การพัฒนาเครื่องหมายพันธุกรรมชนิดไมโครแซเทลไลต์ในหอยเป๋าฮื้อเขตร้อน *Haliotis asinina*

นางสาวสุรีรัตน์ แซ่ตั้ง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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DEVELOPMENT OF MICROSATELLITE MARKERS IN TROPICAL ABALONE *Haliotis asinina*

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สุรีรัตน์ แซ่ตั้ง : การพัฒนาเครื่องหมายพันธุกรรมชนิดไมโครแซเทลไลต์ในหอยเป๋าฮื้อเขตร้อน *Haliotis asinina*. (DEVELOPMENT OF MICROSATELLITE MARKERS IN TROPICAL ABALONE *Haliotis asinina*) อ. ที่ปรึกษา : รศ.ดร.อัญชลี ทัศนาขจร, อ. ที่ปรึกษาร่วม : ดร.ศิราวุธ กลิ่นบุหงา 142 หน้า. ISBN 974-17-1311-8.

ทำการสร้างห้องสมุดยีน 3 แบบ เพื่อค้นหาไมโครแซเทลไลต์ในหอยเป๋าฮื้อ *Haliotis asinina* ห้องสมุดแรกได้จากการตัดดีเอ็นเอของหอยเป๋าฮื้อด้วยเอนไซม์ตัดจำเพาะ *Alu*I ห้องสมุดยีนแบบที่สองได้จากการทำดีเอ็นเอของหอยให้ขาดด้วยการ vortex และ sonication ห้องสมุดยีนแบบที่สามได้จากการตัดดีเอ็นเอของหอยโดยใช้เอนไซม์ตัดจำเพาะรวมกันสามชนิด คือ *Alu* I, *Rsa* I และ *Hinc* II เมื่อตรวจหาไมโครแซเทลไลต์ด้วยตัวตรวจสอบ $(GT)_{15}$ พบว่าห้องสมุดยีนแบบที่สามให้โคลนที่ให้ผลบวกมากที่สุด (1.46%) ขณะที่ห้องสมุดยีนแบบแรก และแบบที่สองให้โคลนที่ให้ผลบวก 0.2% และ 0.43% ตามลำดับ จากการทำ dot blot hybridization พบว่าหอยเป๋าฮื้อมีไมโครแซเทลไลต์ชนิด $(GT)_n$ มากกว่าไมโครแซเทลไลต์ชนิด $(CT)_n$ เมื่อนำโคลนที่ให้ผลบวกมาหาลำดับนิวคลีโอไทด์พบว่ามีไมโครแซเทลไลต์ชนิด perfect, imperfect และ compound ในอัตราส่วนที่ใกล้เคียงกัน จากลำดับนิวคลีโอไทด์บริเวณไมโครแซเทลไลต์ 33 ตำแหน่ง สามารถนำมาออกแบบไพร์เมอร์ได้ 10 คู่ เมื่อทดสอบความหลากหลายของไมโครแซเทลไลต์ทั้ง 10 ตำแหน่ง (Has1-10) จำนวนอัลลีลที่พบอยู่ในช่วง 3-26 อัลลีล และค่าเฮทเทอโรไซโกซิตี้ที่พบ (observed heterozygosity) มีค่าอยู่ในช่วง 0.27-0.85 ยีโนไทป์ของทั้ง 10 ตำแหน่งมีความเกี่ยวข้องกันอย่างสุ่ม ($p > 0.0031$) นอกจากนี้พบว่าไพร์เมอร์ทั้ง 10 คู่นี้ไม่สามารถใช้เพิ่มปริมาณดีเอ็นในหอยเป๋าฮื้อ *H. ovina* และ *H. varia* ความไวในการตรวจสอบจีโนไทป์ของหอยโดยวิธีพีซีอาร์โดยใช้ไพร์เมอร์ที่ติดฉลากด้วยสารรังสี ^{32}P พบว่าสามารถใช้ตรวจสอบตัวอย่างหอยที่มีอายุ 2 สัปดาห์ได้ และปริมาณดีเอ็นเอเพียง 50 พิโคกรัม สามารถใช้ตรวจสอบจีโนไทป์ของตัวอย่างหอยได้

ในการศึกษาความแปรผันทางพันธุกรรมของประชากรหอยเป๋าฮื้อ *H. asinina* 6 แหล่ง ซึ่งแบ่งเป็นจากธรรมชาติ 3 แหล่ง คือ หอยจากทะเลอันดามันซึ่งเก็บตัวอย่างจากบริเวณเกาะตะลิบง จังหวัดตรัง หอยจากอ่าวไทยซึ่งเก็บตัวอย่างจากบริเวณเกาะเสม็ดจังหวัดระยอง และจากประเทศกัมพูชา และตัวอย่างหอยจากโรงเพาะเลี้ยง 3 แห่ง ซึ่งมีพ่อแม่พันธุ์มาจากบริเวณเกาะเสม็ดจังหวัดระยอง จากประเทศกัมพูชา และจากประเทศฟิลิปปินส์โดยใช้ไมโครแซเทลไลต์ที่พัฒนาได้ 3 ตำแหน่ง คือ Has2, Has3 และ Has8 ค่าเฉลี่ยของ effective number of alleles (n_e) และเฮทเทอโรไซโกซิตี้ (h_{obs}) ชี้ให้เห็นว่า ตัวอย่างหอยธรรมชาติจากอ่าวไทย (เสม็ด $n_e = 5.73$, $h_{obs} = 0.78$; กัมพูชา $n_e = 7.65$, $h_{obs} = 0.62$) มีความหลากหลายทางพันธุกรรมที่สูงกว่าหอยจากทะเลอันดามัน(ตะลิบง $n_e = 5.10$, $h_{obs} = 0.58$) ตัวอย่างหอยจากโรงเพาะเลี้ยงเกาะเสม็ด ($n_e = 6.16$, $h_{obs} = 0.82$) และกัมพูชา ($n_e = 6.33$, $h_{obs} = 0.79$) ยังคงมีความหลากหลายทางพันธุกรรมสูง ความถี่ของอัลลีลของกลุ่มประชากรทุกกลุ่มเป็นไปตามกฎของ Hardy-Weinberg ($p > 0.0027$) ยกเว้นกลุ่มตัวอย่างธรรมชาติจากประเทศกัมพูชา (ที่ตำแหน่ง Has2 ($p < 0.0001$) และ Has3 ($p < 0.0001$)) และตัวอย่างจากประเทศฟิลิปปินส์ (ที่ตำแหน่ง Has8 ($p < 0.0001$)) เมื่อทำการวิเคราะห์ทางสถิติเพื่อดูโครงสร้างประชากรพบว่ากลุ่มตัวอย่างหอยจากอ่าวไทยไม่มีความแตกต่างกันทางพันธุกรรมอย่างมีนัยสำคัญ ($p > 0.0027$) แต่กลุ่มตัวอย่างหอยจากอ่าวไทย ทะเลอันดามัน และจากฟิลิปปินส์มีความแตกต่างทางพันธุกรรมอย่างมีนัยสำคัญ ($p < 0.0027$) เมื่อศึกษาgeographic heterogeneity และนำมาแสดงความสัมพันธ์ทางพันธุกรรมในเชิงแผนภูมิ โดยใช้วิธีของ Neighbor-joining สามารถแบ่งกลุ่มหอยเป๋าฮื้อ *H. asinina* ในการศึกษานี้ได้เป็น 3 กลุ่มคือ กลุ่ม (A) จากอ่าวไทย กลุ่ม (B) จากทะเลอันดามัน และกลุ่ม (C) จากประเทศฟิลิปปินส์

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมี ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2545 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

##4272454423: MAJOR BIOCHEMISTRY

KEY WORD: tropical abalone/ *Haliotis asinina*/ microsatellite/ population study/ genetic diversity

SUREERAT TANG : DEVELOPMENT OF MICROSATELLITE MARKERS IN TROPICAL ABALONE *Haliotis asinina.* THESIS ADVISOR : ASSOC. PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS CO-ADVISOR : SIRAWUT KLINBUNGA, Ph.D., 142 p. ISBN 974-17-1311-8

Three genomic libraries were constructed for microsatellite isolation in the tropical abalone *Haliotis asinina*. 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. Dot blot hybridization suggested that $(GT)_n$ repeats were more abundant than $(CT)_n$ repeats. 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 microsatellite 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*. Sensitivity of PCR using ^{32}P labeled primer indicated that genotyping of 2 week-old (spat) samples can be detected and only 50 pg of DNA is enough for detection.

Three microsatellite loci, Has2, Has3 and Has8 were used for examination of genetic variation in natural and hatchery stocks of *H. asinina.* Three natural samples were collected from the Andaman Sea (Talibong Island) and the Gulf of Thailand (Samet Island and Cambodia). Hatchery samples were brought from 3 hatcheries where broodstocks originated from Samet Island, Cambodia and Philippines respectively. The effective number of alleles (n_e) and observed heterozygosity (h_{obs}) indicated that natural samples from the Gulf of Thailand (Semet: $n_e = 5.73$, $h_{obs} = 0.78$; Cambodia: $n_e = 7.65$, $h_{obs} = 0.62$) have higher genetic variation than that of the Andaman sea (Talibong: $n_e = 5.10$, $h_{obs} = 0.58$) and hatchery populations from Samet ($n_e = 6.16$, $h_{obs} = 0.82$) and Cambodia ($n_e = 6.63$, $h_{obs} = 0.79$) still have high genetic variation. Gene frequencies of each population conform to Hardy-Weinberg equilibrium ($p > 0.0027$) except natural population from Cambodia (at Has2 loci ($p < 0.0001$) and Has3 loci ($P < 0.0001$)) and the Philippines samples (at Has8 locus ($p < 0.0001$)). Analysis of geographic heterogeneity suggested that samples within the Gulf of Thailand did not showed significant heterogeneity to one another ($p > 0.0027$) but significant heterogeneity was observed between the Gulf of Thailand, the Andaman Sea and hatchery stock from Philippines ($p < 0.0027$). Phylogenetic reconstruction using the Neighbor-joining approach divided the 6 geographic samples to three different gene pools constituting of the Gulf of Thailand (A), the Andaman Sea (B) and the Philippines (C).

Department Biochemistry Student's signature .....

Filed of study Biochemistry Advisor's signature .....

Academic year 2002 Co-advisor's signature .....

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List of Abbreviations

°C	degree celsius
μCi	microcurie
μl	microlitre
μM	micromolar
bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetraacetic acid (disodium salt)
EtBt	ethidium bromide
h_{exp}	expected heterozygosity
h_{obs}	observed heterozygosity
IPTG	isopropyl-thiogalactoside
kb	kilobase pair
mCi	millicurie
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mmol	millimole
mtDNA	mitochondrial dna
n_e	effective number of allele
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
pg	picogram
RAPD	random amplified polymorphic dna
RFLP	restriction fragment length polymorphism

List of Abbreviations (cont.)

Rnase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sec	second
T_A	annealing temperature
TE	tris EDTA
Tris	tris(hydroxy methyl)aminomethane
U	unit
V	volt
VNTR	variable number of tandem repeats
w/v	weight/volume

Chapter I

Introduction

Abalones are marine gastropods valued as a seafood delicacy. They are the traditional food in many Asian countries, such as China, Japan and Taiwan. They have a traditional place in Chinese society as an item of prestige, often presented as a show of affluence or demonstration of respect. Abalones are categorized as luxurious and high-priced food; in Japan, one kilogram of premium abalone was priced at $45 in January 2000 (Gordon, 2000).

A demand for this unique shellfish is quite consistent whereas supply is decreasing due to many reasons such as predation, competition, loss of habitat, over harvesting and illegal harvesting. In the last two decades, the commercial catch of abalone worldwide has declined from 18,000 metric tons to a little over 10,000 metric tons (Gordon, 2000). In many countries such as Japan, Australia and USA, abalone has been cultured to make restitution to nature and for commercial also (Kojima, 1981). Abalone aquaculture is widespread in several countries including China, Taiwan, South Africa, Mexico, Chile, New Zealand, Ireland, France, Iceland etc. Since the late 1980s, the largest consumer nation of abalone, China, has developed abalone aquaculture rapidly. In the past decades and today, China become a leader of abalone production in the world at 3,500 metric tons whereas the second place is Taiwan at 2,400 metric tons. Outside of Asia, Australia is the largest producer of abalone followed by South Africa and USA (Viana, 2002).

From 75-100 species of abalone, more than 20 species are classified as commercially important species (Table 1.1). In Thailand, three different species of tropical abalone, *Haliotis asinina*, *Haliotis ovina* and *Haliotis varia* are locally found.

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

Scientific name	Common name	Shell length (mm)
H. rufescens	Red	>275
H. fulgens	Green, southern green or blue	125-200
H. corrugata	Pink or corrugated	150-175
H. sorenseni	White or sorensen	125-200
H. assimilis	Threaded	<100
H. cracherodii	Black	75-125
H. walallensis	Flat or northern green	75-125
H. kamtschatkana	Pinto	100
H. discus hannai	Ezo awabi	180-200
H. discus	Kuro awabi, oni or onigai	200
*H. diversicolor supertexta**	Tokobushi	50
H.gigantea	Madaka	250
H. sieboldii	Megae	170
*H. asinina**	Mimigai, donkey's ear	70-100
H. rubra	Black lip	120-140
H. laevigata	Green lip	130-140
H. roei	Roe's	70-80
H. iris	Paua or black	170
H. australis	Silver or queen paua	125
H. virginea	Virgin	70
H. tuberculata	Ormer	120
H. midae	Perlemon	90

* Tropical species

Among these species, *H. asinina* is the most promising species for the culture industry due to the biggest size and environmental tolerance. Compare with some important commercial abalone, *H. asinina* has a very small shell compared with its total weight so it yields more to the consumer (Fig. 1.1). Though *H. asinina* is smaller in size than some temperate abalone species, it is possible to culture this species for the cocktail market, as did *H. diversicolor supertexa* in Taiwan. Besides this, *H. asinina* is one of the fastest growing abalone; it can reach market size (60 mm) and sexual maturity within 1 year (http://www.phuketabalone.com). In addition, this species spawns throughout the year; and at present, the spawning cycle is precisely predictable making it conductive to carry out commercial breeding program (Jarayabhand, personal communication). The general characteristics of *H. asinina* can be briefly summarized as below:

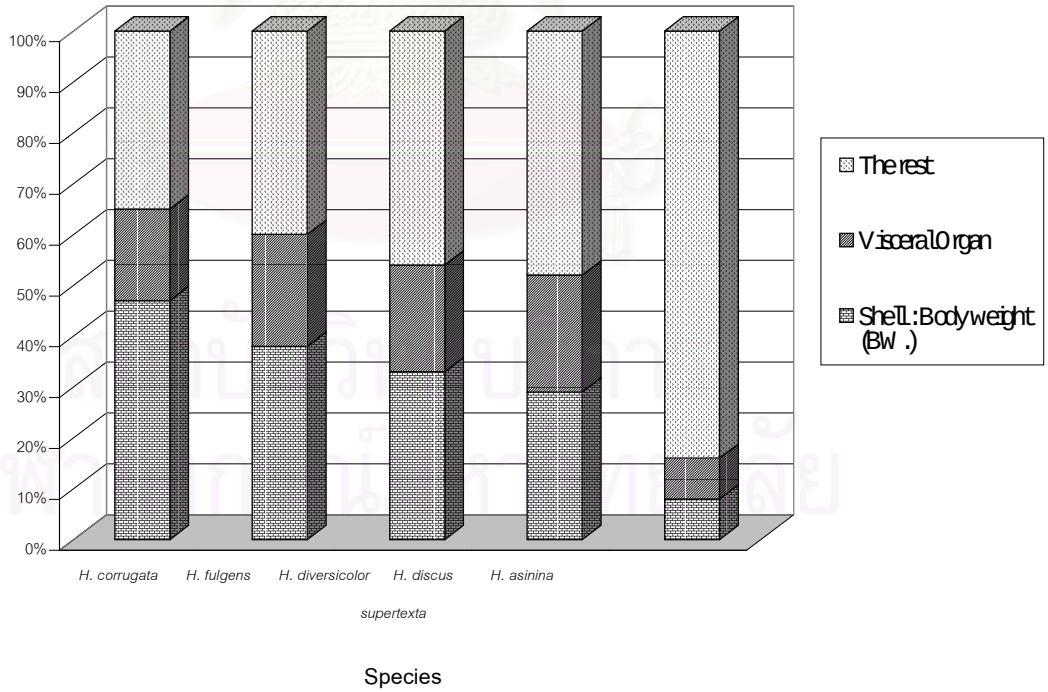

Figure 1.1 Comparison of edible portion of *H. asinina* (the rest) with those of some commercial abalones (http://phuketabalone.com)

1.1 Taxonomy of *Haliotis asinina*

The taxonomic definition of the tropical abalone, *H. asinina* is as follows (Hahn, 1989)

Kingdom Animalia

Phylum Mollusca

Class Gastropoda

Subclass Prosobranchia

Order Archeogastropoda

Suborder Zygobranchia

Superfamily Pleurotomariacea

Family Haliotidae

Genus *Haliotis*

Species *asinina*

Scientific name : *Haliotis asinina* (Linnaeus, 1758)

Common name : Mimigai, donkey's ear

1.2 Morphology and anatomy

Haliotis asinina is a univalve gastropod mollusk. The genus Haliotis, which means "sea ear" referring to the ear shape of the shell (Figure 1.2). The outside of its shell is greenish brown and rougher than the insides part which is smooth and pearly. The shell is on the top and covers most of its body. The shell is in the form of a spiral liked a common snail but quite flatter, about a fifth as high as it is long. Moreover, there is a row of holes found just in from the left-hand side of the shell, extending anteriorly to just over the head. The anterior holes are the biggest and those toward

the back are usually blocked. The holes assist with respiration, removal of wastes and release of gametes.

The foot of an abalone is a large muscle used for crawling onto the seabed in typical snail-like manner. The foot is not suited to crawling over on clinging onto sand because in sandy places, the abalone can be easily turned over and make easy food for predators. As a consequence, abalones are generally found only in areas of hard rock or coral. The part of the foot that is not completely covered by the shell looks similar to a pair of lips and is often referred to as such. Around the outside of the foot, extending from the upper part of the lips, is a series of tentacles, which presumably detect predators and food by touch and taste. At the front of the foot is the head, which is typically snail-like with tentacles similar to those on the lips. The head tentacles are larger and are similar to the eye stalks of land snails. The mouth is at the base of the head underneath the lips. It is a circular orifice in the middle of a circular piece of flesh called the oral disc. There are no teeth, but in the mouth is a tongue-like organ covered with teeth, the radula, which is used to rasp food.

The internal organs are arranged around the foot and under the shell. (Fig. 1.3) The most conspicuous organ, the crescent-shaped gonad, is blue, green or brown in ripe females and cream colored in ripe males. Usually, immature sex glands of either sex are gray. The gonad extends around the side opposite the pores and to the rear of the abalone. The gill chamber is next to the mouth and under the respiratory pores. Water is drawn in under the edge of the shell, and then flows over the gills and out the pores. Waste and reproductive products are carried out in this flow of water. Since abalone has no obvious brain structure, it is considered to be a primitive animal. However, it does have a heart on its left side and blood flows through the arteries, sinuses and veins, assisted by the surrounding tissues and muscles.

Figure 1.2 Tropical abalones *Haliotis asinina*

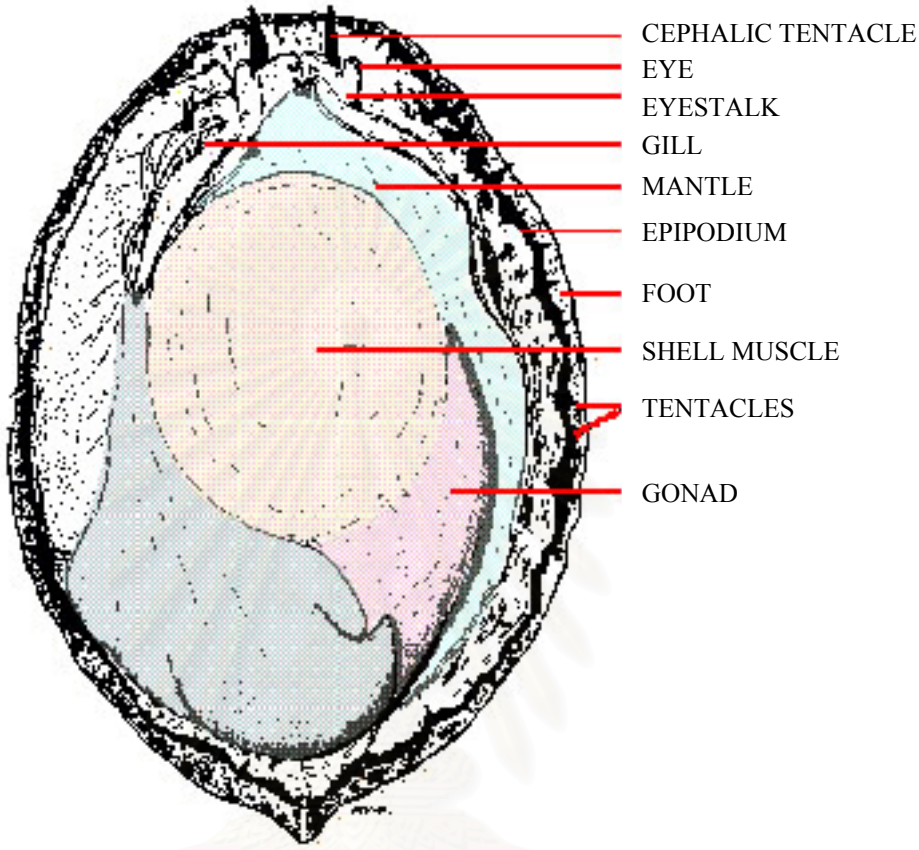

Figure 1.3 Anatomy of an abalone with shell removed (Cox, 1962)

1.3 Life cycle

Abalones have separate sexes. To reproduce, they broadcast sperm and eggs into the sea, relying on high gamete densities for successful fertilization, a reproductive strategy requiring densely aggregated adults for success. When the gametes fuse, the fertilized egg divides repeatedly and forms a larva. The larvae go through a series of changes in body form, through what are termed trochophore and veliger stages. The larvae are free-swimming for only a few days. During this stage, they feed on plankton until their shells begin to form. Once the shell forms, the juvenile abalones sink to the bottom where they cling to rocks and crevice with their single powerful foot. Juvenile abalones feed on rock-encrusting coralline algae and on diatom and bacterial films. Once settled, the abalones eat, grow and develop themselves to be adult abalones who feed primarily on loose pieces of marine algae drifting with the surge or current. After the gonads start to develop and the abalones become sexually mature and the cycle repeats itself. Most abalone species become mature between the first and third year and abalone can live longer than a decade.

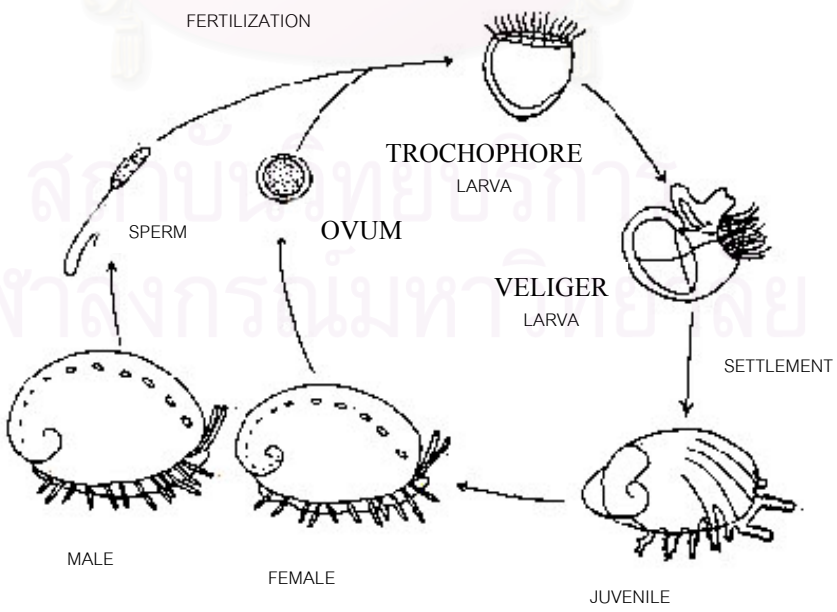

Figure 1.4 Life cycle of abalone (http://www.abalone.net)

1.4 Habitat and distribution

Abalones avoid the light so that in daylight, they are usually found hiding in crevices on rocky reefs and under rocky overhangs. Species of abalone occur in all the major oceans of the world but they appear to be more abundant in the temperate zones. They are not found in all parts of all oceans of the world. The nature of their bodies constrains them to live in certain habitats and adopt appropriate modes of behavior in those habitats. Geiger (2000) reported that *H. asinina* were found in more than 100 localities distributing in South east Asia, Japan and Australia (Figure 1.5). In Thailand, *H. asinina* is found along the coasts of the upper Gulf of Thailand and the Andaman Sea (Nateewathana and Bussarawit, 1988).

Figure 1.5 Worldwide distribution of *Haliotis asinina* (Geiger, 2000)

1.5 Genetic markers

Currently, the artificial propagated breeding program and culture techniques of *H. asinina* are being developed. For sustainable farming approach, basic information on geographic population differentiation and level of genetic variation is necessary for effective stock selection and breeding. In the long term this information is also important for conservation management of the existing natural resources of this species. An initial step toward management of this species is development genetic markers.

Genetic markers especially those inherited in the Mendelian fashion are important for various population genetic studies. They can be applied to fisheries management of *H. asinina* in several aspects including determination of stocks, recognition of genetic tags, analysis of gene flow and reconstruction of intraspecific phylogeny. In selective breeding programs of *H. asinina,* individually and parentage of propagated offspring in large family groups need to be examined to ensure their identification. Presently, several genetic markers have previously been proven to be useful. These include allozyme, mitochondrial DNA, Random amplified polymorphic DNA (RAPD) and Variable Number of Tandem Repeats (VNTR).

1.5.1 Allozymes

Allozymes are the variant protein products of allelic genes in the same chromosomal DNA locus (Park and Moran, 1994). Polymorphism of allozymes can be detected by the different migration of investigated proteins in an electrical field. Protein bands can be visualized by histochemical stains specific for the enzymes. Allozyme electrophoresis has been used as a predominant molecular technique to characterize population level genetic variation in many fishery researches because this

technique is a relatively easy, inexpensive and fairly rapid to perform on a large scale. Furthermore, a number of loci can be screened simultaneously.

However, this technique has certain limitations. Because of redundancy in the DNA code that dictates protein sequences, changes in some base at the DNA level may not change amino acid residues in polypeptide chain. Besides this, since this technique is based on the different net charge of investigated proteins. Theoretically, sixteen of the common amino acids are electrostatically neutral consequently, nonsynonymous mutation from one neutral amino acid to other neutral base does not change the net charge of the polypeptide chain (Nei, 1987). Accordingly, many genetic variants are not detected by protein electrophoresis. The studies of Brown (1991) and Huang (2000) in the blacklip abalone *Haliotis rubra* population had shown that allozymes exhibited low level variability and were not sufficient to discriminate between diverged population.

Interpretation of allozyme data also pose other problem. Since allozyme electrophoresis only detects actively expressed proteins that react to histochemical stain (Hunter and Markert, 1957) and these genes constitute only a small percentage of the whole genome of an animal therefore only a small proportion of DNA sequence variability can be detected.

1.5.2 Mitochodrial DNA

Mitochondrial DNA is DNA that found in mitochondria of eukaryotes. Each mitochondria contains 5-10 copies of double-standed circular DNA. This extra-chromosomal DNA is about 15,700-19,500 bp in length (Brown, 1983) composed of 13 protein coding genes, 2 genes coding for ribosomal RNAs (12S and 16S ribosomal

rRNA), 22 transfer RNA (tRNA) coding genes and the control region which containing initiation site for replication and transcription.

Studies on mtDNA variation are usually by restriction analysis of the entire mtDNA or PCR - amplified mtDNA segment followed by restriction enzyme digestion and/or sequencing. For restriction analysis, the digestion profile can be detected either by chemical staining or radioactive labeling (Chapman and Brown, 1990). Mutation occurring at a restriction site (either by substitutions or deletions) prevents or allows an investigated enzyme to cleave at such position and thus produces the different number of DNA fragments from investigated individuals. At present, analysis of the entire mtDNA by restriction enzyme is increasingly replaced by PCR-RFLP which the specific regions of mtDNA are amplified through the polymerase chain reaction (PCR). The products are then digested with restriction endonuclease before electrophoretically analyzed. Alternatively, the PCR amplified products can be electrophoresed and further analyzed by direct sequencing (Chapman and Brown, 1990).

Mitochondrial DNA evolves 5-10 times faster than single copy nuclear DNA (Brown et al., 1979). This property provides the magnifying ability to distinguish and identify the differences between populations and between closely related taxa. Since evolution rates of each region are various, mtDNA can be used for study in multilevel. For instance, the extremely conserved in protein coding regions, three subunit of cytochrome *c* oxidase (COI, II and III) and cytochrome *b,* are appropriate for the studies of population genetic questions in distantly related taxa. While the evolutionary rate of 12S and 16S rRNA genes is useful for evolutionary studies between distantly related species (Meyer and Wilson 1990; Meyer and Dolven, 1992, cite in Meyer, 1994). In addition, since mtDNA is haploid and uniparentally inherited

therefore effective population size is reduced to one fourth that of nuclear DNA (Nie and Li, 1979; Birky et al., 1989 cited in Ward and Grewe, 1995). However, due to a single-parent inheritance mode of mtDNA, testing against Hardy-Weinberg proportion and other most standard population analyses impossible.

1.5.3 Random amplified polymorphic DNA (RAPD)

RAPD markers are produced by PCR using short oligonucleotide primers (typically 10 bp long) which have random sequence. (Welsh and McClelland 1990 and William et al., 1990). Unlike conventional PCR technique, this technique uses only a single primer for amplification. Because the amplified fragments are those regions of the genome that are flanked by "inward-oriented" sequences complementary to the primer. Under low stringency condition, a number of PCR products are generated from random locations within the genome (Dear, 1997). Allelic variation depends on the presence or absence of these particular amplification products, which can be separated on agarose gels stained with ethidium bromide. Polymorphism of alleles may result from mutation of a primer recognition site which prevent its amplification or from insertion that change the size of DNA segment (William et al., 1990 and Avise, 1994)

RAPD method is quick, simple, relative inexpensive and numerous markers can be developed easily by changing sequences or number of nucleotide in the primer. However, since RAPD markers are mostly inherited as dominant alleles, information on the parental origin of alleles may be inaccessible (Lewis and Snow, 1992). Owing to short length of primer and low stringency of PCR condition, RAPD markers may produce some artifact of amplification products therefore careful control of DNA

quality and amplification conditions is necessary to ensure reproducible banding patterns (Carson et al. 1991, Riedy et al., 1992, Scott et al., 1993).

1.5. 4 Variable Number of Tandem Repeat (VNTR)

VNTR markers are characterized by a core sequence that consists of a number of identical repeated sequences. They can be divided into three categories based on their repeat length including satellite, minisatellite and microsatellite

1.5.4.1 Satellite

Satellite DNA is the first of tandem repeated DNA sequence to be discovered. It was named by its appearance as minor of "satellite" band that separated from the "bulk" DNA upon buoyant density gradient centrifugation (Britten et al., 1968). The basic repeat unit of satellite can vary from hundreds to thousands of base pairs. Satellite DNA may occur as million of copies per genome in some mammals (Alberts et al., 1983). Nevertheless, they are not as variable in size within population as the other members of highly repetitive DNA family are.

1.5.4.2 Minisatellites

Minisatellite is a tandemly repeated nucleotide sequence which ranging between 9-65 bp per repeat unit and typically 0.1 to 20 kb in size (Wright, 1993). Minisatellite is called variable numbers of tandem repeats (VNTR) because increases and decreases in the lengths of the repeat array was found that resulted from changes in the number of repeat copies residing in the region (Avise, 1994). Minisatellites are found within noncoding regions of genomic DNA tending to cluster at telomeric region of chromosome where known as "hot spot" for homologous recombination (Wahls et al.,1990). The mechanism generating variability in minisatellites are still

inconclusive. Several models have been suggested including unequal crossingover between homologous chromosomes at meiois or mitosis, replication, slippage and gene conversion within repeat units (Wolff et al., 1989).

The variation of minisatellite loci can be detected due to difference in length between conserved restriction sites. The approach to detect this was developed first by Jeffrey and colleagues at Leicester University (1985) known as DNA fingerprinting. This method involved hibridizing restriction enzymed digested DNA with repetitive probes. The pattern of DNA is specific for each individual and is inherited in a Mendelian fashion. These characteristics of minisatellites have allowed their application in pedigree construction (Wright, 1993). The principal problem of DNA fingerprinting was that the pattern of DNA generated by multilocus probes (about 20 or more bands per individual). There were no practical ways of knowing which pairs of bands in the fingerprint represented alleles at a particular minisatellite locus. Therefore, it was impossible to calculate allele frequencies. This problem seriously constrain attempts to estimate gene flow or other population parameter employing the usual statistical algorithms that require straightforward Mendelian markers (Avise, 1994).

Two approaches have been developed for single locus analysis, use of refined DNA probes (Georges, 1991) and PCR-based methods (Jeffrey et al., 1988b; Jeffrey et al., 1994 and Galvin et al., 1995). The problems of the first one are finding of a suitable probe and using Southern blotting. The latter is more convenient, using specific primers flanking the array. PCR products are separated by standard gel electrophoresis, and can be visualized by a variety of methods. However, in conventional PCR, fragments much larger than 4 of 5 kb are not readily amplified and detected thus the large minisatellite loci can not be done. Moreover, highly difference

in size of the large allele and small allele make it difficult to separate and determine size of alleles by electrophoresis manner (Budowle et al., 1991 and Taylor et al., 1994).

1.5.4.3 Microsatellites

Microsatellites are short DNA consisting of short repeats (1-6 nucleotides) which arrayed in tandem repeated manner for approximately 10-50 copies (Hearne et al., 1992). Most observed mutations at microsatellite loci involve increase or decrease in copy number of repeat unit (Hastbacka et al., 1992; Mahtani and Willlard, 1993 Weber and Wong, 1993 cited by O'Reilly and Wright 1995). Due mainly to high mutation rate of the microsatellite loci (10^{-6} to 10^{-2} per generation), arrays of the repeat have been found to vary dramatically in length (from several to hundreds of basepairs) providing a plentiful source of allelic polymorphism (Anger and Bernatchez, 1997).

Microsatellite arrays are embedded in unique DNA sequence, sometimes within the coding region of genes, but more commonly in the untranslated regions of the genome (Valdes et al., 1993). Like single locus analysis of minisatellites, variation of these arrays can be determined by using PCR-based method. The method for microsatellite marker developing consists of genomic library construction, screening for microsatellite clones by hybridization with repetitive probe, sequencing and primer design (Tautz, 1989 and Weber and May, 1989). Different from minisatellite, microsatellite alleles vary in size by decrease or increase of small-sized repeat unit therefore the length of a microsatellites allele at a specific locus can be easily determined by size fractionation with acrylamide sequencing gels.

Microsatellites are represented as the markers of choice because there are some advantages of utilizing microsatellites over other markers, which make them

desirable. First, They are abundant widely spread throughout the chromosome and are highly polymorphic in eukaryotic genomes. It was estimated that one microsatellite locus may be found every 10 kb in eukaryotic genomes (Tautz, 1989). This characteristic provides utility in genome mapping. Second, by PCR approach, a very small quantity of DNA (e.g., many social insects) and low quality or degraded DNA can be used for analysis. Besides this, the utility of a PCR approach also allow the possible processing of a large number of samples generally required for population surveys and large breeding programs (Brooker et al, 1994). Third, since microsatellites have a high rate of mutation which causing extensive allelic variation, the power of discrimination between closely related populations is increased (Wright and Bentzen, 1994). Microsatellites have been found to be variable even in populations that have low levels of allozyme and mitochondrial variation (Paetkau and Strobeck, 1994). Nevertheless, there are several report show that some microsatellite loci are conserved enough to allow various applications in closely related species. Finally, co-dominant Mendelian inheritance makes microsatellites more informative in pedigree studies, as well as in population studies (Wright and Bentzen, 1995).

At present, a lot of publications show the application of microsatellite in aquaculture and fisheries management. For instant, microsatellite polymorphism has been successfully used to analyze parental effects on offspring growth and survival in communally reared rainbow trout (Herbinger et al., 1995), analyze genetic diversity in shrimp breeding program (Wolfus et al., 1997), examine genetic population structure of Alaskan Pacific herring, *Clupea pallasi* (O'Connell et al., 1998), determine the early growth performance of Atlantic salmon full-sib families reared in single family tanks versus in mixed family tanks (Herbinger et al., 1999), estimate variation of

stock composition of sockeye salmon *Oncorhynchus nerka* in Barkley Sound British Coulumbia (Beacham et al., 2000) and estimate the long-term effects of stocking domesticated trout into wild brown trout *Salmo trutta* population (Hansen, 2002).

1.6 Genetic marker in abalone

Jang et al., (1995) investigated a physical map of mtDNA of Taiwan abalone *H. diversicolor* Reeve. They found that Taiwan abalone mtDNA showed an extensive length variation, ranging from 17.33 to 19.74 kb. The variation is attributable to the presence of different numbers of tandem repeats. Phylogenic tree constructed by mtDNA analysis show that various populations of Taiwan abalone from discrete localities could be clearly distinguished.

Kirby and Powers (1998) obtained a polymorphic microsatellite locus, Hruf200 by screening size-selected genomic library of red abalone (*H. rufescens*). A total of 21 alleles ranging from 97 to 149 base pairs in length were observed at that locus. Sequencing data from 21 microsatellite loci suggested that the most common microsatellite repeats were GT/TG and AC/CA in this species.

Huang and Hanna (1998) developed three microsatellite loci (RUBGT1, RUBCA1 and RUBGACA1) from screening of randomly amplified polymorphic DNA products and genomic DNA library of blacklip abalone (*H. rubra*). All of these microsatellites were polymorphic when analysed in 100 *H. rubra* individuals. The number of alleles observed was 41, 30 and 8 alleles for RUBGT1, RUBCA1 and RUBGACA1, respectively. They also try to use these markers and other two minisatellite markers (putative growth-hormone-gene-repeat, GHR and putative mollusca-insulin-like peptide-gene-repeat, MIPR) in cross-species amplification of 14 abalone species from the United States, South Africa, South Korea, and Australia.

They found that most amplifications occurred in the Australian species, *Haliotis conicopora*.

Huang et al. (2000) used three types molecular marker to analysis genetic structure of the populations of the blacklip abalone *Haliotis rubra* (Leach) along Victoria, Australia. The DNA markers included 84 randomly amplified polymorphic DNA (RAPD) bands amplified using six random primers, two minisatellites (GHR and MIPR) and three microsatellites (RUBGT1, RUBCA1 and RUBGACA1). All three types of DNA markers revealed significant subdivision in the *H. rubra* populations along the coastline. The genotypes of microsatellites indicated excessive homozygotes across all the populations at all three microsatellite loci.

Eleven microsatellite loci of *H. asinina* were isolated and characterized. (Selvamani et al., 2000). Five of these including 2 highly polymorphic loci (Haμ13 and Haμ2k) and 3 moderately polymorphic loci (Haμ10, Haμ2J and Haμ3K) were used for genotyping of individual abalone larvae produced by 3 separate crosses (Selvamani et al., 2001). The parents of an individual veliger could be determined from as few as 3 loci.

Evans et al. (2001) investigated the interspecific amplification of 22 microsatellite loci developed for *Haliotis rubra* across 12 other species within the Haliotidae. Optimization and analysis of PCR products revealed that of 12 loci examined in the Australian *H. laevigata*, only five were able to be reliably scored. While 6 of 10 for the South African *H. midae* were scoreable and none of the three for the North American species were useable, The assay examines five species from Australia, three from New Zealand, two from South Africa and two from North America. Amplification success varied from 68% for *H. conicopora*, a possible sub-species, to 14% for the distantly separated and related species from North America.

Twelve novel di-, tri- and tetranucleotide microsatellite loci to the pinto abalone *Haliotis kamtschatkana* are described. Over 400 individuals were analysed at each microsatellite locus. Observed heterozygosities ranged from 0.44 to 0.93, and numbers of alleles from 20 to 63. Six of the loci contained excesses in homozygosity indicative of inbreeding, nonrandom mating, population admixture, or null alleles (Miller et al., 2001)

Five novel microsatellite loci were isolated from Pacific abalone *Haliotis discus discus*, and the polymorphisms were examined to estimate genetic variability. The genetic variabilities varied depending on the locus: the number of alleles ranged from 3 to 10, and the observed and expected hetrozygosity ranged from 0.17 to 0.80, and 0.20 to 0.89, respectively. Two loci showed significant Hardy-Weinberg disequilibrium at the $0.01 < P < 0.05$ levels. The high variabilities revealed in this study were evidently higher than that observed in allozyme loci, suggesting that these microsatellites should prove useful for various genetic investigations (Sekino and Hara, 2002)

Conod et al. (2002) examined genetic variation in five geographically isolated samples of the blacklip abalone, *Haliotis rubra*, from south-eastern Australia by using RFLP analysis on the ND3/COIII region of mitochondrial DNA and five independent nuclear DNA microsatellite loci. They found that both techniques showed similar resolving power. They recommended that microsatellite DNA analysis is the preferred molecular technique for the fine scale investigation of blacklip abalone population structure because it makes possible the examination of numerous independent loci with potentially high levels of polymorphism. Both sample and locus specific homozygote excesses were recorded for the microsatellite loci. The most likely

explanation for the locus specific deviations from Hardy-Weinberg expectations is the presence of null alleles.

Genetic diversity of three tropical abalone species in Thailand was observed by using RAPD-PCR. Seventy-two reproducible and polymorphic RAPD fragment were generated using primer UBC101 and OPB11. One hundred percent of polymorphic bands were found for both primers. High genetic diversity levels between *H. asinina, H. ovina and H. varia* was observed. A neighbor-joining tree constructed from the average genetic distance between paired geographic samples indicated phylogenetically clear separation between investigated abalone species. (Popongviwat, 2001)

As cited above, there are a few publications concerning the utilizing of microsatellite markers for genetic diversity analysis in abalone. Despite the difficulties in development of microsatellite markers compared with other markers, they are very useful for numerous applications in aquaculture and fisheries research. The use of different types of markers offers a highly accurate assessment of genetic differentiation among populations of particular species. Therefore, this thesis aims to develop microsatellite marker for use in determination of genetic diversity and differentiation of *H. asinina* in Thailand.

Chapter II

Materials and Methods

2.1 Equipments

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
- Automatic micropipette P20, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)
- Autoradigraphy cassette (Research Products International corp., USA)
- Electrophoresis apparatus
  : Submerged Agarose Gel Electrophoresis System, GelMate™ (Toyobo Co. Ltd., Japan)
  : Vertical sequencing gel electrophoresis apparatus (Hoefer, USA)
  : Power supply: Power PAC 3000 (Bio-RAD Laboratories, USA)
- Cleanser, Ultrasonic (P Selectra®)
- Fisher Stirring Hotplate (F Fisher Scientific)
- -80°C Freezer (REVCO, USA)
- Gel dryer Model 583 (Bio-Rad Laboratories, USA)
- Gene Pulser™ (Bio-Rad Laboratories, USA)
- Gyrotory Water Bath Shaker (New Brunswic Scientific Co. Inc., USA)
- Heating block BD 1761G-26 (Sybron Thermermolyne Co., USA)
- High Speed Microcentrifuge Model MC-15A (Tomy Seiko Co., Ltd., Japan)
- High Speed Microcentrifuge J2-21(Beckman, USA)
- Incubator BM-600 (Memmert GambH, Germany)
- Light box 2859 SHANDON (Shandon / scientific Co. Ltd., England)
- Medical X-ray Film (Kodak, USA)

- Microcentrifuge tube 0.5, 1.5 ml (AxyGen, Inc., USA)
- PCR Thermal cycler : Omnigene-E (Hybaid Limited, England)
- PCR Workstation Model#P-036 (Scientific Co., USA)
- Pipette tips 100, 1000 µl (AxyGen, Inc., USA)
- Refrigerated Microcentrifuge kubota1300 (Kubota, Japan)
- Spectrophotometer DU 650 (Beckman, USA)
- Touch Mixer Model 323 (F Fisher Scientific)
- Ultraviolet Transilluminator 2011 MACROVUE (LKB BROMMA)
- Water bath (HETO BIRKERϕD, Denmark)

2.2 Chemical agents

- Absolute ethanol (Merk, Germany)
- Acrylamide (Merk, Germany)
- Ammonium Persulfate (Promega, USA)
- Bacto-agar (Difco, USA)
- Bacto-tryptone (Difco, USA)
- Bacto-yeast extract (Difco, USA)
- Boric acid (Merk Germany)
- Bromophenolblue (Merck, Germany)
- Chloroform (Merk, Germany)
- dATP, dCTP, dGTP, dTTP (SibEnzyme, Russia)
- Developer (Eastman Kodak Company, USA)
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- Fixer (Eastman Kodak Co., USA)

- Formamide (Gibco BRL, Technologies, Co., USA)
- Glucose (Merck, Germany)
- IPTG (New England Biolabs, Inc., USA)
- Isoamyl alcohol (Merck, Germany)
- Magnesium chloride (BDH, England)
- Magnesium sulfate (Merck, Germany)
- N, N-methylene-bis-acrylamide (Amersham, England)
- N,N.N',N'-tetramethylethylenediamine (Sigma Chemical Co., USA)
- Phenol crystal (Fluka, Germany)
- Potassium choride (Carlo Erba, Italy)
- pUC18/*Sma* I/ BAP (Pharmacia biotech, USA)
- Sodium acetate (Merck, Germany)
- Sodium chloride (BDH, England)
- Sodium citrate (Merck, Germany)
- Sodium dodecyl sulfate (Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)
- Sodium Phosphate (Carlo Erba, Italy)
- Sucrose (Sigma, USA)
- Tretracyclin (Sigma, USA)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- Triton®X-100 (Merck, Germany)
- Urea (APS Ajax Finechem, Australia)
- Xylene cyanol (Sigma, USA)

2.3 Enzymes

- *Alu* I (Promega, USA)
- DNA polymerase, Large (Klenow) Fragment (Biolabs, UK)
- DyNazyme II DNA™ polymerase (Finnzymes, Finland)
- *Hin*c II (Promega, USA)
- Lysozyme (Sigma Chemical Co., USA)
- Proteinase K (Gibco BRL life technologies, Inc., USA)
- Rnase A (Sigma Chemical Co., USA)
- *Rsa* I (Promega, USA)
- *Sma* I (Biolabs, New England)
- T4 Polynucleotide kinase (Biolabs, UK)
- T4 Polynucleotide ligase (Gibco BRL life technologies, Inc., USA)

2.4 Kit

- T7 Sequencing Kit (Amersham Pharmacia Biotech, USA)
- QIAquick™ Gel Extraction kit (Qiagen, Germany)
- QIAquick™ PCR Purification Kit (Qiagen, Germany)

2.5 Oligonucleotide primers suppliers

- Bioservice Unit, Thailand
- Biobasic Inc. Canada
- Biosynthesis Inc., USA
- Genset Singapore Biotech. Pte Ltd

2.6 Radioactive

- [γ-^{32}P]ATP specific activity 3000 Ci/mmol
  (Amersham Pharmacia Biotech, England)
- [α-^{32}P]dATP specific activity 3000 Ci/mmol
  (Amersham Pharmacia Biotech, England)
- [α-^{35}S]dATP specific activity 1000Ci/mmol
  (Amersham Pharmacia Biotech, England)

2.7 Samples

Seven populations of *H. asinina* samples were caught from 4 natural resources and 3 hatcheries. The wild samples were brought form Talibong island in Trang province: NL (N = 28), Samet island in Rayong province: NS, (N = 12) Cambodia: NC (N = 23) and unknown sample from Indonesia: NI (N = 12). The hatcheries samples, P_0 generation samples were obtained from Samet island Hatcheries: HS and Phuket Hatcheries: HC which claimed that broodstocks originated from Samet island and Cambodia, respectively, and F_1 generation were obtained from Aquaculture Department Southeast Asian Fisheries Development Center (SEAFDC) from the Philippines.

Figure 2.1 Map of Thailand illustrating sample collection sites of *H. asinina* in the Gulf of Thailand and the Andaman Sea (sample collection site)

2.8 DNA extraction

Two methods of DNA extraction were performed which depended on the objective of experiments. For cloning purpose, high molecular weight DNA of *Haliotis asinina* was extracted by using the Proteinase K phenol-chloroform extraction. (Davis et al., 1986) with slightly modification. Other method which is more simple and rapid, Chelex based DNA extraction, were carried out for microsatellite amplification (Walsh et al., 1991; Altschmied et al., 1997)

2.8.1 Proteinase K phenol-chloroform extraction

Approximately 50-100 mg of foot of *H. asinina w*as sniped into tiny pieces and then homogenized thoroughly in a 1.5 ml microcentrifuge tube containing 400 µl pre-chilled extraction buffer (100 mM NaCl, 200 mM sucrose, 50 mM EDTA, pH 8.0). The homogenate was added with 40 µl of 10% (w/v) SDS, mixed briefly and incubated at 65°C for 1 hour. After incubation, it was treated with 10 µl of 30 mg/ml Proteinase K and 5 µl of 10 mg/ml Dnase-free Rnase, mixed gently and incubated at the same temperature for 3 hours.

To precipitate protein out of the sample, 5 M of potassium acetate (pH 8.9) was added to a final concentration of 1 M, mixed thoroughly and then held on ice for 45 min. After precipitation, the sample was centrifuged at 12,000 rpm in a refrigerated microcentrifuge for 10 min. The supernatant was transferred to a new 1.5 ml microcentrifuge tube. Protein was removed again by extraction with Tris-saturated phenol (pH 8.0) and chloroform as follows. An equal volume of Tris-saturated phenol was added to the supernatant and mixed gently. The sample was then centrifuged for 5 min at 8,000 rpm. The upper aqueous phase was transferred into a new tube, added an equal volume of phenol:chloroform:isoamyl alcohol mixture

(25:24:1), mixed gently and centrifuged again. After centrifugation, the upper phase was transferred into another new tube to extract again with chloroform:isoamyl (24:1)

The upper phase was removed and mixed gently with 1/10 volume of 3 M ammonium acetate (pH 5.5). The DNA was precipitated out of this solution by adding 2 volumes of cold absolute ethanol. After the tube was inverted several times, it was placed at –20 °C overnight to complete precipitation. The precipitated DNA was removed from the tube, rinsed with 70 % cold ethanol and allowed to air-dry. The DNA was finally dissolved in an appropriate volume of TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). The DNA was incubated overnight at 37 °C for complete solubilization and kept at 4°C until further use.

2.8.2 Chelex-based DNA extraction

Fifty milligrams of foot tissue of *H. asinina* were sniped and ground in 400 µl chelex working solution (5% (w/v) in sterile distilled water). Then the mixture was incubated at 56°C for 3 hours. After that, it was placed in boiling water for 5 minutes to stop reaction. Finally, the mixture was centrifuged to discard cell debris and the chelex resin from solution. The supernatant was transferred to a new tube and kept at 4°C for use as DNA templates in PCR reaction. For 2 week-stage sample, a whole body of *H. asinina* was ground in 400 µl chelex working solution and DNA was extracted as those of the foot tissue sample.

After extraction, DNA concentration was determined by electrophoresis through 0.7 % agarose gel immersed in TBE buffer (89 mM Tris HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3). Each sample was mixed with 1/5 volume of the gel-loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% ficoll) and load into the gel with standard marker DNA (λ/*Hin*d III). The gel was run at 100 volts for 1 hour or

until bromophenol blue tacking dye reached about 1 cm. far from the end of the gel. After electrophoresis, the gel was stained in 2.5 μg/ml ethidium bromide solution for 5 min and then destained in water for 15 min. The ethidium bromide fluorescent DNA bands were visualized under UV light from a UV transilluminator and the gel was photographed through a red filter onto Kodak Tri-X pan 400 film. The amount of DNA sample was estimated by comparing their fluorescence to that of series of standard DNA (Sambrook et. al., 1989). The fluorescent intensity of the standard DNA fragments of 23.1, 9.4, 6.6, 4.3, 2.3, 2.0 and 0.5 kb corresponded to 240, 98, 67, 45, 24, 21 and 6 ng DNA, respectively when 500 ng of λ DNA (48 kb) was digested with *Hin*d III.

For PCR template, DNA quantity was spectrophotometrically measured at the optical density of 260 nanometer (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 μg/ml double-stranded DNA. The purity of extracted DNA sample can be determined by the ratio of OD_{260} and OD_{280}. A ratio of 1.8 indicates pure prepared DNA while higher and lower values of this ration indicate RNA or protein contamination of the isolated DNA samples, respectively (Kirby, 1992).

2.9 Screening of enzyme for library construction

H. asinina genomic DNA was digested with 4 types of blunt ended restriction enzymes *Alu* I, *Rsa* I, *Hin*c II and *Sma* I. In twenty microliters of reaction mixture, 1 μg aliquot of *H. asinina* DNA were digested separately with 5 units each of the 4 restriction enzymes, 2 μl of 10X restriction enzyme buffer and appropriate amount of sterile deionized water. *Alu* I, *Rsa* I and *Hinc* II digested reaction mixture were incubated at 37°C for 2 hr, and *Sma* I digested reaction mixture were incubated at 25°

C for 2 hours. Size and amount of digested DNA were examined by electrophoresis in 1.5% agarose gel with 100 bp ladder marker at 100 volts for 1 hour.

2.10 Construction of *H. asinina* genomic libraries

2.10.1 Preparation of chromosomal DNA for cloning

2.10.1.1 DNA fragmentation

Three genomic libraries were constructed, *Alu* I library, vortex and sonication library (v/s library) and Mixed-enzyme library. The first library, *Alu* I library, 5 μg of genomic DNA of *H. asinina* was digested with 25 units *Alu* I in total volume of 100 μ l at 37°C for 2 hours. Digested DNA was subjected to electrophoresis in 1.5% agarose gel at 100 volt for 1 hour with 100 bp ladder marker. After electrophoresis, 300-800 bp digested DNA were eluted from the gel using the elution procedure as described below. The second library, vortex and sonication library, 1 μg of genomic DNA from *H. asinina* in total volume 22 μl was fragmented by vortex 15 min and then sonication in sonication bath (Cleanser, Ultrasonic BCGR 5139) for 1 hr. After that, the fragmented DNA was subjected to electrophoresis to elute 300-800 bp fragments as well. The third library, Mixed-enzyme library, 6 μg of genomic DNA was digested with mixed enzymes, 20 units each of *Alu* I, *Rsa* I and *Hin*c II in total volume of 200 μl for 2 hours at 37°C. Digested DNA was subjected to electrophoresis as mentioned in the first and second libraries.

2.10.1.2 DNA elution

After electrophoresis, the marker lane (100 ladder marker) was excised from the gel and stained with ethidium bromide solution and visualized the ethidium bromide fluorescent. The distance of the DNA fragments size 300 to 800 bp was marked on

gel. After that the gel of marker lane and the gel of the sample were rejoined and then sample size 300 to 800 bp was excised form the gel at the same distance that marked on the marker lane. The DNA fragments were eluted from the gel using Qiaquick gel extraction kit (Qiagen) as follows. The gel was chopped into small pieces and transferred into 1.5 ml microcentrifuge tube (0.4 g for each tube). The gel in each tube was dissolved in 400 µl of Buffer QG at 50°C. After the gel was dissolved completely, 400 µl of isopropanol was added. The mixture was applied into Qiaquick spin column to bind DNA with the column membrane (1 column for 0.4 g of gel). The columns were centrifuged for 1 min at 12,000 rpm. To discard all traces of agarose, the columns were added 500 µl of Buffer QG and centrifuged again. After that, the columns were washed with 750 µl of Buffer PE and centrifuge twice to remove all washed solution. Finally, the columns were added with 7µl of TE (pH 8.5) and 23 µl of water and stood for 1 min. and then centrifuged to collect the eluted DNA.

The DNA yield was estimated by electrophoresis of the sample with 100 bp ladder marker in 1.5% agarose gel at 100 volts for 1 hour.

2.10.1.3 Blunt ended reaction

As a consequence of vortex and sonication making several different types of the end of DNA fragments, this step was necessarily performed for blunt ended ligation. The reaction mixture contained 300 ng of the eluted DNA, 1X EcoPol Buffer (10 mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 7.5 mM dithiothreitol), 0.2 mM dNTP, 3 U of Klenow, and 0.625 mM ATP in total volume of 10 µl. The reaction was incubated at 37°C for 30 min and then was stopped by chilling on ice and adding with 1 µl of 0.5 M EDTA. The reaction was purified using Qiaquick PCR purification kit as follows.

Five volumes of Buffer PE were added to the reaction, then applied into a spin column and centrifuged at 12,000 rpm for 1 min. To wash the impurity, 750 µl of Buffer PE was added, and column was spun again. The DNA was eluted from the column by adding 10 µl of TE and 40 µl of sterile water, stood for 5 min and spun again to collect the sample.

To improve efficiency of ligation, even though the first and the third libraries were conducted by digestion with blunt ended restriction enzyme, the DNA fragments from these methods were also blunted again.

2.10.2 Ligation

The pUC18 digested with *Sma* I and dephosphorylated, pUC18/*Sma* I BAP (Pharmacia) was used as a vector for blunt-end ligation. The DNA ligation mixture contained 50 ng of vector, 150 ng of the DNA fragments (from 1.3), 1x ligation buffer (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 5 mM dithiothreotol, 25 % (w/v) polyethyleneglycol-8000) and 2 U of T4 polynucleotide ligase in a total volume of 20 µl. The reaction was incubated at 16°C overnight.

2.10.3 Transformation

The portion of the ligation mixture was transformed into host cell *E. coli* stain XL1blue by electroporation procedure (Dower et al., 1988)

2.10.3.1 Preparation of host cell

A single colony of *E. coli* stain XL1blue was cultured as the starter in 15 ml of LB broth medium containing 12.5 µg/ml tetracycline and shaking at 37°C overnight. Two and a half milliliters of the starter was added in 250 ml of L-broth medium (1%

bactrotryptone, 0.5% bacto yeast extract, 0.5% NaCl) and the culture was left for cell growth at 37°C with vigorously shaking for 3-4 hr (OD_{600} ~ 0.5-0.8). The cells were then chilled on ice for 15-30 min, and centrifuged in a cold rotor (Beckman J2-21, USA) at 4,000xg for 15 min. After the supernatant was removed, pellet was washed by resuspending in a total of 250 ml of cold sterile water and centrifuged again. The pellet was washed further with 125 ml of cold water and followed with 5 ml of ice-cold steriled 10% glycerol. The final step, the cells were resuspended to a final volume of 500 – 750 µl. Then it was aliquoted 40 µl into 1.5 ml microcentrifuge tubes and stored at -80°C until use.

2.10.3.2 Electrotransformation

The apparatus were set as follows: 25 µF of Gene Pulser apparatus, 200 Ω of the Pulse controller unit and 2.5kV of the Gene pulser apparatus. Forty microliters of the cell suspension were gently thawed on ice then 1 µl of ligation mixture was added, mixed well and left on ice for 1 min. Then the mixture of cells and DNA was applied into a cold 0.2 cm cuvette. After one pulse was applied, the cells was immediately resuspended with 1 ml of SOC medium (2% bactotryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, $MgSO_4$ and 20 mM glucose). The cell suspension was incubated with shaking at 37°C for 1 hr. After incubation, the suspension was spread on the LB agar plates containing 50 mg/ml ampicillin, and incubated at 37°C overnight. The transformant colonies were counted and screened for microsatellite DNA with colony hybridization as follows.

2.11 Library screening for microsatellite DNA

Screening of microsatellite colonies was carried out by colony hybridization based on the method developed by Grunstein and Hogness (1975).

2.11.1 Colonies lifting

Whatman filter paper #42 (ashless) was used as a membrane for lifting the transformant colonies from plate. The filters were cut 3 asymmetry sites at the edge of circle and labeled certain number of plates with pencil on them. The labeled filter was carefully laid on the agar surface of the plate with the label side down. After the filter was wet thoroughly, the filter was pitched straight through the underneath agar with a pin for several places. After the position of the filters on agar had been drawn on the plates, the filters were lift slowly from the plates. The colonies on the plates were regrew by incubation at 37°C for 3-4 hours and then the plates were kept at 4°C for further use as master plates. The filters were carried on incubation on Whatmann 3 MM paper presoaked with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 7 minutes and followed by neutralization with neutralizing solution (1 M Tris-HCl pH 7.6, 1.5 M NaCl) 3 minutes twice. The filters were washed with 2xSSC (Appendix A), 0.2% [w/v] SDS for 30 seconds to 1 min and then air dried for 15-30 minutes. DNA was fixed on the filter by baking the filter at 80°C for 2 hours in a vacuum oven. After fixation, the pin marks on the filters were spotted intensely with a lead pencil that were making white spot markers on films after autoradiography. Then the filters were rubbed gently in 2xSSC, 0.2%[w/v] SDS with gloved hand to remove the cell debris of bacterial colonies.

2.11.2 Probe labelling reaction

$(GT)_{15}$ and $(CT)_{15}$ synthetic oligonucleotide synthesized from Biosynthesis Inc. USA were used as probe for screening the microsatellite colonies. The labelling reaction consisted of 100 pM of the oligonucleotide, 100 pM of 3,000 Ci/mmol [γ-^{32}P] ATP, 1x kinase buffer (10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100) and 10 U of T4 polynucleotide kinase in a total volume of 10 µl. The reaction was incubated a 37°C for 1 hour. Before using these probes, they were denatured by heating at 95°C for 15 min. and quick-cooled in an ice bucket.

2.11.3 Hybridization

The filters were prehybridized in a prehybridization solution (Appendix A) containing 2x Denhardt's solution, 5x SSPE (Appendix A), 0.5% [w/v] SDS and 100 µg/ml Yeast tRNA at 55°C for 2 hours (four filters for each 10 ml of prehybridization buffer). After that, 5 µl of radiolabeled $(GT)_{15}$ or $(CT)_{15}$ probe was then added to the prehybridization buffer. The hybridization reaction was performed in a hybridization rotor oven (Hybaid, USA) at 55°C overnight. After hybridization the filters were washed twice with 2x SSC, 0.1% [w/v] SDS at hybridization temperature for 10 min per wash, followed with 0.2x SSC, 0.1% [w/v] SDS at room temperature for 10 min. The filters were wrapped between two sheets of saran wrap and then exposed to an autoradiograph film at -80°C with intensifying screens for approximately 2-4 hours. After developing, the film was aligned with the master plates to pick up the positive colonies. The individual positive colonies were cultured overnight in a terrific broth which containing 100 mg/ml ampicillin at 37°C with shaking to extract plasmid.

2.11.4 Dot blot hybridization

As a result of colony hybridization, this experiment was set up to find out that why positive clones were found scantly when use $(CT)_{15}$ as a probe -- Is that probe had been degraded or $(CT)_{15}$ repeats are really less abundant than $(GT)_{15}$ repeats?

The black tiger shrimp (*Penaeus monodon*) DNA was used as controlled DNA for this experiment. Before blotting, *H. asinina* and *P. monodon* DNA were denatured by boiling for 5 min and immediately snap-cooled on ice. Two hundred nanograms of the denatured shrimp and abalone DNA were spotted onto nylon membrane separately then the DNA were fixed to the membrane by exposure for 3 min with UV light (UV trasilluminator 2011 MACROVUE). After that the membrane was hybridized with probes $(GT)_{15}$ and $(CT)_{15}$ as described in colony hybridization.

2.12 Plasmid extraction

A boiling procedure developed by Holmes and Quigley (1981) was used for rapid isolation of plasmid DNA from large numbers of colonies for sequencing.

The culture was applied into 1.5 ml microcentrifuge tube and then centrifuged at 12,000 rpm for 30 second to collect cell pellet. The cell pellet was then resuspened in 180 µl of STET buffer (8% Sucrose (w/v), 5% Triton X-100 (v/v), 50 mM Tris-HCl, 50 mM EDTA). Twenty microliters of 10 mg/ml lysozyme in STET buffer was added, immediately mixed by vortexing for 75 seconds and placing in boiling water for 75 seconds. After centrifugation again at 11,000 rpm for 10 minutes, the cell debris at the bottom of tube was removed by using a toothpick. Plasmid was precipitated from the solution by adding an equal volume of cold isopropanol and

placed at -80°C for at least 10 min. The plasmid was collected after centrifugation at 11,000 rpm for 10 min, then air-dried and resuspended in 20-30 µl of TE.

2.13 DNA sequencing and primer design

The plasmids were sequenced by using T7 sequencing ^{TM} kit (Appendix A) with universal primer, or and reverse primer as described below.

Ten microliters of extracted plasmid DNA (500 ng) was denatured by adding 2.25 µl of 2 N NaOH and incubated for 10 min at room temperature. Then it was added with 1.25 µl of 3 M sodium acetate (pH 4.8) and 1 µl of distilled water. The plasmid was precipitated with 30 µl of absolute ethanol at -80°C for 15 min and then centrifuged at 11,000 rpm for 15 min to collect plasmid pellet. The pellet was washed with ice-cold 70% ethanol and recentrifuged for 5 min. The pellet was air-dried and dissolved in 11 µl of distilled water and 2 µl of annealing buffer. A half volume of the resuspended template was annealed with 1.1 ml of and appropriate primer (5 pmol/µl universal or reverse primer); the remained portion was reserved at 4°C for the next time sequencing. The template and primer were annealed by incubation at 65°C for 5 min and followed by 37°C for 10 min. The annealed template was placed at room temperature for at least 5 minutes and then centrifuged briefly. The annealed template was then incubated for 5 minutes with 3.4 µl of Labeling reaction consisting of sterile water, Labeling Mix-dATP", [α-^{35}S]dATP (1000 Ci/mmol) and 1:5 diluted "T7 DNA polymerase" in the volume proportions of 5 : 15 : 4 : 10 respectively.

After incubation, 2.4 µl of the reaction was added into 1.25 µl each of the four pre-warmed sequencing mixes (A mix-short, C mix-short, G mix-short and T mix-short), and then incubated at 37°C for 5 min. Two microliters of stop solution (10

mM EDTA (pH 7.5), 97.5% deionized formamide and 0.3% each of bromophenol blue xylene cyanol) was added to each tube and mix gently.

Sequencing products were separated in an 8% denaturing polyacrylamide gel containing 7.8 M urea, and using 1x TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA, pH 8.3) at constant 50 watts for approximately 2.5 hours. Sequencing gels were transferred onto Whatmann #1 for vacuum drying with a gel dryer for 2 hours. After drying, the gel was exposed to an autoradiography film without intensifying screens overnight at room temperature. The flanking regions of microsatellite repeats were used to design PCR primer by using computer program OLIGO 4.0.

2.14 PCR amplification of microsatellite loci

One of each pair of PCR primer was labeled at 5'-end with radioactive making detectable PCR products on autoradiogram. The labeling reaction consist of 10 pmol of primer, 10 units of T4 Polynucleotide kinase, 1 μl of 10x kinase buffer and 10 pmol of [γ-^{32}P]ATP (3,000 μCi/mmol). The labelling reaction was performed at 37°C for 30 min and then at 65°C for 15 min to inactivate enzyme.

Five microliters of PCR reaction consisted of 1XPCR buffer (10 mM Tris-HCl, (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100), 200 μM dNTPs, 0.45 μM reverse primer, 0.425 μM forward primer, 0.025 μM labeled forward primer, 0.15 U of DyNAzyme^{TM} DNA polymerase and 15-25 ng of DNA template extracted by proteinase K phenol-chloroform method or 1 μl DNA template extracted by Chelex based method. The reaction mixture was overlaid with a drop of mineral oil and processed in a thermal cycler (Omnigene, Hybaid). The reaction was carried on pre-denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 1

min., annealing at appropriate annealing temperature for 2 min and extension at 72°C for 1 min. and finally, extension at 72°C for 10 min.

Annealing temperature (T_A) of each primer was determined by using the base content in each primer as the following equation.

$$T_A\ (°C) = [4\ (\text{sum of G+C})+2(\text{sum of A+T})]-5$$

The most optimal T_A was further adjusted from the autoradiography results. For instant, when nonspecific bands appeared, T_A was raised up, inversely if no products were visible, the temperature was dropped.

After the amplification process was completed, the reaction was added with 4 μl of stop dye (10 mM NaOH, 99% formamide, 0.1% bromophenol blue, 0.1% Xylene cyanol). Before subjected to electrophoresis, the mixture were denatured by heating at 95°C for 15 min and followed by quick cooling in an ice bucket. Three and a half microliters of the mixture was loaded onto 6 % denaturing polyacrylamide sequencing gel immersed in 1x TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA, pH 8.3). The electrophoresis was run at constant 50 watts for 2.5 – 6 hr. (up to the expected size of PCR products). After that, the gel was transferred onto Whatmann #1 for vacuum drying with a gel dryer. After the gel was dried, it was exposed to an autoradiography film with intensifying screens at -80°C approximately 2 hr. to 2 days or more (depending on product of each locus and the energy of the radioactive at that time).

Sizes of PCR products were determined by loading of M13 sequencing marker along side the products. M13 sequencing marker was prepared using the control template of T7 sequencing kit (single-standed M13mp18 DNA) as a template for

sequencing. A protocol for preparing M13 sequencing marker was described by Yanish-Perron et al., 1985 as follows.

Annealing reaction consisted of 5 μg of the control template, 10 pmol of universal primer and 2 μl of annealing buffer in a total volume of 14 μl. The reaction was performed at 65°C for 15 min then placed at room temperature for 10 min.

Labeling reaction consisted of 1 μl sterile water 3 μl of labeling mix-dATP, 2.0 μl of diluted T7 polymerase (0.3 μl of T7 polymerase and 1.7 μl of enzyme dilution buffer) and 10 pmol of [α-^{32}P]dATP (3000 Ci/mmol). Six microliters of this reaction was added to the annealed template. The labeling reaction was incubated at room temperature for 5 min. Then 5 μl of this reaction was dispensed to 2.5 μl each of pre-warmed short mix A, C, G, and T. After incubation at 37°C for 5 min., 30 μl of stop solution and 1 drop of mineral oil were added into each tube. Before using, the M13 sequencing was denatured as described above; about 1-2 μl of marker was sufficient for loading. In addition, this marker can be stored at -20°C for further use.

2.15 Scoring of microsatellite alleles

Size of microsatellite allele was determined by scoring of size of PCR product that separated onto a 6% denaturing polyacrylamide gel. In general, a microsatellite allele observed from an electrophoretically pattern was not a single band, but 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 given allele was the most intense band located at the biggest in size compared to the neighbor group of stuttered bands (Supungul, 1998).

Since microsatellite is a codominant marker providing 1 or 2 alleles per individual, the genotypes of the individual *H. asinina* for each locus could be divided

into homo- or heterozygotic states. These allelic stages of the individual were necessary for population study in *H. asinina* population, so they were also recorded.

2.16 Polymorphism test of markers

All developed markers were tested with 4 populations of *H. asinina*, NL, NC, HS and HC. Number of alleles per locus was used to determine polymorphism of each loci

2.17 Sensitivity test of the PCR reaction

Sensitivity of the PCR reaction was tested to determine the minimum amount.of DNA that can be detected by this technique, and the stage of the abalone that can be used for microsatellite analysis. Moreover, exposure time was also considered.

To determine the amount of DNA, 25 ng of the DNA template was diluted to 12 different concentrations by two-fold serial dilutions. Each dilutions was subjected to 2 PCR reactions; one of these was performed using primers of the most long exposure time locus while the other one was achieved conversely. This experiment was begun at the day that the radioactive was 3000 Ci/mmol. Exposure time was varied to find the optimum time.

DNA of 3 individuals of 2 weeks *H. asinina* (spat) were extracted DNA in 400 µl Chelex working solution. One microliter of the DNA solution was subjected to PCR with the same pairs of primer.

2.18 Cross amplification

Five individuals of other two species of tropical abalone, *H. ovina* and *H. varia* were tested with all microsatellite loci using the PCR condition as mentioned

2.19 Data analysis

Ten microsatellite loci (Has1-Has10) were used to examine genetic diversity in at least two geographic samples in *H. asinina* but seven geographic samples of *H. asinina* (NC, NI, NL, NS, HC, HP and HS) were analyzed by three loci (Has2, Has3 and Has8). This data set represents the overview of *H. asinina* samples examined in this study.

2.19.1 Genetic variation and heterozygosity

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

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

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.

The effective number of alleles at each locus was calculated by

$$n_e = 1/\Sigma p_i^2$$

where p_i is the frequency of i^{th} allele (Crow and Kimura, 1965).

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

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

Assuming that investigated samples conform Hardy-Weinberg equilibrium.

Practically, those parameters were estimated using GENEPOP Version 2.0 (Raymond and Rousset, 1995).

2.19.2 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 (e.g. selection, mutation or migration) which would change allele frequencies over time and mating is actually occurred at random in a large population. Pairs of alleles are not associated.

In this study, the null hypothesis (H_0) that observed genotypes frequencies of an investigated sample at a given locus conform Hardy-Weinberg equilibrium and an alternative hypothesis (H1) for heterozygote deficiency were tested using permutation version of the exact test based on a Markov chain following the algorithm of Guo and Thomson (1992) routine in GENEPOP version 2.0. The probability to reject null hypothesis was further adjusted using the sequential Bonferroni technique (Rice, 1989).

2.19.3 Genotypic disequilibrium analysis

Population genetic data collected on genotypes, genetic disequilibrium can be estimated. While Hardy-Weinberg equilibrium testing considers two alleles at the same locus but on different gametes, linkage disequlibrium testing looks at two alleles on the same gametes but different loci.

The null hypothesis that genotypes of *H. asinina* at one microsatellite locus are independent from those at the other locus and the alternative hypothesis that genotypes of compared loci of *H. asinina* are in disequilibrium, are tested using the Exact test described by Raymond and Rousset (1995) implemented in GENEPOP. Data on seven samples and three loci were analyzed. In addition, all microsatellite loci (Has1-Has10) analyzed against NL and NK samples were subjected to disequilibrium analysis. The significant level was also adjusted using the Sequential Bonferroni methos (Rice, 1989).

The individual genotype of *H. asinina* of 2 populations, NL and NK, were used to analyze genotypic disequilibrium of all usable loci. The probability to reject null hypothesis (H_0: segregation of each genotype of each locus is in equilibrium, not related with each other) were estimated using GENEPOP version 2.0.

2.19.4 Genetic differentiation between populations

Geographic heterogeneity between pairs of geographic samples 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 geographic samples.

Population differentiation was also analyzed using Wright's *F*-statistics (Wright, 1978). The unbiased F_{ST} estimate was calculated from genotype frequencies based on the assumption that the genotypic distribution is not significantly different across compared samples by GENEPOP. The levels of significance of geographic heterogeneity test and F_{ST}-based exact tests were also further adjusted using the sequential Bonferroni method (RICE, 1995).

2.19.5 Genetic distance and construction of boostrapped tree

Genetic distance between pairs of geographic samples was determined based on Cavalli-Sforza and Edwards (1967)'s chord distance approach. The additive genetic distance estimated from this method was found to be appropriate for microsatellite data obtained from various taxa whether they have undergone the bottleneck events or not (Takezaki and Nei., 1996).

The allelic frequency data was subjected to the chord distance using Gendist implemented in PHYLIP (Felsenstein, 1993). Phylogenetic analysis based on the neighbor-joining approach was carried out using Neighbor. Tree illustration was proper plotted by TREEVIEW. For bootstrapped neighbor-joining tree, the original data was bootstrapped 2000 times using Seqboot and subjected to Cavalli-Sforza and Edwards (1967)'s chord distance estimation for multiple data sets using Gendist. The multiple distance matrices were used to construct 2000 neighbor-joining trees using Neighbor. A consensus tree (bootstrapped) tree was constructed using Consense and illustrated by TREEVIEW. All computational programs mentioned above except TREEVIEW are routine in PHYLIP (Felsenstein, 1993). Significant clustering

between two samples was considered when the percentage of bootstrapped values was greater than 50%.

Chapter III

Results

3.1 DNA extraction

Genomic DNA was extracted from the foot tissue of *H. asinina* using a proteinase K-phenol-chloroform extraction method slightly modified from Davis et al. (1996). From agarose gel electrophoresis, high molecular weight genomic DNA was about 23 kb indicated good quality of extracted DNA (Fig 3.1). The $OD_{260/280}$ ratio was slightly higher than 1.8 indicating its high purity. The yield obtained form this method was about 85 μg per 100 mg of originating tissue. Extraction of genomic DNA using Chelex yielded DNA of 200 μg from 100 mg of foot tissue and about 50 μg from a 2 week old (spat) specimen.

3.2 Screening of appropriate restriction enzymes and library construction methods

Genomic DNA of *H. asinina* was digested with 4 blunt-ended restriction endonucleaes (*Alu* I, *Rsa* I, *Hin*c II and *Sma* I) Results indicated that high yields of desired DNA fragments (300-800 bp) were obtained from *Alu* I and *Rsa* I digestions whereas larger restricted fragments were obtained from *Hin*c II and *Sma* I digestions (Fig. 3.2).

Accordingly, *Alu* I was chosen to establish a partial genomic DNA library in *H. asinina* (*Alu* I library). Nevertheless, only about 100 colonies were obtained reflecting extremely low transformation efficiency. When a blunt-ended step of restricted DNA fragments by the Klenow fragment was performed prior to ligation, a higher

transformation efficiency was obtained and the number of colonies were high enough for colony hybridization using ^{32}P end-labeled GT_{15} probes. Nonetheless, only 0.2% of positive clones were found (5 of 2,510 clones).

The second library (V/S) was constructed using fragments mechanically sheared by vortexing and sonication. It was found that the DNA fragments of desired size were obtained by sonication cooperated with vortexing and the fragments of 300-800 bp were isolated for use in the library construction (Fig. 3.3, lane 6). The third library was established from DNA fragments of 300-800 bp resulted from simultaneous digestion of genomic DNA with *Alu* I, *Rsa* I and *Hin*c II (Fig. 3.4) and called a mixed enzyme library.

After transformation, colony hybridization was carried out using the same $(GT)_{15}$ probe. The mixed enzyme library (3,487 clones) yielded the highest percentage of positive clones (1.46%) whereas the V/S library (4,564 clones) gave a higher percentage than the *Alu* I library (0.43%). Further screening of the V/S library (4,464 clones) with the $(CT)_{15}$ probe provided additional 0.07% of positive clones. An example of colony hybridization results is shown in Fig. 3.5.

Dot blot hybridization using ^{32}P labeled probes, $(GT)_{15}$ and $(CT)_{15}$ indicated that $(CT)_n$ repeats were much less abundant than $(GT)_n$ repeats in *H. asinina* genome (Figure 3.6). The results also indicated that microsatellites were less abundant in *H. asinina* than those in the black tiger shrimp, *P. monodon* (Fig. 3.6).

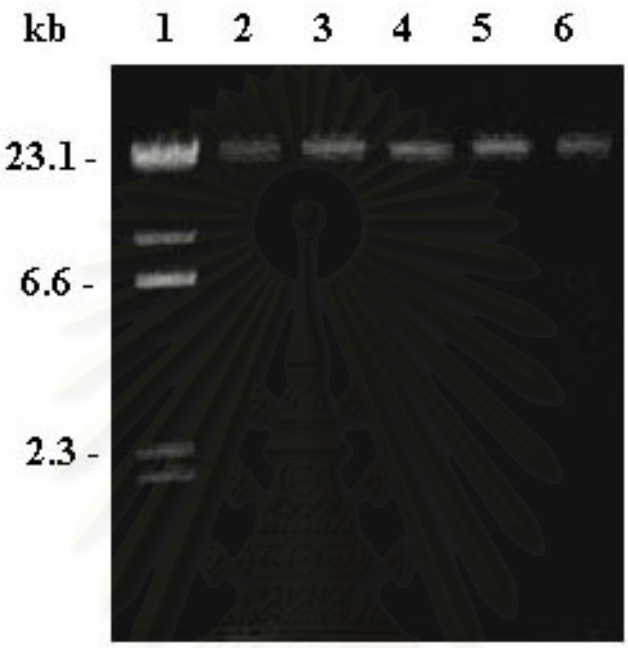

Figure 3.1 A 0.7% ethidium bromide-stained agarose gel showing DNA extracted from the foot tissue of *H. asinina* using a proteinase K-phenol-chloroform method (lanes 2-6). λ DNA/*Hin*d III (lane 1) was used as a standard marker.

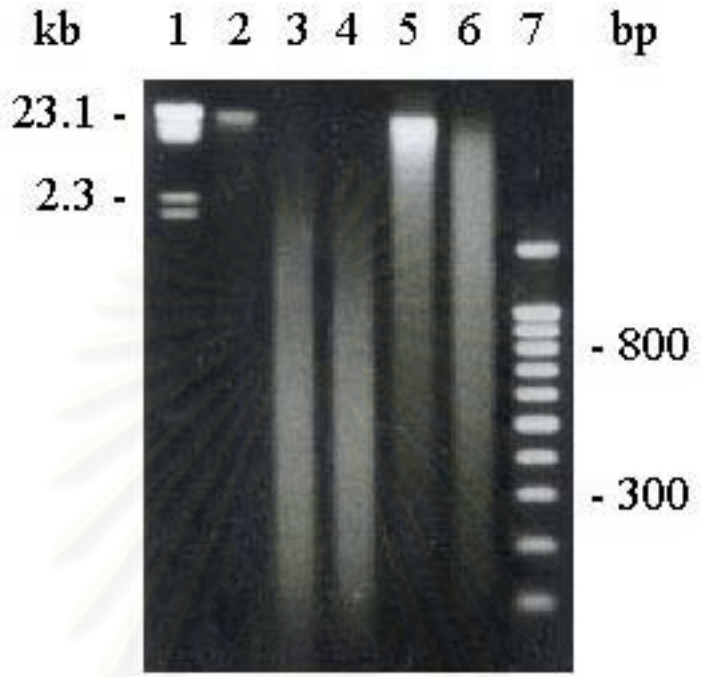

Figure 3.2 A 1.5% ethidium bromide stained agarose gel showing *H. asinina* DNA digested with 4 restriction enzymes.

Lane 1 λ DNA/*Hin*d III standard marker.

Lane 2 undigested *H. asinina* DNA.

Lanes 3-6 *H. asinina* DNA digested with *Alu* I, *Rsa* I, *Sma* I and *Hin*c II, respectively.

Lane 7 A 100 bp DNA ladder.

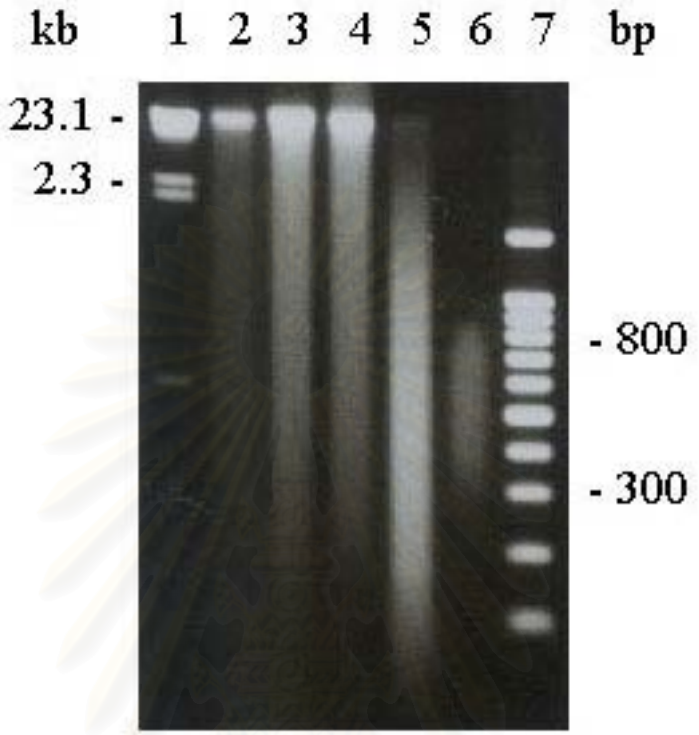

Figure 3.3 A 1.5% ethidium bromide stained agarose gel showing *H. asinina* DNA fragmented by vortexing and sonication.

Lane 1 A λ DNA/*Hin*d III standard marker.

Lane 2 unfragmented *H. asinina* DNA.

Lane 3 *H. asinina* DNA fragmented by vortexing for 15 min.

Lane 4 *H. asinina* DNA fragmented by sonication for 1 hr.

Lane 5 *H. asinina* DNA fragmented by vortexing for 15 min followed by sonication for 1 hr.

Lane 6 DNA fragments (300-800 bp) used for construction of a library.

Lane 7 A 100 bp DNA ladder.

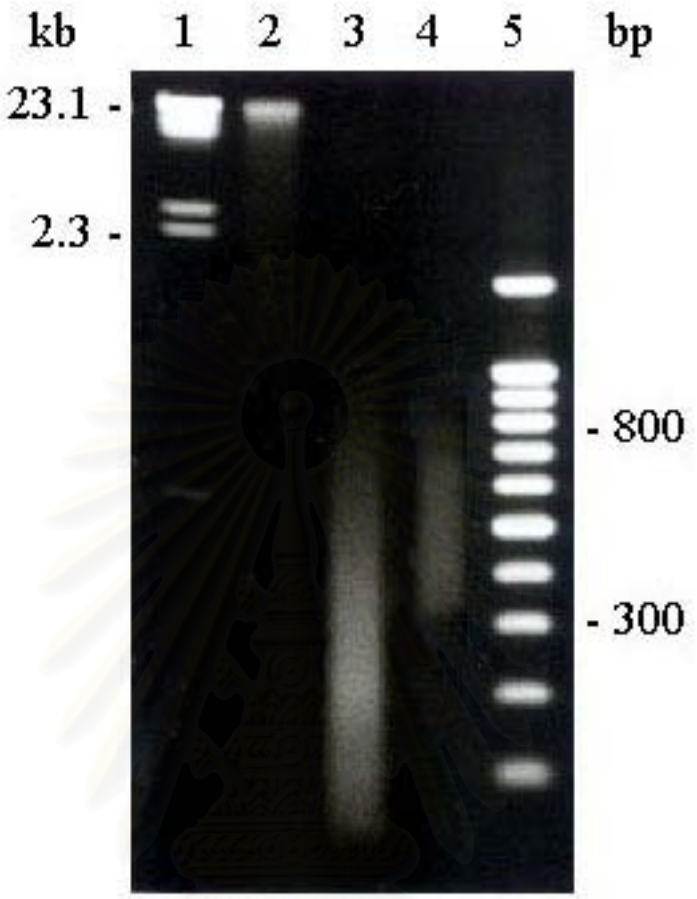

Figure 3.4 A 1.5% ethidium bromide stained agarose gel showing *H. asinina* DNA simultaneously digested with *Alu* I, *Rsa* I, and *Hin*c II.

Lane 1 A λ DNA/*Hin*d III standard marker.

Lane 2 undigested *H. asinina* DNA.

Lane 3 *H. asinina* DNA digested with *Alu* I, *Rsa* I, and *Hin*c II.

Lane 4 DNA fragments (300-800 bp) used for construction of a library.

Lane 5 A 100 bp DNA ladder.

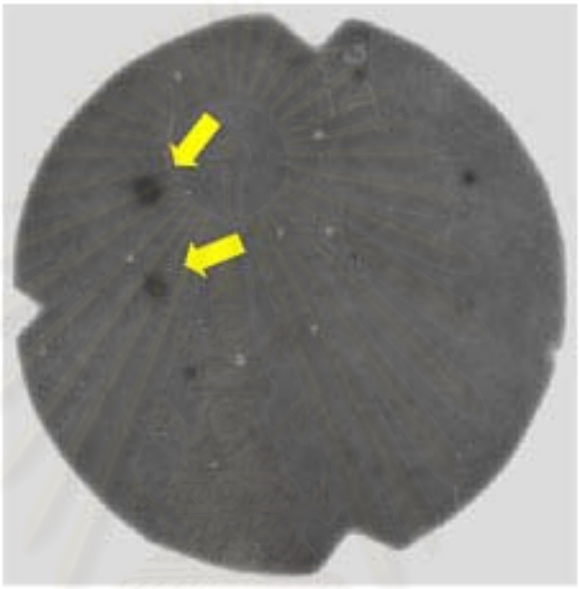

Figure 3.5 Colony hybridization of microsatellites from *H. asinina* genomic library screened with a ^{32}P labeled $(GT)_{15}$ probe. Yellow arrows indicate positive recombinant clones.

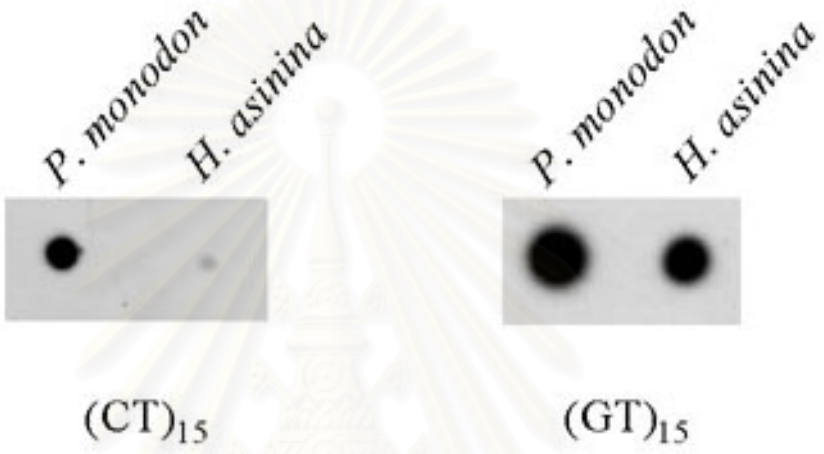

Figure 3.6 Dot blot hybridization of the black tiger shrimp (*P. monodon*) DNA (control) and *H. asinina* DNA (200 ng each) hybridized with ^{32}P labeled $(GT)_{15}$ and $(CT)_{15}$ probes.

3.3 Sequencing analysis of positive hybridizing clones and characteristics of isolated microsatellites

Twenty-six of 68 positive clones sequenced (accounting for 38.2% of investigated clones) contained microsatellites. A total of 33 microsatellite loci were isolated because some clones contained separate microsatellite arrays intervened by three or more bases. Both perfect and imperfect microsatellite repeats (Weber, 1990) were found at the same proportions, 11 loci (32.35%) of each type while 12 compound microsatellites (35.29%) were observed. Examples of perfect, imperfect and compound microsatellite repeated sequences are shown in Figure 3.7.

Although libraries were intentionally screened for $(GT/AC)_n$ microsatellites, several other motifs were also picked up coincidentally (Tables 3.1, 3.2 and 3.3). In this study, 3 types of microsatellite repeats (di-, tetra- and hexanucleotides) were found. The numbers of repeats of di- and tetranucleotide microsatellites were 8-37 repeats and 3-45 repeats, respectively (data not shown). A hexanucleotide repeat with 6 repeated units was found once.

3.4 Levels of polymorphism and characteristics of microsatellites

Fourteen pairs of primer were designed but only 10 pairs of primer could be used to amplify microsatellites of *H. asinina* successfully. The most suitable annealing temperature (T_A) for PCR of each locus was illustrated in Table 3.4. The level of polymorphism of each microsatellite locus was tested against 4 geographical different samples including Talibong (NL), Cambodia (NC), hatchery Samet (HS) and hatchery Cambodia (HC) of *H. asinina*. All of them were polymorphic and have allelic sizes ranging from 104 to 360 bp (Table 3.4). The highest polymorphic locus,

Has1 exhibited 26 alleles and the observed heterozygosity of 0.85 whereas the lowest polymorphic locus was Has7 which possessed 3 alleles and observed heterozygosity of 0.27. The expected heterozygosity of all loci except Has6 and Has7 across these samples were greater than the observed heterozygosity (Table 3.5)

Characteristic of each microsatellite

Has1

Has1 is a combination of 2 $(GT)_n$ loci. The size range of alleles was very large (258-360 bp) therefore enormous differences of band intensity were observed in individuals carrying large and small alleles. Large alleles require longer exposure time for autoradiography. Therefore, genotyping of *H. asinina* at this locus is quite difficult and may possibly interfere by the existence of null alleles.

Has2

This locus is a compound microsatellite containing $(AT)_7(GT)_{37}$ repeats. Sizes of alleles ranged from 286 to 340 bp and could be easily scored even stutter bands were commonly observed in the amplification product. Due to large sizes of alleles, genotyping required a long time for electrophoresis and autoradiography. Highly polymorphic was found at this locus.

Has3

This locus is also a compound microsatellite containing two types of dinucleotide repeats, $(GT)_{24}(GA)_{18.}$ The polymorphic level of this locus is not as high as that of Has1 but the amplification products were small (134-178 bp) and easily scored.

Has4

Microsatellite repeats of the locus Has4 are a combination of $(GT)_6(TGCA)_4 N_{15} (GT)_7$. Sizes of alleles ranged from 220 to 250 bp with a low polymorphic level. This locus showed a high sensitivity of amplification and rapid detection for specimens from the Gulf of Thailand (NC, HC and HS) but not in the Andaman sea sample (NL). Therefore, it was not further used for populaion genetic study of *H. asinina*.

Has5

This locus is a perfect $(GT)_{17}$ repeats microsatllite. This locus was highly polymorphic. Sizes of alleles ranged from 104 to 173 bp with a large number of alleles but a low level of heterzygosity was found. Theoretically, problems from null alleles may have been arisen at this locus.

Has6

Although the microsatellite at this locus is a perfect dinucleotide repeats of $(GT)_{19}$, Sizes of alleles at this locus were 231-240 and could be scored easily. However, only 6 alleles were found from analysis of NC, HC and HS samples but the most serious problem was the failure to amplify any specimen of the Talibong (NL) sample.

Has7

This microsatellite is a perfect tetranucleotide repeats $(ACGC)_6$. Sizes of alleles were small (112-126 bp) so genotyping of of the Gulf of Thailand samples at this locus could be carried out unambiguously. Although patterns of microsatellite alleles were quite sharp and clear without any stutter band, specimens from Talibong (NL, the Andaman sea) could not be amplified at this locus.

Has8

This locus is a perfect tetranucleotide repeats $(AGTG)_{16}$. Sizes of alleles were 148-238 with a high number of alleles and heterozygosity. Patterns of alleles at this locus were sharp and clear with 2 or 3 faint stutter bands therefore, they could be scored easily.

Has9

This locus is an another perfect, $(GT)_{34}$, microsatellite. It was tested only in Talibong (NL) and Cambodian (NC) samples. Patterns of alleles of this locus were sharp with the existence of stutter bands. This locus was highly polymorphic exhibiting a large number of alleles and a high heterozygosity level. Sizes of alleles of the NL sample were smaller (148-162 bp) than those of the NC (184-240 bp). Therefore, differentiation of specimens from these geographic samples was possible.

Has10

This microsatellite is an imperfect dinucleotide repeats. It was also tested for polymorphism only in NL and NC samples. Stutter bands was also found along with the actual alleles. Sizes of alleles were small and easy to be determined (118-160 bp) but level of polymorphism of this locus was quite low.

Table 3.1 Perfect microsatellites found in *H. asinina*

Type	Repeat unit	No. of loci
Dinucleotide	$(GT/CA)_n$	6
Tetranucleotide	$(GGTT)_n$	1
	$(GTGA)_n$	2
	$(GTGC)_n$	1
Hexanucleotide	$(GTGTGC)_n$	1

Table 3.2 Imperfect microsatellites found in *H. asinina*

Type	Repeated unit	No. of loci
Dinucleotide	$(AT/TA)_n$	1
	$(CT/GA)_n$	1
	$(GT/CA)_n$	7
Tetranucleotide	$(GTGA)_n$	2

Table 3.3 Compound microsatellites found in *H. asinina*

Type	Repeated unit	No. of loci
di-di	$(AG)_{21}(GT)_{14}$	1
	$(AT)_{7}(GT)_{37}$	1
	$(GT)_{24}(GA)_{18}$	1
	$(GT)_{17}(AG)_{10}$	1
di-tetra	$(GT)_{11}(CTGT)_{7}$	1
	$(GT)_{37}(CTGT)_{8}$	1
	$(GT)_{6}(TGCA)_{4}$	1
	$(GT)_{9}(TGAG)_{6}$	1
	$(GT)_{13}(AGTG)_{5}$	1
tetra-tetra	$(AGTG)_{7}AG(AGTG)_{19}$	1
tetra-tetra-tetra-di	$(CTAT)_{10}(GTAT)_{23}(CTAT)_{12}(AC)_{20}$	1

Table 3.4 Repeated unit of microsatellite of *H. asinina*, annealing temperature for PCR amplification and expected and observed sizes of alleles

Locus (bp)	Repeated unit (bp)	Annealing Temp. (°C)	Expected size of allele (bp)	Observed $(N)^{\#}$ size of allele (bp)
Has1	$(GT)_{17}N_{36}(GT)_{10}$	53	257	258-360 (72)
Has2	$(AT)_{7}(GT)_{37}$	57	333	286-340 (65)
Has3	$(GT)_{24}(GA)_{18}$	57	175	134-178 (71)
Has4	$(GT)_{6}(TGCA)_{4}N_{15}(GT)_{7}$	57	254	222-250 (67)
Has5	$(GT)_{17}$	49	98	104-173 (72)
Has6	$(GT)_{19}$	57	232	232-240 (48)
Has7	$(ACGC)_{6}$	49	117	112-126 (48)
Has8	$(AGTG)_{16}$	49	191	148-238 (72)
Has9	$(GT)_{34}$	53	117	148-240 (48)
Has10	$(CA)_{16}CG(CA)_{4}$	53	140	118-160 (48)

$(N)^{\#}$ number of samples

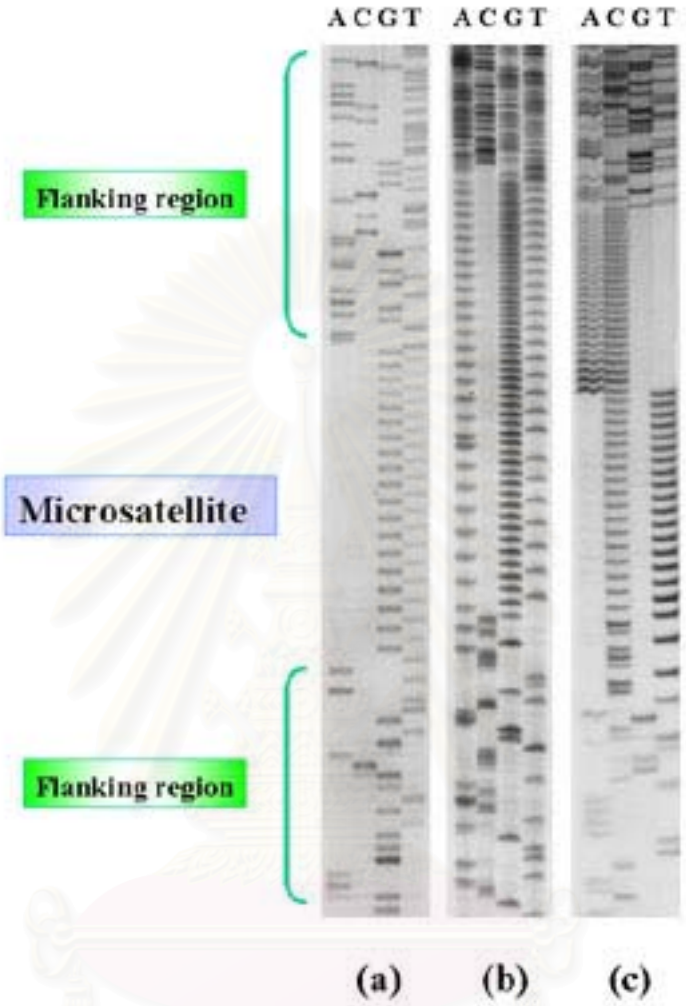

Figure 3.7 DNA sequences of (a) perfect dinucleotide repeats, $(GT)_{19}$, (b) imperfect tetranucleotide repeats, $(AGTG)_7AG(AGTG)_{19}$ and (c) compound dinucleotide repeats, $(CT)_{18}(CA)_{24}$.

Table 3.5 Total number of alleles and expected and observed heterozygosity in geographical different samples of *H. asinina*, Talibong (NL), Cambodia (NC), hatchery Samet (HS) and hatchery Cambodia (HC).

Locus	Number of individuals	Number of alleles	Heterozygosity	
			observed	expected
Has1	72	26	0.85	0.93
Has2	65	21	0.68	0.93
Has3	71	13	0.62	0.82
Has4	67	5	0.40	0.59
Has5	72	19	0.35	0.91
Has6*	48	6	0.75	0.71
Has7*	48	3	0.27	0.24
Has8	76	19	0.71	0.88
Has9#	48	26	0.81	0.92
Has10#	48	9	0.42	0.63

*NL sample could not be amplified and excluded from the analysis

#HS an HC population were not determined and not included

3.5 Sensitivity test and cross-species amplification analysis

Has1 showed the lowest sensitivity for amplification and detection whereas Has4 was the most sensitive locus. Therefore, the sensitivity of these loci was evaluated because sensitivity of other loci should be covered by those of the Has1 and Has4.

As can be seen from Figure 3.8, the PCR product at the locus Has1 could be correctly detected until the ninth dilution (48.83 pg) and the optimal exposure time was one day. Likewise, sensitivity analysis of Has4 indicated that the PCR product could be detected until the eleventh dilution (12.21 pg) with the longest exposure time of 16 hr. At 2 hr exposure time, the PCR product could be detected until the ninth dilution (48.83 pg) which was as sensitive as that of the Has1 locus (Fig. 3.9). In addition, both Has1 and Has4 primers could amplify DNA of two-week old (spat) *H. asinina* extracted by Chelex based method. (Fig. 3.10).

Moreover, five individuals of other tropical abalone, *Haliotis ovina* and *Haliotis varia* were used to test for the success of cross-species amplification. Results showed that all pairs of microsatellite primers developed for *H. asinina* could not be used for cross-amplification in *H. ovina* and *H. varia* (data not shown).

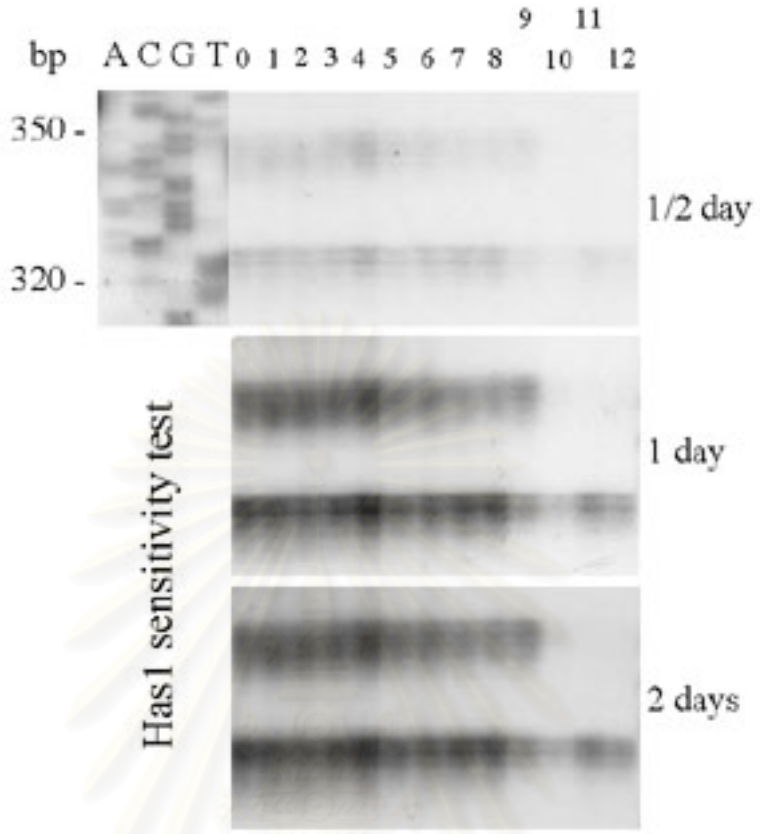

Figure 3.8 The sensitivity test of the locus Has1 (½, 1 and 2 days of exposure time). The size standard is a sequencing ladder of M13 mp 18.

Lane 0 PCR amplification patterns when 25 ng of DNA template were used.

Lanes 1-12 PCR amplification patterns when two-fold serial dilutions of DNA templates were used (12.5 ng - 6.10 pg corresponding to lanes 1-12, respectively).

DNA templates:

1st dilution	= 12.50 ng	2nd dilution	= 6.25 ng
3rd dilution	= 3.13 ng	4th dilution	= 1.56 ng
5th dilution	= 0.78 ng	6th dilution	= 0.39 ng
7th dilution	= 0.20 ng	8th dilution	= 0.10 ng
9th dilution	= 48.83 pg	10th dilution	= 24.41 ng
11th dilution	= 12.21 pg	12th dilution	= 6.10 pg

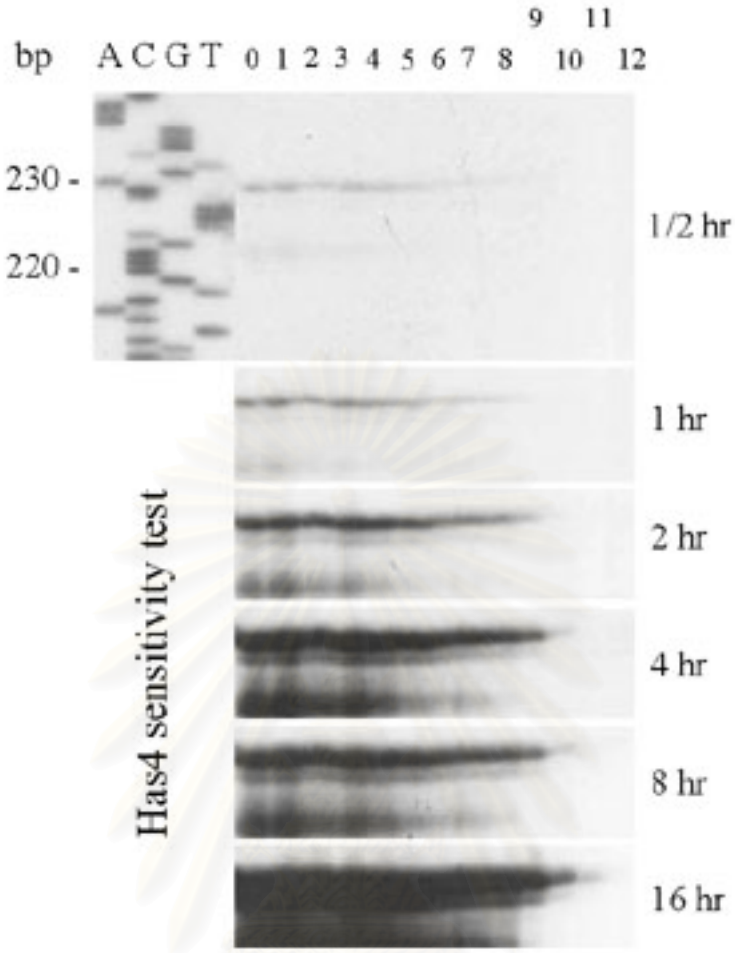

Figure 3.9 The sensitivity test of the locus Has4 (½, 1, 2, 4, 8 and 16 hr of exposure time). The size standard is a sequencing ladder of M13 mp 18.

Lane 0 PCR amplification patterns when 25 ng of DNA template were used.

Lanes 1-12 PCR amplification patterns when two-fold serial dilutions of DNA templates were used (12.5 ng - 6.10 pg corresponding to lanes 1-12, respectively).

DNA templates:

1st dilution	= 12.50 ng	2nd dilution	= 6.25 ng
3rd dilution	= 3.13 ng	4th dilution	= 1.56 ng
5th dilution	= 0.78 ng	6th dilution	= 0.39 ng
7th dilution	= 0.20 ng	8th dilution	= 0.10 ng
9th dilution	= 48.83 pg	10th dilution	= 24.41 ng
11th dilution	= 12.21 pg	12th dilution	= 6.10 pg

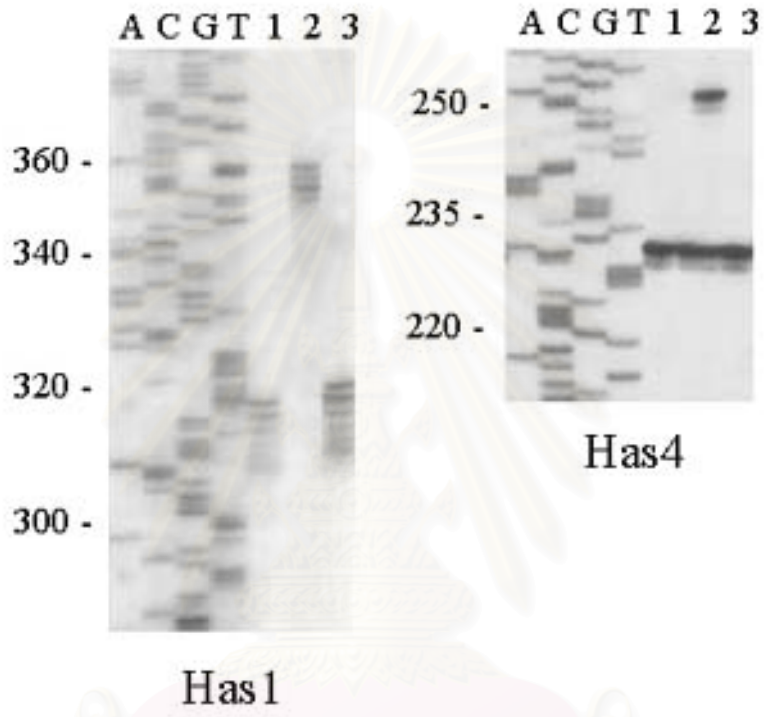

Figure 3.10 Microsatellite patterns at Has1 and Has4 loci of 3 two-week old (spat) *H. asinina* individuals (lanes 1-3). The size standard is a sequencing ladder of M13 mp 18.

3.6. Genotypic disequilibrium analysis

Genotypic disequilibrium of 8 microsatellites in this study was tested against *H. asinina* from Talibong (NL) and Cambodia (NC) samples with the exception of Has6 and Has7 where amplification was not possible in the former sample. Result indicated that genotypes from these microsatellite loci were associated randomly ($p > 0.0031$).

Some loci were further analyzed using more number of samples (Appendix C). Results also indicated a lack of supporting evidence for non-random association of microsatellites in *H. asinina* used in this study ($p > 0.0021$ and $p > 0.0027$).

Table 3.6 Genotypic disequilibrium analysis of *H. asinina* originating from NL and NC at eight microsatellite loci

	Has1	**Has2**	**Has3**	**Has4**	**Has5**	**Has8**	**Has9**	**Has10**
Has1	-							
Has2	1.0000^{ns}	-						
Has3	0.0480^{ns}	1.0000^{ns}	-					
Has4	0.6522^{ns}	0.4474^{ns}	0.2029^{ns}	-				
Has5	0.9816^{ns}	0.1131^{ns}	0.3816^{ns}	0.9826^{ns}	-			
Has8	0.8208^{ns}	1.0000^{ns}	0.2381^{ns}	0.5223^{ns}	0.9902^{ns}	-		
Has9	0.7085^{ns}	1.0000^{ns}	0.6899^{ns}	0.6828^{ns}	0.7118^{ns}	0.7069^{ns}	-	
Has10	0.9994^{ns}	0.3478^{ns}	0.9852^{ns}	0.3326^{ns}	0.0067^{ns}	0.5770^{ns}	0.5229^{ns}	-

*Significant level was adjusted to $p < 0.0031$ using the sequential Bonferroni procedure

ns = not significant

3.7 Genetic diversity and population differentiation of *H. asinina*

Seven geographic samples of *H. asinina* (NL, NS, NC, NI, HP, HS and HC) were tested against 3 microsatellite loci; Has2, Has3 and Has8. These microsatellites could not be successfully amplified DNA isolated from 12 individuals of the Indonesia (NI) sample. Notably, specimens from this geographic sample were not successfully analyzed by PCR analysis of 16S and 18S rDNA. Therefore, population genetic analysis was carried out in the remaining samples. These samples should represent an overview on population genetics of this species accurately. Patterns of amplified microsatellites at these loci are illustrated by Figs. 3.11, 3.12 and 3.13, respectively.

3.7.1 Sizes and allele distribution of microsatellites in H. asinina

A total number of 23, 21 and 18 alleles were observed at Has2, Has8, and Has3 loci against 106 examined individuals of *H.asinina*. Sizes of alleles of Has2 were large (286-340 bp) whereas those of Has3 were smaller (134-196 bp). Distribution of allele sizes at the locus Has8 was between 148-238 bp (Table 3.7 and Figs. 3.14, 3.15 and 3.16).

Allele distribution patterns varied greatly among loci at the locus Has2. At the locus Has3, a 160 bp allele showed the greatest allele frequency (0.760) in Talibong sample and overall (0.310) (Fig 3.15, Table B3). At the Has8 locus, only 2 and 4 alleles were observed in the Philippines and Talibong samples, respectively (Fig. 3.16). Allele distribution frequencies at the 3 loci of *H. asinina* from NS, NC, HC, and HS were similar to one another but were different from those of Talibong and the Philippines. At the locus Has2, natural samples from Talibong showed the highest

number of alleles (17) whereas in the Philippines samples, only 4 alleles were observed (Fig. 3.14)

3.7.2 Genetic variation within geographic samples

Analysis of microsatellites in six geographic samples showed that Has2 was the most polymorphic locus followed by Has3 and Has8 when the average number of alleles and effective number of alleles were considered (9.67 and 7.29, 7.33 and 5.32 and 7.50 and 4.56, respectively) (Table 3.7). The mean observed heterozygosity of a respective locus was 0.63 (Has8), 0.72 (Has2) and 0.78 (Has3). The Philippines samples had the lowest alleles number at all loci but observed heterozygosity at Has2 and Has3 loci were both 1.00. Conversely, a lack of polymorphism was observed in this population at the Has8 locus (h_{obs} = 0.00). The observed heterozygosity in *H. asinina* from Cambodia (Has3) and Philippines (Has2 and Has3) were much higher than the expected heterozygosity

Considering polymorphic levels of each geographic sample across overall loci, *H. asinina* from Cambodia exhibited the highest number of allele and effective number of alleles (12.00 and 7.65) (Table 3.8). Other samples except the Philippines (3.67 and 3.34) had comparable numbers of these parameters (7.67-8.67 and 5.10-6.33). The expected heterozygosity in each sample was not (much) different from the observed heterozygosity. It should be noted that the Philippines samples possessed the lowest number of alleles and effective alleles per locus but observed heterozygosity was greater than the Cambodian sample which showed the greatest levels of those parameters (Table 3.8).

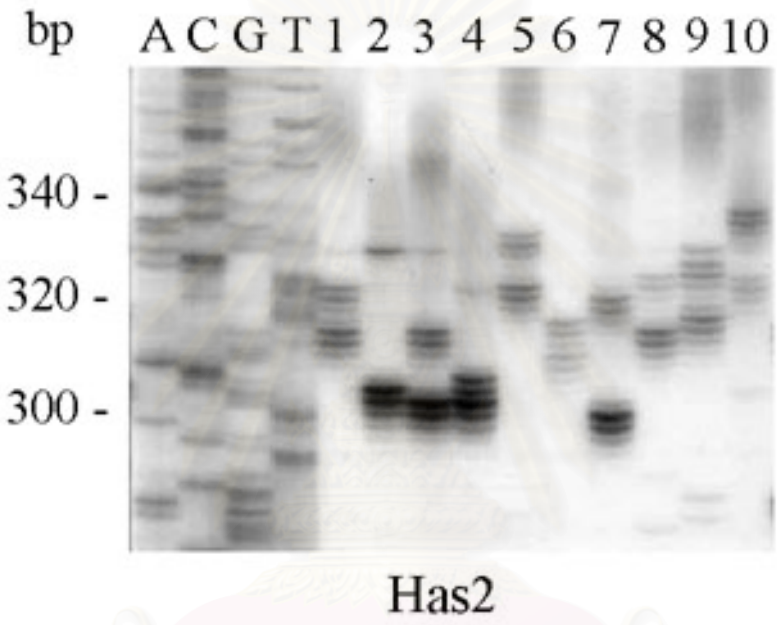

Figure 3.11 Microsatellite patterns resulted from analysis of 10 *H.asinina* individuals at the locus Has2 (lanes 1-10). The size standard is a sequencing ladder of M13 mp 18.

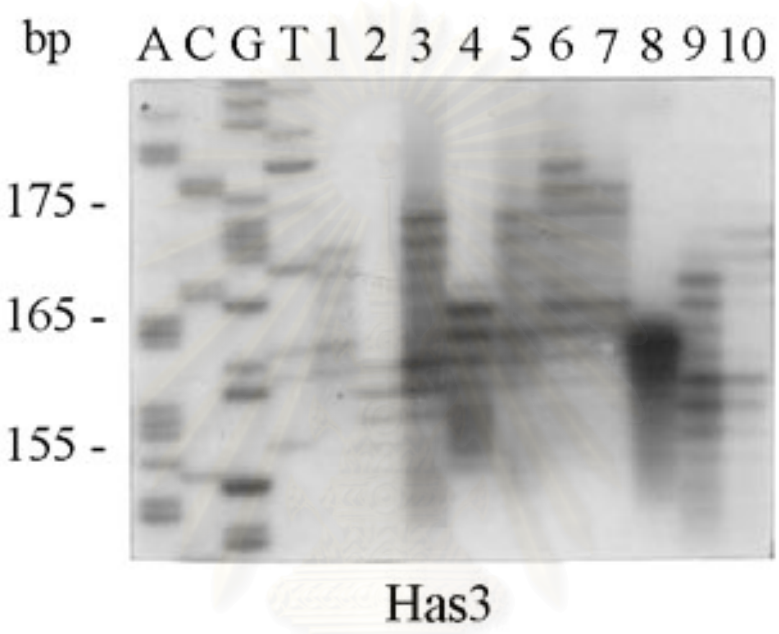

Figure 3.12 Microsatellite patterns resulted from analysis of 10 *H.asinina* individuals at the locus Has3 (lanes 1-10). The size standard is a sequencing ladder of M13 mp 18.

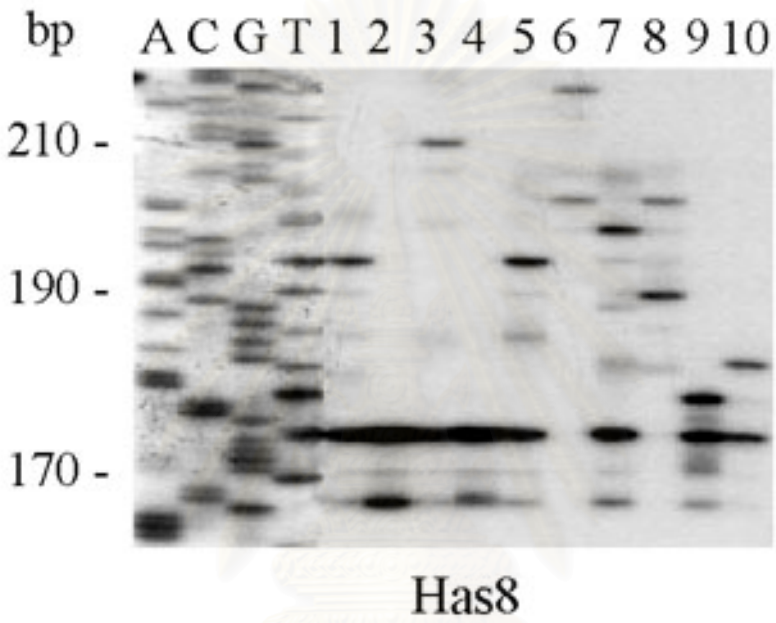

Figure 3.13 Microsatellite patterns resulted from analysis of 10 *H. asinina* individuals at the locus Has8 (lanes 1-10). The size standard is a sequencing ladder of M13 mp 18.

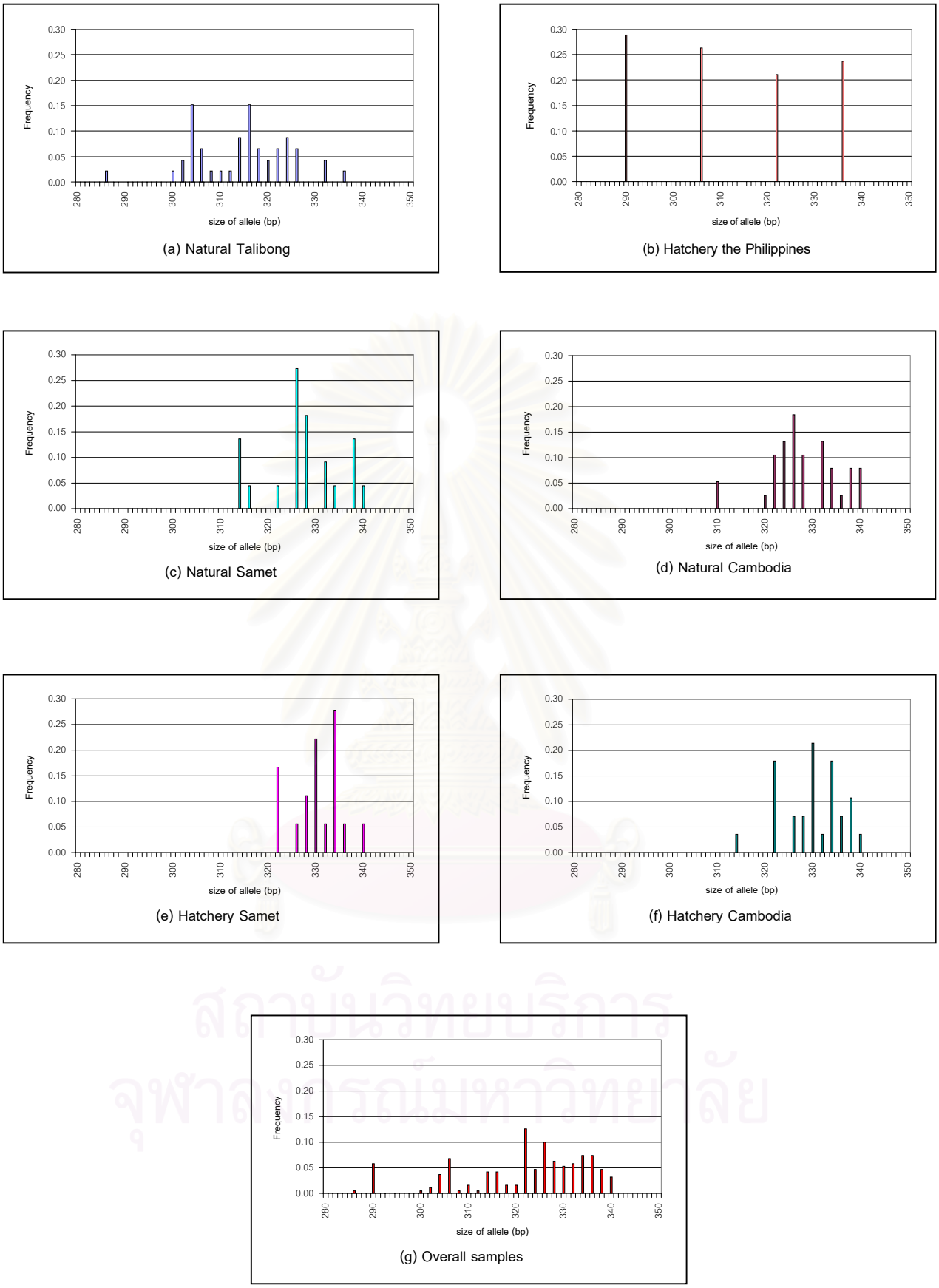

Figure 3.14 Allele distribution frequencies at the Has2 locus of *H. asinina* from Talibong (NL, $N = 23$), Hatchery The Philippines (HP, $N = 19$), Samet (NS, $N = 11$), Cambodia (NC, $N = 19$), Hatchery Samet (HS, $N = 9$), Hatchery Cambodia (HC, $N = 14$) and overall samples ($N = 95$)

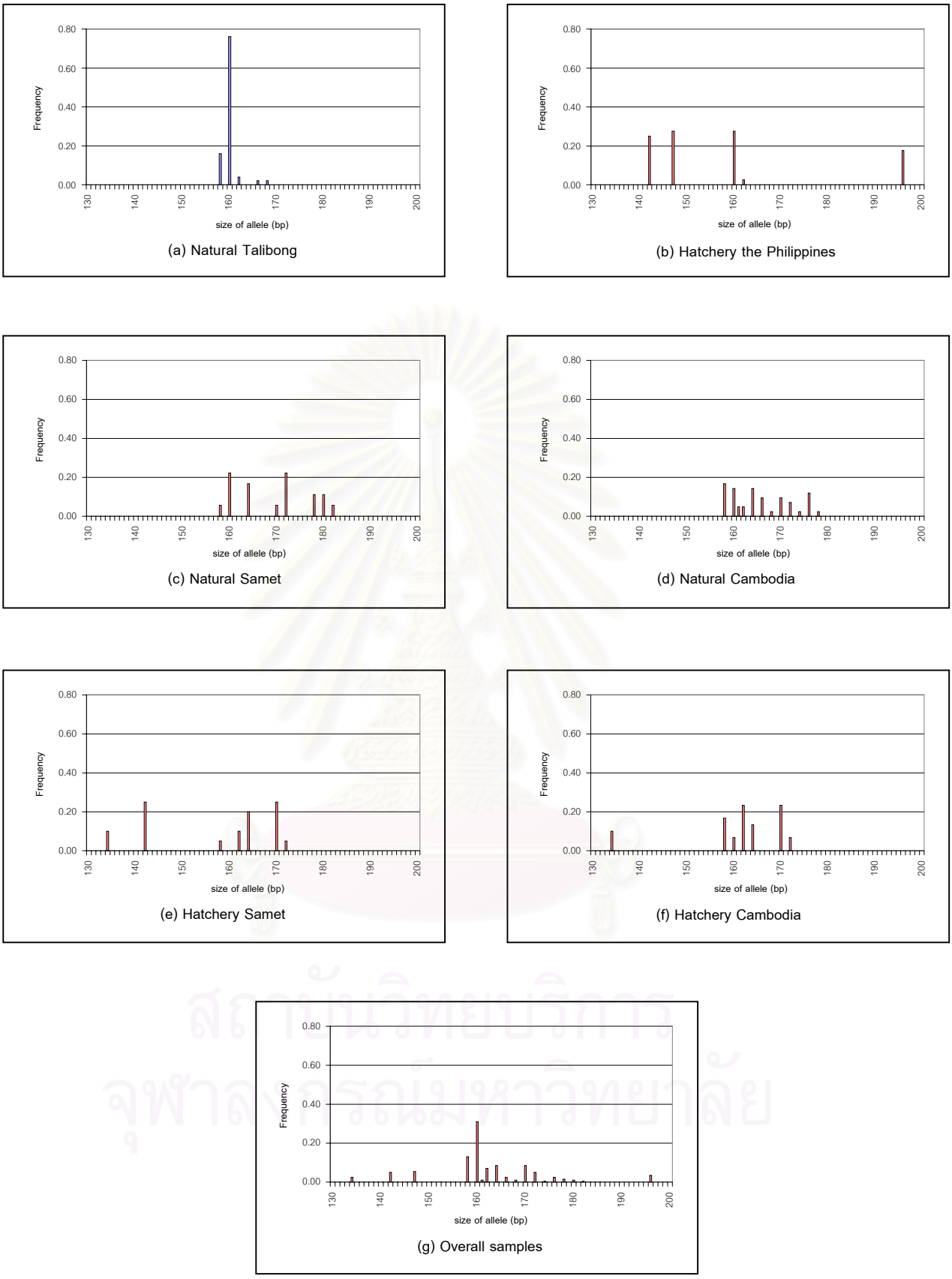

Figure 3.15 Allele distribution frequencies at the Has3 locus of *H. asinina* from Talibong (NL, $N = 25$), Hatchery The Philippines (HP, $N = 20$), Samet (NS, $N = 9$), Cambodia (NC, $N = 21$), Hatchery Samet (HS, $N = 10$), Hatchery Cambodia (HC, $N = 15$) and overall samples ($N = 100$)

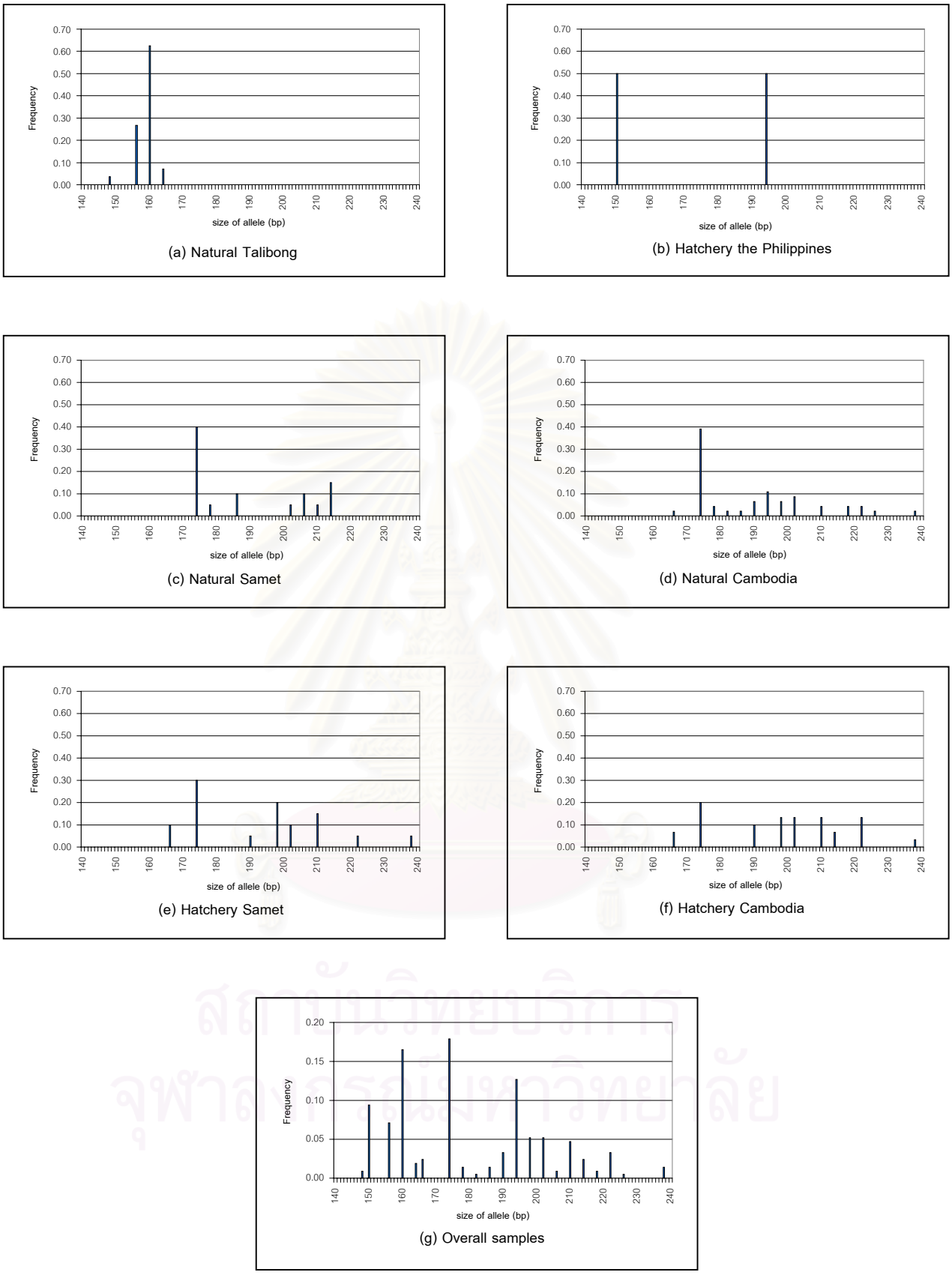

Figure 3.16 Alleles distribution frequencies at the Has8 locus of *H. asinina* from Talibong (NL, $N = 28$), Hatchery The Philippines (HP, $N = 20$), Samet (NS, $N = 10$), Cambodia (NC, $N = 23$), Hatchery Samet (HS, $N = 10$), Hatchery Cambodia (HC, $N = 15$) and overall samples ($N = 95$)

3.7.3 Hardy-Weinberg equilibrium

Three of 18 possible tests (Has2 and Has3 of the Cambodian sample and Has8 of the Philippines sample) were significantly deviated from Hardy-Weinberg expectation ($p < 0.0001$) even though the sequential Bonferroni procedure was applied. Two of the remaining tests (Has2 of the Samet and the hatchery Cambodia) did not conform Hardy-Weinberg equilibrium at $p < 0.05$ but these deviations were not significant after adjusted with the Bonferroni procedure (Table 3.9).

3.7.4 Genetic differentiation within H. asinina

Geographic heterogeneity analysis of allele frequencies of geographically different samples of *H. asinina* showed the existence of genetic population differentiation in this species. Samples within the Gulf of Thailand (natural and hatchery of Samet and Cambodia) did not showed significant heterogeneity when compared ($p > 0.003$) (Table 3.10) suggesting that the gene pool of these samples was not reproductively isolated. In contrast, *H. asinina* from Talibong and the Philippines showed significant geographic heterogeneity with the Gulf of Thailand sample and between each other ($p < 0.0001$ for all three loci). This indicated that *H. asinina* is not a panmictic species but its gene pool was fragmented into 3 stocks (the Andaman Sea, the Gulf of Thailand and the Philippines).

The overall *F*-statstics of each microsatellite locus across overall samples also revealed significant genetic population differentiation of *H. asinina* in this study statistically ($p < 0.0001$ for all investigated loci, Table 3.12). Pairwise comparisons of F_{ST} analysis illustrated similar conclusions as geographic heterogeneity analysis ($p < 0.0027$ except between Talibong-Samet and Talibong-Cambodia, Table 3.11).

3.7.5 Genetic distance and construction of boostrapped tree

Low levels of genetic distance were observed between samples within the Gulf of Thailand (0.0113–0.0871) whereas greater genetic distance was observed between the Talibong and the Philippines samples and those of the Gulf of Thailand (0.1310-0.1556 and 0.1486-0.1716, respectively). A large genetic distance was also observed between Talibong and the Philippines samples. The lowest genetic distance was 0.0113 (hatchery Samet-hatchery Cambodia) while the highest genetic distance was 0.1717 (the Philippines-hatchery Samet) (Table 3.13). The topology of a consensus neighbor-joining tree was significantly supported statistically and provided patterns of differentiation as similar as geographic heterogeneity analysis and F_{ST} statistics.

Therefore, all investigated samples of *H. asinina* in this study could be allocated into three different stocks (populations) including A (the Gulf of Thailand), B (Talibong of the Andaman Sea) and C (the Philippines).

Table 3.7 Sample sizes, size ranges and polymorphism (number of allele per locus, number of effective alleles, and heterozygosity) of Has2, Has3 and Has8 on 6 geographic samples of *H. asinina*

Sample	Sample Size (n)	Size range (bp)	Number of alleles	n_e	Heterozygosity	
					observed	expected
Has2						
NL	23	286-336	17	11.53	0.87	0.91
NS	11	314-340	9	6.21	0.64	0.84
NC	19	310-340	10	8.80	0.47	0.89
HP	19	290-336	4	3.95	1.00	0.75
HS	9	322-340	8	7.65	0.67	0.87
HC	14	314-340	10	5.58	0.64	0.82
Mean	15.83	286-340	9.67	7.29	0.72	0.85
Has3						
NL	25	158-168	5	1.65	0.32	0.39
NS	9	158-182	8	6.23	0.89	0.84
NC	21	158-178	12	8.90	0.62	0.89
HP	20	142-196	5	4.08	1.00	0.76
HS	10	134-172	7	5.26	1.00	0.81
HC	15	134-172	7	5.78	0.87	0.83
Mean	16.67	134-196	7.33	5.32	0.78	0.78
Has8						
NL	28	148-164	4	2.13	0.54	0.53
NS	10	174-214	8	4.76	0.80	0.79
NC	23	166-238	14	5.25	0.78	0.83
HP	20	150-194	2	2.00	0.00	0.50
HS	10	166-238	8	5.56	0.80	0.82
HC	15	166-238	9	7.64	0.87	0.87
Mean	17.67	148-238	7.50	4.56	0.63	0.72

Table 3.8 Number of alleles per locus, effective number of alleles and heterozygosity of each geographic sample averaged over 3 loci (Has2, Has3 and Has8)

Population	Mean no. of alleles per locus	Effective no. of alleles (n_e)	Mean heterozygosity	
			observed ($h_{obs \pm SD}$)	expected ($h_{exp \pm SD}$)
NL	8.67 ± 7.234	5.10	0.58 ± 0.277	0.61 ± 0.27
NS	8.33 ± 0.577	5.73	0.78 ± 0.127	0.82 ± 0.03
NC	12.00 ± 2.000	7.65	0.62 ± 0.155	0.87 ± 0.03
HP	3.67 ± 1.528	3.34	0.67 ± 0.577	0.67 ± 0.15
HS	7.67 ± 0.577	6.16	0.82 ± 0.166	0.83 ± 0.03
HC	8.67 ± 1.528	6.33	0.79 ± 0.133	0.84 ± 0.03

Table 3.9 Hardy-Weinberg expectations in each conspecific *H. asinina* samples at microsatellite loci Has2, Has3 and Has8

Population	P-value*		
	Has2	Has3	Has8
NL	0.1497^{ns}	0.0960^{ns}	0.3759^{ns}
NS	0.0102^{ns}	0.6599^{ns}	0.6267^{ns}
NC	<0.0001	<0.0001	0.3726^{ns}
HP	$1.0000^{ns\ \#}$	$1.0000^{ns\ \#}$	<0.0001
HS	0.0904^{ns}	$1.0000^{ns\ \#}$	0.2456^{ns}
HC	0.0227^{ns}	0.6827^{ns}	0.4427^{ns}

*Significant level was adjusted to $p < 0.0027$ using a sequential Bonferroni technique.
# test for heterozygote excess
ns = not significant

Table 3.10 Geographic heterogeneity analysis of six conspecific samples of *H. asinina* based on three microsatellite loci (Has2, Has3 and Has8)

Population	P-value*		
	Has2	Has3	Has8
NL-NS	0.0009	<0.0001	<0.001
NL-NC	<0.0001	<0.0001	<0.0001
NL-HP	<0.0001	<0.0001	<0.0001
NL-HS	<0.0001	<0.0001	<0.0001
NL-HC	<0.0001	<0.0001	<0.0001
HP-NS	<0.0001	<0.0001	<0.0001
HP-NC	<0.0001	<0.0001	<0.0001
HP-HS	<0.0001	<0.0001	<0.0001
HP-HC	<0.0001	<0.0001	<0.0001
NS-NC	0.2709^{ns}	0.1080^{ns}	0.2206^{ns}
NS-HS	0.0100^{ns}	0.0258^{ns}	0.0188^{ns}
NS-HC	0.0310^{ns}	0.0030^{ns}	0.0093^{ns}
NC-HS	0.0362^{ns}	0.2380^{ns}	0.5050^{ns}
NC-HC	0.0190^{ns}	0.0216^{ns}	0.1098^{ns}
HC-HS	0.9746^{ns}	0.9316^{ns}	0.9273^{ns}

*Significant level was further adjusted to $p < 0.0027$ using a sequential Bonferroni technique.
ns = not significant

Table 3.11 *F*-statistics resulted from analysis of 6 *H. asinina* samples with 3 microsatellites (Has2, Has3 and Has8)

Population	Has2		Has3		Has8	
	F_{ST}	P-value*	F_{ST}	P-value*	F_{ST}	P-value*
NL-NS	0.0471	0.0046^{ns}	0.2569	0.0002	0.3453	< 0.0001
NL-NC	0.0346	0.0043^{ns}	0.2402	< 0.0001	0.3205	< 0.0001
NL-HP	0.1187	< 0.0001	0.2648	< 0.0001	0.4672	< 0.0001
NL-HS	0.0756	< 0.0001	0.3596	< 0.0001	0.3265	< 0.0001
NL-HC	0.0618	< 0.0001	0.3336	< 0.0001	0.2983	< 0.0001
HP-NS	0.1752	< 0.0001	0.1260	0.0002	0.3036	0.0005
HP-NC	0.1340	< 0.0001	0.1213	< 0.0001	0.2810	< 0.0001
HP-HC	0.1259	< 0.0001	0.1706	< 0.0001	0.2943	< 0.0001
HP-HS	0.1491	< 0.0001	0.1844	< 0.0001	0.3204	0.0002
NS-NC	-0.0145	0.7039^{ns}	0.0036	0.3831^{ns}	-0.0047	0.4918^{ns}
NS-HS	0.0536	0.0422^{ns}	0.0552	0.0268^{ns}	0.0266	0.1567^{ns}
NS-HC	0.0333	0.0955^{ns}	0.0593	0.0140^{ns}	0.0385	0.0657^{ns}
NC-HS	0.0205	0.1883^{ns}	0.0031	0.3769^{ns}	-0.0010	0.4265^{ns}
NC-HC	0.0169	0.1734^{ns}	0.0198	0.1074^{ns}	0.0200	0.0934^{ns}
HC-HS	-0.0456	0.9623^{ns}	-0.0211	0.8258^{ns}	-0.0270	0.9062^{ns}

*Significant level was further adjusted to p< 0.0027 using a sequential Bonferroni technique.
ns = not significant

Table 3.12 Average *F*-statistics from analysis of 6 geographic samples of *H. asinina* with 3 microsatellite loci (Has2, Has3 and Has8)

Locus	F_{ST}	P-value*
Has2	0.0738	< 0.0001
Has3	0.1669	< 0.0001
Has8	0.2535	< 0.0001
Overall	0.1647	-

*Significant level was further adjusted to p < 0.0027using a sequential Bonferroni procedure

Table 3.13 Pairwise comparisons of Cavalli-Sforza and Edwards chord distance between pairs of *H. asinina* using three microsatellite loci (Has2, Has3 and Has8)

	NL	NS	HP	NC	HC	HS
NL	-					
NS	0.1393	-				
HP	0.1486	0.1649	-			
NC	0.1310	0.0578	0.1564	-		
HC	0.1494	0.0767	0.1670	0.0531	-	
HS	0.1556	0.0871	0.1716	0.0521	0.0113	-

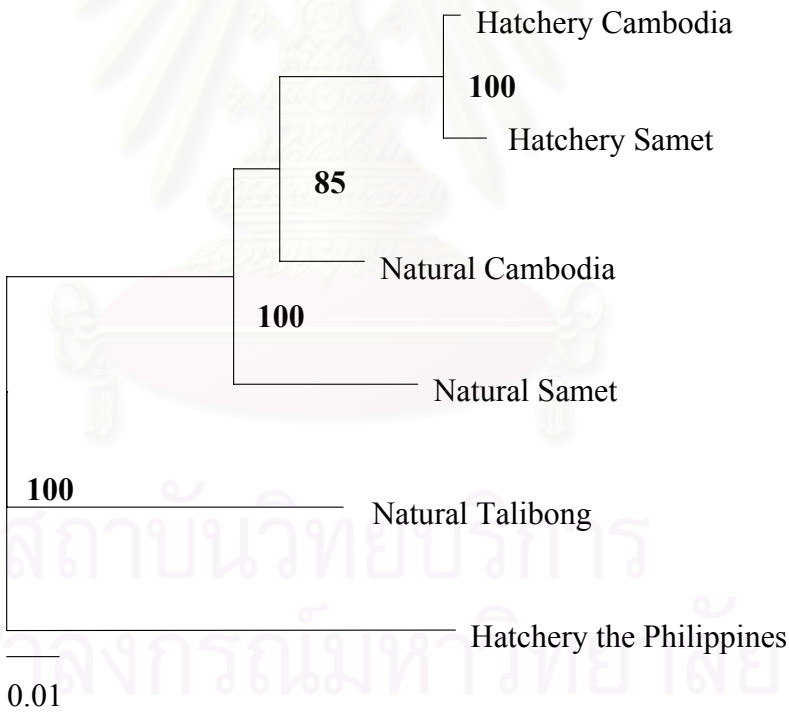

Figure 3.17 A consensus neighbor-joining tree illustrating relationships among six geographic samples of *H. asinina* based on Cavalli-Sforza and Edwards chord distance. Original data was bootstrapped 2000 times. The numbers at the nodes indicate the percentage of bootstrapped values for clustered samples.

Chapter IV

Discussion

Microsatellite polymorphism is a powerful tool for molecular genetic studies of various organisms for example, determination of kinship and clonal identity, analysis of individuality and parentage, identification of stock composition and management units and construction of linkage maps and genome mapping. Highly polymorphic genetic markers such as microsatellites can be used to assist breeding programs of *H. asinina* because the genotype of each individual can be readily determined eliminating problems from traditional selective breeding programs in which offspring of different family lines need to be cultured separately.

The quality of genomic DNA extracted from the foot tissue of *H. asinina* using a proteinase K-phenol-chloroform extraction method was high and suitable for library construction. Among three partial genomic libraries constructed, the mixed enzyme library gave the highest number of positive microsatellite clones. Combination of restriction enzyme digestion caused more efficiency on releasing of microsatellite fragments. Generally, problems from obtaining low numbers of positive microsatellite clones are resolved by enrichment of genomic DNA fragments containing a motif of choices (Rexroad et al., 2002).

Microsatellites have been isolated and characterized in a number of abalone species. Huang and Hanna (1998) isolated three microsatellites; RUBGT1 containing (GT)n repeats, RUBCA1 containing (CA)n repeats and RUBGACA1 containing (GACA)n repeats, from screening of RAPD products and a genomic library of *H. rubra*. Additionally, the first microsatellite in the California red abalone (*H.*

rufescens) was also reported from screening of a size-selected genomic library with dinucleotide and trinucleotide repeats (Kirby et al., 1998).

The $(GT)_n$ and $(CT)_n$ repeats have been reported as the most abundant microsatellites in fish (O'Reilly and Wright, 1995). The former was also highly abundant in the black tiger shrimp, *Penaeus monodon* (Tiptawonnukul, 1996). Therefore, these repeats were used as the DNA probe for screening of microsatellites in this study. A lower level of $(CT)_n$ than $(GT)_n$ microsatellites was observed in *H. asinina* genome. Nevertheless, both types of microsatellites in *H. asinina* were still less abundant than those in *P. monodon*.

Although DNA extraction based on a phenol/chloroform method provided high quality genomic DNA in *H. asinina*, this method was laborious and time-consuming particularly when dealing with large numbers of specimens and investigated loci. As a result, a Chelex-based DNA extraction method was applied to specimens used for population genetic studies of *H. asinina*. Basically, only 1 μl of 300-350 μl of the obtained DNA solution (approximately 150 ng of DNA) was enough for genotyping of each specimen by PCR.

Analysis of polymorphism at 10 successfully isolated microsatellite loci (Has1-10) revealed low polymorphic levels at 4 loci (Has4, Has6, Has7 and Has10). Has6 and Has7 could only amplified microsatellites in specimens originating from the Gulf of Thailand but not the Andaman (Talibong) sample while the Has4 locus showed low sensitivity of amplification and detection. Presumably, this should have resulted from mutations at the priming sites of microsatellites in specimens from Talibong (Andaman).

The remaining microsatellites (Has1, Has2, Has3, Has5, Has8 and Has9) were highly polymorphic. However, the Has1 locus contained a combination of 2 microsatellite motifs. This microsatellite can be used for parental analysis but may not be suitable for population genetic studies of *H. asinina* because convergence of alleles (identical sizes of alleles resulting from length variation of different motifs) may be occurred. The Has5 locus exhibited a large number of alleles but a low level of heterozygosity. This may have resulted from severe occurrence of null alleles. Only Has2, Has3 and Has8 were chosen for population genetic studies of *H. asinina* in details.

The occurrence of null alleles is a serious problem in population genetic studies, segregation analysis and parental analysis. Holm et al. (2001) elucidated the molecular basis of a null allele at an Omy2DIAS microsatellite in the rainbow trout (*Oncorhynchus mykiss*). The presence of a null allele was discovered by analysis of pedigree samples. Sequencing of the null allele amplified by new primers located further from the repeat sequence indicated that a deletion of a 4-5 bp sequence within one of the primer recognition sites caused that circumstance. Therefore, three microsatellite loci (Has2, Has3 and Has8) used in this study should be further analyzed using family materials to ensure the lack of null alleles at these loci.

The sensitivity test using different concentration of DNA template indicated that genotyping of *H. asinina* can be performed using the amount of DNA template as little as 50 pg. Amplification of DNA from 2 week-old (spat) specimens isolated by a Chelex-based method was consistently successful indicating that microsatellite analysis could also be carried out in *H. asinina* larvae. Therefore, correlation between

survival rate and settlement efficiency of abalone larvae and their genotypes can be readily determined.

Hatchery specimens used in this study were established using mass spawning approach. Following this breeding approach, the percentage of polyspermy of *H. asinina* can be evaluated at the early stages of development. Moreover, the possible preferential intraspecific fertilization among different stocks of *H. asinina* can also be examined. Huvet et al.(2001) used microsatellites to examine intraspecific and interspecific fertilization of *Crassostrea gigas* and C. *angulata* and showed no evidence of preferential fertilization between gametes of the same species. Nevertheless, a significant higher contribution of the *C. gigas* males was revealed with the *C. angulata* females but not with the *C. gigas* females. Therefore, the possibility that early heterosis may be occurred between these taxa was illustrated before a large scale breeding programs is performed.

Selvamani et al. (2000) isolated and characterized 11 microsatellites in *H. asinina* from Australia. Five microsatellites composing of 2 highly polymorphic loci (Haμ13 and Haμ2k) and 3 moderately polymorphic loci (Haμ10, Haμ2J and Haμ3K) were used for genotyping of individual abalone larvae produced by 3 separate crosses. The parents of an individual veliger could be precisely determined from only 3 loci.

Considering heterozygosity of 3 microsatellite loci in this study, all loci provided high polymorphic levels in wild (NL, NC and NS) and hatchery (HC, HP and HS) samples. The lowest heterozygosity was observed in the Talibong sample whereas the highest were observed in P_0 of the hatchery stock originating from the Cambodia (HS). Apparently, high heterozygosity was found in all hatchery samples suggesting that founder individuals used to establish those stocks were large.

Positive correlation between numbers of alleles and effective allele per locus and mean heterozygosity was observed in all investigated samples except the Philippines. This F1 sample showed the lowest mean number of alleles (3.67) and effective alleles (3.34) but exhibited greater heterozygosity (0.67) than did Talibong (0.58) and Cambodia (0.62). Therefore, heterozygote advantages and fitness of different microsatellite alleles may be occurred in this stock.

Linkage disequilibrium of Has2, Has3 and Has8 microsatellites was not found across overall investigated samples. Conformation of Hardy-Weinberg equilibrium was found in all geographic samples except the NC (Has2 and Has3) and the Philippines (Has8) samples. Using microsatellite polymorphism, deviation from Hardy-Weinberg expectations was reported in all previously studied abalone including *H. rubra* (Huang et al., 2000), *H. kamtschatkana* (Miller et al., 2001) and *H. discus discus* (Sekino and Hara, 2002).

Huang et al. (2000) examined genetic population structure of *H. rubra* using RAPD, minisatellites (GHR and MIPR) and microsatellites (RUBGT1, RUBCT1 and RUBGACA1). Significant deviation from Hardy-Weinberg equilibrium due to homozygote excess was observed at all microsatellite loci across all geographic samples examined but minisatellites did not showed disequilibrium of that parameter.

Huang and Hanna (1998) reported possible applications of microsatellites (RUBGT1, RUBCT1 and RUBGACA1) developed from *H. rubra* for genetic analysis of a non-target species, *H. conicopore*. Nevertheless, deviation from the expected equilibrium of microsatellites in the target species, *H. rubra* indicated that genetic analysis of non-target abalone species with these microsatellites may provide serious errors of results. In the present study, cross amplification of Has2, Has3 and Has8

developed from *H. asinina* agsinst DNA template of *H. ovina* and *H. varia* was not successful implying large genetic differences between target and non-target abalone species.

Interspecific hybridization between *H. rubra* and *H. laevigata* and introgression of gene of each species into the gene pool of the other was reported using allozyme analysis (Brown, 1991). Moreover, morphological characters evidenced the existence of interspecific hybridization of the commercial catch of the California abalone (Leighton and Lewis, 1982). In addition, hybrids of the California abalone could be produced in laboratory. Accordingly, natural hybridization between different abalone in this study was suspected but no evidences are available at present. The inability to cross-amplify microsatellites in *H. ovina* and *H. varia* indicated that this genetic marker cannot be applied for identification of hybridization and introgression.

Intraspecific genetic differentiation was obviously observed in *H. asinina* across overall loci ($p < 0.0027$). Heterogeneity of the gene pool was not found in different samples within the Gulf of Thailand ($p > 0.0027$). Comparing the Gulf samples with Talibong and Philippines revealed significant genetic differentiation between samples from these three regions ($p < 0.0001$). Phylogenetic analysis between genetic distance between pairs of geographic samples also supported this conclusion. Based on microsatellite polymorphism in this study, the gene pool of *H. asinina* was not panmictic but divided to 3 stocks; the Gulf of Thailand samples, Talibong (the Andaman Sea) and the Philippines sample.

Analysis of genetic differentiation of *H. asinina* in Thai waters by RAPD analysis (OPB11, UBC101, UBC195, UBC197 and UBC271) revealed concordance

results as did microsatellites (Popongviwat, 2001). In contrast, analysis of population differentiation using 16S rDNA revealed a lack of heterogeneity in these species (Pripue, 2001). Including the 18S rDNA into the analysis resulted in an ability to differentiate samples from the Philippines and the remaining locations. Strong genetic differentiation between coastal regions revealed by microsatellites and RAPD analysis but not mtDNA analysis (16S rDNA) should have suggested the biased high female gene flow level in *H. asinina*. Considering life history and larval development of this species, this should not be the case owing to short larval stages and limited ability for migration of *H. asinina* after metamorphosis. As a result, nonequivalent survival rates between genders may have significantly occurred in this species and affected contradictory population subdivision patterns when examined by nuclear (RAPD and microsatellites) and mtDNA (16S rDNA) markers.

Typically, distribution of allele sizes of microsatellites is partially overlapped between conspecific populations of a particular species for example; those in the black tiger shrimp, *P. monodon* (Supungul et al., 2000) and the honey bee, *Apis cerana* (Sittipraneed et al., 2001). Therefore, microsatellites cannot be used as population (or region)-specific markers in various species. Apparently, Has9 revealed non-overlapping allele distributions of specimens originating from the Andaman Sea (NL, 148 bp – 162 bp) and the Gulf of Thailand (NC, 184 bp – 240 bp) indicating the potential of this locus to be used for identification of coastal origins of *H. asinina* in Thai waters. Nevertheless, larger numbers of specimens from additional sites of each coastal region should be genetically examined before drawing an unambiguous conclusion.

The ability to identify population differentiation within a commercially important species is crucial for broodstock management and conservation programs (Holm et al., 2001). In this study, genetic compositions of wild and artificially propagated stocks of *H. asinina* were examined. The findings suggested that different stocks of *H. asinina* should be managed separately. In terms of aquaculture, selective breeding programs of *H. asinina* can be carried out more efficiently by incorporation of microsatellites. Offspring from multiple parents can be reared together allowing a better scrutiny of the genetic effects on each interested phenotype by reducing the confounding effects of environments.

Microsatellite analysis is a powerful and promising approach for evaluation of genetic diversity and differentiation in various taxa (O'Reilly and Wright, 1995). This approach allows several applications in population genetics, breeding programs and aquaculture. Recently, genetic variation and differentiation of five geographic samples of *P. monodon* (Chumphon, Trad, Phangnga, Satun and Trang) in Thailand were reported using 5 microsatellite loci (CUPmo18, Di25, Di27, CUCSPmo1 and CUCSPmo2). The average heterozygosity across all investigated samples was 0.78 indicating high genetic diversity in this species. Geographic heterogeneity analysis of CUPmo18 and Di25 showed significant differences between *P. monodon* from different coastal regions and within the Gulf of Thailand (Trad and Chumphon) but not within the Andaman samples (Supungul et al., 2000). Likewise, intraspecific population differentiation was also observed in *H. asinina*.

Microsatellites play an important role in several aspects of population genetics. Disregarding the Philippines sample where selection of the F1 stock may have occurred, high genetic diversity and strong population genetic structure between *H.*

asinina originating from the west and east coasts of peninsular Thailand were shown based on 3 microsatellite loci (Has2, Has3 and Has8). The present study also showed that microsatellites can be further used to assist genetic improvement and breeding programs of *H. asinina* (e.g., selection of fast-growing and disease-resistant families in the commercial setting conditions, determination of correlation between genotypes and survival rate after settlement of larvae, correlation between growth rate and levels of heterozygosity and dispersing ability of *H. asinina*).

Chapter V

Conclusions

1. Isolation of microsatellites from 3 genomic libraries, *Alu* I library, V/S library and mixed-enzymed library showed that the mix-enzymed library gave the highest yield of positive clones. Dot blot hybridization revealed that $(GT)_n$ microsatellites are more abundant than $(CT)_n$ in *H. asinina* genome. Nucleotide sequencing data showed the same proportion of perfect, imperfect and compound microsatellites.
2. Ten pairs of microsatellite primers were developed and tested for polymorphism analysis. Six microsatellite loci (Has1, Has2, Has3, Has5, Has8 and Has9) showed high level of polymorphism. Two primer pairs of Has6 and Has7 could not amplify samples from Talibong. All primers could not be used for cross-species amplification with the other two tropical abalone species *H. ovina* and *H. varia*
3. Genotypic disequilibrium analysis at all loci (except Has6 and Has7) indicated that genotype from these microsatellites were associated randomly.
4. Sensitivity test showed that the least amount of DNA that could be used for genotyping was about 50 pg; and 2 week-old *H. asinina* could be used for genotyping.
5. Genetic variation of *H. asinina* population determined by three microsatellite loci Has2, Has3 and Has8 indicated that natural populations from the Gulf of Thailand (NC and NS) have higher genetic variation than that of the Andaman Sea (NL).
6. Analysis of gene frequencies investigated at Has2, Has3 and Has8 loci revealed that all populations conform Hardy-Weinberg equilibrium except natural population from Cambodia (at Has2 and Has3 loci) and the Philippines samples (at Has8 locus).

7. Analysis of geographic heterogeneity and phylogenetic reconstruction using the Neighbor-joining approach divided 6 geographic *H. asinina* samples to three different gene pools (stocks) consisting of A (the Gulf of Thailand), B (the Andaman Sea) and C (the Philippines).

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Appendices

Appendix A

Stock solutions for colony hybridization:

50X Denhardt's solution

Ficoll type 400	10 g/l
Polyvinylpyrrolidone	10 g/l
Bovine Serum Albumin (Fraction V)	10 g/l

Dissolve all reagents in deionized water, filter through a 0.22 μm membrane and store at -20°C

20X SSC

3 M NaCl	175.3 g/l
0.3 M $Na_3C_6H_5O_7.2H_2O$	88.2 g/l

Dissolve NaCl and trisodium citrate $2H_2O$ in deionized water and adjust pH to 7.0 with a few drop of HCl. Sterilize by autoclaving and store a room temperature.

20X SSPE, pH 7.4

3 M NaCl	175.3 g/l
0.2 M $NaH_2PO_4H_2O$	27.6 g/l
0.02 M EDTA	7.4 g/l

Dissolve NaCl, sodium phosphate and EDTA in deionized water and adjust pH to 7.4 with NaOH (~ 6.5 ml of a 10 N NaOH), Dispense into aliquots, sterilize by autoclaving and stored at room temperature.

T7 Sequencing™ Kit

'A' Mix-Short:	840 μM each dCTP, dGTP and TTP; 93.5 μM dATP; 14 μM ddATP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.
'C' Mix-Short:	840 μM each dATP, dGTP and TTP; 93.5 μM dCTP; 14 μM ddCTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.
'G' Mix-Short:	840 μM each dATP, dCTP and TTP; 93.5 μM dGTP; 14 μM ddGTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.
'T' Mix-Short:	840 μM each dATP, dCTP and GTP; 93.5 μM dTTP; 14 μM ddTTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.
T7 DNA polymerase:	8 units/μl buffered glycerol solution.
Enzyme Dilution Buffer:	20 mM Tris-HCl (pH 7.5), 5 mM DTT, 100 μg BSA/ml and 5% glycerol.
Universal Primer:	5'-d[GTAAACGACGGCCAGT]-3' in aqueous solution, 0.86 A_{260} units/ml (5 pmol/μl).
Annealing Buffers:	1 M Tris-HCl (pH 7.6), 100 mM $MgCl_2$ and 160 mM DTT.
Labelling Mix-dATP:	1.375 mM each dCTP, dGTP and dTTP and 333.5 mM NaCl.
Stop Solution:	0.3% each Bromophenol Blue and Xylene Cyanol FF, 10 mM EDTA (pH 7.5) and 97.5% deionized formamide.
Control Template:	10 μg of singlep-stranded M13mp18 DNA in 50 μl of Tris-EDTA buffer.

Appendix B

Microsatellite patterns and polymorphism at various polymorphic loci

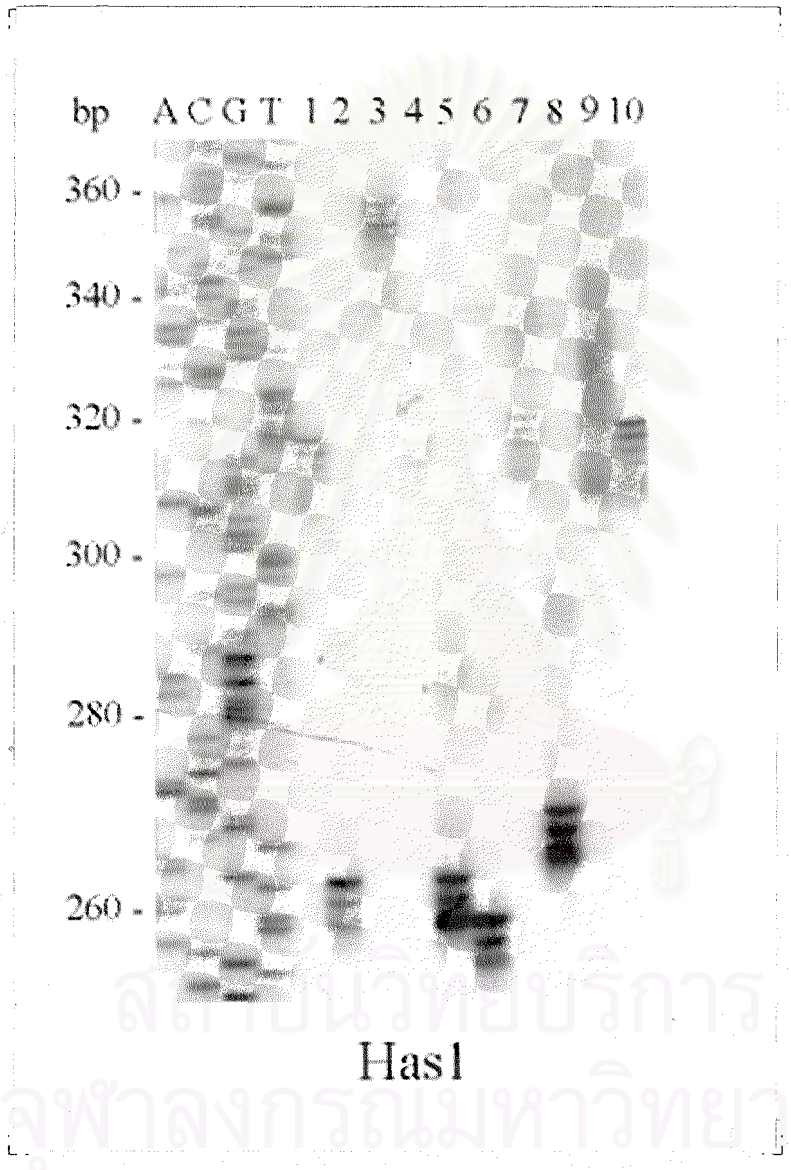


Figure B1 Microsatellite patterns resulted from analysis of 10 *H. asinina* individuals at the locus Has1 (lanes 1-10). The size standard is a sequencing ladder of M13 mp 18.

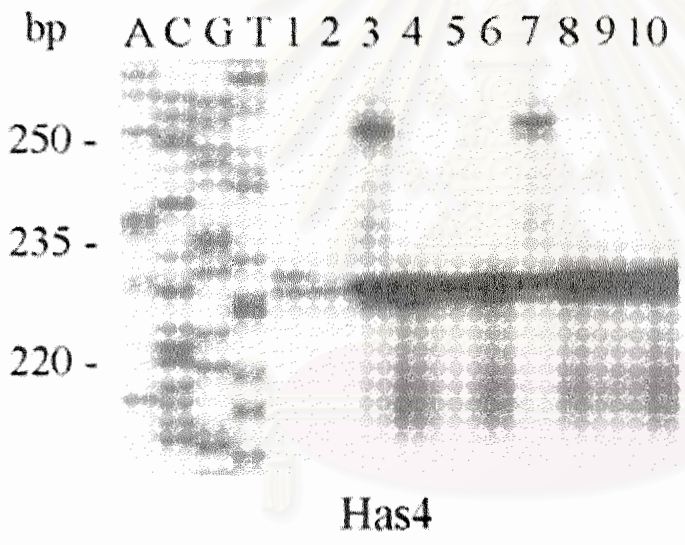


Figure B2 Microsatellite patterns resulted from analysis of 10 *H. asinina* individuals at the locus Has4 (lanes 1-10). The size standard is a sequencing ladder of M13 mp 18.

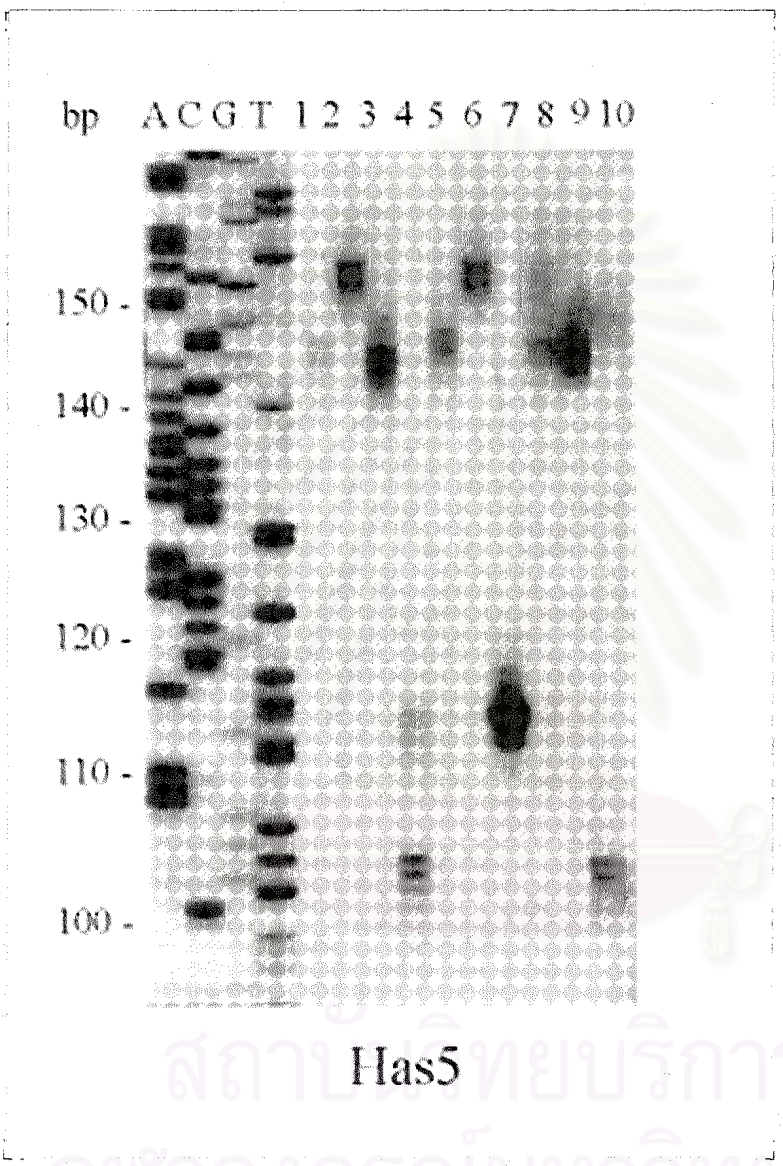


Figure B3 Microsatellite patterns resulted from analysis of 10 *H. asinina* individuals at the locus Has5 (lanes 1-10). The size standard is a sequencing ladder of M13 mp 18.

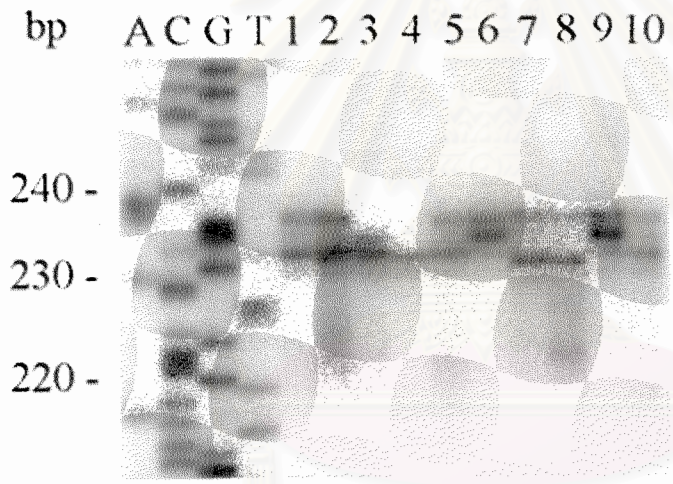


Figure B4 Microsatellite patterns resulted from analysis of 10 *H. asinina* individuals of the NC sample at the locus Has6 (lanes 1-10). The size standard is a sequencing ladder of M13 mp 18.

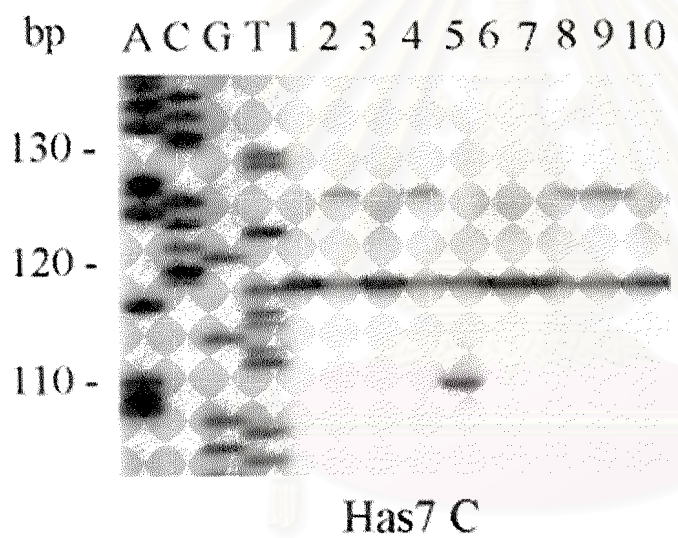
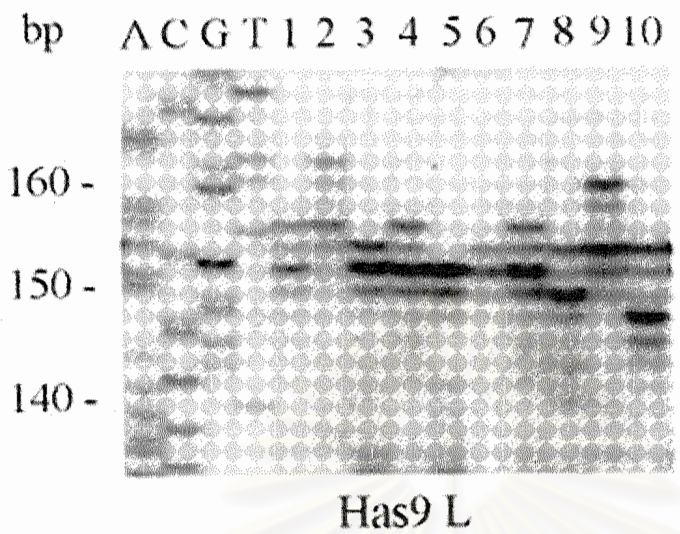


Figure B5 Microsatellite patterns resulted from analysis of 10 *H. asinina* individuals of the NC sample at the locus Has7 (lanes 1-10). The size standard is a sequencing ladder of M13 mp 18.

(a)



(b)

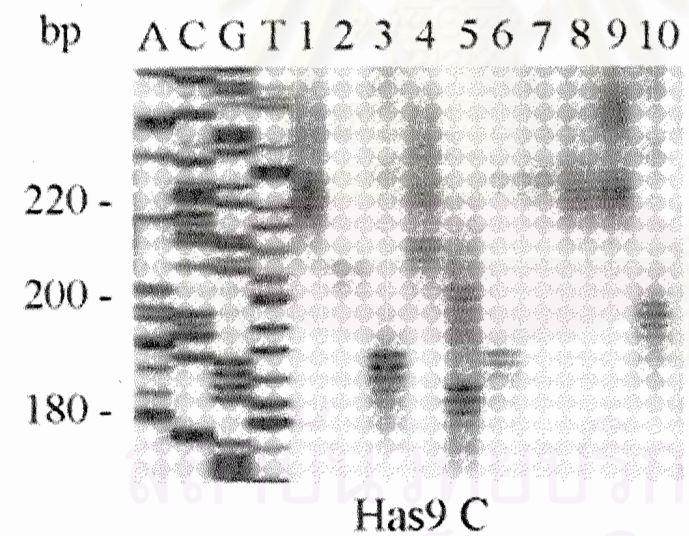
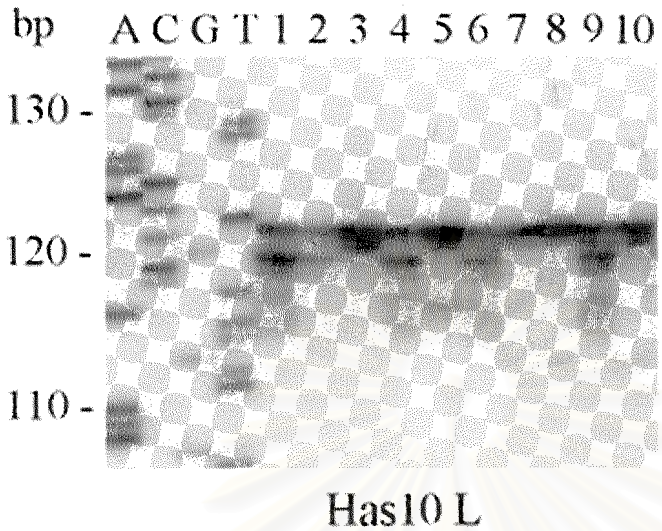


Figure B6 Microsatellite patterns resulted from analysis of 10 *H. asinina* individuals at the locus 9 (lanes 1-10) of the NL sample (a) and the NC sample (b). The size standard is a sequencing ladder of M13 mp 18.

(a)



(b)

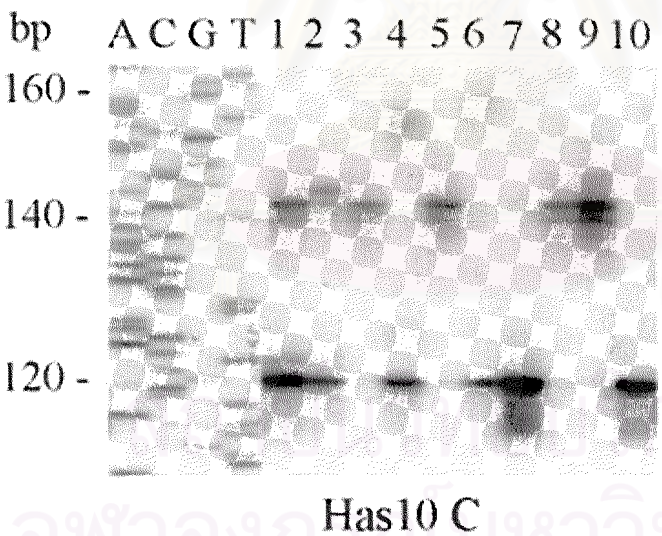


Figure B7 Microsatellite patterns resulted from analysis of 10 *H. asinina* individuals at the locus Has10 (lanes 1-10) of the NL sample (a) and the NC sample (b). The size standard is a sequencing ladder of M13 mp 18.

Table B1 Sizes of alleles (in base pairs) at the Has1 locus and its frequency distribution in four geographic samples of *H. asinina*

Allele (bp)	Frequency distribution				
	NL (*N*=28)	NC (*N*=19)	HS (*N*=10)	HC (*N*=15)	Overall (*N*=72)
258	-	0.026	-	-	0.007
260	-	0.053	-	-	0.014
262	-	0.053	0.150	0.100	0.056
266	-	0.026	-	-	0.007
268	-	0.053	-	-	0.014
272	-	0.026	0.050	0.033	0.021
316	-	0.158	0.250	0.300	0.139
318	-	-	-	0.033	0.007
322	-	0.026	-	-	0.007
324	-	0.026	-	-	0.007
326	-	0.053	0.150	0.133	0.063
328	-	0.132	0.050	0.067	0.056
332	-	0.053	-	-	0.014
334	0.018	0.053	0.100	0.067	0.049
336	-	0.105	0.200	0.167	0.090
338	-	0.026	-	-	0.007
340	-	0.079	-	-	0.021
344	0.036	0.026	-	-	0.021
346	0.036	-	-	0.067	0.028
348	0.089	-	-	-	0.035
350	0.036	0.026	-	-	0.021
352	0.161	-	-	-	0.063
354	0.143	-	-	-	0.056
356	0.339	-	-	-	0.132
358	0.071	-	0.050	0.033	0.042
360	0.071	-	-	-	0.028
Number of alleles	9	18	8	10	26
Number of effective alleles	5.45	12.21	6.06	6.16	13.93
Observed heterozygosity	0.86	0.84	0.80	0.87	0.85
Expected heterozygosity	0.82	0.92	0.84	0.84	0.93

N = Number of sample examined

Table B2 Sizes of alleles (in base pairs) at the Has2 locus and its frequency distribution in seven geographic samples of *H. asinina*

Allele (bp)	Frequency distribution						
	NL (*N*=23)	NS (*N*=11)	NC (*N*=19)	NP (*N*=19)	HS (*N*=10)	HC (*N*=15)	Overall (*N*=95)
286	0.022	-	-	-	-	-	0.005
290	-	-	-	0.289	-	-	0.058
300	0.022	-	-	-	-	-	0.005
302	0.043	-	-	-	-	-	0.011
304	0.152	-	-	-	-	-	0.037
306	0.065	-	-	0.263	-	-	0.068
308	0.022	-	-	-	-	-	0.005
310	0.022	-	0.053	-	-	-	0.016
312	0.022	-	-	-	-	-	0.005
314	0.087	0.136	-	-	-	0.036	0.042
316	0.152	0.045	-	-	-	-	0.042
318	0.065	-	-	-	-	-	0.016
320	0.043	-	0.026	-	-	-	0.016
322	0.065	0.045	0.105	0.211	0.167	0.179	0.126
324	0.087	-	0.132	-	-	-	0.047
326	0.065	0.273	0.184	-	0.056	0.071	0.100
328	-	0.182	0.105	-	0.111	0.071	0.063
330	-	-	-	-	0.222	0.214	0.053
332	0.043	0.091	0.132	-	0.056	0.036	0.058
334	-	0.045	0.079	-	0.278	0.179	0.074
336	0.022	-	0.026	0.237	0.056	0.071	0.074
338	-	0.136	0.079	-	-	0.107	0.047
340	-	0.045	0.079	-	0.056	0.036	0.032
Number of alleles	17	9	10	4	8	10	23
Number of effective alleles	11.53	6.21	8.80	3.95	5.58	7.13	15.09
Observed heterozygosity	0.87	0.64	0.45	1.00	0.67	0.64	0.74
Expected heterozygosity	0.91	0.84	0.89	0.75	0.82	0.86	0.93

N = Number of sample examined

Table B3 Sizes of alleles (in base pairs) at the Has3 locus and its frequency distribution in seven geographic samples of *H. asinina*

Allele (bp)	Frequency distribution						
	NL (*N*=25)	NS (*N*=9)	NC (*N*=21)	NP (*N*=20)	HS (*N*=10)	HC (*N*=15)	Overall (*N*=100
134	-	-	-	-	0.100	0.100	0.025
142	-	-	-	0.250	-	-	0.050
147	-	-	-	0.275	-	-	0.055
158	0.160	0.056	0.167	-	0.250	0.167	0.130
160	0.760	0.222	0.143	0.275	0.050	0.067	0.310
161	-	-	0.048	-	-	-	0.010
162	0.040	-	0.048	0.025	0.100	0.233	0.070
164	-	0.167	0.143	-	0.200	0.133	0.085
166	0.020	-	0.095	-	-	-	0.025
168	0.020	-	0.024	-	-	-	0.010
170	-	0.056	0.095	-	0.250	0.233	0.085
172	-	0.222	0.071	-	0.050	0.067	0.050
174	-	-	0.024	-	-	-	0.005
176	-	-	0.119	-	-	-	0.025
178	-	0.111	0.024	-	-	-	0.015
180	-	0.111	-	-	-	-	0.010
182	-	0.056	-	-	-	-	0.005
196	-	-	-	0.175	-	-	0.035
Number of alleles	5	8	12	5	7	7	18
Number of effective alleles	1.65	6.23	8.90	4.08	5.26	5.78	6.94
Observed heterozygosity	0.32	0.89	0.62	1.00	1.00	0.87	0.72
Expected heterozygosity	0.39	0.84	0.89	0.76	0.81	0.83	0.86

N = Number of sample examined

Table B4 Sizes of alleles (in base pairs) at the Has4 locus and its frequency distribution in four geographic samples of *H. asinina*

Allele (bp)	Frequency distribution				
	NL (*N*= 20)	NC (*N*= 22)	HS (*N*=10)	HC (*N*=15)	Overall (*N*=67)
222	0.450	-	-	0.600	0.134
224	0.225	-	-	-	0.067
228	0.325	0.864	0.550	-	0.597
230	-	0.045	0.100	0.067	0.045
250	-	0.091	0.350	0.333	0.157
Number of alleles	3	3	3	3	5
Number of effective alleles	2.79	1.32	2.30	2.10	2.47
Observed heterozygosity	0.5	0.27	0.50	0.40	0.40
Expected heterozygosity	0.64	0.24	0.57	0.52	0.59

N = Number of sample examined

Table B5 Sizes of alleles (in base pairs) at the Has5 locus and its frequency distribution in four geographic samples of *H. asinina.*

Allele (bp)	Frequency distribution				
	NL (*N*=28)	NC (*N*=20)	HS (*N*=10)	HC (*N*=14)	Overall (*N*=72)
104	-	-	0.100	0.107	0.035
108	0.071	-	-	-	0.028
109	0.482	-	-	-	0.188
110	0.304	-	-	-	0.118
112	0.071	-	-	-	0.028
113	0.018	-	-	-	0.007
114	0.018	0.175	0.150	0.214	0.118
115	-	0.050	-	-	0.014
116	0.036	-	-	-	0.014
117	-	-	-	0.036	0.007
118	-	0.025	-	-	0.007
119	-	0.025	-	-	0.007
145	-	0.025	0.100	0.107	0.042
146	-	-	0.250	0.179	0.069
147	-	0.100	0.100	0.071	0.056
149	-	0.125	0.050	0.036	0.049
150	-	0.050	-	-	0.014
151	-	0.100	-	-	0.028
152	-	0.025	-	-	0.007
154	-	0.150	0.250	0.179	0.111
155	-	0.125	-	-	0.035
162	-	0.025	-	-	0.007
173	-	-	-	0.071	0.014
Number of alleles	7	13	7	9	19
Number of effective alleles	2.97	8.89	5.56	6.88	10.70
Observed heterozygosity	0.46	0.20	0.30	0.36	0.35
Expected heterozygosity	0.66	0.89	0.82	0.85	0.91

N = Number of sample examined

Table B6 Sizes of alleles (in base pairs) at the Has6 locus and its frequency distribution in four geographic samples of *H. asinina*

Allele (bp)	Frequency distribution			
	NC (*N*=23)	HS (*N*=10)	HC (*N*=15)	Overall (*N*=18)
230	-	0.400	-	0.083
232	0.457	0.250	0.433	0.406
234	0.239	0.350	0.200	0.250
236	0.239	-	0.367	0.229
238	0.043	-	-	0.021
240	0.022	-	-	0.010
Number of alleles	5	3	3	6
Number of effective alleles	3.06	2.90	2.76	3.48
Observed heterozygosity	0.74	0.80	0.73	0.75
Expected heterozygosity	0.67	0.66	0.64	0.71

N = Number of sample examined

Table B7 Sizes of alleles (in base pairs) at the Has7 locus and its frequency distribution in four populations.

Allele (bp)	Frequency distribution			
	NC (*N*=23)	HS (*N*=10)	HC (*N*=15)	Overall (*N*=48)
112	-	0.050	0.033	0.021
118	0.935	0.800	0.800	0.865
126	0.065	0.150	0.167	0.115
Number of alleles	2	3	3	3
Number of effective alleles	1.50	1.49	1.14	1.31
Observed heterozygosity	0.13	0.40	0.40	0.27
Expected heterozygosity	0.34	0.33	0.12	0.24

N = Number of sample examined

Table B8 Sizes of alleles (in base pairs) at the Has8 locus and its frequency distribution in seven geographic samples of *H. asinina*

Allele (bp)	Frequency distribution						
	NL (*N*=28)	NS (*N*=10)	NC (*N*=23)	NP (*N*=20)	HS (*N*=10)	HC (*N*=15)	Overall *N*=106)
148	0.036	-	-	-	-	-	0.009
150	-	-	-	0.500	-	-	0.094
156	0.268	-	-	-	-	-	0.071
160	0.625	-	-	-	-	-	0.165
164	0.071	-	-	-	-	-	0.019
166	-	-	0.022	-	0.100	0.067	0.024
174	-	0.400	0.391	-	0.300	0.200	0.179
178	-	0.050	0.043	-	-	-	0.014
182	-	-	0.022	-	-	-	0.005
186	-	0.100	0.022	-	-	-	0.014
190	-	-	0.065	-	0.050	0.100	0.033
194	-	0.100	0.109	0.500	-	-	0.127
198	-	-	0.065	-	0.200	0.133	0.052
202	-	0.050	0.087	-	0.100	0.133	0.052
206	-	0.100	-	-	-	-	0.009
210	-	0.050	0.043	-	0.150	0.133	0.047
214	-	0.150	-	-	-	0.067	0.024
218	-	-	0.043	-	-	-	0.009
222	-	-	0.043	-	0.050	0.133	0.033
226	-	-	0.022	-	-	-	0.005
238	-	-	0.022	-	0.050	0.033	0.014
Number of alleles	4	8	14	2	8	9	21
Number of effective alleles	2.13	4.76	5.25	2.00	5.56	7.64	9.86
Observed heterozygosity	0.54	0.80	0.78	0.00	0.80	0.87	0.58
Expected heterozygosity	0.53	0.79	0.81	0.50	0.82	0.87	0.90

N = Number of sample examined

Table B9 Sizes of alleles (in base pairs) at the Has9 locus and its frequency distribution in two geographic samples of *H. asinina*

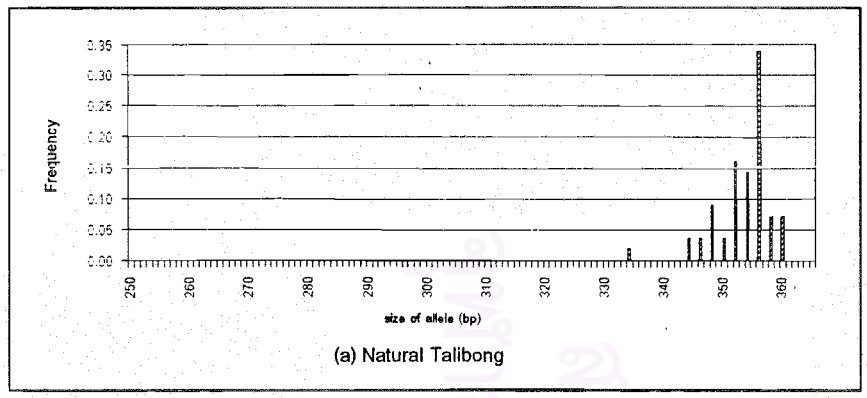
Allele (bp)	Frequency distribution		
	NL (*N*=28)	NC (*N*=20)	Overall (*N*=48)
148	0.143	-	0.083
150	0.018	-	0.010
152	0.286	-	0.167
154	0.268	-	0.156
156	0.161	-	0.094
158	0.054	-	0.031
160	0.018	-	0.010
162	0.054	-	0.031
184	-	0.025	0.010
190	-	0.050	0.021
192	-	0.050	0.021
198	-	0.050	0.021
200	-	0.025	0.010
206	-	0.075	0.031
210	-	0.025	0.010
212	-	0.025	0.010
214	-	0.075	0.031
216	-	0.050	0.021
218	-	0.025	0.010
220	-	0.075	0.031
222	-	0.025	0.010
224	-	0.150	0.063
226	-	0.075	0.031
228	-	0.125	0.052
234	-	0.050	0.021
240	-	0.025	0.010
Number of alleles	8	18	26
Number of effective alleles	4.84	12.90	11.98
Observed heterozygosity	0.86	0.75	0.81
Expected heterozygosity	0.79	0.92	0.92

N = Number of sample examined

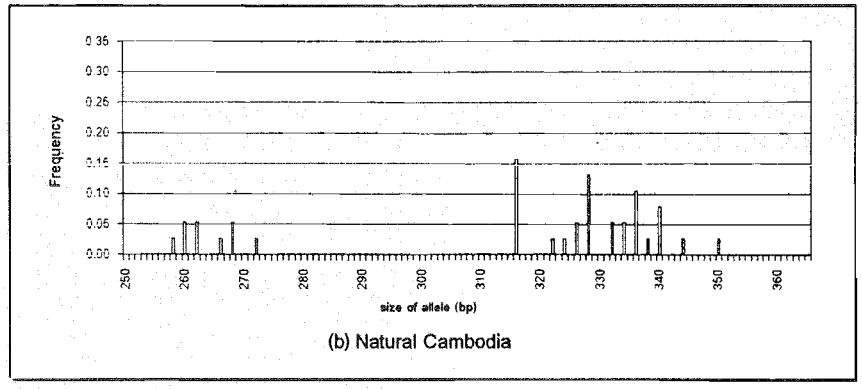
Table B10 Sizes of alleles (in base pairs) at the Has10 locus and its frequency distribution in two geographic samples of *H. asinina*

Allele (bp)	Frequency distribution		
	NL (*N*=28)	NC (*N*=20)	Overall (*N*=48)
118	0.339	-	0.198
120	0.661	0.375	0.542
140	-	0.025	0.010
142	-	0.450	0.188
144	-	0.025	0.010
148	-	0.025	0.010
154	-	0.025	0.010
156	-	0.050	0.021
160	-	0.025	0.010
Number of alleles	2	8	9
Number of effective alleles	1.81	2.87	2.71
Observed heterozygosity	0.39	0.45	0.42
Expected heterozygosity	0.45	0.65	0.63

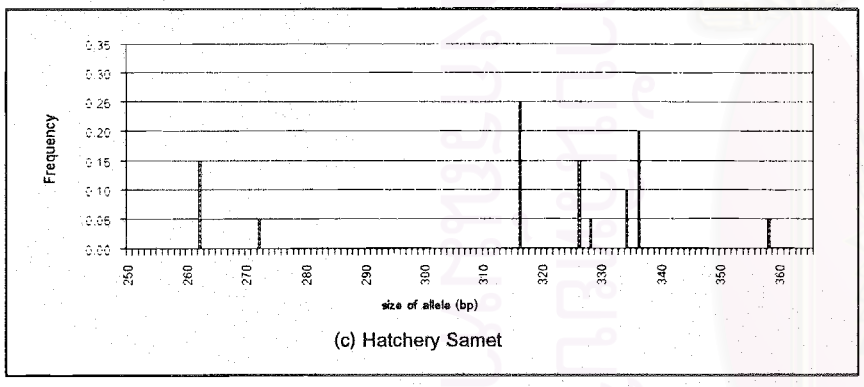
N = Number of sample examined



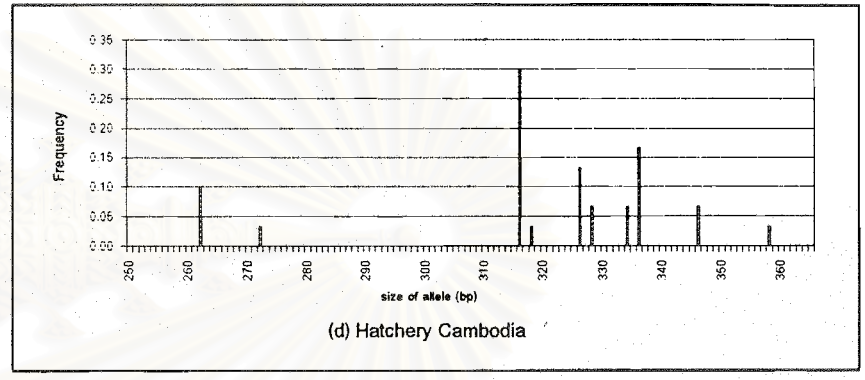
(a) Natural Talibong



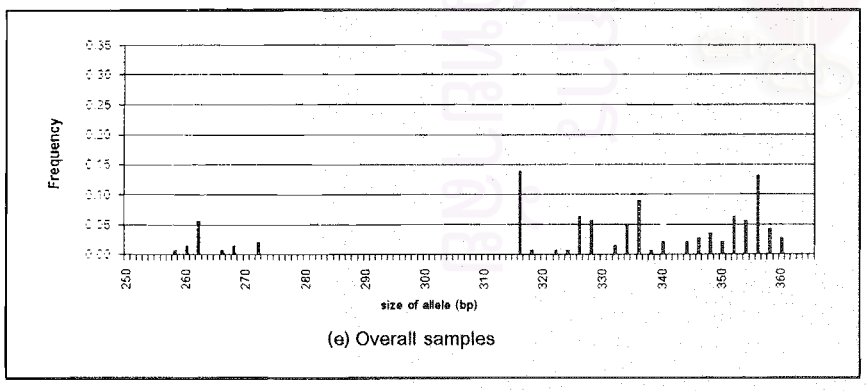
(b) Natural Cambodia



(c) Hatchery Samet

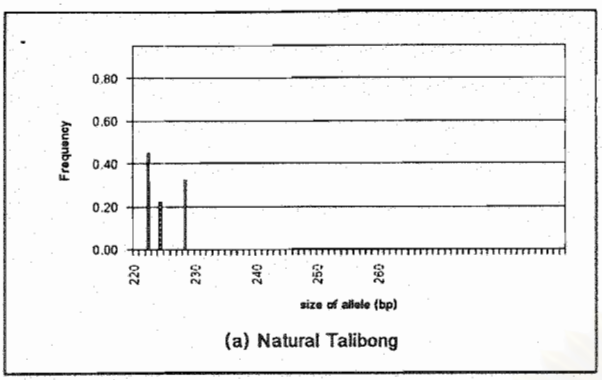


(d) Hatchery Cambodia

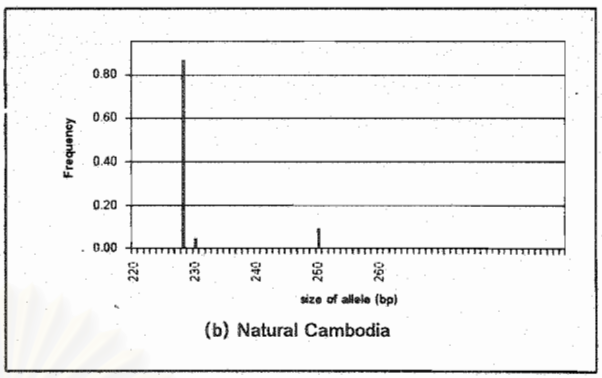


(e) Overall samples

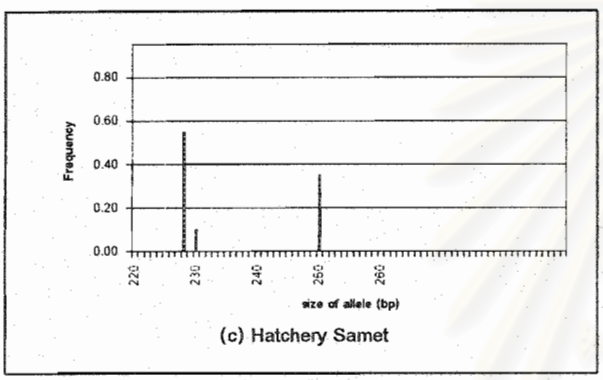
Figure B8 Allele distribution frequencies at the Has1 locus of *H. asinina* from Taibong (NL, $N = 28$), Cambodia (NC, $N = 19$), Hatchery Samet (HS, $N = 10$), Hatchery Cambodia (HC, $N = 15$) and overall samples ($N = 72$)



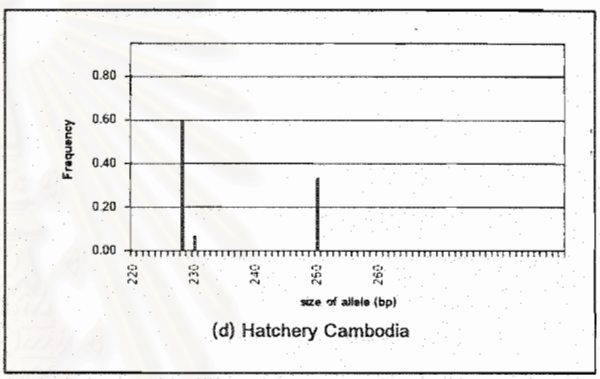
(a) Natural Talibong



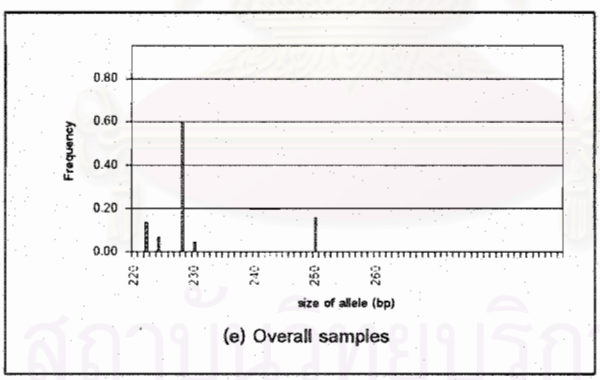
(b) Natural Cambodia



(c) Hatchery Samet



(d) Hatchery Cambodia



(e) Overall samples

Figure B9 Allele distribution frequencies at the Has4 locus of *H. asinina* from Talibong (NL, N = 20), Cambodia (NC, N = 22), Hatchery Samet (HS, N = 10), Hatchery Cambodia (HC, N = 15) and overall samples (N = 67)

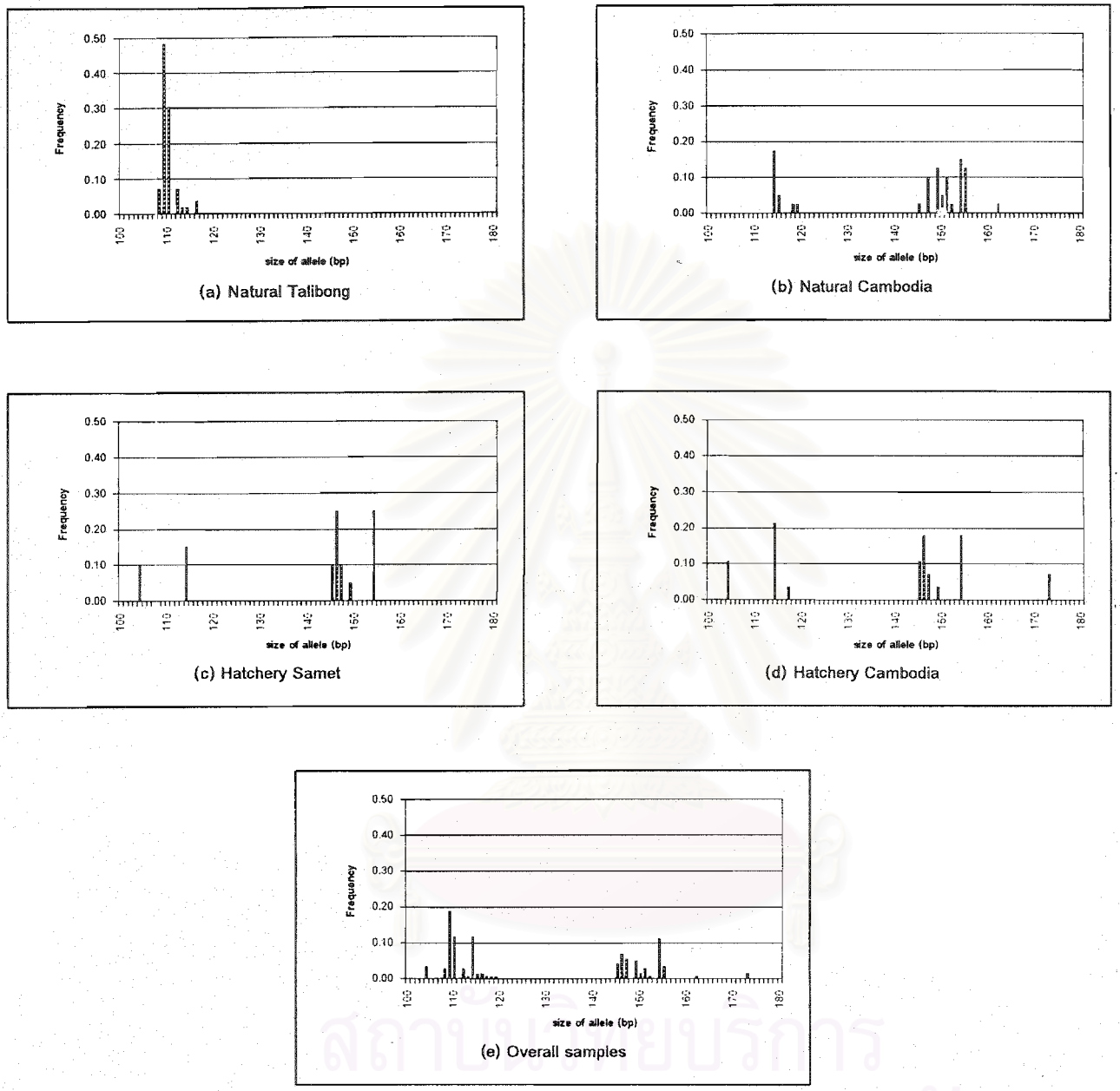


Figure B10 Alleles distribution frequencies at the Has5 locus of *H. asinina* from Talibong (NL, N = 28), Cambodia (NC, N = 20), Hatchery Samet (HS, N = 10), Hatchery Cambodia (HC, N = 14) and overall samples (N = 72)

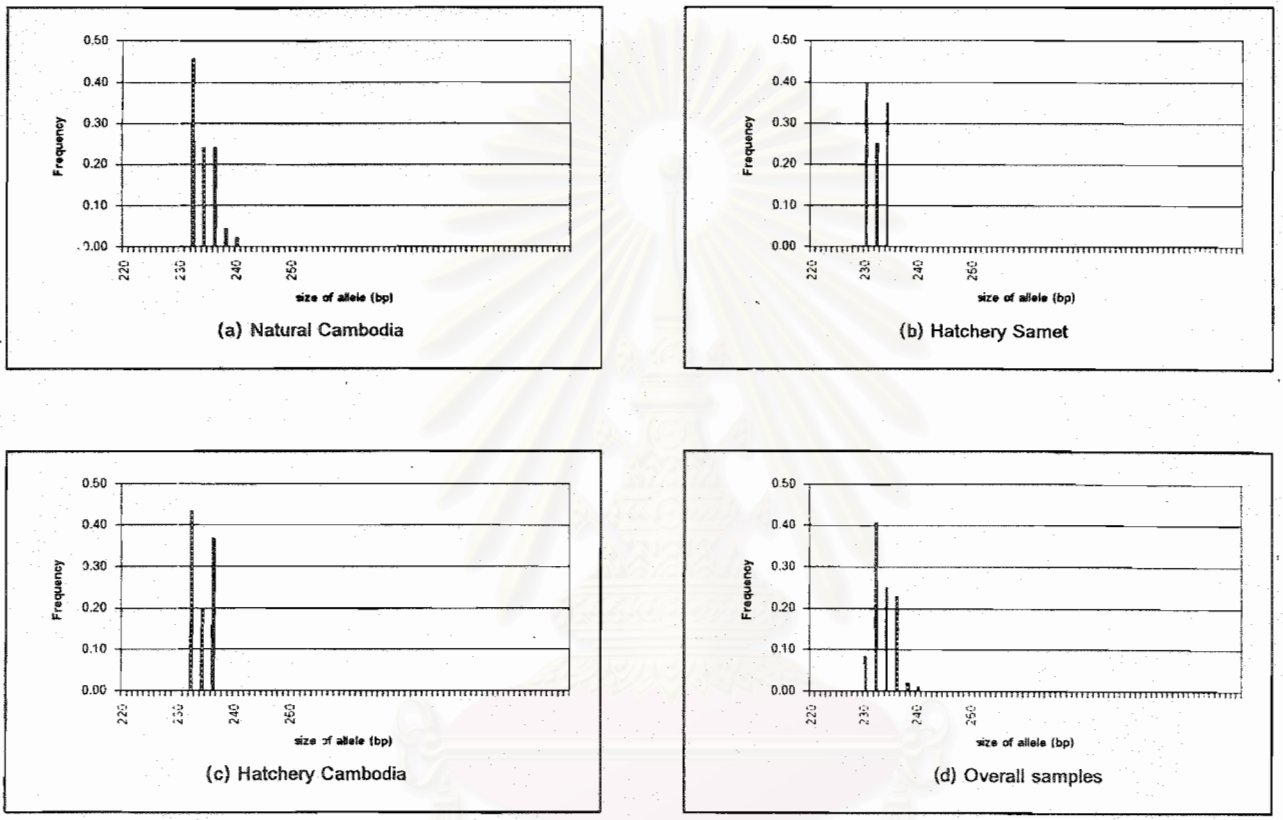


Figure B11 Alleles distribution frequencies at the Has6 locus of *H. asinina* from Cambodia (NC, $N = 23$), Hatchery Samet (HS, $N = 10$), Hatchery Cambodia (HC, $N = 15$) and overall samples ($N = 48$)

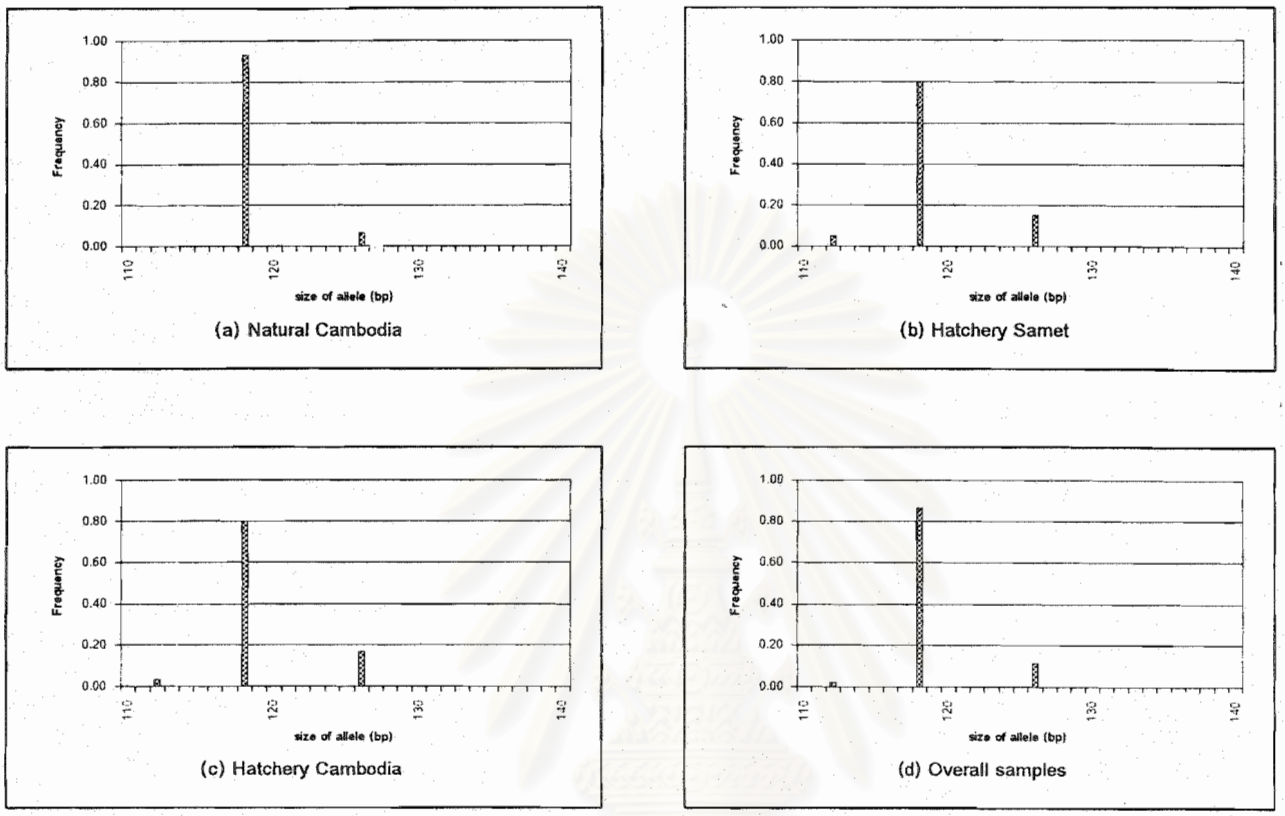


Figure B12 Allele distribution frequencies at the Has7 locus of *H. asinina* from Cambodia (NC, $N = 23$), Hatchery Samet (HS, $N = 10$), Hatchery Cambodia (HC, $N = 15$) and overall samples ($N = 48$)

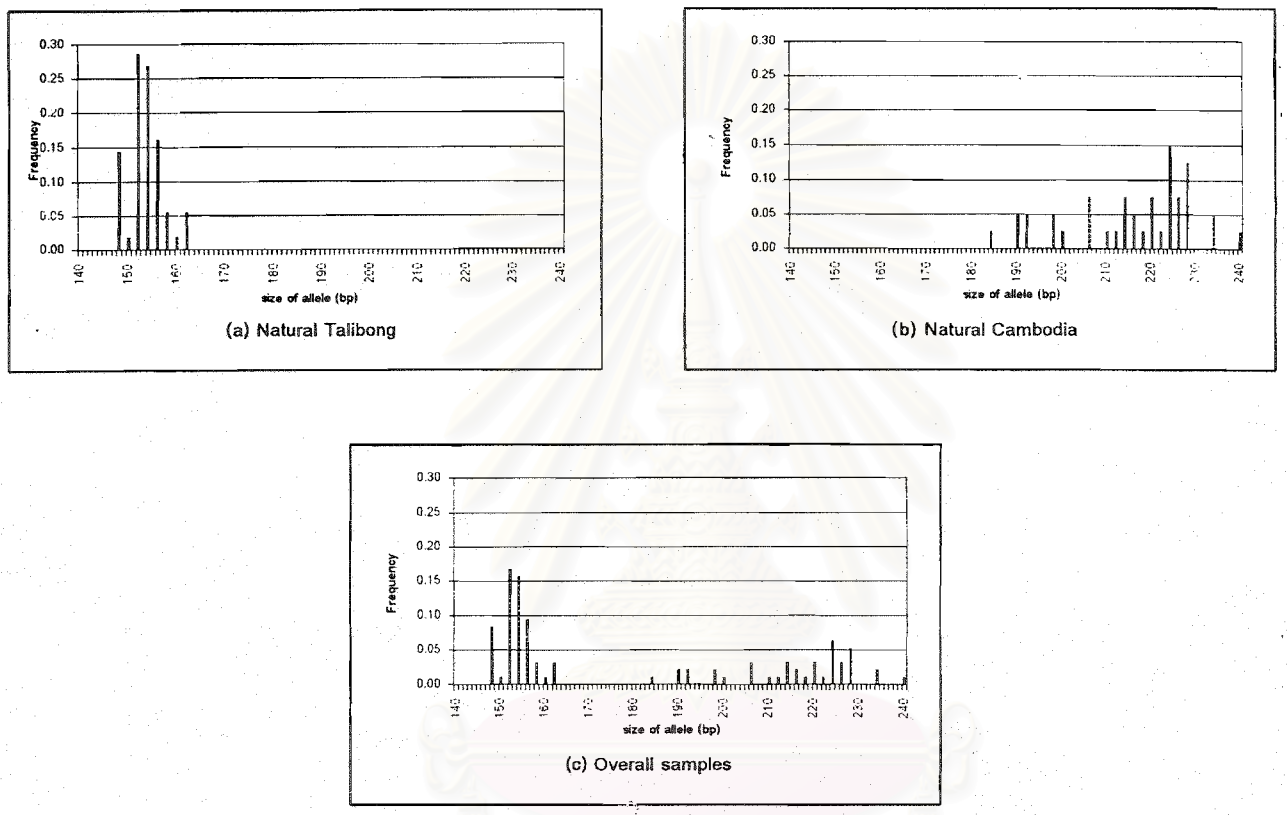


Figure B13 Allele distribution frequencies at the Has9 locus of *H. asinina* from Talibong (NL, $N = 28$) Cambodia (NC, $N = 20$) and overall samples ($N = 48$)

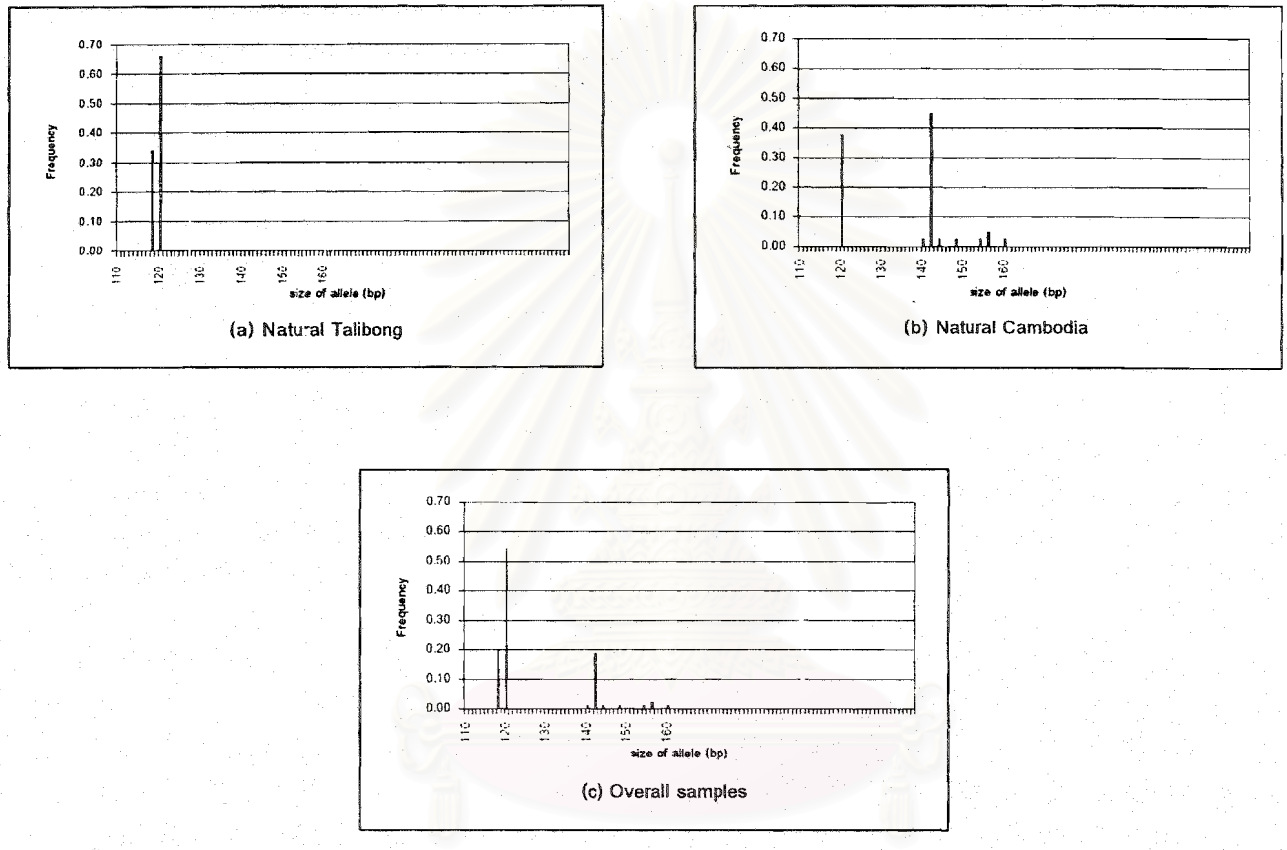


Figure B14 Allele distribution frequencies at the Has10 locus of *H. asinina* from Talibong (NL, $N = 28$) Cambodia (NC, $N = 20$) and overall samples ($N = 48$)

Appendix C

Genetic disequilibrium analysis

Table C1 Genotypic disequilibrium analysis for all possible comparison of six microsatellite loci of *H. asinina* from four geographic samples (NL, NC, HS and HC)

	Has1	Has2	Has3	Has4	Has5	Has8
Has1	-					
Has2	1.0000^{ns}	-				
Has3	0.3261^{ns}	0.1508^{ns}	-			
Has4	0.5235^{ns}	0.1168^{ns}	0.0607^{ns}	-		
Has5	0.9999^{ns}	0.4690^{ns}	0.9308^{ns}	0.0886^{ns}	-	
Has8	0.9924^{ns}	1.0000^{ns}	0.7026^{ns}	0.4040^{ns}	0.1743^{ns}	-

*Significant level was adjusted to $p < 0.0021$ using a sequential Bonferroni technique.
ns = not significant

Table C2 Genotypic disequilibrium analysis of each pair of three microsatellite loci determined by the individual genotype of *H. asinina* from six populations including NL, NS, NC, NP, HS and HC

	Has2	Has3	Has8
Has2	-		
Has3	0.3214^{ns}	-	
Has8	0.9993^{ns}	0.9227^{ns}	-

*Significant level was adjusted to $p < 0.0027$ using a sequential Bonferroni technique.
ns = not significant

Table C3 Pairwise comparisons of genotypic disequilibrium analysis of eight microsatellite loci of *H. asinina* from NL and NC samples

Locus	Population	
	NL	NC
Has1-2	No information	1.0000[ns]
Has1-3	0.0083[ns]	1.0000[ns]
Has1-4	0.4699[ns]	0.6226[ns]
Has1-5	0.8144[ns]	1.0000[ns]
Has1-8	0.4647[ns]	1.0000[ns]
Has1-9	0.3416[ns]	1.0000[ns]
Has1-10	0.9661[ns]	1.0000[ns]
Has2-3	No information	1.0000[ns]
Has2-4	No information	0.4474[ns]
Has2-5	No information	0.1131[ns]
Has2-8	No information	1.0000[ns]
Has2-9	No information	1.0000[ns]
Has2-10	No information	0.3478[ns]
Has3-4	0.0657[ns]	0.7774[ns]
Has3-5	0.3816[ns]	No information
Has3-8	0.0634[ns]	1.0000[ns]
Has3-9	0.6899[ns]	No information
Has3-10	0.8328[ns]	1.0000[ns]
Has4-5	0.8365[ns]	0.9792[ns]
Has4-8	0.4940[ns]	0.4053[ns]
Has4-9	0.3184[ns]	1.0000[ns]
Has4-10	0.4552[ns]	0.2220[ns]
Has5-8	0.8631[ns]	1.0000[ns]
Has5-9	0.7118[ns]	No information
Has5-10	0.6263[ns]	0.0013[ns]
Has8-9	0.3401[ns]	1.0000[ns]
Has8-10	0.2362[ns]	1.0000[ns]
Has9-10	0.2006[ns]	1.0000[ns]

*Significant level was adjusted to $p < 0.0031$ using a sequential Bonferroni technique.
ns = not significant

Table C4 Pairwise comparisons of genotypic disequilibrium analysis of six microsatellite loci of *H. asinina* from NL, NC, HS and HC samples

Locus	Population			
	NL	NC	HC	HS
Has1-2	No information	1.0000 ns	1.0000 ns	1.0000 ns
Has1-3	0.0101 ns	1.0000 ns	1.0000 ns	1.0000 ns
Has1-4	0.4761 ns	0.6228	0.3020 ns	0.3173 ns
Has1-5	0.8032 ns	1.0000 ns	1.0000 ns	1.0000 ns
Has1-8	0.4683 ns	1.0000 ns	1.0000 ns	1.0000 ns
Has2-3	No information	1.0000 ns	0.0903 ns	0.0992 ns
Has2-4	No information	0.4514 ns	0.3637 ns	0.0373 ns
Has2-5	No information	0.1762 ns	1.0000 ns	1.0000 ns
Has2-8	Nc information	1.0000 ns	1.0000 ns	1.0000 ns
Has3-4	0.0616	0.7786 ns	0.1359 ns	0.0881 ns
Has3-5	0.3916	No information	1.0000 ns	1.0000 ns
Has3-8	0.0638 ns	1.0000 ns	1.0000 ns	1.0000 ns
Has4-5	0.8362 ns	0.9803 ns	0.0354 ns	0.0357 ns
Has4-8	0.4925 ns	0.4118 ns	0.4220 ns	0.1834 ns
Has5-8	0.8641 ns	1.0000 ns	0.0842 ns	0.0435 ns

*Significant level was further adjusted to $p < 0.0021$ using a sequential Bonferroni technique.
ns = not significant

Table C5 Pairwise comparison of genotypic disequilibrium analysis of each pair of three microsatellite loci of *H. asinina* from NL, NS, NC, NP, HS and HC samples

Locus	Population					
	NL	NS	NC	NP	HS	HC
Has2-3	No information	1.00000	1.00000	0.22728	0.12009	0.11783
Has2-8	No information	1.00000	1.00000	0.50586	1.00000	1.00000
Has3-8	0.06150	1.00000	1.00000	0.86535	1.00000	1.00000

*Significant level was further adjusted to $p < 0.0027$ using a sequential Bonferroni technique.
ns = not significant

Appendix D

Hardy-Weinberg equilibrium analysis

Table D1 Hardy-Weinberg expectations of *H. asinina* from NL, NC, HS and HC samples at six microsatellite loci

Sample	P-value*					
	Has1	Has2	Has3	Has4	Has5	Has8
NL	0.2426^{ns}	0.1600^{ns}	0.1016^{ns}	0.1225^{ns}	0.0167^{ns}	0.3759^{ns}
NC	0.1206^{ns}	< 0.0001	< 0.0001	1.0000^{ns}	< 0.0001	0.3320^{ns}
HS	0.1129^{ns}	0.0224^{ns}	0.6838^{ns}	0.1590^{ns}	< 0.0001	0.4559^{ns}
HC	0.0975^{ns}	0.0887^{ns}	1.0000^{ns}	0.3160^{ns}	0.0001	0.2551^{ns}

*Significant level was further adjusted to $p < 0.0027$ using a sequential Bonferroni technique.
ns = not significant

Table D2 Hardy-Weinberg expectations of *H. asinina* from NL, NC, HS and HC samples at eight microsatellite loci

Sample	P-value*							
	Has1	Has2	Has3	Has4	Has5	Has8	Has9	Has10
NL	0.2370^{ns}	0.1752^{ns}	0.1022^{ns}	0.1225^{ns}	0.0211^{ns}	0.3759^{ns}	0.7812^{ns}	0.3646^{ns}
NC	0.0830^{ns}	< 0.0001	< 0.0001	1.0000^{ns}	< 0.0001	0.3557^{ns}	< 0.0001	0.0318^{ns}

*Significant level was further adjusted to $p < 0.0031$ using a sequential Bonferroni technique.
ns = not significant

Appendix E

Geographic heterogeneity analysis

Table E1 Geographic heterogeneity analysis of between NL, HC, HS and HC sample determined by six microsatellite loci

Sample	P-value*					
	Has1	Has2	Has3	Has4	Has5	Has8
NL-NC	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
NL-HS	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
NL-HC	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
NC-HS	0.7673^{ns}	0.0378^{ns}	0.2342^{ns}	0.0186^{ns}	0.0155^{ns}	0.5109^{ns}
NC-HC	0.3676^{ns}	0.0192^{ns}	0.0221^{ns}	0.0177^{ns}	0.0026^{ns}	0.1058^{ns}
HS-HC	0.9947^{ns}	0.9745^{ns}	0.9316^{ns}	1.0000^{ns}	0.9759^{ns}	0.9269^{ns}

*Significant level was further adjusted to $p < 0.0021$ using a sequential Bonferroni technique.
ns = not significant

Table E2 Geographic heterogeneity analysis of NL and NC determined by eight microsatellite loci

Sample	P-value*							
	Has1	Has2	Has3	Has4	Has5	Has8	Has9	Has10
NL-NC	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

*Significant level was further adjusted to $p < 0.0031$ using a sequential Bonferroni technique.
ns = not significant

Appendix F

F-statistics

Table F1 *F*-statistics for microsatellite analysis of each pair of six populations of *H. asinina* using by six microsatellite loci

Population	Has1		Has2		Has3		Has4		Has5		Has8	
	F_{ST}	P-value*	F_{ST}	P-value*	F_{ST}	P-value*	F_{ST}	P-value*	F_{ST}	P-value*	F_{ST}	P-value*
NL-NC	0.1133	< 0.0001	0.0396	0.0019^{ns}	0.2402	< 0.0001	0.3728	< 0.0001	0.2001	< 0.0001	0.3205	< 0.0001
NL-HS	0.1426	< 0.0001	0.0756	0.0001^{ns}	0.3596	< 0.0001	0.2272	0.0006	0.2314	< 0.0001	0.3265	< 0.0001
NL-HC	0.1475	< 0.0001	0.0639	< 0.0001	0.3336	< 0.0001	0.2457	< 0.0001	0.2156	< 0.0001	0.2983	< 0.0001
NC-HS	-0.0056	0.5768^{ns}	0.0205	0.1890^{ns}	0.0031	0.3790^{ns}	0.1662	0.0119^{ns}	0.0039	0.3953^{ns}	0.0010	0.4285^{ns}
NC-HC	0.0030	0.3432^{ns}	0.0169	0.1706^{ns}	0.0198	0.1066^{ns}	0.1252	0.0161^{ns}	0.0276	0.3901^{ns}	0.0200	0.0917^{ns}
NS-NC	-0.0366	0.9932^{ns}	-0.0456	0.9635^{ns}	-0.0211	0.8275^{ns}	-0.0501	1.0000^{ns}	-0.0613	0.9901^{ns}	-0.0270	0.9039^{ns}

*Significant level was further adjusted to $p < 0.0021$using a sequential Bonferroni technique.

ns = not significant

Table F2 *F*-statistics of four populations of *H. asinina* (NL, NC, HS and HC) determined by six microsatellite loci

Locus	F_{ST}	P-value*
Has1	0.0867	< 0.0001
Has2	0.0359	0.0010
Has3	0.1828	< 0.0001
Has4	0.2321	< 0.0001
Has5	0.1399	< 0.0001
Has8	0.2109	< 0.0001
Overall	0.1480	< 0.0001

*Significant level was further adjusted to $p < 0.0021$ using a sequential Bonferroni technique.

Table F3 *F*-statistics for microsatellite analysis between NL and NC determined by eight microsatellite loci

Locus	NL-NC	
	F_{ST}	P-value*
Has1	0.1114	< 0.0001
Has2	0.0346	0.0043^{ns}
Has3	0.2402	< 0.0001
Has4	0.3728	< 0.0001
Has5	0.1347	< 0.0001
Has8	0.3205	< 0.0001
Has9	0.1234	< 0.0001
Has10	0.2557	< 0.0001
Overall	0.1991	< 0.0001

*Significant level was further adjusted to $p < 0.0031$ using a sequential Bonferroni technique.
ns = not significant

Biography

Miss Sureerat Tang was born on August 9, 1975. She graduated with the Bachelor of Science from the Department of Biochemistry at Chulalongkorn University in 1998. She has studied for the degree of Master of Science at the department of Biochemistry, Chulalongkorn University since 1999.