การพัฒนาเครื่องหมายโมเลกุลเพื่อศึกษาพหุสัณฐานทางพันธุกรรมและการแสดงออกของยีนที่เกี่ยวข้องกับเมแทบอลิซึมในหอยเป๋าฮื้อไทย *Haliotis asinina*

นางสาวปาริฉัตร พรายภู่

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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DEVELOPMENT OF MOLECULAR MARKERS FOR STUDIES OF GENETIC POLYMORPHISM AND EXPRESSION OF GENES RELATED WITH METABOLISMS IN THAI ABALONE *Haliotis asinina*

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จากการวิเคราะห์ลำดับนิวคลีโอไทด์ของยีนในหอยเป๋าฮื้อจำนวน 2876 อีเอสที พบลำดับเบสซ้ำของไมโครแซทเทไลท์ใน 178 อีเอสที จึงพัฒนาเครื่องหมายไมโครแซทเทไลท์แบบที่หนึ่งจำนวน 4 ตำแหน่ง (*DW455*, *DW503*, *PHe177*, and *PT102*) เพื่อใช้ร่วมกับไมโครแซทเทไลท์แบบที่สองจำนวน 2 ตำแหน่ง (*Haμ9* และ *Haμ10*) สำหรับประเมินความหลากหลายทางพันธุกรรมในหอยเป๋าฮื้อชนิด *H. asinina* จากประชากรธรรมชาติและจากการปรับปรุงพันธุ์ ซึ่งพบว่ามีจำนวนอัลลีลต่อตำแหน่งเท่ากับ 10, 7, 6, 6, 10 และ 12 อัลลีล ตามลำดับ โดยตัวอย่างจากโรงเพาะเลี้ยง มีความถี่อัลลีลที่พบในหอยจากฝั่งอ่าวไทยสูงกว่าอัลลีลที่พบในหอยจากฝั่งอันดามัน พบค่า observed และ expected heterogeneity ในประชากรธรรมชาติระหว่าง 0.0625 – 1.0000 และ 0.0625 – 0.8448 ในขณะที่ประชากรจากโรงเพาะเลี้ยงมีค่าดังกล่าวระหว่าง 0.3441 – 0.7769 และ 0.3333 – 0.8750 ตามลำดับ โดยค่า allele และ genotype discrimination capacity แสดงให้เห็นถึงการลดลงของความหลากหลายทางพันธุกรรมในกลุ่มตัวอย่างจากโรงเพาะเลี้ยง จากการตรวจสอบความแตกต่างทางพันธุกรรมด้วย F_{ST} และ exact test ระหว่างกลุ่มตัวอย่าง แสดงให้เห็นถึงความแตกต่างทางพันธุกรรมระหว่างหอยเป๋าฮื้อธรรมชาติจากฝั่งอ่าวไทย (SAME และ CAME) และฝั่งทะเลอันดามัน (TRGW) และหอยเป๋าฮื้อจากประเทศฟิลิปปินส์อย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) นอกจากนี้ยังพบว่าหอยเป๋าฮื้อจากโรงเพาะเลี้ยงมีความแตกต่างทางพันธุกรรมกับจากหอยฝั่งอันดามันและจากประเทศฟิลิปปินส์ในทุกตำแหน่งของไมโครแซทเทไลท์ที่ทำการศึกษา ($P < 0.05$)

ในการศึกษา AFLP นั้น ได้สืบค้นเครื่องหมายที่มีโพลิมอร์ฟิซึมจาก 64 คู่ไพรเมอร์ ทำการโคลนเครื่องหมาย AFLP จำนวน 9 ชิ้น เพื่อพัฒนาเป็นเครื่องหมาย SCAR จากนั้นใช้เครื่องหมาย $HaSCAR_{320}$, $HaSCAR_{295}$, และ $HaSCAR_{327}$ ตรวจสอบความหลากหลายทางพันธุกรรมของหอยเป๋าฮื้อกลุ่มต่างๆ ด้วยวิธี SSCP พบจำนวนจีโนไทป์เท่ากับ 3, 2 และ 3 จีโนไทป์ต่อตำแหน่งตามลำดับ โดยไม่มีจีโนไทป์ที่ซ้ำกันระหว่างตัวอย่างจากอ่าวไทยและทะเลอันดามันเมื่อตรวจสอบด้วย $HaSCAR_{327}$ ทั้งนี้พบจีโนไทป์ AAA, AAC และ CAA ในหอยเป๋าฮื้อจากฝั่งอ่าวไทยในขณะที่พบจีโนไทป์ ABB, BAB, และ BBB เฉพาะในตัวอย่างธรรมชาติจากฝั่งทะเลอันดามัน และพบว่าตัวอย่างจากโรงเพาะเลี้ยงเกือบทั้งหมด มีจีโนไทป์ที่พบในฝั่งอ่าวไทย (131/135; 97.03%) จากการกระจายความถี่ของจีโนไทป์รวม (composite SSCP genotypes) และการวิเคราะห์ค่าเฮทเทอโรจินิตี้ สรุปได้ว่าหอยจากฝั่งทะเลตะวันออกมีอัตราการรอดที่ดี สามารถปรับตัวต่อภาวะการณ์เพาะเลี้ยงได้ดีกว่าหอยเป๋าฮื้อจากฝั่งทะเลอันดามัน

เมื่อตรวจสอบความสัมพันธ์ระหว่างจีโนไทป์และน้ำหนักตัวของหอยเป๋าฮื้อกลุ่ม B โดยเครื่องหมายไมโครแซทเทไลท์ พบความแตกต่างระหว่างกลุ่ม BL-BS ที่ตำแหน่ง *DW455* และ *Haμ10* ($P < 0.05$) และพบความแตกต่างของน้ำหนักตัวของหอยเป๋าฮื้อที่มีจีโนไทป์ต่างกันของไมโครแซทเทไลท์ที่ตำแหน่ง *Haμ10* ($P < 0.05$)

นอกจากนี้ได้หาลำดับนิวคลีโอไทด์ที่สมบูรณ์ของยีน *α-methylacyl-CoA racemase* (*AMACR*), *carnitine O-palmitoyltransferase 1A* (*CPT-1A*), *hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* (*trifunctional protein*) *α-subunit* และ *vacuolar H^+ ATPase* (*V-ATP*) *14 kDa subunit* โดยเทคนิค RACE - PCR ซึ่งมีขนาด ORF ยาว 1140, 2304, 2292 และ 369 เบส แปลงเป็นโปรตีนที่มี 380, 768, 764, และ 123 กรดอะมิโนตามลำดับ เมื่อวิเคราะห์การแสดงออกของยีนต่างๆ ด้วยวิธี quantitative real-time PCR ไม่พบความแตกต่างระหว่างระดับการแสดงออกของ *AMACR* และ *V-ATP* ใน hepatopancreas ของกลุ่มตัวอย่างที่โตเร็วและโตช้า แต่พบระดับการแสดงออกของยีน *CPT-1A* ในกลุ่มโตเร็วสูงกว่าในกลุ่มโตช้า อย่างมีนัยสำคัญทางสถิติ ($P < 0.05$)

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....ปาริฉัตร พรายภู่.....

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PARICHART PRIAPUE: DEVELOPMENT OF MOLECULAR MARKERS FOR STUDIES OF GENETIC POLYMORPHISM AND EXPRESSION OF GENES RELATED WITH METABOLISMS IN THAI ABALONE *Haliotis asinina*. THESIS ADVISOR: ASSOC. PROF. PADERMSAK JARAYABHAND, Ph.D., THESIS CO-ADVISOR: SIRAWUT KLINBUNGA, Ph.D., 211 pp.

A total of 2876 ESTs were *in silico* analyzed and 178 EST sequences consisting of microsatellite regions were found. Four loci (*DW455*, *DW503*, *PHe177*, and *PT102*) of type I and 2 loci (*Haμ9* and *Haμ10*) of type II microsatellites were used for determination of genetic diversity in wild and domesticated *H. asinina* and generated 10, 7, 6, 6, 10, and 12 alleles across all samples, respectively. Allele distribution frequencies revealed that the domesticated stocks are majorly contributed by the Gulf of Thailand founders. Observed and expected heterogeneity in wild populations ranged from 0.0625 - 1.0000 and 0.0625 - 0.8448 whereas those in CTRGH and CSMaRTH were 0.3441 - 0.7769 and 0.3333 - 0.8750, respectively. Lower allele and genotype discrimination capacity in domesticated than wild stocks suggested slightly reduced genetic diversity even though high observed heterozygosity was found in the domesticated stocks of *H. asinina*. F_{ST}-statistics and the exact test between pairs of samples at each locus clearly revealed significant differences between wild abalone from the east (SAME, and CAME) and west (TRGW) coasts of Thai waters and the Philippines. Both domesticated stocks (CTRGH and CSMaRTH) were genetically different from TRGW (west) and the Philippines (PHI) at all loci.

For AFLP analysis, 64 selective amplification primers were screened. Nine candidates AFLP were converted to sequence-characterized amplified region (SCAR) markers. $HaSCAR_{320}$, $HaSCAR_{295}$, and $HaSCAR_{327}$ exhibited 3, 2, and 3 SSCP genotypes respectively. SSCP genotypes of *H. asinina* from the Gulf of Thailand and Andaman Sea did not overlap at the $HaSCAR_{327}$ locus. Genotypes AAA, AAC, and CAA were restrictively observed in the Gulf of Thailand whereas ABB, BAB, and BBB were only distributed in the TRGW sample. Almost all of the CSMaRTH and CTRGH stocks exhibited the east coast genotypes (131/135; 97.03%). Distribution frequencies of composite SSCP genotypes and genetic heterogeneity analysis suggested that offspring of abalone from the east coast population exhibited better adaptability to the cultivated conditions than those from the west coast population.

Correlation between genotypes and the body weight of *H. asinina* (group B) was tested at each microsatellite. A significant genetic heterogeneity between BL - BS subgroups was observed at *DW455* and *Haμ10* loci ($P < 0.05$). Significant differences between the body weights of the B sample having different genotypes were only observed at *Haμ10* ($P < 0.05$).

The full length cDNA of *α-methylacyl-CoA racemase* (*AMACR*), *carnitine O-palmitoyltransferase 1A* (*CPT-1A*), *hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* (*trifunctional protein*) *α-subunit* and *vacuolar H^+ ATPase* (*V-ATP*) *14 kDa subunit* were successfully characterized by RACE-PCR. *AMACR*, *CPT-1A*, *hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* and *V-ATP* contained the open reading frame (ORF) of 1140, 2304, 2292 and 369 bp corresponding to a polypeptide of 380, 768, 764 and 123 amino acids, respectively. Quantitative real-time PCR analysis indicated that the relative expression level of *AMACR* and *V-ATP 14 kDa subunit* of fast- and slow-growing subgroups of domesticated abalone were not significantly different. In contrast, the expression level of *CPT-1A* in hepatopancreas of the former was significantly greater than that of the latter subgroup ($P < 0.05$).

Field of Study : Biotechnology Student's Signature Parichart Priapue

Academic Year : 2009 Advisor's Signature P. Jarayabhand

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LIST OF ABBREVIATIONS

bp	=	base pair
^{o}C	=	degree celcius
DEPC	=	Diethylpyrocarbonate
dATP	=	deoxyadenosine triphosphate
dCTP	=	deoxycytosine triphosphate
dGTP	=	deoxyguanosine triphosphate
dTTP	=	deoxythymidine triphosphate
DNA	=	deoxyribonucleic acid
EDTA	=	ethylene diamine tetra acetic acid
HCl	=	hydrochloric acid
IPTG	=	isopropyl-thiogalactoside
kb	=	kilobase
KCl	=	potassium chloride
M	=	molar
$MgCl_2$	=	magnesium chloride
$MgSO_4$	=	magnesium sulfate
mg	=	milligram
ml	=	millilitre
mM	=	millimolar
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
ng	=	nanogram

OD	=	optical density
PCR	=	polymerase chain reaction
RNA	=	Ribonucleic acid
pg	=	picogram
RNase A	=	ribonuclease A
rpm	=	revolution per minute
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

CHAPTER I

INTRODUCTION

1.1 General introduction

Abalone are commercially important marine gastropod distributed worldwide in tropical and temperate areas, particularly in the subtidal zones. There are over 100 abalone species and all of them are taxonomically allocated in the genus *Haliotis* (Geiger, 1998; Geiger, 2000; Gruenthal and Burton, 2005). At least 20 species of abalone are commercially important for their edible foot muscle (Table 1.1). Most of which are large size species (except *Haliotis diversicolor supertexta*) harvested from natural stocks (Jarayabhand and Paphavasit, 1996). The abalone products were traded in live, canned, fresh, frozen or dry forms. In addition, the shells were used for decorative purposes whereas their internal organs can use for protein hydrolysate (Lapyernyong, 2005).

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). Overexploitation of natural abalone fishery resulted in the impetus for developing abalone aquaculture in several Asian countries, and others including USA, Mexico, Japan, Australia, New Zealand, Mexico, and South Africa which are major producer of abalone at present (Uki, 1989). There are over 15 species of abalone dominated by temperate species that are farmed and commercially important in many countries and contributes approximately 75% of the world production annually (Hahn, 1989; Jarayabhand and Paphavasit, 1996; Gordon, 2000; Amparyup *et al.*, 2004; Tang *et al.*, 2005 Hara and Sekino, 2007).

The total world abalone fisheries were reduced from 14,830 metric tons (MT) in 1989 to 10,146 MT in 2002 and slightly declined to 9,200 MT in 2007 (Figure 1.1 A). However, the total farm production was dramatically increased from 1,220 MT in 1998 to 8,700 MT and 26,000 MT in 2002 and 2007, respectively (Figure 1.1 B). The estimated world's supply (including fisheries landings, illegal catch and cultured production) in 2007 were 39,600 MT whereas the abalone demands were more than 40,000 MT annually (Figure 1.1 C).

Table 1.1 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.

China is the largest abalone producer with the production of more than 26,000 MT in 2007. More than 300 abalone farms were operated in China. Most products were consumed within the country. Taiwan and Korea anticipated at over 8,000 MT of the total production whereas Chili and South Africa produced about 1,000 MT mostly *H. rufescens* and *H. midae*. In USA, there are 13 abalone farms on the coast of California with the production range from 10 to over 100 MT from each producer. The farmed production in Australia and New Zealand is approximately 600 MT.

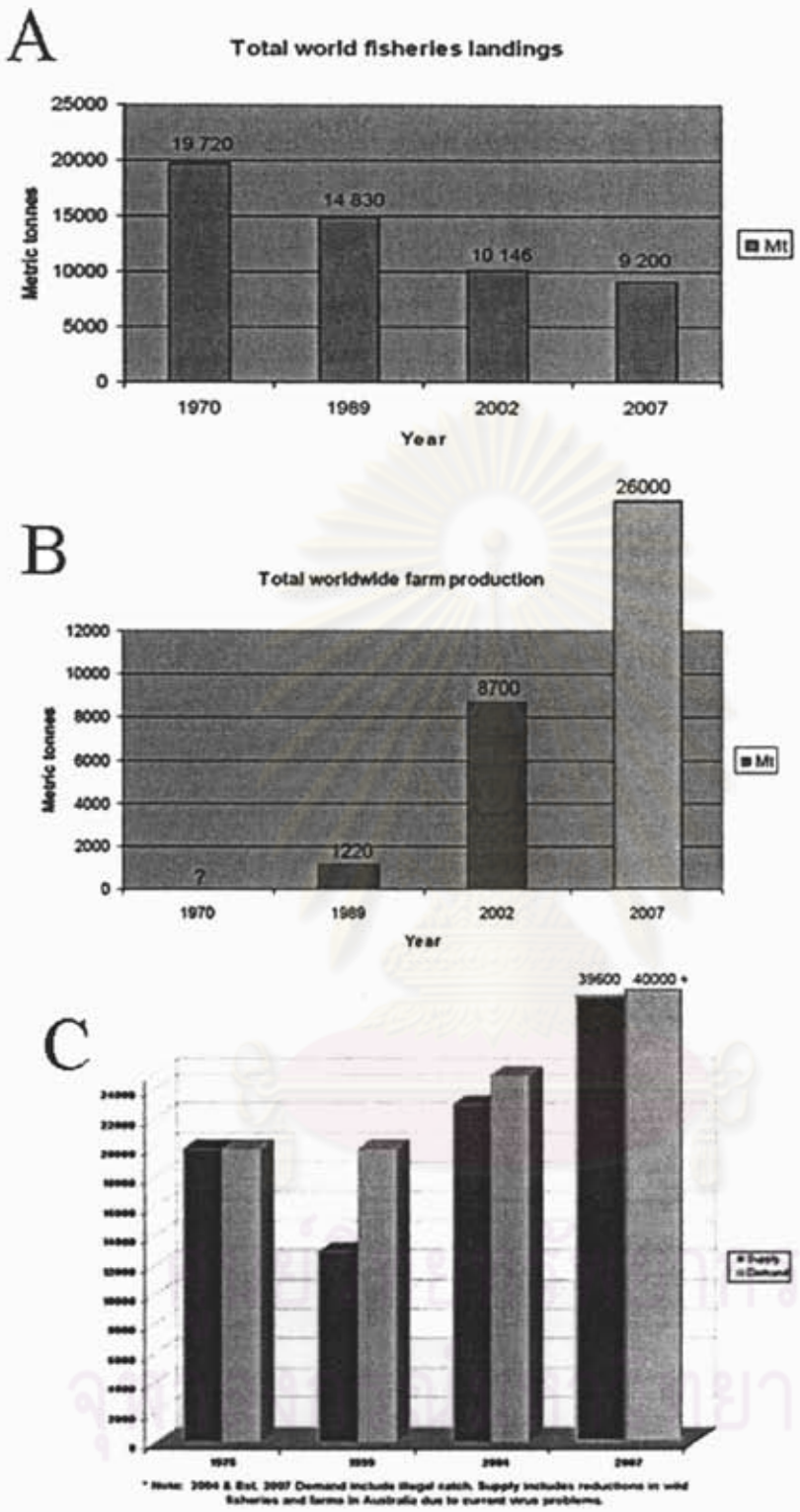


Figure 1.1 The amount of total world fisheries landings (A), total worldwide farm production (B) in 1970, 1989, 2002, and 2007; and demand and supply of abalone in 1975, 1999, 2004, and 2007(C).

The remaining countries including Europe, Iceland and Pacific Rim countries have a farmed production over 300 MT (Fishtech, 2007).

Prices of abalone depend upon sizes, species, forms, location of producers (or fishery), marketing and quality and usually ranging from US$15/kg to US$50/kg or more (Cook and Gordon, 2008).

1.2 Taxonomy, Biology and Distribution of Thai 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)

Haliotis ovina (Gmelin, 1791)

Haliotis varia (Linnaeus, 1758)

Abalones exhibit a very clear nocturnal behavior. Generally, abalones prefer shallow, turbulent waters with high levels of dissolved oxygen and hard surfaces for settlement. 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 and usually venture out of hiding to feed only after dark (Wood and Buxton, 1996). The abalone shell is in a spiral form covers most of the top of the body part of the abalone (Figure 1.3). 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 wastes removal. Abalone holds onto the sea-bed with its foot. A series of tentacles is found around the outside of the foot, presumably for detection predators and food by touch and taste (Figure 1.2). 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. Paired gills are located in a chamber called the mantle cavity. 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. The reproductive glands (or gonads) envelop the tubes of the gut. They form a large cone-shaped appendage between the shell and the foot. 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.

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 and Law, 1974; McShane *et al.*, 1988; Tutschulte and Connell, 1981). Adjacent female and male abalone shed eggs and sperm 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. Initially, an upward swimming larva (trochophore) is 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. Most of abalone are mature between the first and third year. The life span of abalone is longer than a decade.

Taxonomic identification of Thai abalone is based primarily on shell morphology (Figure 1.3). In Thailand, three tropical abalone species; *Haliotis asinina*, *H. ovina*, and *H. varia* were found (Jarayabhand and Paphavasit, 1996). *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). Morphological characteristics, habitat, species distribution and abundance of Thai abalone are shown in Table 1.2.

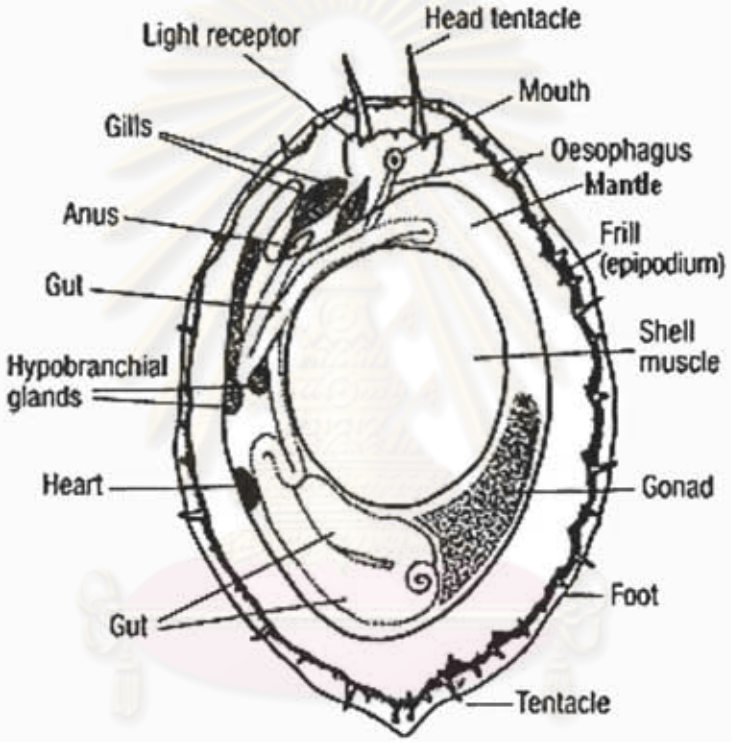


Figure 1.2 Dorsal view anatomy of abalone when shell was removal (http://www.nmfs.noaa.gov/pr/species/invertebrates/blackabalone.htm).

***Haliotis asinina* (Linnaeus, 1758)**

***Haliotis ovina* (Gmelin, 1791)**

***Haliotis varia* (Linnaeus, 1758)**

Figure 1.3 Shell Morphology of *H. asinina*, *H. ovina*, and *H. varia* (Geiger, 2000).

Table 1.2 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).

Among these species, *H. asinina* provides the highest percentage ratio of meat weight to total weight (85%), compared to that of 40% and 30% for *H. ovina* and *H. varia*, respectively (Singhagraiwan and Doi, 1993). In addition, *H. asinina* has a high value for 'cocktail-sized' (40-70 mm) market from their size and meat textures as happening with *H. diversicolor supertexta* in Taiwan (Jarayabhand and Paphavasit,

1996). Moreover, *H. asinina* grows rapidly, the spawning cycle is highly predictable in hatchery conditions, and reach sexual maturity within less than one year when feed with natural and artificial diets. Thus, these species is currently promoted for culture commercially in Thailand (Selvamani *et al.*, 2001; Klinbunga *et al.*, 2004; Tang *et al.*, 2005).

Fishery management has been defined as the application of scientific knowledge to the problems of providing the optimum yield, which is prescribed on the basis of maximum sustainable yield of commercial fisheries products (Allendorf *et al.*, 1987). The proper management requires a basic understanding on biological principles e.g. ecology, population structure and dynamics which are exceptionally important for economically important species.

An effective management of any species requires fundamental understanding of various biological principles such as ecology, physiology, population genetics and dynamics. One of the primary objectives of fishery management is to understand the population structure of any exploited species (Ryman, 1991; Conover *et al.*, 2006).

The recognition of genetically differentiated populations within a species is of importance for their management. Fishery managers need information on size and number of populations in an area to strategize population management and exploitation. Ultimately, the most important goal of fishery management for exploited species is to conserve the existing resources to ensure sustainable yield (Allendorf *et al.*, 1987; Ryman, 1991).

1.3 Domestication and selective breeding program of *H. asinina*

Domestication and subsequently selective breeding programmes (SBPs) are long-term processes used to commercially important trait in selected populations through domestication both (artificial and natural selection). To carry out effective SBPs in *H. asinina*, the integrating of basic knowledge on population genetics for estimation of genetic variation levels, molecular genetics for the identifying genetic markers at different levels and quantitative genetics for selection scheme and estimation of heritability for economically important phenotypes are required. At present, molecular genetic tools in *H. asinina* have been developed to the level that can be directly implemented for practical SBPs approaches.

The breeding program of *H. asinina* has been carried out at Sichang Marine Science Research and Training Station (SMaRT), Chulalongkorn University since the last decade. Founders of the established stocks were originated from various geographic locations of both the Gulf of Thailand (east) and the Andaman Sea (west).

The hatchery-propagated stocks at SMaRT were divided into eight lines. In each breeding, mature males form line 1 were select to cross with female from line 2 and continually bred in such manner to other abalone lines. Male and female used in each spawning time was about 30 - 40 individual per sex. At present, the 5^{th} - 6^{th} generations of abalone is culturing at SMaRT. The levels of genetic diversity, effective population size (N_e), parentage assignment and patterns of gene pools of each domesticating generation should be examined by appropriate genetic markers to reveal possible changes in variability caused by genetic drift, inbreeding, or selection in these stocks (Allendorf and Ryman, 1987; Cruz *et al.*, 2004).

Appropriate genetic markers can be used to elevate culture and management efficiency of economically important species. However, molecular markers indicating the progress of domestication and adaptation of hatchery-propagated *H. asinina* have not been reported. The fundamental controls of growth in *H. asinina* have not been reported. Apart Functional involvement of growth-related genes in *H. asinina* exhibiting different growth rates should be further examined. This information is essential and can be directly applied for the construction of appropriate breeding programmes and for broodstock selection and management scheme leading to sustainable culturing activity of *H. asinina* in Thailand.

1.4 Molecular genetic techniques used in this thesis

Analysis of genetic diversity and relatedness between different species, populations, and individuals is a central task for many biological disciplines. During the past several decades, classical strategies of evaluating genetic variability such as comparative anatomy, morphology, embryology, and physiology have been increasingly complemented by molecular techniques. These techniques are particular powerful when morphological differentiate are ambiguous (Burton, 1996).

Several molecular techniques are commonly used to determine DNA polymorphism for population genetic and systematic purposes. These techniques

included randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (PCR-RFLP), microsatellites and DNA sequencing (Liu and Cordes, 2004). Although DNA sequencing analysis for genetic diversity and intraspecific population differentiation of various organisms is the most direct and reliable technique, other alternative approaches to detect polymorphism are also sufficiently reliable to infer genetic diversity and geographic differentiation of organisms.

1.4.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique widely used in molecular biology introduced by Mullis *et al.* (1987). This method is an *in vitro* technique of DNA replication by a thermostable DNA polymerase, using specific DNA sequences of two oligonucleotide primers, usually 18 - 27 nucleotides in length. Million copies of the target DNA sequence can be synthesized from the low amount of starting DNA template within a few hours.

The PCR components are composed of DNA template, a pair of oligonucleotide primers complementary to the target sequence, deoxynucleoside triphosphates, dNTPs (dATP, dCTP, dGTP, and dTTP), PCR buffer solution and heat-stable DNA polymerase (usually *Taq* DNA polymerase). The amplification reaction typically consists of three steps; denaturation of double stranded DNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal (annealing) temperature, and extension of the annealed primers by heat-stable DNA polymerase. The cycles were repeated for 30 - 40 times (Figure 1.4). The amplification product is analyzed by agarose or polyacrylamide gel electrophoresis.

PCR is used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a part of a gene, or a non-coding sequence (Cheng *et al.*, 1994). The power and selectivity of PCR are primarily due to degrees of selectivity of primers that are highly complementary to the DNA region targeted for amplification, and the thermal cycling conditions used.

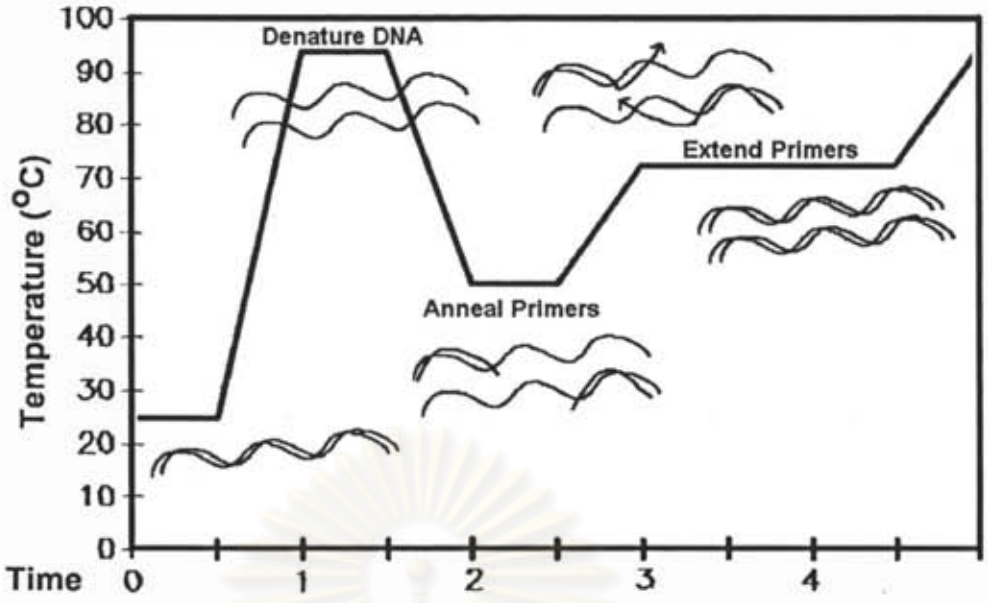


Figure 1.4 General illustration of the polymerase chain reaction (PCR) for amplification of the target DNA (www.mun.ca/biology/scarr/PCR_simplified.html).

1.4.2 Expressed Sequence Tags (EST) analysis

ESTs are large-scale single-pass sequences of randomly picked clones from a cDNA library usually constructed from mRNA at a particular development stage and / or tissue and have proven to be efficient and rapid means to identify novel genes (Adams *et al.*, 1991). This method has been widely employed for discovering novel and uniquely expressed genes, and for characterizing the gene expression profiles of several tissues.

The construction of a cDNA library begins with purification of mRNA from the target tissue that is subsequently transcribed into the first strand cDNA. This step is catalyzed by reverse transcriptase using the oligo (dT) primer as the synthesizing primer. The second strand DNA is then copied from the first strand cDNA using *E. coli* DNA polymerase I. The double strand cDNA is ligated to adapter and subsequently to an appropriate vector using T4 DNA ligase. The recombinant vector-cDNA molecules are packaged (for λ vector) *in vitro* and transfected to the

appropriate host. If a plasmid is used recombinant plasmid is transformed into *E. coli* host cells to generate a cDNA library (Figure 1.5).

ESTs can be sequenced from either 5′ or 3′ ends of cloned cDNA. The 3′ end of the cloned insert is usually marked by the poly A stretch which is often problematic for thermostable polymerase sequencing, and sequencing through poly T can reduce the length and quality of the subsequent sequence. Nevertheless, 3′ UTR usually exhibit reasonably high polymorphism and is a promising location for SNP identification. The 5′ ESTs have the advantage of being more likely to include some of the open reading frame (ORF) of cDNAs and thus facilitate identification of the encoded product.

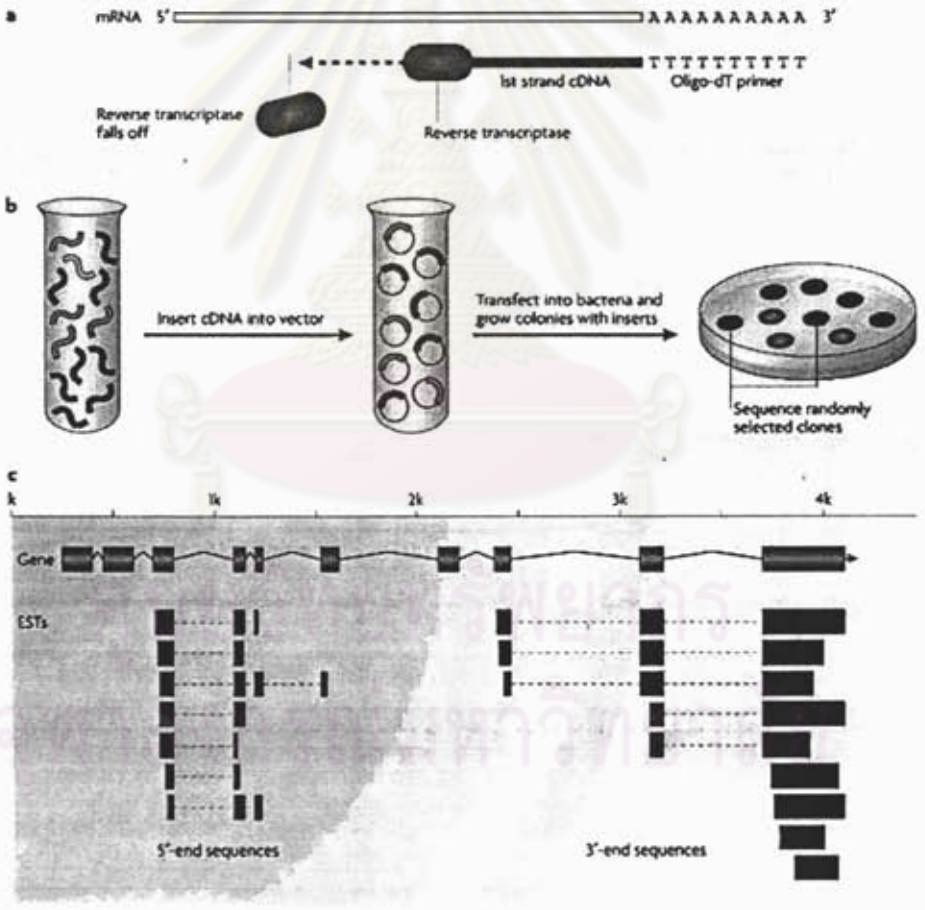


Figure 1.5 Schematic illustrate the synthesis of 1st strand cDNA from mRNA (a), the insertions of cDNA into vector (b) and ESTs results compared with the target gene (c) (http://www.nature.com/nrg/journal/v9/n1/box/nrg2220_BX1.html).

EST sequences are used for homology search for identification of gene homologues through the sequence data in the GenBank (Altschl *et al.*, 1997). EST analysis is an important tool for several applications. They have mainly applied for rapid gene discovery of several animal genome and comparative genomics and functional genomics in various organisms (Zeng, 2002; Satou, 2003; Waterston, 1992; McCombie, 1992; Tugendreich, 1994 and Mironov, 1999).

1.4.3 DNA sequencing

Polymorphism at the DNA level can be studied by several methods but the most direct strategy is determination of nucleotide sequences of a defined region. The sequences obtained can be aligned by comparing an orthologous region in the genome of related organisms (or populations). PCR-amplified fragments can be directly sequenced using the typical chain termination reaction (Sanger, 1977) or alternatively by cycle-sequencing. Nevertheless, more accurate sequences of DNA fragments are obtained through cloning prior to sequencing of the interesting fragments.

The enzymatic sequencing approach has presently been developed to automated method (Figure 1.6). DNA sequences can be detected using a fluorescence-based system following labeling with a fluorescence dye. PCR allow the possibility to isolate homologous DNA sequences from any organism of interest with unprecedented speed. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

1.4.4 Microsatellites

Satellite DNA, a type of tandemly arranged highly repetitive sequence, has been found 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′ untranslated regions of genes (Budowle *et al.*, 1991; Huang *et al.*, 1997). In contrast, microsatellites are located abundantly in both genic and extragenic regions of eukaryotic genomes (Kimpton *et al.*, 1993) (Figure 1.7). Microsatellites are embedded in untranslated genomic DNA sequences (Type II) or the coding region of

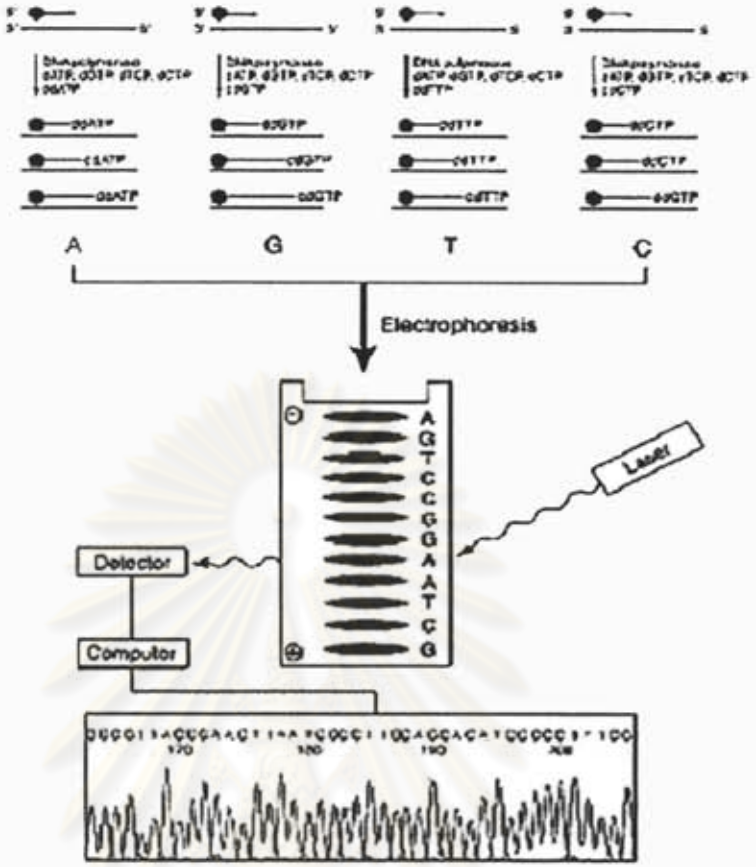


Figure 1.6 A diagram showing the general protocol for DNA sequencing.

genes (Type I) (Valdes *et al.*, 1993). It was estimated that one microsatellite locus may be found every 10 kb in eukaryotic genomes. 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 of various taxa (Bosch *et al.*, 1993; Jeffreys *et al.*, 1991; Budowle *et al.*, 1991; Deka *et al.*, 1995; Primer *et al.*, 1996).

The levels of microsatellite polymorphism vary greatly among loci but are often higher than those observed in allozymes and mtDNA (O'Reilly and Wright, 1995). Highly polymorphic loci exhibiting large numbers of alleles are ideal for gene mapping and pedigree analysis (Pepin *et al.*, 1995), whereas loci with lower levels of

polymorphism can be used for analysis of population differentiation (Estoup *et al.*, 1995, 1996).

Each microsatellite locus is flanked by a unique sequence. As a result, locus-specific primers can be synthesized complementary to the flanking sequences and PCR is then used to amplify the tandem array of the microsatellite locus. The PCR-amplified products are size-fractionated on denaturing polyacrylamide gels. The abundance of di-nucleotide microsatellite in eukaryotic genomes makes them, in general, easily isolated by standard cloning methodologies.

For species that a large number of EST is available, type I microsatellite primers can be designed from cDNA containing simple sequence repeats. The use of type I microsatellites for genetic diversity studies have been reported in several

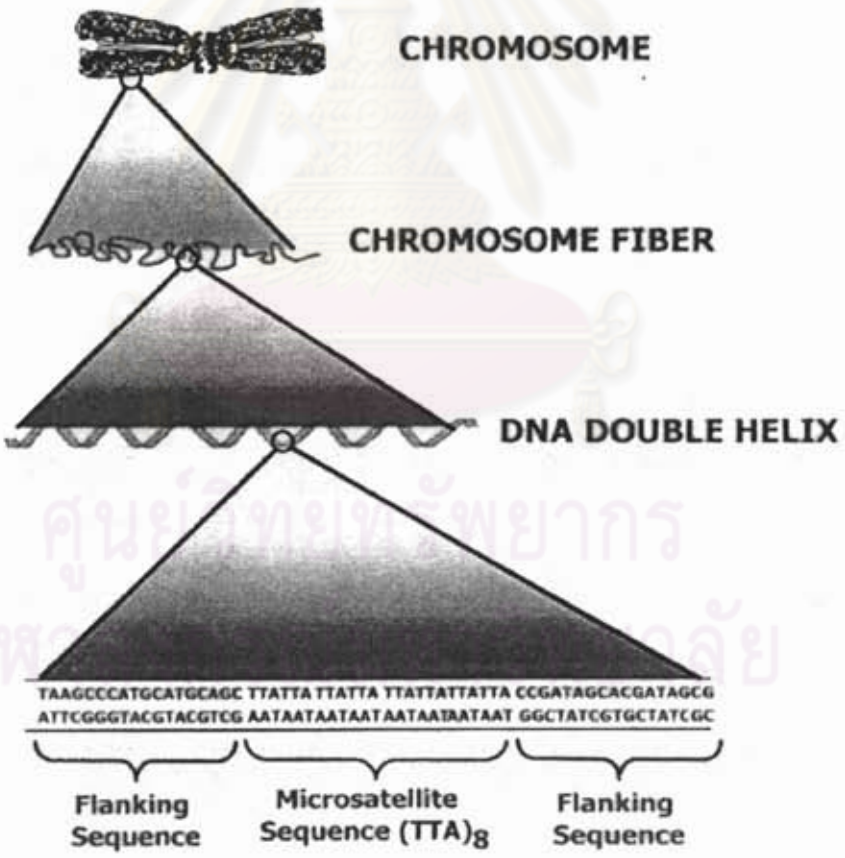


Figure 1.7 A diagram showing microsatellite region

(http://www.asicoaquaticmarkers.com/AnatomyofaMicrosatellite.htm).

species as the data on EST sequences of various species have accumulated rapidly. These sequences represent transcribed sequences of genes. ESTs containing microsatellites offer a relatively simple and efficient means for the development of PCR-based microsatellite markers with function gene information (Scott *et al.*, 2000; Thiel *et al.*, 2003; Park *et al.*, 2005). However, the disadvantage on the use of type I microsatellites for population genetic studies is that they may be selective non-neutrally. Therefore, type I microsatellites should be carefully tested before applied for that application.

When multiple loci of highly polymorphic microsatellites are used for genotyping, the information enabling the distinction between specific individuals can be obtained (Wright and Bentzen, 1995). Moreover, co-dominant Mendelian inheritance makes microsatellites more informative in pedigree studies and suitable for large breeding programs than other molecular markers that segregate dominantly.

1.4.5 Amplified Fragment Length Polymorphism (AFLP)

AFLP was developed by Vos *et al.* (1995) to increase the potential of RFLP and to compensate reproducibility of RAPD by selective amplification of a random array of restriction fragments ligated to linkers of known sequences. The major strengths of the AFLP technique include large (> 100) numbers of polymorphic loci screened, high reproducibility due to high PCR annealing temperatures, and relatively cost effectiveness.

Initially, genomic DNA is digested with two enzymes (a frequent and a rare cutter, for example *Mse* I and *Eco* RI). Double stranded DNA adaptors, with a core sequence and the appropriate overhangs for the enzymes used are ligated to the ends of the digested fragments.

PCR amplification was carried out for twice; pre-selective and selective amplification. Pre-amplification of these fragments is achieved by PCR carried out by adding a single known base to the 3′ end of the primer complementary to each core sequence adaptor. The product from the primary amplification were diluted and further re-amplified by selective primers of core sequence, usually with 3 bases extend to the 3′ end of primers. Optimal amplified genomic DNA fragments are

analyzed by denaturing polyacrylamide gels electrophoresis followed by radioactive (autoradiography) or non-radioactive (silver staining) detection (Figure 1.8).

The main advantages of AFLP are its more reproducibility than RAPD-PCR due to specificity of the PCR primer and high stringency of the amplification reaction. Like RAPD-PCR, AFLP analysis does not require the prior knowledge about genome sequences of species under investigation. The technique offers great flexibility in the number of loci that can be co-amplified in one PCR reaction (typically, 50 to 100

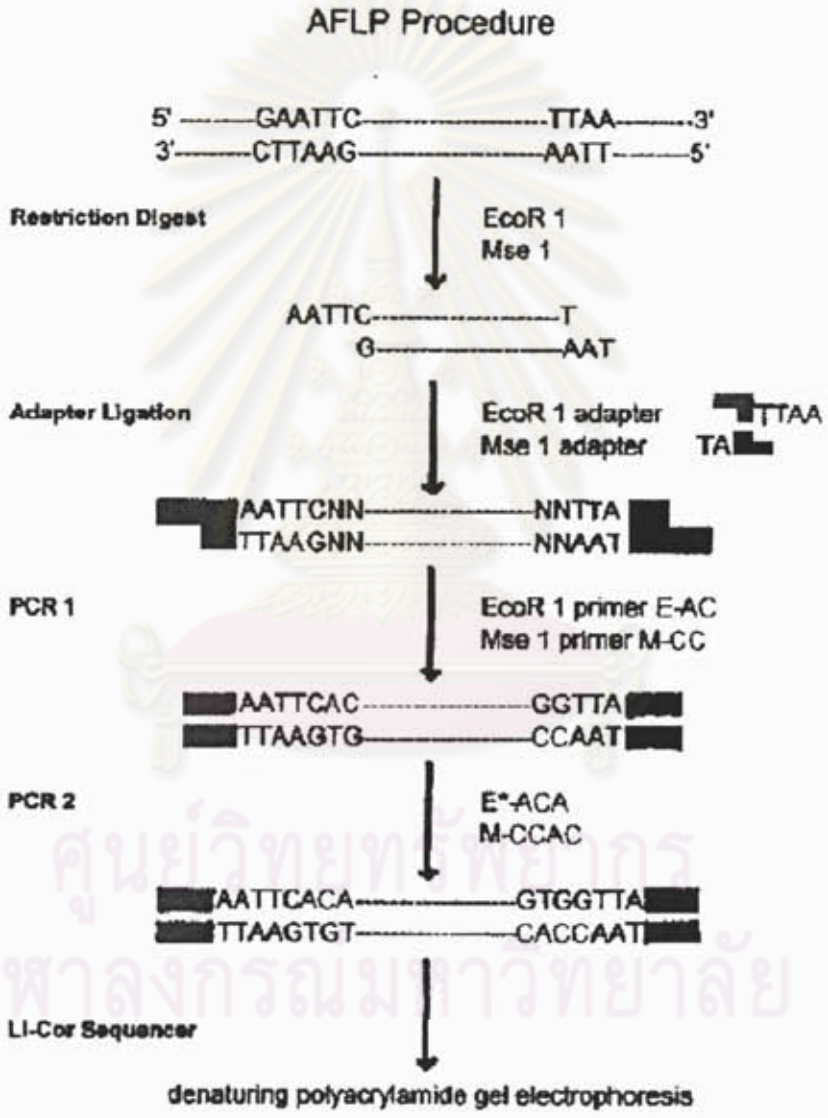


Figure 1.8 A schematic diagram illustrating principles of AFLP procedure (http://sorrel.humboldt.edu/~jlg21/AFLP/principl.html).

restriction fragments). This technique is therefore extremely powerful for identification of DNA polymorphism. AFLP markers can be further converted to SCAR markers by sequencing, cloning and designing of locus-specific primers for direct application for the developed markers.

1.4.6 Single nucleotide polymorphism (SNP)

Single nucleotide polymorphism (SNP) is a single base change in the DNA sequence, with a usual alternative of three possible nucleotides at a given position. For such a base position with sequence alternatives in genomic DNA to be considered as an SNP, the lowest frequent allele should have a frequency of 1% or greater. Although in principle, at each position of a sequence stretch, any of the four possible nucleotide bases can be present.

SNPs are usually bi-allelic. One of the reasons for this is that the low frequency of single nucleotide substitutions at the origin of SNPs, estimated to being between 1×10^{-9} and 5×10^{-9} per site per year at neutral positions in mammals. Therefore, the probability of two independent base changes occurring at a single position is very low. Another reason is due to a bias in mutations, leading to the prevalence of two SNP types (transitions).

1.4.7 Single Stranded Conformational Polymorphism (SSCP)

Single-strand conformational polymorphism (SSCP) analysis was originally described by Orita *et al.* (1989). SSCP analysis is one of the effective techniques used for the detection of mutations and variation of the DNA due to single nucleotide polymorphism (SNP) and/or insertions/deletions (indels). SSCP relies on the principle that the electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is dependent on its structure and size (Orita *et al.*, 1989; Hayashi, 1991). The amplified PCR product (usually less than 400 bp in length) is denatured and loaded into low crosslink non-denaturing polyacrylamide (with or without glycerol supplementation).

Base substitutions (transitions and transversions) result in alteration of the folding affecting the migration of single stranded DNA through polyacrylamide gels.

Therefore, SNP can be conveniently detected through SSCP. The general procedure of SSCP is illustrated by Figure 1.9.

The major advantage of SSCP is that polymorphism of the PCR product of several investigated individuals can be simultaneously examined. SSCP is relatively simple and does not require expensive equipment. Heteroduplex double strand DNA (located at the middle of the gel) can occasionally be resolved from homoduplex double strand DNA (located the bottom of the gel) and give additional information on the presence of variants resulted from single strand DNA (located at the top of the

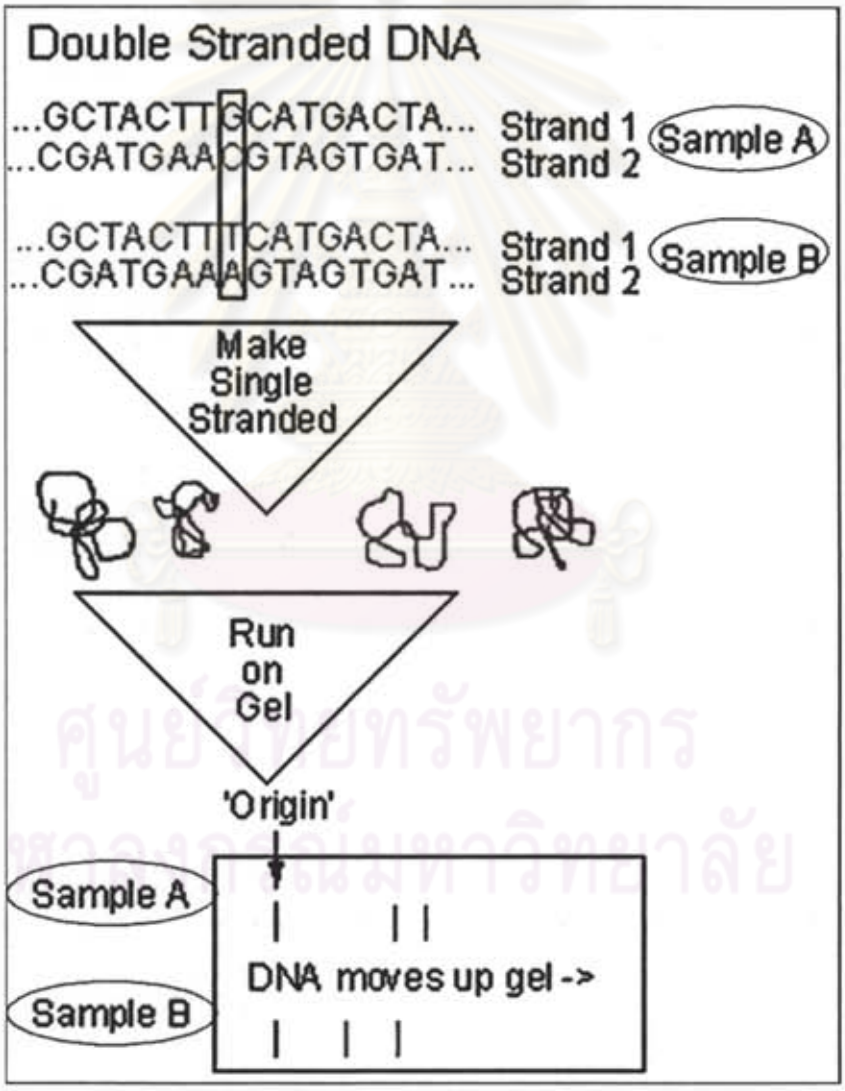


Figure 1.9 A schematic diagram for analysis of DNA polymorphism using SSCP analysis.

gel). Therefore, SSCP is regarded as one of the potential techniques that can be used to detect polymorphism in various species prior to confirmation of the results by nucleotide sequencing. The other advantage of SSCP is that small PCR amplicons are required. Generally, small sizes of PCR products are relative easy to amplify.

The disadvantage of SSCP is that it requires nucleotide sequence data for the design of specific primers. Moreover, reproducibility of the technique seems to be problematic because SSCP patterns are affected by temperature and degree of cross-linking. Additionally, multiallelic patterns of some nuclear DNA markers may cause SSCP patterns too complicated for estimation of allele frequencies precisely.

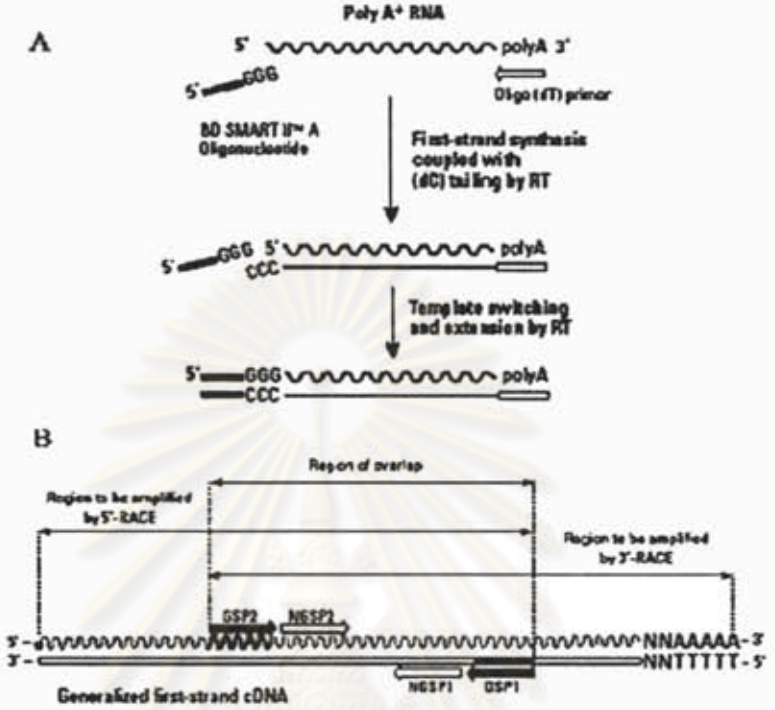
1.4.8 Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR)

RACE-PCR is an approach used for isolation of the full length of characterized cDNA. This method generates cDNA fragments by using PCR to amplify sequences between a single region in the mRNA, either the 3′- or the 5′- end of the transcript. It is necessary to know or to deduce a single stretch of sequence within the mRNA. From this sequence, specific primers are chosen which are oriented in the 3′ and 5′ directions, and which usually produce overlapping cDNA fragments (Primrose, 1998).

Using SMART (Switching Mechanism At 5′ end of RNA Transcript) technology, terminal transferase activity of Powerscript Reverse Transcriptase (RT) adds 3-5 nucleotides (predominantly dC) to the 3′end of the first strand cDNA. This activity is harnessed by the SMART oligonucleotides whose terminal stretch of dG can anneal to the dC-rich cDNA tail and serve as an extended template for reverse transcriptase. A complete cDNA copy of original mRNA is synthesized with the additional SMART sequence at the end (Figure 1.10).

The first strand cDNA of 5′ and 3′ RACE is synthesized using a modified oligo (dT) primers and serve as the template for RACE-PCR reactions. Gene specific primers (GSPs) are designed from the target gene for 5′-RACE PCR (antisense primer) and 3′-RACE PCR (sense primer) and used with the universal primer (UPM)

that recognizes the SMART sequence. RACE products are characterized. The full length cDNA is identified after sequence assembly.



C: Detailed flow chart of 5′-RACE-PCR

D: Detailed flow chart of 3′-RACE-PCR

Figure 1.10 Overview of the SMART™ RACE cDNA Amplification reaction.

1.4.9 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a comparable method of conventional PCR but the first strand cDNA template rather than genomic DNA was used as the template in the amplification reaction (Figure 1.11). It is a direct method for examination of gene expression of known sequence transcripts in the target species. The template for RT-PCR can be the first stranded cDNA synthesized from total RNA or poly A^+ RNA. Reverse transcription of total RNA can be performed with oligo(dT) or random primers using a reverse transcriptase The product is then subjected to the second strand synthesis using a gene-specific forward primer.

RT-PCR can also be used to identify homologues of interesting genes by using degenerate primers and/or conserved gene-specific primers from the original species and the first strand cDNA of the interesting species is used as the template. The amplified product is further characterized by cloning and sequencing.

1.4.10 Quantitative real-time PCR

Real-time PCR is a kinetic approach enabling both detection and quantification (as the absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in the samples. The procedure follows the general principle of PCR. Its key feature is that the amplified DNA is quantified in real time after each amplification cycle as. Two common methods of the product detection are the use of fluorescent dyes that intercalate with double-stranded DNA (e.g. SYBR green), and the use of modified DNA oligonucleotide probes that are fluorescent when hybridized with a complementary DNA. Although more specificity, the later assay is more expensive than the former one.

The general principle of SYBR green PCR is composed of the first denaturation step, at which the unbound dye molecules are weakly fluorescent; the second step, primer annealing, where a few dye molecules bind to the double strand and the last step, product extension, when more dye molecules bind to the newly synthesized DNA, resulting in an increase of the fluorescence signal. Fluorescence

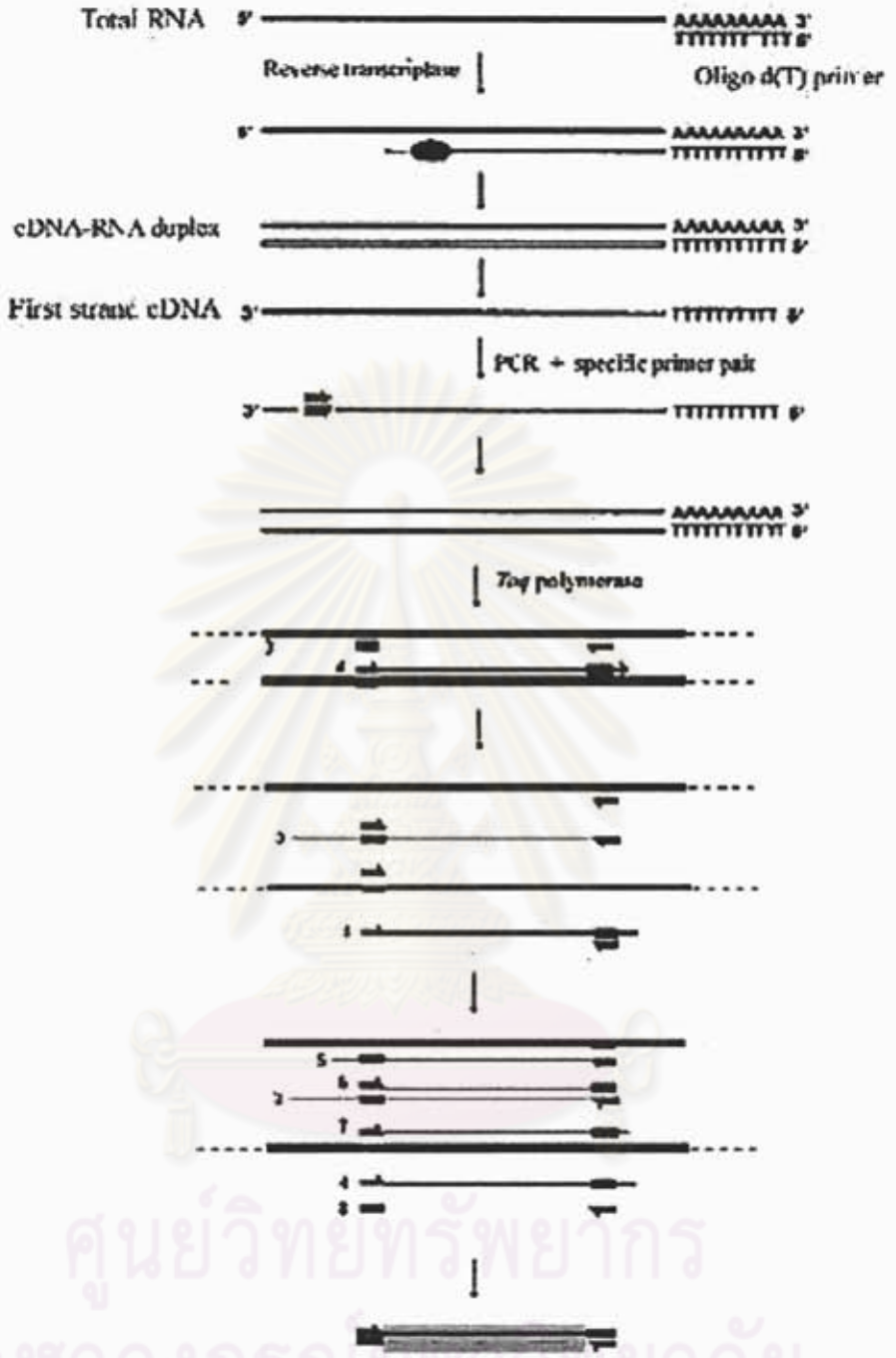


Figure 1.11 Overall concepts of RT-PCR. During the first strand cDNA synthesis, an oligo d(T) (or random primers) primer anneals and extends from sites present within mRNA. The second strand cDNA synthesis primed by the 18 – 25 base specific primer proceeds during a single round of DNA synthesis catalyzed by *Taq* DNA polymerase.

measurement at the end of the elongation step of every PCR cycle is then performed to monitor the increasing amount of amplified DNA (Figure 1.12).

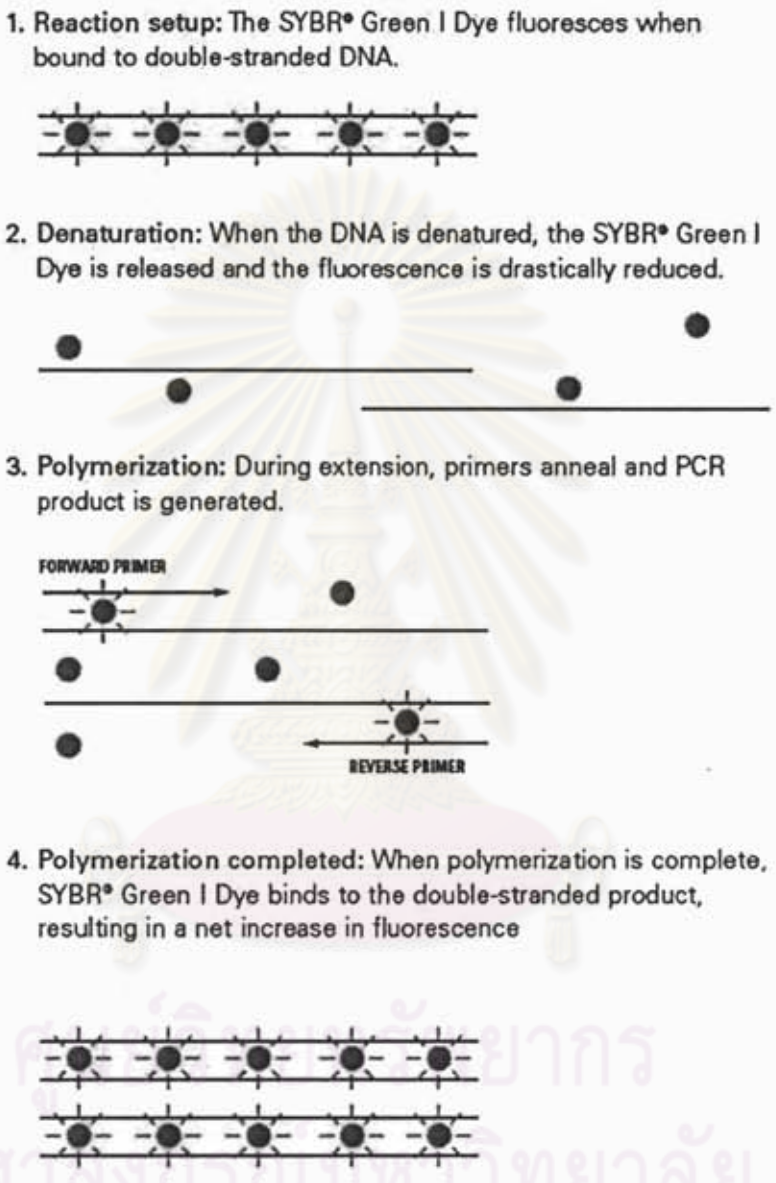


Figure 1.12 A schematic diagram showing real-time PCR procedure using SYBR Green I dye (http://www.scms.utmb.edu/genomics/taqsybr.htm).

1.5 Development of molecular markers and applications

1.5.1 Isolation of microsatellites and population genetic studies

Microsatellites were isolated and applied for genetic studies in several species, for example, investigating genetic variability and genetic structure in the greenshell mussel, *Perna canaliculus* (MacAvoy *et al.*, 2008); the mudflat topshell, *Diloma subrostrata* (Donald and Spencer, 2006); the land snail, *Arianta arbustorum* (Locher and Baur, 2001); the black tiger shrimp, *Penaeus mododon* (Tassanakajon *et al.*, 1998; Pan *et al.*, 2004); the Pacific white shrimp, *Litopenaeus vannamei* (Meehan *et al.*, 2003; Cruz *et al.*, 2004; Valles-Jimenez *et al.*, 2005); the pencilfish, *Nannostomus unifasciatus* (Beheregaray *et al.*, 2004); the shortfin mako, *Isurus oxyrinchus* (Schrey and Heist, 2003) and the blue carb, *Callinectes sapidus* (Steven *et al.*, 2005), for comparing genetic diversity of natural and cultivated population of Atlantic cod, *Gadus morhua* L. (Pampoulie *et al.*, 2006) and the mud carp, *Cirrhina molitorella* (Yang *et al.*, 2008), for evaluating growth responses in different families of the rainbow trout, *Oncorhynchus mykiss* (Palti *et al.*, 2006) and parentage determination in the kuruma shrimp, *Marsupenaeus japonicus* (Jerry *et al.*, 2004, 2006), the Chinese shrimp , *Fenneropenaeus chinensis* (Dong *et al.*, 2006) and *G. morhua* (Herlin *et al.*, 2007, 2008).

Microsatellites were also applied for genetic studies in various abalone species. Selvamani *et al.* (2000) isolated and characterized 11 microsatellites in *H. asinina*. Genetic variability of these microsatellites was examined in the Heron reef population of Australia (N = 21 – 41 per locus). The numbers of alleles per locus ranged from 2 to 25 alleles and the expected heterozygosity ranged from 0.29 to 0.96. The level of allelic variability for all except two loci (*Haμ13* and *HaμK*) was high. Two loci (*HaμJ* and *HaμL*) significantly deviated from Hardy-Weinberg expectations owing to heterozygote deficiencies (P = 0.003 and $P = < 10^{-6}$, respectively).

In addition, three genomic libraries were constructed for microsatellite isolation in the tropical abalone *Haliotis asinina* in Thai waters. The first genomic library was constructed from DNA fragments obtained from *Alu*I digestion. The second library was from vortexed and sonicated genomic DNA. The third library was

from mixed-enzyme (*Alu* I, *Rsa* I and *Hinc* II) digested genomic DNA. After screening with $(GT)_{15}$, it was found that the third library gave the highest percentage of positive clones (1.46%) whereas the first and the second libraries gave 0.2% and 0.43% of positive clones, respectively. Nucleotide sequencing of positive clones revealed that perfect, imperfect and compound microsatellites were found in the same proportion. From 33 microsatellite loci, 10 pairs of specific primers were designed for microsatellie amplification. Preliminary analysis of polymophism of 10 loci (*Has1 - 10*) showed that numbers of alleles ranged from 3-26 alleles and observed heterozygosity were 0.27 - 0.85. Genotypes from these microsatellites were associated randomly ($P > 0.0031$). All pairs of primers could not be used for cross-amplification in other tropical abalone species, *H. ovina* and *H. varia* (Tang, 2002).

Twelve of di-, tri- and tetranucleotide microsatellites were isolated and used for survey over 400 individuals in pinto abalone (*H. kamtschatkana*) from the coast of British Columbia. The number of alleles identified at each of the loci ranged from 20 to 63. Observed heterozygosities ranged from 0.44 to 0.93, Expected heterozygosities ranged from 0.68 to 0.96. Significant deviations from Hardy-Weinberg equilibrium were observed for six of the loci (*Hka*3, *Hka*6, *Hka*28, *Hka*48, *Hka*65, and *Hka*85) (Miller *et al.*, 2001).

Sekino and Hara (2001) isolated and characterized 5 microsatellite loci (*Hdd6C, Hdd108C, Hdd114B, Hdd115B*, and *Hdd229*) for examining genetic variability of the Pacific abalone *H. discus discus*. The number of alleles of all five loci ranged from 3 to 10, and the observed and expected heterozygosity ranged from 0.17 to 0.80, and 0.20 to 0.89, respectively. Two loci, *Hdd114B* and *Hdd229*, showed significant deviations of the observed genotype frequencies from Hardy–Weinberg's expectations ($P < 0.05$).

An and Han (2006) developed 17 microsatellites in *H. discus hannai*. All loci were polymorphic with an average of 13.1 alleles per locus (range 3 - 28). The mean observed and expected heterozygosities were 0.77 (0.17 - 1.00) and 0.79 (0.42 - 0.96), respectively. Six loci (*KHdh46, KHdh50, KHdh53, KHdh55, KHdh57*, and *KHdh64*) deviated significantly from Hardy–Weinberg proportions, and thus should be used with caution. The high polymorphic levels of these microsatellites suggest that they

should provide useful markers for studies of trait mapping, kinship and population genetics.

The microsatellite-enriched library of *H. discus hannai* was also constructed using magnetic bead hybridization selection method. Three hundred and fifty white colonies were screened by PCR, 84 clones were identified to potentially contain microsatellite repeat motif. After sequenced, 42 microsatellites and 4 minisatellites with a minimum of five repeats were found and could be categorized in to perfect repeats (73.3%), imperfect repeats (13.3%), and compound repeats (13.4%). The relative short arrays (< 20 repeats) were most abundant (75%). The largest length was 48 repeats and the average number of repeats was 13.4. These data can be useful for choosing the repeat motifs for microsatellite isolation in other abalone species (Li and Kijima, 2007).

Tang *et al.* (2004) examined genetic diversity and differentiation of natural *H. asinina* in coastal waters of Thailand using 3 microsatellite loci (*CUHas2, CUHas3,* and *CUHas8*). Genetic variation in terms of the effective number of alleles (n_e), H_o, and H_e were higher in 2 samples from the Gulf of Thailand (n_e = 9.37, 7.66; H_o = 0.62, 0.78; and H_e = 0.87, 0.86) than those of a sample (n_e = 6.04; H_o = 0.58; and H_e = 0.62) from the Andaman Sea. Assessment of genetic heterogeneity, including allele frequency comparison and pairwise F_{ST} analysis, indicated interpopulational differentiation, between natural *H. asinina* from the Gulf of Thailand and the Andaman Sea ($P < 0.0001$).

Subsequently, Tang *et al.*, (2005) extended genetic heterogeneity studies of *H. asinina* of within the Gulf of Thailand and the Andaman Sea using *CUHas1, CUHas4* and *CUHas5*. Results indicated relatively high genetic diversity in *H. asinina* (total number of alleles = 26, 5, 23, effective alleles per locus = 13.93, 2.47, 10.70 and observed heterozygosity = 0.84, 0.42 and 0.33, respectively). Significant population differentiation was also found between samples from different coastal regions ($P <$ 0.0001). This further confirmed that the gene pool of natural *H. asinina* in coastal Thai waters can be genetically divided to 2 different populations; the Gulf of Thailand and the Andaman Sea.

Microsatellites DNA markers were also potential for species-assignment in members of Pacific abalone species complex (*H. discus hannai*, *H. discus discus*, *H. madaka*, and *H. gigantea*) and five additional species *(H. diversicolor aquatilis*, *H. midae*, *H. corrugate*, *H. fulgens*, and *H. rubra*. The amplification success of 24 microsatellites was tested in various species and 4 of these were not transferable to *H. gigantea*, suggesting a solid genetic boundary between *H. gigantea* and other three species. Among the three latter abalones, both assignment tests achieved approximately 90% or more success rate to assignment (Sekino and Hara, 2007).

Gruenthal and Burton (2005) developed five nuclear microsatellite loci and partial sequences of one nuclear (VERL) and two mitochondrial (*COI* and *CytB*) genes, to assess genetic variability in the endangered white abalone (*H. sorenseni*). All five microsatellite loci were polymorphic and followed expectations of simple Mendelian inheritance in laboratory crosses. Each of wide-caught adult abalone exhibited a unique composite microsatellite genotype, suggesting that significant genetic differentiation still remains in natural populations. A combination of nuclear and mitochondrial gene sequencing demonstrated that one of the original wild-caught animals was not *H. sorenseni* but *H. kamtschatkana*. Similarly, another animal of uncertain identity accidentally collected was also shown to be *H. kamtschatkana*.

Thirty eight microsatellite markers that worked well in 3 full-sib families established from wild caught *H. discus hannai* were developed. Nine loci with easy scoring alleles were applied to 2 groups of samples derived from coastal areas where intensive releases of hatchery-produced abalone seedlings have been carried out. Multilocus genotypes based on the 9 markers rejected a null hypothesis of genetic homogeneity between populations. Although both populations conformed to Hardy Weinberg's equilibrium at almost all of the 9 loci, results of simulation analysis for variance of relatedness coefficient provided evidence of nonrandom mating in each population, possibly caused by the cumulative effect of stocking on the genetic make-up. The results consider that the microsatellite markers are potentially and efficient means to examine the reproductive contribution of released abalone to natural resources (Sekino *el al*., 2005).

Multiple genetic markers were used to evaluate genetic structure of natural populations of red abalone (*H. rufescens*). Mitochondrial *cytochrome oxidase subunit*

I (*COI*) sequence, nuclear microsatellites, and amplified fragment length polymorphisms (AFLPs) were analyzed the connectivity among 9 abalone populations samples along approximately 1,300 km of California coastline from Crescent City to San Miguel Island. *COI* sequences and microsatellite genotypes did not show significant genetic divergence among 9 sampled populations. A subset of 5 populations spanning the geographic range of the study was scored for 163 polymorphic AFLP markers. Of these, 41 loci showed significant divergence ($P < 0.001$) among populations. Although the AFLP data suggested significant genetic differentiation among California red abalone populations, the discordance between the different genetic markers needs further study before unambiguous conclusions can be drawn with respect to connectivity among the populations (Gruenthal and Burton, 2007).

In another candidate for, population genetic analyses of *COI* DNA sequences, four nuclear microsatellites, and 142 amplified fragment length polymorphisms (AFLPs) of endangered California black abalone, *H. cracherodii* were carried out in samples from the central California coast and four islands in the Southern California Bight. Global divergence among populations was significant based on *COI* and AFLP polymorphism. The *Hka28* microsatellite locus and AFLP showed significant divergence in multiple pairwise population comparisons and exhibited a signal of isolation by distance. The observed level of interpopulation genetic divergence suggests that larval dispersal is restricted, and natural recovery of decimated *H. cracherodii* populations along the coast of California is unlikely to occur in the near future (Gruenthal and Burton, 2008).

1.5.2 Detecting reduced genetic diversity in cultured abalone stocks

Li *et al.* (2004) used 6 microsatellite loci (*Hdh1321, Hdd114B, Hdd108C, Hdh513, Hdh57*, and *Hdh145*) to estimate the level of genetic diversity of *H. discus hannai* within 3 hatchery strains and 2 wild populations of Pacific abalone. All loci were polymorphic with varying degrees of variation. *Hdh513* had the highest number of alleles (45) in the Iwate Prefecture population (IKI), while the number of alleles at *Hdh1321, Hdh57, Hdh145, Hdd114B*, and *Hdd108C* was 42, 11, 8, 29, and 6, respectively. The average number of alleles per locus in the hatchery and wild populations was 5.4 and 22.4. The mean number of alleles per locus detected in the

three hatchery strains ranged from 3.5 to 8.5 which were significantly lower than those in the two wild populations (21.8 and 23.0). The mean expected heterozygosity was 0.793 in both of the wild populations and ranged from 0.559 to 0.715 in the hatchery strains. Compared to the wild populations, expected heterozygosities were significantly reduced in the hatchery strains ($P < 0.05$). The results showing differentiation was found between the hatchery strains (F_{ST} range 0.243 – 0.427) and between the hatchery strains and wild populations (F_{ST} range 0.059 – 0.252) but no obvious difference was detected between the wild populations ($F_{ST} = 0.004$). The reduction in genetic variation in these hatchery strains may be caused by a small number of parent abalone increasing the effect of genetic drift in hatchery-reared broodstocks.

Genetic variations within and between 9 hatchery stocks and 7 natural populations of abalone including *H. discus hannai*) and *H. discus discus* were examined with 9 microsatellite markers. Devistions from HWE were observed in hatchery stocks owing to heterozygotes excess, whereas all natural populations did not show such a tendency. Highly significant F_{ST} values were observed for all cases between the hatchery stocks, and between the hatchery stocks and natural populations. Genetic distance between each hatchery stock and the geographically proximal population (0.108 ± 0.035) were similar to those estimated for between the natural Ezo-abalone and Kuro-abalone (0.101 ± 0.021). The self-assignment test, which allocated individuals to their own stock with a high success rate, provided evidenee of solid genetic differences among the 9 hatchery stocks. Results suggest that the allelic composition and diversity in the natural populations was not necessarily reflected in the hatchery stocks owing to population bottleneck and genetic drift through seedling process. Therefore, the seedling and stocking practice of these hatchery stocks should take much notice of the results to conserve the genetic diversity of natural populations (Hara and Sekino, 2007).

Microsatellite DNA markers were also used to investigate levels of genetic diversity within cultured populations of *H. midae* and *H. rubra* in South Africa and Australia, respectively. The cultured populations examined were F1 progeny of wild caught broodstock. All populations show a decline in genetic diversity, measured as the number of alleles per locus (35 – 62% allele loss) when compared to wild stocks

in respective broodstock population. There was no associated loss of heterozygosity. Changes in the frequency of alleles between farmed and wild samples were observed in both species. Mean levels of genetic relatedness for the cultured *H. midae* were not significantly different to zero, while those for the cultured *H. rubra* were significantly higher. The estimated effective population size of *H. midae* broodstock was between 75.3 ± 57.6 and 43.5 ± 29.8 for a west coast farm and between 18.5 ± 8.4 and 16.8 ± 8.0 for an east coast farm. The observed loss of alleles in both farmed samples was significantly greater than that expected due to genetic drift based on such effective population size estimates. The effective population size of a farm sample of *H. rubra* was estimated at between 27.2 ± 3.8 and 22.4 ±4.7 (Evans *et al.*, 2004).

Gutierrez-Gonzalez and Perez-Enriquez (2005) analyzed the genetic diversity in 2 hatcheries and devised a method to monitor released *H. fulgens* in the wild based on 2 microsatellite loci. The genetic diversity of the hatchery-reared abalone was high (mean *Ho* = 0.865, number of alleles per locus = 14) and comparable to that of the broodstock. The number of spawners that contributed genetically to the progeny was more than 80% of the total, indicating that management practices appear adequate to avoid significant reduction in genetic diversity. The presence of released larvae in the wild, assessed by recapture samplings 6, 12, and 18 months after release, was low, indicating that the stock enhancement strategy should be modified to release older juveniles that will have better survival. Low probability of identity ($I = 1.7 \times 10^{-4}$), estimated with the combination of the 2 microsatellites, indicates their potential for identification of individuals in the wild in stock enhancement programs.

1.5.3 Parentage assignment

Selvamani *et al.* (2001) applied microsatellite DNA markers to genotype parentage, to assess fertilization success, and to maintain pedigree information for selective breeding of *H. asinina.* At least 10 loci can be analyzed from a single abalone veliger larva. Five polymorphic loci; 2 highly polymorphic loci (*Haμ13* and *Haμ2K*) and 3 moderately polymorphic loci (*Haμ10, Haμ2J*, and *Haμ3E*); were used to identify parents of individual larvae produced from 3 separate crosses. The numbers of alleles per locus ranged from 12 to 25 and the expected heterozygosity was between 0.77 - 0.96. In all cases, the parents of an individual veliger could be examined from as few as 3 loci. The microsatellite analysis revealed that, in all

crosses, a single male fathered most of the veligers. These observations suggest that highly controlled breeding practices may be required to ensure that genetic diversity of an abalone population produced for aquaculture is maintained at the level of diversity of the original broodstock.

Li *et al.* (2003) examined the mode of inheritance of 7 microsatellite loci of *H. discus hannai* in 4 families with a reciprocal cross of 2 females x 2 males. All loci segregated codominantly, but only 3 loci (*Hdh1321, Hdh78,* and *Hdd108C*) conformed to Mendelian segregation and can be used for parental analysis and population genetic studies. When null alleles were considered, 2 loci (*Hdh1761* and *Hdh1457*) confirmed Mendelian expectations in all families, while the remaining 2 loci (*Hdd114B* and *Hdd229*) showed deviation from Mendelian segregation in at least one family even though null alleles were considered. These results indicated the need to test the inheritance pattern for microsatellite markers in abalones before using them for population genetic or parentage analysis.

The heritability for growth-related traits at 12 months of age of *H. asinina* by creating a single cohort of 84 families in a full-factorial mating design consisting of 14 sires and 6 dams were estimated. Of 500 progeny sampled, 89% were successfully assigned to their parents based on shared alleles at 5 polymorphic microsatellite loci (*Haμ2J, Haμ10, Haμ13, Haμ2K*, and *Haμ3E*). Heritability estimates for shell length, shell width and weight were 0.48 ± 0.15, 0.38 ± 0.13 and 0.36 ± 0.13 respectively. Genetic correlations were > 0.98 between shell parameters and weight, indicating that breeding for weight gains could be successfully achieved by selecting for shell length (Lucas *et al.*, 2006).

The parentage of *H. discus hannai* families reared together in a mixed single tank from settle stage to juvenile stage (7 months of age) was detected using eight microsatellite loci. The selected families were derived from 7 parents (3 females and 4 males) selected for larger size in shell length at about 1-year-old, and the non-selected families originated from 5 wild captives (3 females and 2 males). Results from microsatellite markers unambiguously allocated 170 juveniles sampled to the 17 parental pairs. The family size was highly heterogeneous among families, as 2 males in the selected families and 1 male in non-selected families dominated the contribution to the offspring pool (80%). The mean shell length of the selected

families was approximately 23% larger than that of the non-selected families ($P < 0.001$). This study suggested that the use of microsatellite markers is effective for parentage determination in the mixed family farming commonly used in abalone hatcheries, and selective operations for larger size could improve the growth of the next generation (Hara and Sekino, 2007).

1.5.4 Type I microsatellites and their applications in genetic and fishery management

Yue *et al.* (2004) identified 36 new microsatellites from the common carp (*Cyprinus carpio*) by screening through genes found in the GenBank and EST sequences from a testis cDNA library and a genomic DNA library enriched for CA repeats. Eleven of 28 type I microsatellites identified were AT repeats, suggesting their high abundance in the genome of *C. carpio*. Characterization of these microsatellites on 18 unrelated individuals revealed that all, except 2, were polymorphic with an average number of alleles per locus of 7.3 (range: 2 – 15 alleles). Type I microsatellites showed higher allele number than those of type II microsatellites (8 loci) (7.7/locus vs. 4.9/locus df = 32, $P < 0.05$). Crossspecies amplification showed that 41.7% (15/34) of primer pairs generated polymorphic PCR products in the silver crucian carp (*Carassius auratus gibelio*). Interestingly, the success rate of cross-species amplification was lower for type I than type II microsatellites. These novel microsatellites will be very useful for genome mapping and population genetic studies in both species.

Wang *et al.* (2007) compared genetic variation in cultured populations of *Argopecten irradians* from China and that in a natural population from the east coast of USA using 11 microsatellites. Although the difference in heterozygosity was small, the Chinese populations lost 9 of the 45 alleles (20%) found in the wild population. The reduced allele diversity suggests that the Chinese bay scallop populations experienced a bottleneck effect despite several recent introductions of new stocks aimed at expanding the gene pool. The loss of allele diversity may affect future efforts in selective breeding and domestication. Results of this study highlight the need for additional introductions, advanced breeding programs that minimize inbreeding and continued genetic monitoring.

Likewise, genetic diversity in 3 hatchery populations of *Patinopecten yessoensis* in China was compared with 2 wild Japanese populations using 6 microsatellite loci. The hatchery populations were substantially less variable than wild populations with the average numbers of alleles per locus were 5.7 and 7.9, and the average expected heterozygosities were 0.582 and 0.671, respectively. This loss of variation is attributable to about 20 years of isolation and genetic drift. F_{ST} values showed significant genetic differentiation among the 5 populations. Genetic differences between populations were also detected by pairwise comparison based on allelic distribution. The neighbor-joining tree topology constructed from genetic distances among populations showed a clear division between the wild and hatchery populations. The information on the genetic variation and differentiation in cultured and wild populations is useful for setting up suitable guidelines for founding and maintaining of cultured stocks, and for future genetic improvement by selective breeding (Li *et al.*, 2007).

The EST database of *H. discus* (1476 ESTs) was determined. Fifty sequences (approximately 3.4%) contained one or more microsatellites. Based on the length and GC content of the flanking regions, cluster analysis and BLAST*N*, primers were designed from 13 microsatellite-containing ESTs. The results showed that 10 out of 13 primer pairs successfully amplified scorable PCR products and showed polymorphism. The number of alleles per locus ranged from 2 to 13 and H_o and H_e varied from 0.1222 to 0.8611 and 0.2449 to 0.9311, respectively. No significant linkage disequilibrium (LD) between pairs of loci was found, and 6 of 10 loci conformed to the Hardy-Weinberg equilibrium (HWE) (Aibin *et al.*, 2008). However, there has been no publication concerning isolation and characterization of type I in *H. asinina*.

1.5.5 Amplified Fragment Length Polymorphism (AFLP) Studies

Amplified fragment length polymorphism (AFLP) analysis (Vos *et al.*, 1995) is a PCR-based multilocus fingerprinting technique that combines the strengths and overcomes the weaknesses of PCR-RFLP and RAPD-PCR (Welsh and McClelland 1990; Williams *et al.*, 1990). AFLP analysis has been used for indirect examination of levels of genetic diversity in several species (Li *et al.*, 2003; Felip *et al.*, 2005; Li *et al.*, 2006; Khamnamtong *et al.*, 2006; Yu and Chu, 2006; Sriphairoj *et al.*, 2007;

Zhang *et al.*, 2007; Gruenthal and Burton, 2008; Zhao *et al.*, 2009). The major strengths of the AFLP method include simultaneous screening of a large number of polymorphic loci, high reproducibility due to high stringency of PCR, and relative cost effectiveness (Liu and Cordes, 2004). Moreover, it does not require any prior molecular information about sequences under investigation and is thus especially applicable to species in which the genome sequences are not well characterized, like *H. asinina*. More important, taxonomically specific fragment and/or polymorphic fragment can be converted to sequence-characterized amplified region (SCAR) markers for different applications (Khamnamtong *et al.*, 2006; Klinbunga *et al.*, 2007).

AFLP genotypes were examined in 3 full-sib families of Zhikong scallop *Chlamys farreri*. Overall, 9.3% and 0.8% of analyses exhibited non-Mendelian segregation ratios before and after the Bonferroni correction, respectively. AFLP markers were used to investigate levels of genetic diversity within the cultured stocks in comparison with the wild populations. Six pairs of primers generated 293 loci among 139 individuals in 4 cultured and 2 wild populations. High polymorphism was found within both cultured and wild populations. The expected heterozygosity ranged from 0.291 (hatcheries) to 0.295 (wild), while the percentage of polymorphic loci ranged from 92.1% (hatcheries) to 93.9% (wild), respectively. This suggests that more than 30 years of artificial cultivation have not substantially reduced the genetic variability of the cultured stocks of *C. farreri*. Significant genetic differentiation was observed between the cultured stocks, and between the cultured and wild populations (Zhao *et al.*, 2009).

Kong and Li (2007) used AFLP markers to investigate levels of genetic diversity within cultured and wild populations of the clam *Coelomactra antiquata*. Seven pairs of primers generated 365 fragments (loci) among 125 individuals in 3 cultured and 3 wild populations. High polymorphism at the AFLP markers was found within both cultured and wild populations. Although not statistically significant, reductions in the expected heterozygosity and percentage of polymorphic loci were observed in the cultured populations (2.8% and 8.3% reduction, respectively), and higher frequency of private alleles within the wild compared to the cultured populations indicated that rare alleles in some loci were lost in the cultured

populations. Significant genetic differentiation was observed between the cultured populations, and between the cultured and wild populations. Northern populations were genetically distinct from southern populations (F_{ST} = 0.696 - 0.746). Therefore, continued genetic monitoring of the cultured populations is warranted and the northern and southern populations of *C. antiquata* should be managed separately in hatchery practices for the preservation of genetic diversity in wild populations.

Seven AFLP primer sets were used to investigate the genetic structure of a wild base population and 3 generations of the Chinese shrimp *Fenneropenaeus chinensis* selected for fast growth (F5 – F7). More than 500 bands were generated in total. On average, each primer set detected 33.7 polymorphic AFLP fragments. The expected average heterozygosity ranged from 0.085 to 0.097. As time under selection increased, genetic diversity tended to reduce (Li *et al.*, 2006).

Yu and Chu (2006) studies genetic variation in 5 populations of the pearl oyster *Pinctada fucata*, from China (Daya Bay, Sanya Bay and Beibu Bay), Japan (Mie Prefecture) and Australia (Port Stephens). Three primer combinations generated 184 AFLP fragments among which 91.8 – 97.3% is polymorphic. An overall genetic diversity of 0.38 among populations and an average of 0.37 within populations (ranging from 0.35 in Japanese population to 0.39 in Beibu Bay population) were observed. Genetic differentiation among 5 populations is low but significant as indicated by pairwise G_{ST} (0.0079 – 0.0404). AMOVA further shows that differentiation was significant among the 5 populations but was not significant among Chinese, Japanese and Australian populations or among the two groups of Australian and north Pacific populations. Among the 5 populations, the Australian one is more differentiated from others, based on both pairwise AMOVA and G_{ST} analyses, and is genetically isolated by distance as indicated by Mantel test. However, genetic differences among the 3 Chinese populations are not correlated with the geographic distances, suggesting that Hainan Island and Leizhou Peninsula may act as barriers blocking gene flow.

Qin *et al.* (2007) constructed genetic linkage maps for the bay scallop *Argopecten irradians* using AFLP and microsatellite markers and conducted composite interval mapping (CIM) of body size-related traits. Three hundred and seventeen AFLP and 10 microsatellite markers were used for the map construction.

The female parent map contained 120 markers allocated in 15 linkage groups, spanning 479.6 cM with an average interval of 7.0 cM. The male parent map had 190 markers allocated in 17 linkage groups, covering 883.8 cM with 7.2 cM between pairs of markers. The observed coverage was 70.4% for the female and 81.1% for the male map. Six size-related traits, shell length, shell height, shell width, total weight, soft tissue weight, and shell weight were measured for QTL mapping. The size data were significantly correlated with each other. CIM analysis revealed one significant QTL (LOD = 2.69, 1000 permutation, $P < 0.05$) in linkage group 3 on the female parent map. The mapping of size-related QTL in this study raises the possibility of improving the growth of bay scallops through marker-assisted selection.

Low efficiency for characterization of microsatellites in *M. japonicus* leads to the use of AFLP analysis as an alternative approach for construction of the genetic linkage map in this species. Over 570 polymorphic loci were defined using different primer combinations. A primary linkage map based on a three generation pedigree, genotyped at 246 AFLP loci has been constructed. It incorporates 129 markers in 44 linkage groups with a genome coverage of approximately 57% (Moore *et al.*, 1999).

AFLP fingerprinting approach has been used for identification of sex-linked markers in the rainbow trout. Using a sex-typed pool strategy, 486 different primer combinations of which 19 gave sex-associated amplification. Segregation analysis showed that 18 out of 19 AFLPs (95%) were informative for individual sexing and genetic mapping. Fifteen AFLPs that showed sex-associated amplification in the pooled segregant analysis were converted into SCARs markers. One of these, *Omy*-163, was Y chromosome-linked and mapped near the sex locus in all of 4 genetically diverse male clonal lines of the rainbow trout examined. Three microsatellites, *Ots517NWFSC, OmyFGT19TUF*, and *Omm1026*, which were previously mapped on the sex linkage group in rainbow trout, were also analyzed. The SCAR *Omy*-163, *Ots517NWFC* and *OmyFGT19TUF* showed low levels of intraspecific allelic polymorphism. Y chromosome-linked AFLP markers were relatively strain-specific suggesting an intraspecific genetic polymorphism of the rainbow trout Y-chromosome. An AFLP approach in combination with a sex-type pool strategy resulted in a quick analysis for identifying sex-linked loci in the rainbow trout.

Similar approaches may be useful for molecular mapping of markers linked to phenotypes of interest in other species (Felip *et al.*, 2005).

1.5.6 β-oxidation

The fatty acid degradation pathway is referred to as β-oxidation. In animal cells, β-oxidation takes place in both the mitochondria and peroxisomes whereas in lower eukaryotes, it appears to be solely a peroxisomal process (Bhaumik *et al.*, 2005). Mitochondrial fatty acid β-oxidation occurs through a series of cyclic steps within the mitochondria (Figure 1.13).

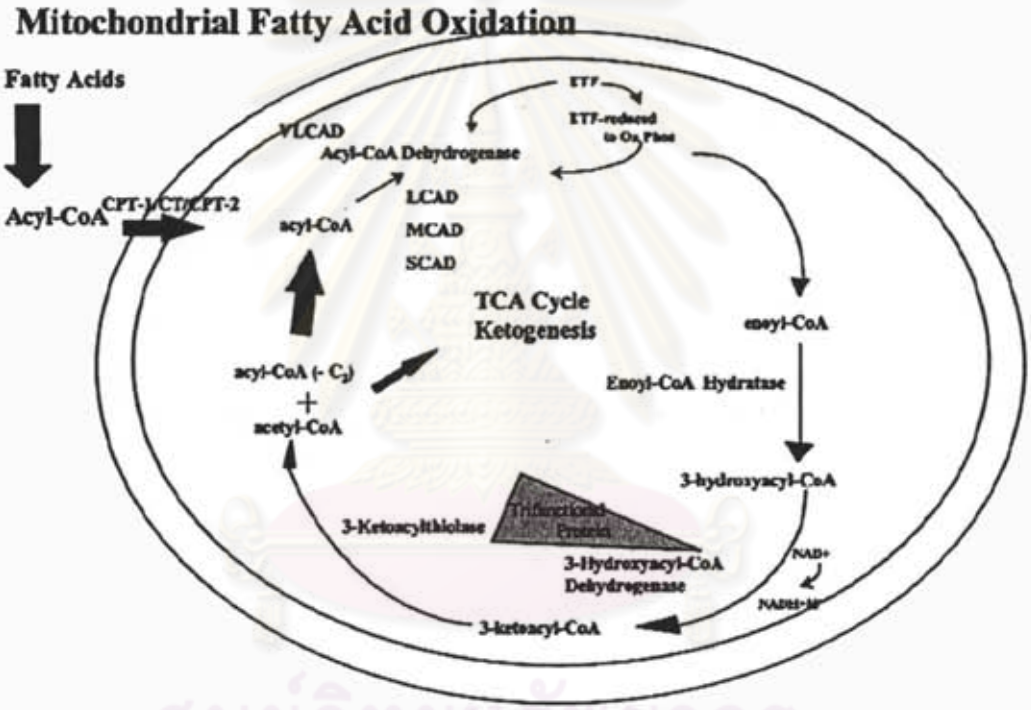


Figure 1.13 Overview of mitochondrial β-oxidation of fatty acids. CPT-1, carnitine palmitoyltransferase-1; CPT-2, carnitine palmitoyltransferase-2; CT, carnitine/acyl-carnitine translocase; ETF, electron transport flavoprotein; LCAD, long-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase; TCA, tricarboxylic acid cycle; VLCAD, very long-chain acyl-CoA dehydrogenase (Schuler and Wood, 2002).

1.5.6.1 Genes/proteins involved in lipid metabolism

The first step of β-oxidation pathway concerns the introduction of a trans-2 double bond into the acyl-CoA substrate and then transported into the mitochondrial matrix. Fatty acids are first bound by fatty acid binding proteins and then "activated" to the acyl-CoA form in the cytosol by acyl-CoA synthases that are associated with the endoplasmic reticulum or the outer mitochondrial membrane. After activation of the fatty acid, the acyl-CoA is transported across the double mitochondrial membrane as an acyl-carnitine via a process requiring carnitine palmitoyltransferase- 1 (CPT-1 or carnitine acyltransferase I, CAT-1) which regulated through inhibition by malonyl-CoA, carnitine/acyl-carnitine translocase (CT), and carnitine palmitoyltransferase-2 (CPT-2 or carnitine acyltransferase II, CAT-II) (Kashfi and Cook, 1995). The two isoforms of CPT-1 are known as the liver isoform (CPT-1A) and the muscle isoform (CPT-1B). The primary structures of the lier and muscle isoforms predicted from the cDNAs show a high degree of homology with calculated molecular masses of 88,227 Da (772 amino acids) and 88,150 Da (773 amino acids) for the rat muscle and rat liver isoforms, respectively (Distler *et al.*, 2009).

Carnitine-dependent transport of the long-chain acyl- CoA occurs in four steps. First, carnitine accepts the acyl group of the fatty acid via CPT-1. Second, CT translocates the acyl-carnitine across the outer and inner mitochondrial membranes. Then, CPT-2 catalyzes the carnitine release from the acyl group, which is then reattached to a CoA from within the mitochondrial matrix forming the acyl-CoA that will be β-oxidation. Finally, CT, which mediated the transfer of the acyl-carnitine, simultaneously exchanges free carnitine back into the cytosol (Figure 1.14).

In a complete cycle of β-oxidation (Figure 1.15), fatty acids are catabolized to generate an acetyl-CoA and an acyl-CoA, which is two carbon units shorter. The first reaction within the mitochondrial matrix is the acyl-CoA dehydrogenation step that catalyzes the formation of a trans-α, β double bond. The acyl-CoA dehydrogenase step is catalyzed by a group of enzymes named for the chain length of substrate on which they act: very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCADC), and short-chain acyl-CoA dehydrogenase (SCAD).

Next, enoyl-CoA hydratase catalyzes the hydration of the trans double bond, which results in the formation of 3-hydroxyacyl-CoA. This process is followed by the 3-hydroxyacyl-CoA dehydrogenases (long-chain hydroxyacyl-CoA dehydrogenase [LCHAD] or medium-/short-chain hydroxyacyl-CoA dehydrogenase [M/SCAHD]), which catalyze the formation of 3-ketoacyl-CoA through an NAD+/NADH-dependent dehydrogenation of 3-hydroxyacyl-CoA. Finally, 3-ketoacyl- CoA thiolase catalyzes the reformation of the shorter acyl- CoA and the newly generated acetyl-CoA through a thiolysis reaction. These last three steps for long-chain substrates are accomplished by long-chain mitochondrial trifunctional protein (MTP), which is composed of MTPα and β proteins. MTPα and MTPβ are encoded by two separate but linked genes, *HADHA* and *HADHB,* respectively. MTPα contains the long-chain enoyl-CoA hydratase and LCHAD activities, whereas, MTPβ contains the 3-ketoacyl-CoA thiolase activity (Ibdah *et al.*, 2001).

The summary of one round of the β-oxidation pathway:

$$\text{Fatty acyl-CoA} + \text{FAD} + \text{NAD}^+ + \text{HS-COA} \longrightarrow \text{Fatty acyl-CoA (2 C shorter)} + \text{FADH}_2 + \text{NADH} + \text{H}^+ + \text{acetyl-CoA}$$

1.5.6.2 Trifunctional protein

The trifunctional protein catalyzes the 2-enol-CoA hydration, 3-hydroxyacyl-CoA dehydrogenation and 3-ketoacyl-CoAthiolysis of long and medium chain acyl-CoA esters, but is inactive towards short chain (C_4) substrates. The trifunctional protein is a multienzyme complex, a hetero-octomer made up of four molecules of α-subunits (HADHA) with long-chain-enoyl-CoA hydratase and 3- hydroxyacyl-CoA dehydrogenase domains (78 kDa) and four molecules of β-subunits (HADHB) with long-chain 3-ketoacyl-CoA thiolase domain (50 kDa), is closely associated with the inner mitochondrial membrane (Jackson *et al.*, 1995; Orii *et al.*, 1997; Eaton *et al.*, 1998) (Figure 1.16).

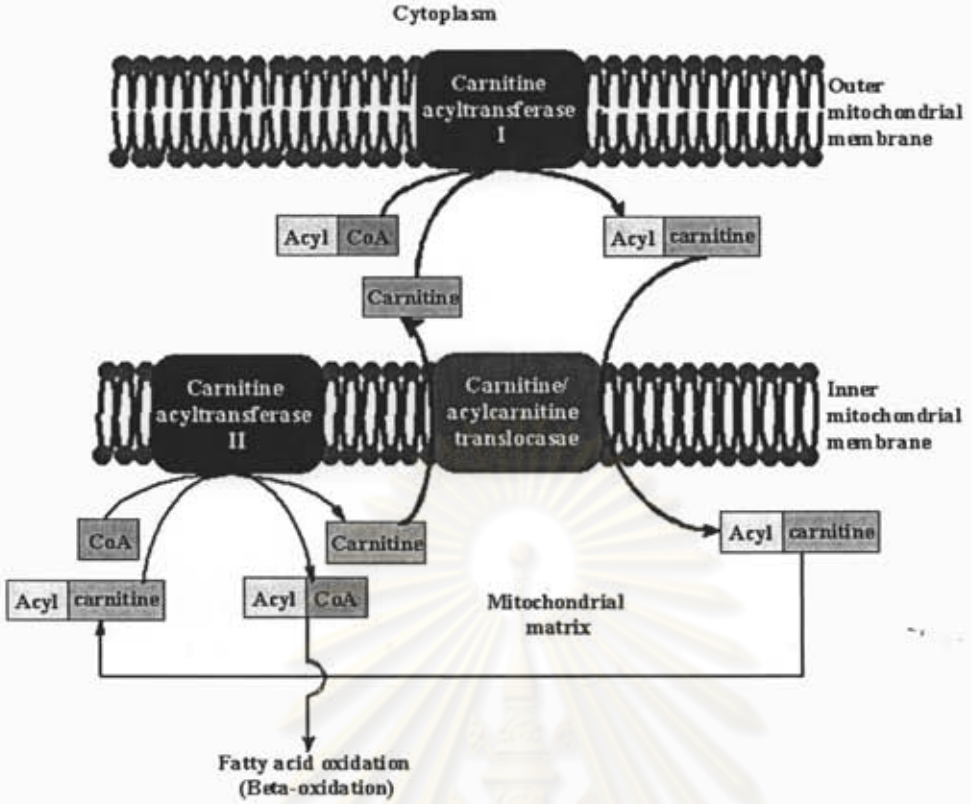


Figure 1.14 Diagram shows transportation of fatty acid from cytoplasm to mitochondria matrix with carnitine acyltransferase I (CAT-1), carnitine acyltransferase II (CAT-II), and carnitine/ acylcarnitine translocase (CT). (http:// www.dentistry.leeds.ac.uk/biochem/lecture/faox/faox.htm).

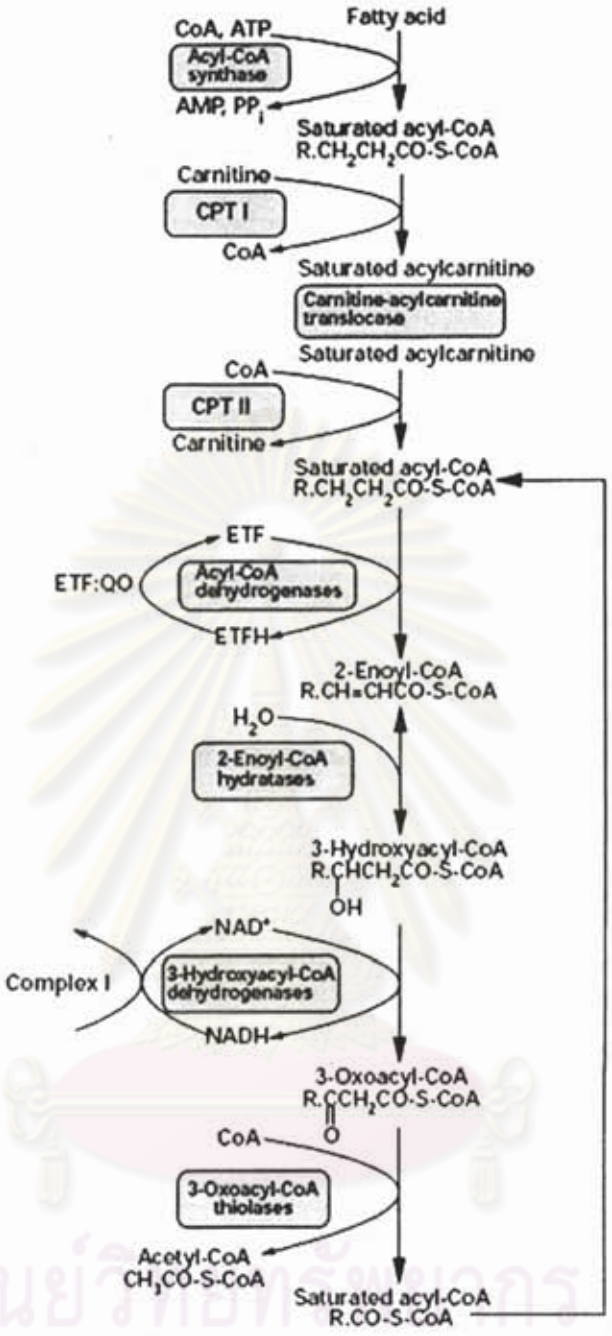


Figure 1.15 Enzyme of mitochondrial β-oxidation. CPT, carnitine palmitoyl transferase; ETF, electron transfer flavoprotein; ETF: QO, ETF: ubiquinone oxidoreductase; ETFH, reduced ETF (Eaton *et al.*, 1996).

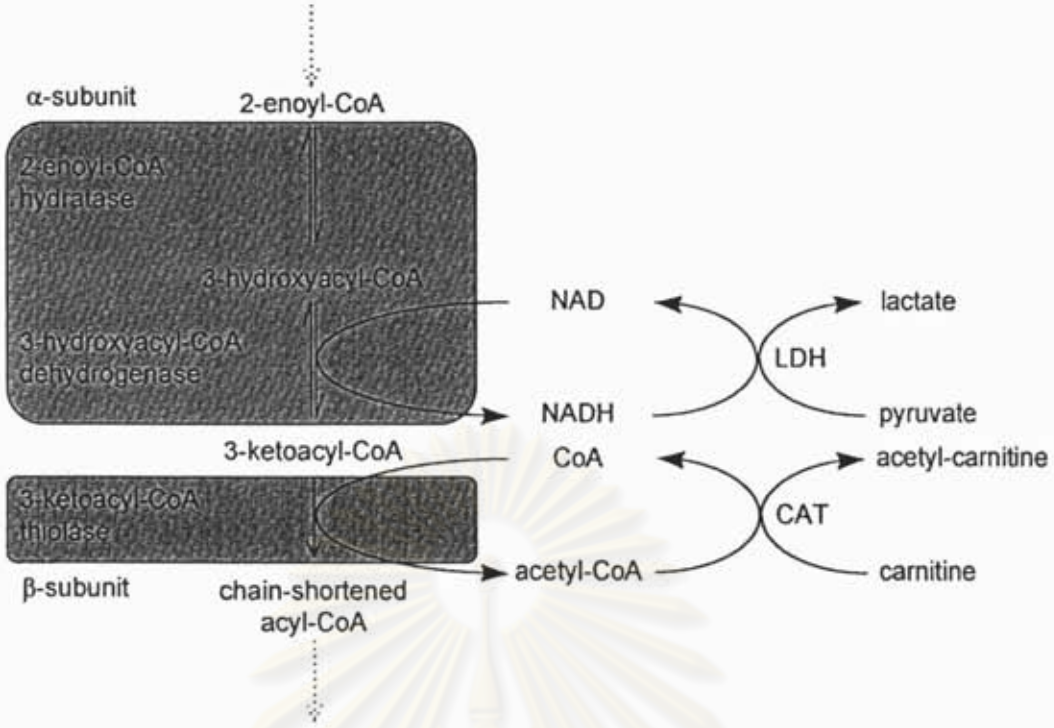


Figure 1.16 Diagram showing the activities and subunits of the trifunctional protein of β-oxidation, together with the recycling systems used in the experimental section. LDH, L-lactate dehydrogenase; CAT carnitine acetyl transferase.

1.5.6.3 α-methylacyl-CoA racemase

α-methylacyl-CoA racemase (AMACR or α-methylacyl-CoA epimerase) originally identified in rat liver as a mitochondrial and peroxisomeal enzyme. It is known as a linking enzyme play an important role in metabolic degradation of branched-chain fatty acids and fatty acid derivatives (Jiang *et al.*, 2003; Ferdinandusse *et al.*, 2000). The α-oxidation cycle yields both (*R*)- and (*S*)-isoforms of α-methyl-branched chain compounds, whereas the first enzymes in the β-oxidation cycle are sterospecific for (*S*)- isoforms of their CoA-activated substrantes. AMACR catalyzes the conversion of several (2*R*)-methyl branched-chain fatty acyl-CoAs to their (*S*)-stereoisomers (Figure 1.17). As a result, AMACR is a required component of the oxidative metabolism and biosynthetic parthways of branched-chain fatty acids and bile acids, respectively. It is expressed at appreciable levels and is transported to both the peroxisomal and mitochondrial compartments in a variety of tissues.

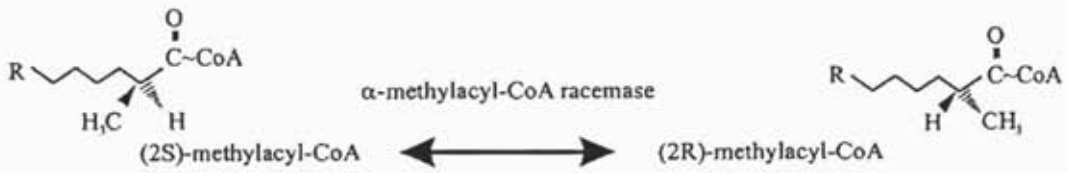


Figure 1.17 The mechanism of α-methylacyl-CoA racemase in the conversion of (2R)-methylacyl-CoA to (2S)-methylacyl-CoA (Hiltunen and Qin, 2000).

1.5.6.4 Vacuolar H+ ATPase

The vacuolar H^+-ATPases (or V-ATPases) are a family of ATP-dependent proton pumps responsible for acidification of intracellular compartments in eukaryotic cells. Vacuolar acidification is essential for a variety of cellular processes, including ligand-receptor dissociation and receptor recycling following receptor-mediated endocytosis, intracellular targeting of newly synthesized lysosomal enzymes, protein processing and degradation, and coupled transport of small molecules, such as neurotransmitters. Acidification of endosomal compartments is also necessary for budding of transport vesicles and infection by certain envelope viruses, such as influenza virus. V-ATPases have also been identified in the plasma membrane of certain cells where they function in such processes as renal acidification, pH homeostasis, bone resorption and coupled K^+ transport. They have also been implicated in a variety of disease processes, including renal tubular acidosis, osteopetrosis and tumor metastasis (Kawasaki-Nishi *et al.*, 2003).

The V-ATPases from fungi, plants, and animals are structurally very similar. Biological and genetic analysis revealed that the the V-ATPases are composed of a peripheral V1 domain responsible for ATP hydrolysis and Vo domain responsible for proton translocation (Figure 1.18). The approximately 640 kDa V1 domain consists of eight subunits (subunit A–H) and the approximately 260 kDa Vo domain consists of at least four different subunits (subunits a, c, d, and e) and two newly discovered Ac45 and M8-9 accessory subunits (Yao *et al.*, 2007).

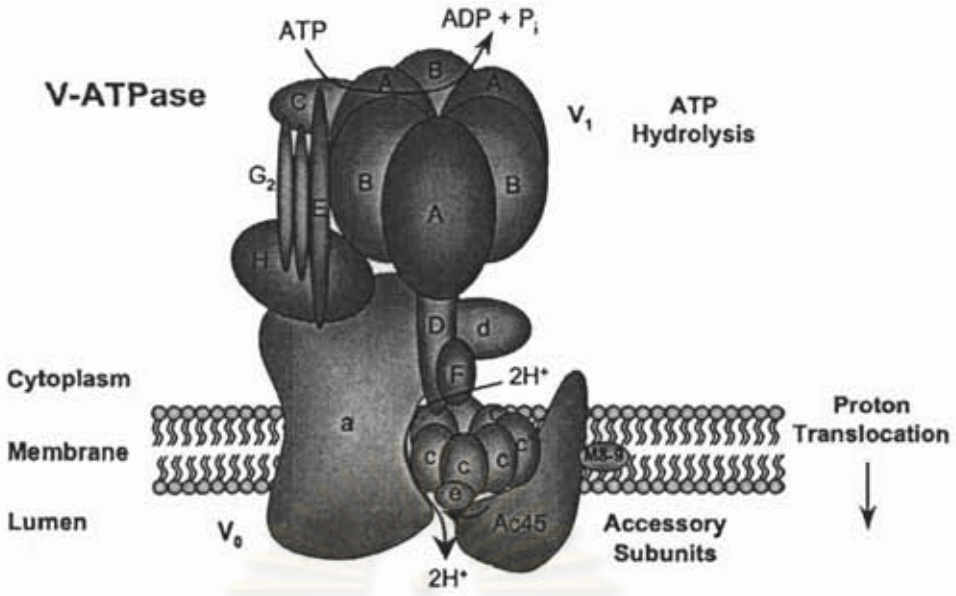


Figure 1.18 Schematic depiction of the V-ATPases. V-ATPase complex is composed of a peripherally located V1 domain and a integral membrane associated V0 domain. The V1 domain consists of eight different subunits (A–H) and is responsible for ATP hydrolysis. The V0 domain consists of four different subunits (a, c, d, and e) responsible for proton translocation. Furthermore two accessory subunits, Ac45 and M8-9, also exist but their structural location and association with other subunit remains to be determined.

Guo *et al.* (1996) reported a *Drosophila melanogaster (Dm)* cDNA *(vhal4)* encoding the 14-kDa F-subunit of the vacuolar H+-ATPase (V-ATPase) has been cloned via homology with the corresponding *Manduca sexta (MS)* gene. Its deduced translation product is a 124 amino acid polypeptide sharing 90% identity with the *MS* polypeptide and 50% identity with an analogous polypeptide of *Saccharomyces cerevisiae,* and a more distant similarity to a subunit of the Na+-transporting ATPase of *Enterococcus hirae.* Homology was also found with expressed sequence tags from man, *Arabidopsis thaliana, Caenorhabditis elegans* and C. *briggsiae, Oryza sativa* and *Plasmodium falciparum,* indicating that the subunit is phylogenetically conserved. The *Dm* gene *(vhal4)* is present as a single copy at cytological position 52B on the second chromosome, and gives rise to an mRNA species of 0.65 kb. Expression of the latter shows relatively little variation during development, or between adult head, thorax and abdomen, suggesting that the F-subunit is a relatively ubiquitous component of the V-ATPase.

1.6 Objectives

The objectives of this study are determination of domesticated status of hatchery-propagated stocks and characterization of genes functionally related with growth of *H. asinina*. Type I microsatellites were developed from abalone ESTs and AFLP fingerprints of hatchery-propagated stocks were also carried out. Polymorphic AFLP fragments were converted to SCAR markers. The developed markers were tested against hatcheries and wild stocks of Thai *H. asinina*. In addition, the full length cDNA and expression profiles of metabolism-related genes were isolated and characterized. The basic information is useful for improving the efficiency of breeding and domestication of *H. asinina*.

CHAPTER II

MATERIALS AND METHODS

The experimental procedures carried out in this thesis are divided in to 3 parts:

1. Development of type I microsatellites for determining genetic studies of hatchery propagated and natural stocks of Thai abalone, *H. asinina*

2. Development of stock-specific markers in *H. asinina* using AFLP-derived markers

3. Isolation, characterization and expression of genes related with metabolisms in *H. asinina*.

2.1 Development of type I microsatellites for determining genetic studies of hatchery propagated and natural stocks of Thai abalone, *H. asinina*

2.1.1 Experimental animals

Specimens of *H. asinina* were collected from different geographic locations in Thailand including Talibong Island, Trang province (TRGW, N = 25), Cambodia (CAME, N = 20), and the P_0 progeny established from founders originating from Samet Island, Rayong province (SAME, N = 16). Moreover, the abalone stocks bred from those founders by mass spawning were maintained at Sichang Marine Research and Training Station, Chonburi province (SMaRT). Subsequently, the propagated stocks were bred and cultured separately at SMaRT (CSMaRTH, N = 101) and at Trang province (CTRGH, N = 48). These specimens were also included in the experiments (Table 2.1 and Figure 2.1). Foot tissue or epipodial tentacles were dissected and kept at -20°C or in absolute ethanol until needed. In addition, the F_1 progeny cultured at the Southeast Asian Fisheries Development Center-Aquaculture Department (SEAFDEC/AQD), Iloilo, Phillipines (PHI, N = 20) were placed in absolute ethanol and stored at -20 °C until required.

To examine the possible correlation between genotypes of microsatellites and the body weight of the hatchery-propagated samples, the body weight of 280

individuals of the group B sample (11 months old) cultured at SMaRT was weighed. Fifteen percent of abalone exhibiting from the top (BL, $N = 40$, $\overline{X} = 12.2 \pm 1.451$ g) and the bottom (BS, $N = 40$, $\overline{X} = 3.90 \pm 0.580$ g) according to the body weight were selected and used for association analysis between microsatellite genotypes and the body weight of these abalone (Figure 2.2). The foot muscle of each abalone was dissected out and kept in absolute ethanol at -30 °C.

2.1.2 DNA extraction

Total DNA was extracted from the epipodial tentacles or food muscles of each abalone using a phenol-chloroform-proteinase K method. A piece of muscle was dissected from each specimen, homogenized with a micropestle in a prechilled 1.5 ml. microcentrifuge tube containing 500 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

Table 2.1 Sample collection sites and sample sizes of *H. asinina* specimens

Geographic origin	Abbreviation	Sample size (*N*)	
		micros atellite	**AFLP**
Wild stocks			
Talibong Island, Trang (Andaman Sea)	TRGW	25	25
Cambodia (east of peninsular Thailand)	CAME	20	22
P_0 stock, Samet Island, Rayong (Gulf of Thailand)	SAME	16	20
F1 generation, the Philippines	PHI	20	20
Domesticated stocks			
Trang hatchery	CTRGH	48	40
Sichang Marine Research and Training Station, Chonburi	CSMaRTH	181	95
Total		310	222

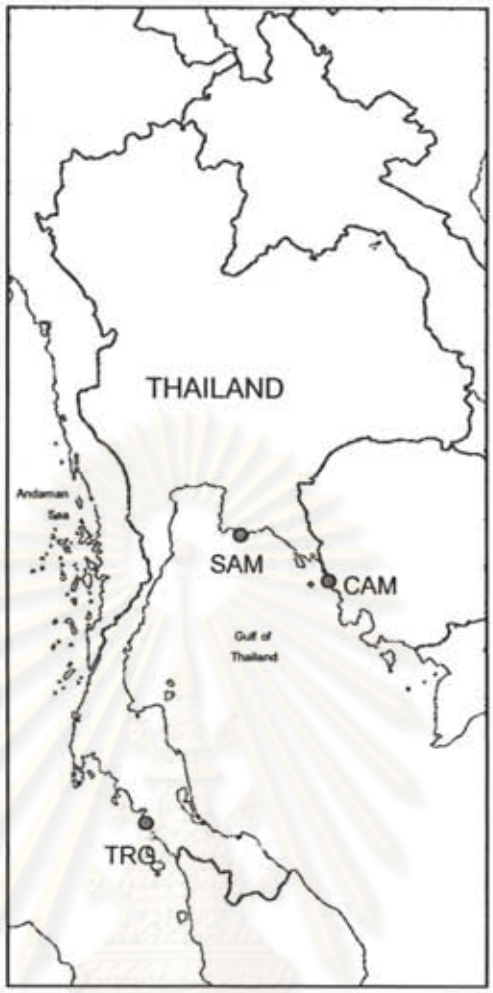


Figure 2.1 Map of Thailand indicating sampling sites of wild *H. asinina* used in this study. Dots represent sample locations from which *H. asinina* was collected. Abbreviations; CAM = Cambodia, SAM = Samet Island, TRG = Trang.

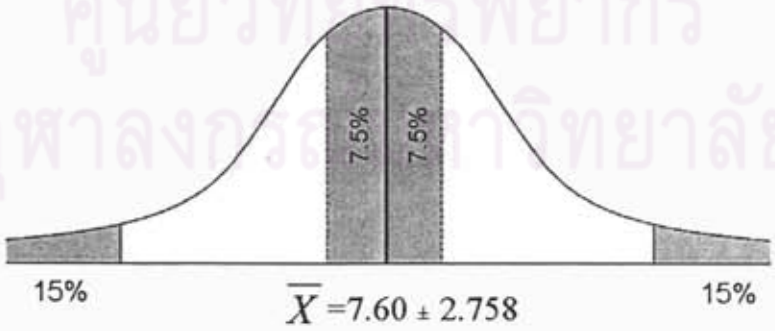


Figure 2.2 Normal distribution curve illustrating the sampling collection scheme of group B sample used for association analysis between microsatellite genotype and the body weight of *H. asinina*.

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 -80°C for 30 min. 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.

2.1.3 Measuring concentrations of extracted DNA using spectrophotometry and electrophoresis

2.1.3.1 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;

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

2.1.3.2 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. The gel was destained in water. DNA concentration was estimated by comparing the fluorescent intensity of interested band with that of λ/*Hin*d III and undigested λ-DNA.

2.1.4 Polymerase Chain Reaction (PCR) of type I and type II microsatellites

2.1.4.1 Identification of ESTs containing microsatellites

EST sequences from testis, ovary, and hemocyte cDNA libraries of *Haliotis asinina* comprising a total of 649 sequences (Amparyup, 2004) and additional 2,227 ESTs from other *Haliotis* species deposited in the GenBank (http://ncbi.nlm.nih.gov) were *in silico* examined for the existence of simple repeats using the Tandem Repeat Finder 2.2 (Benson, 1999). Motifs that contained the number of repeats greater than 10 for mononucleotide, 6 for dinucleotide, 4 for trinucleotide, and 3 for tetranucleotide, pentanucleotide, and hexanucleotide repeats were regarded as microsatellite-containing ESTs (Stalling *et al.*, 1991; Estroup *et al.*, 1993; Tassanakajon *et al.*, 1998).

2.1.4.2 Development of EST-derived microsatellites and genotyping

Twelve primer pairs that flank microsatellites in ESTs were designed using Primer Premier 5.0 (Table 2.2). The major parameters for primer design were set as follows: PCR product size 150 - 200 bp, primer length 18 - 25 bp, annealing temperature approximately of 50°C, and GC content of approximately 50%.

The amplification reaction was performed against genomic DNA of a few individuals of natural and cultivated abalone in a 15 µl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, 1 unit of DyNazyme™ II DNA Polymerase (Finnzymes) and 50 ng of DNA template. PCR was carried out by predenaturation at 94°C for 3 minutes,

followed by 10 cycles of 94°C for 45seconds, 10°C above the annealing temperature of each primer pair for 1 minute (Table 2.2), with a reduction of 2°C in every other cycles, and extension at 72°C for 45 seconds and 30 cycles of 94°C for 45 seconds, at the annealing temperature for 1 minute and 72°C for 45 seconds. The final extension was carried out at 72°C for 10 minutes. The PCR products were electrophoretically analyzed through 1.5 - 2.0% agarose gel. The successfully amplified products were further electrophoresed through 6% denaturing polyacrylamide gels and visualized by silver staining.

In addition, six loci of type II microsatellites; *Haμ1M*, *Haμ2K*, *Haμ3C*, *Haμ3E*, *Haμ9* and *Haμ10* (Selvamani *et al.*, 2000); were screened against cultured and natural abalone specimens. Locus that successfully amplifications were selected for examine genetic diversity of abalone samples. The reverse primer of loci *Haμ9* and *Haμ10* were 5′-terminally labeled with HEX and FITC, respectively. The PCR products of those loci were detected by a fluorescent scanner (PhosphorFX, BioRad).

2.1.5 Agarose gel electrophoresis

The amplification products were electrophoresed through 1.2 - 1.5% agarose gels. The appropriate amount of agarose was weighed and mixed with 1XTBE (89 mM Tris-HCl, 8.91 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. When needed, the gel was placed in the electrophoretic chamber containing and enough amount of 1XTBE buffer covering the gel for approximately 0.5 cm and the comb was gently removed.

The product was mixed with one-fourth volume of a loading dye solution (0.25% bromophenol blue and 25% ficoll) and then loaded into the well. A 100 bp DNA ladder was used as standard DNA marker. Electrophoresis was carried out at 4 - 5 V/cm until the tracking dye migrated about three-quartered of the gel. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) for 5 minutes and destained to remove unbound EtBr by submerged in water for 15 minutes. The DNA fragments were visualized under the UV light using a UV transilluminator.

Table 2.2 Names of loci, characteristics of repeats primer sequences, and Ta of type I and type II microsatellite loci use in this study

Locus	Gene homologue	Repeat motif	Primer Sequences (5' – 3')	Ta (°C)
Type I microsatellite				
PHe4	*B-cell translocation gene 2*	$(CAG)_4(CAA)_4$	F: ACAAGGAAAGCAGTCTCAGC R: TCTTACACGAATACCCAATC	50
PHe24	Unknown	$(CCGA)_8$	F: TATTCCGCTTCATAGATTGT R: AGAGCCTATTCTGCCACG	50
PHe156	Unknown	$(TG)_4(GTGTGC)_6$	F: ACCCCACGAAACAAGCAAT R: ATTGGCTTCCCCTTTTACAT	49
PHe177	Unknown	$(TA)_7$	F: AACACATTCACTGATTTAGG R: ATTATTATCTGACCGAGCA	50
PHe221	*Thrombospondin 1*	$(TAC)_5$	F: CTGTTGGCGGTTGTCCTT R: TATCTGGTGTAGCCGTATCC	50
PO97	Unknown	$(TAG)_{10}$	F: CAGGGGCTTGGTGGATGGAG R: CAAACAGTGAACAGAGGGGG	58
PO179	*Cysteine proteinase 1 precursor*	$(TGAG)_5$	F: CACGCATACGCTAGCAGTCA R: GGGAAGCAGGGGTCTTTGTT	53
PT101	*Ribosomal protein L41*	$(CAG)_7$	F: CTTGGTGTTGTGGCGTGTG R: TCTACTGACTATCTCATTCTCTGG	50
PT102	*Ribosomal protein L41*	$(ATG)_{10}$	F: TGCTTGTGAAGGACAGTATC R: ACCAACACGCACTGACAT	50
DW404	Unknown	$(GAT)_8$	F: TCCCTATCGGTGTGGTCG R: GCTCTCCTGAGGAATGCGT	57
DW455	Unknown	$(TG)_{13}(GC)_7$	F: CGGACAAAGATACTTGCTCA R: ATCAATGCTGGTCAAACGGA	59
DW503	Unknown	$(ATG)_{12}$	F: AGTCCCAGGTGTAAGAGCAT R: TGAACAGAGGGGTTGTCGTG	57

Table 2.2 (cont.)

Locus	Gene homologue	Repeat motif	Primer Sequences (5' – 3')	Ta (°C)
Type II microsatellite				
Haμ1M	-	$(CA)_{23}$	F: ATAATATACTCGTGAAGTTGATAG R: TCATATACTTCATACATTACTCAT	48
Haμ2K	-	$(CA)_{30}$	F: CCATGAGGTTTGTAAAATGGTG R: ATATGTTCGCCCTCGTAGGA	55
Haμ3C	-	$(CA)_{33}$	F: AAGTATGTGTGTAAAGGGTC R: CAAGAAACTCAAGAGAAAAC	55
Haμ3E	-	$(CA)_6TA(CA)_5TA(CA)_2TACATA(CA)_4TA(CA)_4TA(CA)_4TACACATA(CA)_4TACATA(CA)_5TA(CA)_6TA(CA)_7(TA)_2$	F: TCACTAAAATAACCATGCATTC R: GTAACTGATTTGGCATATTCGT	60
Haμ9	-	$(CA)_8CG(CA)_4AA(CA)_3$	F: CCACCCCCATCCCACCCCT R: ATTGCATCACCTACGAAAACAAA	57
Haμ10	-	$(CA)_{10}AA(CA)_2(GA)_6CA(GA)_6$	F: CTAATTGCAGTTATGGGCGTTC R: AAGGCTTTCGGAGGACGTGTC	63

2.1.6 Denaturing Polyacrylamide gel electrophoresis

2.1.6.1 Preparation of glass plates

One milliliter of 95% commercial grade ethanol was thoroughly wiped on the long glass plate with a piece of the tissue paper in one direction. This process was repeated twice. After that, 1 ml of freshly prepared Binding solution (10 µl of Bind silane, GE Healthcare, 980 µl of 95% ethanol and 10 µl of 5% glacial acetic acid) was coated on the long glass plate and left for approximately 10 – 15 minutes. Excess binding solution was removed with a piece of the tissue paper. The long glass plate was further cleaned with 95% ethanol for 3 times.

The short glass plate was treated as described above with the exception that the binding solution was replaced with the Repel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The cleaned glass plates were assembled with a pair of 1.5 mm spacers.

2.1.6.2 Preparation of denaturing polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels at desired concentrations (4.5 - 6.0%) were prepared by combining 80 ml of 30 minutes degassed acrylamide solution (19:1 acrylamide: bisacrylamide with 7 M urea in 1xTBE buffer) with 480 µl of freshly prepared 10 % ammonium persulphate and 48 µl of TEMED. The acrylamide solution was gently swirled. The assembled plate sandwich was hold at a 45 degree angle on the bottom corner. The acrylamide solution was then gently injected into one side of the assembled plates. The filled plate sandwich was left in the horizontal position. The flat edge of the shark-tooth comb was then inserted. The gel was left at room temperature for 1 hour. Then the polymerized gel was covered by the water-soaked tissue papers and left at room temperature for 4 hours (or overnight) for complete polymerization. When required, the spring clips and the sealing tape were carefully removed. The top of the gel was rinsed with deionized water.

2.1.6.3 Electrophoresis

The complete gel sandwich was placed in the vertical sequencing apparatus with the short glass plate inward. The gel sandwich was securely clamped with the integral gel clamps along the sides of the sequencing apparatus. The upper and lower

buffer chambers were filled with approximately 300 ml of 1XTBE. The shark-tooth comb was reinserted into the gel until the teeth just touched the surface of the gel. Six microlitres of the polyacrylamide gel loading dye (98 % formamide, 200 µl EDTA, 0.25 % bromophenol blue and 0.25 % xylene cyanol) was loaded by put one well between for checking for the leaking well. The gel was prerun at 35 W for 20 - 30 minutes.

Six microlitres of the successfully amplification products were mixed with 3 µl of the loading buffer and heated at 95°C for 5 minutes before put on ice for at least 3 minutes. The sample was carefully loaded into the well. Electrophoresis was carried out at 35 W for approximately 2.5 - 3 hours (XC moved to the end of the gels).

2.1.6.4 Silver staining

After finish electrophorized, the gel plates were carefully separated using a plastic wedge. The long glass plate with the attached gel was placed in a plastic tray containing 3 litres of the fix/stop solution (10% glacial acetic acid) and agitated well for 30 minutes. The gel was briefly soaked with shaking 3 times for 3 minutes with deionized water. The gel was transferred in the 3 litres tray of 0.1% silver nitrate solution and incubated with agitation at room temperature for 30 minutes. After that, the gel was soaked in 3 litres of deionized water with shaking (10 forward and 10 backward agitations) and immediately placed in the tray containing 3 litres of the chilled developing solution (Sodium carbonate). This step is crucial and the time taken to soak the gel in the water and transfer it to chilled developing solution should be no longer than 10 seconds. The gel was well agitated until the first bands are visible (usually 1.5 - 2 minutes). The gel was then transferred to another tray containing 3 litres of chilled developing solution and shaken until bands from every lane were observed (usually 2 – 3 minutes). Two liters of the fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was placed to room temperature for air-dried.

2.1.7 Data analysis

2.1.7.1 Scoring of microsatellite alleles

Sizes of microsatellite allele were determined by scoring the PCR product separated in denaturing polyacrylamide gels. In general, a microsatellite allele from an electrophoretically pattern did not reveal a single band, but displayed a ladder of bands called stutter bands. Therefore, scoring of a particular band was carried out by making an assumption that an actual band of a given allele was the most intense band located at the biggest in size compared to the neighbor group of stuttered bands.

Since microsatellite is a co-dominant marker providing 1 or 2 alleles per individual, the genotype of the individual *H. asinina* for each locus could be divided into homozygotic or heterozygotic states. For microsatellites detected by silver staining, both alleles of a particular locus were visualized and only one allele was scored.

2.1.7.2 Genetic variation and heterozygosity

The number of alleles per locus was directly counted from obtained data. The frequency of a particular allele in a sample at a given locus was calculated as

$$P = (2N_{AA} + N_{Aa})/2N$$

where P is the frequency of the A allele. N is a total number of investigated individuals within a sample. N_{AA} and N_{Aa} are numbers of homozygotes and heterozygotes for such a locus.

Heterozygosity can be calculated as observed (direct-counted, h_{obs}) and expected heterozygosity (h_{exp}). The former is a proportion of heterozygous individuals and overall investigated specimens in a sample or species. The latter is computed from allele frequencies of a locus using the formulae;

$$h_{exp} = 1 - \Sigma p_i^2$$

Assuming that investigated samples conform with Hardy-Weinberg equilibrium.

Practically, those parameters were estimated using GENEPOP Ver. 3.2 (Raymond and Rousset, 1995).

2.1.7.3 Discrimination capacity (DC)

The discrimination capacity was introduced to eliminate the possible bias on using the number of genotype as the diversity index. The DC can be calculated as

DC = numbers of genotype/number of investigated individuals.

2.1.7.4 Hardy-Weinberg equilibrium

Once allele and genotype frequencies have been estimated, association of two alleles that an individual receives at a locus should be considered. Without significantly disturbing forces (i.e. selection, mutation or migration) which would change allele frequencies over time and mating is actually occurred at random in a large population. Pairs of allele are not associated.

In this study, the null hypothesis (H_0) that observed genotypes frequencies of an investigated sample at a given locus conform to the Hardy-Weinberg equilibrium and an alternative hypothesis (H_1) for heterozygote deficiency were tested using permutation version of the exact test based on a Markov chain following the algorithm of Guo and Thomsom (1992) routine in GENEPOP.

2.1.7.5 Genetic heterogeneity between subgroups of the hatchery-propagated H. asinina

Genetic heterogeneity between subgroups of *H. asinina* was tested based on the null hypothesis that the allelic distribution of paired samples at a given locus is not statistically significant difference using the exact test described by Raymond and Rousset (1995) implemented in GENEPOP. Results are expressed as the probability of homogeneity between compared samples.

2.1.7.6 Association between microsatellite genotypes and the body weight in the hatchery sample (group B) of H. asinina

Association between genotypes (fix effect) and the body weight (variable effect) was initially tested at each locus using simple linear regression analysis.

Significant differences between the body weights of the BL and BS samples having different genotypes were analyzed using ANOVA followed by Duncan's new multiple range test. Significant comparisons were considered when the *P* value was < 0.05.

2.2 Development of stock-specific markers in *H. asinina* using AFLP-derived markers

2.2.1 Experimental animals

Wild and cultured abalone used in the experiment are shown in Table 2.1. Genomic DNA were extracted from each abalone using a phenol-chloroform-proteinase K extraction method and determined for the quality and quantity as described above.

2.2.2 Amplified Fragment Length Polymorphism (AFLP) analysis

2.2.2.1 Restriction enzyme digestion and adaptor ligation

Approximately 250 ng of genomic DNA of two individuals each from TRGW and SAME was digested with 4 units of *Eco* RI in a 25 µl reaction mixture containing 1 X OPA buffer (10 mM Tris-acetate; pH 7.5, 100 mM magnesium acetate, 500 mM potassium acetate) at 37°C for 3 hours. The reaction was terminated by incubated at 65°C for 15 minutes. The reaction mixture was adjusting to a final volume of 40 µl by adding 1 X OPA buffer, deionized water and 2.5 units of *Tru*9I (an isochizomer of *Mse* I) and further incubated at 65°C for 3 hours. Double stranded *Eco* RI and *Mse* I adaptors (Table 2.3) were ligated to restricted products in a 30 µl volume composing of 20 µl of the digested product, 1 X OPA buffer, 0.25 µM of *Eco* RI and 2.5 µM of *Mse* I adaptors, 1 mM of ATP and 3 Weiss units of T4 DNA ligase. The reaction mixtures were incubated at 4°C for 16 hours.

2.2.2.2 Preamplification

Preamplification was carried out utilizing adaptor-specific primers with a single selective base on each primer; E_{+A} (5′-GAC TGA GTA CCA ATT C**A**-3′) and M_{+C} (5′-GAT GAG TCC TGA GTA A**C**-3′). The reaction was carried out in a 25 µl volume containing 10 mM Tris-HCl; pH 8.8, 50 mM KCL, 0.1% Triton X-100, 200

μM of each dNTP, 1.5 nM $MgCl_2$, 37.5 ng of each of E_{+A} and M_{+C} primers, 1.5 units of DyNazyme^{TM}II DNA Polymerase (Finnzymes) and 1 μl of the ligation product. PCR was performed consisting of denaturation at 94°C for 30 seconds, annealing at 56°C for 1 minute and extension at 72°C for 1 minute for 20 cycles and final extension at 72°C for 5 minutes.

2.2.2.3 Selective Amplification

The preamplification product was diluted five fold and further used for selective amplification. Sixty-four of selective amplification primer combinations were screened (Table 2.4). The selective amplification was carried out in a 25 μl volume containing 10 mM Tris-HCl; pH 8.8, 50 mM KCL, 0.1% Triton X-100, 200 μM of each dNTP, 1.5 nM $MgCl_2$, 30 ng of each of E_{+ANN} and M_{+CNN} primers, 1.5 units of DyNazyme^{TM}II DNA Polymerase (Finnzymes) and 5 μl of the preamplified diluted products. PCR was performed consisting of denaturation at 94°C for 30 seconds, annealing at 65°C for 45 seconds and extension at 72°C for 90 seconds for 2 cycles followed by 12 cycles of a touch down phase with lowering of the annealing temperature for 0.7°C in every cycle. The amplification consisting of 94°C for 30 seconds, 56°C for 45 seconds and 72°C for 90 seconds for 25 cycles. The final extension was carried out at 72°C for 5 minutes.

2.2.3 Gel electrophoresis

The selective amplification products were electrophorized through 1.5% agarose gel to determine the amplification success. AFLP fragments were size-fractionated by 4.5 - 5.0% denaturing polyacrylamind gel electrophoresis and visualized by silver staining as described previously.

2.2.4 Cloning and Sequencing of AFLP Fragments

2.2.4.1 Elution and reamplification of AFLP fragment from polyacrylamind gel

Candidate population-specific AFLP fragments were excised from the polyacrylamind gel using sterile razor blade and washed 3 times for 2 hours each at room temperature with 500 μl of sterile deionized water. Twenty microlitres of TE

buffer was added and incubated at 50°C for 30 minutes and transfer to 37°C for overnight and kept at 4°C.

Table 2.3 Double strand adaptor and preamplification primers for AFLP analysis

Primer	Sequence (5′-3′)
Adaptor sequences	
Eco RI adaptor	CTCGTAGACTGCGTACC
	AATTGGTACGCAGTCTAC
Mse I adaptor	GACGATGAGTCCTGAG
	TACTCAGGACTCAT
Preamplification primers	
E_{+A}	GACTGAGTACCAATTC**A**
M_{+C}	GATGAGTCCTGAGTAA**C**

Table 2.4 Primer combinations used for screening of polymorphic AFLP fragment in *H. asinina*

	E_{AAG}	E_{ACA}	E_{ACC}	E_{AGG}	E_{AGA}	E_{ATG}	E_{ATC}	E_{ATA}
M_{CAC}	+	+	-	+	+	+	+	+
M_{CAG}	+	+	-	+	+	-	+	-
M_{CTA}	+	+	-	+	+	+	+	+
M_{CTG}	+	+	-	+	+	-	+	-
M_{CTT}	+	+	-	+	+	+	+	+
M_{CGA}	+	+	-	+	+	+	+	-
M_{CGT}	+	-	-	+	-	-	+	-
M_{CGC}	+	-	-	+	+	-	+	-
M_{CGG}	+	-	+	-	+	-	+	-
M_{CCA}	+	-	+	-	+	+	+	-
M_{CCT}	+	-	+	-	+	+	+	-
M_{CCG}	+	-	+	-	-	+	+	-
M_{CCC}	-	-	-	-	-	+	+	-

+ = primer combinations used in this study.

The target fragment was reamplified using the same PCR recipes as those for selective amplification with the exception that 100 ng of each primer and 5 µl of the eluted AFLP product were used. The amplification condition were composed of predenaturation at 94°C for 3 minutes, followed by 5 cycles of 94°C for 30 seconds, 42°C for 45 seconds and 72°C for 1 minute. The amplification consisting of 94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute. The final extension was carried out at 72°C for 7 minutes. The reamplification products were electrophorized through 1.5 - 1.8% agarose gel for examined the success of the amplification fragment. The DNA fragments were excised and eluted out from the agarose gels using a Hiyield™ gel Extraction kit (Real Genomincs) according to the protocol recommended by the manufacture.

2.2.4.2 Ligation of AFLP fragment to pGEM®-T Easy vector

The gel-eluted PCR products (3 µl) was ligated to pGEM®-T easy vector (Promega) 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®-T easy vector. The reaction mixture was incubated at 4°C overnight before electrotransformed into *E. coli* JM109.

2.2.4.3 Transformation of ligated products to E. coli host cells

2.2.4.3.1 Preparation of competent cells

A single colony of *E. coli* JM109 was inoculated in 10 ml of LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl), vigorous shaking overnight at 37°C. The starting culture was inoculated to 50 ml 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 10 minutes and transferred to a centrifuge bottle and centrifuged in a prechilled rotor at 3,000xg at 4°C for 10 minutes. The pellets were resuspended in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged as above. The supernatant was discarded and the pellet was resuspended in 2 - 3 ml of ice-cold 0.1 M $CaCl_2$ and divided in to 200 aliquots. These cells could be used immediately or stored at -70°C until used.

2.2.4.3.2 Electrotransformation

The competent cells were thawed on ice for 5 minutes. Approximately 2 - 4 µl of ligation product were added, mixed by pipetting and left on ice for 30 minutes. During the incubation period, the ice box was gently moved forward and backward a few times every 10 minutes. The reaction tube was placed at 42°C water bath for 45 seconds for heat-shocked. The reaction tube was then immediately snapped on ice fore 5 minutes. All the reaction were pipetted into 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5mM KCL, 10 mM $MgCl_2$, 10 mM $MgSO_3$ and 20 mM glucose). The cell suspension was shaking incubated at 37°C for 1.5 hours. Then the cell suspension was centrifuged at 6000 rpm for 1 minute at room temperature. The cell pellet was resuspended in 100 µl of SOC medium and spread on a LB agar plate containing 50 µg/ml of ampicillin, 25 µg/ml of IPTG and 20 µg/ml of X-gal. The plate was incubated at 37°C for overnight (Sambrook *et al.*, 1989). The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.2.4.4 Detection of recombinant clone by colony PCR and restriction endonucleases

A recombinant clone was scraped by the micropipette tip and mixed well in the amplification reaction which performed in a 25 µl containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µM of dNTPs, 1.5 mM MgCl2, 0.2 µM of pUC1 (5'-TCCGGCTCGTATGTTGTGTGGA-3') and pUC2 (5'-GTGGTGCAAGGCGATTAAGTTGG-3') primers and 0.5 unit of DyNazyme™II DNA Polymerase (Finnzymes). The PCR was performed consisting of denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute. The final extension was carried out at 72°C for 7 minutes. The amplification products were analyzed through 1.2 - 1.5% agarose gel electrophoresis.

The colony PCR products containing the insert were separately digested with *Hin*d III and *Rsa* I (Promega). The digestion reaction was set up in total of 15 µl composed of 1X of restriction enzyme buffer of each enzyme, 5 µl of amplification products and 2 units of either *Hin*d III or *Rsa* I. The restriction mixture was incubated at 37°C for 3 - 4 hours and analyzed by agarose gel electrophoresis.

2.2.4.5 Extraction of plasmid DNA and estimation of the insert size

A single recombinant 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. Plasmid was isolated using HiYield™ Plasmid Mini Kit. The culture was transferred into a new 1.5 ml microcentrifuge tube and centrifuged at 13,000xg for 1 minute. The supernatant was carefully removed. Cell pellet was resuspended with 200 μl of PD1 Buffer (containing RNaseA) and missed by vortexed. Cells were lysed by addition of 200 μl of PD2 Buffer and mixed gently by inverting the tube 10 times. The mixture was stood for 2 minutes at room temperature. After that, 300 μl of PD3 Buffer were added to neutralize the alkaline lysis step and mixed immediately by inverting the tube fore 10 times. The mixture was centrifuged at 14,000 rpm for 15 minutes. The supernatant was transferred into a new microcentrifuge tube and to the PD column and centrifuged at 6,000xg (8,000 rpm) for 1 minute. The flow-through was discarded. The PD column was paced back in the collection tube, The column was washed by adding 400 μl of W1 Buffer and centrifuged at 6,000xg (8,000 rpm) for 1 minute. After discarding the flow-through, 600 μl of the ethanol-added Wash Buffer was added and centrifuged as above. The flow-through was discarded. The spin tube was centrifuge for and additional 2 minutes at full speed to remove the residual Wash Buffer. The dried column was placed in a new 1.5 ml microcentrifuge tube and 30 - 50 μl of the Elution Buffer or water was added at the centre of the column and left at room temperature for 2 minutes, centrifuged at full speed for 2 minutes and kept at -20°C. The concentration of extracted plasmid DNA was spectrophotometrically measured.

The extracted plasmid was digested with *Eco*R I for estimating the insert size of each recombinant plasmid. The digestion was carried out in a 12 μl volume composed of 1X restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl and 50 mM MgCl2), 3 units of *Eco*R I (Promega) and 1 μl of extracted plasmid. The reaction mixture were incubated at 37°C for 4 hours and analyzed by agarose gel electrophoresis.

2.2.4.6 DNA sequencing

The recombinant plasmids were unidirectional sequenced using the M13 reverse or M13 forward primers on an automated DNA sequencer at Macrogen (Korea). The nucleotide sequence of an AFLP-derived fragment was compared with those previously deposited in the GenBank using Blast*N* and Blast*X* (Altschul *et al.*, 1990, available at http://www.ncbi/nlm/nih.gov). Significant probabilities of matched nucleotides and proteins were considered when the *E*-value was < 1e-04.

2.2.5 Development of SCAR Markers and SSCP Analysis

2.2.5.1 Primer designed and PCR ampification

Nine sequences were obtained from candidate population-specific AFLP fragments. Sequence-specific primers were designed from six AFLP-derived markers using Primer premier 5.0 (Table 2.5). The amplification reaction was carried out against genomic DNA of each abalone in a 15 µl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, 1 unit of DyNazyme^{TM} II DNA Polymerase (Finnzymes) and 50 ng of genomic DNA. PCR was performed by predenaturation at 94°C for 3 min, followed by 10 cycles of 94°C for 45 s, at 10°C above the annealing temperature of each primer pair for 1 min (Table 2.4), with a reduction of 2°C in every other cycles and extension at 72°C for 45 s, and 30 cycles of 94°C for 45 s, at the appropriate annealing temperature for 1 min (Table 2.5) and 72°C for 45 s. The final extension was carried out at 72°C for 10 min. The PCR product was electrophoretically analyzed through 1.5% agarose gels visualized under a transilluminator after ethidium bromide staining. Sizes of products were determined by comparing with a 100 bp marker.

2.2.5.2 Single strand conformational polymorphism (SSCP) analysis

2.2.5.2.1 Preparing of glass plates and non-deturing polyacrylamind gel

The glass plates (PROTEAN II xi Cell, BioRad) were cleaned and prepared in essentially identical to that described for AFLP analysis. Different concentration of low cross link non-denaturing polyacrylamide gels (37.5:1 or 75:1) were prepared by dilution of a 40% stock solution to required gel concentration. After degassed, 30 ml of the acrylamide gel solution was mixed with 300 µl of 10% ammonium persulphate

and 30 µl of tetramethylethylenediamine (TEMED). The analytical comb was inserted into the prepared gel and allowed to complete polymerization for 4 hours (or overnight).

Table 2.5 Nucleotide sequences, length and annealing temperature of primers designed from candidate population specific AFLP fragments of *H. asinina*

Name of SCAR marker	Primer Sequence (5′ – 3′)	Length (bp)	Ta (°C)
$HaSCAR_{328}$	F: TACGAATTCAGACTACACGCAA	22	57
	R: GATTAACGCTCTCAATGAGAGA	22	
$HaSCAR_{167}$	F: GCTCTCACATTGTAGCCTTTG	21	50
	R: ATTGAAATGGAAAGAAAAGTG	21	
$HaSCAR_{339}$	F: TACGAATTCATCAAAGGGAC	20	57
	R: GATTAACAGACTGCTGCCCA	20	
$HaSCAR_{320}$	F: TACGAATTCATCCTCGATAGTC	22	50
	R: GATTAACTTGGCAACAAGTGA	21	
$HaSCAR_{295}$	F: TACGAATTCATCGCTCTAATCAT	23	55
	R: AACAGACCGGAACTCTCATG	20	
$HaSCAR_{327}$	F: TACGAATTCATCCAACAAAGG	21	50
	R: GATTAACCCTAACCTATCGTAAA	23	

2.2.5.2.2 SSCP gel electrophoresis

For SSCP analysis, the amplification product of each specimen (6 µl) was mixed with 4 volume of SSCP loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH). The mixture then denatured at 95°C for 5 minutes, and immediately put on ice for at least 3 minutes. Non-denaturing polyacrylamide gel (12.5 - 17.5%; 37.5:1 crosslink) was used for analyzed at 12.5 V/cm for 16 hr at 4°C. SSCP patterns were visualized by silver staining. Nucleotide sequence of each SSCP genotype of $HaSCAR_{320}$, $HaSCAR_{295}$, $HaSCAR_{327}$ was direct-sequenced. Nucleotide divergence between sequences was calculated based on the two parameter method (Kimura, 1980).

2.2.6 Data Analysis

The percentage of monomorphic (> 95% of investigated specimens) and polymorphic (< 95% of investigated specimens) bands and gene diversity (Nei, 1987) was estimated for each geographic sample. Unbiased genetic distance between pairs of samples was determined (Nei, 1978). Genetic heterogeneity in allele distribution frequencies between compared geographic samples was examined using the exact test. The F_{ST}-based statistics (θ) between pairs of samples, bootstrapping 10000 iterations to generate the 95% confidence interval, was estimated. The chi-square value was calculated and tested to determine whether θ was statistically different from zero (Weir and Cockerham, 1984) using $\chi^2 = 2N\theta(k-1)$ and df = (k-1) (s-1) where N = number of investigated individuals, k = number of allele per locus and s = number of geographic samples. Population genetic parameters described above were computationally analyzed by TFPGA (Miller, 1997). The significance level of multiple comparisons was further adjusted using a sequential Bonferroni method (Rice, 1989).

2.2.7 Screening of genes for further studies on single nucleotide polymorphism (SNP) using heterospecific markers from the giant tiger shrimp, P. monodon

One hundred and thirty five pairs of gene-specific primers designed from cDNA sequences of *P. monodon* (Table 2.6) were tested with one individual each of *H. asinina* from Samet Island (Po generation, SAME), Talibong Island, Trang province (TRGW) and the Philippines (PHI). The amplification reaction was carried out in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer, 1 unit of DyNazyme™ II DNA Polymerase (Finnzymes) and 100 ng of genomic DNA. PCR was performed by predenaturation at 94°C for 3 min, followed by 25 cycles of 94°C for 30 seconds, 50°C for 45 seconds, and 72°C for 45 seconds. The final extension was carried out at 72°C for 7 min. The PCR product was analyzed through 1.5% agarose gels electrophoresis. Bands were visualized under a transilluminator after ethidium bromide staining. Sizes of products were determined by comparing with a 100 bp marker.

The PCR products that showed the major band in all specimens were further characterized. Bands were excised from the agarose gel. DNA fragment were eluted by HiYield™ Gel/PCR DNA Extraction Kit (RBC; Real Biotech Corporation) and

Table 2.6 Gene specific primers, primer sequences and length cDNA libraries of *Peneaus monodon*

Gene homologue	Sequence (5'-3')	Length (bp)
Adenosylhomocysteinase (O)	F: CCATCCAGACTGCTGTGCTC	20
	R: TCCCGTGGTGGTTTCTTCCG	20
AgCP13148 (O)	F: CATACCTCGCATCATCAGTG	20
	R: CCTCAGGAGACGATACAAAGC	21
Arginyl-tRNA-protein transferase1 (T)	F: GGGATGGAGACGGAGTGGAA	20
	R: TGGCATCTGGAGGGATACACC	21
Aspartate aminotransferase (O)	F: AGGAATGCCTATGCTGTGCG	20
	R: CTGGATTACTAAGAATGGTGG	21
ATP-dependent RNA helicase (O)	F: GAAGGGAAGGCAGCCAGGAG	20
	R: CGACGGATTTCAGCCATTACATAG	24
Calcineurin B (O)	F: TGGGTAACGAAGCCTCATTG	20
	R: ATTCGGAAAGCAAAACGCAA	20
Calcium independent phospholipase A2 isoform1 (H)	F: TGTCCCACAGCACCAGTAAT	20
	R: CCAGCCTTCACCTCCTCTTC	20
Calcium regulated heat stable protein (H)	F: CCAAACAAGGGCTTTCTGCTG	21
	R: CGCTTCGGTGGGATGGGACA	20
Calponin I (H)	F: GCCGCCGAGTGCTTGGAATG	20
	R: CCTTCCTGCCCAGAGACT	18
Carbamoyl phosphate synthetase (H)	F: TGTCACTTCACGCAGGATTG	20
	R: ATAGTTGTCATTTGAGGCACC	21
Cardiomyopathy associated 4 sterile muscle (H)	F: CCTGACGCTGGATGCTGATGTT	22
	R: TGTCCACAAGAGCGGAAGCAGC	22
Carnitine palmitoyl transferase II (O)	F: CAAGAGCAGTGGCATCATCC	20
	R: AGAACTATTCCTAAGGCGGTCAT	23
Casein kinase II beta chain (O)	F: TGAACCAGATGAGGAGGAAGA	21
	R: GGAAGCCAGTGCCGAAGTAA	20
Cell divition protein kinase2 (T)	F: CGACTGCTGGATGTGGCGTA	20
	R: CCGAGGAGAATCTGTGGGGC	20
Cell divition protein kinase 7 (T)	F: CGGAAGACAGGATGGAAGTAGAA	23
	R: ATGTTGGATGGGCGTGAGGATG	22

Table 2.5 (cont.)

Gene homologue	Sequence (5'-3')	Length (bp)
Chk1 checkpoint kinase (H)	F: TTTTATGGAAGTCGCCGATGAA	22
	R: ATTCTCGTCAAGCAGCAAGTTCT	23
Chloride intracellular channel 6-like protein (H)	F: TCTCTCTGAAGGTCACCACTGTCG	24
	R: CGCAACATCCTTATCCTGAACAAA	24
Chromobox protein (O)	F: TGGGAACCTGAATCTCATCTTG	22
	R: ACGAACATTTGCCTGCCTTG	20
Clatrin adaptor protein AP 50 (H)	F: TCCCAACTCCACTGAATACTT	21
	R: TGAGCTTCGACTCTAAACACCTT	23
C-myc binding protein (AMY-1) (H)	F: GTCTGGCGTAATGTCGGCACTG	22
	R: CCTCTGGTGCTGCTTTGGGTTC	22
Contractile ring component anillin (T)	F: TGTTTGAGGATGTTGGGGGCT	21
	R: AACTGGAAGGTATGCTGACGGG	22
Cop 9 constitutive photomorphogenic homolog subunit 6 (O)	F: CAGTGATGTTAGCCCAGGAA	20
	R: CCAGCCAAGGAGGTCAAGGT	20
Cyclic AMP regulated protein (H)	F: AAGGGACCCCGTCAATCCACTG	22
	R: CGTGATTTTATCCGAACGAAGCCG	24
Cyclin A (O)	F: CAGCAAGTATGAGGTGGATTCT	22
	R: TCTGCCCAACTCTGTAGGTATT	22
Death box protein 15 (O)	F: AATCCTTTTACGAATCTGCCAT	22
	R: CGGTCTCTCCAACTAATACAAT	22
Dendritic cell protein (H)	F: AAGCCAGCAAAGTGATGATT	20
	R: CAAGAGGTCGTGGATGAGTTC	21
Diphenoloxidase A2 (H)	F: ACAATAATGAGGTAGCACGGT	21
	R: CGGAAAATCTGTCTCTCTGGGA	22
DNA primase (O)	F: GAGCCGCAGAGTTGAGGTTG	20
	R: TCGTTTCTTGTCCAGGTTTTTG	22
Dolichyl diphosphooligosaccharide protein glycotransferase (H)	F: TTCTGGCAACGGCAAAGTAG	20
	R: ATGGGTCAATGCGAACAAAG	20
Domain family member (O)	F: GCTGGCCCTTTTCAATGTCCTA	22
	R: GAAGCAAACATCGTCAGGAACC	22
Endothelial cell growth factor 1(H)	F: GGAGGTCGGCTGCTGTATCG	20
	R: GCCACATCCACCATTTTCTT	20
ETS1 protein (H)	F: CGTGGACTGGCGACGGCTGGGA	22
	R: GGGGCGGGGAGGGCAAGGCA	20

Table 2.5 (cont.)

Gene homologue	Sequence (5'-3')	Length (bp)
Ferrochelatase (O)	F: GCTGCCATTCCAAGATTACAT	21
	R: TTTCAACTCCATCCTCCTCCA	21
FK 506 binding protein 4 (H)	F: ACAGGGAGCGAGGAGGGG	18
	R: AGGGTGGCATTTGGCGGG	18
Fructose 1,6-bisphosphate aldolase (H)	F: CTCGCTGCTGATGAGTCTGTCT	22
	R: TTCTGGTAAAGGGTTTCGTGGA	22
Fus prove protein (H)	F: GAGATTGGAAGTGCCCTGTGCC	22
	R: GTGTTTCGCCATTCAGGCAGTT	22
Gelsolin, Cytoplasmic (actin depolymerizing factor) (H)	F: GCAGAGACTTACATCAAGACCG	22
	R: CTGAAGGAGACACCCCATTTAG	23
Gene flightless-I protein (H)	F: AGAGAAAATCGGATAAATACGGA	23
	R: TCTGGAGTTTGTTGTGGCGGAC	22
Glutathione peroxidase (H)	F: AACTGGCTTCCTCCGCTATC	20
	R: TGAGTTGGTTCATCAGGTGG	20
Glycogen phospholipase (H)	F: GGCTTCCTTGACCGTAACTT	20
	R: CGAAATCCGTGCGAACCTGG	20
Hepatocarcinogenesis-related transcription factor (X-box protein) (O)	F: TGATGAACTTCGGGACCTAA	20
	R: CCTCAACGACAACTGCTGCG	20
Heterogeneous nuclear ribonucleo protein 87F (O)	F: TTTTCCTACAATGGAGGCAAGTGGCT	26
	R: AGGCTACAACAACCCTGGTCCTAACA	26
Heterogeneous nuclear RNA protein clone (H)	F: ACTTCCAGGGCAACGGTATG	20
	R: ATGGCGTGCTTTGGCTTTCT	20
Heterotrimeric GTP binding protein alpha subunit G-alpha-q (H)	F: CAAACAGTGAACACGCAGAT	20
	R: GCTCGGTCGGTAAGAAGTCC	20
High mobility group protein DSP1 (O)	F: CGCTGGAAGACGATGACTGATA	22
	R: TGGTGGTGTGTTACTCCAGGCA	22
HLA-B associated transcript 1A, nuclear RNA helicase bat 1 (O)	F: AGGCAGGAGTAGGAGAAACG	20
	R: AAGGATGCTCAAAGCCACAG	20
Hyaluronan receptor (H)	F: TGTGCTCCTCCTCCAACAAAAT	22
	R: CGCCTCCTGACACTGATGAACT	22
Hydroxyacyl-CoA-dehydrogenase (H)	F: CGATTCCCTTCGTAATGTTG	20
	R: ATAGCTCACTGATGTCAAGTCA	22
Hypothetical protein XP 207715 cyclin nucleotide(cyclic nucleotide gated channel beta subunit 1) (O)	F: TCTCCCTTCTATGCCTGTGTCC	22
	R: GCTCGCTTCAACCAAACTGC	20

Table 2.5 (cont.)

Gene homologue	Sequence (5'-3')	Length (bp)
Immonophilin FKBP 52 (O)	F: GAGAAGAAAGACGGAGGAGTG	21
	R: TATCAGGGTGGCATTTGGCG	20
Inhibitor of apoptosis protein (T)	F: CCTGAAGAGTTAGCAGCAGATGG	23
	R: TACTTGCTTTTGGAGGATTGTCAC	24
Innexin 2 (O)	F: CCTTCACGATACCCTCCTTG	20
	R: ACCTTACCACCTTCCCAGAT	20
Integrin beta 4 binding protein (O)	F: GCACTCGGAAATGTGGTTG	19
	R: CCTGGTTGGAAATGACTGAAT	21
Interleukin 1 receptor like 1 ligand precursor (H)	F: CTTCTTTGGTGTTGTGATTGAG	22
	R: AAGCGTGACTTTGTGAGATGGT	22
Keratinocyte associated protein 2 (O)	F: AGGGAAGGGGAGGAGGACCAGT	22
	R: TAGGACGAGACACAGGGCGACC	22
Kin protein (H)	F: CCAGGCTGTCCTTGACCGTAAC	22
	R: AATCCGTGCGAACCTGGTCTTT	22
L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain (O)	F: GTGGCAGCAGCCGTAGATAG	20
	R: GGAGGAGAGATGTTCAAGATGT	22
Leukemia virus receptor (H)	F: GATGGCGGTCTTCAGGTGTC	20
	R: ATGGAAAGTGCGTGATGGGT	20
Metaxin2 (T)	F: AGATACTGCTCCCTGATAATGCCCA	25
	R: GCCGTCTGTCAAGGTCACTCCC	22
Methyl CpG binding protein 2 (H)	F: ATTTCCGCTTCAGACACTCCA	21
	R: CATCATTCCATTATGTTTCCTT	22
Methylcytosine (H)	F: ATGTCTTTACCACGACCCTTG	21
	R: CGGTGATATCCCATAGCCAG	20
MINUTE protein (H)	F: AGGAGAGGGACACCCAGACA	20
	R: TAGCATACCAAACATTAGACC	21
Mitocondrial oxodicarboxylates (H)	F: GGAAGATGGCAAGAAATGAGG	21
	R: AAGGGTTTGACCAGGATAGC	20
Mitotic checkpoint (T)	F: CGAGTCTGAAGTCGGCAAAATG	22
	R: GCTGACCATCTAAGCCTCCACT	22
mRNA splicing factor (deahbox) (H)	F: AATCCTTTTACGAATCTGCCAT	22
	R: TTAGGAAAATGCTTAGAGGGTA	22
Multicatalytic endopeptidase (O)	F: GCCGAGTGTTCCAAGTAGAGT	21
	R: ACATCAACGATAGCACGAGCA	21
Multiprotein bridging factor (T)	F: TGCCACCACCTTCAACACAG	20
	R: CATCCCAATAGCCTTCTCAATC	22

Table 2.5 (cont.)

Gene homologue	Sequence (5'-3')	Length (bp)
Muskelin 1 (O)	F: GTCACGATGGTCTTCCGATTC	21
	R: GTTCCAGTCCTCACAGCCTCC	21
Myosin regulatory light chain polypeptide 91 (H)	F: ATCCCGTAAGGCAGGAAAGA	20
	R: CACCAAACAGAGTGAGGAACAT	22
NADP-dependent leukotriene B4 12-hydroxydehydrogenase (O)	F: AGAGGGGATGCCCAAGAAGG	20
	R: AGCCAGACGGTGAAACAACG	20
Nit protein 2 (H)	F: TTGGTTGCCCTGCCTGAGTG	20
	R: CGGGTGATAGGACATCGGAC	20
Nm2 protein non-histone chromosome protein 2-like (H)	F: AGGCAACACTCACCCAGAAG	20
	R: TTGATACACCACAGGCACGG	20
Nme2 protein (H)	F: TTGCTGACAAGCCCTTCTACCC	22
	R: TGTGCCACTAATCCACCTACTG	22
Nuclear autoantigenic sperm protein (O)	F: AGGAAATGGAAACTGATGTCGC	22
	R: TTCTTAGCCATCTCTGGGTTGT	22
Nucleolar phosphatase (H)	F: ACAGAACCCTCAAGGACACTCG	22
	R: ATTCAACAAATAAACAACTCCCACA	25
Nudix-type motif9 isofrom A (T)	F: CGCACTGATGATAAGACTCCTCG	23
	R: CCAGCATCCATTTTCCACCG	20
O-methyltransferase (O)	F: AGCACCGTAGAGCGGCGATGTT	22
	R: CGAAGGCGATGACTCCACCAGA	22
Ornithine decarboxylase (H)	F: GCAAACGGCACGCTTCTTTCCA	23
	R: CAGCCAGTCTCCACAAGCCAGC	22
Ovarian lipoprotein receptor (O)	F: CGGGATGAGTGCGAGAAGTGC	21
	R: CAGGGGCTCCGAGTCAAAGA	20
Phenylalanine-tRNA synthesis-B-subunit (H)	F: TCAGCCATTCACTGCGGAGC	20
	R: TAGGAGGGACATCGACCACAA	21
Phosphatidylinositol-4 kinase (H)	F: CAACGCCATCAACTCCATCAC	21
	R: CTTCCAGCACCACACAGTTTTAT	23
Phosphoglucose isomerase (H)	F: TTCTGGGACTGGGTTGGTGG	20
	R: TGGCAATAAGGCATGGGTTT	20
Phospholipase C (O)	F: TCTTTGTCCATTTGAAGGTCGG	22
	R: TCATTGGCTGTCGTTGTAGTGT	22
Phosphopyruvate hydratase (O)	F: GGAGAACTGGACCAAGATGACC	22
	R: CTCACCAGACCTATGGGAAACC	22

Table 2.5 (cont.)

Gene homologue	Sequence (5'-3')	Length (bp)
Phosphopyruvate hydratase (O)	F: GGAGAACTGGACCAAGATGACC	22
	R: CTCACCAGACCTATGGGAAACC	22
Poliferating cell nucleolar antigen p120 (H)	F: ACCAGTGACCATCCGCACCAAC	22
	R: TCCATTTTTGGGTGCCAGAGCC	22
Postsynaptic density protein (citron) (H)	F: TAGAGGAAGCAGGGATGGTC	20
	R: GTCTGAAGCGGCAGCACACA	20
Pre B cell colony enhancing factor (O)	F: GAACGACGACCCTCTACTCC	20
	R: TCCCTCTGGCACAGCCTTGA	20
Prefold in beta subunit 2 (H)	F: TCCAGAAGTTCCAGCAGATG	20
	R: TGGTCCCTATTGTTTGTGAGA	21
Presenilin enhancer (O)	F: CAAGCCAAGCGACCATCCAT	20
	R: TAATCCAACGAGCCATACAA	20
Proactivator polypeptide precursor (Prosaposin) (O)	F: GCCATAAAGTTCTGCCCCCACC	22
	R: GCCCTCCCAATATCTACATCCA	22
Programmed cell death protein6 (H)	F: CAGGGTGCCACCGCGACTCTTT	22
	R: GGCTTGCCATTGTCCACCTCAG	22
Programmed cell death7 (T)	F: CCCTGACAGCCCTGCGACA	19
	R: GCACTTTCACCCATCATAACCCG	23
Proteasome (prosome, macropain)26S subunit, ATPase2 (T)	F: AACTCTCCAGAATGAGCAGCCA	22
	R: TACTTATTACGATGCCACACCCAC	24
Proteosome (proteosome subunit alpha type3) (H)	F: GAGTATGCCCAAAAAGCAGTCG	22
	R: AGGTGCTCACTCGTTCTGCCAA	22
Proteosome (prosome,macropain) subunit,alpha type3 (O)	F: AAAGATGGTGTTGTGTTTGCTGTAG	25
	R: AGAGGGTATAGGCATGAAGGTAGG	24
Putative cold-induced protein (O)	F: TGTGTTCTTCATTGCCTCTCCG	22
	R: TATTTGGAAGTGGGGCATCTGA	22
Ras interacting protein RIPA (O)	F: GTGAGAGTGAGGGGGAAGAGGGTAA	25
	R: GGGAGGTTTGGTTTTTGGGGG	21
Rho protein (H)	F: CTGGCTCGTCTCCCTCTTCGTG	22
	R: TCGTAGTCCTCCTGACCTGCTG	22
Ribophorin I (O)	F: CGACTTCCAGAGAGAGCACA	20
	R: GGTCTTCCATCCTCCAAACA	20
Semaphorin 2A precursor (H)	F: CTTGGTGGTGATGAATGTAA	20
	R: GGATGGCTGTTCTGCTGGCT	20

Table 2.5 (cont.)

Gene homologue	Sequence (5'-3')	Length (bp)
Serine palmitoyl transferase LCB2 subunit (O)	F: ATCCAACAACTGTCTCGCAATG	22
	R: ATCCAACCCCTACGCCAGCCAC	22
Serine proteinase inhibitor (O)	F: GGAAGAAAGAAGCAAAGTC	19
	R: CCAGTCCTCCAATGTCAGCA	20
Signal recognition particle 72 kDa (H)	F: AGAAGAGGAAGTGGCTGATGAG	22
	R: GCAAGTAAGCAGTGATTGAAGG	22
Singed protein (H)	F: TGTCCGTGACCGACGACGAGCA	22
	R: ATCTCGGTGCCCTCCTCGTTCA	22
Small androgen receptor interacting protein (O)	F: ATGACAGACTACAAGGAAGAACAGAA	26
	R: CTCCAATGAGTATGAGACAAGCG	23
Small ubiquitin-like modifier (O)	F: GGAAGGGAACGAATACATCAAA	22
	R: GCCTGGTCTGTGCGAAAATCTC	22
Solute carrier family 3 member 2 (O)109	F: GCACGAAGCCCTACCGAGCC	20
	R: CGTCTTCCTGCGGAGCCATC	20
Solute carrier family25 ,member 5;2F1: adenine nucleotide transiocation2 (O)	F: GTCCGCATCCCAAAGGAACGAG	22
	R: CGAGCGAAGTCAAGGGGGTAGA	22
SPAPC (O)	F: AGCGTATCACCCCTCGCCATCT	22
	R: TCTCAAACACCTCCCTACTCCATCA	25
Sperm tail specific protein mst 101 (O)	F: AGCCAAAGATTAGGAAAACGAA	22
	R: GCCAGGGGTGCTTAGAATAGGT	22
Splceosome-association protein9 (H)	F: TCTACCTCTGCTGACCGCTAAGT	23
	R: AACCGTTACCCTATTATCACAATCTAT	27
Splicing factor 3a,subunit 1 (O)	F: AGTTGAAAGAACGAGGTGGACG	22
	R: TACCCAATACACGCAAATAACC	22
SRPK2 protein (T)	F: CACTGTTTGGCTGTGTTGGG	20
	R: CCTGGTAATTGGAGCGGATG	20
SRY-box 7; SOX7 transcription factor (H)	F: TCATCGGGTCAAAAAGGTCCAA	22
	R: TGCTGAAGTGCCTGTGCCATCT	22
Stromal membrane associated protein (H)	F: CTTTCTTACCTGACGCTGGATGCTGA	26
	R: TCGGTGTCCACAAGAGCGGAAGCAGC	26
Survival motor neuron (O)	F: TAACGATAAGGAACTCACCCAT	22
	R: CATCACCTGTGTGCCACTCAAT	22
TATA box binding protein (TBP)-associated factor, 68kDa, isoform CRA_c (H)	F: ATGGGCATCACGGACTACGAG	21
	R: CAGGCAGAGAGGCAATAACGA	21

Table 2.5 (cont.)

Gene homologue	Sequence (5'-3')	Length (bp)
TERA protein (chromosome 12 open reading frame 14) (H)	F: GGACCCGTGACCTCGCTCGCCT	22
	R: CTTCATACTTCTTAGAGTCTGTAAACCG	28
Tetrasparin D 107 (H)	F: GTTCTTTGATGGCAAGTTCG	20
	R: CGTTCCACAGCAATGGTAGTTC	22
Tetratricopeptide repeat domain 5 (H)	F: TGCGGCTGTCAAAAAGAACCAA	22
	R: GGCACTCTCCAAGCTCGTTCCA	22
Tissue specific transplantation antigen p35B like protein (O)	F: ATGGTTTTTGGGCTCATTAGTG	22
	R: ATTGAATCGTAGGAAATCGCAG	22
Translationally controlled tumor protein (H)	F: AGTATGAGGAGGTGGATGATGC	22
	R: GAAGCCGGTTTCCTGCAGACGC	22
Translocon associated protein gamma (H)	F: CAGGACTTCAGTCGGAGTGTGT	22
	R: ATCTTCTTGTCAGCATCCAGG	21
Ubiquitin isopeptidase (O)	F: CAAGTTGGCTGCCCCTGAAG	20
	R: GTTGCCTGCTCTCGTGTGAATC	22
Vacuolar type H+ ATPase subunit A (H)	F: GACCTCACAGAGTCCATTTACA	22
	R: GGAGTTCTCAGGCACAATACCA	22
Vitellogenin (O)	F: AGCCGAAGAAACGAAGGGA	19
	R: CACTACATCCAAAGCAACTGTCTCG	25
Voltage-dependent anion-selective channel protein 2 (H)	F: CAAGATGTGTTTGGCAAGGGAT	22
	R: GAAGTCGGGGTTGGTGAAGC	20
Wolf hirschorn syndrome candidate 1 protein (H)	F: ATGGCACCACTGTGATGAATGT	22
	R: GGTCAACTGATAACAAAGCCTC	22
Y-box protein Ct50 (O)	F: CGGAGACACAAGCCAAGCCT	20
	R: GGTGGAACCCAACCAGCAAC	20
Zeta 1 cop (H)	F: TGGTGTCAGTGTTGAGTTGTCT	22
	R: GCTCTCCTCCAGCCTTAGTGC	21
Zonadhesin isoform 4 (O)	F: CCTGGGCGTAGCTAATCTTAAC	22
	R: TCGGTAGGGCCATATCCTCTCC	22
ZZZ3 (H)	F: AGGAGCAAGGCAGTTCGGGACA	22
	R: GTTTGGGGTTTACTCTCATCGT	22
CG1681-PA (H)	F: ATCAAAGCCATTCATTGCGAGC	22
	R: AACCAGACAAAAATAAAACCAAAT	24
Chaperon subunit 8 (O)	F: CTATTCTGGGCTGGAGGAGGC	21
	R: CAACATCTGTGGGAGTGAGGC	21

H, O, and T = primers designed from ESTs found in the hemocyte, ovarian and testes cDNA library, respectively.

ligated to pGEM®-T Easy vector. The ligation product was transformed to *E. coli* JM109. Recombinant clone was identified by colony PCR. Plasmid DNA was extracted by a QIAprep® Spin Miniprep Kit and unidirectional sequenced. The nucleotide sequence of DNA fragment was compared with those previously deposited in the GenBank using Blast*N* and Blast*X*, (http://www.ncbi/nlm/nih.gov). Significant probabilities of matched nucleotides and proteins were considered when the *E*-value was < 1e-04.

Six gene-specific primers of *H. asinina* were designed. In addition, four primer pairs for amplification of *Isocitrate dehydrogenase*, *Arginine kinase*, *ADP/ATP carrier* and *NADH dehydrogenase subunit II* previously identified by sequencing *H. asinina* cDNA libraries were also designed and tested against genomic DNA of a few samples from each *H. asinina* population (Table 2.7). The amplification were carried out in 15 µl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, 1 unit of DyNazyme™ II DNA Polymerase (Finnzymes) and 50 ng of DNA template. PCR was carried out by predenaturation at 94°C for 3 minutes, followed by 10 cycles of 94°C for 45 seconds, 10°C above the annealing temperature of each primer pair for 1 minute (Table 2.6), with a reduction of 2°C in every other cycles, and extension at 72°C for 45 seconds and 30 cycles of 94°C for 45 seconds, at the annealing temperature for 1 minute and 72°C for 45 seconds. The final extension was carried out at 72°C for 10 minutes. The PCR products were electrophoretically analyzed through 1.5-2.0% agarose gel. After analyzed with agarose gel electrophoresis, the amplification products were further analyzed using SSCP analysis.

For polymorphic genes, the PCR product representing each SSCP pattern were eluted from the agarose gel, ligated, cloned and sequenced. The nucleotides were further compared to characterize the fixed single nucleotide polymorphisms (SNPs).

2.3 Isolation, characterization and expression of genes related with metabolisms in H. asinina.

2.3.1 Experimental animals

The hatchery-propagated abalone (group C) were bred from broodstock at SMART by mass spawning and cultured at SMaRT until 5 month-old. Subsequently,

juvenile abalone was transferred to the grow-out farm at Trang province and continued culturing for 6 months. Three hundred individuals of the group C sample (11 months old) were weighted. Ten percent of abalone exhibiting from the top (CL, N = 30, $\overline{X}$ = 24.52 ± 2.545 g) and the bottom (CS, N = 30, $\overline{X}$ = 7.59 ± 1.468 g) according to the body weight were selected. Hepatopancreas, gills and heart of each abalone were dissected and kept in RNAlater® Tissue Collection: RNA Stabilization Solution (Ambion) and stored at -30 °C until used for RNA extraction.

Table 2.7 Nucleotide sequences, primer length and annealing temperature of gene-specific primers of *H. asinina*

Gene	Sequence (5'-3')	Length (bp)	Ta (°C)
HaSCAR$_{U1}$	F: GCTCCCCACAGTGCTCTTGC	20	57
	R: TGTAATAATGTCCTCCTCGG	20	
HaSCAR$_{U2}$	F: TCCGAAGCATCAATGTCTCC	20	57
	R: TTCCGTCCTGGCAAAGCACT	20	
HaSCAR$_{U3}$	F: CTTTGGCTTTCTTGGAGTTA	20	57
	R: ACATCCTTCCTTCATTCGTG	20	
HaSCAR$_{U4}$	F: CATTTTCAGGCTAAGTGGTG	20	57
	R: TGTATCCTTCCTTTGTGTGC	20	
HaSCAR$_{U5}$	F: AATGTTGTAATGAGTATGAGTGG	23	57
	R: CTGATGATGCTATTGTGACCT	21	
HaSCAR$_{U6}$	F: AACCTCGCACGCTTCTCCGT	20	57
	R: TCCTGCTTCTGTGGTGTAGT	20	
IcDh	F: GAGGCTTTGTATGGGCTTGC	20	50
	R: AAGGTCAGCATTTCCGTCCA	20	
AK	F: AAGCGGTCTTTCATTTGCCA	20	50
	R: CGTAAACACCTCCCACACTC	20	
ATPCa	F: GTGAGCGTCAGTTCAATGGT	20	50
	R: TCAATAGGGTAGGACAGCAAT	21	
ND-II	F: ATCGGGTTCGTGTTCTCCTT	20	55
	R: GGGCTCGTTTGGTGTTGTCA	20	

2.3.2 Total RNA preparation

Total RNA was extracted from hepatopancreas of each abalone using TRI REAGENT® (Molecular Research Center). A piece of tissues (50 - 100 mg) was placed in a mortar containing liquid nitrogen and ground to the fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 µl of TRI REAGENT® and homogenized. Additional 500 µl of TRI REAGENT® were added. The homogenate was standed at room temperature for 10 minutes and centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a new 1.5 ml microcentrifuge tube. Phase separation was performed by the addition of 200 µl of chloroform. The mixture was vortexed for 15 minutes. After centrifugation at 12,000 rpm for 10 minutes at 4°C, the upper phase was transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by an addition of 250 µl of prechilled isopropanol and 250 µl of 1.6 M sodium acetate. The mixture was stored on ice for 30 minutes. Total RNA was recovered by and centrifugation at 12,000 rpm for 10 minutes at 4°C. The supernatant was removed. The RNA pellet was briefly washed with 1 ml of 75% prechilled ethanol and centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was discarded. The RNA pellet was air-dried for 10 minutes. Total RNA was dissolved in DEPC-treated H_2O for immediately used. Alternatively, the extracted total RNA was kept under absolute ethanol in a -80° C freezer for long storage.

2.3.3 Purification of mRNA

Messenger RNA was purified from the total RNA using a QuickPrep *micro* mRNA purification Kit (GE Healthcare). RNA pellet were dissolved in 25 µl of DEPC-treated H_2O was added with 400 µl of the extraction buffer, mixed by pipetting. Two volumes (800 µl) of the elution buffer were added to the solution. The mixture was centrifuge at 16,000xg for 1 minute. In the same period, the tube containing 1 ml of oligo(dT)-cellulose for each purification was centrifuges for 1 minute. The supernatant was removed. The homogenate was transferred into the microcentrifuge tube containing the oligo(dT)-cellulose pellet. The tube was gently inverted for 3 minutes and centrifuged at 16,000xg for 10 seconds at room temperature. The supernatant was carefully removed. One milliliter of the high salt

buffer was added and spun for 15 seconds at 16,000xg. The supernatant was carefully removed. The wash and repeated four more times, as described above. One milliliter of the low salt buffer was added to the pellet. The tube was inverted and spun at 350xg for 2 minutes. This process was repeated twice. The pellet was resuspended in 300 µl of the low salt buffer. The slurry was transferred to MicroSpin column and centrifuged at full speed for 5 - 10 seconds. The flow-through solution was discarded. The low salt buffer (500 µl) was added and centrifuged at full for 5 - 10 seconds. The column was centrifuged for another 10 seconds to dry the column. The column was placed into a sterile 1.5 ml microcentrifuge tube. The pre-warmed (65°C) elution buffer (200 µl) was added to the top of column and centrifuged at 16,000xg for 5 seconds. This step was repeated twice for elute residual of mRNA. The eluted mRNA was precipitated by the addition of one-tenth volume of 3 M sodium acetate and 2 volumes of prechilled absolute ethanol. The mixture was kept at –80°C for at least 30 minutes before centrifugation at 12,000 rpm at 4°C for 15 minutes. The mRNA pellet was kept under ethanol at –80°C until used.

2.3.4 Measuring concentrations of RNA using spectrophotometry

The concentration of total RNA was determined as described for DNA but an OD unit at 260 nm corresponds to 40 µg/ml of RNA (Sambrook *et al.*, 1989).

2.3.5 Isolation and characterization of the full length cDNA using Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR)

2.3.5.1 First strand RACE-cDNA synthesis

The full length cDNA of interesting gene homologues were characterized using a SMART™ RACE cDNA Amplification Kit (BD Bioscience Clontech). The RACE-Ready DNA template was prepared by combining 1 µg of purified mRNA with 1 µl of 5′-CDS primer and 1 µl of 10 µM SMART II A oligonucleotide for 5′-RACE-PCR or 1 µg of mRNA with 1 µl of 3′-CDS primer A for 3′-RACE-PCR (Table 2.8). The component were mixed and spun briefly. The reaction was incubated at 70°C for 2 minutes and immediately put on ice for at least 2 minutes. Two microliters of 5X First-Strand buffer, 1µl of 20 mM DTT, 1µl of dNTP mix (10 mM each) and 1µl of PowerScript Reverse Transcriptase were added. The mixture was

incubated at 42°C for 2 hours in thermocycler. The first strand products were diluted with 125 µl of TE buffer and heated at 72°C for 7 minutes and kept at -20°C.

Table 2.8 Primers used for the first strand cDNA synthesis and RACE – PCR

Primers	Sequence (5'-3')
SMART II™ A Oligonucleotide	AAGCAGTGGTATCAACGCAGAGTACGCGGG
3'-RACE CDS Primer A	AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}N_{-1}N$ (N = A, C, G or T; N_{-1} = A, G or C)
5'-RACE CDS Primer	$5(T)_{25}$ N_{-1} N (N = A, C, G or T; N_{-1} = A, G or C)
10x Universal Primer A Mix (UPM)	Long: CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT Short: CTAATACGACTCACTATAGGGC
Nested Universal Primer A (NUP)	AAGCAGTGGTATCAACGCAGAGT

Table 2.9 Name of primers, nucleotide sequences and melting temperature of gene-specific primers, nested primer and internal primer-walking used for isolation of the full length cDNA of various genes by RACE-PCR

Gene/ Primer	Sequence (5'-3')	Tm (°C)
Alpha-methylacyl CoA racemase		
3'-RACE	GTC AAT CTG TTG GGT GAC TTT GCT G	74
Nested 3'RACE	CCA ACA TGG TGC AGG CTC AGC TTG T	76
Carnitine O-palmitoyltransferase I		
5'-RACE	GCT GGA TCA TTC GCA TCA TAG CCA TGT G	84
3'-RACE	TGT CGA TCA TGC ACT GTG GAG TCT TGT G	84
Walk 3'-RACE	TGA TGA TGG GTA TGG AGT GTC CTA TG	76
hydroxyacyl CoA dehydrogenase/ 3-ketoacyl CoA thiolase/ enoyl CoA hydratase (trifunctional protein)		
3'-RACE	GGC CTT GTT GAC AGC TTG GTA GAT CC	80
Walk 3'-RACE	CAC TCC CTA TCG CCA GAA TTG C	68
Vacuolar H+ ATPase 14kDA subunit		
5'-RACE	AAT GAT GGC AAT GTC ATC CCG CTT C	74
3'-RACE	TGC CTG GAG TAC AGT GCT CAG TGT GA	80

2.3.5.2 Primer design

The 5′ and 3′ gene-specific primer (GSP) and 5′ and 3′ nested GSP were designed from homologues of 4 metabolism-related genes; *alpha-methylacyl CoA racemase* and *Carnitine O-palmitoyltransferase I* found in the testis cDNA library, *hydroxyacyl CoA dehydrogenase/ 3-ketoacyl CoA thiolase/ enoyl CoA hydratase* (trifunctional protein) found in the ovary cDNA library and *vacuolar H+ ATPase 14kDA subunit* found in the hemocyte cDNA library (Table 2.10).

2.3.5.3 Rapid Amplification of cDNA Ends (RACE)-PCR

The master mix of 5′-RACE and 3′-RACE reactions was prepared in 25 µl volume. Each amplification reaction included 16 µl of PCR-Grade Water, 2.5 µl of 10X BD Advantage 2 PCR buffer, 0.5 µl of 10 mM dNTP mix, 0.5 µl of 50X BD Advantage 2 polymerase mix, 5 µl of 10X UPM, 1 µl of 10µM 5′-RACE or 3′-RACE specific primer and 1.5 µl of 5′-RACE-Ready or 3′-RACE-Ready cDNA.

Amplification was initiaslly carried out using UPM and GSP of each gene. The amplification reaction was carried out for 5 cycles composing of a 94°C for 30 seconds, 70°C for 1 minute and 72°C for 2 minutes followed by 20 cycles of a 94°C for 30 seconds, 68°C for 1 minute and 72°C for 2 minutes. The final extension was carried out at 72°C for 7 minutes. Nested PCR was performed using primers described in Table 2.9. The primary PCR product was 50-fold-diluted (1 µl of the product + 49 µl of TE buffer). The amplification reaction was performed using 5 µl of the diluted PCR product as the template using the same condition for the first PCR except the NUP primer was used instead of the UPM primer. The amplification product was analyzed by agarose gel electrophoresis.

2.3.5.4 Cloning and sequencing of 5′ and 3′-RACE-PCR products

The RACE-PCR product of each reaction were eluted from the agarose gel by HiYield™ Gel/PCR DNA Extraction Kit (RBC; Real Biotech Corporation) and ligated to pGEM®-T Easy vector. The ligation product was transformed to *E. coli* JM109. Recombinant clone was identified by colony PCR. Plasmid DNA was extracted by a QIAprep® Spin Miniprep Kit and unidirectional sequenced. The

nucleotide sequence of RACE-PCR fragment was compared with those previously deposited in the GenBank using Blast*N* and Blast*X*, (http://www.ncbi/nlm/nih.gov). Significant probabilities of matched nucleotides and proteins were considered when the *E*-value was < 1e-04.

The transmembrane helices, protein domain, p*I* and molecular weight and hydrophobicity of deduced amino acids of transcripts were searched using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), SMART (http://smart.embl-heidelberg.de/), Protparam (http://www.expasy.org/tools/protparam.heml) and ProtScale (http://www.expasy.org/tools/protscale.html), respectively.

2.3.6 Examination of expression levels of interesting genes in hepatopancreas of H. asinina by quantitative real-time PCR

2.3.6.1 Experimental animals

Hapatopancreas of 40 individuals of cultured *H. asinina* (8-month-old); 20 samples exhibiting from the top (CL) and 20 samples from the bottom (CS); were collected. Total RNA was extracted from each specimen. The quality and quantity of extracted RNA was evaluated by spectrophotometry and agarose gel electrophoresis, respectively.

2.3.6.2 First strand cDNA synthesis

One and a half microgram of total RNA was reverse-transcribed to the first strand cDNA using an ImProm- II™ Reverse Transcription System Kit (Promega). First, total RNA was combined with 0.5 µg of oligo dT_{12-18} and an appropriate amount of DEPC-treated H_2O to make up of 5 µl final volume. The mixture was incubated at 70°C for 5 minutes and then immediately put on ice for at least 5 minutes. Afterwards, the mixture (15 µl) of 5X reaction buffer, $MgCl_2$ (25 mM), dNTPs Mix (1mM), Recombinant RNasin Ribonuclease Inhibitor and ImProm- II™ Reverse transcriptase were added to final concentrations of 1X, 2.5 mM, 0.8 mM and 20 units and 1 µl, respectively. The reaction mixture was incubated at 25°C for 5 minutes, 42°C for 90 minutes, followed by 70°C for 15 minutes to terminate the reverse transcriptase activity. Concentration and quality of the synthesized first strand cDNA were examined by spectrophotometry and 1.2 % agarose gel electrophoresis.

2.3.6.3 Primer design

Forward and reverse primers were designed from cDNA sequence of each interesting gene and *β-actin* (house-keeping gene) using Primer Premier 5.0 (Table 2.10).

2.3.6.4 Quantitative real-time PCR analysis

The target transcripts (*alpha-methylacyl CoA racemase*, *Carnitine O-palmitoyltransferase I*, *hydroxyacyl CoA dehydrogenase/ 3-ketoacyl CoA thiolase/ enoyl CoA hydratase* and *vacuolar H+ ATPase 14 kDA subunit*) and the internal control (*β-actin*) were amplified in 10 µl reaction volume using 2X LightCycler® 480 SYBR Green I Master (Roche, Germany).

For construction of the standard curve of each gene, the DNA segment covering the target gene and *β-actin* were amplified using cDNA as the template. The PCR products were ligated to pGEM®-T Easy vector. The ligation product was transformed to *E. coli* JM109. Plasmid DNA was extracted and unidirectional sequenced as previously described. The nucleotide sequence of fragment was compared with those previously deposited in the GenBank using Blast*N* and Blast*X*, (http://www.ncbi/nlm/nih.gov). Plasmid DNA was used as the template for estimation of the copy number. A 10 fold-serial dilution was prepared corresponding to 10^3-10^8 molecules/µl. The copy number of standard DNA molecules can be calculated using the following formula

$$X \text{ g/ µl DNA} / [\text{plasmid length in bp} \times 660] \times 6.022 \times 10^{23} = Y \text{ molecules/ µl}$$

The standard curves (correlation coefficient = 0.995-1.000 or efficiency higher than 95%) were drawn for each run. Each standard point was run in duplicate.

Quantitative real-time PCR of each specimen was carried out using the final concentration of 0.1 - 0.4 µM and 100 ng the first strand cDNA template. The thermal profile for SYBR Green real-time PCR was 95°C for 10 minutes followed by 40 cycles of a 95°C for 15 seconds, 58°C for 30 seconds and 72°C for 30 seconds. Cycles for the melting curve analysis was 95°C for 15 seconds, 65°C for 1 minute and

98°C for continuation and cooling was 40°C for 10 seconds. Each sample was run in duplicate.

Table 2.10 Nucleotide sequences, length and Tm of primers used for quantitative real-time PCR analysis of various genes in *H. asinina*

Gene/ Primer	Sequence (5'-3')	Length (bp)	Tm (°C)
Alpha-methylacyl CoA racemase			
Forward	CTC CAA GGT ATC CGA GTC AT	20	60
Reverse	GTT GTC AGC CAT CAG CGT CT	20	62
Carnitine O-palmitoyltransferase I			
Forward	AGT GGG ACA TCT CGG AAC A	19	58
Reverse	CTT CTC TGA ACA GTC GGG TC	20	62
Hydroxyacyl CoA dehydrogenase/ 3-ketoacyl CoA thiolase/ enoyl CoA hydratase (trifunctional protein)			
Forward	GAA TAA CAG CAG CGT TAC AGC G	22	66
Reverse	TGA TGG CAG CGA CAA TCG GTT T	22	66
Vacuolar H+ ATPase 14kDA subunit			
Forward	GAC ATT GCC ATC ATT CTC AT	20	56
Reverse	GCC TAC ATC ACA CTG AGC AC	20	62
β-actin			
Forward	TGA TGG TCG GTA TGG GTC AGA A	22	66
Reverse	GCA ACA CGG AGC TCG TTG TAG	21	66

2.3.6.5 Data analysis

A ratio of the absolute copy number of the target gene and that of *β-actin* was calculated. The relative expression level between the large and small *H. asinina* were statistically tested using one way analysis of variance (ANOVA) followed by a Duncan's new multiple range test. Significant comparisons were considered when the *P* value was < 0.05.

CHAPTER III

RESULTS

3.1 DNA extraction

Total genomic DNA extracted from the epipodial tentacles and foot muscle of each abalone showed acceptable quality for further used on population genetic analysis. The approximate quality of extracted genomic DNA was electrophoretically estimated using a 1.0% agarose gel. High molecular weight DNA at approximately 23.1 kb along with slightly sheared DNA was observed (Figure 3.1).

The ratio between the optical density at 260 and 280 nm ($OD_{260/280}$)was 1.6 - 2.2, indicated that extracted DNA from some individuals was contaminated with protein or phenol (samples having $OD_{260/280} < 1.8$) or with RNA (samples having OD260/280 > 2.0). DNA samples showed possible contamination with residual proteins was re-extracted once with phenol/chloroform followed by ethanol precipitation before used. However, RNA contamination did not affect the PCR amplification. Therefore, the extracted DNA with $OD_{260}/OD_{280} > 2.0$ was used without further elimination of RNA.

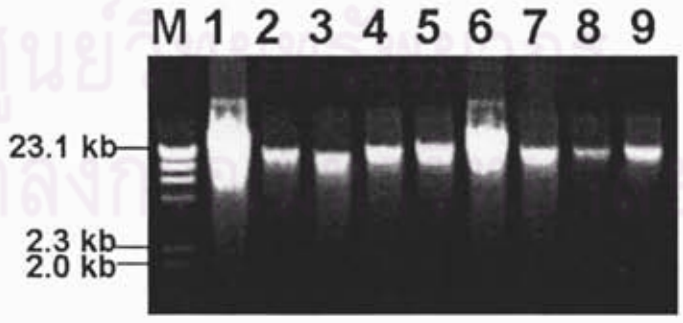


Figure 3.1 Genomic DNA extracted from the foot muscle of nine individuals of *H. asinina* (lanes 1 - 9). The extracted DNA was electrophoresed through 1.0 % agarose gel and stained with ethidium bromide. Lane M = λ-*Hin*d III DNA.

3.2 Development of type I microsatellites from microsatellite-containing ESTs in testis, ovary and hemocyte cDNA libraries of *H. asinina*

3.2.1 Identification of ESTs containing microsatellites

A total of 2,876 EST sequences composing of 649 EST sequences from testis, ovary, and hemocyte cDNA libraries of *H. asinina* (Amparyup, 2004) and additional of 2,227 ESTs from *H. asinina*, *H. discus*, and *H. diversicolor supertexta* deposited in the GenBank (http://ncbi.nlm.nih.gov) before February 6th, 2007 were *in silico* analyzed for the existence of simple repeats motifs using the Tandem Repeat Finder 2.2 (Benson, 1999).

Motifs that contained the number of repeats greater than 10 for mononucleotide, 6 for dinucleotide, 4 for trinucleotide, and 3 for tetranucleotide, pentanucleotide, and hexanucleotide repeats were regarded as microsatellite-containing ESTs. Results of microsatellite-containing motif were shown in Appendix A, B. Twelve EST-containing microsatellite sequences were selected and a primer pair that flank microsatellites regions of each EST was designed (Table 3.1).

Type I microsatellite primers: 9 loci from local cDNA libraries and 3 loci (*DW404*, *DW455*, and *DW503*) from the GenBank, together with six loci of type II microsatellites; *Haμ1M*, *Haμ2K*, *Haμ3C*, *Haμ3E*, *Haμ9*, and *Haμ10* (Selvamani *et al.*, 2000) were screened against few individuals of *H. asinina.* PCR products were initially analyzed by agaose gel electrophoresis.

All type I microsatellite were successfully amplified against genomic DNA of *H. asinina.* Non-specific amplification products were found at *PHe156*, *PO97* and *PO179* loci (Figure 3.2). The PCR products of loci *PHe221* (approximately 700 bp) and *DW404* (approximately 1700 bp) were much longer than that of the expected prodict (121 and 289 bp, respectively) suggesting that the amplified region contained the relatively large intron (s) (Figure 3.3 D and Figure 3.4 A). Accordingly, PCR products of 7 loci of type I microsatellites (*PHe4*, *PHe24*, *PHe177*, *PT101*, *PT102*, *DW455*, and *DW503*) were further analyzed with denaturing polyacylamide gel elexctrophoresis.

Table 3.1 Names of loci, characteristics of repeats, primer sequences, and annealing temperature (Ta) of type I microsatellite loci used in this study

Locus	Gene homologue	Repeat motif	Primer Sequence (5' – 3')	Expected size (bp)
PHe4	*B-cell translocation gene 2*	$(CAG)_4(CAA)_4$	F: ACAAGGAAAGCAGTCTCAGC R: TCTTACACGAATACCCAATC	141
PHe24	Unknown	$(CCGA)_8$	F: TATTCCGCTTCATAGATTGT R: AGAGCCTATTCTGCCACG	198
PHe156	Unknown	$(TG)_4(GTGTGC)_6$	F: ACCCCACGAAACAAGCAAT R: ATTGGCTTCCCCTTTTACAT	196
PHe177	Unknown	$(TA)_7$	F: AACACATTCACTGATTTAGG R: ATTATTATCTGACCGAGCA	182
PHe221	*Thrombospondin 1*	$(TAC)_5$	F: CTGTTGGCGGTTGTCCTT R: TATCTGGTGTAGCCGTATCC	121
PO97	Unknown	$(TAG)_{10}$	F: CAGGGGCTTGGTGGATGGAG R: CAAACAGTGAACAGAGGGGG	120
PO179	*Cysteine proteinase 1 precursor*	$(TGAG)_5$	F: CACGCATACGCTAGCAGTCA R: GGGAAGCAGGGGTCTTTGTT	127
PT101	*Ribosomal protein L41*	$(CAG)_7$	F: CTTGGTGTTGTGGCGTGTG R: TCTACTGACTATCTCATTCTCTGG	160
PT102	*Ribosomal protein L41*	$(ATG)_{10}$	F: TGCTTGTGAAGGACAGTATC R: ACCAACACGCACTGACAT	183
DW404	Unknown	$(GAT)_8$	F: TCCCTATCGGTGTGGTCG R: GCTCTCCTGAGGAATGCGT	289
DW455	Unknown	$(TG)_{13}(GC)_7$	F: CGGACAAAGATACTTGCTCA R: ATCAATGCTGGTCAAACGGA	221
DW503	Unknown	$(ATG)_{12}$	F: AGTCCCAGGTGTAAGAGCAT R: TGAACAGAGGGGTTGTCGTG	202

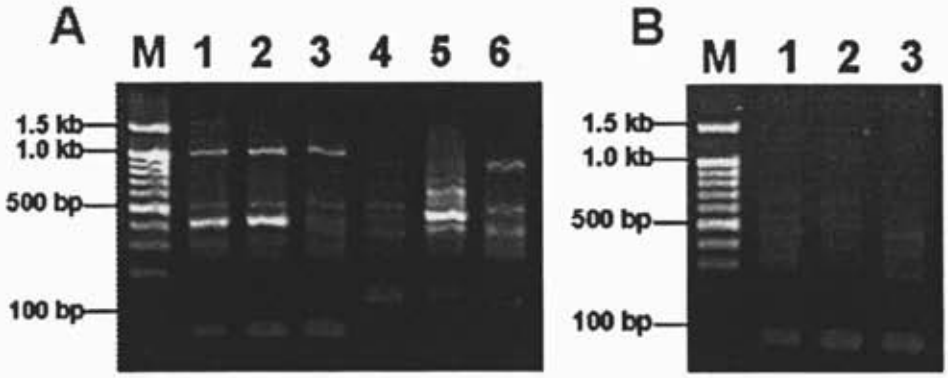


Figure 3.2 Agarose gel electrophoresis showing the PCR products of *PHe156* (lanes 1 - 3; A) and *PO97* (lanes 4 - 6; A), and *PO179* (B) against genomic DNA of *H. asinina* from SAME (lanes 1 and 4, A), PHI (lanes 2 and 5, A), and TRGW (lanes 3 and 6, A). Lanes M is a 100 bp DNA marker.

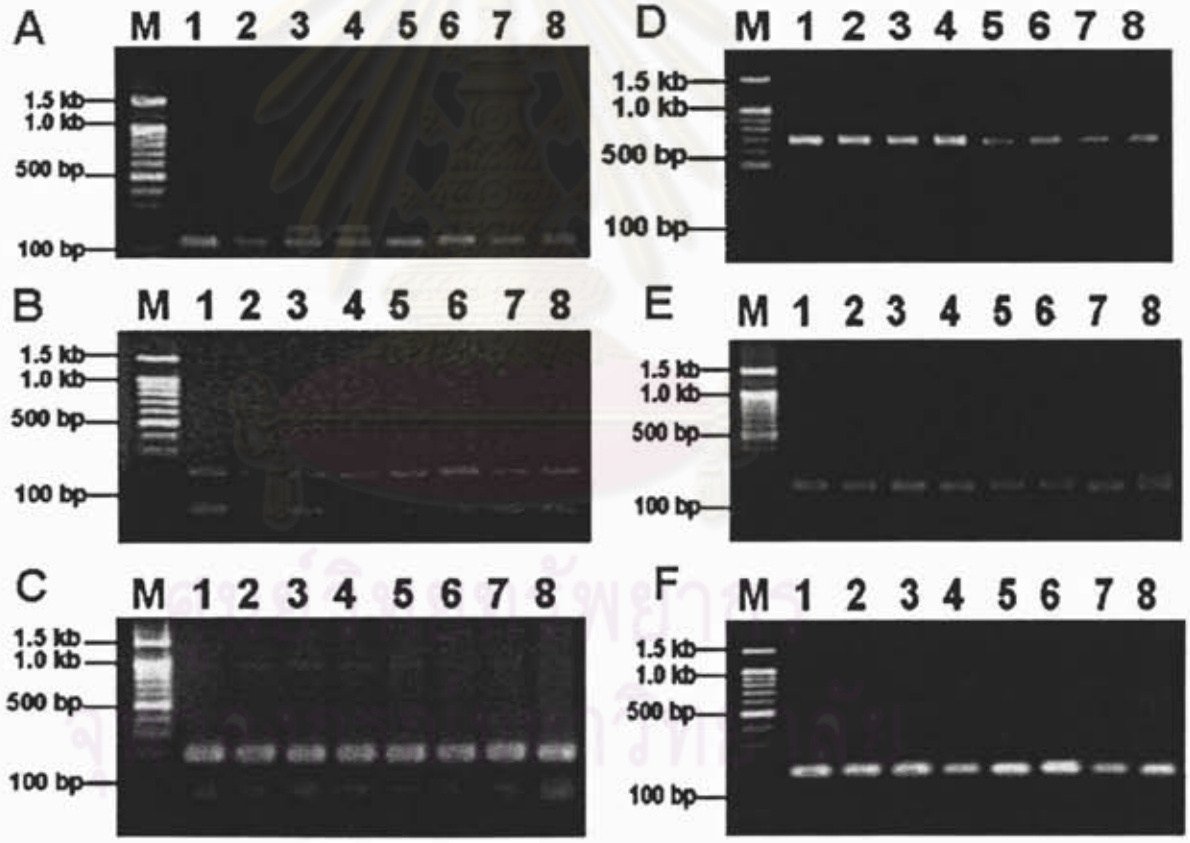


Figure 3.3 Agarose gel electrophoresis illustrating the amplification products of type I microsatellites at *PHe4* (A), *PHe24* (B), *PHe177* (C), *PHe221* (D), *PT101* (E), and *PT102* (F) loci against genomic DNA of *H. asinina* from CSMaRTH (lanes 1 - 2), SAME (lanes 3 - 4), TRGW (lanes 5 - 6), CAME (lanes 7 - 8). Lanes M are a 100 bp DNA marker.

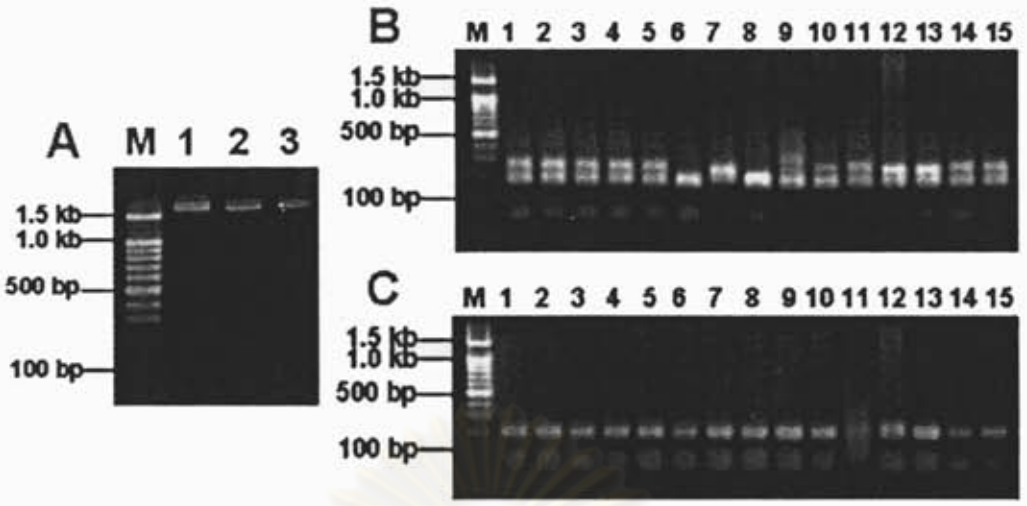


Figure 3.4 Agarose gel electrophoresis illustrating the amplification products of type I microsatellites; *DW404* (A) against genomic DNA of abalone from SAME, PHI, and TRGW (lanes 1 - 3, respectively) and *DW455* (B), and *DW503*(C) with specimens from CSMaRTH (lanes 1 - 2), SAME (lanes 3 - 5), TRGW (lanes 6 - 7), CAME (lanes 9 - 11), PHI (lanes 12 - 13), and CTRGH (lanes 14 - 15). Lanes M are a 100 bp DNA marker.

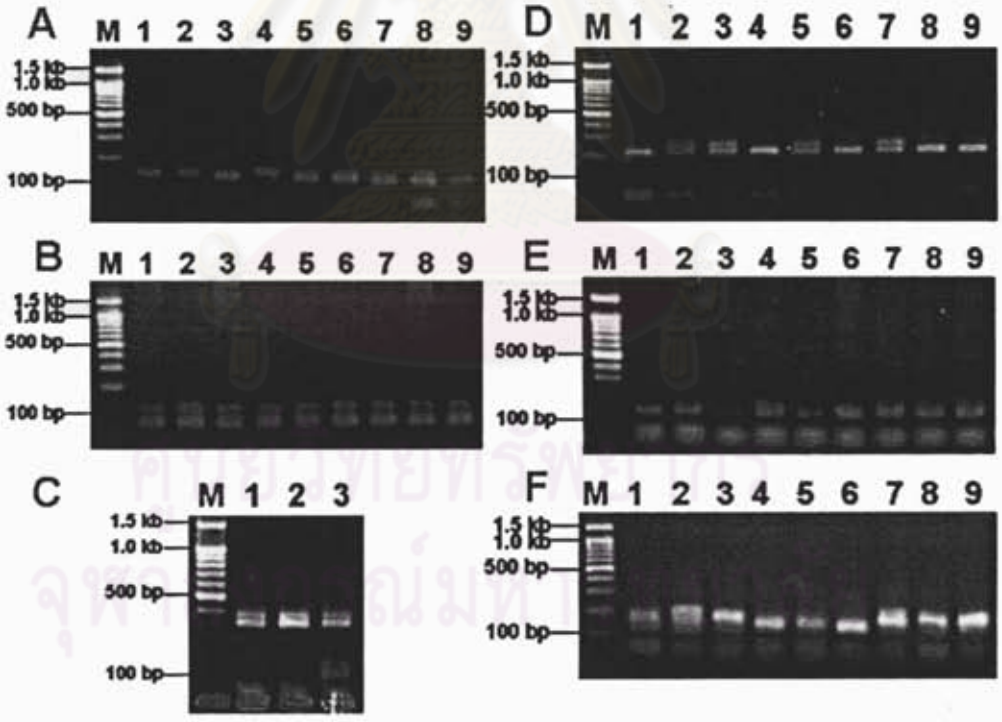


Figure 3.5 Agarose gel electrophoresis illustrating the amplification products of type II microsatellites; *Haμ1M* (A), *Haμ2K* (B), *Haμ3E* (D), *Haμ9* (E), and *Haμ10* (F) against genomic DNA of *H. asinina* from SAME (lanes 1 - 3), TRGW (lanes 4 - 6), and CAME (lanes 7 - 9) and *Haμ3C* using specimens from SAME, PHI and TRGW (lanes 1 - 3, C).

For type II microsatellite, *Haμ3C* generated the non-specific amplification products whereas 5 loci (*Haμ1M*, *Haμ2K*, *Haμ3E*, *Haμ9*, and *Haμ10*) were further characterizede by denaturing polyacrylamind gel electrophoresis (Figure 3.5).

Four type I (*DW455*, *DW503*, *PHe177*, and *PT102*) and 2 type II microsatellite loci (*Haμ9* and *Haμ10*) that gave polymorphic and easily scorable patterns were selected for determination of genetic diversity in wild and domesticated

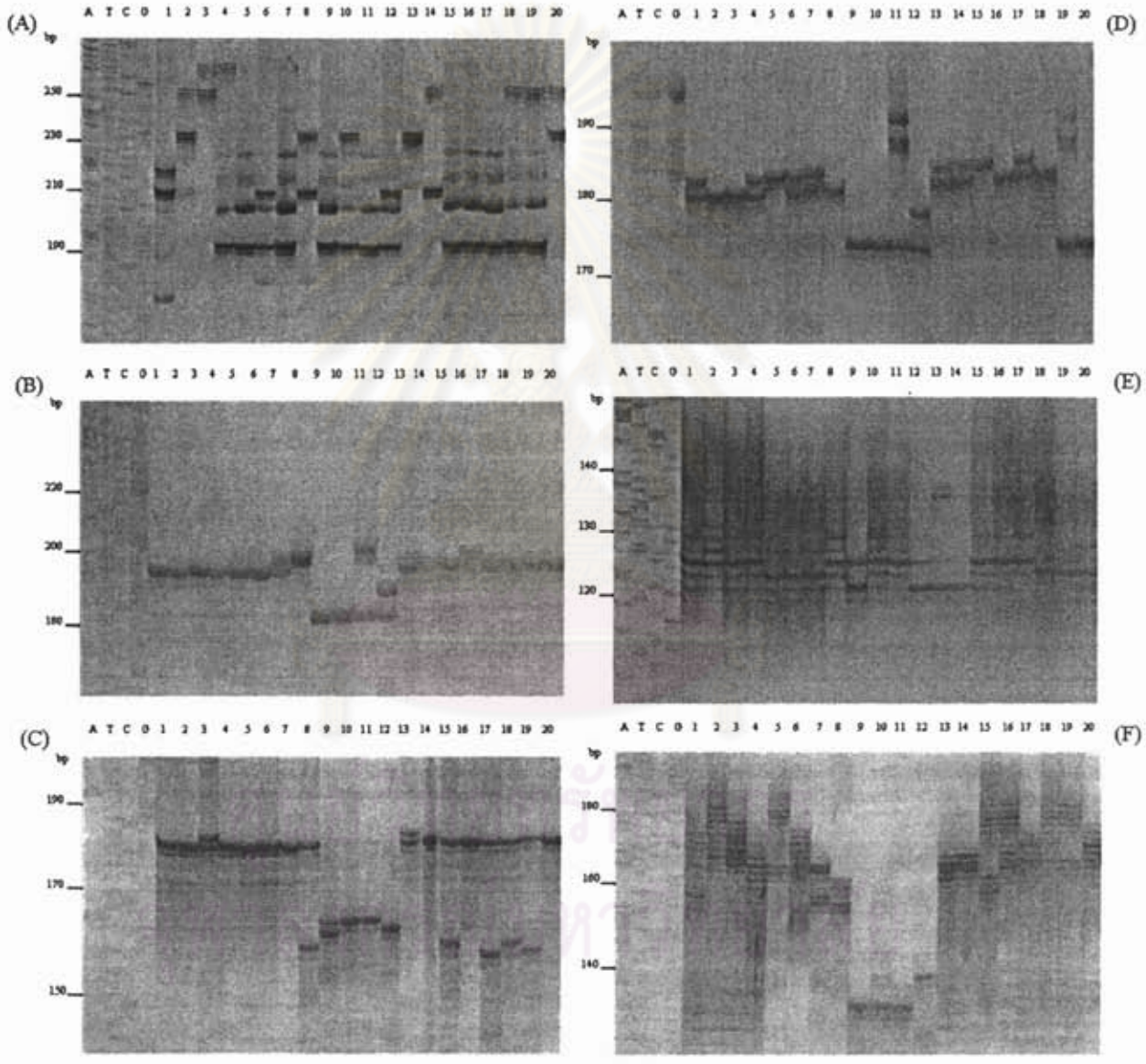


Figure 3.6 Examples of microsatellite patterns resulted from analysis at *DW455* (A) and *Haμ9* (E) using abalone samples from CTRGH (lanes 1 - 10) and CSMaRTH (lanes 11 - 20) and *DW503* (B), *PHe177* (C), *PT102* (D), and *Haμ10* (F) using genomic DNA of *H. asinina* from SAME (lanes 1 - 4), CAME (lanes 5 - 8), TRGW (lanes 9 - 12), CTRGH (lanes 13 - 16), and CSMaRTH (lanes 17 - 20).

H. asinina. Patterns of *DW455*, *DW503*, *PHe177*, and *PT102* were visualized by silver staining whereas those of *Haμ9* (HEX) and *Haμ10* (FITC) were fluorescently detected (Figure 3.6).

DW455, *DW503*, *PHe177*, *PT102*, *Haμ9*, and *Haμ10* generated 10, 7, 6, 6, 10, and 12 alleles, respectively. Sizes of allele distribution for respective loci were 191 - 272, 174 - 201, 157 - 183, 173 - 190, 107 - 137, and 134 - 181 bp, respectively (Table 3.2).

At the *DW455* locus, 7, 6 5, and 3 alleles were found in wild samples (SAME, CAME and TRGW) and the F1 generation from the Philippines. The domesticated stocks (CTRGH and CSMaRTH) had 7 and 8 alleles, respectively. A 191 bp allele was the most common allele in wild abalone from the Gulf of Thailand (0.4063 and 0.5500 in SAME and CAME, respectively) whereas a 267 bp alleles had the highest frequency (0.5200) in the Andaman sample (TRGW). A 233 bp allele (0.5000) had the greatest frequency from the Philippines. Only two alleles (233 and 252 bp) possessed low frequencies were found in both coastal populations of Thai abalone. For cultured samples, the most common allele for both CTRGH and CSMaRTH samples was the allele 191 bp). Like wild stock from the Gulf of Thailand, the most common alleles in the domesticated stocks was the 191 bp allele. A 267 bp alleles commonly found in wild abalone from the Andaman Sea was found in only 0.0104 and 0.0138 of CTRGH and CSMaRTH, respectively. Only TRGW and CSMaRTH did not conform to Hardy-Weinberg equilibrium ($P < 0.05$).

At the locus *DW503*, seven alleles were found in abalone from the Andaman Sea for which a 174 bp allele is the most common allele (0.5400). This allele was not found in the Andaman samples. A lower number of alleles per locus was observed in SAME, CAME, PHI and domesticated stocks. The most common allele in CTRGH and CSMaRTH was the same as that of the Gulf of Thailand samples (196 bp). A 174 bp allele commonly found in TRGW was found at extremely low frequencies (0.0208 and 0.0166) in domesticated stocks. All examined samples conform to Hardy-Weinberg expectations ($P > 0.05$).

Limited numbers of alleles of *PHe177* was observed in wild abalone (2, 2 and 3 in SAME, CAME and TRGW) and the Philippines (3 alleles) but a greater number

Table 3.2 Allele frequencies, number of alleles per locus (NA), number of specimens (*N*), observed and expected heterozygosity and Hardy-Weinberg expectations of each microsatellite locus in wild and hatchery stocks in this study.

Locus	Allele (bp)	Wild				Hatchery	
		SAME	CAME	TRGW	PHI	CTRGH	CSMaRTH
DW455	191	**0.4063**	**0.5500**	-	-	**0.4479**	**0.4696**
	194	0.0313	0.0750	-	-	-	0.0276
	203	0.0313	0.0500	-	0.2000	0.1458	0.0276
	209	0.0938	0.1500	-	0.3000	0.1979	0.1851
	217	0.3438	0.0750	-	-	0.0208	0.0718
	233	0.0625	0.1000	0.0400	**0.5000**	0.1354	0.0442
	252	0.0313	-	0.1200	-	0.0417	0.1602
	256	-	-	0.1800	-	-	-
	267	-	-	**0.5200**	-	0.0104	0.0138
	272	-	-	0.1400	-	-	-
NA		7	6	5	3	7	8
N		16	20	25	20	48	181
HW		0.9554	0.8058	0.0228*	1.0000	0.9999	0.0112*
DW503	174	-	-	**0.5400**	-	0.0208	0.0166
	177	-	-	0.0600	-	-	-
	180	0.0313	-	0.1000	0.2000	0.0938	0.1768
	183	-	-	0.1400	0.1750	-	-
	188	-	-	0.0200	-	-	-
	196	**0.9375**	**0.7000**	0.1200	-	**0.8021**	**0.7624**
	201	0.0313	0.3000	0.0200	**0.6250**	0.0833	0.0442
NA		3	2	7	3	4	4
N		16	20	25	20	48	181
HW		1.0000	0.5672	0.9878	0.7859	0.2726	0.3541
PHe177	157	-	-	0.1600	0.2000	0.0417	0.0856
	163	-	0.0750	-	**0.5250**	0.0833	0.1216
	166	-	-	0.2600	0.2750	0.0938	0.0083
	170	-	-	**0.5800**	-	-	0.0249
	180	**0.9688**	**0.9250**	-	-	**0.7188**	**0.6934**

Table 3.2 (cont.)

Locus	Allele (bp)	Wild				Hatchery	
		SAME	CAME	TRGW	PHI	CTRGH	CSMaRTH
	183	0.0313	-	-	-	0.0625	0.0663
NA		2	2	3	3	5	6
N		16	20	25	20	48	181
HW		Not possible	1.0000	0.1257	0.9486	0.4840	0.0276*
PT102	173	-	-	**0.4600**	-	0.0208	0.0249
	177	0.0313	-	0.1200	0.2000	0.1563	0.1879
	183	-	-	0.0800	0.1750	-	-
	186	**0.9375**	**0.7250**	0.1000	-	**0.7500**	**0.7376**
	188	0.0313	0.2750	0.2200	-	0.0729	0.0497
	190	-	-	0.0200	**0.6250**	-	-
NA		3	2	6	3	4	4
N		16	20	25	20	48	181
HW		1.0000	0.4409	0.0286*	0.7839	0.2596	0.9920
Haμ9	107	-	-	-	**0.2000**	-	-
	110	-	-	-	**0.2000**	-	-
	122	-	0.3250	0.6000	0.3000	0.0313	0.0884
	123	0.2188	0.2500	-	-	0.4896	0.2679
	126	0.1563	0.3250	0.0800	0.3000	0.4167	0.2707
	127	0.3750	0.0500	-	-	0.0417	0.2072
	129	0.2500	0.0500	-	-	0.0208	0.1602
	132	-	-	0.2600	-	-	0.0028
	137	-	-	0.0600	-	-	0.0028
NA		4	5	4	4	5	7
N		16	20	25	20	48	181
HW		1.0000	0.0338*	0.9025	1.0000	0.1284	0.0000*
Haμ10	134	-	-	**0.2200**	-	-	-
	140	-	-	**0.2800**	-	-	-
	141	-	-	**0.2600**	-	-	-
	143	-	-	**0.2400**	-	-	0.0083
	154	0.0625	0.0500	-	-	-	0.0359

Table 3.2 (cont.)

Locus	Allele (bp)	Wild				Hatchery	
		SAME	CAME	TRGW	PHI	CTRGH	CSMaRTH
	158	0.1250	0.1500	-	0.3500	0.0417	0.0469
	161	0.0313	0.1000	-	0.6500	-	0.1298
	163	0.2813	0.0250	-	-	0.2708	0.3204
	167	0.1563	0.2750	-	-	0.0208	0.2209
	173	0.2188	0.1000	-	-	0.3750	0.0663
	176	0.0313	0.2750	-	-	0.1146	0.0773
	181	0.0938	0.0250	-	-	0.1771	0.0939
NA		8	8	4	2	6	9
N		16	20	25	20	48	181
HW		0.1865	0.4466	0.0009*	0.4258	0.0018*	0.0000*

*Significant at $P < 0.05$; HW, Hardy Weinberg equilibrium; **heterozygote excess test and significant at $P < 0.05$, a = heteroztgote deficiency test.

of alleles per locus was observed in CTRGH and CSMaRTH (5 and 6 alleles, respectively). The most common alleles in SAME, CAME and domesticated stocks (CTRGH and CSMaRTH) was a 180 bp alleles found at 0.9688, 0.9250, 0.7188 and 0.6934, respectively. The TRGW and PHI samples possessed the non-overlapped common allele of 170 and 163 bp in length. Only CSMaRTH deviated from Hardy-Weinberg expectations ($P < 0.05$).

A greater number of alleles per locus in TRGW (6 alleles) than the Gulf of Thailand samples (2 - 3 alleles) were observed. The most common allele in the Gulf of Thailand (SAME and CAME, 186 bp with the frequencies of 0.9375 and 0.7250), the Andman Sea (TRGW, 173 bp with the frequency of 0.4600) and the Philippines (PHI, 190 bp with the frequency of 0.6250) was different. A 186 bp allele was also common in domesticated stocks. Only TRGW deviated from Hardy-Weinberg expectations ($P < 0.05$).

Comparable polymorphic levels were observed when the same sample set was screened with type II microsatellites. At the locus *Haμ9*, approximately equal frequencies of 107 (0.2000), 110 (0.2000), 122 (0.3000) and 126 (0.3000) bp alleles

were observed. Likewise comparable distribution frequencies between 4 alleles in SAME were also noticed. Five and seven alleles were found in CTRGH and CSMaRTH whereas only 4 - 5 alleles were observed in other groups of samples. TRGW and CSMaRTH deviated from Hardy-Weinberg expectations ($P < 0.05$).

Haμ10 showed non-overlapping alleles between wild abalone originating from the east (CAME and SAME) and the west (TRGW) coasts. Only 3 individuals (0.83%) of the domesticated CSMaRTH stock exhibited the Andaman allele (143 bp). Unlike other loci, *Haμ10* generated more numbers of alleles in the Gulf of Thailand than the Andaman Sea samples. The domesticated stocks possessed comparable numbers of alleles with wild samples from the east coast of Thailand.

The expected and observed heterogeneity in wild populations ranged from 0.0625 - 0.8448 and 0.0625 - 1.0000 whereas those values in CTRGH and CSMaRTH were 0.3441 - 0.7769 and 0.3333 - 0.8750, respectively. The highest expected heterogeneity values among wild populations were observed at the locus *Haμ10* (0.8448) and the lowest value was found at the locus *Phe177* (0.0625) in SAME. The greatest observed heterozygosity was observed in this population at *DW455* (1.0000) and *Haμ9* (1.0000) whereas the lowest observed heterozygosity was found at the locus *PHe177* (0.0625 at locus, Table 3.3).

Relatively high genetic diversity was found in the domesticated stocks. The highest and lowest expected heterozygosity was observed at *Haμ10* in CSMaRTH (0.7769) and at *DW503* in CTRGH (0.3441). The lowest and highest observed heterozygosity in domesticated stocks were found at *DW455* and *DW503* loci in CTRGH (0.3333 and 0.8750). No rare allele was observed in domesticated samples suggesting the loss of low frequency alleles through domestication of *H. asinina*.

To eliminate the effects of different sample sizes, a ratio between the number of genotypes/genotype and the number of investigated specimens in a particular population (genotype/allele discrimination capacity), which is more reliable than the number of genotypes alone was introduced (Klinbunga *et al.*, 2005).

The allele discrimination capacity which was deduced from the number of identified allele/number of investigated individuals, showed the greater values in natural population than hatchery populations. The greatest allele discrimination

Table 3.3 The expected and observed heterogeneity of different groups of *H. asinina* at six microsatellite loci.

Samples		Locus					
		DW455	***DW503***	***PHe177***	***PT102***	***Haμ9***	***Haμ10***
Wild							
SAME	H_e	0.7238	0.1229	**0.0625**	0.1229	0.7480	**0.8448**
	H_o	0.8750	0.1250	**0.0625**	0.1250	**1.0000**	0.6875
TRGW	H_e	0.6751	0.6735	0.5820	0.7233	0.5739	0.7633
	H_o	0.4400	0.8000	0.3600	0.5200	0.6800	0.4800
CAME	H_e	0.6679	0.4308	0.1423	0.4089	0.7397	0.8231
	H_o	0.7000	0.4000	0.1500	0.3500	0.4500	0.8500
PHI	H_e	0.6359	0.5526	0.6244	0.5526	0.7590	0.4667
	H_o	**1.0000**	0.5500	0.7500	0.5500	**1.0000**	0.4000
Hatchery							
CTRGH	H_e	0.7259	**0.3441**	0.4669	0.4116	0.5897	0.7471
	H_o	**0.8750**	**0.3333**	0.4583	0.3958	0.4583	0.6250
CSMaRTH	H_e	0.6901	0.4015	0.5746	0.4159	0.7546	**0.7769**
	H_o	0.6913	0.4404	0.4629	0.4757	0.5637	0.5256

H_e = expected heterogeneity; H_o = observed heterogeneity.

Table 3.4 Allele discrimination capacity of different groups of *H. asinina* populations at six microsatellite loci.

Samples	Locus					
	DW455	***DW503***	***PHe177***	***PT102***	***Haμ9***	***Haμ10***
Wild						
SAME	0.4375	0.1875	0.1250	0.1875	0.2500	0.5000
TRGW	0.2000	0.2800	0.1200	0.2400	0.1600	0.1600
CAME	0.3000	0.1000	0.1000	0.1000	0.2500	0.4000
PHI	0.1500	0.1500	0.1500	0.1500	0.2000	0.1000
Average	0.2719	0.1794	**0.1238**	0.1694	0.2150	**0.2900**
Hatchery						
CTRGH	0.1458	0.0830	0.1042	0.0830	0.1042	0.1250
CSMaRTH	0.0442	0.0221	0.0331	0.0221	0.0331	0.0497
Average	**0.0950**	**0.0526**	0.0687	**0.0526**	0.0687	0.0874

capacity among each locus was 0.5000 for *Haμ10* whereas the lowest value was 0.0221 for *DW503* and *PT102*. This suggested more discrimination power of the former than the latter and the remaining loci (*DW455*, *PHe177,* and *Haμ9*) in this study (Table 3.4).

Higher levels of the genotype discrimination capacity (no. of identified genotypes/number of examined specimens) in wild than domesticated stocks were also observed and showed the higher level in wild population than cultured samples. In wild samples, the greatest genotype discrimination capacity was observed at *Haμ10* (0.6500) whereas the lowest value was observed at *PHe177* (0.1463) (Table 3.5). In contrast, *PHe177* and *PHe177* revealed the greatest (0.1649) and lowest (0.0681) genotype discrimination capacity for domesticated stocks, respectively.

Comparable numbers of alleles and heterozygosity but lower levels of allele and genotype discrimination capacity suggested low degrees of genetic diversity reduction in *H. asinina* after domesticated for 5-6 generations.

Table 3.5 Genotype discrimination capacity of different groups of *H. asinina* populations at six microsatellite loci

Samples	Locus					
	DW455	***DW503***	***PHe177***	***PT102***	***Haμ9***	***Haμ10***
Wild						
SAME	0.3750	0.1875	0.1250	0.1875	0.2500	0.6250
TRGW	0.2800	0.3600	0.1600	0.4000	0.2400	0.3200
CAME	0.4500	0.1500	0.1000	0.1500	0.3500	0.6500
PHI	0.1000	0.2000	0.2000	0.2000	0.1000	0.1500
Average	0.3013	0.2244	**0.1463**	0.2344	0.2350	**0.4363**
Hatchery						
CTRGH	0.2083	0.1250	0.1667	0.1250	0.1250	0.1458
CSMaRTH	0.1215	0.0112	0.0773	0.0387	0.0773	0.1657
Average	**0.1649**	**0.0681**	0.1220	0.0819	0.1012	0.1558

F_{ST}-statistics and the exact test between pairs of samples at each locus clearly revealed significant differences between wild abalone from the east (SAME, and CAME) and west (TRGW) coasts of Thai waters and the Philippines (Table 3.6). Both domesticated stocks (CTRGH and CSMaRTH) were genetically different from TRGW (west) and PHI at all loci.

Genetic heterogeneity between the Gulf of Thailand samples was observed at *DW503*, *PT102*, *Haμ9*, and *Haμ10* loci ($P < 0.05$). CTRGH was genetically different from SAME at 4 loci (*DW455, PHe177*, *Haμ9*, and *Haμ10*) and from CAME for 4 loci (*DW455*, *DW503*, *PT102*, and *Haμ10*). Interestingly, the domesticated CTRGH and CSMaRTH bred from the same founder group but cultured at different hatcheries also exhibited genetic heterogeneity at 4 loci (*DW455, PHe177*, *Haμ9* and *Haμ10*).

Table 3.6 Genetic heterogeneity analysis based on the exact test and F_{ST}-statistics between pairs of *H. asinina* samples based on six microsatellite loci.

Samples	*DW455*		*DW503*		*PHe177*		*PT102*		*Haμ9*		*Haμ10*	
	F_{ST}	Exact test	F_{ST}	Exact test	F_{ST}	Exact test	F_{ST}	Exact test	F_{ST}	Exact test	F_{ST}	Exact test
TRGW-SAME	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*
CAME-SAME	**0.1261**^{ns}	**0.0919**^{ns}	0.0059*	0.0039*	**0.1559**^{ns}	**0.1593**^{ns}	0.0132*	0.0096*	0.0000*	0.0000*	0.0105*	0.0022*
CAME-TRGW	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*
PHI-SAME	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*
PHI-TRGW	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*
PHI-CAME	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*
CTRGH-SAME	0.0000*	0.0000*	0.3107^{ns}	0.4358^{ns}	0.0086*	0.0525*	0.1057^{ns}	0.1662^{ns}	0.0000*	0.0000*	0.0060*	0.0011*
CTRGH-TRGW	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*
CTRGH-CAME	0.0166*	0.0394*	0.0016*	0.0016*	0.0027*	**0.0454**^{ns}	0.0004*	0.0006*	0.0005^{ns}	0.0000*	0.0000*	0.0000*
CTRGH-PHI	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*
CSMaRTH-SAME	0.0035*	0.0015*	0.0433*	**0.1226**^{ns}	0.1112^{ns}	0.0371*	0.0133*	0.0681^{ns}	**0.0820**^{ns}	0.0924*	**0.1742**^{ns}	0.0420*
CSMaRTH-TRGW	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*
CSMaRTH-CAME	0.0223*	0.0151*	0.0000*	0.0000*	0.0048*	**0.0681**^{ns}	0.0000*	0.0000*	**0.0037**^{ns}	**0.0005**^{ns}	0.0000*	0.0000*
CSMaRTH-PHI	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*
CSMaRTH-CTRGH	0.0000*	0.0000*	**0.1627**^{ns}	**0.1050**^{ns}	0.0016*	0.0006*	**0.4937**^{ns}	**0.4468**^{ns}	0.0000*	0.0000*	0.0000*	0.0000*

*Significant at $P < 0.05$; ns, not significant.

3.3 Development of population-specific SCAR markers in *H. asinina* using AFLP technique

3.3.1 Screening of candidate population-specific AFLP

Genomic DNA of *H. asinina* from the *Po* of Samet founders (SAME, $N = 2$) and Trang (TRGW, $N = 2$) were used for identification of candidate population-specific markers. Total DNA were digested with *Eco* RI and *Mse* I. After ligated with double stranded *Eco* RI and *Mse* I adaptor, preamplification was carried out using a single pre-selective nucleotide of *Eco* RI primer (E_{+A}) and *Mse* I primer (M_{+C}). The smear preamplification products indicated successful digestion/ligation of *H. asinina* genomic DNA with *Eco* RI and *Mse* I adaptors (Figure 3.7).

A total of 64 primer combinations were screened against representative individuals of *H. asinina*. All screened selective primers combinations were successfully amplified. The selective amplification products were electrophoresd through 1.8% agarose gel (Figure 3.8) for the primary screening. The products were further size fractionated through 4.5 % denaturing polyacrylamind gel electrophoresis (crosslink = 19:1) (Figure 3.9).

Results from denaturing polyacrylamind gel electrophoresis of selective amplification products show bands ranging from 100 - 600 bp in size. A total of 1,128 bands were found (666 monomorphic bands and 462 polymorphic bands). Of these, 166 and 174 bands were only observed in representative individuals of TRGW or SAME, respectively (Appendix D).

3.3.2 Cloning, characterization and development of SCAR markers

A total of nine candidate AFLP bands (four candidate population-specific AFLP fragments from TRGW and SAME and one polymorphic AFLP fragment) were converted to sequence-characterized amplified region (SCAR) markers for more reliable amplification by PCR. AFLP fragments were excised from the polyacrylamind gel, reamplified, cloned and unidirectional sequenced.

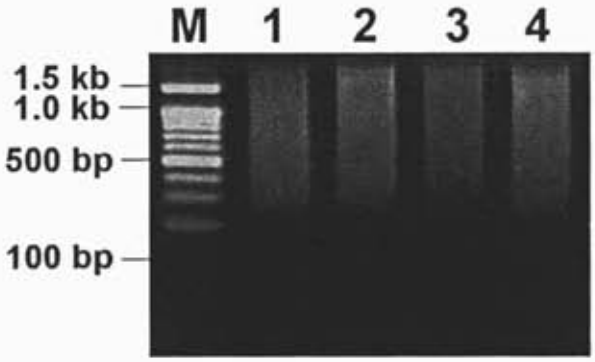


Figure 3.7 Agarose gel electrophoresis showing preamplification products of SAME (lanes 1 - 2) and TRGW (lanes 3 - 4) using preamplified *Eco* RI (E_{+A}) and *Mse* I primers (M_{+C}). Lane M is a 100 bp DNA marker.

Figure 3.8 A 1.8% agarose gel electrophoresis showing the selective amplification products of SAME (lanes 1, 2, 5, 6, 9, 10, 13, 14) and TRAG (lanes 3, 4, 7, 8, 11, 12, 15, 16) using primer E_{AAG}/M_{CAC} (lanes 1 - 4), E_{ACA}/M_{CAG} (lanes 5 - 8), E_{ACC}/M_{CTA} (lanes 9 - 12), E_{AGG}/M_{CTG} (lanes 13 - 16). Lane M is a λ/*Hin*d III DNA marker.

Results from homology search and the characteristic of each SCAR marker is illustrated by Table 3.1. Nucleotide sequences of all fragments did not match to any previously deposited sequence in the GenBank (*E*-value > 10^{-4}) and were regarded as novel (unknown) DNA of *H. asinina* (Figure 3.10).

Six SCAR primers were designed from E_{AGA}/M_{CGC}-345, E_{AGA}/M_{CCA}-380, E_{ATC}/M_{CAG}-356, E_{ATC}/M_{CTT}-351, E_{ATC}/M_{CGT}-336 and E_{ATC}/M_{CCC}-344 (hereafter called *HaSCAR*$_{328}$, *HaSCAR*$_{167}$, *HaSCAR*$_{339}$, *HaSCAR*$_{320}$, *HaSCAR*$_{295}$, *HaSCAR*$_{327}$,

respectively) and tested against genomic DNA of *H. asinina*. Sequences of E_{ATG}/M_{CCG}, E_{ATC}/M_{CGC}, and E_{ATC}/M_{CGG} were 164, 102, and 127 bp, therefore, they were not converted to SCAR markers (Figure 3.11).

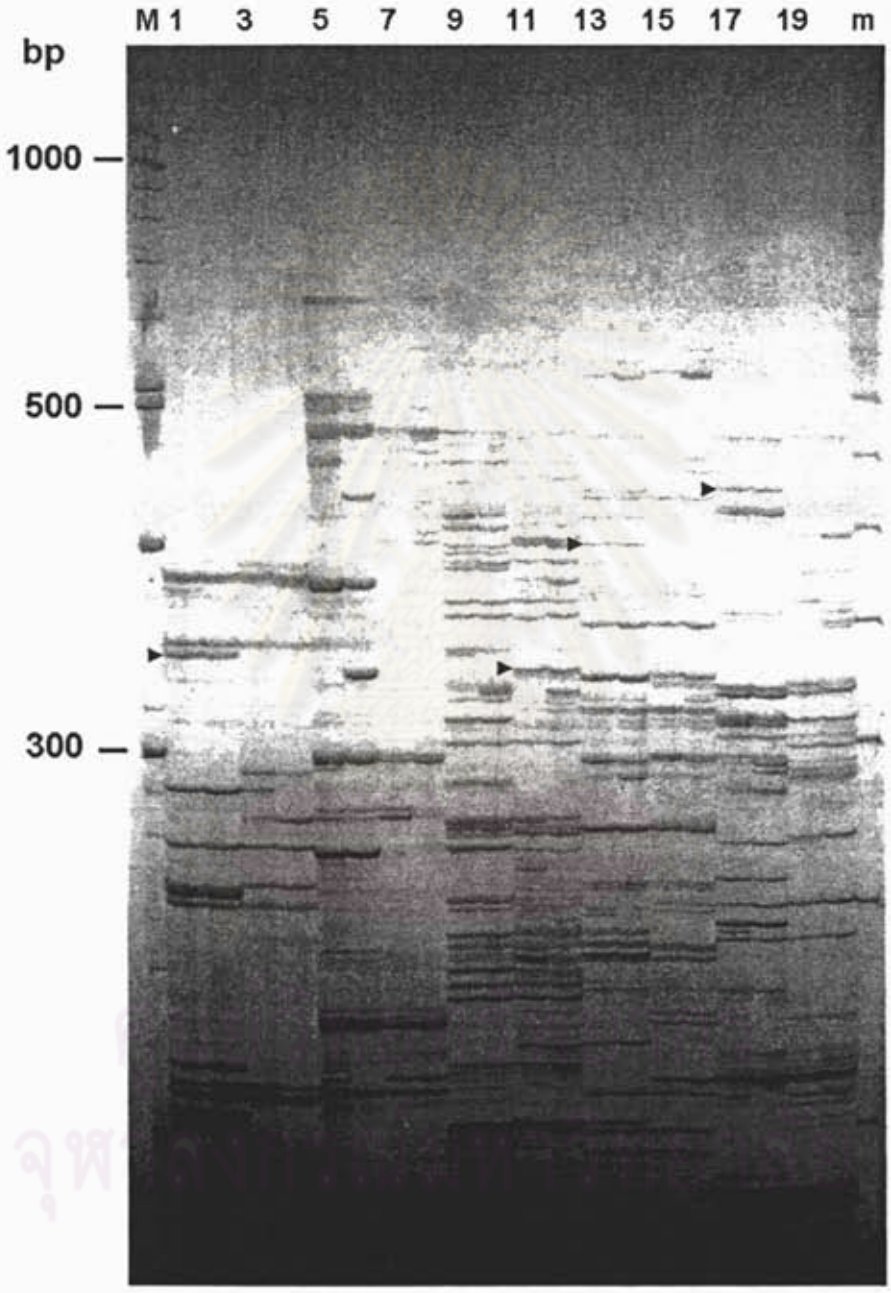


Figure 3.9 A 4.5% denaturing polyacrylamide gel electrophoresis showing AFLP patterns from five selective primer combinations; E_{AAG}/M_{CGT}, E_{AAG}/M_{CGC}, E_{AGA}/M_{CTA}, E_{AGA}/M_{CAG}, and E_{AGA}/M_{CTT} corresponding to lanes 1 - 4, 5 - 8, 9 - 12, 13 - 16, and 17 - 20, respectively. Arrowheads indicate candidate population-specific AFLP markers. Lanes M and m are 100 bp and 50 bp DNA markers, respectively.

Table 3.7 Name of SCAR marker, blast analysis, primer sequence, the annealing temperature and the expected product size of AFLP-derived markers of *H. asinina*

AFLP primers	Type of AFLP Band	Size of fragment (bp)	Blast analysis	Name of SCAR marker	Primer Sequence (5′ – 3′)	Ta (°C)	Expected product (bp)
E_{AGA}/M_{CGC}	Polymorphic	345	Unknown	$HaSCAR_{328}$	F: TACGAATTCAGACTACACGCAA R: GATTAACGCTCTCAATGAGAGA	57	328
E_{AGA}/M_{CCA}	TRGW specific	380	Unknown	$HaSCAR_{167}$	F: GCTCTCACATTGTAGCCTTTG R: ATTGAAATGGAAAGAAAAGTG	50	167
E_{ATC}/M_{CAG}	SAME specific	356	Unknown	$HaSCAR_{339}$	F: TACGAATTCATCAAAGGGAC R: GATTAACAGACTGCTGCCCA	57	339
E_{ATC}/M_{CTT}	**TRAG specific**	**351**	**Unknown**	**$HaSCAR_{320}$**	**F: TACGAATTCATCCTCGATAGTC R: GATTAACTTGGCAACAAGTGA**	**50**	**320**
E_{ATC}/M_{CGT}	**TRAG specific**	**336**	**Unknown**	**$HaSCAR_{295}$**	**F: TACGAATTCATCGCTCTAATCAT R: AACAGACCGGAACTCTCATG**	**55**	**295**
E_{ATC}/M_{CCC}	**SAME specific**	**344**	**Unknown**	**$HaSCAR_{327}$**	**F: TACGAATTCATCCAACAAAGG R: ATTAACCCTAACCTATCGTAAA**	**50**	**327**
E_{ATG}/M_{CCG}	SAME specific	164	Unknown	ND	ND	ND	ND
E_{ATC}/M_{CGC}	SAME specific	102	Unknown	ND	ND	ND	ND
E_{ATC}/M_{CGG}	TRAG specific	127	Unknown	ND	ND	ND	ND

*ND = not determined. SCAR markers further used for SSCP analysis are illustrated in boldface.

A

GACTGCG**TACCAATTCAGACTACACGCAA**TTATAAATGAATCGGCTAATGAAAGACTGA
AAGTCTGTGTTGGCATCATTATGTTTTCAGCGACTGGAATAAGACACCATAGATTGATAAA
AAGGTCCCGTGATATAACTGAATGTGAGATGGATCATAGAATAGATTCAGTTCATAGGCT
GAATCATGATGATACTGTTGGGCGGAAAGGAAACTATGGTCATAAACTTTGATTGAATGA
CGGCAATAATGATGATCTGACGATACATTTAGATCGCCTTGTGTCACAGCAAATCCTGGCC
AGCCTAAGAAAA**TCTCTCATTGAGAGCGTTACTC**AGGACTCATC

B

TACCAATTCAGAGTGTTCAAGATGTACTATGTCGTGTCTGGTTACTCAGGACTCATCATGA
CTGCGTACGAATTCAGAATGTTACATTATTACCTAAAAGGTTCATGGCCATGGTGGTCATT
GATAAAGACTCTTTGAACTCGTCGTTTGAGTAT**GCTCTCACATTGTAGCCTTTG**TGTAGG
CACATGACTCTGCACACTACTTTTGATTATAAAGCACATCTTACTTGAAAGAACTAACGTT
GCTAAAGGATTCAAATTATTGTGACGGCCCTGTGCCAGACTGTAAAATGTCAACATAT**CA**
CTTTTCTTTCCATTTCAATTGAACTCAAATGTTCACGTCAGAATCAGCTGGAGTACATTG
ACATTGGTTAATCAGGA

C

GACTGCG**TACCAATTCATCAAAGGGAC**AATACTCTGAAGTGAGTTGCAGCTTCAAACAG
GGCATAAGAACTGTTACAAGTAGTCGAAGAATGTCTGGTGCTCCCCTATCTTGCATTTGTG
TCATGTACAGTGATATGGGGCCAGTATAAAACTGAAGAAAAATACCTGTACATCGGAACA
TATACCTATATATACTGTCATAGACATAATATTACTGGCAATAACTGAGGTTTGTACATTG
TGAACATTCATAACATGTCCAGGTCAACACAATTCATTCAGAGTGAGTGAGTGAGTCTGC
ACTCAGCAATATTCCAGCTATATTA**TGGGCAGCAGTCTGTTACTC**AGGACTCATC

D

GACTGCG**TACCAATTCATCCTCGATAGTC**TCAATGGTATCTATTACGATTACACTCAACT
ATACCCTGGATGCATCTATGGCTCAACCTGAGAATGTTATTACCTCCCTTTGCTGGCTGTG
CTTTCTTACTATAGGCAATTAGCTAAGCTGTTACAACCGGTCTTGCCAATGTCAACAAAGA
TGGTGGTTGCAAGTGCAGAGGTTTGTTCACAGTGCAACCCAGATTTTGGGGAAATCATAC
AGACAAACTGACAGGTCCAAAACTTGAAAGATATATATGTTCGAGAGCTCCTACCTCCTC
TTTCAGAGTACCTCTGCA**TCACTTGTTGCCAAGTTACTC**AGGACTCATC

E

GACTGCG**TACCAATTCATCGCTCTAATCAT**ATTTTCATCGTCGTGATGTCATCTCATTGA
AATCTAATTGTATTTGACAGAATGGACTTGACACAACATTATGTTCTTTACACAAATGTCT
TACGGCTGCTGTTGTTTGACAGGCAGATTTCCAGCGCTACTTCCTCCACATGAATGTTCCA
GTCTGTTCTTGTTTGACAGGCAGATTTCCACCGCTACTTTCTTCACATAAATGCATGTTTGC
TGTTGGTTGACAAGGAGATTTCGTCGTTACTTCCTTCA**CATGAGAGTTCCGGTCTGTT**CT
TGTTTGATTTCCAACGTTACTCAGGACTCATC

F

GACTGCG**TACCAATTCATCCAACAAAGG**TCATGACACGTTATCATCCATCCTGTCAGCAT
ATCATTA**G**ATCCTGGTGAGACGGCGCTTCAAGAACCTTCGTTACCCCAATATACGTTCTGA
CAAACTGTTTGTAACAGTTCTCAGTAACATTTACCCGTAAAAACAGCCCAATACTGGGTAG
TAACTGCTCCGACATTTCGCTTTATTGTCCCACTATTGCCTTCGATCCGCTTCAATTCCGAG
AGCACGCAATGAACAGATGTCAAGGGGAGGCTGTTATTGGGTGGAGGCCCTAGGTAAACT
ATGGTAT**TTTACGATAGGTTAGGGTTACTC**AGGACTCATC

Figure 3.10 Nucleotide sequence of *HaSCAR*$_{328}$ (A), *HaSCAR*$_{167}$ (B), *HaSCAR*$_{339}$ (C), *HaSCAR*$_{320}$ (D), *HaSCAR*$_{295}$ (E) and *HaSCAR*$_{327}$ (F) derived from E_{AGA}/M_{CGC}-345, E_{AGA}/M_{CCA}-380, E_{ATC}/M_{CAG}-356, E_{ATC}/M_{CTT}-351, E_{ATC}/M_{CGT}-336 and E_{ATC}/M_{CCC}-344 AFLP markers, respectively. Positions of the forward and those complementary to the reverse primer of each SCAR marker are boldfaced and underlined.

A

GATGAGTCCTGAGTAACCGTGTTCACTCATTCAAACCATCAACCGCGTAATCGTGAGTGG
GCTTCGTTCCTATCTTTCAACAGAAGTTGGTGGACATATGAGATTGTGACTTTGCTGGTTA
CAGATATATTGCATTTATAATTTTCATGAATTGGTACGCAGTC

B

GACTGCGTACCAATTCATCTACCGTTATCTATTGACAAACGTGATTGGATGGCACCGTCTG
GAATGCATGGTGCGTTACTCAGGACTCATCAACCTAAATAG

C

GACTGCGTACCAATTCATCACGCGGTTGTTATCAACTTACAGCACAGGACGATGTAGCCGT
TACTCAGGACTCATCATCACATTAATTGCGTTGCGCTCACTGCCCGCTTCGTGCCAGCTGC
ATTAA

Figure 3.11 Nucleotide sequence of E_{ATG}/M_{CCG}-164 (A), E_{ATC}/M_{CGC}-102 (B) and E_{ATC}/M_{CGG}-127 (C) generated from AFLP analysis.

3.3.3 Amplification of SCAR markers, agarose gel electrophoresis and SSCP analysis

All primer pairs generated the positive amplification product (Figures 3.12-3.17). Size polymorphism of *HaSCAR*$_{167}$, *HaSCAR*$_{339}$, *HaSCAR*$_{320}$, *HaSCAR*$_{295}$, *HaSCAR*$_{327}$ was not observed. Accordingly, sequence polymorphism of each SCAR marker was further determined by single strand conformational polymorphism (SSCP). In contrast, length polymorphism of *HaSCAR*$_{328}$ were found and it was genotyped by denature polyacrylamind gel electrophoresis.

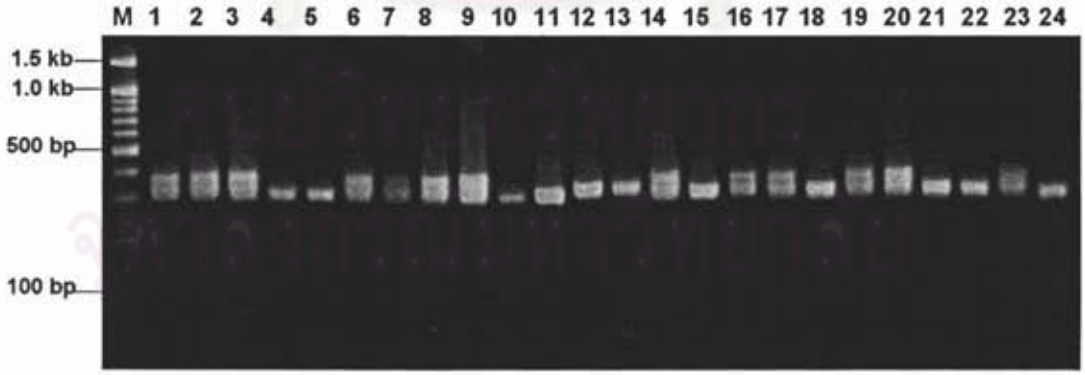


Figure 3.12 Agarose gel electrophoresis showing the amplification product of *HaSCAR*$_{328}$ against genomic DNA of *H. asinina* from SAME (lanes 1 - 5), CAME (lanes 6 - 10), TRGW (lanes 11 - 15), CTRGH (lanes 16 - 20), and CSMaRTH (lanes 21 - 24). Lanes M is a 100 bp DNA marker.

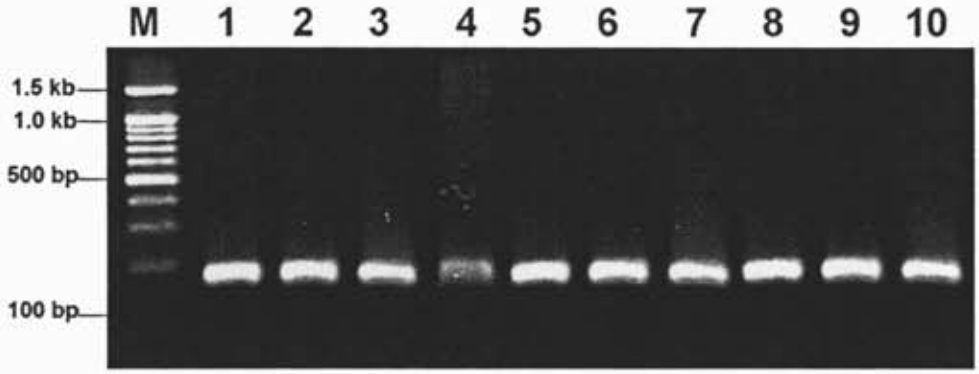


Figure 3.13 Agarose gel electrophoresis showing the amplification product of *HaSCAR*$_{167}$ against genomic DNA of *H. asinina* from SAME (lanes 1 - 2), CAME (lanes 3 - 4), TRGW (lanes 5 - 6), CTRGH (lanes 7 - 8), and CSMaRTH (lanes 9 - 10). Lane M is a 100 bp DNA marker.

Figure 3.14 Agarose gel electrophoresis showing the amplification product of *HaSCAR*$_{339}$ against genomic DNA of *H. asinina* from SAME (lanes 1 - 3), CAME (lanes 4 - 5), TRGW (lanes 6 - 7). Lane M is a 100 bp DNA marker.

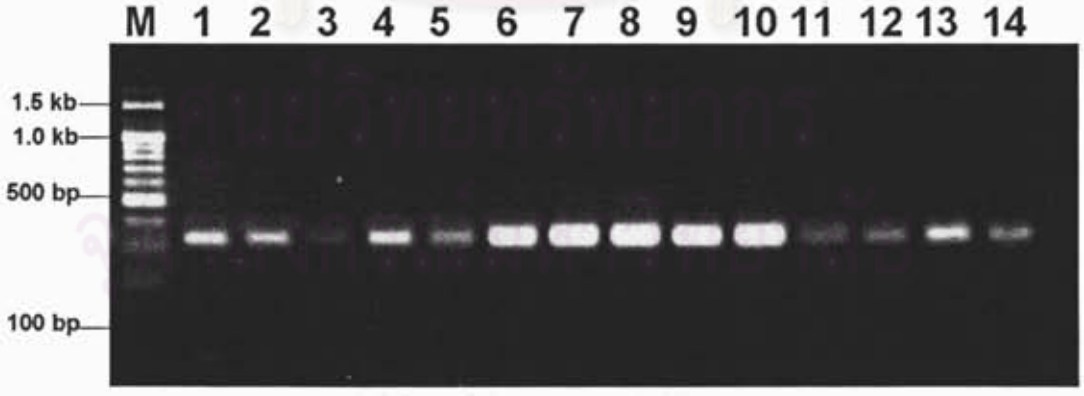


Figure 3.15 Agarose gel electrophoresis showing the amplification product of *HaSCAR*$_{320}$ against genomic DNA of *H. asinina* from SAME (lanes 1 - 2), CAME (lanes 3 - 5), TRGW (lanes 6 - 10), CTRGH (lanes 11 - 12), and CSMaRTH (lanes 13 - 14). Lane M is a 100 bp DNA marker.

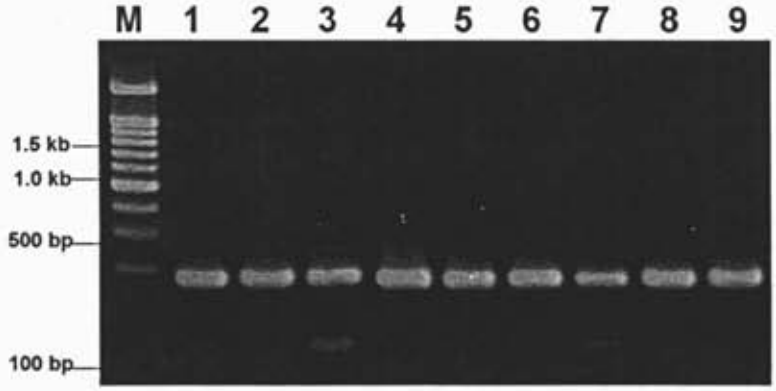


Figure 3.16 Agarose gel electrophoresis showing the amplification product of *HaSCAR*$_{295}$ against genomic DNA of *H. asinina* from SAME (lanes 1 - 3), CAME (lanes 4 - 6), TRGW (lanes 7 - 9). Lane M is a 100 bp DNA marker.

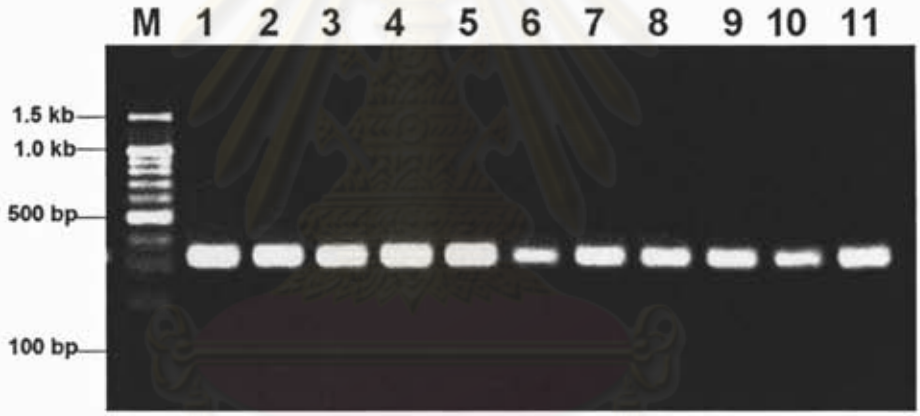


Figure 3.17 Agarose gel electrophoresis showing the amplification product of *HaSCAR*$_{327}$ against genomic DNA of *H. asinina* from SAME (lanes 1 - 3), CAME (lanes 4 - 5), TRGW (lanes 6 - 11). Lane M is a 100 bp DNA marker.

The PCR products of *HaSCAR*$_{167}$, *HaSCAR*$_{339}$, *HaSCAR*$_{320}$, *HaSCAR*$_{295}$, *HaSCAR*$_{327}$ were denatured and electrophoresed through low crosslink non-denaturing polyacrylamind gels (37.5:1).

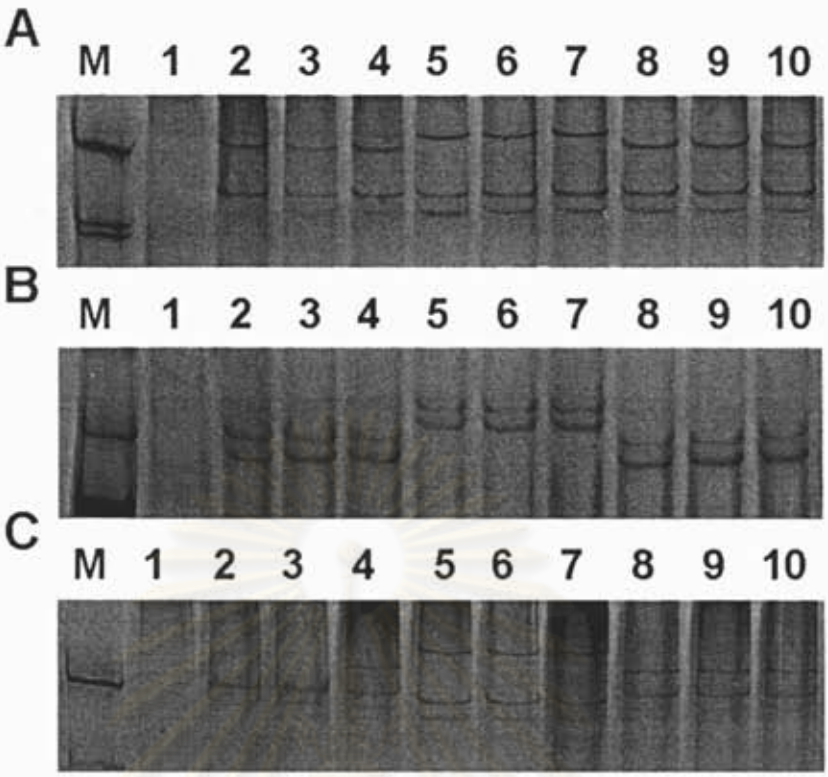


Figure 3.18 SSCP patterns resulted from size fractionation of three SCAR markers; *HaSCAR*$_{320}$ (A), *HaSCAR*$_{295}$ (B) and *HaSCAR*$_{327}$ (C), of *H. asinina* from the domestication stocks (genotypes A for *HaSCAR*$_{320}$ and *HaSCAR*$_{295}$, lanes 2 - 4 and genotypes A, lane 4 and C, lanes 2 - 3 for *HaSCAR*$_{327}$), Talibong Island (lanes 5 - 7, genotypes B, B and B), and Cambodia (lanes 8 - 10, genotypes A, A and C) through 15% non-denaturing polyacrylamide gel electrophoresis of. Lanes M and 1 are 100 bp DNA ladder and the non-denatured PCR product (double standard control), respectively.

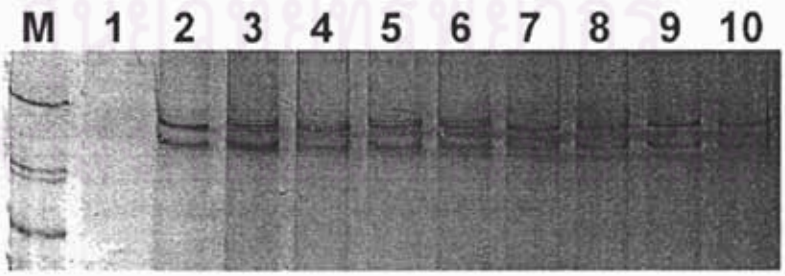


Figure 3.19 SSCP patterns resulted size fractionation of *HaSCAR*$_{339}$ marker of *H. asinina* from SAME (lane 2 - 4), TRGW (lane 5 - 7) and CAME (lane 9 - 10) though 15% non-denaturing polyacrylamide gel electrophoresis. Lanes M and 1 are 100 bp DNA ladder and the non-denatured PCR product (double standard control), respectively.

Three, two and three SSCP genotypes were found from *HaSCAR*$_{320}$, *HaSCAR*$_{295}$, *HaSCAR*$_{327}$, respectively (Figure 3.18). The amplification products of *HaSCAR*$_{167}$ was not successfully analyzed with SSCP whereas the product of *HaSCAR*$_{339}$ shows monomorphic patterns in samples from different geographic locations of *H. asinina* (Figure 3.19). Allelic polymorphism of *HaSCAR*$_{328}$ of wild and hatchery *H. asinina* was observed and showed in Figure 3.20.

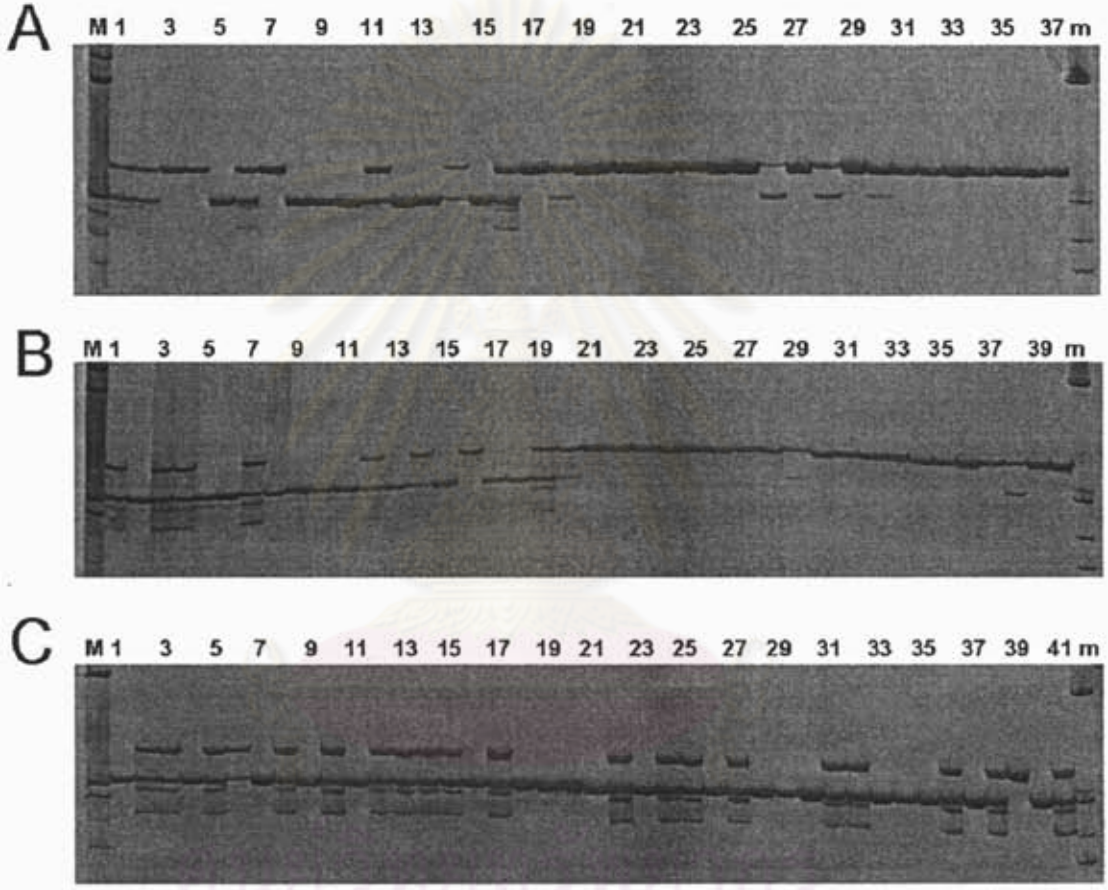


Figure 3.20 A 5% denaturing polyacrylamide gel electrophoresis of *HaSCAR*$_{328}$ from SAME and TRGW (A; lanes 1 - 16 and 17 - 37, respectively), CAME and PHI (B; lanes 1 - 19, and 20 - 40, respectively) and hatchery CTRGH and CSMaRTH (C; lane 1 - 20 and 21 - 41, respectively) of *H. asinina*. Lanes M and m are denatured 100 bp and 50 pb DNA markers, respectively.

SSCP genotypes of *H. asinina* from the Gulf of Thailand and Andaman Sea did not overlap at the $HaSCAR_{327}$ locus whereas a single individual from the west coast (TRGW) exhibited genotype A at $HaSCAR_{320}$ and $HaSCAR_{295}$ loci which was carried by all examined individuals of CAME and SAME samples (Table 3.8). Almost all of the hatchery-propagated samples possessed the Gulf of Thailand genotypes except only 3 specimens from the CSMaRTH hatchery at $HaSCAR_{320}$ exhibited genotype B which was typically found in the Andaman Sea population. In addition, 3 and 4 individuals of CTRGH showed SSCP genotype B at $HaSCAR_{295}$ and $HaSCAR_{327}$ loci typically found in the Andaman Sea population, respectively.

Table 3.8 Distribution frequencies of SSCP genotypes of $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$ in wild and domesticated *H. asinina*

Sample	$HaSCAR_{320}$			$HaSCAR_{295}$		$HaSCAR_{327}$		
	A	B	C	A	B	A	B	C
SAME	18	-	2	20	-	18	-	2
CAME	14	-	8	22	-	22	-	-
TRGW	1	**24**	-	1	**24**	-	**25**	-
CTRGH	32	-	8	37	3	25	4	11
CSMaRTH	84	3	8	95	-	79	-	16
Total	149	27	26	175	27	144	29	29

Six composite SSCP genotypes were found in wild abalone. Genotypes AAA, AAC and CAA were restrictively observed in the Gulf of Thailand whereas ABB, BAB and BBB were only distributed in the TRGW sample (Table 3.9).

Eight composite genotypes were found in the domesticated samples and only three of which were observed in CAME and SAME. Composite genotypes BAA and BAC were found only in CSMaRTH while AAB and ABC were restrictively observed in CTRGH. The disappearance of these composite genotypes reflected greater genetic diversity of the domesticated than wild stocks of *H. asinina* in this study. PCR products of each individual representing to each SSCP genotype of three SCAR markers were amplified and directly sequencing. Multiple alignment of SSCP

genotype of $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$ were illustrated in Figure 3.21. Nucleotide divergence between these composite genotypes was 0.10% (between ABB-BBB) - 1.46% (between ABB-CAA).

Table 3.9 Distribution frequencies of composite SSCP genotypes of $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$ markers in wild and domesticated stocks of *H. asinina*

Composite haplotype	Wild			Domestication	
	SAME	CAME	TRGW	CTRGH	CSMaRTH
AAA	16 (0.80)	14 (0.64)	-	18 (0.45)	70 (0.74)
AAB	-	-	-	4 (0.10)	-
AAC	2 (0.10)	-	-	7 (0.18)	14 (0.15)
ABB	-	-	1 (0.04)	-	-
ABC	-	-	-	3 (0.07)	-
BAA	-	-	-	-	2 (0.02)
BAB	-	-	1 (0.04)	-	-
BAC	-	-	-	-	1 (0.01)
BBB	-	-	23 (0.92)	-	-
CAA	2 (0.10)	8 (0.36)	-	7 (0.18)	7 (0.07)
CAC	-	-	-	1 (0.02)	1 (0.01)
Total (*N*)	20	22	25	40	95

An unrooted maximum parsimony network inferred from polymorphic sites of examined sequences revealed 2 different lineages of composite genotypes perfectly corresponding to specimens from the Gulf of Thailand (lineage I) and Andaman Sea (lineage II), respectively (Figure 3.22). Nucleotide differences between within lineage genotypes (2 - 4 mutation steps) were lower than those between lineage genotypes (8 mutation steps) where an interconnection between the common genotype AAA (lineage I) and BBB (lineage II) required 14 mutation steps. Interestingly, composite genotypes ABB, BAB and BBB found in wild abalone from the Andaman Sea and regarded as members of the lineage II were not found in the domesticated stocks (CTRGH and CSMaRTH). All individuals of CSMaRTH (95/95; 100%) carried lineage I composite genotypes. Only 4 individuals of CTRGH (4/40, 10%) possessed

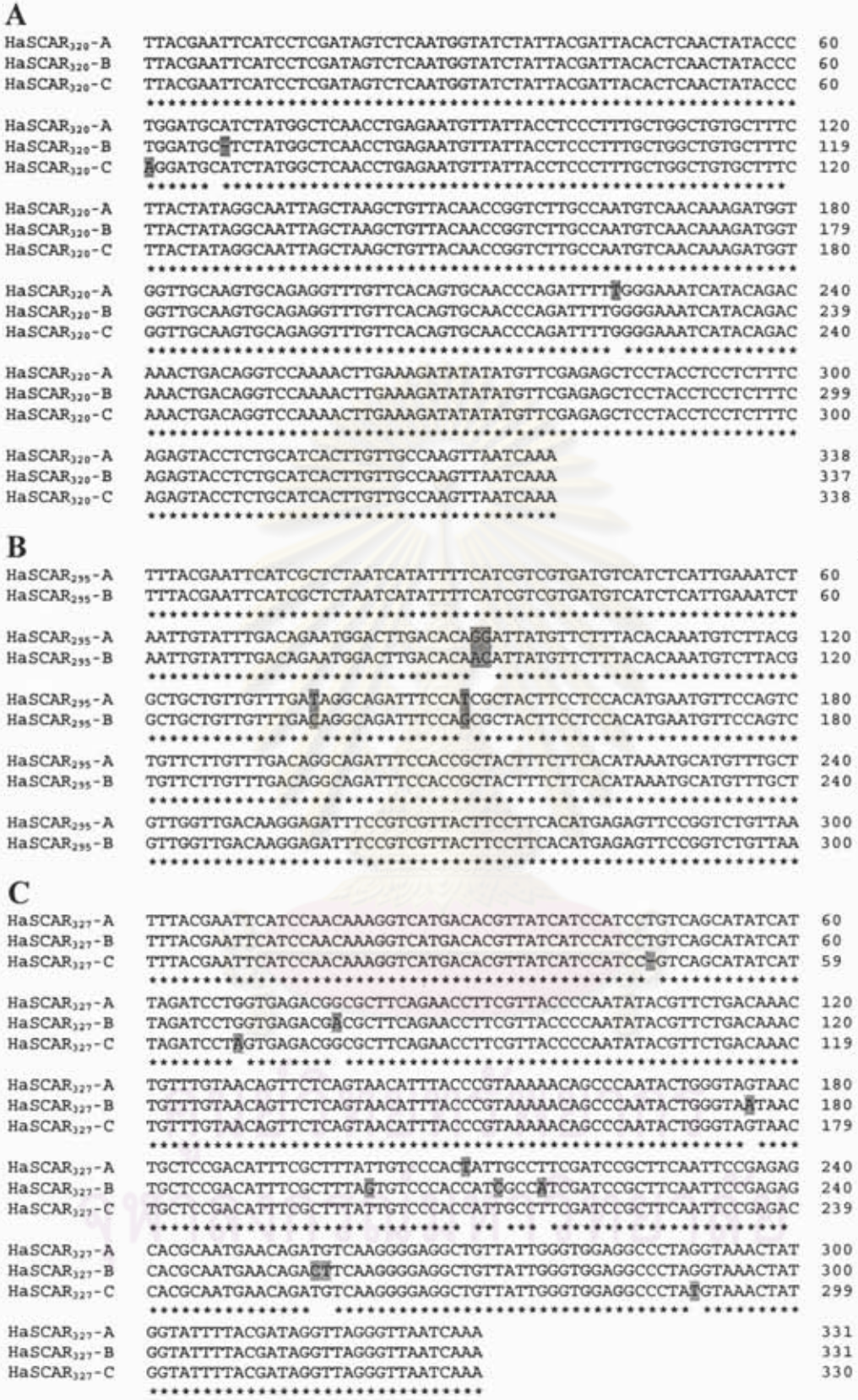


Figure 3.21 Nucleotide sequence of an individual representing each genotype of *HaSCAR*$_{320}$ (A), *HaSCAR*$_{295}$ (B) and *HaSCAR*$_{327}$ (C).

the AAB genotype which was parsimoniously recognized as a member of the lineage II genotypes. This indicated that the gene pools of domesticated stocks have been genetically contributed by almost all of the brooders from the Gulf of Thailand.

A UPGMA dendogram constructed from genetic distance between pairs of composite genotypes (Figure 3.23) allocated all composite haplotypes to 2 clusters; I and II. Interestingly, wild abalone from the Gulf of Thailand were phylogenetically recognized as members of cluster I whereas those of the Andaman Sea were allocated to cluster II, respectively. All genotypes identified in cultured stock (CTRGH and CSMaRTH) are located in cluster I except AAB which was allocated in a member of cluster II.

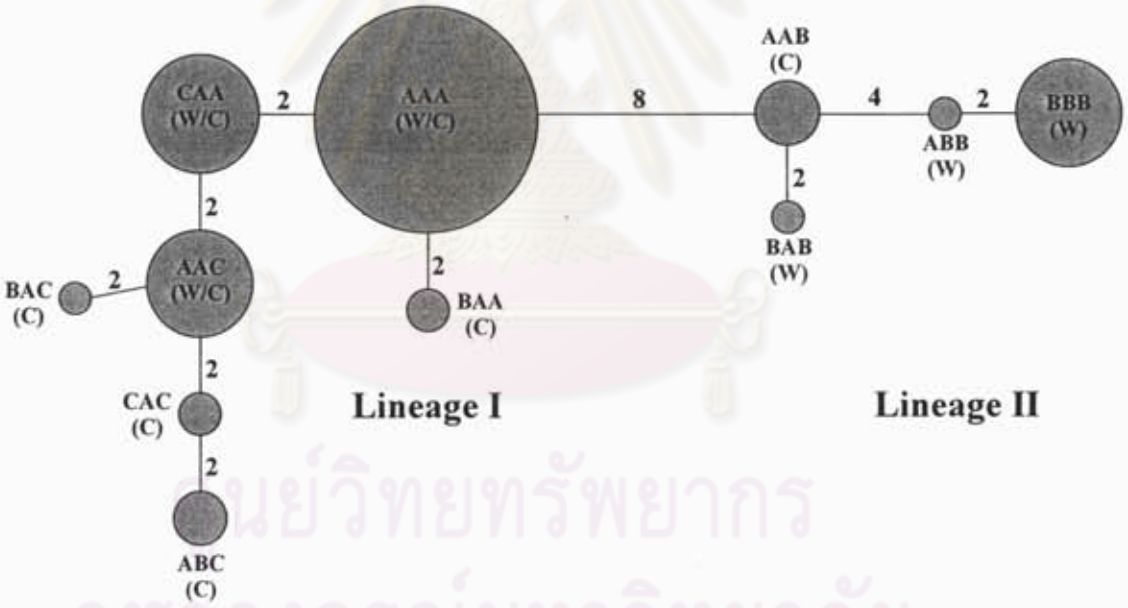


Figure 3.22 An unrooted maximum parsimony network illustrating genetic relationships between composite SSCP genotypes of $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$. Numbers between pairs of composite SSCP genotypes indicate the number of mutation steps required for their connections.

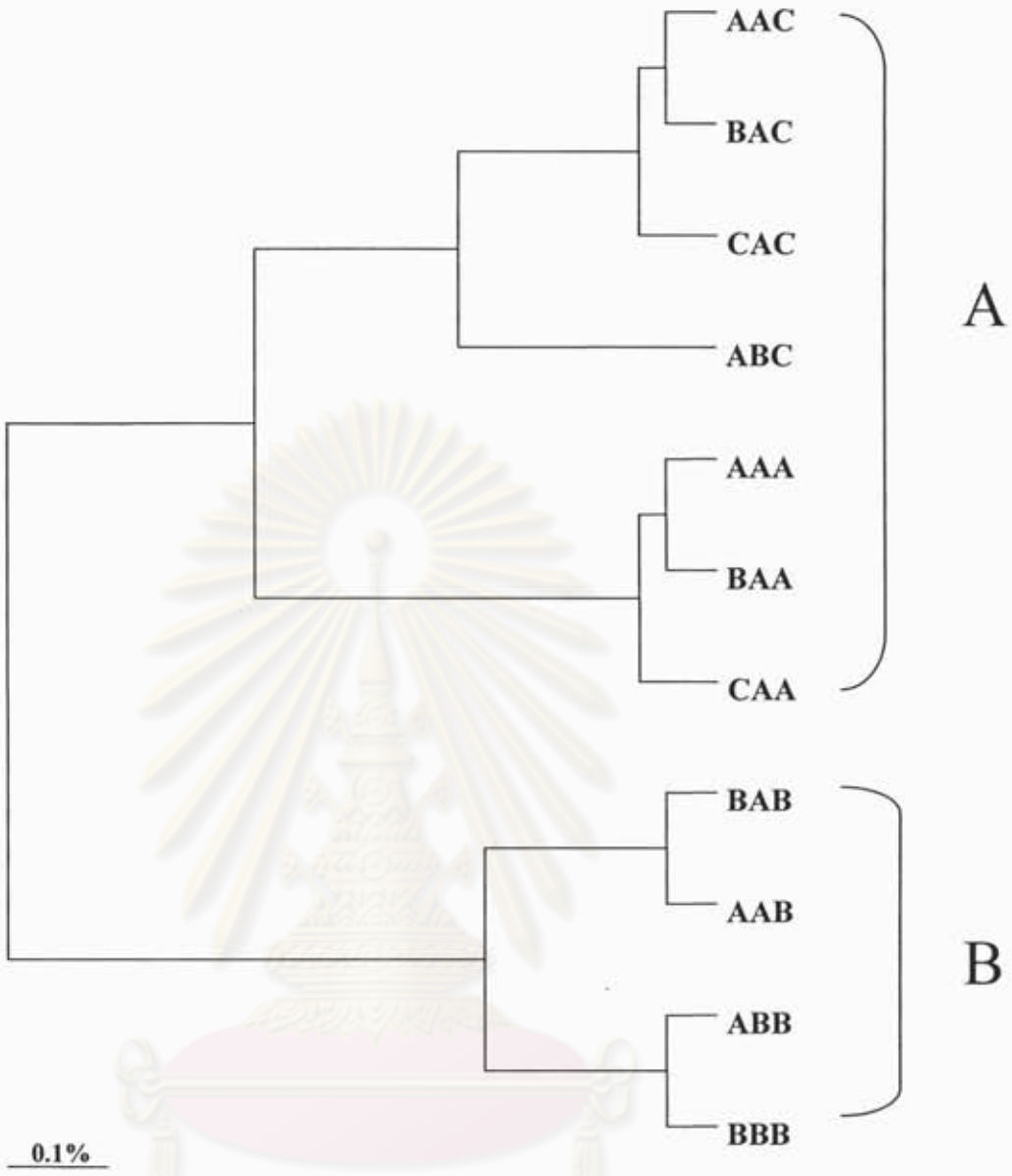


Figure 3.23 A UPGMA dendrogram constructed from genetic distance (Nei, 1978) between pairs of composite genotypes of $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$ in *H. asinina* in wild and cultured populations.

3.3.4 Genetic diversity and heterogeneity of cultured and wild population of H. asinina

Limited genetic diversity was observed in wild samples. The average polymorphic bands and gene diversity of all primers in TRGW (23.08% and 0.0847) was slightly lower than that of SAME (30.77% and 0.0836) but greater than that of CAME (15.38% and 0.0617). Genetic distance between pairs of wild samples were 0.0154 (CAME and SAME), 1.0473 (TRGW and SAME) and 1.0926 (TRGW and CAME), respectively (Table 3.10).

Modest genetic diversity was observed in the domesticated stocks. The average polymorphic bands and gene diversity of all primers in CSMaRTH (69.23% and 0.2322) was comparable to but those of CTRGH (23.08% and 0.0934) were greater than those of wild stocks. Limited genetic distance were observed between these and SAME and CAME (-0.0005 - 0.0565) but large genetic distance were observed when compared with the Andaman Sea sample (TRGW; 0.6951 - 1.0187).

Genetic heterogeneity was observed between abalone from different coastal regions ($P < 0.0001$ for the exact test and $P < 0.001$ for F_{ST} statistics) but not within the Gulf of Thailand ($P > 0.05$) (Table 3.11). Genetic heterogeneity based on the exact test indicated that CSMaRTH and CTRGH were genetically different ($P < 0.001$). The gene pool of CSMaRTH was not different from wild abalone from the Gulf of Thailand (CAME and SAME; ($P > 0.05$). However, genetic heterogeneity was observed between CTRGH and CAME ($P < 0.017$) and between the domesticated samples and TRGW ($P < 0.001$).

Table 3.10 Gene diversity within samples (diagonal) and genetic distances between (below diagonal) pairs of *H. asinina* samples

	SAME	CAME	TRGW	CSMaRTH	CTRGH
SAME	**0.0836**				
CAME	0.0154	**0.0617**			
TRGW	1.0473	1.0926	**0.0847**		
CSMaRTH	-0.0005	0.0217	1.0187	**0.2322**	
CTRGH	0.0279	0.0565	0.6951	0.0241	**0.0934**

Table 3.11 Genetic heterogeneity analysis based on the exact test (above diagonal) and F_{ST} estimate (below diagonal) between pairs of *H. asinina* samples

	SAME	CAME	TRGW	CSMaRTH	CTRGH
SAME	-	$P = 0.9243^{ns}$	$P < 0.0001$*	$P = 1.0000^{ns}$	$P = 0.5125^{ns}$
CAME	0.1635*	-	$P < 0.0001$*	$P = 0.1461^{ns}$	$P = 0.0115$*
TRGW	0.8759*	0.8919*	-	$P < 0.0001$*	$P < 0.0001$*
CSMaRTH	-0.0058^{ns}	0.1826*	0.8641*	-	$P < 0.0001$*
CTRGH	0.1220*	0.2245*	0.7064*	0.1478*	-

ns = not significant, * = significant at $P < 0.017$ following the sequential Bonferroni adjustment (Rice, 1989).

3.3.5 Development of SCAR markers in H. asinina using sequence-specific primers of P. monodon

3.3.5.1 Penaeus monodon libraries

A total of 135 gene-specific primers from testis, ovary and hemocyte cDNA libraries of *P. monodon* were screened using genomic DNA of an individual from TRGW, SAME, and PHI. Six amplified products from five primers for amplification of *P. monodon glycogen phosphorylase* (*GlyP*), *DNA primase* (*DNAP*), *herterogeneus nuclear ribonucleoprotein 87F* (*HNRNP*), *programmed cell death protein 6* (*PCDP6*), and *Wolf-Hirschorn syndrome candidate 1 protein* (*WHSC*, 1400 and 700 bp, respectively) were cloned and sequenced (Figure 3.24).

Nucleotide sequences of all characterized fragments did not match any sequences in the GenBank (E value > 10^{-4}) and regarded as unknown sequences (Table 3.12). Sequence-specific primers were designed from these unknown transcripts and called $HaSCAR_{U1}$, $HaSCAR_{U2}$, $HaSCAR_{U3}$, $HaSCAR_{U4}$, $HaSCAR_{U5}$, and $HaSCAR_{U6}$, respectively. Primer pairs were amplified with few individuals of genomic DNA of *H. asinina* from wild and cultured stocks. All designed primers successfully generated the positive amplification products (Figure 3.25) and subjected for further analysis using non-denaturing polyacrylamind gels.

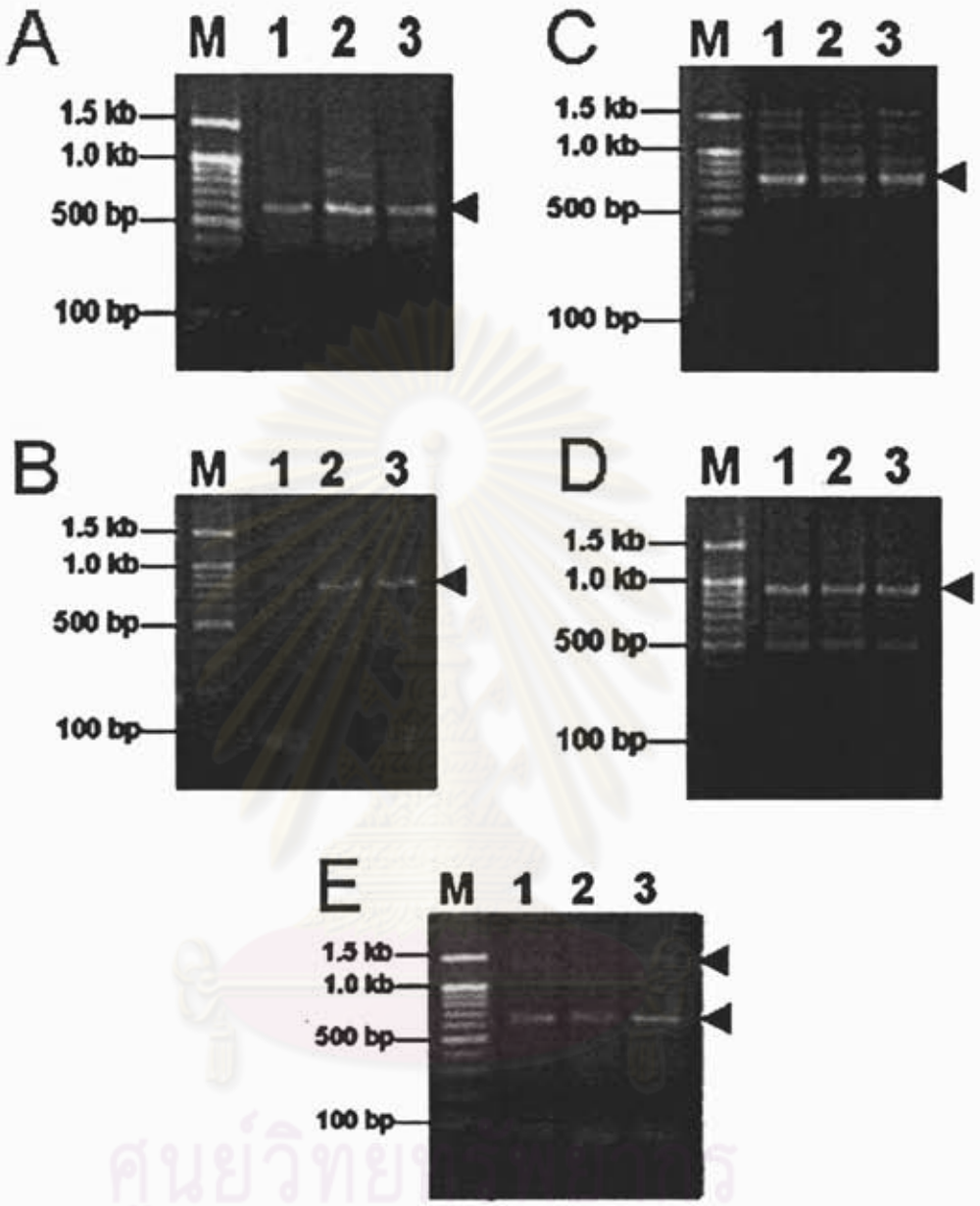


Figure 3.24 Agarose gel electrophoresis showing the amplification products from *P. monodon* primers for amplification of *glycogen phosphorylase* (*GlyP*, A), *DNA primase* (*DNAP*, B), *herterogeneus nuclear ribonucleoprotein 87F* (*HNRNP*, C), *programmed cell death protein 6* (*PCDP6*, D), and *Wolf-Hirschorn Syndrome candidate 1 protein* (*WHSC*, E) tested against genomic DNA of *H. asinina* (lanes 1 - 3 = specimens from SAME, PHI, and TRGW, respectively).

Table 3.12 Name of original gene-specific primers, blast analysis, primer sequence, the annealing temperature of *H. asinina* obtained from characterization of the PCR products using heterospecific primers from *P. monodon*

Original gene-specific primers	Size of fragment (bp)	Blast analysis	Name of SCAR marker	Primer Sequence (5′ – 3′)	Ta (°C)	Expected product* (bp)
GlyP	663	Unknown	*HaSCAR*$_{U1}$	F: GCT CCC CAC AGT GCT CTT GC R: TGT AAT AAT GTC CTC CTC GG	57	294
DNAP	898	Unknown	*HaSCAR*$_{U2}$	F: TCC GAA GCA TCA ATG TCT CC R: TTC CGT CCT GGC AAA GCA CT	57	224
HNRNP	699	Unknown	*HaSCAR*$_{U3}$	F: CTT TGG CTT TCT TGG AGT TA R: ACA TCC TTC CTT CAT TCG TG	57	263
PCDP6	1007	Unknown	*HaSCAR*$_{U4}$	F: CAT TTT CAG GCT AAG TGG TG R: TGT ATC CTT CCT TTG TGT GC	57	313
WHSC-1400	1496	Unknown	*HaSCAR*$_{U5}$	F: AAT GTT GTA ATG AGT ATG AGT GG R: CTG ATG ATG CTA TTG TGA CCT	57	319
WHSC-700	680	Unknown	*HaSCAR*$_{U6}$	F: AAC CTC GCA CGC TTC TCC GT R: TCC TGC TTC TGT GGT GTA GT	57	246

*The expected size from the original EST sequences of *P. monodon*.

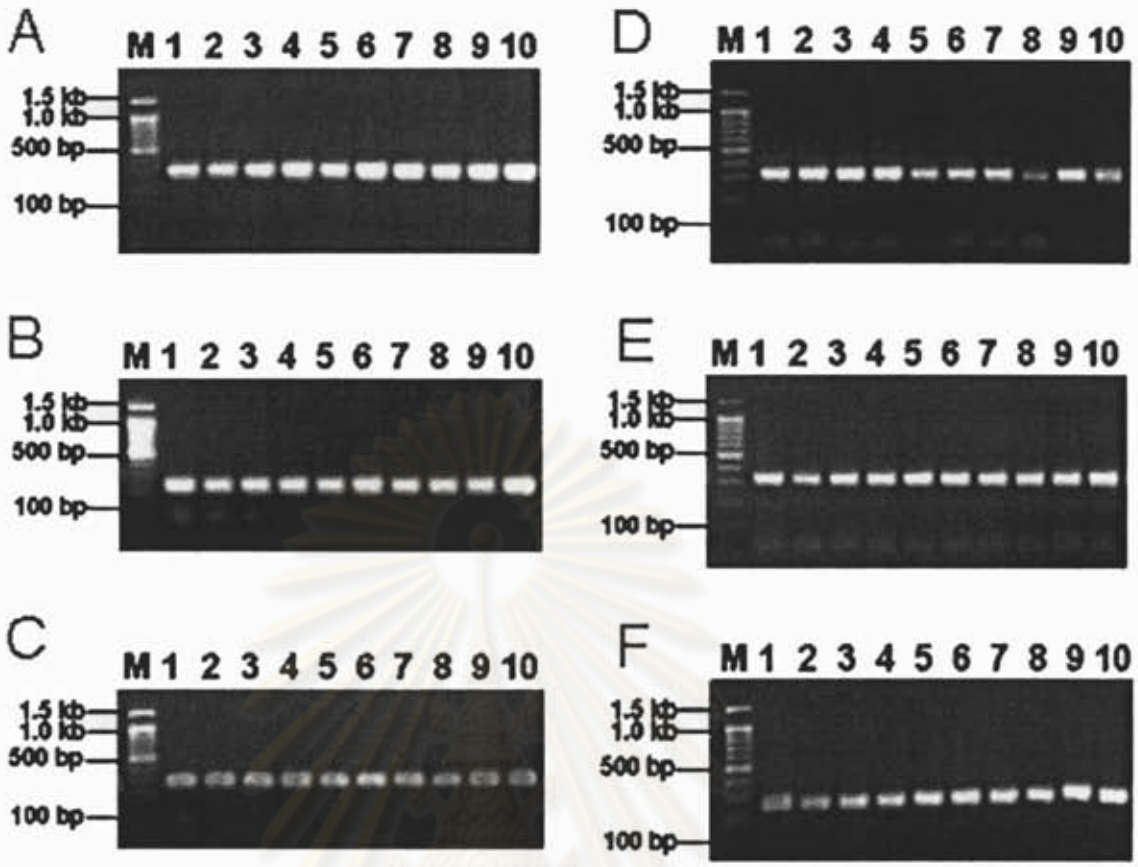


Figure 3.25 Agarose gel electrophoresis showing the amplification products of *HaSCAR*$_{U1}$, *HaSCAR*$_{U2}$, *HaSCAR*$_{U3}$, *HaSCAR*$_{U4}$, *HaSCAR*$_{U5}$, and *HaSCAR*$_{U6}$ (A - F, respectively) using genomic DNA of abalone from CSMaRTH (lanes 1 - 2), SAME (lanes 3 - 5), TRGW (lanes 6 - 8) and PHI (lanes 9 - 10), respectively. Lanes M are a 100 bp DNA marker.

SSCP analysis revealed that *HaSCAR*$_{U1}$ was monomorphic (Figure 3.26A). *HaSCAR*$_{U3}$, *HaSCAR*$_{U4}$, and *HaSCAR*$_{U5}$ illustrated that genotypes of abalone from TRGW was different from other geographic samples. The SSCP pattern of PHI was clearly different from *H. asinina* in Thai waters revealed by polymorphism of *HaSCAR*$_{U3}$.

3.3.5.1 Amplification of functional important gene homologues of H. asinina

Four primer pairs were designed from ESTs representing *isocitrate dehydrogenase* (*IcDh*), *arginine kinase* (*AK*), *ADP/ATP carrier* (*ATPCa*) and *NADH dehydrogenase subunit II* (*ND-II*) of *H. asinina* and tested against genomic DNA of a few individuals of abalone from wild and cultured stocks. Non-specific products were obtained from amplification of *AK* and *IcDh* genes (Figure 3.27A). In contrast, *ATPCa* and *ND-II* were successfully amplified by PCR (Figure 3.27B - C). Polymorphism of the amplified gene segments were further examined by SSCP analysis.

Figure 3.26 Non-denaturing polyacrylamind gel electrophoresis illustrating SSCP patterns of $HaSCAR_{U1}$ (A), $HaSCAR_{U3}$ (B), $HaSCAR_{U4}$, (C) and $HaSCAR_{U5}$ (D) using genomic DNA of abalone from CSMaRTH (lanes 2 - 3). SAME (lanes 4 - 5), TRGW (lanes 6 - 9), CAME (lanes 10 - 13) and PHI (lanes 14 - 17), respectively. Lanes M and 1 are 100 bp DNA ladder and the non-denatured PCR product (double standard control), respectively.

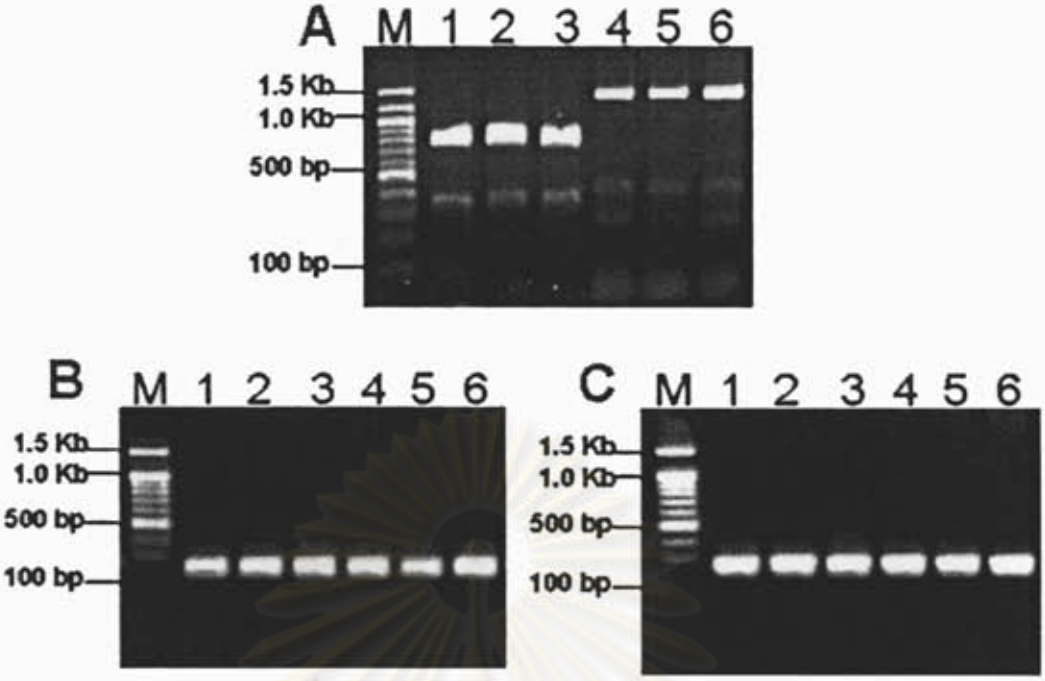


Figure 3.27 Agarose gel electrophoresis showing the amplification products of *H. asinina* samples from SAME, PHI, and TRGW using heterospecific primers for *arginine kinase* (*AK*) and *isocitrate dehydrogenase* (*IcDh*) (A lane 1 - 3 and 4 - 6, respectively), *ADP/ATP carrier* (*ATPCa*) (B) and *NADH dehydrogenase subunit II* (*ND-II*, C). Lane M is a 100 bp DNA marker.

Nevertheless, *ATPCa* was monomorphic in all examined abalone individuals. Only *ND-II* polymorphism was examined in all specimens (Figure 3.28). The Gulf of Thailand and Philippines samples exhibited an identical pattern. Two different genotypes (B and C) were found in the TRGW sample (Table 3.13).

Nucleotide sequences of representative individuals carrying each genotype of *ND-II* were clearly different owing to base substitutions (15, 13 and 2 positions between A - B, A - C and B - C, Figure 3.29). $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$, *ND-II* polymorphism could be used to infer geographic origin of *H. asinna* in this study.

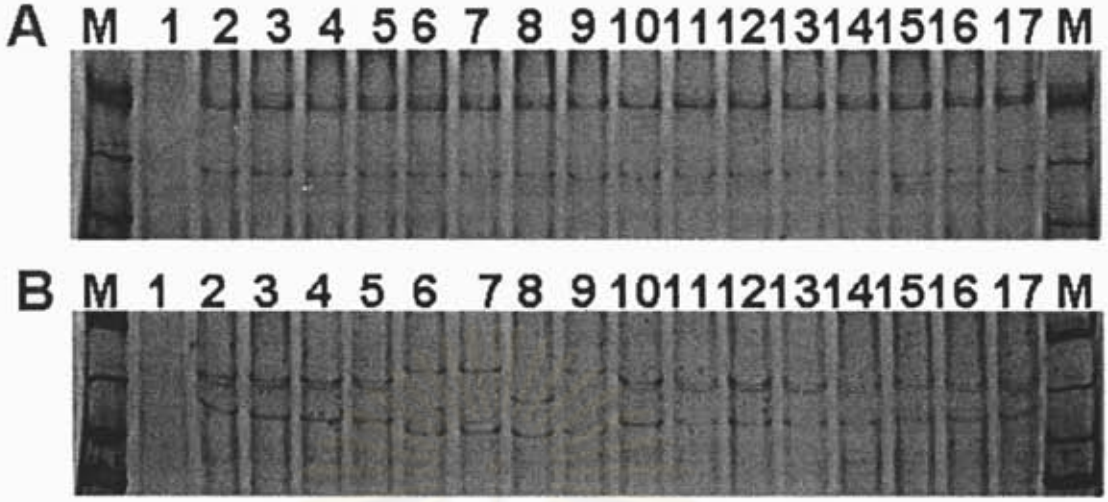


Figure 3.28 Non-denaturing polyacrylamide gel electrophoresis illustrating SSCP patterns of *ATPCa* (A) and *ND-II* (B) using genomic DNA from CSMaRTH (lanes 2 - 3), SAME (lanes 4 - 5), TRGW (lanes 6 - 9), CAME (lanes 10 - 13) and PHI (lanes 14 - 17). Lanes M and 1 are a 100 bp DNA ladder and the non-denatured PCR product (double standard control), respectively.

Table 3.13 Distribution frequencies of SSCP genotypes of *ND-II* in wild and domesticated *H. asinina*

Sample	*ND-II*		
	A	B	C
SAME	20	-	-
CAME	22	-	-
TRGW	-	18	7
PHI	20	-	-
CTRGH	40	-	-
CSMaRTH	95	-	-
Total	197	18	7

```
ND-IIA   ATCGGGTTCGTGTTCTCCTTTATCACATTTTTAGGCTCATCATCTCCCTATCCTCAGTC   60
ND-IIC   ATCGGGTTCGTGTTCTCCTTTATCACATTTTTAGGCTCTATCATCTCCCTGTCCTCAGTC   60
ND-IIB   ATCGGGTTCGTGTTCTCCTTTATCACATTTTTAGGCTCTATCATCTCCCTGTCCTCAGTC   60
         ************************************ ********** *********

ND-IIA   CACTGACTAAGGATCTGAGTCGGATTAGAAATTAACATAATAGGATTCATCCCCCTTTA  120
ND-IIC   CATTGACTAAGGATCTGAGTCGGGTTAGAAATTAACATAATAGGATTCATCCCCCTCTTA  120
ND-IIB   CATTGACTAAGGATCTGAGTCGGGTAGAAATTAACATAATAGATTCATCCCCCTCTTA  120
         ** ******************  ********** ************ ***

ND-IIA   ATTTACCGGGCCTGACCATAGAAACAGAATCTAGGATTAAATACTTCATTATCAGGCG  180
ND-IIC   ATTTACCGAGGCCTAACCATAGAGACAGAATCTAGGATTAAATACTTCATTATACAAGCG  180
ND-IIB   ATTTACCGAGGCCTAACCATAGAGACAGAATCTAGGATTAAATACTTCATTATACAAGCG  180
         ******** ***** ******** ****************************** ** ***

ND-IIA   CTAGGTCAAGAATACTTATATTTGGAAGACTTTTATCATTTAATCTCTCCCTGTCATGG  240
ND-IIC   CTAGGATCAAGAATACTCATATTTGGAAGACTTTTATCATTTAATCTCTCCCTGTCATGG  240
ND-IIB   CTAGGATCAAGAATACTCATATTTGGAAGACTTTTATCATTTAATCTCTCCCTGTCATGG  240
         ***** ********** ********** **************************

ND-IIA   GAGGCATGACAACACCAAACGAGCCC                                    266
ND-IIC   GAGGCATGACAACACCAAACGAGCCC                                    266
ND-IIB   GAGGCATGACAACACCAAACGAGCCC                                    266
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Figure 3.29 Multiple alignments showing nucleotide sequence of an individual representing each genotype of *ND-II*.

3.3.6 Association between microsatellite and SCAR genotypes and the body weight in domesticated stocks (B) of H. asinina

3.3.6.1 Regression analysis between genotypes and phenotype (growth) in domesticated H. asinina

Correlation between genotypes (fix effect) and the body weight (variable effect) was initially tested at each locus using regression analysis. A simple linear regression indicated significant correlation between those factors at *DW455* and *Haμ10* loci ($P < 0.05$) (Table 3.14). SCAR marker ($HaSCAR_{328}$) developed from AFLP analysis was also analyzed but did not show significant results ($P > 0.05$).

3.3.6.2 Genetic differences between fast and slow growing domesticated H. asinina

Genetic heterogeneity in allele and genotype distribution frequencies between BL and BS groups exhibiting high and low growth rates collected from the domesticated B sample is shown in Table 3.15. Significant genetic differences between subgroups BL-BS were observed at *DW455* and *Haμ10* loci ($P < 0.05$). Nevertheless, the F_{ST}-statistics and the exact test between subgroup BL and BS examined by $HaSCAR_{328}$ were not significantly different ($P > 0.05$).

Table 3.14 Correlation between genotype and the body weight in the domesticated sample (group B) of *H. asinina* based on six microsatellite loci and a $HaSCAR_{328}$ SCAR marker

Locus	Regression value	P-value
DW455	0.246	0.031*
DW503	0.016	0.892 ^{ns}
PHe177	0.003	0.981 ^{ns}
PT102	0.103	0.368 ^{ns}
Haμ9	0.139	0.220 ^{ns}
Haμ10	0.265	0.023*
$HaSCAR_{328}$	0.103	0.361 ^{ns}

*Significant at $P < 0.05$

Table 3.15 Genetic differences between fast (BL) and slow (BS) growing *H. asinina* cultured in the same environments analyzed by F_{ST}-statistics and the exact test

Locus	F_{ST}	Exact test
DW455	0.0237*	0.0170*
DW503	0.5336 ^{ns}	0.5121 ^{ns}
PHe177	0.9713 ^{ns}	0.9677 ^{ns}
PT102	0.0504 ^{ns}	0.0690 ^{ns}
Haμ9	0.4580^{ns}	0.3028 ^{ns}
Haμ10	0.0766 ^{ns}	0.0211*
All microsatellite loci	0.0402 ^{ns}	0.0119 ^{ns}
$HaSCAR_{328}$	0.3982^{ns}	0.4003^{ns}

*Significant at $P < 0.05$ for single locus comparisons and $P < 0.01$ for multiple locus comparisons according to the sequential Bonferroni adjustment (Rice, 1989)

Significant differences between the body weights of the B sample having different genotypes were further analyzed using ANOVA followed by Duncan's new multiple range test. Significant differences were only observed at the *Haμ10* locus ($P < 0.05$).

At the *Haμ10* locus, heterozygotic of allele 163 had a higher body weight than exhibit in homozygote whereas the appearance of allele 158 showed the lower weight of examined abalone (Table 3.16). Abalone exhibiting the 161/163 genotype had the highest body weight whereas those carrying the 158/158 genotype had the lowest body weight. Homozygote and heterozygote individuals carrying the 176 bp allele had greater or approximately equal body weight to the mean body weight for this group of samples. Interestingly, abalone possessed 176/176 and 153/153 were found in 21.25% and 20.00% of examined individuals with the average body weight of 9.252 ± 4.113 and 5.314 ± 3.934 g. This suggested that the 176 bp allele may be advantage for growth and survive of this domesticated stock.

Table 3.16 Statistical analysis to indicate differences in body weight of the hatchery sample (B) of *H. asinina* carrying different *Haμ10* genotypes

Genotype (*N*)	Average Weight ± SD (g)
158/167 (1)	3.200 ± 4.000^{ND}
158/176 (1)	4.600 ± 3.700^{ND}
158/173 (1)	11.300 ± 4.000^{ND}
158/163 (1)	11.500 ± 3.900^{ND}
161/173 (1)	11.700 ± 4.100^{ND}
167/173 (1)	12.800 ± 5.200^{ND}
158/158 (2)	2.990 ± 4.604^{a}
163/176 (2)	$4.035 \pm 3.313^{a,b}$
163/163 (16)	$5.314 \pm 3.934^{a,b}$
161/161 (5)	$7.226 \pm 3.877^{a,b}$
163/181 (5)	$7.224 \pm 4.148^{a,b}$
173/181 (2)	$7.435 \pm 3.406^{a,b}$
167/176 (2)	$7.505 \pm 4.301^{a,b}$
163/167 (7)	$7.860 \pm 4.162^{a,b}$
161/167 (2)	$8.615 \pm 4.415^{a,b}$
163/173 (3)	$8.983 \pm 3.512^{a,b}$
167/167 (17)	$9.252 \pm 4.113^{a,b,c}$
167/181 (5)	$10.308 \pm 4.986^{a,b,c}$
176/176 (2)	$11.460 \pm 3.932^{b,c}$
176/181 (2)	$11.530 \pm 3.962^{b,c}$
161/163 (2)	16.460 ± 8.952^{c}

The same superscripts indicated non-significant differences between genotypes. ND = not determined.

3.4 Isolation, characterization and expression analysis of genes functionally related with metabolisms in *H. asinina*

3.4.1 RNA extraction

Total RNA was extracted from hepatopancreas of each *H. asinina*. The quality and quantity of total RNA were determined by spectrophotometry and agarose gel electrophoresis (Figure 3.30). The ratio of OD_{260}/ OD_{280} of the extracted RNA was 1.8-2.0 indicating that its quality was acceptable for further applications. Agarose gel electrophoresis showed discrete *ribosomal RNA* bands reflecting good quality of the extracted RNA. Total RNA was reverse-transcribed to the first strand cDNA for quantitative real-time PCR analysis (Figure 3.31). Messenger (m) RNA was purified for further used for the construction of template for RACE-PCR analysis.

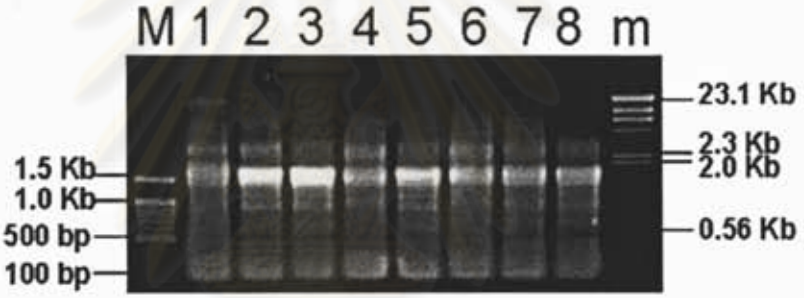

Figure 3.30 A 1.0% ethidium bromide-stained agarose gel showing the quality of total RNA extracted from hepatopancreas of 8 individuals of *H. asinina* (lanes 1 – 8). Lanes M and m are 100 DNA ladder and λ-*Hin*d III markers, respectively.

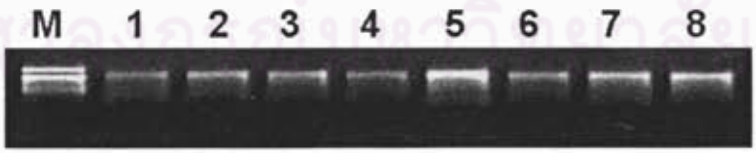

Figure 3.31 A 1.0% agarose gel electrophoresis illustrating the quality of first strand cDNA synthesized from total RNA of *H. asinina* (lanes 1 – 8). Lane M is λ-*Hin*d III marker.

3.4.2 Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE - PCR)

Normal and subtractive complementary DNA library were constructed from ovaries, testes and hemocyte of *H. asinina* and sequenced (Amparyup, 2004). ESTs sequences were divided to 7 categories, which are functionally related to sex and reproduction, stress response, detoxification and cell defense proteins, protein synthesis and ribosomal proteins, cell division and DNA replication, metabolism, unidentified function and unknown.

Four genes homologues involving the β-oxidation: *α-methylacyl-CoA racemase* (*AMACR*), *carnitine O-palmitoyltransferase 1A* (*CPT-1A*), *hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* (*trifunctional protein*) *α-subunit* and *vacuolar H^+ ATPase* (*V-ATP*) *14 kDa subunit*, were selected for further characterized by RACE-PCR.

The 3′-end gene-specific primer was designed from an EST of the testes cDNA library of *H. asinina* (clone no. 069, 726 bp insert) significantly matched *α-methylacyl-CoA racemase* (*AMACR*) *isoform 3* of *Macaca mulatta* (*E*-value = 4 x 10^{-53}) (Table 3.17) and used to characterize 3′-RACE of this gene.

The smear amplification results were obtained from the initial 3′-RACE-PCR of *AMACR*. Therefore, the 3′-nested primer were designed and use in nested-PCR with Nested Universal Primer A (NUP). Approximately a 1200 bp fragment was obtained. This band was cloned and sequenced (Figure 3.32). Nucleotide sequences of EST and 3′ RACE products (Figure 3.33) of this gene were assembled. The full length cDNA of *H. asinina-AMACR* was 1729 bp in length consisting of an ORF of 1140 bp corresponding to a polypeptide of 380 amino acids and the 5′ and 3′ UTRs of 106 and 483 bp, respectively (Figure 3.34). It significantly matched *α-methylacyl-CoA racemase* (*AMACR*) *isoform 3* of *Gallus gallus* (E value = 5 x 10^{-127}). The predicted CoA_transf_3 domain was found at positions 53 – 244 amino acids (*E*-value = 1.3 x 10^{-82}, Figure 3.35) of the deduced AMACR protein of *H. asinina*.

Table 3.17 Names, nucleotide sequences and melting temperature of gene- and nested gene-specific primers and internal primers for isolation of the full length cDNA of various genes by RACE-PCR

Gene/ Primer	Sequence (5'-3')	Tm (°C)
Alpha-methylacyl CoA racemase		
3′-RACE	GTC AAT CTG TTG GGT GAC TTT GCT G	74
Nested 3′RACE	CCA ACA TGG TGC AGG CTC AGC TTG T	76
Carnitine O-palmitoyltransferase I		
5′-RACE	GCT GGA TCA TTC GCA TCA TAG CCA TGT G	84
3′-RACE	TGT CGA TCA TGC ACT GTG GAG TCT TGT G	84
Walk 3'-RACE	TGA TGA TGG GTA TGG AGT GTC CTA TG	76
Hydroxyacyl CoA dehydrogenase/ 3-ketoacyl CoA thiolase/ enoyl CoA hydratase (trifunctional protein)		
3′-RACE	GGC CTT GTT GAC AGC TTG GTA GAT CC	80
Internal 3′-RACE	CAC TCC CTA TCG CCA GAA TTG C	68
Vacuolar H+ ATPase 14kDA subunit		
5′-RACE	AAT GAT GGC AAT GTC ATC CCG CTT C	74
3′-RACE	TGC CTG GAG TAC AGT GCT CAG TGT GA	80

In addition, gene-specific primers for 5′- and 3′ RACE-PCR were designed from an EST significantly matched *Carnitine O-palmitoyltransferase 1A* (*CPT-1A*, of *Danio rerio* (clone no. 075 having an insert of 729 bp, E-value = 5×10^{-70}).

The amplification fragments of approximately 2.0 and 1.5 kb in length were obtained from 5′- and 3′ RACE-PCR. The gel-eluted DNA was cloned and sequenced (Figure 3.36). Primer walking at the 3′ end was applied for further sequenced of the cloned fragments using internal sequencing primers. Nucleotide sequences of EST 5′- and 3′ RACE-PCR of this gene were then assembled (Figure 3.37).

The full length cDNA of *H. asinina-CPT-1A* was 4067 bp in length containing an ORF of 2304 bp corresponding to a polypeptide of 768 amino acids and the 5′ and

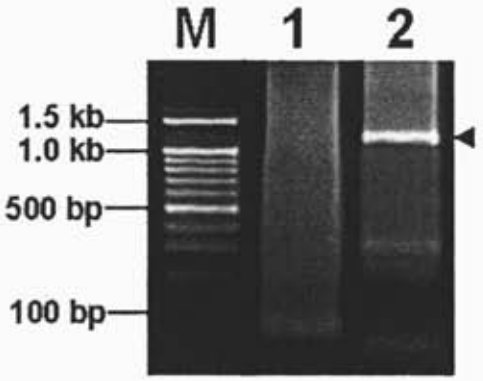

Figure 3.32 3′ RACE-PCR products of *α-methylacyl-CoA racemase*. An arrowhead indicates bands selected for further analysis. Lane 1 and 2 are amplification products from 3′-RACE and nested 3′-RACE, respectively. Lane M is a 100 bp DNA marker.

A.

GTCAATCTGTTGGGTGACTTTGCTGGGGGTGGATTGATGTGTGCCCTGGGGGTGGTGCTGGCCCTGTTT
GAGAGGACCCGGTCAGGCAGGGGCCAAGTGGTTGATGCCAACATGGTGCAGGGCTCAGCCTATGTCTCT
AACTTCCTGTGGGCAATGAAAGACACATTTGTGTGGGGCGCAGAGAGAGGGGAGAACCTACTGGACACA
GGCTCTGCATTTTATGAGACATACAAGACTGCTGATGGAAAGTTCATGTCTGTTGGTGCCTTGGAACCA
CAGTTTTATGCTGCACTGCTCAAAGGCCTTCAGCTTGATCCAGACAAAGTACCACAGTATAGCGATAGA
AAAGAAATGAAAAAGTTGTTCACAGACATTTTCTTGTCAAAGACTCGGGATGAGTGGACTGAGGCCTTT
AGAGATTTAGACGCATGTGTGGAACCCATCATGGATATGGATGAGGCACCCATGCACCCTCATAATCAA
GCTAATAATACATTCTTGATGAACCCCAGAGGTGATTATGAGGCTGGACCTGCACCTGCTCTGTCACGG
ACACCAGGGGTCAGCACCTTGCTACCTCAGCCTGTGATTGGTGAACACACACTGGAGGTACTGCAGGAT
GCTGGCTACAGCCAGGATGACATCAGGAGTCTGGTGGATGCTGGGGTAGTTCAACAGACAAGCACTAGC
TCCAAGTTGTGAGAGGCATGAAATCTTGTACAATTATTGAACACCTCAACGTTAATTAGTACATTGAAC
CCTGTCAGGGAAAGATTCAATAATTTCAAGATGAAAGACAGTTTGTGTTGATGCTGAGCATTGGCTTCT
CAGCCCAAACAGAGCTGTTTTCTTTTTCTTCATGTTACAGAAACTTCCATTTGAGTTAATAACGATAAT
AATGGACAATGGATATTTGGTGGTGTTCCAATCTACTCCATAAGCCATGCCCAAAGTGGAACATTACTG
ATGTCAACAACTTGATCACAATGTACATGGTTGCAGCACATAATGTCCATACGTGACATGATGGTTGAA
TTTTTTATGCTATGAACCCCCCAGTGATCCTATCAAGCATGTCTACATACCGAGGTGCCTTGTATTAGT
CGGCAAACTCAGAAGAGTCGGTATGGAAATAAAAGTATATGATTTTCTGCCATAAAAAAAAAAAAAAAA
AAAAAAAAAAAA

B.

GTCTGCTTGGGTTCAAAGTTCCTCAAACGTGGTCACAGATTAAGCTCCACAAGATCCAAGTGGCTGGCA
CTGTAGAAACTGACTCATGCAAGGGAAGATCTCGAACATGGCCCTCCAAGGTATCCGAGTCATCGAGAT
GGCAGGGCTGGCACCAGCCCCTTCTGTGGAATGGTCTTGTCAGACTTTGGAGCTAAAGTTATCAGAATA
GACAGGACAAGGAATCCTCCTGACACCGACCGCTTAGGTCAGGGAAGAGGTCAGTAGCTGTAGACTTGA
AGCAAAAGAAGGGAACTGAAGTTGTGAAAAAACTCTGCTCAGCAGCTGATGTGCTGATAGAACCTTTCC
GCCCAGGTGTGATGGAGAAGCTTGGACTGGGACCGGAGACGCTGATGGCTGACAACCCACGCCTTGTGT
ATGCCAGACTGACTGGCTATGGACAGAAGGGAAGTCTGTCCCATCGAGCTGGACATGACATCAATTACA
TTGCTACATCAGGTGTTCTTTCTACTCTGGGTCGTAAGCATGAGAATCCTTGTGCTCCTGTCAATCTGT
TGGGTGACTTTGCTGGGGGTGGATTGATGTGTGCCTGGGGGTGGTGCTGGCCCTGTTTGAAAGGACCGG
TCAGGCAGGGGGCCAAGTGGTGATGCCAACATGGTGCAGGCTCAGCTTATGTCTCTACTTCCTGTGGGC
AATGAAGACACATTGTGTGGGGCGCAGAGAGAGGGG

Figure 3.33 Nucleotide sequence of 3′ RACE-PCR (A) and EST (B) of *α-methylacyl-CoA racemase* of *H. asinina*.

```
GTCTGCTTGGGTTCAAAGTTCCTCAAACGTGGTCACAGATTAAGCTCCACAAGATCCAAG   60
TGGCTGGCACTGTAGAAACTGACTCATGCAAGGGAAGATCTCGAACATGGCCCTCCAAGG  120
                                              M  A  L  Q  G     5
TATCCGAGTCATCGAGATGGCAGGGCTGGCACCAGCCCCCTTCTGTGGAATGGTCTTGTC  180
 I  R  V  I  E  M  A  G  L  A  P  A  P  F  C  G  M  V  L  S    25
AGACTTTGGAGCTAAAGTTATCAGAATAGACAGGACAAGGAATCCTCCTGACACCGACCG  240
 D  F  G  A  K  V  I  R  I  D  R  T  R  N  P  P  D  T  D  R    45
CTTAGGTCGAGGGAAGAGGTCAGTAGCTGTAGACTTGAAGCAAAAGAAGGGAACTGAAGT  300
 L  G  R  G  K  R  S  V  A  V  D  L  K  Q  K  K  G  T  E  V    65
TGTGAAAAAACTCTGCTCAGCAGCTGATGTGCTGATAGAACCTTTCCGCCCAGGTGTGAT  360
 V  K  K  L  C  S  A  A  D  V  L  I  E  P  F  R  P  G  V  M    85
GGAGAAGCTTGGACTGGGACCGGAGACGCTGATGGCTGACAACCCACGCCTTGTGTATGC  420
 E  K  L  G  L  G  P  E  T  L  M  A  D  N  P  R  L  V  Y  A   105
CAGACTGACTGGCTATGGACAGAAGGGAAGTCTGTCCCATCGAGCTGGACATGACATCAA  480
 R  L  T  G  Y  G  Q  K  G  S  L  S  H  R  A  G  H  D  I  N   125
TTACATTGCTACATCAGGTGTTCTTTCTACTCTGGGTCGTAAGCATGAGAATCCTTGTGC  540
 Y  I  A  T  S  G  V  L  S  T  L  G  R  K  H  E  N  P  C  A   145
TCCTGTCAATCTGTTGGGTGACTTTGCTGGGGGTGGATTGATGTGTGCCCTGGGGGTGGT  600
 P  V  N  L  L  G  D  F  A  G  G  G  L  M  C  A  L  G  V  V   165
GCTGGCCCTGTTTGAGAGGACCCGGTCAGGCAGGGGCCAAGTGGTTGATGCCAACATGGT  660
 L  A  L  F  E  R  T  R  S  G  R  G  Q  V  V  D  A  N  M  V   185
GCAGGGCTCAGCCTATGTCTCTAACTTCCTGTGGGCAATGAAAGACACATTTGTGTGGGG  720
 Q  G  S  A  Y  V  S  N  F  L  W  A  M  K  D  T  F  V  W  G   205
CGCAGAGAGAGGGGAGAACCTACTGGACACAGGCTCTGCATTTTATGAGACATACAAGAC  780
 A  E  R  G  E  N  L  L  D  T  G  S  A  F  Y  E  T  Y  K  T   225
TGCTGATGGAAAGTTCATGTCTGTTGGTGCCTTGGAACCACAGTTTTATGCTGCACTGCT  840
 A  D  G  K  F  M  S  V  G  A  L  E  P  Q  F  Y  A  A  L  L   245
CAAAGGCCTTCAGCTTGATCCAGACAAAGTACCACAGTATAGCGATAGAAAAGAAATGAA  900
 K  G  L  Q  L  D  P  D  K  V  P  Q  Y  S  D  R  K  E  M  K   265
AAAGTTGTTCACAGACATTTTCTTGTCAAAGACTCGGGATGAGTGGACTGAGGCCTTTAG  960
 K  L  F  T  D  I  F  L  S  K  T  R  D  E  W  T  E  A  F  R   285
AGATTTAGACGCATGTGTGGAACCCATCATGGATATGGATGAGGCACCCATGCACCCTCA 1020
 D  L  D  A  C  V  E  P  I  M  D  M  D  E  A  P  M  H  P  H   305
TAATCAAGCTAATAATACATTCTTGATGAACCCCAGAGGTGATTATGAGGCTGGACCTGC 1080
 N  Q  A  N  N  T  F  L  M  N  P  R  G  D  Y  E  A  G  P  A   325
ACCTGCTCTGTCACGGACACCAGGGGTCAGCACCTTGCTACCTCAGCCTGTGATTGGTGA 1140
 P  A  L  S  R  T  P  G  V  S  T  L  L  P  Q  P  V  I  G  E   345
ACACACACTGGAGGTACTGCAGGATGCTGGCTACAGCCAGGATGACATCAGGAGTCTGGT 1200
 H  T  L  E  V  L  Q  D  A  G  Y  S  Q  D  D  I  R  S  L  V   365
GGATGCTGGGGTAGTTCAACAGACAAGCACTAGCTCCAAGTTGTGAGAGGCATGAAATCT 1260
 D  A  G  V  V  Q  Q  T  S  T  S  S  K  L  *                  380
TGTACAATTATTGAACACCTCAACGTTAATTAGTACATTGAACCCTGTCAGGGAAAGATT 1320
CAATAATTTCAAGATGAAAGACAGTTTGTGTTGATGCTGAGCATTGGCTTCTCAGCCCAA 1380
ACAGAGCTGTTTTCTTTTTCTTCATGTTACAGAAACTTCCATTTGAGTTAATAACGATAA 1440
TAATGGACAATGGATATTTGGTGGTGTTCCAATCTACTCCATAAGCCATGCCCAAAGTGG 1500
AACATTACTGATGTCAACAACTTGATCACAATGTACATGGTTGCAGCACATAATGTCCAT 1560
ACGTGACATGATGGTTGAATTTTTTATGCTATGAACCCCCCAGTGATCCTATCAAGCATG 1620
TCTACATACCGAGGTGCCTTGTATTAGTCGGCAAACTCAGAAGAGTCGGTATGGAAATAA 1680
AAGTATATGATTTTCTGCCATAAAAAAAAAAAAAAAAAAAAAAAAAA              1729
```

Figure 3.34 The full length cDNA and deduced animo acid sequence of *α-methylacyl-CoA racemase* of *H. asinina*. The putative start and stop codons are boldfaced and underlined. The polyadenyltion signal (AATAAA) are boldfaced.

Figure 3.35 Diagram illustrating a predicted CoA_transf_3 domain functionally important in fatty acid metabolism and transport was found in the deduced α-methylacyl-CoA racemase protein (residues 53 - 244) of *H. asinina*.

3′ UTRs of 156 and 1607 bp, respectively (Figure 3.38). The *H. asinina CPT-1A* show the highest similarity to that of *Monodelphis domestica* (*E*-value = 0.0). The deduced carnitine palmitoyltransferase IA protein of *H. asinina* contained an intrinsic disorder (residues 8 - 19), three transmembrane domains (residues 50 - 72, 87 - 109 and 114 - 136, respectively) and a Carn_acyltransf domain (residues 170 - 761, *E*-value = 4.6 x 10^{-272}) (Figure 3.39). This strongly indicated that the *H. asinina* carnitine palmitoyltransferase IA is a membrane protein.

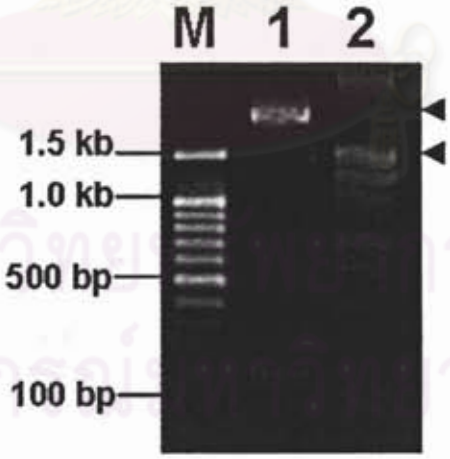

Figure 3.36 Agarose gel electrophoresis of 3′ RACE-PCR (lane 1), and 5′ RACE-PCR (lane 2) products of *carnitine O-palmitoyltransferase 1A*. Arrowheads indicate bands selected for further analysis. Lane M is a 100 bp DNA marker.

A.

CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACGGGGGGAGATTCGTCGGACGCT
GGTACGTTTTGGTTCACGTCGTGTTGTTGTCGGAATAAATAGCTTATTGCTTTGATTTTGGACTGCACA
CGTTTGAAAACAAAACGCTCATCAAGTATTTGGCAACAAGACCTTGGTTTCTGCGCCGTGATAATGGCA
GAAGCTCATGCCGCTGTAGCCTTCTCCTTCACTGTCACCCCAGATGGAGTCAATGTCAACCTCAACCAT
GAGGCTATCAAGGCAGTATGGGACAGTGGTTTACTGTCCTGGAAGAAGAGAATAGGACGTATGAAAAAC
AACTTCCATAATGGAGTTTACCCGGCGTCCCCTGTCTCACTGATCTTCACAATTACATTGATACCGGCT
CTTGTCCTTGGAGGATATGACCCTTCCTACGGCTGTATCTCCTACATCCAGGCCCATATCCCACTCCTG
GGTGTAGCCAGTACGTCCATGTGCCTATATGTGAGCTGTGTGTTGTTCTCTACACTTCTGTGGCTGGTG
GTAATCTTTGCCATACAGTACACCCTGCGTCTGCTCCTTACCTACCATAACTGGATATATGAGGTGCGC
GGAACCATCTCACTCAGGACCAAAATCTGGATGGGCCTAGTGCGAGTGTTTGGAGGTCGAAAACCTCTC
CTTAACAGCTACCAGGGTTCTCTGCCAAAGCTGTTTGTCCCACCACTGGATGGCACATTGTCAAGGTAC
CTGAAGTCAGTTCAACCTCTGCTGGATGATGAGAAGTATGCCCGTATGGAAAAGTTGGGTCAGGAGTTC
CGTAATGGAATTGGACCCAAATTACAGCGTTACCTTTTGCTCAAGTCATGGTGGTCTGCCAACTATGTG
TCTGACTGGTGGGAACAGTATGTCTACCTTCGTGGGCGTTCACCTATCATGATCAACAGCAACTTCTAT
GGAATTGATGCTGTGCTGGAGAAACCAACAGGCGTGCAGGTGTCACGTGCAGCCAACATCATCTACGCC
ATCCTGCAGTACAGACGTGAGGTGGATAGGGAAGAGCTCAAGCCGATTTTATTGAACAAGACTGTTCCA
CTGTGTTCTGCTCAGTATGAGAGACAGTTCAGCACGACACGTATTCCTGGCATCGAAACAGACACCTTG
GTACATCACAAAGACAGTAACCACATAGCTGTGTATTATCGTGGCCGATACTTTAAGGTCTACATCAGG
CATAAGGGCAGACTACTCAAGCCCATTCAGATTGAATCAATGCTGCAGAAGATTTTAGATGACACCACT
GAGCCATCATATGGTGAAGAGCATCTGGCTGCCCTGACTGCCGGGGAGAGGGTCAACTGGGCGAAGGCA
AGGAAGGAGTTTTTCTCAAAGGGCAAGAACAAGGCTTCACTGGATGCTATTGAGAAGGCTGCCTTCTGT
GTGACATTGGATGAAGAAGCACATGGCTATGATGCGAATGATCCAGCG

B.

TGTCCGATCATGCACTGTGGAGTCTTGTGCATTTGTGAGAGCAATGGATGACAAGAATAACACTAATGC
TGGACGTATCAAACTGCTGAAGGAGGCCGCAGAGGTCCATCAGCATGCGTATAGAGAGTGCATGACTGG
ACTTGGTATTGACAGGCATCTCTTCTGTCTGTATGTCGTCTCTAAATACCTTGGTGTTGACTCTCCATT
CTTGGCTGAAGTTCTCAGTGGGCCCTGGAGGCTGTCAACAAGTCAGACTCCTCACCAGCAGACAGACAA
GCTGGACCTGAAGAAATTCCCAGACAGAATCTGTGCAGGAGGTGGCTTTGGGCCGGTTGCTGATGATGG
GTATGGAGTGTCCTATGTTGTAGCTGGGGAGGACACCATCTTCTTCCATATATCGTCCAAGAAGTCCTG
TCCACTTACTAATTCTCCGAAGTTTGGCATTGAATTGAGGCGCTCCCTTAATGACATGCGAGAGCTGTT
CACGAAAGAGGATTGACATGAGTAAAGCAGGTAGATAGACATCCAGCAGATACCTAGACACACCAGCCA
ACCACATGATCCACTTGAAATGTCCTGATATGCAGGTGGAGTCCGAGTGTGTGTAGGTGATATAGATCT
GAAGAGCCATGTTTGACTGTGATGCAGTGAAGATGTACAGGTGTTATATTTTATGTGTTAACTGTTTTT
TACATTTATCATTTCTGTCGTACAAACCACATGGTTTGGTGGATATTATGAACGTGCACATCAAATGCA
ATCTTTTTACTCAATGCATCCTGTACAATTTTAATTTATTTGAGAGATGACATTTTAGCGGAATATCCT
GCATCTTCAGCAATGCCTGCTTAGAGATATGTGCCATGCTTAAATTAACACTAAGTCGACTGGTTCTCA
GATGGAGTGCTGCTCTATCAGGGGAGCTAACTCTTACTAACTTACCAGCACCAAACTGGTGCTATTTTT
CTGTCATACATATCTGTATCCATCACGATTGGAATTTATTTATCTTAGAATGCTGAAAGCAATGTGCCA
TGGAATATTCAGCTATTTGTACCAAGATATGAGATATGTATTTTATCACAGCTTTAGCATGCTGTGTCT
TTAGTTTTATCTTCAATGCTGGCATGAGAGTCTTTCCTTTATTATGACATGTTTAACAAAAGAAATACT
TTTATTGTGTATCATTTGAATTGCCATTTTCATACAGATATTATCTTGAAAGTCAGGTTTTTACTTCAC
TGAATTACATTTCAAATTGTACTTTTATTCTTTATGCAAACACATAAAATTTAACATAGATCATTTGTA
TATTTATTAAAAGAACTGTTTTAATTGTACATATTGGTGTGCGATGTAGTGAGAGTGTTCACTCATAAT
TTAAAGCATAATGTGTATCACTTAAGTCTGGGTTTCTCTCTTCCTTCATTTTGGTTATTGGGTTAGCAT
AACCAGTAAGGGAATAGGTATGATGTCCCTGTTGGCTAGGTGTCACATGTTTACTGTCTACTGTGTCTC
AACAGATCTCATGTTTATCATATTTCCTGTACCAAGAGAGGGTAGATGAAATACAGATATAATATCAAT
GGTATCAGGATTTTTAGAGATTATTGTCCAGGTAAAACTGGAAATGTACATAATTTTAAGGAAATGTCA
AGTTTATTCATACTGGATATTCTAAAATCAATTTTGACAAAAATATTTCATTTTATTTACAAAACAATT
ACAACTAAAATGTTCATGTTTTAACCCTTTTAGGAATTTTTAAAAGATAAAAAATATGAGACAGAATCT
TCAATCCACATACTACAGAACTGTGACCTTGTTACCATGGGAATAAGAACAGGTTTAGTAACAACAGCT
GTTGTAGCACTGATAACTGGCGTAGCCACATCATCATCAAAAATGCATCATCATTGTCATCTGTATGTT
CAGAATGTATGTTTCTGTTTATGTGCACTGTTTTCACATTGGTACAAATTTTATTCTTCTGCTTGAGGC
AGGGTGATTTTAGTTAATTTATTGACAAAAAAACAACAACAGATGGTTCTAGTTTTGTGAAAATAAAAA
GGTGTACTACAAAAAAAAAAAAAAAAAAAAAAAA

C.

GGCGAAGGCAAGGAAGGAGTTTTTCTCAAAGGGCAAGAACAAGGCTTCACTGGATGCTATTGAGAAGGC
TGCCTTCTGTGTGACATTGGATGAAGAAGCACATGGCTATGATGCGAATGATCCAGCCAGTCTGGACAG
GTTTGGAAAAGCCATGCTTCATGGAAAAGGCCATGACCGGTGGTTTGACAAGTCTTTCACTCTGGTTGT
GTGTTCTAACGGTCATATTGGCTTCAATGCAGAGCATTCCTGGGCTGATGCCCAATCATGGCTCATATG
TGGGAGTACGCAATCAGTGATGAAGTACATTATGCAGGATACACCTCAGAAGGCAAGTGTGTTGGTGAA
GCTGATCCTAACACACCTAACCCCATAAGACTGGAGTGGGACATCTCGGAACAGGCCAACTCGGTGATA
GAGAACAGCTTGCAGATTGCACACCAGCTCATTAGTGATGTTGATCTCAATGTGATCATGTTCAAAGAT
TATGGTAAAGGTTTCATCAAGAAGTGCAAAGTGTCCCCTGATGCCTTCATACAGATGGCTCTACAGCTC
ACATACCACAGGTCTACCAACAAGTTCTGTCTTATCTATGAGTCCTCCATGACCCGACTGTTCAGAGAA
GGATGAACTGAGAATGTCGATCATGCACTGTGGAGTCTTGTGCATTTGTGAGAGCAATGGATGACAAGA
ATAACACTAATGCTGGACGTATCAAACTGCTGAAGGAGG

Figure 3.37 Nucleotide sequence of 5′ RACE-PCR (A), 3′ RACE-PCR (B) and EST (C) of *H. asinina carnitine O-palmitoyltransferase*.

```
ACGGGGGGAGATTCGTCGGACGCTGGTACGTTTTGGTTCACGTCGTGTTGTTGTCGGAAT  60
AAATAGCTTATTGCTTTGATTTTGGACTGCACACGTTTGAAAACAAAACGCTCATCAAGT  120
ATTTGGCAACAAGACCTTGGTTTCTGCGCCGTGATAATGGCAGAAGCTCATGCCGCTGTA  180
                                      M  A  E  A  H  A  A  V    8
GCCTTCTCCTTCACTGTCACCCCAGATGGAGTCAATGTCAACCTCAACCATGAGGCTATC  240
A  F  S  F  T  V  T  P  D  G  V  N  V  N  L  N  H  E  A  I    28
AAGGCAGTATGGGACAGTGGTTTACTGTCCTGGAAGAAGAGAATAGGACGTATGAAAAAC  300
K  A  V  W  D  S  G  L  L  S  W  K  K  R  I  G  R  M  K  N    48
AACTTCCATAATGGAGTTTACCCGGCGTCCCCTGTCTCACTGATCTTCACAATTACATTG  360
N  F  H  N  G  V  Y  P  A  S  P  V  S  L  I  F  T  I  T  L    68
ATACCGGCTCTTGTCCTTGGAGGATATGACCCTTCCTACGGCTGTATCTCCTACATCCAG  420
I  P  A  L  V  L  G  G  Y  D  P  S  Y  G  C  I  S  Y  I  Q    88
GCCCATATCCCACTCCTGGGTGTAGCCAGTACGTCCATGTGCCTATATGTGAGCTGTGTG  480
A  H  I  P  L  L  G  V  A  S  T  S  M  C  L  Y  V  S  C  V    108
TTGTTCTCTACACTTCTGTGGCTGGTGGTAATCTTTGCCATACAGTACACCCTGCGTCTG  540
L  F  S  T  L  L  W  L  V  V  I  F  A  I  Q  Y  T  L  R  L    128
CTCCTTACCTACCATAACTGGATATATGAGGTGCGCGGAACCATCTCACTCAGGACCAAA  600
L  L  T  Y  H  N  W  I  Y  E  V  R  G  T  I  S  L  R  T  K    148
ATCTGGATGGGCCTAGTGCGAGTGTTTGGAGGTCGAAAACCTCTCCTTAACAGCTACCAG  660
I  W  M  G  L  V  R  V  F  G  G  R  K  P  L  L  N  S  Y  Q    168
GGTTCTCTGCCAAAGCTGTTTGTCCCACCACTGGATGGCACATTGTCAAGGTACCTGAAG  720
G  S  L  P  K  L  F  V  P  P  L  D  G  T  L  S  R  Y  L  K    188
TCAGTTCAACCTCTGCTGGATGATGAGAAGTATGCCCGTATGGAAAAGTTGGGTCAGGAG  780
S  V  Q  P  L  L  D  D  E  K  Y  A  R  M  E  K  L  G  Q  E    208
TTCCGTAATGGAATTGGACCCAAATTACAGCGTTACCTTTTGCTCAAGTCATGGTGGTCT  840
F  R  N  G  I  G  P  K  L  Q  R  Y  L  L  L  K  S  W  W  S    228
GCCAACTATGTGTCTGACTGGTGGGAACAGTATGTCTACCTTCGTGGGCGTTCACCTATC  900
A  N  Y  V  S  D  W  W  E  Q  Y  V  Y  L  R  G  R  S  P  I    248
ATGATCAACAGCAACTTCTATGGAATTGATGCTGTGCTGGAGAAACCAACAGGCGTGCAG  960
M  I  N  S  N  F  Y  G  I  D  A  V  L  E  K  P  T  G  V  Q    268
GTGTCACGTGCAGCCAACATCATCTACGCCATCCTGCAGTACAGACGTGAGGTGGATAGG  1020
V  S  R  A  A  N  I  I  Y  A  I  L  Q  Y  R  R  E  V  D  R    288
GAAGAGCTCAAGCCGATTTTATTGAACAAGACTGTTCCACTGTGTTCTGCTCAGTATGAG  1080
E  E  L  K  P  I  L  L  N  K  T  V  P  L  C  S  A  Q  Y  E    308
AGACAGTTCAGCACGACACGTATTCCTGGCATCGAAACAGACACCTTGGTACATCACAAA  1140
R  Q  F  S  T  T  R  I  P  G  I  E  T  D  T  L  V  H  H  K    328
GACAGTAACCACATAGCTGTGTATTATCGTGGCCGATACTTTAAGGTCTACATCAGGCAT  1200
D  S  N  H  I  A  V  Y  Y  R  G  R  Y  F  K  V  Y  I  R  H    348
AAGGGCAGACTACTCAAGCCCATTCAGATTGAATCAATGCTGCAGAAGATTTTAGATGAC  1260
K  G  R  L  L  K  P  I  Q  I  E  S  M  L  Q  K  I  L  D  D    368
ACCACTGAGCCATCATATGGTGAAGAGCATCTGGCTGCCCTGACTGCCGGGGAGAGGGTC  1320
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T  T  E  P  S  Y  G  E  E  H  L  A  A  L  T  A  G  E  R  V    388
AACTGGGCGAAGGCAAGGAAGGAGTTTTTCTCAAAGGGCAAGAACAAGGCTTCACTGGAT  1380
N  W  A  K  A  R  K  E  F  F  S  K  G  K  N  K  A  S  L  D    408
GCTATTGAGAAGGCTGCCTTCTGTGTGACATTGGATGAAGAAGCACATGGCTATGATGCG  1440
A  I  E  K  A  A  F  C  V  T  L  D  E  E  A  H  G  Y  D  A    428
AATGATCCAGCCAGTCTGGACAGGTTTGGAAAAGCCATGCTTCATGGAAAAGGCCATGAC  1500
N  D  P  A  S  L  D  R  F  G  K  A  M  L  H  G  K  G  H  D    448
CGGTGGTTTGACAAGTCTTTCACTCTGGTTGTGTGTTCTAACGGTCATATTGGCTTCAAT  1560
R  W  F  D  K  S  F  T  L  V  V  C  S  N  G  H  I  G  F  N    468
GCAGAGCATTCCTGGGCTGATGCCCCAATCATGGCTCATATGTGGGAGTACGCAATCAGT  1620
A  E  H  S  W  A  D  A  P  I  M  A  H  M  W  E  Y  A  I  S    488
GATGAAGTACATTATGCAGGATACACCTCAGAAGGCAAGTGTGTTGGTGAAGCTGATCCT  1680
D  E  V  H  Y  A  G  Y  T  S  E  G  K  C  V  G  E  A  D  P    508
AACACACCTAACCCCATAAGACTGGAGTGGGACATCTCGGAACAGGCCAACTCGGTGATA  1740
N  T  P  N  P  I  R  L  E  W  D  I  S  E  Q  A  N  S  V  I    528
GAGAACAGCTTGCAGATTGCACACCAGCTCATTAGTGATGTTGATCTCAATGTGATCATG  1800
E  N  S  L  Q  I  A  H  Q  L  I  S  D  V  D  L  N  V  I  M    548
TTCAAAGATTATGGTAAAGGTTTCATCAAGAAGTGCAAAGTGTCCCCTGATGCCTTCATA  1860
F  K  D  Y  G  K  G  F  I  K  K  C  K  V  S  P  D  A  F  I    568
CAGATGGCTCTACAGCTCACATACCACAGGTCTACCAACAAGTTCTGTCTTACCTATGAG  1920
Q  M  A  L  Q  L  T  Y  H  R  S  T  N  K  F  C  L  T  Y  E    588
TCCTCCATGACCCGACTGTTCAGAGAAGGACGAACTGAGACTGTCCGATCATGCACTGTG  1980
S  S  M  T  R  L  F  R  E  G  R  T  E  T  V  R  S  C  T  V    608
GAGTCTTGTGCATTTGTGAGAGCAATGGATGACAAGAATAACACTAATGCTGGACGTATC  2040
E  S  C  A  F  V  R  A  M  D  D  K  N  N  T  N  A  G  R  I    628
AAACTGCTGAAGGAGGCCGCAGAGGTCCATCAGCATGCGTATAGAGAGTGCATGACTGGA  2100
K  L  L  K  E  A  A  E  V  H  Q  H  A  Y  R  E  C  M  T  G    648
CTTGGTATTGACAGGCATCTCTTCTGTCTGTATGTCGTCTCTAAATACCTTGGTGTTGAC  2160
L  G  I  D  R  H  L  F  C  L  Y  V  V  S  K  Y  L  G  V  D    668
TCTCCATTCTTGGCTGAAGTTCTCAGTGGGCCCTGGAGGCTGTCAACAAGTCAGACTCCT  2220
S  P  F  L  A  E  V  L  S  G  P  W  R  L  S  T  S  Q  T  P    688
CACCAGCAGACAGACAAGCTGGACCTGAAGAAATTCCCAGACAGAATCTGTGCAGGAGGT  2280
H  Q  Q  T  D  K  L  D  L  K  K  F  P  D  R  I  C  A  G  G    708
GGCTTTGGGCCGGTTGCTGATGATGGGTATGGAGTGTCCTATGTTGTAGCTGGGGAGGAC  2340
G  F  G  P  V  A  D  D  G  Y  G  V  S  Y  V  V  A  G  E  D    728
ACCATCTTCTTCCATATATCGTCCAAGAAGTCCTGTCCACTTACTAATTCTCCGAAGTTT  2400
T  I  F  F  H  I  S  S  K  K  S  C  P  L  T  N  S  P  K  F    748
GGCATTGAATTGAGGCGCTCCCTTAATGACATGCGAGAGCTGTTCACGAAAGAGGATTGA  2460
G  I  E  L  R  R  S  L  N  D  M  R  E  L  F  T  K  E  D  *    768
CATGAGTAAAGCAGGTAGATAGACATCCAGCAGATACCTAGACACACCAGCCAACCACAT  2520
GATCCACTTGAAATGTCCTGATATGCAGGTGGAGTCCGAGTGTGTGTAGGTGATATAGAT  2580
CTGAAGAGCCATGTTTGACTGTGATGCAGTGAAGATGTACAGGTGTTATATTTTATGTGT  2640
TAACTGTTTTTTACATTTATCATTTCTGTCGTACAAACCACATGGTTTGGTGGATATTAT  2700
GAACGTGCACATCAAATGCAATCTTTTTACTCAATGCATCCTGTACAATTTTAATTTATT  2760
TGAGAGATGACATTTTAGCGGAATATCCTGCATCTTCAGCAATGCCTGCTTAGAGATATG  2820
TGCCATGCTTAAATTAACACTAAGTCGACTGGTTCTCAGATGGAGTGCTGCTCTATCAGG  2880
GGAGCTAACTCTTACTAACTTACCAGCACCAAACTGGTGCTATTTTTCTGTCATACATAT  2940
CTGTATCCATCACGATTGGAATTTATTTATCTTAGAATGCTGAAAGCAATGTGCCATGGA  3000
ATATTCAGCTATTTGTACCAAGATATGAGATATGTATTTTATCACAGCTTTAGCATGCTG  3060
TGTCTTTAGTTTTATCTTCAATGCTGGCATGAGAGTCTTTCCTTTATTATGACATGTTTA  3120
ACAAAAGAAATACTTTTATTGTGTATCATTTGAATTGCCATTTTCATACAGATATTATCT  3180
TGAAAGTCAGGTTTTTACTTCACTGAATTACATTTCAAATTGTACTTTTATTCTTTATGC  3240
AAACACATAAAATTTAACATAGATCATTTGTATATTTATTAAAAGAACTGTTTTAATTGT  3300
ACATATTGGTGTGCGATGTAGTGAGAGTGTTCACTCATAATTTAAAGCATAATGTGTATC  3360
ACTTAAGTCTGGGTTTCTCTCTTCCTTCATTTTGGTTATTGGGTTAGCATAACCAGTAAG  3420
GGAATAGGTATGATGTCCCTGTTGGCTAGGTGTCACATGTTTACTGTCTACTGTGTCTCA  3480
ACAGATCTCATGTTTATCATATTTCCTGTACCAAGAGAGGGTAGATGAAATACAGATATA  3540
ATATCAATGGTATCAGGATTTTTAGAGATTATTGTCCAGGTAAAACTGGAAATGTACATA  3600
ATTTTAAGGAAATGTCAAGTTTATTCATACTGGATATTCTAAAATCAATTTTGACAAAAA  3660
TATTTCATTTTATTTACAAAACAATTACAACTAAAATGTTCATGTTTTAACCCTTTTAGG  3720
AATTTTTAAAAGATAAAAAATATGAGACAGAATCTTCAATCCACATACTACAGAACTGTG  3780
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ACCTTGTTACCATGGGAATAAGAACAGGTTTAGTAACAACAGCTGTTGTAGCACTGATAA  3840
CTGGCGTAGCCACATCATCATCAAAAATGCATCATCATTGTCATCTGTATGTTCAGAATG  3900
TATGTTTCTGTTTATGTGCACTGTTTTCACATTGGTACAAATTTTATTCTTCTGCTTGAG  3960
GCAGGGTGATTTTAGTTAATTTATTGACAAAAAAACAACAACAGATGGTTCTAGTTTTGT  4020
GAAAATAAAAAGGTGTACTACAAAAAAAAAAAAAAAAAAAAAAAA                 4067
```

Figure 3.38 The full length cDNA and deduced amino acid sequence of *carnitine palmitoyltransferase IA* of *H. asinina*. The putative start and stop codons are boldfaced and underlined. The polyadenyltion signal (AATAAA) are boldfaced. The predicted Carn_acyltransf domain is highlighted.

Figure 3.39 Diagram illustrating 3 transmembrane domains (residues 50 - 72, 87 - 109 and 114 - 136, respectively) and a predicted Carn_acyltransf domain (residues 170 - 761) found in the deduced carnitine palmitoyltransferase IA protein of *H. asinina*.

An EST (ovarian cDNA library, clone no. 149, 785 bp insert) significantly matched *hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* (*trifunctional protein*) *α-subunit* of *Mus musculus* (*E*-value = 1 x 10^{-75}) was further characterized. A gene-specific primer was designed for 3′ RACE-PCR of this gene (Table 3.17).

The amplification fragment of approximately 1300 bp in length was cloned and sequenced (Figure 3.40). Internal primer was designed for primer walking of the cloned fragment. Nucleotide sequences of EST and 3′ RACE s of this gene were assembled (Figure 3.41). The full length cDNA of *H. asinina hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* was 2548 bp in length consisting of an ORF of 2292 bp corresponding to a polypeptide of 764 amino acids and the 5′ and 3′ UTRs of 16 and 240 bp, respectively (Figure 3.42). This transcript

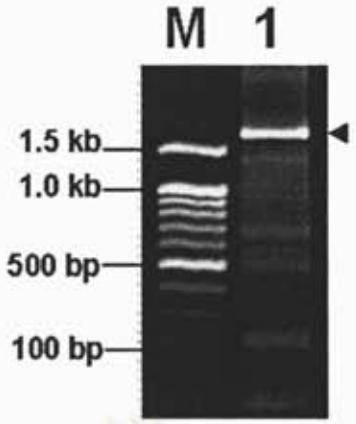

Figure 3.40 3′ RACE-PCR products (lane 1) of *hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase*. An arrowhead indicates bands selected for further analysis. Lane M is a 100 bp DNA marker.

A.

GGCCTTGTTGACAGCTTGGTAGATCCTCTAGGTCCTGGCATTGCTCCCCCGGAGGAAAGAACTCTTCAG
TATTTGGAGGACGTGGGCATTCAAACAGCCAGGGGTCTAGCAAATGGGCACTGAAGAAGACACCTAGAA
AAAAAGGTTTACAAGATAAGATCATGGACACCATGTTACAGTATGACTTTGGCAAGAACTTTGTTTTCA
AGAAAGCTCGTGGGCAGGTGATGAAATTGTCACAGGGACTTTATCCAGCACCTCTGAAGATACTGGAAG
TGGTGAGGACTGGTCTTGATGAAGGGAAGGAAGCTGGATACAAGGCAGAGGCAAAGGGATTTGCTCAGC
TGGGAATGACATCTCAGTCCAAGGGCCTTATAGGACTGTACTATGGGCAGACAGCTTGCAAAAAGAATG
CGTTTGGAGAGCCCAAGAAAGAAGTAAAGACGCTAGCAGTGCTTGGTGCTGGTCTGATGGGCGCCGGCA
TTGCTCAGGTGAGCATCGACAAAGGCATGAGCGTTATCCTGAAGGACATGTCCACCCAGGGTCTGGCTC
GGGGACAGGACCAGGTACAGAAGGGCTTTGACATGCAGGTCAAAAAGAGGAAAATCACAACCGCTGACA
GAGATTTAATCATGTCTAATCTGGACTGTACCTTGTCGTATGAGAACTTCAAGAATTGTGACATGGTGA
TTGAAGCTGTGTTTGAAGACATTGGTATCAAGCACAAGGTTGTCAAAGAAGTGGAGCAGCACCTGCCTG
AACACTGTATCTTTGCATCCAAAACTTCTGCACTCCCTATCGCCAGAATTGCAAAGGCCTCCTCCCGAC
CAGACAGGTTCATTGGAATGCACTATTTCTCCCCCAGTTGACAAGATGCAGTTCTTGAGATTTCACCCC
GGACAAGAAGGTGTTCTAACCCACGTCAAGAATGGGCCTGGTTTTTACACAACCAAGAATTATCGCTCC
CATGTTGGGCGAGAGGGCAATCAGGTGGTGCAGGAAGGGTGTTTAGTTCCAACAAGACCTTTGACAGAC
CCAATAAGAAATTTGGCTTCCCAGTGGGGAGCTGCCACCACCTGCAGATGAAGTGGGCGTTGATTGGGG
CTGCACCTGTAGCAGAAGATCTAGGAAAGGCTTTTGGGGAGAGGTTTGGAGGCGGAAGCCCTGAAGTGT
TGAAGGACATGGTTGCCAGTGGTTTCCTAGGCCGCAAGAGCGGAAAAGGCTGCTATGGGTATGATAAGG
GATCCAAGGATAGACCAGAAAATGAAGAAGCAAAGAAGATTGTTCAGAAGTACCATCTTGAACCTAAAC
TAGTGAACACGGATGAAGATGTGCAGTACCGACTTGTGTCCAGATTTGTCAATGAGGCAGTAATGTGTC
TCCAAGAAGGAATCCTCAGAAATCCTCTGGAAGGGGACATTGGTGCTGTGTTTGGTCTTGGATTCCCTC
CATTCCTTGGTGGCCCGTTCCGCTACATGGACCTGCATGGAGCCAAGCCCCTGGTGGATCGCATGCTCA
AGTACAGAGACTTGTATGGAATCCAGTTCCAGCCATGTCAGTTGCTCCTAGACCATGCCAACGATCCAT
CTAAGAAGTTCCACTCTTGAGCCTGAACAAAATGGACGTTAGGATTATGACTGTGATGTTATCCATGAC
GTGCAGCAGTTGCCTCCCTTGTTGAGGAGTGTCAGATTTTCATCATGTGCTTATCAAGTTGAATGAGTG
GTGTACACATTTCATGCAAGGTGCTACATCTATTGTAAATAATATTGTATGAATGAATATCAGAGTTTT
CCAATAAAACTTGCATATGTAACAGAAAAAAAAAAAGAAAAAAAAAAAAAAAAA

B.

ACCGGCTTCTTTGGAAATGGCCGGCATGAGGGGTGTTGGGCTGCGAGACAGCTAATCCGTGCCAGGAGG
GCTAGCGTTTCAGGGAGACAAGAAATGCAGGCTTCTCTCCACTTCTGCTGTGATGTGTGCTCAGAAACA
CTTCAAAGTTGATCAGAAGGGTGATGTGGCTGTTGTACGGATTGATAGCCCAAACTCAAAGGTTAATAC
CTTGTCAGTGGAGGTCCAGACGGAGTTCTTGGAAGTCCTAAATGAGGTCCAGAATAACAGCAGCGTTAC
AGCGTTGTTCTCATATCGCTCAAAACCAGGATGTTTCATTGCAGGCGCAGATATTGGGATGATTGATTC
TTGCAAGACTGAAGAAGAAGTGATGAGAATATCTCAGGCCGGACAACAAGCCTTCCAGACGATGGAGGA
AAGTCCCAAACCGATTGTCGCTGCCATCATGGGATCGTGCCTTGGTGGTGGTCTTGAGGTGGCCTTGCA

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TGTCAGTACCGGCTGGCTGTGAAGGATCGCGAGACCAGTCTTGCTTTACCGGAAGTGATGCTTGGTCTT
CTACCGGGAGCAGGGGGCACACAAAGACTGCCAAGACTTATTAGTGTCCCAAATGCCCTTGACATGATG
TTGACTGGGAAAGACATTCGTCCAGACAAAGCCAAGAAGATGGGCCTTGTTGACAGCTTGGTAGATCCT
CTAGGTCCTGGCATTGCTCCCCCGGAGGAAAGAACTCTTCAGTATTTGGAGGACGTGGGCATTCAAACA
GCCAGGGGTCTAGCAAATGGGCACTG
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Figure 3.41 Nucleotide sequence of 3' RACE-PCR (A) and EST (B) of *H. asinina hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase*. The putative stop codin is highlighted.

was significantly similar to *hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* of *Xenopus tropicalis* (*E*-value = 0.0). Three functional domains including ECH domain (residues 51 – 224, E-value = 3.2 x 10^{-63}), 3HCDH_N domain (residues 365 – 543, E-value = 2.5 x 10^{-65}), and 3HCDH domain (residues 545 – 640, *E*-value = 1.7 x 10^{-40}) were found in the deduced protein of this gene (Figure 3.43).

```
ACCGGCTTCTTTGGAAATGGCCGGCATGAGGGGTGTTGGGCTGCGAGACAGCTTATCCGT  60
                  M  A  G  M  R  G  V  G  L  R  D  S  L  S  V  15
GCCAGGAGGGCTAGCGTTTCAGGGAGACAAGAAATGCAGGCTTCTCTCCACTTCTGCTGT  120
 P  G  G  L  A  F  Q  G  D  K  K  C  R  L  L  S  T  S  A  V  35
GATGTGTGCTCAGAAACACTTCAAAGTTGATCAGAAGGGTGATGTGGCTGTTGTACGGAT  180
 M  C  A  Q  K  H  F  K  V  D  Q  K  G  D  V  A  V  V  R  I  55
TGATAGCCCAAACTCAAAGGTTAATACCTTGTCAGTGGAGGTCCAGACGGAGTTCTTGGA  240
 D  S  P  N  S  K  V  N  T  L  S  V  E  V  Q  T  E  F  L  E  75
AGTCCTAAATGAGGTCCAGAATAACAGCAGCGTTACAGCGTTGTTCTCATATCGCTCAAA  300
 V  L  N  E  V  Q  N  N  S  S  V  T  A  L  F  S  Y  R  S  K  95
ACCAGGATGTTTCATTGCAGGCGCAGATATTGGGATGATTGATTCTTGCAAGACTGAAGA  360
 P  G  C  F  I  A  G  A  D  I  G  M  I  D  S  C  K  T  E  E  115
AGAAGTGATGAGAATATCTCAGGCCGGACAACAAGCCTTCCAGACGATGGAGGAAAGTCC  420
 E  V  M  R  I  S  Q  A  G  Q  Q  A  F  Q  T  M  E  E  S  P  135
CAAACCGATTGTCGCTGCCATCATGGGATCGTGCCTTGGTGGTGGTCTTGAGGTGGCCTT  480
 K  P  I  V  A  A  I  M  G  S  C  L  G  G  G  L  E  V  A  L  155
GGCATGTCAGTACCGGCTGGCTGTGAAGGATCGCGAGACCAGTCTTGCTTTACCGGAAGT  540
 A  C  Q  Y  R  L  A  V  K  D  R  E  T  S  L  A  L  P  E  V  175
GATGCTTGGTCTTCTACCGGGAGCAGGGGGCACACAAAGACTGCCAAGACTTATTAGTGT  600
 M  L  G  L  L  P  G  A  G  G  T  Q  R  L  P  R  L  I  S  V  195
CCCAAATGCCCTTGACATGATGTTGACTGGGAAAGACATTCGTCCAGACAAAGCCAAGAA  660
 P  N  A  L  D  M  M  L  T  G  K  D  I  R  P  D  K  A  K  K  215
GATGGGCCTTGTTGACAGCTTGGTAGATCCTCTAGGTCCTGGCATTGCTCCCCCGGAGGA  720
 M  G  L  V  D  S  L  V  D  P  L  G  P  G  I  A  P  P  E  E  235
AAGAACTCTTCAGTATTTGGAGGACGTGGCCATTCAAACAGCCAGGGGTCTAGCAAATGG  780
 R  T  L  Q  Y  L  E  D  V  A  I  Q  T  A  R  G  L  A  N  G  255
GACACTGAAGAAGACACCTAGAAAAAAAGGTTTACAAGATAAGATCATGGACACCATGTT  840
 T  L  K  K  T  P  R  K  K  G  L  Q  D  K  I  M  D  T  M  L  275
ACAGTATGACTTTGGCAAGAACTTTGTTTTCAAGAAAGCTCGTGGGCAGGTGATGAAATT  900
 Q  Y  D  F  G  K  N  F  V  F  K  K  A  R  G  Q  V  M  K  L  295
GTCACAGGGACTTTATCCAGCACCTCTGAAGATACTGGAAGTGGTGAGGACTGGTCTTGA  960
 S  Q  G  L  Y  P  A  P  L  K  I  L  E  V  V  R  T  G  L  D  315
TGAAGGGAAGGAAGCTGGATACAAGGCAGAGGCAAAGGGATTTGCTCAGCTGGGAATGAC  1020
```

```
 E  G  K  E  A  G  Y  K  A  E  A  K  G  F  A  Q  L  G  M  T   335
ATCTCAGTCCAAGGGCCTTATAGGACTGTACTATGGGCAGACAGCTTGCAAAAAGAATGC  1080
 S  Q  S  K  G  L  I  G  L  Y  Y  G  Q  T  A  C  K  K  N  A   355
GTTTGGAGAGCCCAAGAAAGAAGTAAAGACGCTAGCAGTGCTTGGTGCTGGTCTGATGGG  1140
 F  G  E  P  K  K  E  V  K  T  L  A  V  L  G  A  G  L  M  G   375
CGCCGGCATTGCTCAGGTGAGCATCGACAAAGGCATGAGCGTTATCCTGAAGGACATGTC  1200
 A  G  I  A  Q  V  S  I  D  K  G  M  S  V  I  L  K  D  M  S   395
CACCCAGGGTCTGGCTCGGGGACAGGACCAGGTACAGAAGGGCTTTGACATGCAGGTCAA  1260
 T  Q  G  L  A  R  G  Q  D  Q  V  Q  K  G  F  D  M  Q  V  K   415
AAAGAGGAAAATCACAACCGCTGACAGAGATTTAATCATGTCTAATCTGGACTGTACCTT  1320
 K  R  K  I  T  T  A  D  R  D  L  I  M  S  N  L  D  C  T  L   435
GTCGTATGAGAACTTCAAGAATTGTGACATGGTGATTGAAGCTGTGTTTGAAGACATTGG  1380
 S  Y  E  N  F  K  N  C  D  M  V  I  E  A  V  F  E  D  I  G   455
TATCAAGCACAAGGTTGTCAAAGAAGTGGAGCAGCACCTGCCTGAACACTGTATCTTTGC  1440
 I  K  H  K  V  V  K  E  V  E  Q  H  L  P  E  H  C  I  E  A   475
ATCCAAAACTTCTGCACTCCCTATCGCCAGAATTGCAAAGGCCTCCTCCCGACAGACGGT  1500
 S  K  T  S  A  L  P  I  A  R  I  A  K  A  S  S  R  Q  T  V   495
TCATTGGATGCACTATTTCTCACCAGTTGACAAGATGCAGTTGCTTGAGATTATCACCAC  1560
 H  W  M  H  Y  F  S  P  V  D  K  M  Q  L  L  E  I  I  T  T   515
AGACAAGACTTCAAAGGATACCACAGCCTCAGCTGTGAAGGTTGGGCTGAGACAGGGAAA  1620
 D  K  T  S  K  D  T  T  A  S  A  V  K  V  G  L  R  Q  G  K   535
GGTTGTCATCACAGTCAAGGATGGGCCTGGCTTTTACACAACCAGAATACTGGCTCCCAT  1680
 V  V  I  T  V  K  D  G  P  I  F  Y  T  R  L  A  P  M        555
GTTGGCAGAGGCAATCAGGCTGCTGCAGGAAGGTGTTAGTCCAACAGACCTTGACAGACT  1740
 L  A  E  A  I  R  I  L  Q  T  L  V  S  P  T  D  L  D  R  L   575
CACTAAGAAATTTGGCTTCCCAGTGGGAGCTGCAACACTTGCAGATGAAGTGGGCGTTGA  1800
 T  K  F  G  F  P  V  G  A  T  C  A  D  F  V  G  V  D        595
TGTGGCTGCACATGTAGCAGAAGATCTAGGAAAGGCTTTTGGGGAGAGGTTTGGAGGCGG  1860
 V  A  A  H  Y  R  E  D  L  G  K  R  E  T  F  R  E  G  G  G   615
AAGCCCTGAAGTGTTGAAGGACATGGTTGCCAGTGGTTTCCTAGGCCGCAAGAGCGGAAA  1920
 S  P  K  V  L  K  D  M  V  A  S  G  F  L  G  R  K  S  G  K   635
AGGCTGCTATGTGTATGATAAGGGATCCAAGGATAGACCAGAAAATGAAGAAGCAAAGAA  1980
 G  C  Y  V  Y  D  K  G  S  K  D  R  P  E  N  E  E  A  K  K   655
GATTGTTCAGAAGTACCATCTTGAACCTAAACTAGTGAACACGGATGAAGATGTGCAGTA  2040
 I  V  Q  K  Y  H  L  E  P  K  L  V  N  T  D  E  D  V  Q  Y   675
CCGACTTGTGTCCAGATTTGTCAATGAGGCAGTAATGTGTCTCCAAGAAGGAATCCTCAG  2100
 R  L  V  S  R  F  V  N  E  A  V  M  C  L  Q  E  G  I  L  R   695
AAATCCTCTGGAAGGGGACATTGGTGCTGTGTTTGGTCTTGGATTCCCTCCATTCCTTGG  2160
 N  P  L  E  G  D  I  G  A  V  F  G  L  G  F  P  P  F  L  G   715
TGGCCCGTTCCGCTACATGGACCTGCATGGAGCCAAGCCCCTGGTGGATCGCATGCTCAA  2220
 G  P  F  R  Y  M  D  L  H  G  A  K  P  L  V  D  R  M  L  K   735
GTACAGAGACTTGTATGGAATCCAGTTCCAGCCATGTCAGTTGCTCCTAGACCATGCCAA  2280
 Y  R  D  L  Y  G  I  Q  F  Q  P  C  Q  L  L  L  D  H  A  N   755
CGATCCATCTAAGAAGTTCCACTCTTGAGCCTGAACAAAATGGACGTTAGGATTATGACT  2340
 D  P  S  K  K  F  H  S  *                                     764
GTGATGTTATCCATGACGTGCAGCAGTTGCCTCCCTTGTTGAGGAGTGTCAGATTTTCAT  2400
CATGTGCTTATCAAGTTGAATGAGTGGTGTACACATTTCATGCAAGGTGCTACATCTATT  2460
GTAAATAATATTGTATGAATGAATATCAGAGTTTTCCAATAAAACTTGCATATGTAACAG  2520
AAAAAAAAAAAAAAAAAAAAAAAAAAA                                   2548
```

Figure 3.42 The full length cDNA and deduced amino acid sequence of *hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase (trifunctional protein)* of *H. asinina*. The putative start and stop codons are boldfaced and underlined. The polyadenyltion signal (AATAAA) are boldfaced.

Figure 3.43 Diagram illustrating predicted ECH (residues 51 – 224), 3HCDH_N (residues 365 – 543), and 3HCDH (residues 545 – 640) domains found in the deduced *hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* of *H. asinina*.

In addition, an EST exhibiting significant similarity to *vacuolar H^+ ATPase (V-ATP) 14 kDa subunit* of *Apis mellifera* previously identified from the hemocyte cDNA library of *H. asinina* (clone no. 130, 486 bp insert) was also characterized for both 5′ and 3′ directions. Gene-specific primer were designed (Table 3.17) and used for 5′ and 3′ RACE-PCR analysis.

The amplification product of approximately 1450 bp obtained from 5′ RACE-PCR and 290 and 500 bp fragments obtained from 3′ RACE-PCR were cloned and sequenced (Figure 3.44). Nucleotide sequences of EST, 5′ and 3′ RACE-PCR of this gene were assembled (Figure 3.45). The full length cDNA of *H. asinina V-ATP* was 1723 bp in length composing of an ORF of 369 bp corresponding to a polypeptide of 123 amino acids and the 5′ and 3′ UTRs of 20 and 1334 bp, respectively (Figure 3.46). It significantly matched *V-ATP* of *Apis mellifera* (*E*-value = 1 x 10^{-48}). The ATP-synt_F was found at positions 11 – 111 of the deduced V-ATP protein (*E*-value = 1.4 x 10^{-47} (Figure 3.47).

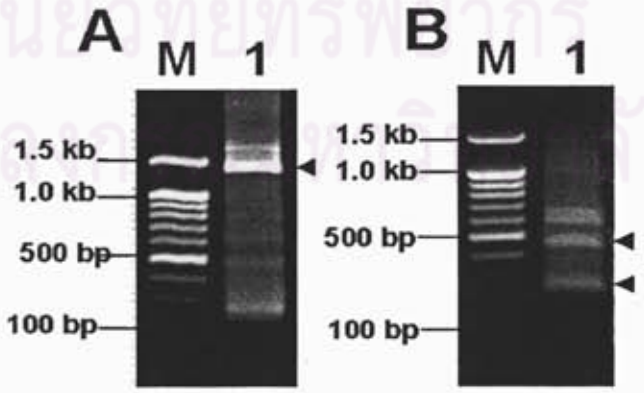


Figure 3.44 Agarose gel electrophoresis of 3′ RACE-PCR (A), and 5′ RACE-PCR (B) products of *vacuolar H^+ ATPase (V-ATP), 14 kDa subunit*. Arrowheads indicate bands selected for further analysis. Lane M is a 100 bp DNA marker.

A.

ACCGGGGGCACTTGTTCAAGATGGCGATATCAGCAGCAAGGGGACGGCTAATAGCTGTCATTGGTGATG
AGGACACCTGCACTGGTTTCGTGCTGGGTGGAATTGGAGAAATGAACAAACACAGACACCCAAATTTCC
TGATTGTTGACAAGAACACAAGTGTTACAGAGATAGAGGATGTATTCCGGTCTTTTTTGAAGCGGGATG
ACATTGCCATCATT

B.

TGCCTGGAGTACAGTGCTCAGTGTGATGTAGGCTGGCCTACCTCCGGTCCCTTGGACTCACCTCCATGT
TGAGGGACACAGTCAATATATATTAGCAGAGGACATTGTGTGCAGGGGGCAGACAACAAGAAGCAAATT
TAATAAATACTACTCTGTAACTCTACAGCCTTAAACATTTGTTTCTATTATTGTTGCTGGCCCAGCTTG
AATGAGATGTCTCTGAGCATTATCTTTGTTTACATTTTTGTCTGTTTAATTTCATTCCCCTTTTAATTA
AAACTGGTTCAGAATTCTGCTCTAACTCTATGAGTCAATAAGGACCAACACTGCTGGTGTTCTCCATAC
AATTGTTTTATTTTAGGCGAGAGGAAGTTCATGAATTTCAAAGGCTGTTTCATTAGTTGCAAAAATGTT
TAACAAATGAAGATAATATTGGCATGTTGTGACATTGGATATTTCACATTTATGATTGTTCAAAGTTTA
CATGACAATATGAATTGATGTTATTAAGTGAAGGTTAAATTGCCTGTCTACAAATGTCATTGAAGTGAA
CATAACATTTTGTCTATGACCTGAATTTGAATACAGATTGTAGGCTGAATCTCTCTCTGCACACATGCC
TTGGTATGGGGTATTATATAATTTTGAAATCATTCTTTTAAGTTCTTTAAGAGTAATCAAAACTAGATT
GTAGAAATCTTTTCTCATTTTCAGCCATAACATTCAAAATATTTTTTGCTTTGATTCAGATTATCACTC
TACTTCTCTTTCGTCCAACTTGTGTGAATTTTCAGTACAGCTTTTCATTTCGTACTGATTCACATAGAA
GCAAACATTTCTTTTTGCTTCATTTCATTTTAGAGGAATTATTTATGCAAGCTATAATATGATCTCATG
AGACCAAGGAACACTAGTGTGATGTCAAGTGTCACTGTTTTCATGTCACATGTCTGGGTTGGTTAATAT
TACCTGATACCACGGATGTACCAACAAAAACGTTGCAATCTAGTCAAGACATTATAAATGACTTGTGTG
ACTTGTGAAAACAAGGGAGGTAACTCATGTTTTTCACATCGTAGCACTAAAGGCAAATAAATGTTACAC
CTAGTAACTCTGAAACAAGGCTTAGATCAGTGATAGATATCTGTTAGTGTTATCACATCTTTGTTGTTT
ACAGATTGTTTTGACTACATGTAGTTACACAGGAGGGTTGACTGTTCTTTGGTGTAAGTGTCTGTTTCT
CTTCATGTTATTTGAATAAGGTATAAGTATGAAAACTGAAATAAAATAGATCAGTACAAAAAAAAAAAA
AAAAAAAAAAAAAAAAA

C.

TGGCGATATCAGCAGCAAGGGGACGGCTAATAGCTGTCATTGGTGATGAGGACACCTGCACTGGTTTCG
TGCTGGGTGGAATTGGAGAAATGAACAAACACAGACACCCAAATTTCCTGATTGTTGACAAGAACACAA
GTGTTACAGAGATAGAGGATGTATTCCGGTCTTTTTTGAAGCGGGATGACATTGCCATCATTCTCATCA
ACCAAACTGTAGCTGAGATGATTCGTCATGTGATTGACTCCCATCTCGAACCCATTCCTGCTGTGCTAG
AGATCCCCAGCAAGGACGCACCATACGATGCTTCAAAAGATTCCATTTTGCGACGTGCTAAGGGCATGT
TTTCTGCAGAAGACTTGCGGTAGAAGATCTGCCTGGAGTACAGTGCTCAGTGTGATGTAGGCTGGCCTA
CCTCCGGTCCCTTGGACTCACCTCCATGTTGAGGGACACAGTCAATATATATTAGCAGAGGACATTGTG
TGCAGGGGGCAG

Figure 3.45 Nucleotide sequence of 5′ RACE-PCR (A), 3′ RACE-PCR (B) and EST (C) of *H. asinina vacuolar H⁺ ATPase* (*V-ATP*), *14 kDa subunit*.

```
ACCGGGGGCACTTGTTCAAGATGGCGATATCAGCAGCAAGGGGACGGCTAATAGCTGTCA  60
                     M  A  I  S  A  A  R  G  R  L  I  A  V  I  14
TTGGTGATGAGGACACCTGCACTGGTTTCGTGCTGGGTGGAATTGGAGAAATGAACAAAC  120
  G  D  E  D  T  C  T  G  F  V  L  G  G  I  G  E  M  N  K  H  34
ACAGACACCCAAATTTCCTGATTGTTGACAAGAACACAAGTGTTACAGAGATAGAGGATG  180
  R  H  P  N  F  L  I  V  D  K  N  T  S  V  T  E  I  E  D  V  54
TATTCCGGTCTTTTTTGAAGCGGGATGACATTGCCATCATTCTCATCAACCAAACTGTAG  240
  F  R  S  F  L  K  R  D  D  I  A  I  I  L  I  N  Q  T  V  A  74
CTGAGATGATTCGTCATGTGATTGACTCCCATCTCGAACCCATTCCTGCTGTGCTAGAGA  300
  E  M  I  R  H  V  I  D  S  H  L  E  P  I  P  A  V  L  E  I  94
TCCCCAGCAAGGACGCACCATACGATGCTTCAAAAGATTCCATTTTGCGACGTGCTAAGG  360
  P  S  K  D  A  P  Y  D  A  S  K  D  S  I  L  R  R  A  K  G  114
GCATGTTTTCTGCAGAAGACTTGCGGTAGAAGATCTGCCTGGAGTACAGTGCTCAGTGTG  420
  M  F  S  A  E  D  L  R  *                                    123
```

```
ATGTAGGCTGGCCTACCTCCGGTCCCTTGGACTCACCTCCATGTTGAGGGACACAGTCAA  480
TATATATTAGCAGAGGACATTGTGTGCAGGGGGCAGACAACAAGAAGCAAATTTAATAAA  520
TACTACTCTGTAACTCTACAGCCTTAAACATTTGTTTCTATTATTGTTGCTGGCCCAGCT  600
TGAATGAGATGTCTCTGAGCATTATCTTTGTTTACATTTTTGTCTGTTTAATTTCATTCC  660
CCTTTTAATTAAAACTGGTTCAGAATTCTGCTCTAACTCTATGAGTCAATAAGGACCAAC  720
ACTGCTGGTGTTCTCCATACAATTGTTTTATTTTAGGCGAGAGGAAGTTCATGAATTTCA  780
AAGGCTGTTTCATTAGTTGCAAAAATGTTTAACAAATGAAGATAATATTGGCATGTTGTG  840
ACATTGGATATTTCACATTTATGATTGTTCAAAGTTTACATGACAATATGAATTGATGTT  900
ATTAAGTGAAGGTTAAATTGCCTGTCTACAAATGTCATTGAAGTGAACATAACATTTTGT  960
CTATGACCTGAATTTGAATACAGATTGTAGGCTGAATCTCTCTCTGCACACATGCCTTGG 1020
TATGGGGTATTATATAATTTTGAAATCATTCTTTTAAGTTCTTTAAGAGTAATCAAAACT 1080
AGATTGTAGAAATCTTTTCTCATTTTCAGCCATAACATTCAAAATATTTTTTGCTTTGAT 1140
TCAGATTATCACTCTACTTCTCTTTCGTCCAACTTGTGTGAATTTTCAGTACAGCTTTTC 1200
ATTTCGTACTGATTCACATAGAAGCAAACATTTCTTTTTGCTTCATTTCATTTTAGAGGA 1260
ATTATTTATGCAAGCTATAATATGATCTCATGAGACCAAGGAACACTAGTGTGATGTCAA 1320
GTGTCACTGTTTTCATGTCACATGTCTGGGTTGGTTAATATTACCTGATACCACGGATGT 1380
ACCAACAAAAACGTTGCAATCTAGTCAAGACATTATAAATGACTTGTGTGACTTGTGAAA 1440
ACAAGGGAGGTAACTCATGTTTTTCACATCGTAGCACTAAAGGCAAATAAATGTTACACC 1500
TAGTAACTCTGAAACAAGGCTTAGATCAGTGATAGATATCTGTTAGTGTTATCACATCTT 1560
TGTTGTTTACAGATTGTTTTGACTACATGTAGTTACACAGGAGGGTTGACTGTTCTTTGG 1620
TGTAAGTGTCTGTTTCTCTTCATGTTATTTGAATAAGGTATAAGTATGAAAACTGAAATA 1680
AAATAGATCAGTACAAAAAAAAAAAAAAAAAAAAAAAAAAAA                    1723
```

Figure 3.46 The full length cDNA and deduced amino acid sequence of *vacuolar H⁺ ATPase* (*V-ATP*), *14 kDa subunit* of *H. asinina*. The putative start and stop codons are boldfaced and underlined. The polyadenyltion signal (AATAAA) are boldfaced.

Figure 3.47 Diagram illustrating a predicted ATP-synt_F (residues 11 – 111) found in the deduced *Vacuolar H⁺ ATPase* (*V-ATP*) *14 kDa subunit* protein of *H. asinina*.

3.5 Examination of expression levels of interesting genes related with metabolisms of *H. asinina* by Quantitative real-time PCR

Total RNA was extracted from hepatopancreas of each individual of *H. asinina* of the group C sample classified into fast (large)- and slow (snall)-growing subgroups (CL, $\overline{X} = 24.52 \pm 2.545$ g and CS, $\overline{X} = 7.59 \pm 1.468$ g, respectively). Gene specific primers for amplification of *α-methylacyl CoA racemase*, *Carnitine O-palmitoyltransferase I*, *hydroxyacyl CoA dehydrogenase/ 3-ketoacyl CoA thiolase/*

enoyl CoA hydratase (trifunctional protein) and *Vacuolar H+ ATPase 14kDA subunit* were designed and tested against the first strand cDNA of hepatopancreas of *H. asinina* (Table 3.18).

All primers generated the positive amplification product. Accordingly, the expression levels of these genes were quantitatively estimated by real-time PCR. *β-actin* was included as the control. The standard curve for the quantitative assay of each gene was constructed (Figure 3.48 – 3.52). The high amplification efficiency and low error was observed from amplification of each gene segment.

Table 3.18 Nucleotide sequences, length and Tm of primers used for quantitative real-time PCR analysis of various genes in *H. asinina*

Gene/ Primer	Sequence (5'-3')	Length (bp)	Tm (°C)
Alpha-methylacyl CoA racemase			
Forward	CTC CAA GGT ATC CGA GTC AT	20	60
Reverse	GTT GTC AGC CAT CAG CGT CT	20	62
Carnitine O-palmitoyltransferase I			
Forward	AGT GGG ACA TCT CGG AAC A	19	58
Reverse	CTT CTC TGA ACA GTC GGG TC	20	62
Hydroxyacyl CoA dehydrogenase/ 3-ketoacyl CoA thiolase/ enoyl CoA hydratase (trifunctional protein)			
Forward	GAA TAA CAG CAG CGT TAC AGC G	22	66
Reverse	TGA TGG CAG CGA CAA TCG GTT T	22	66
Vacuolar H+ ATPase 14kDA subunit			
Forward	GAC ATT GCC ATC ATT CTC AT	20	56
Reverse	GCC TAC ATC ACA CTG AGC AC	20	62
β-actin			
Forward	TGA TGG TCG GTA TGG GTC AGA A	22	66
Reverse	GCA ACA CGG AGC TCG TTG TAG	21	66

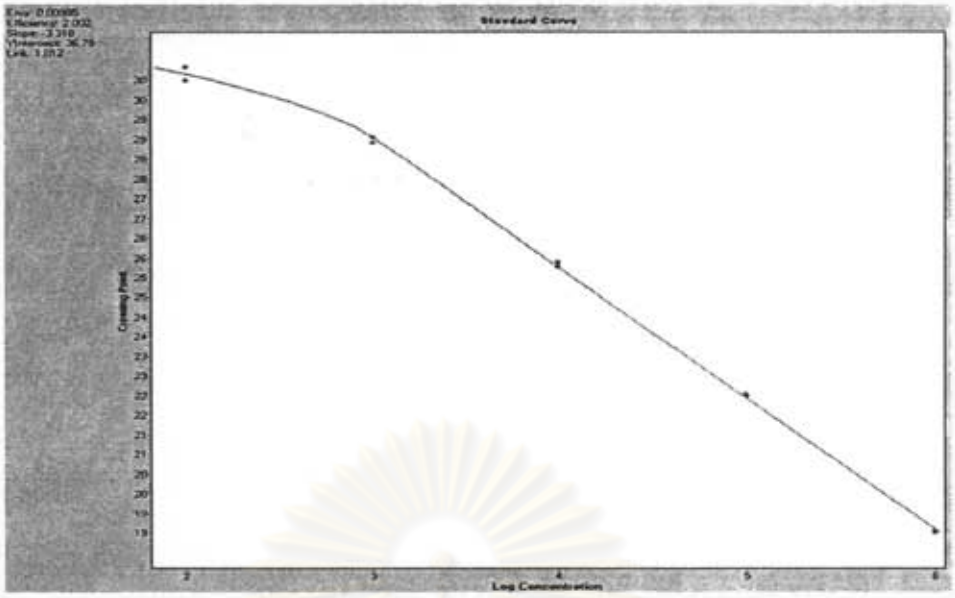


Figure 3.48 A standard curve of *α-methylacyl-CoA racemase* (*AMACR*) (error = 0.00985, efficiency = 2.002 and equation; Y = -3.318 * log(X) + 36.79) using 10-fold dilution of (10^2 – 10^8 copy) plasmid DNA of *AMACR*.

Figure 3.49 A standard curve of *carnitine O-palmitoyltransferase 1A* (*CPT-1A*) (error = 0.00385, efficiency = 1.954 and equation; Y = -3.437 * log(X) + 38.13) using 10-fold dilution (10^2 – 10^8 copy) of plasmid DNA of *CPT-1A*.

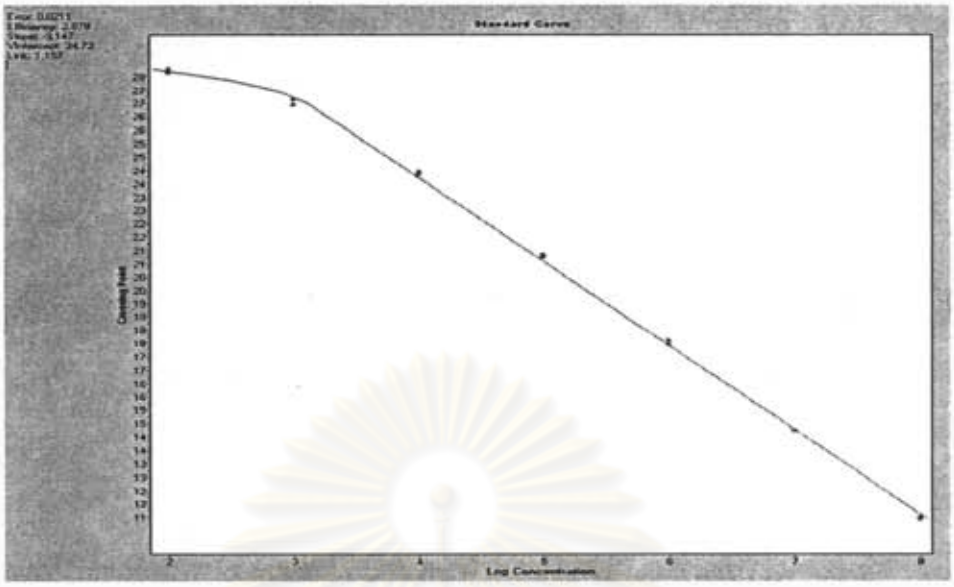


Figure 3.50 A standard curve of *hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* (*trifunctional protein*) (Error = 0.0211, efficiency = 2.078 and equation; Y = -3.147 * log(X) + 34.72) using 10-fold dilution (10^2 – 10^8 copy)of plasmid DNA of *trifunctional protein*.

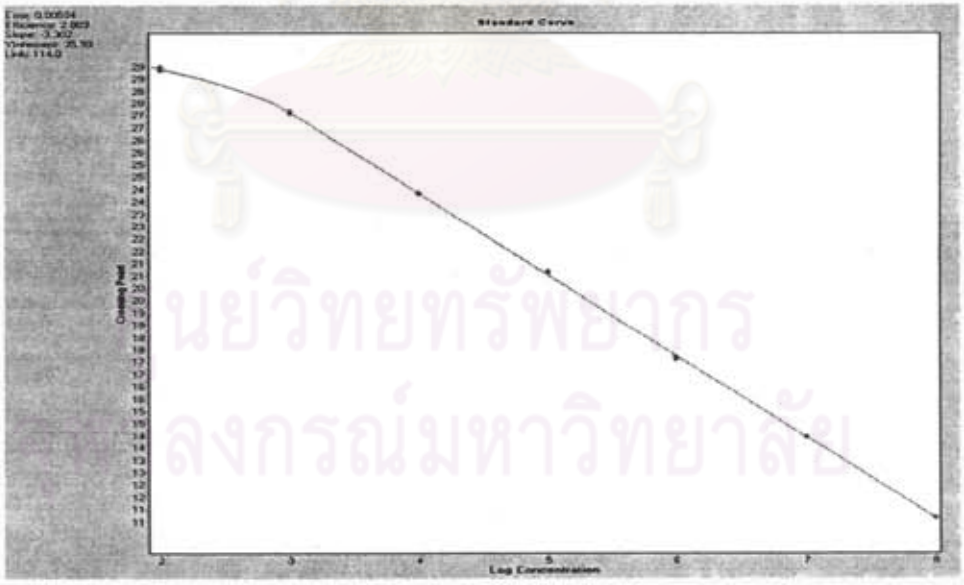


Figure 3.51 A standard curve of *Vacuolar H^+ ATPase* (*V-ATP*) (Error = 0.00594, efficiency = 2.009 and equation; Y = -3.302 * log(X) + 35.99) using 10-fold dilution (10^2 – 10^8 copy) of plasmid DNA of *V-ATP*.

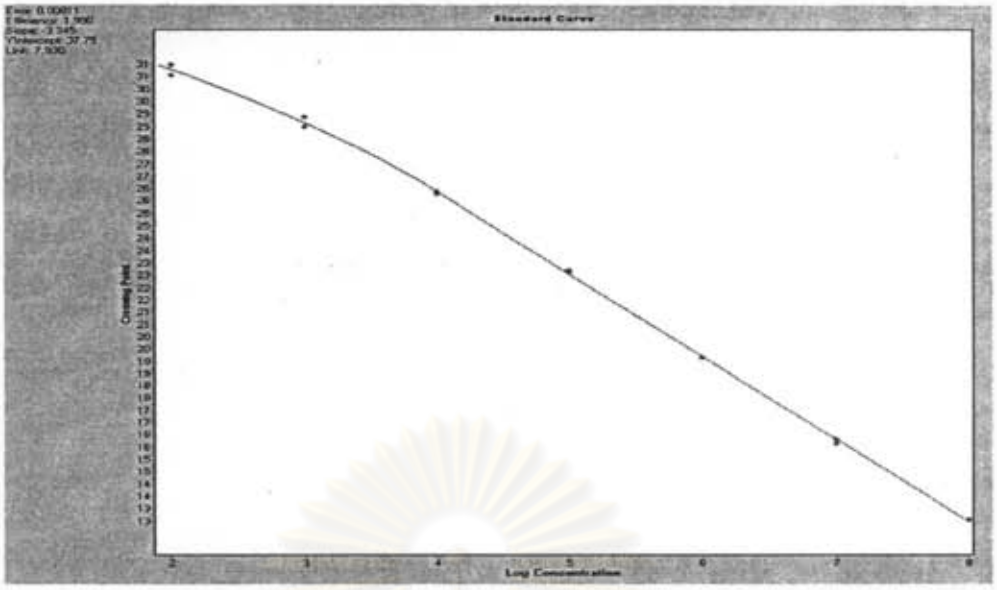
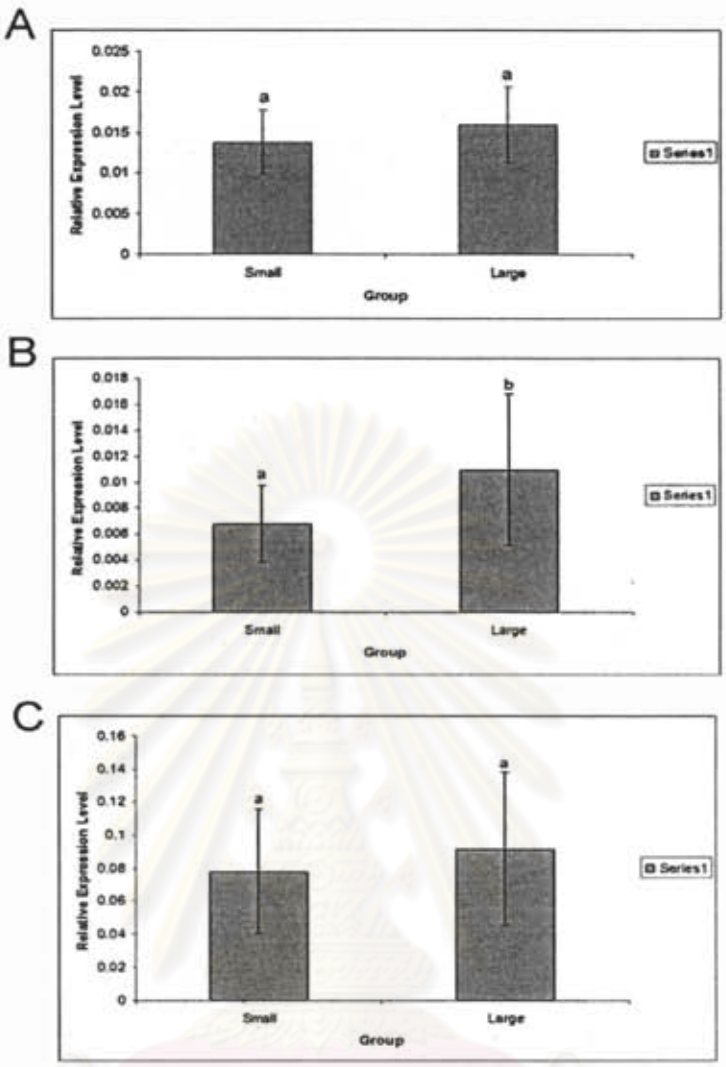


Figure 3.52 A standard curve of *β-actin* (Error = 0.00811, efficiency = 1.990 and equation; Y = -3.345 * log(X) + 37.75) using 10-fold dilution (10^2 – 10^8 copy) of plasmid DNA of *β-actin*.

The relative expression level of *α-methylacyl-CoA racemase* and *vacuolar H^+ ATPase* in large and small subgroups was not significantly different whereas *carnitine O-palmitoyltransferase 1A* was more abundantly expressed in the large than the small subgroups ($P < 0.050$) (Figure 3.53). Quantitative real-time PCR of *hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* (*trifunctional protein*) was not carried out owing to its limited expression levels (< 10^3 copy).



Gene	Group	Relative Expression Level	*N*
AMACR	Small	0.01378 ± 0.0039^{a}	10
	Large	0.01589 ± 0.0046^{a}	10
CPT-1A	Small	0.0068 ± 0.0029^{a}	15
	Large	0.0120 ± 0.0059^{b}	15
V-ATP	Small	0.0777 ± 0.0379^{a}	15
	Large	0.0913 ± 0.0466^{a}	15

Figure 3.53 Histograms showing the relative expression profiles of *α-methylacyl-CoA racemase* (A), *carnitine O-palmitoyltransferase 1A* (B), and *vacuolar H^+ ATPase* (C) in hepatopancreas of large and small subgroups of *H. asinina* cultured in the same pond. Expression levels were measured as the absolute copy number of mRNA (100 ng template) and normalized by that of *β-actin* mRNA (100 ng template). The same superscripts indicate non-significant difference between groups of samples.

CHAPTER IV

DISCUSSION

Domestication and selective breeding programs aim to increase commercially important traits in selected populations (Cock *et al.*, 2009). Basically, short generation time and high fecundity of abalone suggested that genetic improvement of *H. asinina* is promising. This will in turn lead to the sustainable aquaculture of *H. asinina*.

The benefits of heterosis have been demonstrated in aquatic species (Goyard *et al.*, 2003; Dixon *et al.*, 2009). Domestication and selective breeding programs of Thai *H. asinina* should take the advantage of population genetic differentiation (genetic differences among geographically different samples previously reported by molecular genetic markers between major stocks of *H. asinina*: the Andaman Sea and the Gulf of Thailand; Klinbunga *et al.*, 2003; Tang *et al.*, 2004 and 2005). In terms of aquaculture, the establishment of appropriate domesticated stocks requires samples from different geographic locations as the founder stocks for genetic improvement through selective breeding programs.

Loss of genetic variation in small populations as a consequence of genetic drift and inbreeding is commonly observed within captive stocks (Sbordoni *et al.*, 1986). The low levels of genetic variability may reduce the mean fitness of a population, affecting its viability. Inbreeding increases homozygosity, which in some species can reduce growth, viability and reproductive performance (Hansson and Westerberg, 2002). Inbreeding has also been linked to biochemical disorders and deformities from lethal and sub-lethal recessive alleles (Dunham, 2004). Inbreeding can be avoided if a wide genetic variation is secured in the base or founder population, and even better if parentage of animals is known avoiding mating among relatives.

In general, the effective number of founders (N_e) = 50, which is not a census number of male and female in the hatcheries, is minimally required for producing an inbreeding coefficiency of 1% per generation. Practically, as high N_e as possible should be maintained through generations.

To carry out effective breeding programs in *H. asinina*, high genetic diversity stocks should be established. Integrated knowledge on population genetics for estimation of genetic variation levels, molecular genetics for the identifying genetic markers at different levels and quantitative genetics for selection scheme and estimation of heritability for economically important phenotypes are required to improve the efficiency of genetic improvement in this species.

4.1 Genetic diversity in wild and domesticated stocks of *H. asinina* analyzed by microsatellites

Microsatellites have been used for identification of population structure, parentage assignment of different families reared together in the same pond, construction of genetic linkage map and monitoring changes in genetic diversity in domesticated stocks of *H. asinina* (Selvanami *et al.*, 2004). The ability to track large numbers of individuals and families is a key determinant of the power and precision of breeding programs, including the capacity to quantify interactions between genotypes and their environment. Almost all of the microsatellites developed in abalone were type II (non-coding microsatellites). Although it is believed that type II microsatellites are selective neutrally, type I (coding) microsatellites may also useful for various applications when used with cautions. Type I markers developed from ESTs which contain short tandem repeats may less polymorphic but more conserved than non-coding regions making them more transferable across species for comparative analysis, an aspect which would increase the value in breeding programs, than typical type II microsatellites (Fraser *et al.*, 2004).

The number of allele per locus of type I microsatellite developed in this study (*DW455*, *DW503*, *PHe177*, and *PT102*) varied from 6 to 10 which is comparable to 2 - 13 alleles for ten loci of EST-derived microsatellite in the Pacific abalone, *H. discus* (Aibin *et al.*, 2008). However, higher numbers of alleles per locus were observed in type II microsatellites of several abalone species; for instance, 3 - 26 alleles in *H. asinina* (Tang *et al.*, 2004 and 2005), 2 - 32 alleles in *H. midae* (Evans *et al.*, 2004); 6 - 45 alleles in *H. discus hannai* (Li *et al.*, 2004); and 10 -24 alleles in *H. fulgens* (Gutierrez-Gonzalez and Perez-Enriquez, 2005).

The domesticated stocks in this study were initially established from abalone covering both coastal sides of Thai waters (Gulf of Thailand and Andaman Sea). Therefore, genotyping of the maintained stocks was carried out and wild specimens originating from Samet Island, Talibong Island were included to identify whether the domesticated stocks equally contributed from founders originating from different coastal regions.

The allele distribution frequencies of microsatellites in this study indicated that the present generation of abalone stocks has been contributed by founders from both the Andaman and Gulf of Thailand. However, greater levels of the "Gulf of Thailand alleles" than the "Andaman alleles" were observed at all loci. More importantly, *Haμ10* showed non-overlapping alleles between wild abalone originating from the east and the west coasts and only 3 individuals (0.83%) of the domesticated CSMaRT stock exhibited the Andaman allele (143 bp). This clearly indicated that the Gulf of Thailand founders more genetically contributed to the present stocks than those from the Andaman Sea. The information opens the possibility that abalone from the east coast should have better survival rates and be more adaptable to the cultivated conditions than those from the west coast. Additional molecular markers exhibiting fixed genotypes (or alleles) in either coastal sample are required to confirm this circumstance.

Reduction in allelic diversity (i.e. number of alleles per locus) relative to wild populations seems to be a characteristic of hatchery-propagated species (*H. discus hannai*). The reduction in genetic variation in these hatchery samples may be caused by suboptimal of parent abalone contributing in the previous generation increasing the effect of genetic drift, as has previously been reported for other hatchery-reared abalone (Li *et al.*, 2004).

Genetic diversity of hatchery-propagated stocks (G8, $N = 102$ and B, $N = 120$) and a wild sample originating from Talibong Island ($N = 14$ - 25) of the same stock of *H. asinina* were examined at 5 microsatellites loci. A total number of alleles per locus at *CUHas2, CUHas3, CUHas8, Haμ2J* and *Haμ13* across all samples were 9, 14, 14, 7 and 6 alleles, respectively. Sizes of allele distribution were 310 - 352, 155 - 183, 144 - 220, 228 - 240 and 124 - 135 bp, respectively. The number of *CUHas2, CUHas3, CUHas8*, and *Haμ13* alleles in the hatchery-propagated abalone was greater

than that of the Talibong sample. In contrast, those of the *Haμ2J* locus in the wild sample (6 alleles) were greater than in the hatchery samples (4 alleles). The allele discrimination capacity was 0.175 ± 0.096, 0.216 ± 0.133 and 0.349 ± 0.201 for G8, B hatchery samples and the Talibong sample, respectively. Observed heterozygosity in the respective hatchery samples was 0.69 ± 0.308 and 0.56 ± 0.241 which is slightly greater than that of the Talibong sample (0.52 ± 0.180). The estimated effective number of population size (N_e) and inbreeding coefficient of each hatchery stock was 20 individuals and 2.5% per generation. Results indicated relatively high levels of genetic diversity but limited numbers of contributed founders from the previous generation of the hatchery stock of *H. asinina* (Rungpituckmana, 2007).

Considering the number of alleles per locus and heterozygosity, comparable levels of genetic diversity were observed in *H. asinina* examined in the present study. When allele and genotype discrimination capacity were taken into the account, these parameters in the domesticated stocks were slightly lower than those of wild stocks suggesting moderate reduction of genetic diversity in domesticated *H. asinina* after propagated for 5 - 6 generations.

Selvamani *et al.* (2001) carried out parentage assignment of *H. asinina* veliger larva produced from the Heron Island Reef (Great Barrier Reef, Australia) founders using 5 microsatellite loci (2 highly polymorphic, *Haμ13* and *Haμ2K*, and 3 moderately polymorphic loci, *Haμ10*, *Haμ2J*, and *Haμ3E)*. The allele number per locus range between 14 - 25 alleles and the expected heterogeneity were 0.7700 – 0.9600. The parents of an individual veliger could be determined from as few as 3 loci. The locus *Haμ10* gave 4 - 9 alleles when genotype each of wild abalone populations whereas it gave 14 alleles when examined with hatchery samples.

Recently, 3 hatchery strains and 2 wild population of *H. discus hannai* were investigated with six microsatellite loci. The hatchery strains possessed less genetic variation as revealed in lower number of alleles and lower expected heterozygosity (2 – 14 alleles per locus and 0.238 – 0.876, respectively) than natural populations (5 – 45 alleles and 0.525 – 0.973, respectively). This implied that bottleneck effects occurred when each strain was founded (Li *et al.*, 2004). Comparing to this study, the number alleles per locus and expected heterozygosity was 2 – 8 and 0.3441 – 0.7769 in the domesticated stocks and was 4 – 9 alleles and 0.0625 – 0.8448 in wild stocks,

respectively. Therefore, severe reduction of genetic diversity in domesticated *H. asinina* is not observed at present.

There are obviously significant differences between wild samples from the east coast (SAME, and CAME) and the west coast (TRGW) at all loci ($P < 0.05$). Significant differences were observed between samples from the same coastal region (SAME and CAME). The Philippines sample is recognized as a different stock. Result from this study strongly supported the previous reports on the existence of intraspecific population differentiation of *H. asinina* in Thai waters based on microsatellite (Tang *et al.*, 2004 and 2005) but not PCR-RFLP of *16S* and *18S rDNA* (Klinbunga et al., 2003). Moreover, the present study provided the further insights on differentiation between CAME and SAME samples originated from the same coastal region.

The F_{ST}-statistics and exact test also indicated significant genetic heterogeneity between the domesticated stocks. Based on the fact that these stocks were bred from the same groups of founders but separated maintained at SMaRT (CSMaRTH) and at the farm in Trang (CTRGH), high mortality rates reflected the occurrence of genetic drift in these stocks. As a result, a large number of brooders should be used for breeding of *H. asinina* in each generation.

Although results on genetic diversity and heterogeneity of *H. asinina* based on type I microsatellites (this study) were concordant with those from type II microsatellites (Rungpituckmana, 2007). Theoretically, the neutrality concepts predict the amount of genetic variability within a given population as a function of mutation rate, gene flow (where applicable) and effective population size (Kimura and Ohta, 1971). The absence from assumptions is selection coefficient, because alleles are assumed to be neutral. Under the strict neutral theory, molecular variability is a function of the neutral mutation rate and the evolutionary effective population size, N_e. Therefore, type I molecular markers must be used with cautions due to the functional constraints of the translated gene products.

Significant association of microsatellite polymorphism and phenotypes (e.g. growth rates) of aquatic species was reported. Recently, Xu and Zhu (2006) characterized two *parvalbumim* genes and their association with the growth traits in

the Asian sea bass (*Lates calcarifer)*. Expression of *PVALB2* was detected only in muscle, brain, and intestine, was up to 10-fold lower than *PVALB1* expression. A $(CT)_{17}$ microsatellite was identified in the 3′-untranslated region of *PVALB1* and three single nucleotide polymorphism (SNP) were identified in the third intron of *PVALB2*. The microsatellite in *PVALB1* was significantly associated with body weight and body length at 90 days post-hatch ($P < 0.01$) whereas the SNPs in *PVALB2* were not associated with these traits ($P > 0.05$).

Considering association between microsatellite genotypes and the body weight of domesticated stocks, low (but significant) degrees of correlation was observed in the small sample size of cultured *H. asinina* based on the simple regression analysis ($P < 0.05$). Genetic heterogeneity analysis indicated that BL and BS subgroups were genetically different at two microsatellite loci (*DW455* and *Haμ10*).

Significant differences between the body weight of the B sample having different genotypes were found ($P < 0.05$). Homozygote and heterozygote individuals carrying the 176 bp allele had greater or approximately equal body weight to the mean body weight for this group of samples. Interestingly, abalone possessed 176/176 were found in 21.25% of examined individuals. Therefore, breeding of founders carrying a 176 allele should be considered for the subsequent generation of this domesticated stock. However, these preliminary results should be confirmed in larger sample sizes of domesticated *H. asinina*.

4.2 Genetic diversity in wild and domesticated stocks of *H. asinina* examined by SSCP analysis of AFLP-derived SCAR markers

Population and/or region-specific markers for identification the origin of abalone in Thai waters can be utilized for identification of seed stocks, and distribution and recruitment of abalone larvae, leading to increasing efficiency in management of local *H. asinina* stocks in Thailand (Klinbunga *et al.*, 2003).

SSCP which is favored for examining genetic diversity of various species owing to its convenience and cost effectiveness was applied to determine polymorphism of the developed SCAR markers. The major advantage of SSCP is that

a large number of individual could be simultaneously genotyped (Khamnamtong *et al.*, 2005) and variations according to one or a few substitutions could be detected (Orita *et al.*, 1989). Therefore, SSCP is one of the potential techniques for detection of genetic polymorphism at different taxonomic levels of organisms.

Jarayabhand *et al.* (2002) examined genetic diversity in *H. ovina* originating from Chon Buri ($N = 24$) and Rayong ($N = 18$) located in the Gulf of Thailand and Trang ($N = 18$) and Phangnga located in the Andaman Sea ($N = 11$) by PCR-RFLP of *16S rDNA* (approximately 580 bp in length) with *Bam* HI, *Eco* RI, *Hae* III and *Alu* I. Three composite haplotypes (ABBB, AAAB and AABB) were found across overall specimens. No overlapping haplotypes were found between *H. ovina* originating from the east (ABBB) and west (AAAB and AABB) coasts of peninsular Thailand.

In contrast, distribution patterns of composite haplotypes of *16S rDNA* based on the same approach (AAAA and AAAE) indicated a lack of intraspecific population structure in Thai *H. asinina*. The combined data from restriction analysis of *18S* (nuclear) rDNA with *Alu* I, *Taq* I, and *Hae* III and *16S* (mitochondrial) rDNA with *Bam* HI, *Eco* RI, *Hae* III, and *Alu* I did not reveal differentiation between geographically different populations of *H. asinina* in Thai waters but showed partial differentiation between those and the Philippines samples ($P < 0.0021$, Klinbunga *et al.*, 2003). Under the presumption of selective neutrality of molecular markers and life history of *H. asinina*, it was proposed that founder effects may have occurred in Thai *H. asinina*. (Klinbunga *et al.*, 2003).

In this study, a total of 11 composite genotypes were generated from all single genotypes generated from $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$. All composite genotypes could be related by at least two mutations. No direct intermediate genotypes were observed suggesting that the sample sizes should be increased from 222 individuals used in the present study to identify direct intermediate genotypes between their evolutionary pathways.

Two lineages of composite genotypes were clearly observed. Wild sample in this study did not reveal shared genotypes between abalone from different coastal regions. Distributions of *H. asinina* composite SSCP genotypes clearly indicated the existence of strong intraspecific genetic differentiation within *H. asinina* in Thai

waters. The information also suggests that *H. asinina* from the Gulf of Thailand and Andaman Sea may have been colonized by two independent ancestral populations, one from the west, one from the east.

Unlike results from PCR-RFLP of *16S* and *18S rDNAs*, SSCP analysis of *ND2* also showed significant population differentiation between *H. asinina* from the Gulf of Thailand (HASAME and HASAME) and Andaman Sea (HATRGW). Accordingly, patterns of genetic differentiation of *H. asinina* in Thai waters could be finally confirmed. Based on the fact that PCR-RFLP examined polymorphism only at restriction sites whereas SSCP is able to detect variations according to one or a few substitutions could be detected (Orita *et al.*, 1989). Moreov er, strong degrees of coastal differentiation were found based on microsatellites and SSCP analysis of AFLP-derived SCAR. The contradictory reports on patterns of genetic differentiation of *H. asinina* in Thai waters clearly arose from variation in the sensitivity of different analysis approaches rather than from the influence of founder effects or female-biased gene flow per sex.

Shephred and Brown (1993) predicted that microgeographic population differentiation within each abalone species should be occurred due to its short planktonic larval stages and limited dispersal ability. Accordingly, intraspecific genetic differentiation of abalone may be found within the scale of a few kilometers.

Recently, phylogeographical structure of *H. asinina* collected from 16 geographically discrete sites throughout the Indo-Malay Archipelagoes, and eastern Indian and western Pacific Oceans was examined by sequence polymorphism of the mitochondrial *cytochrome oxidase II* (*COII*) gene segment (482 bp, N = 206). Limited sequence divergence among geographic samples ranged from 1.1% between Indian and Indo-Malay sites and 3.7% between Indian and Pacific sites and 3.0% between Pacific and Indo-Malay sites, was observed. No finer scale phylogeographical structure was resolved within the respective geographical regions (Imron *et al.*, 2007).

Genetic heterogeneity of *H. asinina* in Thai waters was reported based on polymorphism of six microsatellite loci (*CUHas1*, *CUHas2, CUHas3, CUHas4*, *CUHas5* and *CUHas8*). Relatively high genetic diversity and strong population

differentiation in *H. asinina* was found between samples from the Gulf of Thailand (Samet Island and Cambodia) and Andaman Sea (Talibong Island) ($P < 0.0001$; Tang *et al.*, 2004 and 2005).

On the basis of genetic distance values in this study, clear genetic differences were observed between pairwise comparisons of the east (CAME and SAME) and west (TRGW) coastal samples. Like results from microsatellites, genetic heterogeneity analysis and F_{ST} statistics revealed significant genetic differentiation between abalone from different coastal regions ($P < 0.0001$). This suggested that the gene pool of Thai *H. asinina* is not panmictic but fragmented to two genetic populations; the Gulf of Thailand and the Andaman Sea.

Genetic differentiation of *H. asinina* could be explained by a major physical barrier as the main current in the Straits of Malacca moves from the south to the north throughout the year (Dale, 1956). This should have affected the gene flow levels of *H. asinina* in Thai waters. In addition, *H. asinina* needs to settle on specific species of coralline algae after 3 - 4 days in the planktonic larval stage. The failure to contact suitable algae within approximately 10 days of hatching usually results in death. These life cycle characteristics suggest a limited dispersal level of *H. asinina* (Imron *et al.*, 2007).

Considering the life history, larval development, and dispersing ability of *H. asinina*, contradictory results on patterns of genetic differentiation of natural *H. asinina* in Thai waters based on analysis of mitochondrial DNA *16S rDNA* (panmictic gene pool; Jarayabhand *et al.*, 2002; Klinbunga *et. al.*, 2003) and microsatellites and nuclear $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$ (fragmented gene pool; Tang *et al.*, 2004 and 2005 and this study) are likely to have resulted from the sensitivity of the techniques. Moreover, population differentiation within the Gulf of Thailand detected by microsatellites but not by $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$ should have resulted from a greater polymorphic level of the former than the latter. Nevertheless, AFLP-derived markers ($HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$) are more appropriate for detecting population origin of *H. asinina* in Thai waters owing to non-overlapping composite SSCP genotypes between abalone from different coastal regions generated by these markers.

Therefore, the previous assumption of a large single breeding stock in Thai *H. asinina* previously reported based on *16S* and *18S rDNAs* polymorphism must be changed (Ward and Grewe, 1994; Klinbunga *et al.*, 2003). Geographically and genetically different populations of Thai *H. asinina* should be recognized as different stocks and managed separately by fishery managers and government organizations (Carvalho and Hauser, 1994; Conover *et al.*, 2007).

Higher genetic diversity of the domesticated stock (CTRGH and CSMaRTH) than wild samples based on polymorphism of developed SCAR markers suggested that inbreeding is not a major concern for the established stocks at present. The domesticated *H. asinina* stocks were initially established from mass spawning of founders from different geographic locations in Thai waters. The finding that the gene pools of domesticated stocks of *H. asinina* have been maintained by progeny bred from the east coast rather than those bred the west coast founders strongly reflects better adaptability of *H. asinina* from the Gulf of Thailand than the Andaman Sea to aquacultural conditions. Nevertheless, this circumstance may be biased by the including of larger number of founders from the east than the west coasts of Thai waters (approximately 70:30) at the beginning of our breeding program.

Significant genetic heterogeneity between CSMaRTH and CTRGH (domesticated stocks) and between CTRGH and wild CAME (east) were observed. Regarding that both domesticated stocks were bred from the same group of domesticated founders and offspring was cultured separately in the hatcheries located at different geographic areas (CSMaRTH at SMaRT and CTRGH at Trang province), the possibility of randomly high mortality rates in cultured abalone may have occurred. Accordingly, $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$ should be further used to confirm whether mixed progeny from multiple parents exhibiting different survival rates under the same cultivated conditions using a new cultured stock established from approximately equal founders from either coastal region.

Population-specific SCAR markers developed in this study can be further applied to assist genetic improvement and breeding programs of *H. asinina*; for example, determination of correlation between genotypes and survival rates after settlement of larvae. In addition, $HaSCAR_{320}$, $HaSCAR_{295}$ and $HaSCAR_{327}$ may be

used to monitor effects of aquacultural activity on the long-term levels of population differentiation of natural *H. asinina* in Thai waters.

4.3 Isolation and characterization of genes functionally involved with growth of *H. asinina*

An EST approach (single-pass sequencing of randomly selected clones from cDNA libraries) has been successfully applied and recognized as an effective method for discovery of novel genes in various species (Amparyup *et al.*, 2004; Preechaphol *et al.*, 2007).

In *H. asinina*, normal and subtractive complementary DNA libraries were established from ovaries and testes. A total of 588 randomly selected clones were unidirectionally sequenced. Results indicated that 109 (54.5%) and 73 (61.9%) of normal cDNA libraries from *H. asinina* ovaries and testes significantly matched with known genes (E value $< 10^{-4}$). Of these, vitelline coat proteins (VCPs; 40 clones, 20.0%) were predominant in the former library, but sperm lysin (9 clones, 7.6%) was the most abundant transcript in the latter library. For subtractive cDNA libraries, 71 clones (64.5%) and 56 clones (35.0%) of ovary (forward subtraction) and testis (reverse subtraction) libraries were known transcripts. Expression of sex-related genes was further examined using reverse transcriptase polymerase chain reaction. Gender-specific expression was found from homologues of *VCP1*, *VCP3*, *VCP7*, *VCP49*, and *VTG1* and *axonemal p66.0*, *tektin A1*, and *sperm lysin* in female and male *H. asinina*, respectively.

At present, the basic knowledge of abalone biology, particularly with regard to the control of growth, reproduction and the immune system, is limited and hamstrung by the lack of genome information. In this study, the full length cDNA of four metabolic related transcripts, *α-methylacyl-CoA racemase*, *carnitine O-palmitoyltransferase 1A*, *hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase* (*trifunctional protein*) α-subunit, and *vacuolar H^+ ATPase* (*V-ATP*) 14 kDa subunit were successfully identified allowing the examination of expression levels of these genes in abalone cultured in the same conditions but exhibiting different growth rates.

Quantitative real-time PCR analysis indicated that expression of *carnitine O-palmitoyltransferase 1A* but not *α-methylacyl-CoA racemase* and *vacuolar H^+ ATPase* ($P > 0.05$), in fast-growing abalone was significantly greater than that in slow-growing *H. asinina*. This suggested that a higher level of *carnitine O-palmitoyltransferase 1A* mRNA may be required for lipid metabolism in the fast-growing subgroup.

The information on correlations of genotypes and phenotypes through genetic linkage maps in this species are not available at present. It is interesting to study the possible correlation between single nucleotide polymorphism (SNP) of this gene and the growth rates of domesticated abalone for development growth-related biomarkers in *H. asinina*. SNP markers in genes can be treated as similar as other bi-allelic co-dominantly segregated DNA markers. The advantage of coding SNP (type I) is that they are located in regions that code for functionally important proteins. Therefore, they are more likely to be near QTL that affect commercially important traits. Therefore, analysis of gene-based SNP is one of the efficient approaches for discovery of genes which are significantly contributed in complex traits of *H. asinina*.

In conclusions, levels of genetic diversity in wild and the present generation of domesticated stocks of *H. asinina* were examined and provided the useful information to elevate management efficiency of breeding program of this economically important species. Appropriate breeding plans could be implemented to reduce the inbreeding coefficient of the hatchery stock. Genetic diversity of the established stocks should be maximized and regularly monitored by highly polymorphic microsatellites. Different contribution of founders from different coastal regions of abalone in Thai waters should be further verified to reduce biased mass mortality in this species leading to the sustainability of abalone aquaculture. The possible correlation between microsatellite genotypes and a phenotype (i.e. the body weight) require further studies. In addition, different expression levels of *carnitine O-palmitoyltransferase 1A* mRNA in the fast- and slow-growing subgroups were observed. Results from this study demonstrated the possibility to apply molecular markers (different alleles of microsatellites and/or differential expression levels of *carnitine O-palmitoyltransferase 1A*) for genetic selection of *H. asinina* in the future.

CHAPTER V

CONCLUSION

1. Type I microsatellites (*DW455*, *DW503*, *PHe177* and *PT102*) were successfully developed from ESTs that contain simple repeat sequences. These and type II microsatellites (*Haμ9* and *Haμ10*) were used for genetic studies of *H. asinina*. The expected and observed heterogeneity in wild populations ranged from 0.0625 - 0.8448 and 0.0625 - 1.0000 whereas those values in domesticated CTRGH and CSMaRTH stocks were 0.3441 - 0.7769 and 0.3333 - 0.8750, respectively.

2. F_{ST}-statistics and the exact test between pairs of examined samples clearly revealed significant differences between wild abalone from the east (SAME, and CAME) and west (TRGW) coasts of Thai waters and the Philippines. Both domesticated stocks (CTRGH and CSMaRT) were genetically different from TRGW (west) and PHI at all loci.

3. Correlation between genotypes and the body weight of the group B sample was initially tested at each locus. Significant differences between the body weights of abalone having different genotypes were only observed at *Haμ10* ($P < 0.05$).

4. AFLP markers for genetic analysis of *H. asinina* were identified. A total of 64 primer combinations were screened against representative individuals of *H. asinina*. Nine AFLP bands were converted to sequence-characterized amplified region (SCAR) markers. Six primer pairs ($HaSCAR_{328}$, $HaSCAR_{167}$, $HaSCAR_{339}$, $HaSCAR_{320}$, $HaSCAR_{295}$, $HaSCAR_{327}$) were designed.

5. $HaSCAR_{320}$, $HaSCAR_{295}$, and $HaSCAR_{327}$ were further used for genotyping of *H. asinina*. SSCP genotypes of *H. asinina* from the Gulf of Thailand and Andaman Sea did not overlap at the $HaSCAR_{327}$ locus whereas a single individual from the west coast (TRGW) exhibited genotype A at $HaSCAR_{32}$ and $HaSCAR_{295}$ loci.

6. Six composite SSCP genotypes were found in wild abalone. Genotypes AAA, AAC and CAA were restrictively observed in the Gulf of Thailand whereas ABB, BAB and BBB were only distributed in the TRGW sample. Eight composite genotypes were found in the domesticated samples and only three of which were observed in CAME

and SAME. Composite genotypes BAA and BAC were found only in CSMaRTH while AAB and ABC were restrictively observed in CTRGH.

7. An unrooted maximun parsimony network inferred from polymorphic sites of examined SSCP sequences revealed 2 different lineages of composite genotypes perfectly corresponding to specimens from the Gulf of Thailand (lineage I) and Andaman Sea (lineage II), respectively. Composite genotypes ABB, BAB and BBB found in wild abalone from the Andaman Sea and regarded as members of the lineage II were not found in the domesticated stocks (CTRGH and CSMaRTH).

8. Genetic heterogeneity between the Andaman Sea and Gulf of Thailand samples was also found when analyzed by *ND-II* polymorphism.

9. The full length cDNAs of *α-methylacyl-CoA racemase* (*AMACR*), *carnitine O-palmitoyltransferase 1A* (*CPT-1A*), *hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase (trifunctional protein) α-subunit*, and *vacuolar H+ ATPase (V-ATP) 14 kDa subunit* were successfully characterized the full length by RACE-PCR.

10. The relative expression levels of *α-methylacyl-CoA racemase* and *vacuolar H+ ATPase* in large and small subgroups of abalone were not significantly different (0.2871 and 0.3883, respectively). Nevertheless, *carnitine O-palmitoyltransferase 1A* in hepatopancreas of the large subgroup was more abundantly expressed than that of the small subgroup ($P < 0.05$).

REFERENCES

Adams, M. D., Kelley, J. M., Gocayne, J. D., Dubnick, M., Polymeropoulos, M. H., Xiao, H., Merril, C. R., Wu, A., and Venter, J. C. 1991. Complementary DNA sequencing : expressed sequence tags and human genome project. Science. 252: 1651-1656.

Aibin, Z., Zhenmin, B., Mingling, W., Dan, C., Jian, Y., Xiaolong, W., Xiaoli, H., Chengzhu, H., and Jingjie1, H. 2008. Development and characterization of microsatellite markers for the Pacific abalone (*Haliotis discus*) via EST database mMining. J. Ocean. Univ. Chin. 7(2): 219-222.

Allendorf, F. W., and N. Ryman. 1987. Genetic management of hatchery stocks. In: N. Ryman & F Utter, editors. Population genetics and fishery management. Seattle, WA: Washington Sea Grant Program, University of Washington Press. pp. 141– 159.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Amparyup, P. 2004. Development of genetic markers for increasing production efficiency of the tropical abalone, *Haliotis asinina* in Thailand. PhD.' Thesis. Programme of Biotechnology, Faculty of Science, Chulalongkorn University.

Amparyup, P., Klinbunga, S., Preechaphol, R., Tassanakajon, A., Hirono, I., Aoki, T., and Jarayabhand, P. 2004. Expressed sequence tag analysis of ovaries and testes from tropical abalone (*Haliotis asinina*). Mar. Biotechnol. 6: S1-S6.

An, H. S., and Han, S. J. 2006. Isolation and characterization of microsatellite DNA markers in the Pacific abalone, *Haliotis discus hannai*. Molecular Ecology. 6: 11–13.

Anderson, I., and Brass, A. 1998. Searching DNA databases for similarities to DNA sequence: when is a match significant. Bioinformatics. 14(4): 349-356.

Beheregaray, L. B., Schwartz, T. S., Moller, L. M., Call, D., Chao, N. L., and Caccone, A. 2004. A set of microsatellite DNA markers for the one-lined pencilfish *Nannostomus unifasciatus*, an Amazonian flooded forest fish. Molecular Ecology. 4: 333–335.

Benson, G. 1999. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. 27: 573-580.

Beridze, T. 1986. Detection of satellite DNAs. In: Satellite DNA, Beridze, T. (ed.). Springer-Verlag, Berlin, 1-5 pp.

Bhaumik, P., Koski, M. K., Glumoff, T., Hiltunen, J. K., and Wierenga, R. K. 2005. Structural biology of the thioester-dependent degradation and synthesis of fatty acids. Curr. Opin. Struct. Biol. 15: 621-628.

Bosch, A., Nunes, V., Patterson, D., and Estivill, X. 1993. Isolation and characterisation of 14 CA- repeat microsatellites from human chromosome 21. Genomics. 18: 151-155.

Budowle, B., Chakraborty, R., Giusti, A. M., Eisenberg, A. J., and Allen, R.C. 1991. Analysis of the VNTR locus DIS80 by the PCR followed by high-resolution PAGE. Am. J. Hum. Genet. 48: 137-144.

Burton, R. S. 1996. Molecular tools in marine ecology. J. Exp. Mar. Biol. Ecol. 1200 (1-2): 85-101.

Cheng, S., Fockler, C., Barnes, W. M., and Higuchi, R. 1994. Effective amplification of long targets from cloned inserts and human genomic DNA. Proc. Natl. Acad. Sci. 91: 5695-5699.

Cook, P., and Gordon, R., 2008. The world abalone market and economic reasons for environmental accreditation. http://www.worldwildlife.org/what/globalmarkets/ aquaculture/ WWFBinaryitem11677.pdf.

Cox, L. R. 1960. Gastropoda, General characteristics of gastropoda. In: Treastise on Invertebrate Paleontology, Part I. Mollusca 1, Moore, R.C., and Pitrat, C.W. (eds.). The University of Kansas Press and the Geological Society of America, Inc., 84-69 pp.

Crisp, D. S. 1974. Factors affecting settlement of marine invertebrate larvae. In: Chemoreception in Marine Organisms, Grant, P., and Mackie, A.M. (eds.). New York and London: Academic Press, 177-265 pp.

Cruz, P., Ibarra, A. M., Mejia-Ruiz, H., Gaffney, P.M., and Perez-Enriquez, R. 2004. Genetic variability assessed by microsatellites in a breeding program of Pacific white shrimp (*Litopenaeus bannamei*). Mar. Biotechnol. 6: 157-164.

Deka, R., Jin, L., Shriver, M. D., Yu, L. M., DeCroo, S., Hundrieser, J., Bunker, C. H., Ferrell, R. E., and Chakraborty, R. 1995. Population genetics of dinucleotide $(dC\text{-}dA)_n$ $(dG\text{-}dT)_n$ polymorphisms in world populations. Am. J. Hum. Genet. 56: 461-474.

Distler, A. M., Kerner, J., and Hoppel, C. L. 2009. Mass spectrometric demonstration of the presence of liver carnitine palmmitoyltransferase-I (CPT-I) in heart mitochondria of adult rats. Biochim. Biophys. Acta. 1794: 431-437.

Dixon, T. J., Coman, G. J., Arnold, S. J., Sellarrs, M. J., Lyons, R. E., Dierens, L., Preston, N. P., Li, Y. 2009. Shifts in genetic diversity during domestication of Black Tiger shrimp, *Penaeus monodon*, monitored using two multiplexed microsatellite systems. Aquaculture. 283: 1-6.

Donald, K. M., and Spencer, H. G. 2006. Polymorphic microsatellite DNA markers in the mudflat topshell *Diloma subrostrata* (Gastropoda, Trochidae). Molecular Ecology. 6: 762–764.

Dong, S., Kong, J., Zhang, T., Meng, X., and Wang, R. 2006. Parentage determination of Chinese shrimp (*Fenneropenaeus chinensis*) based on microsatellite DNA markers. Aquaculture. 258: 283–288.

Dunham, R. A. 2004. Aquaculture and Fisheries Biotechnology: Genetic Approaches. Oxford University Press, Oxford, UK.

Eaton, S., Bartlett, K., and Pourfarzam, M. 1996. Mammalian mitochondrial β-oxidation. Biochem. J. 320: 345-357.

Eaton, S., Middleton, B., and Bartlett, K. 1998. Control of mitochondrial β-oxidation: sensitivity of the trifunctional protein to $[NAD^+]/[NADH]$ and [acetyl-CoA]/[CoA]. Biochim. Biophys. Acta. 1429: 230-238.

Estroup, A., Solignac, M., Harry, M., and Cornut, J.M. 1993. Characterization of (GT)n and (CT)n microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*. Nucleic Acids Res. 21: 1427-1431.

Evans, B., Bartletta, B. J., Sweijde, N., Cooke, P., and Elliotta, N. G. 2004. Loss of genetic variation at microsatellite loci in hatchery produced abalone in Australia (*Haliotis rubra*) and South Africa (*Haliotis midae*). Aquaculture. 233: 109-127.

Fallu, R. 1991. Abalone Farming. United Kingdom: Fishing News Books, Oxford, 195 pp.

Felip, A., Young, W. P., Wheeler, P. A., and Thorgaard, G. H. 2005. An AFLP-based approach for the identification of sex-linked markers in rainbow trout (*Oncorhynchus mykiss*). Aqaculture. 247: 35-43.

Ferdinandusse, S., Denis, S., and Jlst. I. L. 2000. Subcellular localization and physiological role of a α-methylacyl-CoA racemase. J. Lipid Res. 41: 1890-1896.

Fishtech. 2007. Worldwide abalone farming production. http://www.fishtech.com/faming. html.

Geiger, D. L. 1998. Recent Genera and Species of the Family Haliotidae Rafinesque, 1815 (Gastropoda: *Vetigastropoda*). Nautilus 111(3): 85-116.

Geiger, D. L. 2000. Distribution and biogeography of the recent Haliotidae (Gastropoda: *Vetigastropoda*) worldwide. Boll. Malacol. 35: 57–120.

Gordon, H. R. 2000. World abalone supply, markets and pricing: historical, current and future prospective. The 4th International Abalone Symposium, Cape Town, South Africa. University of Cape Town, 6-11th February 2000.

Goyard, E., Arnaud, S., Vonau, V., Bishoff, V., Mouchel, O., Pham, D., Wyban, J., Boudry, P., AQUACOP. 2003. Residual genetic variability in domesticated populations of the Pacific blue shrimp (*Litopenaeus stylirostris*) of New Caledonia, French Polynesia and Hawaii and some management recommendations. Aquat. Living Resour. 16: 501–508.

Griffiths, A. J. F., Miller, J. H., Suzuki, D. T., Lewontin, R. C., and Gelbart, W. M. 1993. The structure and function of eukaryotic chromosome. In: An introduction to genetic analysis, 5th ed, Griffiths, A. J. F., Miller, J. H., Suzuki, D. T.,

Lewontin, R. C., and Gelbart, W. M. (eds.). New York: Freeman, W. H. and company, 82-487 pp.

Gruenthal, K. M., Acheson, L. K., and Burton, R. S. 2007. Genetic structure of natural populations of California red abalone (*Haliotis rufescens*) using multiple genetic markers. Mar Biol. 152: 1237-1248.

Gruenthal, K. M., and Burton, R. S. 2005. Genetic diversity and species identification in the endangered white abalone (*Haliotis sorenseni*). Conserv. Genet. 6: 929-939.

Gruenthal, K. M., and Burton, R. S. 2008. Genetic structure of natural populations of the California black abalone (*Haliotis cracherodii* Leach, 1814), a candidate for endangered species status. J. Exp. Mar. Biol. Ecol. 355: 47-58.

Guo, S. W., and Thompson, E. A. 1992. Performing the extract test of Hardy-Weinberg proportion for multiple alleles. Biometrics. 48: 361-372.

Guo, Y., Kaiser, K., Wieczorek, H., and Dow, J. A. T. 1996. The *Drosophila melanogaster* gene *vha14* encoding a 14-kDa –subunit of the vacuolar ATPase. Gene. 172: 239-243.

Gutierrez-Gonzalez, J. L., and Perez-Enriquez, R. 2005. A genetic evaluation of stock enhancement of blue abalone *Haliotis fulgens* in Baja California, Mexico. Aquaculture. 247: 233-242.

Hahn, K. O. 1989. Survey of the commercially important abalone species in the world. Pp. 3-10 in: K.O. Hahn (ed.). Handbook of Culture of Abalone and Other Marine Gastropods. CRC Press, Boca Raton. FL.

Hansson, B., and Westerberg, L. 2002. On the correlation between heterozygosity and fitness in natural populations. Mol. Ecol. 11: 2467-2474.

Hara, M., and Sekino, M. 2007. Genetic differences between hatchery stocks and natural populations in Pacific abalone (*Haliotis discus*) estimated using microsatellite DNA markers. Mar. Biotechnol. 9: 74-81.

Herlin, A., Taggart, J. B., McAndrew, B. J., and Penman, D. J. 2007. Parentage allocation in a complex situation: Alarge commercial Atlantic cod (*Gadus morhua*) mass spawning tank. Aquaculture. 272S1: S195-S203.

Herlin, M., Delghandi, M., Wesmajervi, M., Taggart, J. B., McAndrew, B. J., and Penman, D. J. 2008. Analysis of the parental contribution to a group of fry from a single day of spawning from a commercial Atlantic cod (*Gadus morhua*) breeding tank. Aquaculture. 274: 218-224.

Hiltunen, J. K., and Qin, Y.-M. 2000. β-oxidation-strategies for the metabolism of a wide variety of acyl-CoA esters. Biochim. Biophys. Acta. 1484: 117-128.

Hinman, V. F., and Degnan, B. M. 2002. *Mox* homeobox expression in muscle lineage of the gastropod *Haliotis asinina*: evidence for a conserved role in bilaterian myogenesis. Dev. Genes Evol. 212: 141-144.

Huang, B. X., Chai, Z. L., Hanna, P. J., and Gough, K. H. 1997. Molecular sequences of two minisatellites in blacklip abalone, *Haliotis rabra*. Electrophoresis 18(9): 1653-1659.

Ibdah, J. A., Paul, H., Zhao, Y., Binford, S., Salleng, K., Cline, M., Matern, D., Bennett, M. J., Rinaldo, P., and Strauss, A. W. 2001. Lack of mitochondrial trifunctional protein in mice causes neonatal hypoglycemia and sudden death. J. Clin. Invest. 107: 1403-1409.

Jackson, S., Schaefer, J., Middleton, B., and Tumbull, D. M. 1995. Characterisation of a novel enzyme of human fatty acid β-oxidation: a matrix-associated, mitochondrial 2-enoyl-CoA hydratase. Biochem. Biophys. Res. Commun. 214: 247-253.

Jarayabhand, P., and Paphavasit, N. 1996. A review of the culture of tropical abalone with special reference to Thailand. Aquaculture. 140: 159-168.

Jarayabhand, P., Piyateeratitivorakul, S., Choonhabandit, S., and Rungsupa, S. 1991. Final report on research and development on some aspects of abalone culture. Presented to the Toray Science International Research Grant 1990, Bangkok, 52 pp.

Jeffreys, A. J., Royle, N. J., Patel, I., Armour, J. A. L., MacLeod, A., Collick, A., Gray, I. C., Neumann, R., Gibbs, M., Crosier, M., Hill, M., Singer, E., and Monckton, D. 1991. Principals and recent advances in human DNA fingerprinting. In: DNA fingerprinting: Approaches and applications. Burke, T., Jeffreys, A.J., and Wolff, R. (eds.). Berlin: Brikhauser Verlag, 1-19 pp.

Jerry, D. R., Preston, N. P., Crocos, P. J., Keys, S., Meadow, J. R. S., and Li, Y. 2004. Parentage determination of Kuruma shrimp *Penaeus* (*Marsupenaeus*) *japonicus* using microsatellite markers (Bate). Aquaculture. 235: 237-247.

Jerry, D. R., Preston, N. P., Crocos, P. J., Keys, S., Meadow, J. R. S., and Li, Y. 2006. Application of DNA parentage analyses for determining relative growth rates of *Penaeus japonicus* families reared in commercial ponds. Aquaculture. 254: 171-181.

Jiang, Z. 2007. Does α-methylacyl-CoA-racemase play an important role in perineural tumer invasion?- reply. Hum. Pathol. 2007: 1289-1291.

Jiang, Z., Fanger, G. R., Woda, B. A., Banner, B. F., Algate, P., Dresser, K., Xu, J., and Chu, P. G. 2008. Expression of α-methylacyl-CoA-racemase (P504S) in various malignant neoplasms and normal tissues: a study of 761 cases. Hum. Pathol. 34: 792-796.

Kakhai, N., and Petjamrat, K. 1992. Survey on species and broodstock collection of abalone (Haliotis spp.) in Chon Buri, Rayong and Trad Provinces. Technical Paper No. 6/1992, Rayong Coastal Aquaculture Station, Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand, 31 pp. (in Thai, with English abstract).

Kashfi, K., and Cook, G. A. 1995. Temperature effects on malonyl-CoA inhibition of carnitine palmitoyltransferase I. Biochim. Biophys. Acta. 1257: 133-139.

Kawasaki-Nishi, S., Nishi, T., and Forgac, M. 2003. Proton translocation driven by ATP hydrolysis in V-ATPases. FEBS Letters. 545: 76-85.

Khamnamtong, B., Thumrungtanakit, S., Klinbunga, S., Aoki, T., Hirono, I., and Menasveta, P. 2006. Identification of sex-specific expression markers in the giant tiger shrimp (*Penaeus monodon*). J. Biochem. Mol. Biol. 39: 37-45.

Kimpton, C. P., Gill, P., Walton, A., Urquhart, A., Millican, E. S., and Adams, M. 1993. Automated DNA profiling employing multiplex amplification of short tendem repeat loci. PCR Methods Applications 3: 13-22.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111-120.

Kimura, M. and Ohta, T. 1971. Theoretical Aspects OF Population Genetics. Princeton University Press, Princeton, New York.

Kirby, L. T. 1992. DNA Fingerprinting: An introduction. New York: W. H. Freeman.

Klinbunga, S., Amparyup, P., Leelatanawit, R., Tassanakajon, A., Hirono, I., Aoki, T., Jarayabhand, A., and Menasveta, P. 2004. Species-identification of the tropical abalone (*Haliotis asinina*, *Haliotis ovina* and *Haliotis varia*) in Thailand using RAPD and SCAR markers. J. Biochem. Mol. Biol. 37: 213-222.

Klinbunga, S., Khamnamtong, B., Puanglarp, N., Jarayabhand, P., Yoosukh, W. and Menasveta, P. 2005. Molecular taxonomy of cupped oysters (*Crassostrea*, *Saccostrea* and *Striostrea*) in Thailand based on COI, 16S and 18S rDNA polymorphism. Mar. Biotechnol. 7: 306-317.

Klinbunga, S., Khetpu., K., Khamnamtong, B., and Menasveta, P. 2007. Development of a species-diagnostic SCAR marker of the blue swimming crab (*Portunus pelagicus*). Biochem. Genet. 45: 755-760.

Kong, L., and Li, Q. 2007. Genetic comparison of cultured and wild populations of the clam *Coelomactra antiquate* (Spengler) in China using AFLP markers. Aquaculture. 271: 152-161.

Lapyernyong, D. 2005. Use of protease in the production of protein hydrolysate from abalone Haliotis asinina Linnaeus. Master's Thesis. Department of Food Science. Faculty of Science, Chulalongkorn University.

Li, Q., and Kijima, A. 2007. Sequences characterization of microsatellite DNA sequences in Pacific abalone (*Haliotis discus hannai*). J. Ocean Univ. Chin. 6: 47-52.

Li, Q., Park, C., Endo, T., and Kijima, A. 2004. Loss of genetic variation at microsatellite loci in hatchery strains of the Pacific abalone (*Haliotis discus hannai*). Aquaculture. 235: 207-222.

Li, Q., Park, C., Kobayashi, T., and Kijima, A. 2003. Inheritance of microsatellite DNA markers in the pacific abalone *Haliotis discus hannai*. Mar. Biotechnol. 5: 331-338.

Li, Z., Li, J., Wang, Q., He, Y., and Liu, P. 2006. The effects of selective breeding on the genetic structure of shrimp *Fenneropenaeus chinensis* populations. Aquaculture. 258: 278-282.

Li, Z., Xu, K., and Yu, R. 2007. Genetic variation in Chinese hatchery populations of the Japanese scallop (*Patinopecten yessoensis*) inferred from microsatellite data. Aquaculture. 269: 211-219.

Liu, Z. F., and Cordes, J. F. 2004. DNA marker technologies and their applications in aquaculture genetics. Aquaculture. 238: 1–37.

Locher, R., and Baur, B. 2001. Isolation of microsatellite markers in the hermaphroditic land snail Arianta arbustorum (Gastropoda). Molecular Ecology Notes. 1: 39-40.

Lucas, T., Macbeth, M., Degnan, S. M., Knibb, W., and Degnan, B. M. 2006. Heritability estimates for growth in the tropical abalone *Haliotis asinina* using microsatellites to assign parentage. Aquaculture. 259: 146–152.

MacAvoy, E. S., Wood, A. R., and Gardner, J. P. A. 2008. Development and evaluation of microsatellite markers for identification of individual Greenshell mussels (*Perna canaliculus*) in a selective breeding programme. Aquaculture. 274: 41-48.

McCombie, W. R., Adams, M. D., Keller, J. M., Fitzgerald, M. G., Utterback, T. R., Khan, M., Dubnick, M., Kerlavage, A. R., Venter, J. C., and Fields, C. 1992. *Caenorhabditis elegans* expressed sequence tags identify gene families and potential disease homologues. Nature Genet. 1: 124-131.

McShane, P. E., Black, K. P., and Smith, M. G. 1988. Recruitment Processes in *Haliotis rabra* (Mollusca: Gastropoda) and regional hydrodynamics in southeastern Australia imply localized dispersal of larvae. J. Exp. Mar. Biol. Ecol. 124: 175-203.

Meechan, D., Xu, Z., Zuniga, G., and Alcuvar-Warren, A. 2003. High frequency and large number of polymorphic microsatellites in cultured shrimp, *Penaeus* (*Litopenaeus*) *vannamei* [Crustacea: *Decapoda*]. Mar Biotechnol. 5: 311-330.

Mileikovsky, S. A. 1971. Types of larval development in marine bottom invertebrates, their distribution and ecological significance. Mar. Biol. 10: 193-213.

Miller, K. M., Laberee, K., Kaukinen, K. H., Li, S., and Withler, R. E. 2001. Development of microsatellite loci in pinto abalone (*Haliotis kamtschatkana*). Molecular Ecology Notes. 1: 315-317

Miller, M. P. 1997. Tools for population genetic analyses TFPGA 1.3: a window program for the analysis of allozyme and molecular population genetic data. Department of Biological Sciences, Northern Arizona University.

Moore, S. S., Whan, V., Davis, P. G., Byrne, K., Hetzel, J. S. D., and Preston, N. 1999. The development and application of genetic markers for the Kuruma prawn *Penaeus japonicus*. Aquaculture. 173: 19-32.

Mullis, K. B., and Faloona, F. A. 1987. Specific synthesis of DNA in vitro via a polymerase-catalysed chain reaction. Methods Enzymol. 155: 335-350.

Nateewathana, A., and Bussarawit, S. 1988. Abundance and distribution of abalones along the Andaman Sea coast of Thailand. Kasetsart Hournal (Natural Science) 22: 8-15.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics. 89: 583-590.

Nei, M. 1987. Molecular Evolutionary Genetics, Columbia University Press, New York, USA.

Ngow, O., and Jarayabhand, P. 1993. Distribution and habitat selection of the abalone, *Haliotis ovina* (Gmelin, 1791), at the eastern coast of Thailand. Presented at the 19th Congress on Science and Technology of Thailand, 27-29 October 1993, Bangkok, 472-473 pp. (in Thai, with English abstract).

O'Brien, E. K., and Degnan, B. M. 2002. Pleiotropic developmental expression of *HasPOU-III*, a class III *POU* gene, in the gastropod *Haliotis asinina*. Mech. Dev. 114: 129-132.

O'Brien, E. K., and Degnan, B. M. 2000. Expression of *POU*, *Sox*, and *Pax* genes in the brain ganglia of the tropical abalone *Haliotis asinina*. Mar. Biotechnol. 2: 545-557.

Orii, K. E., Aoyama, T., Wakui, K., Fukushima, Y., Miyajima, H., Yamaguchi, S., Orii, T., Kondo, N., and Hashimoto, T. 1997. Genomic and mutational analysis

of the mitochondrial trifunctional protein β-subunit (HADHB) gene in patients with trifuctional protein deficiency. Hum. Mol. Genet. 6(8): 1215-1224.

Orita, M., Iwahana, H. K., Kanazawa, H., Hayashi, K., Sekiya, T. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc. Natl. Acad. Sci. USA. 86: 2766–2770.

Palti, Y., Silverstein, J. T., Wieman, H., Phillips, J. G., Barrows, F. T., and Parsons, J. E. 2006. Evaluation of family growth response to fishmeal and gluten-based diets in rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 255: 548–556.

Pampoulie, C., Jorundsdottir, T.D., Steinarsson, A., Petursdottir, G., Stefansson, M.O., and Danielsdottir, A.K. 2006. Genetic comparison of experimental farmed strains and wild Icelandic populations of Atlantic cod (*Gadus morhua L.*). Aquaculture. 261: 556-564.

Pan, Y. W., Chou, H. H., You, E. M., and Yu, H. T. 2004. Isolation and characterization of 23 polymorphic microsatellite markers for diversity and stock analysis in tiger shrimp (*Penaeus monodon*). Molecular Ecology Notes 4: 345–347.

Park, Y. H., Alabady, M. S., Ulloa, M., Sickler, B., Wilkins, T. A., Yu, J., Stelly, D. M., Kohel, R. J., El-Shihy, O. M., and Cantrell, R. G. 2005. Genetic mapping of new cotton fiber loci using EST-derived microsatellites in an interspecific recombinant inbred line cotton population. Mol. Gen. Genomics. 274: 428-441.

Primmer, C. R., Moller, A. P., and Ellegren, H. 1996. A wide-range survey of cross-species microsatellite amplification in birds. Mol. Ecol. 5: 365-378.

Qin, Y., Liu, X., Zhang, H., Zhang, G., and Guo, X. 2007. Genetic mapping of size-related quantitative trait loci (QTL) in the bay scallop (*Argopecten irradians*) using AFLP and microsatellite markers. Aquaculture. 272: 281-290.

Raymond, M., and Rousset, F. 1995. GENEPOP (Version 1.2): a population genetics solfware for exact tests and ecumenicism. J. Heredity. 86: 248-249.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution. 41: 223-225.

Rungpituckmana, T. 2007. Genetic diversity and microsatellite patterns in atchery-propagated Thai abalone, *Haliotis asinina*. Master's Thesis. Department of Marine Science, Faculty of Science, Chulalongkorn University.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory manual, 2nd ed. New York: Cold Spring Habor Laboratory Press.

Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chainterminating inhibitors. Proc. Natl. Acad. Sci. USA. 74: 5463-5467.

Satou, Y., Kawashima., K., and Satoh., N. 2003. Large scale EST analyses in *Ciona intestinalis*: Its application as Northern blot analyses. Dec. Genes. Evol. 213: 314-318.

Sbordoni, V., De Matthaeis, E., Cobolli-Sbordoni, M., La Rosa, G., Mattoccia, M., 1986. Bottleneck effects and the depression of genetic variability in hatchery stocks of *Penaeus japonicus* (Crustacea, Decapoda). Aquaculture. 57: 239–251.

Schrey, A. W., and Heist, E. J. 2003. Microsatellite analysis of population structure in the shortfin mako (*Isurus oxyrinchus*). Can. J. Fish. Aquat. Sci. 60: 670–675.

Schuler A. M., and Wood, P. A. 2002. Mouse models for disorders of mitochondrial fatty acid β-oxidation. ILAR. J. 34: 57-65.

Scott, D. D., Eggler, P., Seaton, G., Rossetto, M., Ablett, E. M., Lee, L. S., and Henry, R. J. 2000. Analysis of SSRs derived from grapes EST. Theor Appl Genet. 100: 723-726.

Sekino, M., and Hara, M. 2001. Microsatellite DNA loci in Pacific abalone *Haliotis discus discus* (Mollusca, Gastropoda, Haliotidae). Molecular Ecology Notes. 1: 8–10.

Sekino, M., and Hara, M. 2007. Individual assignment tests proved genetic boundaries in a species complex of Pacific abalone (genus *Haliotis*). Conserv. Genet.8: 823-841.

Sekino, M., Saido, T., Fujita, T., Kobayashi, T., and Takami, H. 2005. Microsatellite DNA markers of Ezo abalone (*Haliotis discus hannai*): a preliminary assessment of natural populations samples from heavily stocked areas. Aquaculture. 243: 33-47.

Selvamani, M. J. P., Degnan, S. M., and Degnan, B. M. 2001. Microsatellite genotyping of individual abalone larvae: parentage assignment in aquaculture. Mar Biotechnol. 3: 478-485.

Selvamani, M. J. P., Degnan, S. M., Paetkau, D., and Degnan, B. M. 2000. Highly polymorphic microsatellite loci in the Heron Reef population of the tropical abalone *Haliotis asinina*. Mol. Ecol. 9: 1184-1186.

Shepherd, S. A., and Laws, H. M. 1974. Studies on southern Australian abalone (genus *Haliotis*). II. Reproduction of five species. Aust. J. Mar. Freshw. Res. 25(1): 49-62.

Shepherd, S. A., Lowe, D., and Partington, D. 1992. Studies of southern Australian abalone (genus *Haliotis*) XIII: larval dispersal and recruitment. J. Exp. Mar. Biol Ecol. 164: 247-260.

Singhagraiwan, T., and Doi, M. 1993. Seed production and culture of a tropical abalone, Haliotis asinina Linne. The research project of fishery resource development in the Kingdom of Thailand. Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand, 32 pp.

Sriphairoj, K., Na-Nakorn, U., Brunelli, J. P., and Thorgaard, G. H. 2007. No AFLP sex-specific markers detected in *Pangasianodon gigas* and *P. hypophthalmus*. Aquaculture. 273: 739-743.

Stalling, R. L., Ford, A. F., Nelson, D., Torney, D. C., Hildebrand, C. E., and Moyzis, R. K. 1991. Evolution and distribution of (GT)n repetitive sequences in mammalian grnomes. Genomics. 10: 807-815.

Steven, C. R., Hill, J., Masters, B., and Place, A. R. 2005. Genetic markers in blue carbs (*Callinectes sapidus*) I: isolation and characterization of microsatellite markers. J. Exp. Mar. Biol. Ecol. 319: 3-14.

Tang, S., Popongviwat, A., Klinbunga, S., Tassanakajon, A., Jarayabhand, P., and Menasveta, P. 2005. Genetic heterogeneity of the tropical abalone (*Haliotis asinina*) revealed by RAPD and microsatellite analyses. J. Biochem. Mol. Biol. 38: 182-190.

Tang, S., Tassanakajon, A., Klinbunga, S., Jarayabhand, P., and Menasveta, P. 2004. Population structure of tropical abalone (*Haliotis asinina*) in coastal waters of Thailand determined using microsatellite markers. Mar. Biotechnol. 6: 604-611.

Tassanakajon, A., Pongsomboon, S., Jarayabhand, P., Klinbunga, S., and Boonsaeng, V. 1998. Genetic structure in wild populations of the black tiger shrimp

(*Penaeus monodon*) using randomly amplified polymorphic DNA analysis. J. Mar. Biotechnol. 6: 249-254.

Tharin, S., Dziak, E., Michalak, M., and Opas, M. 1992. Widespread tissue distribution of rabbit calreticulin, a non-muscle functional analogue of calsequestrin. Cell Tissue Res. 269: 29-37.

Thiel, T., Michalek, W., Varshney, R. K., and Graner, A., 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.) Theor Appl Genet. 106: 411-422.

Thorson, G. 1964. Light as an ecological factor in the dispersal and settlement of larvae of marine bottom invertebrates. Ophelia. 1: 167-208.

Tookvinas, S., Leknim, V., Donyadol, Y., Predalampabut, Y., and Paengmark, P. 1986. A survey of species and distribution of abalone (*Haliotis* spp) in Surat Thani, Nakhon Si Thammarat and Songkla. Tech. Rep. No. 1/1986 NICA, 16 pp.

Tutschulte, T., and Connell, J. H. 1981. Reproductive biology of three species of abalones (*Haliotis*) in southern California. Veliger. 23: 195-206.

Uki, N. 1989. Abalone seeding production and its theory (1). International Journal of Aquaculture Fish Technology 1: 3-15.

Valdes, A. M., Slatkin, M., and Freimer, M. B. 1993. Allele frequencies at microsatellite loci: the stepwise mutation model revisited. Genetics. 133: 737-740.

Valles-Jimenez, R., Cruz, P., and Perez-Enriquez, R. 2005. Population genetic structure of Pacific white shrimp (*Litopenaeus vannamei*) from Mexico to Panama: microsatellite DNA variation. Mar Biotechnol. 6: 475-484.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407-4414.

Wang, L., Zhang, H., Song, L., and Guo, X. 2007. Loss of allele diversity in introduced populations of the hermaphroditic bay scallop *Argopecten irradians*. Aquaculture. 271: 252-259.

Waterston, R., Martin, C., Craxton, M., Huynh, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Shownkeen, R., Halloran, N., Metzstein, M., Hawkins, T., Wilson, R., Berks, M., Du, Z., Thomas, K., Thierry-Mieg, J., and Sulston, J. 1992. A survey of expressed genes in *Caenorhabditis elegans*. Nature Genet. 1: 114-123.

Weir, B. S., and Cockerham, C. C. 1984. Estimation *F*-statistics for the analysis of population structure. Evolution. 38: 1358-1370.

Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18: 7213–7218.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531–6535.

Wood, A. D., and Buxton, C. D. 1996. Aspects of biology of the abalone *Haliotis midae* (Linne', 1758) on the east coast of South Africa. I. Feeding biology. S. Afr. J. Mar. Sci. 17: 61-68.

Wright, J. M., and Bentzen, P. 1995. Microsatellites: genetic markers for the future. In Carvalho, G.R. and Pitcher, T.J. (eds.), molecular genetic in fisheries. p.117-122. Great Britain: Chapman & Hall.

Yang, C., Zhu, X., and Sun, X. 2008. Development of microsatellite markers and their utilization in genetic diversity analysis of cultivated and wild populations of the mud carp (*Cirrhina molitorella*). J. Genet. Genomics. 35: 201-206.

Yao, G. F., Feng, H. T., Cai, Y, L., Qi, W. L, and Kong, K. M. 2007. Characterization of vacuolar-ATPase and selective inhibition of vacuolar-H(+)-ATPase in osteoclasts. Biochem. Biophys. Res. Commun. 357: 821-827.

Yu, D. H., and Chu, K. H. 2006. Low genetic differentiation among widely separated populations of the pearl oyster *Pinctada fucata* as revealed by AFLP. J. Exp. Mar. Biol. Ecol. 333: 140-146.

Yue, H. G., Ho, M. Y., Orban, L., and Komen, J. 2004. Microsatellites within genes and ESTs of common carp and their applicability in silver crucian carp. Aquaculture. 234: 85-98.

Zeng, S., and Gong, Z. 2002. Expressed sequence tag analysis of expression profiles of zebrafish testis and ovary. Gene. 294: 45-53.

Zhang, J., Wang, H., and Cai, Z. 2007. The application of DGGE and AFLP-derived SCAR for discrimination between Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Food Cont. 18: 672-676.

Zhao, C., Li Q., and Kong, L. 2009. Inheritance of AFLP makers and their use for genetic diversity analysis in wild and farmed scallop (*Chlamys farreri*). Aquaculture. 287: 67-74.

APPENDICES

APPENDIX A

Microsatellites containing EST found in testis, ovary, and hemocyte cDNA library of *H. asinina*

clone	motif	No. of repeats	SSR start	SSR end	Length	cDNA library	Gene homologue
PHe 004	cag	4	636	647	1073	Haemocyte	*B-cell translocation gene 2*
	caa	4	648	659	1073		
PHe 024	ccga	8	877	908	1022	Haemocyte	Unknown
PHe 027	tta	4	113	124	850	Haemocyte	*Lysin precursor, gene, exons 4 and 5 and partial*
PHe 041	ga	5	511	520	913	Haemocyte	*Choline cotransporter*
PHe 061	ttg	4	457	468	651	Haemocyte	Unknown
PHe 085	ttg	4	449	460	625	Haemocyte	Unknown
PHe 104	tg	5	594	603	696	Haemocyte	Unknown
PHe 140	ta	5	373	382	578	Haemocyte	Unknown
PHe 150	tg	5	386	395	511	Haemocyte	Unknown
	gt	9	416	433	511		
	gt	6	452	463	511		
	gtgc	5	432	451	511		
PHe 156	tg	6	139	150	676	Haemocyte	Unknown
	tca	8	644	667	676		
	gtgtgc	6	146	181	676		
PHe 166	ta	5	889	898	934	Haemocyte	*Mannose-binding lectin-associated serine protease-3b*
PHe 177	ta	7	634	647	749	Haemocyte	Unknown
PHe 198	cat	10	689	718	766	Haemocyte	Unknown
PHe 221	tac	5	124	138	835	Haemocyte	*Thrombospondin 1*
PHe 224	ga	5	170	179	195	Haemocyte	Unknown
PHe 238	at	6	794	805	1001	Haemocyte	*Neurexin IV*
ST 099	tc	5	321	330	399	Testis Subtraction	Unknown
ST 119	tc	5	287	296	389	Testis Subtraction	Unknown
SO 056	tcc	4	87	98	216	Ovary Subtraction	*Mitotic Apparatus Protein P62*

Appendix A (cont.)

Clone	motif	No. of repeats	SSR start	SSR end	Sequence Length	cDNA library	*Predicted*
SO 108	gtg	4	527	538	665	Testis Subtraction	*Vitelline coat protein 41*
PT 010	cag	7	457	477	766	Testis	*Ribosomal protein L41*
	atg	10	703	732	766		
PT 036	ga	5	487	496	761	Testis	*ENSANGP00000014099 [Anopheles gambiae]*
PT 039	gat	4	591	602	702	Testis	*High mobility group protein 1; HMG 1*
PT 041	aag	4	99	110	423	Testis	*Ribosomal protein S25*
PT 044	tg	5	608	617	810	Testis	*Unknown (protein for IMAGE: 4309224) [Homo sapiens]*
PT 061	aag	4	405	416	529	Testis	*Ribosomal protein S24*
PT 083	ga	5	487	496	718	Testis	*ENSANGP00000014099 [Anopheles gambiae]*
PT 093	gat	4	52	63	735	Testis	Unknown
PT 103	ag	5	609	618	789	Testis	Unknown
PO 022	tca	4	294	305	504	Ovary	Unknown
PO 052	tg	5	510	519	533	Ovary	*Ribosomal protein L10A*
PO 067	gac	4	331	342	822	Ovary	*Elongation factor 1 delta*
PO 097	atg	10	130	159	346	Ovary	Unknown
PO 104	gat	4	342	353	774	Ovary	*Eukaryotic translation elongation factor 1 beta 2*
PO 111	gac	4	898	909	956	Ovary	*Vitelline coat protein 41*
PO 114	tca	4	216	227	418	Ovary	Unknown
PO 138	aat	4	251	262	685	Ovary	Unknown
PO 179	tgag	5	49	68	555	Ovary	*Cysteine proteinase 1 precursor*
PO 216	ag	6	521	532	678	Ovary	*Ribosomal protein L13*

APPENDIX B

Microsatellites containing ESTs of *Haliotis spp.* analyzed from those deposited to the GenBank

Clone	motif	No. of repeats	SSR start	SSR end	Length	Organism	Gene
DW986507	att	4	556	567	727	*H. asinina*	Unknown
DW986495	tga	6	742	759	823	*H. asinina*	*Tigger transposable element ML8F6*
DW986470	at	5	207	216	514	*H. asinina*	Unknown
DW986435	caa	4	216	227	741	*H. asinina*	Unknown
DW986434	gaa	4	556	567	850	*H. asinina*	Unknown
DW986410	aaaac	4	215	234	278	*H. asinina*	Unknown
DW986401	tg	5	31	40	722	*H. asinina*	Unknown
DW986374	ag	5	112	121	443	*H. asinina*	Unknown
DW986302	cact	9	459	494	512	*H. asinina*	Unknown
DW986291	ccg	4	89	100	540	*H. asinina*	*ribosomal protein S12 ML5B12*
DW986231	gagt	4	98	113	488	*H. asinina*	Unknown
DW986221	gat	5	132	146	297	*H. asinina*	*clathrin light polypeptide ML1E8*
DW986503	atg	12	165	200	402	*H. asinina*	Unknown
DW986499	ag	4	271	278	876	*H. asinina*	*Eukaryotic translation initiation factor ML8G1*
	gct	4	542	553	876		
DW986498	cca	4	339	350	578	*H. asinina*	Unknown
DW986463	caa	4	156	167	473	*H. asinina*	Unknown
DW986455	tg	7	365	378	819	*H. asinina*	Unknown
	tg	13	383	408	819		
	gc	7	408	421	819		
DW986443	tcg	4	183	194	413	*H. asinina*	Unknown
DW986440	ca	5	367	376	487	*H. asinina*	*hypothetical protein ML7F6*
DW986404	gat	8	545	568	850	*H. asinina*	Unknown
DW986393	tcac	8	709	740	794	*H. asinina*	*lysin-like ML6G9*
DW986392	tc	5	69	78	797	*H. asinina*	*FERM domain containing ML6G4*

Appendix B (cont.)

Clone	motif	No. of repeats	SSR start	SSR end	Length	Organism	Predicted
DW986382	aga	5	121	135	566	*H. asinina*	Unknown
DW986345	aatg	4	727	742	871	*H. asinina*	Unknown
DW986307	ga	5	255	264	497	*H. asinina*	*ribosomal protein S17 ML5D3*
DW986295	ca	5	183	192	914	*H. asinina*	*solute carrier family 39 ML5B7*
DW986239	caa	11	248	280	527	*H. asinina*	Unknown
DW986222	agtg	5	51	70	211	*H. asinina*	*hemocyanin ML1E9*
DY403075	agg	4	323	334	588	*H. asinina*	Unknown
DY403054	at	5	344	353	802	*H. asinina*	Unknown
	tg	5	406	415	802		
	gc	6	423	434	802		
DY402936	aag	5	570	584	618	*H. asinina*	*node of Ranvier*
DY403115	tg	5	31	40	720	*H. asinina*	Unknown
DY403055	tgta	4	245	260	778	*H. asinina*	Unknown
DY403047	ct	4	295	302	500	*H. asinina*	Unknown
	caa	4	219	230	500		
DY403027	ga	5	45	54	222	*H. asinina*	Unknown
DY403023	att	4	244	255	335	*H. asinina*	Unknown
DY403013	cagcaa	4	279	302	500	*H. asinina*	Unknown
DY402968	ta	5	768	777	821	*H. asinina*	*Selenoprotein W, 2a*
	tta	4	524	535	821		
DY402914	ctac	4	76	91	1039	*H. asinina*	*inhibitor of differentiation 2*
DY402903	tg	6	451	462	653	*H. asinina*	*Heat shock protein 10*
DY402865	gat	4	482	493	600	*H. asinina*	*beta-NAC-like protein*
DN856388	tga	4	118	129	777	*H. discus discus*	Unknown
DN856373	cac	4	34	45	640	*H. discus discus*	Unknown
DN856343	cat	8	176	199	776	*H. discus discus*	Unknown
DN856331	at	8	155	170	746	*H. discus discus*	Unknown
CX727307	aaca	4	588	603	740	*H. discus*	Unknown

Appendix B (cont.)

Clone	motif	No. of repeats	SSR start	SSR end	Sequence Length	Organism	Predicted
CX727232	ggaacg	5	696	725	725	*H. discus*	Unknown
CX727231	ct	5	112	121	731	*H. discus*	Unknown
CX727214	cac	4	284	295	545	*H. discus*	Unknown
	aca	4	330	341	545		
CX727208	cac	4	469	480	681	*H. discus*	Unknown
	aca	4	515	526	681		
CX727202	aaca	5	506	525	587	*H. discus*	Unknown
CX727188	cta	4	90	101	533	*H. discus*	Unknown
	ctg	6	117	134	533		
CX727179	atc	4	79	90	410	*H. discus*	Unknown
CX727163	atc	4	520	531	608	*H. discus*	Unknown
CX727104	tg	5	266	275	616	*H. discus*	Unknown
CX727102	actc	4	246	261	716	*H. discus*	Unknown
CX727092	tga	8	662	685	699	*H. discus*	Unknown
CX727068	ta	5	80	89	680	*H. discus*	Unknown
CX727064	ta	5	224	233	715	*H. discus*	Unknown
CX727058	actc	4	267	282	615	*H. discus*	Unknown
CX727054	gt	4	242	249	701	*H. discus*	Unknown
	gt	4	393	400	701		
	gat	7	303	323	701		
CX727034	gag	4	499	510	650	*H. discus*	Unknown
CX727009	gaa	19	496	552	798	*H. discus*	Unknown
CX727008	tgc	4	246	257	435	*H. discus*	Unknown
CX726959	gtga	4	117	132	601	*H. discus*	Unknown
CX726955	tc	5	124	133	686	*H. discus*	Unknown
CX726917	agg	4	94	105	501	*H. discus*	Unknown
CX726905	ga	4	292	299	679	*H. discus*	Unknown
	aag	4	484	495	679		

Appendix B (cont.)

Clone	motif	No. of repeats	SSR start	SSR end	Sequence Length	Organism	Predicted
CX726902	at	4	87	94	688	*H. discus*	Unknown
	agtg	4	338	353	688		
CX726888	tga	4	588	599	669	*H. discus*	Unknown
CX726880	ta	5	499	508	763	*H. discus*	Unknown
CX726869	ag	4	96	103	707	*H. discus*	Unknown
	gca	5	158	172	707		
CX726838	tg	4	403	410	816	*H. discus*	Unknown
	gt	5	418	427	816		
	gtga	5	426	445	816		
	acgcc	11	67	121	816		
CX726806	gca	5	654	668	737	*H. discus*	Unknown
CX726794	ta	8	6	21	477	*H. discus*	Unknown
	tta	11	443	475	477		
CX726787	ga	12	21	44	349	*H. discus*	Unknown
CX726786	ga	12	23	46	791	*H. discus*	Unknown
CX726752	agc	4	556	567	679	*H. discus*	Unknown
CX726751	cca	5	411	425	541	*H. discus*	Unknown
CX726742	tga	6	620	637	639	*H. discus*	Unknown
CX726732	agc	8	675	698	733	*H. discus*	Unknown
CX726686	gt	5	456	465	569	*H. discus*	Unknown
CX726647	tg	5	333	342	479	*H. discus*	Unknown
CX726610	ag	5	181	190	651	*H. discus*	Unknown
CX726598	actc	13	481	532	762	*H. discus*	Unknown
CX726578	tgat	4	578	593	643	*H. discus*	Unknown
CX726573	ga	6	24	35	617	*H. discus*	Unknown
CX726549	aat	4	459	470	488	*H. discus*	Unknown
CX726539	ag	4	329	336	790	*H. discus*	Unknown
	gca	4	391	402	790		

Appendix B (cont.)

Clone	motif	No. of repeats	SSR start	SSR end	Sequence Length	Organism	Predicted
CX726536	att	4	460	471	543	*H. discus*	Unknown
CX726518	actc	11	462	505	713	*H. discus*	Unknown
CX726495	ag	5	150	159	755	*H. discus*	Unknown
CX726491	gt	5	57	66	653	*H. discus*	Unknown
CX726485	cta	4	24	35	390	*H. discus*	Unknown
	tac	5	55	69	390		
CX726473	at	4	258	265	766	*H. discus*	Unknown
	caa	4	590	601	766		
CX726464	agc	4	597	608	756	*H. discus*	Unknown
CX726427	tga	9	665	691	710	*H. discus*	Unknown
CX726423	caac	4	702	717	769	*H. discus*	Unknown
CX726415	at	15	308	337	656	*H. discus*	Unknown
CX726410	gt	6	176	187	704	*H. discus*	Unknown
CX726390	ta	6	591	602	747	*H. discus*	Unknown
	at	4	605	612	747		
CX726361	at	9	47	64	753	*H. discus*	Unknown
CX726346	at	4	306	313	668	*H. discus*	Unknown
	aga	4	315	326	668		
	atg	4	402	413	668		
CX726310	actc	4	416	431	744	*H. discus*	Unknown
CX726305	ct	6	26	37	711	*H. discus*	Unknown
CX726261	tgt	5	275	289	645	*H. discus*	Unknown
CX726244	gcc	4	124	135	722	*H. discus*	Unknown
CX726241	at	8	247	262	719	*H. discus*	Unknown
	ta	13	264	289	719		
	ta	7	292	305	719		
	ta	7	324	337	719		
	ca	4	547	554	719		

Appendix B (cont.)

Clone	motif	No. of repeats	SSR start	SSR end	Sequence Length	Organism	Predicted
CX726240	ga	5	24	33	726	*H. discus*	Unknown
CX726239	tg	4	256	263	563	*H. discus*	Unknown
	ca	5	475	484	563		
CX726226	tga	14	672	713	725	*H. discus*	Unknown
CX726222	cgt	4	620	631	646	*H. discus*	Unknown
CX726221	tc	5	485	494	600	*H. discus*	Unknown
CX726204	ga	5	26	35	761	*H. discus*	Unknown
CX726188	ga	6	28	39	817	*H. discus*	Unknown
CX726187	cgt	4	303	314	733	*H. discus*	Unknown
CX726186	ct	8	26	41	737	*H. discus*	Unknown
	aac	4	685	696	737		
CX726174	tca	4	243	254	731	*H. discus*	Unknown
CX726170	cag	7	123	143	650	*H. discus*	Unknown
CX726151	actc	4	694	709	726	*H. discus*	Unknown
CX726147	aag	4	507	518	736	*H. discus*	Unknown
CX726144	gt	5	461	470	743	*H. discus*	Unknown
CX726136	actc	4	32	47	722	*H. discus*	Unknown
CX726125	gct	4	101	112	704	*H. discus*	Unknown
	agc	5	535	549	704		
	gca	4	557	568	704		
CX726118	ag	5	742	751	800	*H. discus*	Unknown
CX726105	ag	5	108	117	714	*H. discus*	Unknown
CX726072	aag	5	133	147	720	*H. discus*	Unknown
	aag	4	373	384	720		
CX726055	ac	4	330	337	752	*H. discus*	Unknown
	at	4	596	603	752		
	tca	5	452	466	752		
CX726042	ac	4	88	95	751	*H. discus*	Unknown

Appendix B (cont.)

Clone	motif	No. of repeats	SSR start	SSR end	Sequence Length	Organism	Predicted
CX726041	tgag	4	545	560	749	*H. discus*	Unknown
CX726030	ga	6	23	34	668	*H. discus*	Unknown
CX726027	tga	6	657	674	675	*H. discus*	Unknown
CX726020	at	5	353	362	765	*H. discus*	Unknown
	at	4	521	528	765		
CX726017	gcc	4	265	276	772	*H. discus*	Unknown
CX726016	tga	6	667	684	759	*H. discus*	Unknown
CX725994	ctgc	4	111	126	338	*H. discus*	Unknown
CX725957	gt	5	24	33	303	*H. discus*	Unknown
CX725941	acc	4	223	234	423	*H. discus*	Unknown
AY449741	gttt	4	528	543	676	*H. diversicolor supertexta*	Unknown

APPENDIX C

Genotypes of *H. asinina* in this study at 6 microsatellite loci

Samples	Locus					
	DW455	*DW503*	*PHe177*	*PT102*	*Haμ9*	*Haμ10*
***Po* generation, Samet Island founders (east)**						
SAME1	191/217	196/196	180/180	186/186	126/127	158/173
SAME2	191/217	196/196	180/180	186/186	123/127	158/173
SAME3	191/217	196/196	180/180	186/186	123/127	154/167
SAME4	191/217	196/196	180/180	186/186	123/127	158/181
SAME5	191/217	196/196	180/180	186/186	127/129	158/173
SAME6	191/191	196/196	180/180	186/186	127/129	167/181
SAME7	203/209	196/196	180/180	186/186	126/129	167/176
SAME8	191/194	196/196	180/180	186/186	123/127	154/163
SAME9	191/252	196/196	180/180	186/186	126/129	161/173
SAME10	191/217	196/196	180/180	186/186	127/129	167/181
SAME11	191/217	196/201	180/183	186/188	126/129	167/173
SAME12	209/233	180/196	180/180	177/186	123/127	163/163
SAME13	209/233	196/196	180/180	186/186	123/127	163/163
SAME14	191/217	196/196	180/180	186/186	127/129	163/163
SAME15	191/217	196/196	180/180	186/186	123/127	163/163
SAME16	217/217	196/196	180/180	186/186	126/129	173/173
Talibong Island, Trang province (west)						
TRGW1	267/267	174/177	157/166	173/173	122/122	140/140
TRGW2	267/267	174/177	170/170	173/173	122/122	140/140
TRGW3	256/272	174/180	170/170	173/177	122/132	141/141
TRGW4	256/272	174/177	157/166	173/173	122/132	140/140
TRGW5	267/267	174/174	170/170	173/173	122/132	141/143
TRGW6	233/267	174/174	157/166	173/173	122/132	134/134
TRGW7	252/252	174/174	170/170	173/173	122/132	134/143
TRGW8	233/267	174/201	170/170	173/190	132/137	134/134
TRGW9	267/267	174/180	157/166	173/177	122/126	140/141
TRGW10	267/267	174/196	170/170	188/188	122/132	143/143
TRGW11	267/267	180/183	157/166	177/183	132/132	134/134
TRGW12	267/267	174/196	170/170	186/188	122/126	141/143
TRGW13	252/256	174/183	170/170	173/183	122/126	134/140

Samples	Locus					
	DW455	*DW503*	*PHe177*	*PT102*	*Haμ9*	*Haμ10*
TRGW14	252/252	174/180	166/166	173/177	122/122	134/134
TRGW15	267/267	174/183	157/166	186/188	122/122	134/140
TRGW16	267/267	174/188	166/166	173/188	122/132	141/143
TRGW17	252/256	174/180	170/170	173/177	122/137	141/143
TRGW18	256/267	174/196	170/170	188/188	122/122	140/140
TRGW19	267/267	174/183	157/166	173/183	122/137	141/143
TRGW20	267/267	174/174	170/170	173/173	122/122	141/143
TRGW21	256/267	174/196	170/170	188/188	122/122	141/141
TRGW22	256/272	183/196	170/170	177/183	122/132	140/141
TRGW23	256/272	174/174	157/166	173/186	122/132	140/140
TRGW24	272/272	174/183	166/170	186/186	122/126	143/143
TRGW25	256/272	183/196	170/170	188/188	122/132	141/143
Cambodia (east)						
CAME1	191/191	196/201	180/180	186/188	126/126	158/176
CAME2	191/191	196/196	180/180	186/186	126/126	167/181
CAME3	191/209	196/196	180/180	186/186	122/123	173/176
CAME4	191/233	196/201	180/180	186/188	122/123	161/176
CAME5	194/233	201/201	163/180	188/188	126/126	158/167
CAME6	191/191	196/201	180/180	186/188	126/126	154/161
CAME7	191/217	196/201	180/180	186/188	122/123	173/176
CAME8	191/209	196/196	180/180	186/186	122/122	167/176
CAME9	191/194	196/201	180/180	186/186	123/123	154/176
CAME10	191/209	196/196	163/180	186/186	123/123	158/167
CAME11	191/191	196/196	180/180	186/186	122/122	176/176
CAME12	209/217	196/196	163/180	186/186	126/126	167/167
CAME13	209/217	196/196	180/180	186/186	127/129	158/176
CAME14	191/233	196/201	180/180	186/188	123/126	161/167
CAME15	191/191	201/201	180/180	186/188	127/129	158/176
CAME16	209/233	196/196	180/180	188/188	122/122	163/167
CAME17	191/203	196/201	180/180	186/186	122/123	161/167
CAME18	191/191	196/201	180/180	186/188	123/126	158/167
CAME19	191/203	196/196	180/180	186/186	122/126	173/173
CAME20	191/194	196/196	180/180	186/186	122/122	167/176

Samples	Locus					
	DW455	*DW503*	*PHe177*	*PT102*	*Haμ9*	*Haμ10*
F1 generation, the Philippines						
PHI1	209/233	201/201	157/163	190/190	122/126	161/161
PHI2	203/233	180/201	157/166	177/190	122/126	161/161
PHI3	209/233	201/201	157/163	190/190	107/110	158/161
PHI4	209/233	183/201	157/163	183/190	122/126	161/161
PHI5	209/233	201/201	163/166	190/190	107/110	161/161
PHI6	203/233	180/183	163/166	177/183	122/126	161/161
PHI7	209/233	201/201	163/163	190/190	107/110	158/158
PHI8	209/233	201/201	163/166	190/190	122/126	158/161
PHI9	209/233	201/201	163/163	190/190	122/126	161/161
PHI10	209/233	201/201	163/163	190/190	107/110	158/158
PHI11	203/233	180/201	163/163	177/190	107/110	158/158
PHI12	203/233	180/201	157/163	177/190	107/110	158/161
PHI13	209/233	183/201	163/163	183/190	107/110	161/161
PHI14	203/233	183/201	163/166	183/190	122/126	158/161
PHI15	203/233	180/201	157/166	177/190	107/110	158/161
PHI16	203/233	180/183	157/166	177/183	122/126	158/161
PHI17	203/233	180/183	163/166	177/183	122/126	161/161
PHI18	209/233	180/183	157/166	177/183	122/126	161/161
PHI19	209/233	201/201	163/166	190/190	122/126	158/161
PHI20	209/233	201/201	163/166	190/190	122/126	158/161
Hatchery-propagated cultured at Trang						
CTRGH1	209/217	196/196	180/180	186/186	126/126	176/181
CTRGH2	233/252	180/196	166/166	177/186	126/127	173/176
CTRGH3	252/267	196/196	166/180	186/186	126/126	173/181
CTRGH4	191/203	196/196	180/180	186/186	126/126	173/176
CTRGH5	191/203	196/196	180/180	186/186	123/123	173/181
CTRGH6	191/209	196/196	180/183	186/186	123/123	173/173
CTRGH7	191/203	196/196	180/180	186/186	123/123	163/163
CTRGH8	209/233	196/196	163/180	186/186	126/129	163/163
CTRGH9	191/203	196/196	180/180	186/186	122/126	158/181
CTRGH10	191/233	196/201	180/180	186/188	126/129	163/181
CTRGH11	191/203	201/201	166/180	188/188	126/126	158/176
CTRGH12	191/209	174/196	180/180	173/186	122/126	163/163
CTRGH13	191/191	196/196	180/180	186/186	123/123	173/176

Samples	Locus					
	DW455	*DW503*	*PHe177*	*PT102*	*Haμ9*	*Haμ10*
CTRGH14	191/209	180/196	166/180	177/186	123/126	173/176
CTRGH15	233/252	196/196	163/183	186/186	123/123	173/181
CTRGH16	233/252	196/196	163/183	186/186	123/123	173/181
CTRGH17	191/209	180/196	163/166	177/186	123/127	173/173
CTRGH18	203/217	196/196	157/180	177/186	123/127	173/181
CTRGH19	191/209	180/201	163/180	177/188	123/123	163/163
CTRGH20	191/209	180/196	163/180	177/186	123/126	173/173
CTRGH21	209/233	196/196	157/180	186/186	123/123	163/163
CTRGH22	191/209	196/196	163/180	186/186	123/126	163/163
CTRGH23	191/209	180/201	180/180	186/186	123/126	163/163
CTRGH24	209/233	196/196	180/183	186/186	123/126	158/176
CTRGH25	191/209	196/201	180/180	186/186	123/126	163/163
CTRGH26	191/209	196/201	180/183	186/186	123/126	173/173
CTRGH27	191/203	196/196	180/180	186/186	122/126	173/181
CTRGH28	191/203	196/196	180/180	186/186	123/126	173/181
CTRGH29	191/209	174/196	180/180	177/186	126/127	173/173
CTRGH30	191/209	196/196	157/180	186/186	123/123	173/173
CTRGH31	191/191	180/196	163/180	177/188	126/126	163/173
CTRGH32	191/203	196/201	180/183	177/186	123/126	176/181
CTRGH33	191/203	196/196	180/180	186/186	126/126	163/173
CTRGH34	209/233	196/196	180/180	186/186	126/126	173/173
CTRGH35	191/203	196/196	180/180	186/186	123/123	163/181
CTRGH36	191/203	180/196	180/180	177/186	126/126	163/173
CTRGH37	191/209	196/196	166/180	177/186	123/126	173/181
CTRGH38	191/209	196/196	180/180	186/186	123/123	163/173
CTRGH39	203/233	196/196	166/180	186/186	123/126	158/176
CTRGH40	191/191	180/196	180/180	177/186	123/123	167/167
CTRGH41	191/233	196/196	166/180	177/186	126/126	163/163
CTRGH42	191/233	196/196	180/180	177/188	123/123	173/181
CTRGH43	191/233	196/196	180/180	186/188	126/126	176/181
CTRGH44	191/191	196/196	180/180	186/186	123/126	176/181
CTRGH45	191/233	196/196	180/180	186/186	123/123	173/181
CTRGH46	191/191	196/196	180/180	186/186	123/126	163/173
CTRGH47	191/191	180/196	157/180	177/186	123/123	163/173
CTRGH48	191/203	196/196	180/180	186/186	123/123	173/173

Samples	Locus					
	DW455	*DW503*	*PHe177*	*PT102*	*Haμ9*	*Haμ10*
Hatchery-propagated cultured at Sichang Marine Science Research and Traning Station						
CSMaRTH1	233/233	196/196	180/180	186/186	122/122	161/163
CSMaRTH2	209/252	196/196	180/183	186/186	122/122	161/181
CSMaRTH3	191/203	196/196	180/180	186/186	126/129	158/176
CSMaRTH4	191/203	180/196	163/180	177/186	126/129	167/167
CSMaRTH5	191/203	180/196	180/180	177/186	126/129	158/163
CSMaRTH6	191/252	180/196	163/180	177/186	123/123	163/167
CSMaRTH7	191/203	196/196	180/183	186/186	123/127	176/176
CSMaRTH8	233/252	180/196	180/183	177/186	123/127	163/163
CSMaRTH9	209/252	196/196	166/180	186/186	126/126	176/176
CSMaRTH10	191/209	180/196	163/180	177/186	123/126	158/167
CSMaRTH11	191/209	180/196	163/180	177/186	123/127	167/181
CSMaRTH12	191/203	196/196	166/183	186/186	123/127	158/167
CSMaRTH13	191/203	196/196	157/180	186/186	123/127	161/176
CSMaRTH14	217/252	196/196	163/180	186/186	123/129	161/181
CSMaRTH15	191/203	196/196	157/180	186/186	123/127	161/181
CSMaRTH16	191/217	196/196	180/180	186/186	123/127	167/176
CSMaRTH17	191/217	196/196	180/180	186/186	126/129	163/181
CSMaRTH18	194/194	196/196	180/180	186/186	126/129	158/167
CSMaRTH19	217/217	196/196	180/180	186/186	126/129	163/176
CSMaRTH20	191/217	196/196	157/180	186/186	123/127	163/181
CSMaRTH21	191/217	196/196	180/180	186/186	126/126	158/161
CSMaRTH22	191/217	196/196	157/180	186/186	126/129	161/176
CSMaRTH23	194/252	196/196	180/180	186/186	123/126	167/181
CSMaRTH24	209/209	196/196	180/180	186/186	126/126	167/167
CSMaRTH25	217/217	180/196	180/183	177/186	123/126	176/176
CSMaRTH26	209/209	196/196	157/180	186/186	122/123	176/176
CSMaRTH27	191/217	180/196	157/183	177/186	123/127	163/167
CSMaRTH28	191/217	196/196	180/180	186/186	123/126	161/167
CSMaRTH29	191/191	196/196	180/180	186/186	123/126	167/176
CSMaRTH30	209/233	196/196	180/180	186/186	123/129	161/173
CSMaRTH31	191/209	196/196	180/180	186/186	126/129	143/163
CSMaRTH32	203/209	180/196	170/180	177/186	123/127	154/167
CSMaRTH33	191/191	196/196	180/180	186/186	122/126	143/167
CSMaRTH34	191/191	196/196	180/180	186/186	126/129	143/167

Samples	Locus					
	DW455	*DW503*	*PHe177*	*PT102*	*Haμ9*	*Haμ10*
CSMaRTH35	209/252	180/196	170/180	177/186	126/127	161/181
CSMaRTH36	203/252	180/180	170/170	177/177	123/127	161/181
CSMaRTH37	191/191	174/196	166/180	173/186	126/129	163/181
CSMaRTH38	191/191	196/196	180/180	186/186	126/129	154/167
CSMaRTH39	191/209	180/196	170/180	177/186	122/126	154/167
CSMaRTH40	191/252	196/196	180/180	186/186	123/126	154/167
CSMaRTH41	194/252	196/196	180/180	186/186	126/129	161/181
CSMaRTH42	191/191	196/196	180/180	186/186	123/127	163/163
CSMaRTH43	191/252	180/196	170/180	177/186	126/126	163/163
CSMaRTH44	191/203	180/196	170/180	177/186	123/127	154/163
CSMaRTH45	191/191	196/196	180/180	186/186	122/126	154/163
CSMaRTH46	209/252	196/196	180/180	186/186	126/126	173/173
CSMaRTH47	191/194	180/201	170/183	177/188	127/129	163/163
CSMaRTH48	252/252	180/196	170/180	177/186	123/127	163/173
CSMaRTH49	191/209	196/196	180/183	186/186	126/126	163/163
CSMaRTH50	191/209	196/196	180/180	177/186	127/127	163/163
CSMaRTH51	191/252	180/196	180/180	177/186	126/126	161/161
CSMaRTH52	191/252	180/196	163/180	177/186	127/129	163/163
CSMaRTH53	191/252	180/196	180/180	177/186	126/126	163/163
CSMaRTH54	191/209	196/196	163/183	186/186	126/126	163/163
CSMaRTH55	191/252	196/196	163/180	186/186	129/129	163/173
CSMaRTH56	252/252	196/196	163/180	186/186	126/126	163/173
CSMaRTH57	191/252	180/196	180/180	177/186	126/126	161/161
CSMaRTH58	191/252	180/196	180/180	177/186	127/129	161/161
CSMaRTH59	191/209	196/196	180/180	186/186	127/129	163/176
CSMaRTH60	252/252	180/196	163/180	177/186	127/129	163/176
CSMaRTH61	191/191	196/196	163/180	186/186	126/126	163/163
CSMaRTH62	191/209	196/196	180/180	186/186	127/129	161/163
CSMaRTH63	217/252	196/196	180/180	186/186	126/126	154/173
CSMaRTH64	191/252	196/196	163/180	186/186	129/129	163/163
CSMaRTH65	191/191	196/196	180/180	186/186	126/129	163/163
CSMaRTH66	252/252	180/196	163/180	177/186	126/129	163/173
CSMaRTH67	191/252	180/196	163/180	177/186	126/126	163/163
CSMaRTH68	191/252	180/196	180/180	177/186	129/129	163/163
CSMaRTH69	194/252	196/201	163/163	186/188	127/127	161/173

Samples	Locus					
	DW455	*DW503*	*PHe177*	*PT102*	*Haμ9*	*Haμ10*
CSMaRTH70	191/233	196/196	180/180	186/186	127/127	167/181
CSMaRTH71	217/252	180/196	180/180	177/186	126/126	161/167
CSMaRTH72	233/252	201/201	157/163	177/186	126/126	161/173
CSMaRTH73	191/209	180/196	180/180	177/186	123/123	176/181
CSMaRTH74	191/252	180/196	157/180	177/186	122/126	161/173
CSMaRTH75	209/233	196/196	180/180	186/186	123/126	163/163
CSMaRTH76	191/191	196/196	157/163	186/186	127/129	163/163
CSMaRTH77	191/209	196/196	180/180	186/188	127/129	173/173
CSMaRTH78	191/194	196/196	180/180	186/186	127/132	154/161
CSMaRTH79	191/209	196/201	180/180	186/186	126/126	161/167
CSMaRTH80	191/252	180/196	163/180	177/186	126/126	161/161
CSMaRTH81	191/191	180/196	163/180	177/186	126/126	161/161
CSMaRTH82	191/233	180/196	180/180	186/186	122/123	154/154
CSMaRTH83	191/252	180/196	163/180	177/186	126/129	163/163
CSMaRTH84	209/252	180/196	163/180	177/186	122/123	163/181
CSMaRTH85	191/252	196/201	180/180	186/188	122/126	181/181
CSMaRTH86	191/233	196/201	157/163	186/188	122/123	163/163
CSMaRTH87	191/191	180/196	180/180	177/186	126/129	167/167
CSMaRTH88	191/191	180/196	180/180	177/186	123/123	161/161
CSMaRTH89	191/233	196/201	180/180	186/188	123/127	158/181
CSMaRTH90	191/209	196/196	180/180	186/186	123/127	163/163
CSMaRTH91	191/217	196/196	157/180	186/186	123/127	154/154
CSMaRTH92	191/252	180/201	163/180	177/188	123/127	158/163
CSMaRTH93	191/209	196/196	180/180	186/186	123/127	163/163
CSMaRTH94	191/209	196/196	157/180	186/186	123/127	154/181
CSMaRTH95	191/209	196/196	163/163	186/186	126/126	161/181
CSMaRTH96	252/252	196/196	180/180	177/186	129/129	173/181
CSMaRTH97	191/252	180/196	180/180	177/186	122/122	163/173
CSMaRTH98	191/209	196/196	180/180	186/186	123/123	163/167
CSMaRTH99	209/209	180/196	163/180	177/186	126/127	167/167
CSMaRTH100	191/191	196/196	157/180	186/186	122/122	161/173
CSMaRTH101	191/217	196/196	180/180	186/186	122/126	163/163

Samples	Locus					
	DW455	*DW503*	*PHe177*	*PT102*	*Haμ9*	*Haμ10*
15% higher weight of group B						
BL1	209/209	180/196	180/180	177/186	123/126	167/181
BL2	191/194	180/196	163/180	177/186	122/123	167/167
BL3	191/209	180/196	157/180	186/186	127/132	167/181
BL4	191/209	196/196	180/180	186/186	126/126	167/176
BL5	191/233	196/196	157/180	177/186	123/127	161/161
BL6	191/209	180/196	180/180	186/186	123/127	161/163
BL7	209/217	196/196	180/180	186/186	123/127	176/181
BL8	191/209	196/196	163/183	186/186	127/127	163/163
BL9	191/209	196/196	180/180	186/188	123/127	176/176
BL10	191/252	196/201	180/180	177/186	122/122	167/167
BL11	191/191	180/196	183/183	186/186	127/127	158/173
BL12	191/252	196/196	180/180	177/186	127/127	167/167
BL13	191/252	180/196	180/180	177/188	126/127	163/167
BL14	191/252	180/201	180/183	177/186	123/123	176/176
BL15	191/209	180/196	180/180	177/186	123/123	167/181
BL16	191/252	180/196	180/180	186/186	127/127	167/167
BL17	191/217	196/196	180/180	177/186	122/126	163/173
BL18	191/194	180/196	180/180	177/186	123/123	163/173
BL19	191/191	196/196	180/180	186/186	123/123	176/181
BL20	191/209	196/196	180/180	186/186	126/127	167/167
BL21	191/191	174/196	180/183	186/186	127/129	163/167
BL22	191/191	196/196	180/180	186/186	129/129	173/181
BL23	191/191	196/196	180/180	186/186	126/129	167/167
BL24	191/191	196/196	157/163	186/186	123/127	167/167
BL25	191/191	196/196	157/180	177/186	127/127	161/161
BL26	209/209	196/196	180/183	186/186	123/127	167/167
BL27	191/209	180/196	163/180	177/186	126/126	167/167
BL28	209/209	174/196	157/180	177/186	126/129	167/181
BL29	191/209	180/196	180/183	177/186	126/129	167/173
BL30	191/191	196/196	180/180	186/186	123/127	158/163
BL31	191/217	196/196	157/180	186/186	126/129	161/163
BL32	191/209	196/196	163/180	173/186	126/129	163/163
BL33	191/209	196/196	163/180	186/186	123/123	163/181

Samples	Locus					
	DW455	*DW503*	*PHe177*	*PT102*	*Haμ9*	*Haμ10*
BL34	191/209	196/196	163/180	186/186	123/127	163/181
BL35	209/209	196/196	163/180	186/186	122/122	161/173
BL36	191/191	196/196	157/180	177/186	123/127	167/167
BL37	191/191	196/196	163/180	186/186	123/123	163/163
BL38	191/191	180/196	157/180	177/186	129/129	167/167
BL39	191/191	180/196	180/183	177/186	127/129	163/167
BL40	191/209	196/196	180/180	186/186	123/126	161/167
15% lower weight of group B						
BS1	233/252	196/196	180/180	177/186	127/132	163/176
BS2	217/217	180/196	180/180	177/186	123/123	163/176
BS3	191/209	180/196	183/183	186/186	127/127	163/167
BS4	191/191	196/196	180/180	186/186	122/123	161/167
BS5	191/209	196/196	180/183	186/186	127/129	163/163
BS6	191/191	180/196	163/180	186/186	122/126	167/181
BS7	191/267	196/196	180/180	173/188	123/123	163/181
BS8	191/233	201/201	157/180	186/186	123/123	167/167
BS9	191/267	196/196	163/180	173/188	123/126	163/163
BS10	191/267	174/201	180/180	173/186	123/123	163/181
BS11	191/191	174/196	180/180	173/186	123/123	158/158
BS12	209/209	196/196	157/180	186/186	123/123	163/163
BS13	209/209	196/196	180/180	186/186	123/123	158/176
BS14	194/233	196/196	180/180	173/186	122/122	161/161
BS15	191/191	196/196	180/180	177/186	127/127	163/181
BS16	191/209	180/196	180/180	177/186	122/122	167/167
BS17	191/191	180/196	180/180	173/186	122/122	161/161
BS18	191/209	174/196	180/180	186/186	123/129	158/158
BS19	191/191	196/196	180/180	186/186	123/129	167/167
BS20	191/191	196/196	157/163	186/186	123/127	167/167
BS21	191/191	196/196	180/180	186/186	126/129	163/167
BS22	191/209	196/196	163/180	186/186	129/129	163/167
BS23	217/233	196/201	157/157	186/188	126/129	163/173
BS24	191/191	180/196	180/180	177/186	123/126	163/167
BS25	209/252	180/196	163/183	177/186	123/123	173/181
BS26	191/191	196/201	180/180	186/188	126/129	167/167
BS27	252/252	180/196	157/180	177/177	126/129	163/163

Samples	Locus					
	DW455	*DW503*	*PHe177*	*PT102*	*Haμ9*	*Haμ10*
BS28	191/217	196/196	163/180	186/186	123/127	163/163
BS29	191/252	196/196	157/180	177/186	126/129	163/163
BS30	191/209	196/196	163/183	186/186	126/129	163/163
BS31	191/217	196/196	157/157	186/186	123/127	163/163
BS32	252/252	196/196	157/180	186/186	123/123	163/163
BS33	209/217	180/196	180/183	177/188	126/129	158/167
BS34	191/191	196/196	180/180	186/186	126/126	163/163
BS35	233/252	196/196	180/180	186/186	123/127	163/163
BS36	209/252	180/196	180/180	177/186	123/123	161/161
BS37	209/252	180/196	180/183	177/186	123/129	167/167
BS38	217/252	196/196	180/180	186/186	123/123	167/176
BS39	191/267	174/196	163/180	173/186	123/123	163/163
BS40	191/267	196/196	180/180	186/186	127/127	163/163

APPENDIX D

Primer combinations used for screening of polymorphic AFLP markers of *H. asinina*

Primer Pair	No. of monomorphic band	No. of bands only in TRGW	No. of bands only in SAME	No. of polymorphic bands		
				1 Band	2 Bands	3 Bands
E_{AAG}/ M_{CAC}	17	1	2	-	-	-
E_{AAG}/ M_{CAG}	15	4	3	-	1	3
E_{AAG}/ M_{CTA}	8	5	10	3	-	-
E_{AAG}/ M_{CTG}	5	11	7	-	2	1
E_{AAG}/ M_{CTT}	9	5	4	3	1	1
E_{AAG}/ M_{CGA}	8	-	2	1	-	1
E_{AAG}/ M_{CGT}	8	1	3	1	-	1
E_{AAG}/ M_{CGC}	10	1	3	1	-	2
E_{AAG}/M_{CGG}	9	6	6	2	-	1
E_{AAG}/ M_{CCA}	15	1	1	-	-	-
E_{AAG}/ M_{CCT}	13	1	5	2	-	1
E_{AAG}/ M_{CCG}	3	5	1	-	1	1
E_{ACA}/ M_{CAC}	13	3	2	-	-	1
E_{ACA}/ M_{CAG}	16	1	3	-	-	-
E_{ACA}/ M_{CTA}	7	4	2	1	-	-
E_{ACA}/ M_{CTG}	11	5	7	1	2	1
E_{ACA}/ M_{CTT}	10	1	4	2	-	1
E_{ACA}/ M_{CGA}	11	-	4	1	-	-
E_{ACC}/ M_{CGG}	5	4	-	3	-	-
E_{ACC}/ M_{CCA}	13	3	5	1	-	-
E_{ACC}/ M_{CCT}	3	3	-	1	-	2
E_{ACC}/ M_{CCG}	5	-	3	2	-	-
E_{AGG}/ M_{CAC}	10	1	1	-	-	-
E_{AGG}/ M_{CAG}	10	1	1	2	-	1
E_{AGG}/ M_{CTA}	8	-	1	-	-	-
E_{AGG}/ M_{CTG}	16	2	2	-	2	1
E_{AGG}/ M_{CTT}	9	3	3	1	-	2

Primer Pair	No. of monomorphic band	No. of bands only in TRGW	No. of bands only in SAME	No. of polymorphic bands		
				1 Band	2 Bands	3 Bands
E_{AGG}/ M_{CGA}	2	4	4	1	1	3
E_{AGG}/ M_{CGT}	7	3	2	1	-	1
E_{AGG}/ M_{CGC}	7	5	-	3	-	1
E_{AGA}/ M_{CAC}	20	2	2	1	-	1
E_{AGA}/ M_{CAG}	10	4	4	1	-	-
E_{AGA}/ M_{CTA}	7	6	3	1	-	-
E_{AGA}/ M_{CTG}	8	2	1	-	-	-
E_{AGA}/ M_{CTT}	17	-	6	1	-	-
E_{AGA}/ M_{CGA}	10	2	3	-	-	1
E_{AGA}/ M_{CGC}	3	-	3	3	-	1
E_{AGA}/ M_{CGG}	9	1	3	-	-	-
E_{AGA}/ M_{CCA}	14	10	2	2	-	1
E_{AGA}/ M_{CCT}	15	4	5	1	-	3
E_{ATG}/ M_{CAC}	12	4	-	-	-	-
E_{ATG}/ M_{CTA}	6	4	4	2	-	2
E_{ATG}/ M_{CTT}	9	-	3	-	-	-
E_{ATG}/ M_{CGA}	9	2	4	-	-	1
E_{ATG}/ M_{CCA}	15	2	-	-	1	1
E_{ATG}/ M_{CCT}	11	2	4	-	-	1
E_{ATG}/ M_{CCG}	8	2	1	-	-	-
E_{ATG}/ M_{CCC}	8	4	3	3	-	-
E_{ATC}/ M_{CAC}	13	1	3	-	-	2
E_{ATC}/ M_{CAG}	15	3	4	-	-	1
E_{ATC}/ M_{CTA}	9	3	2	-	-	2
E_{ATC}/ M_{CTG}	11	2	1	1	-	1
E_{ATC}/ M_{CTT}	15	2	2	1	-	-
E_{ATC}/ M_{CGA}	8	1	-	3	-	1
E_{ATC}/ M_{CGT}	8	1	2	-	-	-
E_{ATC}/ M_{CGC}	12	-	3	2	1	1
E_{ATC}/ M_{CGG}	12	2	1	-	-	-

Primer Pair	No. of monomorphic band	No. of bands only in TRGW	No. of bands only in SAME	No. of polymorphic bands		
				1 Band	2 Bands	3 Bands
E_{ATC}/ M_{CCA}	15	4	3	1	-	-
E_{ATC}/ M_{CCT}	14	2	1	1	-	1
E_{ATC}/ M_{CCG}	10	2	3	-	-	-
E_{ATC}/ M_{CCC}	13	4	4	1	-	-
E_{ATA}/ M_{CAC}	16	1	-	2	-	-
E_{ATA}/ M_{CTA}	11	1	2	1	-	-
E_{ATA}/ M_{CTT}	10	2	1	2	-	-

APPENDIX E

Genotype of of *H. asinina* examined by 4 AFLP-derived SCAR markers and mtDNA (*ND-II*) polymorphism

Samples	SCAR markers				
	HaSCAR$_{320}$	*HaSCAR*$_{295}$	*HaSCAR*$_{327}$	*HaSCAR*$_{328}$	*ND-II*
***Po* generation, Samet Island founders (east)**					
SAME1	A	A	A	B	A
SAME2	A	A	A	B	A
SAME3	A	A	A	C	A
SAME4	A	A	A	C	A
SAME5	A	A	A	A	A
SAME6	A	A	A	B	A
SAME7	A	A	C	C	A
SAME8	A	A	A	A	A
SAME9	A	A	A	A	A
SAME10	A	A	A	A	A
SAME11	A	A	C	B	A
SAME12	A	A	A	A	A
SAME13	A	A	A	A	A
SAME14	A	A	A	C	A
SAME15	A	A	A	B	A
SAME16	A	A	A	A	A
SAME17	A	A	A	A	A
SAME18	D	A	A	B	A
SAME19	D	A	A	B	A
SAME20	A	A	A	A	A
Talibong Island, Trang province (west)					
TRGW1	B	B	B	C	B
TRGW2	B	B	B	C	C
TRGW3	B	B	B	C	B
TRGW4	B	B	B	C	B
TRGW5	B	B	B	C	C
TRGW6	B	B	B	B	B
TRGW7	B	A	B	C	C
TRGW8	B	B	B	C	B
TRGW9	A	B	B	C	B

Samples	SCAR markers				
	HaSCAR$_{320}$	*HaSCAR*$_{295}$	*HaSCAR*$_{327}$	*HaSCAR*$_{328}$	*ND-II*
TRGW10	B	B	B	C	C
TRGW11	B	B	B	C	C
TRGW12	B	B	B	C	B
TRGW13	B	B	B	B	C
TRGW14	B	B	B	C	B
TRGW15	B	B	B	B	C
TRGW16	B	B	B	C	B
TRGW17	B	B	B	C	B
TRGW18	B	B	B	C	B
TRGW19	B	B	B	C	B
TRGW20	B	B	B	C	B
TRGW21	B	B	B	B	B
TRGW22	B	B	B	C	B
TRGW23	B	B	B	B	B
TRGW24	B	B	B	C	B
TRGW25	B	B	B	C	B
Cambodia (east)					
CAME1	D	A	A	B	A
CAME2	D	A	A	A	A
CAME3	D	A	A	B	A
CAME4	A	A	A	B	A
CAME5	A	A	A	A	A
CAME6	A	A	A	A	A
CAME7	A	A	A	A	A
CAME8	A	A	A	A	A
CAME9	D	A	A	A	A
CAME10	D	A	A	A	A
CAME11	A	A	A	A	A
CAME12	C	A	A	B	A
CAME13	C	A	A	A	A
CAME14	A	A	A	B	A
CAME15	A	A	A	A	A
CAME16	A	A	A	C	A
CAME17	A	A	A	A	A
CAME18	D	A	A	A	A

Samples	SCAR markers				
	HaSCAR$_{320}$	*HaSCAR*$_{295}$	*HaSCAR*$_{327}$	*HaSCAR*$_{328}$	*ND-II*
CAME19	A	A	A	B	A
CAME20	A	A	A	C	A
CAME21	A	A	A	B	A
CAME22	A	A	A	A	A
F1 generation, the Philippines					
PHI1	E	A	A	C	A
PHI2	E	A	A	C	A
PHI3	F	A	A	C	A
PHI4	F	A	A	C	A
PHI5	F	A	A	C	A
PHI6	F	A	A	C	A
PHI7	E	A	A	C	A
PHI8	E	A	D	C	A
PHI9	F	A	D	B	A
PHI10	B	A	D	C	A
PHI11	E	A	D	C	A
PHI12	E	A	A	C	A
PHI13	E	A	D	C	A
PHI14	B	A	A	C	A
PHI15	B	A	D	C	A
PHI16	E	A	D	C	A
PHI17	E	A	D	C	A
PHI18	F	A	A	B	A
PHI19	F	A	D	C	A
PHI20	E	A	D	C	A
Hatchery-propagated cultured at Trang					
CTRGH1	C	A	A	B	A
CTRGH2	A	A	A	A	A
CTRGH3	D	A	A	B	A
CTRGH4	A	A	A	C	A
CTRGH5	A	A	A	A	A
CTRGH6	A	A	A	B	A
CTRGH7	A	A	A	A	A
CTRGH8	A	A	A	B	A
CTRGH9	A	A	C	B	A

Samples	SCAR markers				
	HaSCAR$_{320}$	*HaSCAR*$_{295}$	*HaSCAR*$_{327}$	*HaSCAR*$_{328}$	*ND-II*
CTRGH10	A	A	A	B	A
CTRGH11	A	A	C	B	A
CTRGH12	A	A	C	B	A
CTRGH13	A	A	A	A	A
CTRGH14	D	A	A	A	A
CTRGH15	A	A	B	B	A
CTRGH16	A	A	A	A	A
CTRGH17	A	A	A	A	A
CTRGH18	C	A	A	B	A
CTRGH19	A	A	A	A	A
CTRGH20	A	B	C	B	A
CTRGH21	A	A	A	A	A
CTRGH22	A	A	A	A	A
CTRGH23	A	A	B	B	A
CTRGH24	A	A	A	B	A
CTRGH25	A	A	A	C	A
CTRGH26	A	A	B	B	A
CTRGH27	A	A	B	B	A
CTRGH28	A	B	C	A	A
CTRGH29	A	A	A	B	A
CTRGH30	D	A	C	B	A
CTRGH31	A	A	C	A	A
CTRGH32	A	A	C	A	A
CTRGH33	A	A	A	B	A
CTRGH34	A	A	C	A	A
CTRGH35	A	A	C	B	A
CTRGH36	C	A	A	A	A
CTRGH37	C	A	A	A	A
CTRGH38	A	A	A	A	A
CTRGH39	C	A	A	B	A
CTRGH40	A	B	C	A	A
Hatchery-propagated cultured at Sichang Marine Science Research and Traning Station					
CSMaRTH1	C	A	A	B	A
CSMaRTH2	C	A	A	A	A
CSMaRTH3	A	A	A	B	A

Samples	SCAR markers				
	HaSCAR$_{320}$	*HaSCAR*$_{295}$	*HaSCAR*$_{327}$	*HaSCAR*$_{328}$	*ND-II*
CSMaRTH4	A	A	A	B	A
CSMaRTH5	C	A	A	B	A
CSMaRTH6	A	A	C	B	A
CSMaRTH7	A	A	A	B	A
CSMaRTH8	A	A	C	B	A
CSMaRTH9	A	A	A	A	A
CSMaRTH10	A	A	C	A	A
CSMaRTH11	A	A	A	A	A
CSMaRTH12	A	A	A	B	A
CSMaRTH13	A	A	A	B	A
CSMaRTH14	A	A	A	A	A
CSMaRTH15	A	A	A	A	A
CSMaRTH16	A	A	A	B	A
CSMaRTH17	A	A	A	A	A
CSMaRTH18	A	A	A	B	A
CSMaRTH19	A	A	A	A	A
CSMaRTH20	A	A	A	B	A
CSMaRTH21	D	A	A	A	A
CSMaRTH22	A	A	A	B	A
CSMaRTH23	A	A	A	B	A
CSMaRTH24	A	A	A	A	A
CSMaRTH25	A	A	A	A	A
CSMaRTH26	A	A	A	A	A
CSMaRTH27	A	A	A	B	A
CSMaRTH28	A	A	A	B	A
CSMaRTH29	A	A	A	A	A
CSMaRTH30	A	A	A	A	A
CSMaRTH31	A	A	A	B	A
CSMaRTH32	A	A	A	A	A
CSMaRTH33	A	A	A	B	A
CSMaRTH34	A	A	A	A	A
CSMaRTH35	A	A	A	A	A
CSMaRTH36	A	A	A	A	A
CSMaRTH37	A	A	C	A	A
CSMaRTH38	A	A	A	B	A

Samples	SCAR markers				
	HaSCAR$_{320}$	*HaSCAR*$_{295}$	*HaSCAR*$_{327}$	*HaSCAR*$_{328}$	*ND-II*
CSMaRTH39	A	A	A	B	A
CSMaRTH40	A	A	A	A	A
CSMaRTH41	A	A	A	B	A
CSMaRTH42	A	A	A	B	A
CSMaRTH43	A	A	A	C	A
CSMaRTH44	A	A	A	A	A
CSMaRTH45	A	A	A	B	A
CSMaRTH46	A	A	A	C	A
CSMaRTH47	A	A	A	B	A
CSMaRTH48	C	A	A	B	A
CSMaRTH49	A	A	C	A	A
CSMaRTH50	C	A	C	A	A
CSMaRTH51	A	A	C	A	A
CSMaRTH52	A	A	A	A	A
CSMaRTH53	A	A	C	A	A
CSMaRTH54	A	A	C	A	A
CSMaRTH55	A	A	A	B	A
CSMaRTH56	A	A	C	B	A
CSMaRTH57	A	A	A	C	A
CSMaRTH58	A	A	A	A	A
CSMaRTH59	A	A	C	B	A
CSMaRTH60	A	A	C	A	A
CSMaRTH61	A	A	A	A	A
CSMaRTH62	A	A	A	B	A
CSMaRTH63	A	A	A	B	A
CSMaRTH64	A	A	A	B	A
CSMaRTH65	C	A	A	B	A
CSMaRTH66	A	A	A	B	A
CSMaRTH67	A	A	C	A	A
CSMaRTH68	A	A	A	B	A
CSMaRTH69	A	A	A	A	A
CSMaRTH70	B	A	C	A	A
CSMaRTH71	A	A	C	A	A
CSMaRTH72	B	A	A	B	A
CSMaRTH73	A	A	A	A	A

Samples	SCAR markers				
	HaSCAR$_{320}$	*HaSCAR*$_{295}$	*HaSCAR*$_{327}$	*HaSCAR*$_{328}$	*ND-II*
CSMaRTH74	A	A	A	B	A
CSMaRTH75	A	A	A	B	A
CSMaRTH76	A	A	A	C	A
CSMaRTH77	A	A	A	B	A
CSMaRTH78	A	A	A	B	A
CSMaRTH79	A	A	A	A	A
CSMaRTH80	A	A	A	A	A
CSMaRTH81	C	A	A	B	A
CSMaRTH82	A	A	A	A	A
CSMaRTH83	A	A	A	A	A
CSMaRTH84	A	A	A	C	A
CSMaRTH85	A	A	A	A	A
CSMaRTH86	A	A	A	B	A
CSMaRTH87	A	A	A	B	A
CSMaRTH88	A	A	A	A	A
CSMaRTH89	A	A	A	A	A
CSMaRTH90	A	A	A	B	A
CSMaRTH91	A	A	C	B	A
CSMaRTH92	A	A	A	A	A
CSMaRTH93	A	A	A	B	A
CSMaRTH94	B	A	A	A	A
CSMaRTH95	A	A	A	A	A

APPENDIX F

I. Hatchery-propagated specimens used in correlation analysis between genotypes of microsatellites and the body weight *H. asinina*

Sample	Large	Body Weight (g)	Small	Body Weight (g)
Group B	BL1	15.14	BS1	4.58
	BL2	12.36	BS2	4.03
	BL3	12.09	BS3	4.58
	BL4	11.81	BS4	4.31
	BL5	12.92	BS5	3.61
	BL6	15.14	BS6	2.50
	BL7	10.84	BS7	4.31
	BL8	11.39	BS8	4.03
	BL9	10.97	BS9	4.31
	BL10	12.92	BS10	4.58
	BL11	11.25	BS11	3.20
	BL12	11.81	BS12	4.31
	BL13	10.97	BS13	4.58
	BL14	11.95	BS14	4.03
	BL15	10.84	BS15	2.92
	BL16	10.97	BS16	4.17
	BL17	10.97	BS17	4.03
	BL18	11.53	BS18	2.78
	BL19	12.22	BS19	4.03
	BL20	11.95	BS20	4.45
	BL21	11.81	BS21	4.31
	BL22	10.84	BS22	4.31
	BL23	13.34	BS23	4.45
	BL24	11.25	BS24	4.31
	BL25	10.84	BS25	4.03
	BL26	10.84	BS26	4.45
	BL27	11.95	BS27	4.31
	BL28	10.97	BS28	3.47
	BL29	12.78	BS29	3.75
	BL30	11.53	BS30	4.45
	BL31	17.78	BS31	3.89
	BL32	11.81	BS32	3.75
	BL33	13.34	BS33	3.20

Sample	Large	Body Weight (g)	Small	Body Weight (g)
	BL34	10.97	BS34	2.92
	BL35	11.67	BS35	2.78
	BL36	12.36	BS36	4.31
	BL37	12.36	BS37	4.31
	BL38	12.09	BS38	3.20
	BL39	14.73	BS39	3.75
	BL40	12.92	BS40	4.17

II. *H. asinina* samples from hatchery-propagated used for the expression level of metabolism- related genes by quantitative Real – time PCR

Sample	Large	Body Weight (g)	Small	Body Weight (g)
Group C	CL1	24.16	CS1	8.67
	CL2	28.98	CS2	4.80
	CL3	23.27	CS3	7.46
	CL4	20.64	CS4	9.95
	CL5	30.29	CS5	9.81
	CL6	24.70	CS6	7.84
	CL7	28.77	CS7	8.80
	CL8	25.51	CS8	7.90
	CL9	24.53	CS9	7.84
	CL10	26.57	CS10	8.47
	CL11	21.55	CS11	7.16
	CL12	20.83	CS12	6.80
	CL13	24.26	CS13	6.13
	CL14	26.60	CS14	8.67
	CL15	26.49	CS15	8.32
	CL16	24.31	CS16	8.17
	CL17	25.14	CS17	8.72
	CL18	27.98	CS18	4.23
	CL19	24.52	CS19	6.15
	CL20	25.22	CS20	7.09
	CL21	22.36	CS21	9.97
	CL22	22.43	CS22	4.06
	CL23	25.02	CS23	7.80
	CL24	22.52	CS24	7.24
	CL25	20.78	CS25	6.47

Sample	Large	Body Weight (g)	Small	Body Weight (g)
	CL26	21.02	CS26	8.61
	CL27	23.61	CS27	6.37
	CL28	27.15	CS28	8.31
	CL29	25.04	CS29	7.85
	CL30	21.37	CS30	8.01

BIOGRAPHY

Miss Parichart Praipue was born on Nov 23, 1976 in Bangkok, Thailand. She graduated with the degree of Bachelor of Science in Biotechnology from Mahidol University in 1997 and the degree of Master of Science (Biotechnology) at the Program of Biotechnology, Chulalongkorn University in 2001. She has studied for the Degree of Doctor of Philosophy in Biotechnology, Chulalongkorn University since 2004 (second semester).

Publications during graduate study

International Publications

1. **Praipue, P.**, Klingunga, S., and Jarayabhand, P. 2009. Development of populations-specific SCAR markers in Thai abalone, *Haliotis asinina* using AFLP analysis (submitted).
2. **Praipue, P.**, Klingunga, S., and Jarayabhand, P. 2009. Development of EST-derived microsatellites and applications for determining genetic diversity of natural and cultured stocks of the tropical abalone *Haliotis asinina* (in preparation).

International Conferences

1. **Praipue, P.**, Khamnamtong, B., Klinbunga, S., Hirono, I., Aoki, T., and Jarayabhand, P. 2006. Genetic diversity of wild and hatchery-propagated stocks of the tropical abalone *Haliotis asinina*. JSPS-NRCT Joint Seminar