Chapter I

Introduction

Farming of the black tiger shrimp (*Penaeus monodon*) is one of the common aquacultural industry in many tropical countries. In 1996 the world's shrimp production was increased, for approximately 10% compared with that in 1995 (Table 1.1). Indonesia, Ecuador and India showed dramatically higher shrimp production than that of the previous year for approximately 32%, 20%, and 33%, respectively.

In Thailand, due to an outbreak of the white spot disease across vast culturing areas, the total cultured shrimp (mainly, *P. monodon*) production was decreased from 225,000 metric tons (MT) in 1995 to 205,000 MT in 1996 corresponding to approximately 9% decreasing in the shrimp production. Although, Thailand has confronted difficulties resulted from diseases, it is still the biggest *P. monodon* producer for six consecutive years.

The world's leading importing countries of shrimps are the United States of America and Japan. Approximately two-third of *P. monodon* exported from Thailand is imported to these countries, the remaining market are Europe, Asian countries, Australia, and others (Table 1.2). The production sources of *P. monodon* are from the captured fisheries and from an aquaculture sector. Previously, the former was the main contributor for the total production. Nevertheless, the number of farmed *P. monodon* has consistently increased since the last decade and has become more importance contribution than that of the captured fisheries (Table 1.3).

The farming activity of *P. monodon* in Thailand has rapidly increased reflecting large annual production. The reasons for this are supported by several factors including the appropriate farming areas without the serious disturbing from typhoons or cyclone, small variable of sea water during seasons, and ideal soils and terrain for pond construction. Culture of *P. monodon* causes increasing national revenue, therefore this penaeid shrimp species is economically important species in Thailand.

Table 1.1 World Cultured Shrimp Production : 1994-1996.

Country	Head-on Production (MT)			Variance 96/95	
	1994	1995	1996	MT	%
Thailand	250,000	225,000*	205,000*	-20,000	-9
Indonesia	100,000	100,000*	132,000*	+32,000	+32
Ecuador	100,000	100,000	120,000	+20,000	+20
India	70,000	60,000	80,000*	+20,000	+33
Vietnam	50,000	45,000*	45,000*	-	-
Bangladesh	35,000	30,000	35,000	+ 5,000	+17
China	35,000	70,000	80,000	+10,000	+14
Philippines	30,000	20,000	25,000	+ 5,000	+25
Other	88,000	82,000	83,000	+ 1,000	+1
TOTAL	758,000	732,000	805,000	+73,000	+10

Source : ASIAN SHRIMP NEWS, 4th Quarter 1996

Table 1.2 The annual shrimp exports of Thailand between 1992-1995.

	1995		1994		1993		1992	
	MT	%	MT	%	MT	%	MT	%
- USA	77,955	39	80,955	42	66,955	45	53,955	38
- JAPAN	48,725	24	49,455	26	51,635	35	47,000	33
- EUROPE /OTHER	75,320	37	60,240	32	30,299	20	39,477	28
TOTAL	202,000*	100	190,650	100	148,889	100	140,432	100

* = estimated   Source : ASIAN SHRIMP NEWS, 1st Quarter 1996

Table 1.3 Shrimp production from fisheries and aquaculture sectors and culture area in Thailand.

Year	Production by normal fisheries (MT)	Production by aquaculture (MT)	Total Production (MT)	Culture Area (Rai)*
1981	122,706	10,729	133,435	171,619
1982	156,523	10,091	166,614	192,453
1983	127,584	11,550	139,134	222,107
1984	104,394	13,007	117,401	229,949
1985	91,632	15,840	107,472	254,805
1986	102,227	17,886	120,113	283,548
1987	128,100	23,566	151,666	279,812
1988	110,200	55,633	165,833	342,364
1989	110,800	93,495	204,295	444,785
1990	107,400	118,227	225,627	403,787
1991	129,100	162,070	291,170	470,826
1992	116,800	184,884	301,684	454,975
1993	100,000	225,514	325,514	449,292

* 6.25 rai = 1 ha

Source : ASIAN SHRIMP NEWS, 3rd Quarter 1996

1.1 Taxonomy of *P. monodon*

The taxonomic definition of the giant tiger shrimp, *P. monodon* is as follows (Bailey-Brook and Moss, 1992):

Phylum Arthropoda

Subphylum Crustacea

Class Malacostraca

Subclass Eumalacostraca

Order Decapoda

Suborder Natantia

Infraoder Penaeidea

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1985

Genus Penaeus Fabricius, 1798

Subgenus Penaeus

Species monodon

Scientific name : *Penaeus monodon* (Fabricius),1798

Common name : giant tiger shrimp or prawn

1.2 Morphololgy

Externally, the shrimp can be basically divided into thorax and abdomen (Fig. 1.1). The thorax is covered by single immobile carapace which protects internal organs and supports muscle origins. The internal organs in this part consist of eyes and eye stalks, sensory antennules, antennae, and walking legs (pereopods). The abdomen has segmentation commonly observed in invertebrates. It consists of swimming legs (pleopods), which arise from each of six abdominal segments, and a tail. The tail fans comprises a telson, which bears the anus, and two uropods attach to

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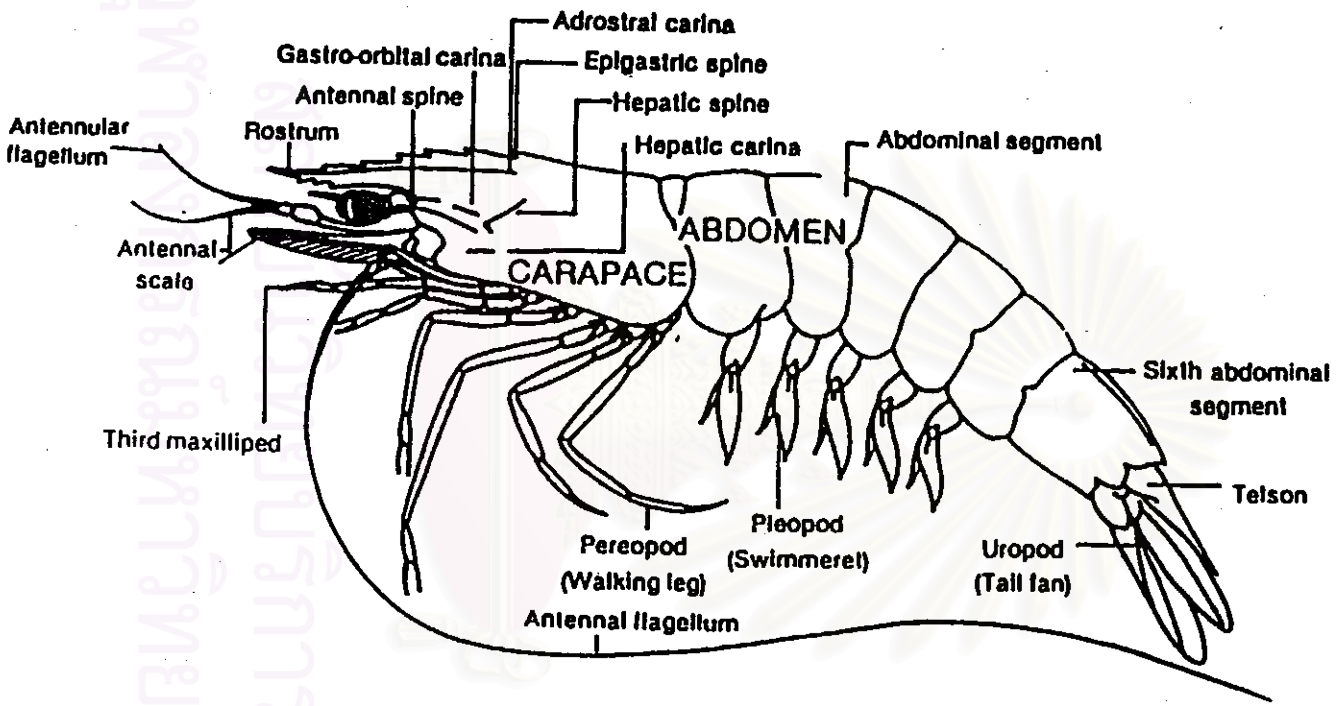

Fig.1.1 Lateral view of *P.monodon* showing important parts.

the last abdominal segment. A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of prawns.

Shrimps grow by periodically releasing their cuticle secreted by the epidermal cell layer, consisting of chitin and proteins. Molting starts when the epidermis detaches from the cuticle layer and begins to secrete a new cuticle. The new cuticle is soft and is stretched to accommodate the increased size of the prawn immediately after molting(Anderson, 1993).

A live giant tiger shrimp has the following characteristic coloration : carapace and abdomen are transversely banded with red and white, the antennae are greyish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow brackishwaters or when cultured in ponds, the color changes to dark and, often, to blackish brown (Motoh, 1981 : cited in Solis, 1988).

1.3 Life cycle

Development of penaeid shrimp is complex. It begins with a larvae hatching from the fertilized egg to the first stage, nauplius, followed by protozoea, mysis, and post larval stages (Fig 1.2). These require the developmental times about 1-5 days, 5 days, 4-5 days, and 6-15 days, respectively (Solis, 1988). Larvae exhibit planktonic behavior with antennal propulsion for swimming in nauplius, antennal and thoracic propulsion in mysis, and abdominal propulsion in megalopa. Nauplii utilize yolk granules within their body while the feeding starts in protozoea and mysis. At mysis stage, Lervae has five pairs of functioning pereiopods. The carapace now covers all the thoracic segments. The mysis swims like adults. After this stage, lervae metamorphoses to the post-larvae with a full complement of functioning appendages. The post-larvae continue to molting as they grow. They migrate shoreward and settle in nursery areas closed to shore or estuaries, before develop to juvenile and sub-adults, which more tolerate to variety of environmental factors. Sub - adults migrate back to the sea where they finally mature and have the first corpulation and spawn. The life span of penaeid shrimp are approximately 2 years (Anderson, 1993 ; Soils, 1988).

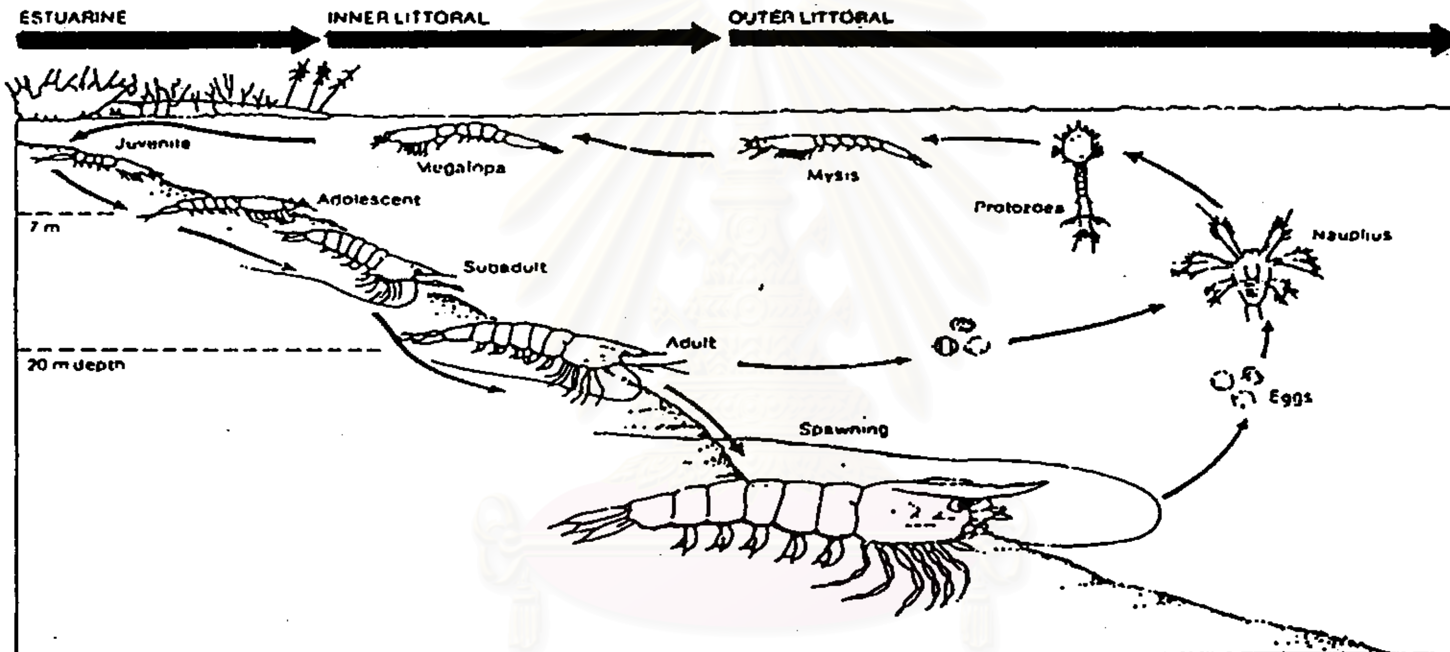

Figure 1.2 The life cycle of the giant tiger prawn, *P.monodon*, with stages in different habitats

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1.4 Distribution

The black tiger prawn (*P. monodon*) is principally distributed in the major part of the Indo-West Pacific region. It is commonly found in the East and Southeast Africa, through the Red Sea and Arabian Gulf, around the Indian subcontinent, and throughout the Malasian Archipelago to Northern Australia and Japan (Fig. 1.3). It is a marine species inhabits in mud or sand bottoms at all depths from shallows to 110 meters (360 feet), so it can be caught from offshore or inshore as well as from tidal zones (or ponds). The species is one of the most important aquaculture shrimp species in Asia (Dore and Frimodt, 1987).

1.5 Exploitation

Due mainly to the strong demand and the high price of *P. monodon* in the international markets, *P. monodon* industry in Thailand has rapidly expanded. The farming activity across the country has carried out without good management practice producing serious mismanagement problems in several areas.

The high demand on broodstock particularly from the Andaman Sea has stimulated the movement of broodstock and leading to overfishing in this area. The broodstock was also declining in the wild population. The government try to increase the prawns by releasing them from the farm origin into this area (mainly from the Andaman Sea sources, into the Gulf of Thailand). It leads to stock mixing and breaks down of any natural population structures.

We must have good genetic management to maintain genetic variation, increase the prawns productions and solve the other problems about it. The basic principle of all molecular genetics is to employ inherited, discrete and stable markers to identify the levels and the distribution of genetic variable in relation to life history, population size, mating patterns and migration of them. Besides this, genetic information can help us to find or select the species which give more and more yields per area. It is better than the expanded the prawn farms which can destroy the mangrove forests.

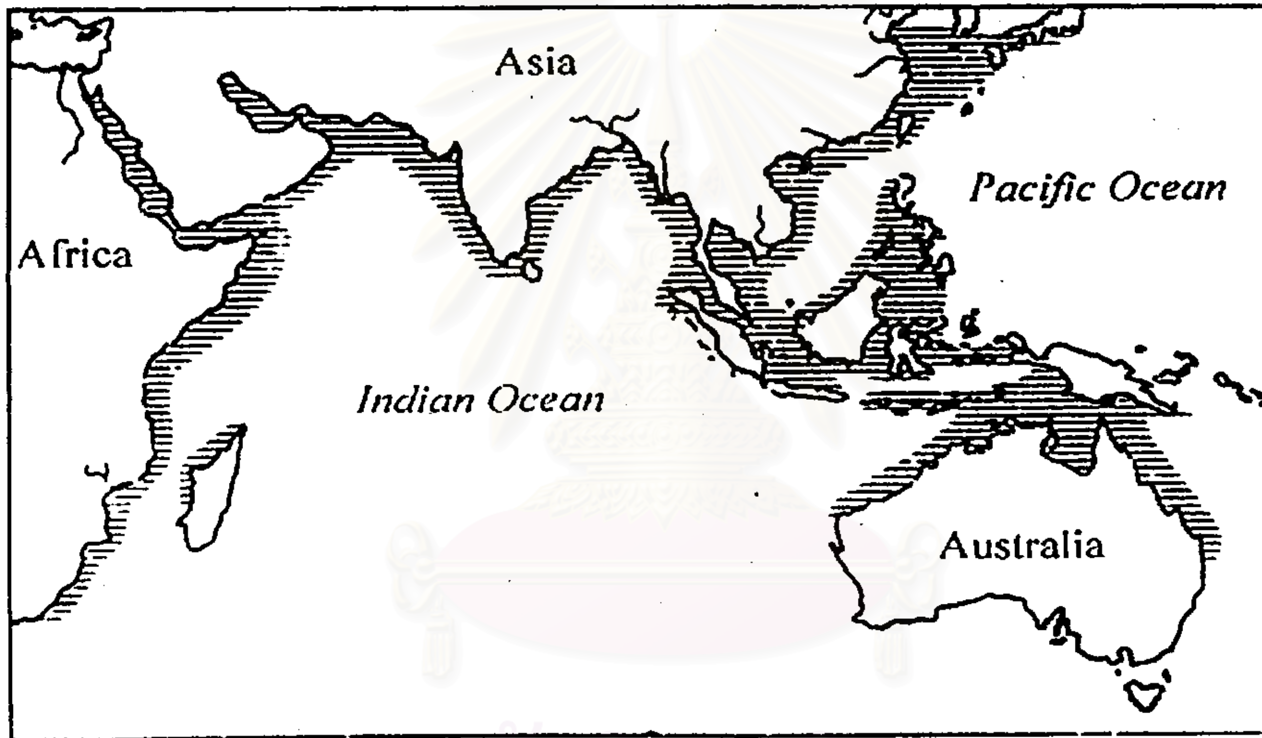

Figure 1.3 Geographic distribution of *P. monodon* in Indo-West Pacific region (Grey et al., 1983)

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1.6 Genetic markers

Genetic markers especially those inherited in the Mendelian fashion are important for various population genetic studies. Based on the neutral theory, the polymorphisms observed are assumed to be primarily generated by mutation, migration, gene flow, and genetic drift. Therefore, analysis of population genetic problems involved with these factors can be answered using molecular markers. These include allozymes, mitochondrial DNA, and variable number of tandem repeat (VNTR).

1.6.1 Allozymes

Allozyme refers to the enzyme that is produced by different alleles at the same chromosomal DNA locus (Park and Moran, 1994). The polymorphic allozyme can be detected by electrophoresis of proteins (usually enzymes) observed based on the property that proteins with different net charges migrate at the different rate through a gel matrix when exposed to an electric field (Avise, 1994). The charge characteristics of proteins vary with pH of the running buffer reflecting their movements towards the positive or negative poles (Morizot and Schmidt, 1990). Therefore, proteins are electrically separated according to their net charges, sizes and shapes (Cooper, 1977). The advantages of allozyme analysis are that this technique is convenient and cost-effective. Large number of specimen can be analysed within a limitation of time. Moreover, several allozyme loci can be simultaneously examined. Accordingly, allozyme analysis is a technique of choice to begin with when the species under investigation has not been reported for any molecular data. The systems are generally useful across various taxonomic levels (Memzies, 1981 ; Mitton and Koehn, 1985 ; Todd and Hatcher, 1993 ; Ward and Elliott, 1993 all cited in Park and Moran, 1994). The allozyme markers are transmitted in a co-dominant manner. Unfortunately, this technique possesses the technical limitations. Only histochemical stains available for investigated enzymes can be carried out. The most serious problems to applied this approach to a practical selective breeding program are due mainly to the level of detected polymorphisms and the broodstock, in most cases, need to be sacrified for the

analysis. Morever, isoloci can cause difficulties resulted from co-migration of bands originating from different loci (Buth, 1990). The products of protein coding genes have the lowest level of evolutionary rate so analysis of these may not be sensitive enough for descrimination of intraspecifically closely related populations which seem to be the case for population genetic studies of *P. monodon*.

1.6.2 Animal mitochondrial DNA

Mitochondrion is the cell organelle found in cytoplasm of eukaryotes. Each mitochondrion contains 5 - 10 copies of double - stranded circular DNA. The animal mitochondrial genome is approximately 16,000 - 20,000 bp in length coding for 13 protein coding genes (NADH dehydrogenase, ND, subunits 1, 2, 3, 4, 4L, 5 and 6 ; cytochrome b, three subunits of cytochrome oxidase, COI, II and III, and two subunits of ATP synthetase (ATPase 6 and 8), 2 genes coding for ribosomal RNAs (16S and 12S rRNA), 22 transfer RNA coding genes) (King and Stansfield, 1985; Park and Moran, 1994). Generally, mitochondria are inherited martiarchally except in some species (e.g. *Mytilus edulis*, *M. galloprovencialis*) whose contribution of paternal mitochondria is observed (Gyllensten et al., 1991 ; Margoulas and Zouros, 1993). Basically, the mutation rate of mtDNA is much more rapidly than that of single - copy nuclear genes reflecting its potential to be used for determination of intraspecific genetic variation among geographically different populations (Brown et al., 1979 ; Lynch and Jarrell, 1993).

Studies on mtDNA variation are usually by restriction analysis of the entire mtDNA or PCR - amplified mtDNA segment followed by RE 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. In addition, variation in copy number of localized tandem repeats, usually located in or near the control region of the mtDNA molecule, causes changes in size of mtDNA (length heteroplasmy)and, thus,

creating differences in digestion profiles (Harrison et al. , 1985). 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).

1.6.3. Variation number of tandem repeats (VNTR)

VNTR markers are characterized by a core sequence which consists of a number of identical repeated sequences. They can be divided into three categories; satellite, minisatellite, and microsatellite, based on the repeat length. (O'Reilly and Wright, 1995).

1.6.3.1 Satellites

Satellite DNA is a repetitive DNA that contains tandemly repeated short nucleotide sequences. The repeat unit may be from one to a few hundred nucleotides long. In some mammals, certain satellite DNAs may occur as millions of copies per genome (Alberts et al. , 1983). However, they are not as variable in size within populations as the other members of highly repetitive DNA family.

1.6.3.2 Minisatellites

Minisatellites is a repeating DNA sequence ranging between 15 - 70 bp per unit and 0.5 - 30 kb in size (Koreth et al., 1996). Minisatellites are found within noncoding regions of genomic DNA. Increases and decreases in the lengths of these result from changes in the number of repeat copies residing in the region and, hence, it is called variable numbers of tandem repeats (Avise, 1994). The variation of this DNA can be detected which is due to differences in length between conserved restriction sites. The mechanisms generating variability in minisatellites are still inconclusive. Several models have been suggested including unequal crossing over between homologous

chromosomes during meiosis, replication slippage, and gene conversion (Wolff et al., 1989). Differences in length of minisatellites can be not only from the number of copies of repeats, but from the interspersion patterns of different types of repeats for which comprise the array.

Jeffreys et al. (1991) first developed a more expedient PCR-based method from the repeat unit sequence variation within single molecules, termed minisatellite variant repeat-PCR (MVR-PCR also referred as digital DNA fingerprinting). In this strategy, the sequence of repeat units in both arrays is simultaneously determined by PCR using a primer complementary to different repeat unit types, and the other primed nearby unique flanking DNA. The result in the rapid attenuation of signal strength of bands representing increasingly distal repeat positions. The problem was circumvented by using "the tagged" repeat primers. In this strategy, initial PCR amplifications were performed using the flanking primer, and lower concentrations of a repeat unit primer with approximately 20 bp extensions. After several cycles, a series of PCR products differing in length by integral numbers of repeat units are generated.

Preliminary MVR-PCR surveys of the Atlantic salmon (*Salmo solar*) Ssa 197 minisatellite locus revealed limited variation resulted from repeat units in the first 10 repeats at one end of the tandem array. Due to internal annealing of repeat unit primers, band intensity diminishes rapidly when the repeat position increases (Jeffreys et al., 1991).

1.6.3.3 Microsatellites

Microsatellites are short DNA consisting of short repeats (1-6 nucleotides) which arrayed in tandemly repeated manner for approximately 10-50 copies (Hearne et al., 1992). Microsatellite are highly abundant and randomly dispersed in most eukaryote genomes (Valdes et al., 1993 ; Weissenbach et al. , 1992 ; Wright, 1993). It was estimated that one microsatellite locus may be found every 10 kb in eukaryotic genomes (Tautz, 1989). Due mainly to high mutation rate of the microsatellite loci (1×10^{-5}- 5×10^{-4} per generation), they exhibit high allelic variation and heterozygosity levels (Hearne et al., 1992; Wright and Bentzen, 1994). Like minisatellite, the

variability in microsatellites regions arise from changing in the number of repeated sequences which is proposed to be from slipped - strand misparing or slippage during DNA replication (Schlotter and Taulz, 1992). Microsatellite alleles exhibit codominant inheritance fashion (Queller et al., 1993). As the result, microsatellites are potential for several applications particularly when homozygotes are needed to be dissociated from heterozygotes.

Allelic variations of a particular microsatellite locus are detected through polymerase chain reaction. After amplification, the products are fractionated for their length polymorphisms using agarose (usually for tetranucleotide microsatellites) or polyacrylamide gels (di or trimeric microsatellites) with either non-radioactive or radioactive methods. Generally, detection of amplified microsatellites by autoradiography (labeled 5' end of the primers, electrophoresed of the products and exposed the gel with the X-ray films) are more sensitive and common. Radioactive detection of microsatellite gives clean results but less cost-effective than does the non-radioactive approach.

Non-radioactive detection are composed of ethidium bromide, silver stain and fluorescence. Staining of the electrophoretic gels with ethidium bromide is the simplest visualization approach but the lowest sensitivity compared to the remaining techniques. At least 10 ng of double standard DNA fragments are required for unambiguous detection (Bethwaite et al., 1995). Silver staining offers better sensitivity over ethidium bromide staining (pg quantities of DNA) and has been widely used for qualitative assessment of microsatellite allelic bands (Love, 1990). However, silver stain produce high but variable background caused by non-linear deposition of the silver. Detection of microsatellites using fluorescence dyes in coupling with the automated DNA sequencer yield significantly more rapid and reliable results. This technique is suitable for detection of multiplex amplification of multiple microsatellite loci. Nevertheless, the use of this system is limited by the cost.

Several recent studies used microsatellite marker for studies of population structure for example Tam and Kornfield (1996) characterized microsatellite markers

in the American lobster (*Homarus americanus*), Wolfus et al. (1997) studied application of the microsatellite loci for genetic diversity study in shrimp breeding program, while Lanzaro et al. (1995) studied microsatellite variability in a West African population of *Anopheles gambiae*. Furthermore, many microsatellite loci can be amplified in a variety of closely related species. For example, microsatellite developed in the rainbow trout (*Oncorhychus mykis*) and the Atlantic salmon (*S. salar*) were able to cross-analyze in several other *Salmo* and *Onchorynchus* species (Morris et al., 1996 ; McConnell et al.,1995a).

The aim of this thesis is to study genetic variation and population structures of the black tiger shrimp *P. monodon* in Thailand using microsatellite markers.