CHAPTER I

INTRODUCTION

1.1 Introduction

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

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

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

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

* Tropical species

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

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

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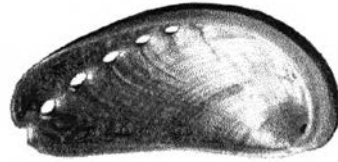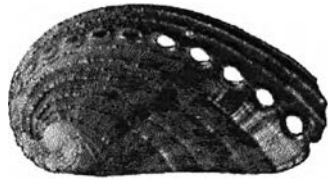
H. asinina

H. ovina

H. varia

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

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

1.2 Biology and Life history of Abalone

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

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

1.2.1 Classification

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

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

Phylum: Mollusca

Class: Gastropoda

Subclass: Prosobranchia

Order: Archeogastropoda

Suborder: Zygobranchia

Superfamily: Pleurotomariacea

Family: Haliotidae

Genus: *Haliotis*

Scientific name of Thai abalone:

Haliotis asinina (Linnaeus, 1758)

Haliotis ovina (Gmelin, 1791)

Haliotis varia (Linnaeus, 1758)

1.2.2 Morphology and Anatomy

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

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

1.2.3 Reproduction

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

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

with the shell length of 41.8 mm. The sex ratio was not significantly different from 1:1 with respect to months and size classes.
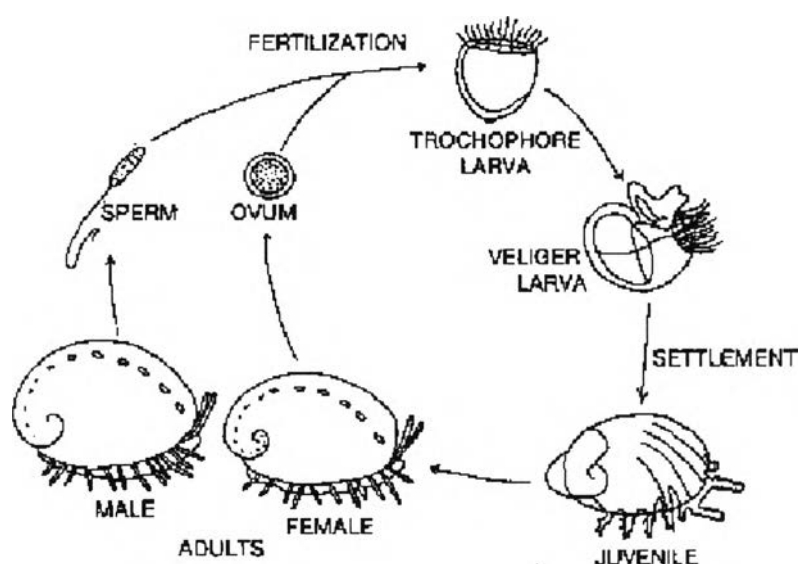
Figure 1.2 Life cycle of abalone (www.abalone.net).

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

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

1.2.4 Food

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

1.2.5 Age and growth

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

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

1.2.6 Habitats

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

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

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

1.2.7 Distributions

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

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

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

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

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

Basically, *H. asinina* and *H. ovina* are found around the islands along the eastern coasts of upper Gulf of Thailand, and all three species occur in the Andaman Sea.
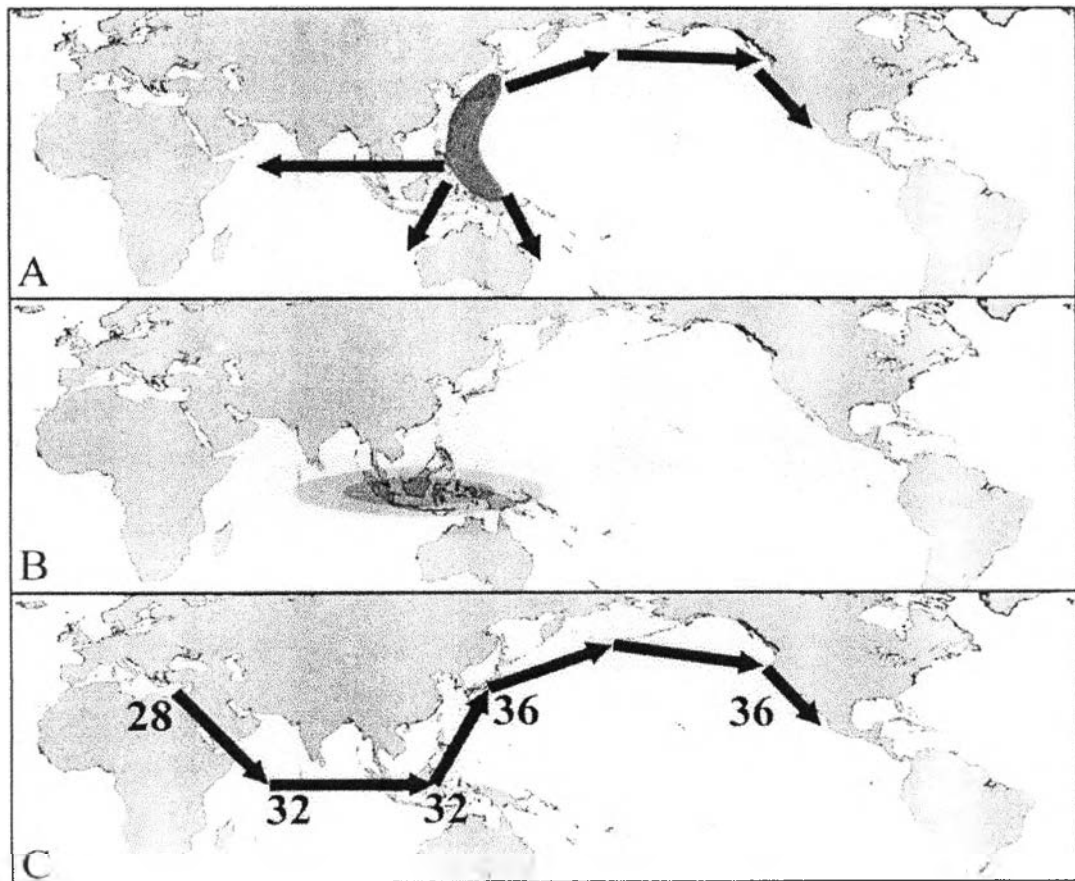
Figure 1.3 Three bio-geographical models proposing the origin of the *Haliotidae*

(Geiger, 2000).

A) Pacific Rim origin (Talmadge, 1963)

B) Indo-malayan origin (Lindberg, 1992)

C) Tethys origin (Geiger and Groves, 1999)
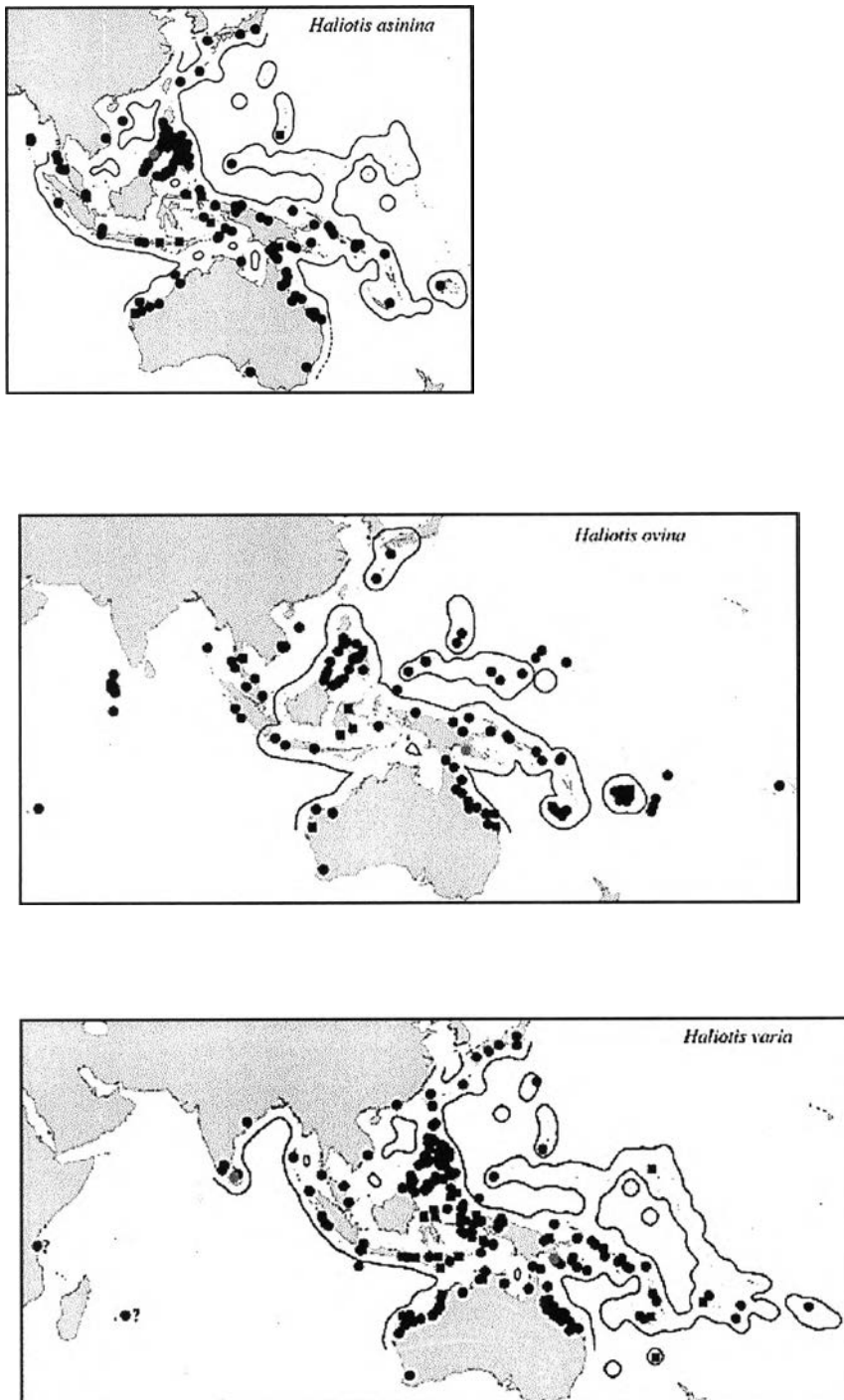
Figure 1.4 Worldwide distributions of abalone, *Haliotis asinina, H. ovina* and *H. varia* (Geiger, 2000).

1.3 DNA Markers

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

1.3.1   Hybridization-based (non-PCR) techniques

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

1.3.1.1 Restriction fragment length polymorphisms (RFLPs)

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

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

1.3.1.2 DNA fingerprinting with VNTR sequences

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

1.3.1.2.1 Multilocus DNA fingerprints

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

1.3.1.2.2 Single-locus minisatellite

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

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

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

1.3.2 PCR based techniques

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

1.3.2.1 PCR amplification of specific sequence and microsatellite primers

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

1.3.2.2 PCR - RFLP

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

1.3.2.3 DNA sequencing

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

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

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

1.3.2.4 Randomly Amplified Polymorphic DNA (RAPD)

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

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

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

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

1.4 Genetic study in abalone

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1.5 Objectives

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