, ...	125	5e-26

Composite haplotype AABC (HV 013)

Sequences producing significant alignments:	Score (bits)	E Value
gi|1272393|gb|U51989.1|HDU51989 Haliotis diversicolor 16S r...	363	1e-97
gi|15637191|gb|AF338145.1|AF338145 Dendropoma gregaria smal...	147	1e-32
gi|15637195|gb|AF338146.1|AF338146 Dendropoma maxima small ...	145	6e-32
gi|10281749|gb|AF101007.2|AF101007 Cacozeliana lacertina tR...	137	1e-29
gi|16554868|gb|AY010519.1| Tarebia granifera tRNA-Thr, tRNA...	129	3e-27
gi|16554864|gb|AY010515.1| Clypeomorus sp. tRNA-Thr, tRNA-G...	129	3e-27
gi|15637188|gb|AF338143.1|AF338143 Dendropoma sp. Chile sma...	129	3e-27
gi|16554863|gb|AY010514.1| Cerithium coralium tRNA-Thr, tRN...	127	1e-26
gi|15637212|gb|AF338156.1|AF338156 Vasum muricatum small su...	127	1e-26
gi|16554906|gb|AY010316.1| Cerithidea anticipata tRNA-Thr, ...	125	5e-26

Composite haplotype BABF (HV 011)

Sequences producing significant alignments:	Score (bits)	E Value
gi|1272393|gb|U51989.1|HDU51989 Haliotis diversicolor 16S r...	349	2e-93
gi|15637191|gb|AF338145.1|AF338145 Dendropoma gregaria smal...	163	2e-37
gi|15637195|gb|AF338146.1|AF338146 Dendropoma maxima small ...	161	9e-37
gi|10281749|gb|AF101007.2|AF101007 Cacozeliana lacertina tR...	137	1e-29
gi|4511931|gb|AF058240.1|AF058240 Cymbula canescens 16S rib...	133	2e-28
gi|5852868|gb|AF161178.1|AF161178 Amathia lendigera 16S rib...	131	8e-28
gi|16554868|gb|AY010519.1| Tarebia granifera tRNA-Thr, tRNA...	129	3e-27
gi|16554864|gb|AY010515.1| Clypeomorus sp. tRNA-Thr, tRNA-G...	129	3e-27
gi|15637188|gb|AF338143.1|AF338143 Dendropoma sp. Chile sma...	129	3e-27
gi|16554863|gb|AY010514.1| Cerithium coralium tRNA-Thr, tRN...	127	1e-26

Composite haplotype AABB (HT 010)

Sequences producing significant alignments:	Score (bits)	E Value
gi|1272393|gb|U51989.1|HDU51989 Haliotis diversicolor 16S r...	622	e-176
gi|15637191|gb|AF338145.1|AF338145 Dendropoma gregaria smal...	163	2e-37
gi|15637195|gb|AF338146.1|AF338146 Dendropoma maxima small ...	161	9e-37
gi|16554868|gb|AY010519.1| Tarebia granifera tRNA-Thr, tRNA...	137	1e-29
gi|10281749|gb|AF101007.2|AF101007 Cacozeliana lacertina tR...	137	1e-29
gi|4511931|gb|AF058240.1|AF058240 Cymbula canescens 16S rib...	133	2e-28
gi|16554869|gb|AY010520.1| Thiara amarula tRNA-Thr, tRNA-Gl...	131	8e-28
gi|5852868|gb|AF161178.1|AF161178 Amathia lendigera 16S rib...	131	8e-28
gi|15637188|gb|AF338143.1|AF338143 Dendropoma sp. Chile sma...	129	3e-27
gi|1857668|gb|U86349.1|LSU86349 Lepetodrilus sp. 16S riboso...	129	3e-27

Composite haplotype AAAB (HO 086)

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|1272393|gb|U51989.1|HDU51989  Haliotis diversicolor 16S r...   622   e-176
gi|15637191|gb|AF338145.1|AF338145  Dendropoma gregaria smal...   163   2e-37
gi|15637195|gb|AF338146.1|AF338146  Dendropoma maxima small ...   161   9e-37
gi|16554868|gb|AY010519.1|  Tarebia granifera tRNA-Thr, tRNA...   137   1e-29
gi|4511931|gb|AF058240.1|AF058240  Cymbula canescens 16S rib...   133   2e-28
gi|16554869|gb|AY010520.1|  Thiara amarula tRNA-Thr, tRNA-Gl...   131   8e-28
gi|5852868|gb|AF161178.1|AF161178  Amathia lendigera 16S rib...   131   8e-28
gi|15637188|gb|AF338143.1|AF338143  Dendropoma sp. Chile sma...   129   3e-27
gi|10281749|gb|AF101007.2|AF101007  Cacozeliana lacertina tR...   129   3e-27
gi|1857668|gb|U86349.1|LSU86349  Lepetodrilus sp. 16S riboso...   129   3e-27
```

Composite haplotype ABBB (HO 049)

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|1272393|gb|U51989.1|HDU51989  Haliotis diversicolor 16S r...   339   2e-90
gi|15637195|gb|AF338146.1|AF338146  Dendropoma maxima small ...   139   3e-30
gi|16554868|gb|AY010519.1|  Tarebia granifera tRNA-Thr, tRNA...   137   1e-29
gi|10281749|gb|AF101007.2|AF101007  Cacozeliana lacertina tR...   137   1e-29
gi|15637191|gb|AF338145.1|AF338145  Dendropoma gregaria smal...   133   2e-28
gi|16554869|gb|AY010520.1|  Thiara amarula tRNA-Thr, tRNA-Gl...   131   8e-28
gi|15637212|gb|AF338156.1|AF338156  Vasum muricatum small su...   129   3e-27
gi|15637211|gb|AF338155.1|AF338155  Vasum caestus small subu...   129   3e-27
gi|15637188|gb|AF338143.1|AF338143  Dendropoma sp. Chile sma...   129   3e-27
gi|1857668|gb|U86349.1|LSU86349  Lepetodrilus sp. 16S riboso...   129   3e-27
```

Composite haplotype AAAA (HA 005)

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|1272393|gb|U51989.1|HDU51989  Haliotis diversicolor 16S r...   379   e-102
gi|15637191|gb|AF338145.1|AF338145  Dendropoma gregaria smal...   178   4e-42
gi|16944721|emb|AJ390328.1|NPO390328  Nautilus pompilius par...   165   6e-38
gi|16215596|emb|AJ416578.1|NPO416578  Nautilus pompilius par...   165   6e-38
gi|4583049|gb|AF098298.1|AF098298  Branchiostoma floridae mi...   159   4e-36
gi|16215598|emb|AJ416579.1|NPO416579  Nautilus pompilius par...   157   1e-35
gi|5852868|gb|AF161178.1|AF161178  Amathia lendigera 16S rib...   155   6e-35
gi|3292989|emb|Y16474.1|MTY16474  Branchiostoma lanceolatum ...   151   9e-34
gi|16554864|gb|AY010515.1|  Clypeomorus sp. tRNA-Thr, tRNA-G...   135   5e-29
gi|16554863|gb|AY010514.1|  Cerithium coralium tRNA-Thr, tRN...   135   5e-29
```

Composite haplotype AAAE (HL 006)

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

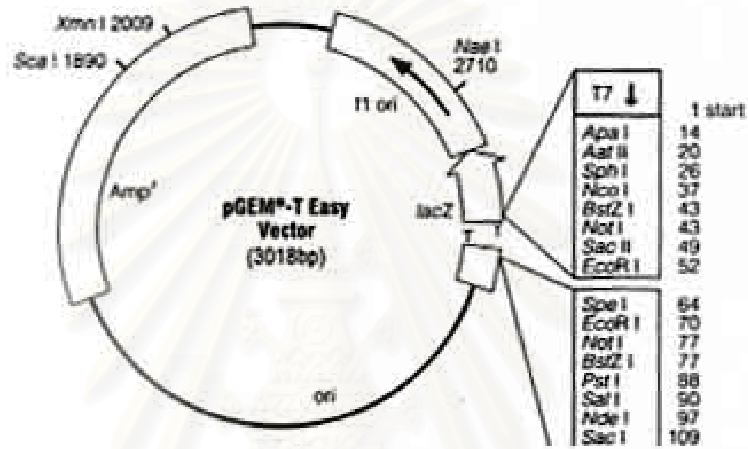
gi|1272393|gb|U51989.1|HDU51989  Haliotis diversicolor 16S r...   373   e-100
gi|15637191|gb|AF338145.1|AF338145  Dendropoma gregaria smal...   186   2e-44
gi|16215596|emb|AJ416578.1|NPO416578  Nautilus pompilius par...   167   1e-38
gi|4583049|gb|AF098298.1|AF098298  Branchiostoma floridae mi...   167   1e-38
gi|16944721|emb|AJ390328.1|NPO390328  Nautilus pompilius par...   165   6e-38
```

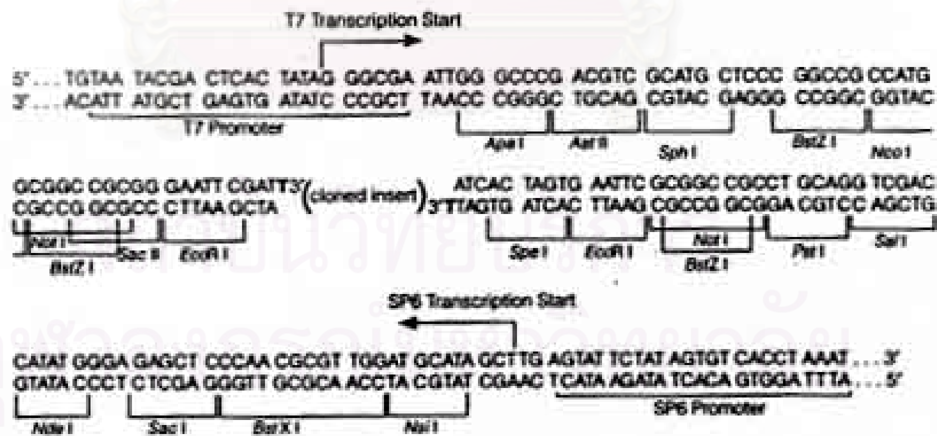
gi|5852868|gb|AF161178.1|AF161178 Amathia lendigera 16S rib... 163 2e-37
gi|16215598|emb|AJ416579.1|NPO416579 Nautilus pompilius par... 159 4e-36
gi|3292989|emb|Y16474.1|MTY16474 Branchiostoma lanceolatum ... 159 4e-36
gi|16554863|gb|AY010514.1| Cerithium coralium tRNA-Thr, tRN... 143 2e-31
gi|16554864|gb|AY010515.1| Clypeomorus sp. tRNA-Thr, tRNA-G... 139 3e-30

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix H

Restriction mapping of plasmid pGEM^R–T easy vector





Appendix I

International publication from this thesis:

Jarayabhand, P., Praipue, P., Khamnumtong, N., Klinbunga, S., and Tassanakajon, A. 2002. Fisheries Science. 68 (in press).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Identification of Species-Diagnostic Markers of Abalone in Thailand Using PCR-RFLP of 16S rDNA

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SUMMARY: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of 16S ribosomal (r) DNA was used to identify species-specific markers of three abalone species; *Haliotis asinina*, *H. ovina* and *H. varia* in Thailand. A total of 10 composite haplotypes were found across geographically different samples of these abalone. Species-specific composite haplotypes of each abalone were found. Intraspecific genetic differentiation was clearly observed in *H. ovina* but not in *H. asinina* and *H. varia*. The 16S rDNA of an individual representing major composite haplotypes AAAA, ABBB, AAAB, BABG and BABC were cloned and sequenced. Comparisons of 16S rDNA sequences suggest the possibility of developing a species-specific PCR for each abalone species.

KEY WORDS: **genetic markers, PCR-RFLP, 16S rDNA, abalone**

INTRODUCTION

Abalone are economically important marine gastropods currently being cultured worldwide. Three species of tropical abalone; *Haliotis asinina*, *H. ovina* and *H. varia* are found in Thai waters.[1)] Of these, *H. asinina* is the most promising species being initially cultured in Thailand at present. Nevertheless, relatively little is known about the basic knowledge of the genetic diversity and population structure of this species. This information is essential for the construction of an appropriate management scheme leading to sustainable culturing activity of *H. asinina* in Thailand.

Appropriate genetic markers can be used to elevate the culture and management efficiency of abalone in Thailand. The success of aquacultural activity of commercially important species requires the basic knowledge on stock structure and the use of suitable molecular genetic markers to establish broodstock management programmes in wild populations of exploited species.[2,3)]

Restriction analysis of mtDNA has been successfully used to estimate levels of genetic diversity and to identify population differentiation in several commercially important marine species, for example; the mussels *Mytilus edulis* and *M. galloprovincialis*[4)], the American oyster *Crassostrea virginica*[5)] and the giant tiger shrimp *Penaeus monodon*.[6)]

Since mtDNA is haploid and transmitted maternally, the effective population size estimated from mtDNA is generally smaller than that estimated from nuclear markers, such as allozymes and nuclear DNA.[6)] This increases its sensitivity to inbreeding and bottleneck effects compared to nuclear DNA markers.[7)]

Species-specific markers also play the important roles to prevent supplying incorrect abalone larvae for the industry and for quality control of cultured abalone from Thailand. These markers are necessary for the development of monospecific farming of *H. asinina* in Thailand.

The objectives of this study were determination of intraspecific genetic differentiation and molecular genetic markers showing species-specific nature with *H. asinina*, *H. ovina* and *H. varia* in Thailand.

MATERIALS AND METHODS

Sampling

Specimens representing *H. asinina* (*N* = 47) and *H. ovina* (*N* = 64) were collected from the east (Gulf of Thailand) and the west (The Andaman Sea) coasts of peninsular Thailand. Additional *H. asinina* specimens were collected from Cambodia (*N* = 23) and Philippines (*N* = 14). The *H. varia* abalone (*N* = 25) were also collected from the Andaman Sea.

DNA extraction

Total DNA was extracted from the adductor muscle of each abalone using a phenol-chloroform-proteinase K method described by Klinbunga *et al.*.[2] DNA concentration was spectrophotometrically determined and kept at 4° C until required.[8]

Polymerase chain reaction (PCR) and restriction analysis

The 16S ribosomal (r) DNA of each abalone was amplified by PCR using primers 16S-F; 5'-CGCCTGTTTAACAAAAACAT-3' and 16S-R1; -5'-CCGGTCTGAACTCAGATCATGT-3'.[9] Specimens which were not successfully amplified with those primers were then amplified with 16S-F and 16S-R2; 5'-CCGGTCTGAACTCAGATCAGATCACGT-3'[10] according to the conditions described by Klinbunga *et al.*.[3]

Eight microlitres (approximately 250 ng) of the amplification product were separately digested with *Bam* HI, *Eco* RI, *Hae* III and *Alu* I, using standard conditions.[8] The digests were electrophoretically analysed through 2.0% agarose (*Bam* HI, *Eco* RI, *Hae* III) or 3.0 % MetaPhor agarose gels (*Alu* I) and visualised under a UV light after ethidium bromide staining.

Restriction profiles of 16S rDNA digested with each restriction enzyme were alphabetically coded in order of appearance. Each abalone was then assigned a four letter code to describe its composite haplotype.

Cloning of 16S rDNA fragments

The 16S rDNA gene segment was amplified from representative individuals having major composite haplotypes; AAAA, ABBB, AAAB, BABG and BABC. A 580 bp fragment was excised and recovered from the electrophoresed gel individually. The gel-eluted DNA was digested with proteinase K (50 µg/ml in the presence of 0.5% SDS) at 65°C for 1h followed by phenol/chloroform extraction and ethanol precipitation. DNA was cloned using a T-A cloning method.[11]

One-tenth volume of each ligation reaction was electrotransformed to *E. coli* XL-1 BLUE. Recombinant clones were selected by a *lac Z'* system following standard protocols.[8] Five recombinant clones were unidirectional sequenced. DNA sequences were aligned using Clustal W.[12] The divergence between pairs of sequences was estimated using Kimura's two-parameter model.[13]

RESULTS

Digestion of 16S rDNA (approximately 580 bp in length) with *Bam* HI, *Eco* RI, *Hae* III and *Alu* I provided 2, 2, 2 and 7 restriction patterns, respectively (Table 1). A total of 10 composite haplotypes were found across overall specimens (Table 2). These composite haplotypes could differentiate the species origins of abalone in Thailand unambiguously. No overlapping haplotypes were found between different abalone species. Two composite haplotypes; AAAA and AAAE, were specifically found in *H. asinina* whereas haplotypes ABBB, AAAB and AABB were restricted to *H. ovina*.

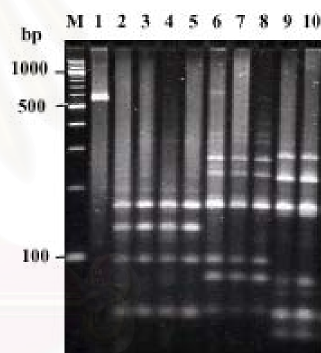

Fig. 1 RFLP patterns of 16S rDNA of *H. asinina* (pattern A, lanes 1-5), *H. ovina* (pattern B, lanes 6-8) and *H. varia* (patterns G, lanes 9-10) digested with *Alu* I. Lanes M and 1 were a 100 bp DNA ladder and undigested 16S rDNA, respectively.

The remaining composite haplotypes (BABG, BABC, BABD, BABF and AABG) were only found in *H. varia*. A lack of geographic heterogeneity was observed in *H. asinina* even though samples originating from Cambodia and Philippines were included. Conversely, genetic differentiation between *H. ovina* from the Andaman Sea (west) and Gulf of Thailand (east) were clearly observed. Differentiation of *H. varia* could not be examined because it is found only in the Andaman Sea.

The 16S rDNA gene segment of representatives of *H. asinina*, *H. ovina* and *H. varia* showed relatively high diversity. Interspeciefic sequence divergence between pairs of 16S rDNA sequences was 10.65%

(between AAAA and AAAB) to 13.42% (between AAAA and BABC).

Table 1 Restriction patterns of 16S rDNA of *H. asinina*, *H. ovina* and *H. varia* digested with *Alu* I, *Bam* HI, *Eco* RI and *Hae* III

Enzyme	Pattern observed (bp)
Bam HI	A: 580
	B: 380, 200
Eco RI	A: 580
	B: 300, 280
Hae III	A: 375, 125, 80
	B: 500, 80
Alu I	A:175, 140, 95, 50, 35
	B: 175, 175, 95, 80, 50
	C: 175, 175, 50
	D: 175, 140, 50, 35
	E: 175, 140, 95, 95, 50
	F: 220, 140, 80, 50, 35
	G: 220, 175, 80, 50, 35

Table 2 Geographic distribution of composite haplotypes (arranged from 16S rDNA digested with *Bam* HI, *Eco* RI, *Hae* III and *Alu* I, respectively) among geographically different samples of three species of abalone

Haplotype	Geographic distribution						
	H. asinina				*H. ovina*		*H. varia*
	A	G	C	P	A	G	A
AAAA	25	17	23	14			
AAAE	3	2	-	-			
ABBB	-				38	-	-
AAAB	-	-	-	-	-	25	-
AABB	-	-	-	-	-	1	-
BABG	-	-	-	-	-	-	15
BABC	-	-	-	-	-	-	7
BABD	-	-	-	-	-	-	1
BABF	-	-	-	-	-	-	1
AABG	-	-	-	-	-	-	1

Abbreviations: A = the Andaman sea, G = Gulf of Thailand, C = Cambodia, P = Philippines

DISCUSSION

Species-diagnostic markers of three species of abalone (*H. asinina*, *H. ovina* and *H. varia*) in Thailand were successfully identified based on restriction analysis of the amplified 16S rDNA with *Bam* HI, *Eco* RI, *Hae* III and *Alu* I.

Common composite haplotypes with high frequencies were observed in each species allowing the use of these RFLP markers as species-diagnostic markers for classification of *H. asinina*, *H. ovina* and *H. varia* at different stages of development.

Distribution patterns of composite haplotypes in *H. asinina* indicated a lack of intraspecific population structure of this species over vast geographic areas. Our results also suggest that *H. asinina* is probably a high gene flow species. In contrast, region-specific composite haplotypes were observed in *H. ovina* originating from different coastal sides of peninsular Thailand, but not within each coast (data not shown).

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BABC    CGCCTGTTNACCAAAAACA-GGCTCCTCGGTCAATTAGTGTGGATGGGAAGTCGGACCTG
BABG    CGCCTGTTNAACAAAAACATGGCTCCTCGGTCAATTAGTG-GGATGGGAAGTCGGACCTG
AAAA    CGCNTGTTNACCAAAA-CATGGCTCCTTG--TGTTTAGNC-GNATANGNAGTCGGACCTG
AAAB    CGCCTGTTTAACAAAAACATGGCTCCTTGGTTGTCTGAGT-GGATGAGGAGTCGGACCTG
ABBB    CGCCTGTTTAACAAAAACATGGCTCCTCGGTTGTTTGAGT-GGATGGGGAGTCGGACCTG
        *** **** * ***** ** ******* *      *      * **   * ***********
BABC    CCCGGTGACCTACGGGTTAAACGGGCCGCGGTACACTGACCGTGCAAAGGTAGCACAAT-
BABG    CCCGGTGACCTACGGGTTAACCGG-CCCCGGTACACTGCCGG--CAAAGGTAGCACAATT
AAAA    CCCGGTGACCTACGGGT-AACCGG-CCGCGG-ACACTGACCGTGCAAAGGTAGCACAAT-
AAAB    CCCGGTGACTTACGGGTTAAACGGCCGCGGGTACACTGACCGTGCAAAGGTAGCACAAT-
ABBB    CCCGGTGACTTACGGGTTAAACGGCCGCGG-TACACTGACCGTGCAAAGGTAGCACAAT-
        ********* ******* ** *** *    *   ****** * *  ***************
BABC    CACTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTT
BABG    CACTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTT
AAAA    CACTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTT
AAAB    CACTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTC
ABBB    CACTTGCCTTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGGGCTGAGCTGTCTCTTC
        ***********************************************************
BABC    TGAAATATTTAAAAATTAACTTCT-AGGTGAAAAGGCTTAGATTAAGCTGAGGGACGAGA
BABG    TGAAATATTTAAAAATTAACTTCTTAGGTGAAAAGGCTTAGATTAAGCTGAGGGACGAGA
AAAA    TGGAATATTTAAAAATTAACTTCT-AGGTGAAAAGGCCTAGATTGAGCTGAGGGACGAGA
AAAB    TGAAATATTTAAAAATTAACTTCT-AGGTGAAAAGGCCTAGATTAGGCTGAGGGACGAGA
ABBB    TGAAATATTTAAAAATTAACTTCT-AGGTGAAAAGGCTTAGATTAGGCTGAGGGACGAGA
        ** ********************** *********** ******  **************
BABC    AGACCCTGTTGAGCTTTAGTGTGGAATGAAGGGGTGTGCTCCTGAAATAAGTTAGAGAGC
BABG    AGACCCTGTTGAGCTTTAGTGTGGAATGAAGGGGTGTGCTCCTGAAATAAGTTAGAGAGC
AAAA    AGACCCTGTTGAGCTTTAGTGTTGAATAAAGGTTTATATTTCCTAGTTGAATTTAGAGGT
AAAB    AGACCCTGTTGAGCTTTAGTGTGGAGTGAAGGTTC-TAATTTCTAGTTGTACTAGAGAAT
ABBB    AGACCCTGTTGAGCTTTAGTGTGGAGTGAAGGTTTATAATTTCTAGTTGTACTAGAGAAT
        ********************* ** * ****     *  *    *  *    *
BABC    TCAGGTTTGTTTTTACATCTTTAGTTGGGGTGACTGGGGAACATAAGAAGCTTCCCTGTT
BABG    TCAGGTTTGTTCTTACATCTTTAGTTGGGGTGACTGGGGAACATAAGAAGCTTCCCTGTT
AAAA    TTAAGTTTATTCTTACATCTTTAGTTGGGGTGACTGGGGAACAAAAGTAGCTTCTCTACT
AAAB    TTAAATTCATTTTTACATCTTTAGTTGGGGTGACTGGGGAACATAGGTAGCTTCTCTGTT
ABBB    TCAAATTCATTTTTACATCTTTAGTTGGGGTGACTGGGGAACATAGGTAGCTTCTCTGTT
        * *  **  ** ****************************** * * ****** **  *
BABC    TATAGTTTAATTATTTTCGGTTTTCTGACTAAGGATCCAGCATTGCTGATTGTCGGAAAA
BABG    TATAGTTTAATTATTTTCGGTTTTCTGACTAAGGATCCAGCATTGCTGATTGTCGGAAAA
AAAA    TATTACTGGGTT-----TGGCTTGCTAGCTAATGATCCGGCATTGCTGATTATTGGAAAA
AAAB    T-TTAGTAAATTAAATTTGGTCTGCTGACTGATGATCCGGCATTGTCGATTATCGGAAAA
ABBB    T-TTAGTAGATTGAATTTGGTCTGCCGACTGATGATCCGACATTGTCGATTATCGGAAAA
        * *   *   **       **  * *   ** * *****  *****  **** * ******
BABC    AGTTACCACAGGGATAACAGC-GTAATCTTTCTGG-AGAGTTCACATTGAAAGAA-GGGT
BABG    AGTTACCACAGGGATAACAGC-GTAATCTTTCTGG-AGAGTTCACATTGAAAGAA-GGGT
AAAA    AGTTACCACAGGGATAACAGC-GTAATCTTTTTGG-AGAGTTCATATTGAAAAAA-GGGT
AAAB    AGTTACCACAGGGATAACAGC-GTAATCTTTCTGG-AGAGTTCACATTGAAAGAA-GGGT
ABBB    AGTTACCACAGGGATAACAGCCGTAATCTTTCTGGGAGAGTTCACATTGAAAGAAAGGGT
        ********************* ********* *** ******** ******* ** ****
BABC    TTGCGACCTCGATGTTGGATTAAGGTGTCCTGAGGGTGTAGCAGCTTTCGTTGG-TTGGT
BABG    TTGCGACCTCGATGTTGGATTAAGGTGTCCTGAGGGTGTAGCAGCTTTCGTTGG-TTGGT
AAAA    TTGCGACCTCGATGTTGGATTAAGGTGTCCTAAGGGTGTAGCAGCTCTTGTTGG-TTGGT
AAAB    TTGCGACCTCGATGTTGGATTAAGGTGTCCTGAGGGTGTAGCAGCTTTCGTTGG-TTGGT
ABBB    TTGCGACTCCNATGTTGGATAAAGGTGTCTGGGGGGNGTAGCAACTTTCGTTGGGTTGGN
        *******  * ********* ********    *** ****** ** * ***** ****
BABC    CTGTTCGACCATTAAAACCTTACGTGATCTGATCTGAGTTCAGACCGG
BABG    CTGTTCGACCATTAAAACCTTACATGATCTGA-----GTTCAGACCGG
AAAA    CTGTTCGACCATTAAAACCTTACATGATCTGA-----GTTCAGACCGG
AAAB    CTGTTCGACCATTAAAACCTTACATGATCTGA-----GTTCAGACCGG
ABBB    CTGTTCNACCATTAAAACCTTACGTGATCTGATCTGAGTTCAGACCGG
        ****** *************** ********     ***********
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Fig. 2 Sequences of 16S rDNA amplified from *H. asinina* individuals possessing AAAA, *H. ovina* possessing ABBB and AAAB, and *H. varia* possessing BABC and BABG composite haplotypes.

Distributions of *H. ovina* composite haplotypes clearly indicated the existence of intraspecific genetic differentiation in this species. The most common haplotypes ABBB and AAAB in *H. ovina* were different by 2 restriction sites while the rare composite haplotype AABB was the intermediate haplotype between those composite haplotypes. The highest level of genetic diversity was found in *H. varia*. Five composite haplotypes were found in a relatively small sample size (N = 25) of *H. varia* compared to 2 and 3 composite haplotypes from *H. asinina* (N = 84) and *H. ovina* (N = 64), respectively.

The cDNA sequences of the sperm lysin protein previously used for systematic studies of *Haliotis* species showed unusual divergence between species but was highly conserved within species suggesting its high species-specific nature.[14,15)] Recently, Sweijd *et al.* [16)] successfully developed species-specific PCR for *H. midae* and *H. spadicea* based on lysin sequences described by Youn-Ho and Vacquier.[14)] Our results indicated that PCR-RFLP can also be unambiguously used for identification of species origins of Thai abalone. Moreover, large genetic divergence was observed between different species but lower divergence was observed intraspecifically (3.19% between ABBB and AAAB in *H. ovina* and 1.11% between BABC and BABG in *H. varia*). Sequences of the amplified 16S rDNA of individuals showing major composite haplotypes indicated the possibility of developing rapid and reliable species-specific PCR of abalone in Thailand.

The ability to identify the species origins of Thai abalone is crucial for broodstock management and conservation programmes in these taxa. Following which, these molecular markers can also be used for comparisons of growth performance among three abalone species in communal setting conditions. Our results illustrated the existence of population subdivisions in *H. ovina* but not in *H. asinina* suggesting that levels of gene flow in these species are different. High genetic diversity in a discontinuously distributed species like *H. varia* was surprisingly observed.

PCR-RFLP analysis is a promising approach for population genetic and systematic studies in various taxa.[3, 17, 18)] Genetic markers found in this study can also identify the species origin of abalone seed accurately. In addition, the PCR-RFLP approach can be further used for evaluation of genetic diversity levels of three abalone species in Thailand.

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BIOGRAPHY

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