CHAPTER I

INTRODUCTION

The giant tiger shrimp, *Penaeus monodon* Fabricius, is the economically important penaeid shrimp species. It is the most commonly cultured species in the Southeast Asian regions. The reasons in favour of widespread culture of this taxon are its high growth rate, general tolerance to the environment, and omnivorous in behavior. As a result, simple cultured facilities are sufficient for *P. monodon*. In Thailand, *P. monodon* industry rapidly increased in the past 13 years as shown in Table 1.1. Although, the production by capture had decreased since 1981 but the total production of shrimp production increased because the production by cultivation and the increase in shrimp culture area. The main production of *P. monodon* for aquaculture section are from China, Indonesia, Ecuador, Philippines, Taiwan and Thailand. Thailand has become the largest *P. monodon* producer since 1994. The total production was estimated to be approximately 225,000 MT accounting for a quarter of the total production in 1996 (Table 1.2).

Therefore, the methods for selection and improve of pedigree by study genetic of *P. monodon* for using in the aqauculture. The genetic marker is very useful for the detecting DNA polymorphism, marker-assisted selection programs, genetic typing of brood stocks, monitoring levels of inbreeding, parentage and the levels of gene flow among populations.

Taxonomy of *P. monodon*

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

Phylum Arthropoda

Subphylum Crustacea

Class Malacostraca

Subclass Eumalacostraca

Superorder Eucarida

Order Decapoda

Suborder Natantia

Infraorder Penaeidea

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1815

Genus Penaeus Fabricius, 1798

Subgenus *Penaeus*

Species *monodon*

Its scientific name is *Penaeus monodon* Fabricius, 1798 and its common name is giant tiger shrimp.

Distribution

P. monodon is commonly found in tropical countries, It distributed all over the greater part of the Indo West Pacific regions (Figure 1.1). It is a local species in Thailand and found in both sides of the Thai-Malaysian Peninsula (the Andaman Sea on the west coast and the Gulf of Thailand on the east coast. The breeding grounds of *P. monodon* is usually 30-45 m and 26-50 m in depth for the

Table 1.1 Shrimp production from fisheries and aquaculture sectors in Thailand.

Year	Production by Capture	Production by Culture	Total Production	Shrimp Culture Area
	(MT)	(MT)	(MT)	(MT)
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

Andaman and the Gulf of Thailand, respectively. These correspond to approximately 11-15 miles from the shore lines in which the salinity of seawater is greater than 31 ppt (Sirirat, 1995). The life cycle of *P. monodon* can be roughly devided into 4 stages including planktome larval stage (nauplii, protozoa, mysis, megatopor and postlarval substages), juvenile which inhabit near the shore to 160 m indepth, subadult and adult. The life history of *P. monodon* is illustrated in Fig. 1.2. The life-span of this species is about 3 years (Liao, 1992).

Table 1.2 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

Exploitation

Based on the fact that wild *P. monodon* females are strongly required for breeding, the broodstock is over-exploited resulting in a rapid decrease of *P. monodon* in natural population. Accordingly, a supplement of local founders from artificially propagated programmes are needed in the future. Therefore, the basic knowledge on genetic variation and population structure of *P. monodon* in Thailand is essential. Generally, broodstock used for farming activity in Thailand are available from both the Gulf of Thailand and the Andaman sea. *P. monodon* broodstock from the latter is requried by shrimp farmers. Therefore, the broodstocks in some fisheries areas are heavily exploited. Apparently, the government organizations have supplemented large amounts of the hatchery-reared *P. monodon* larvae into several geographic areas without the prior knowledges on the genetic diversity, population structure and the most important

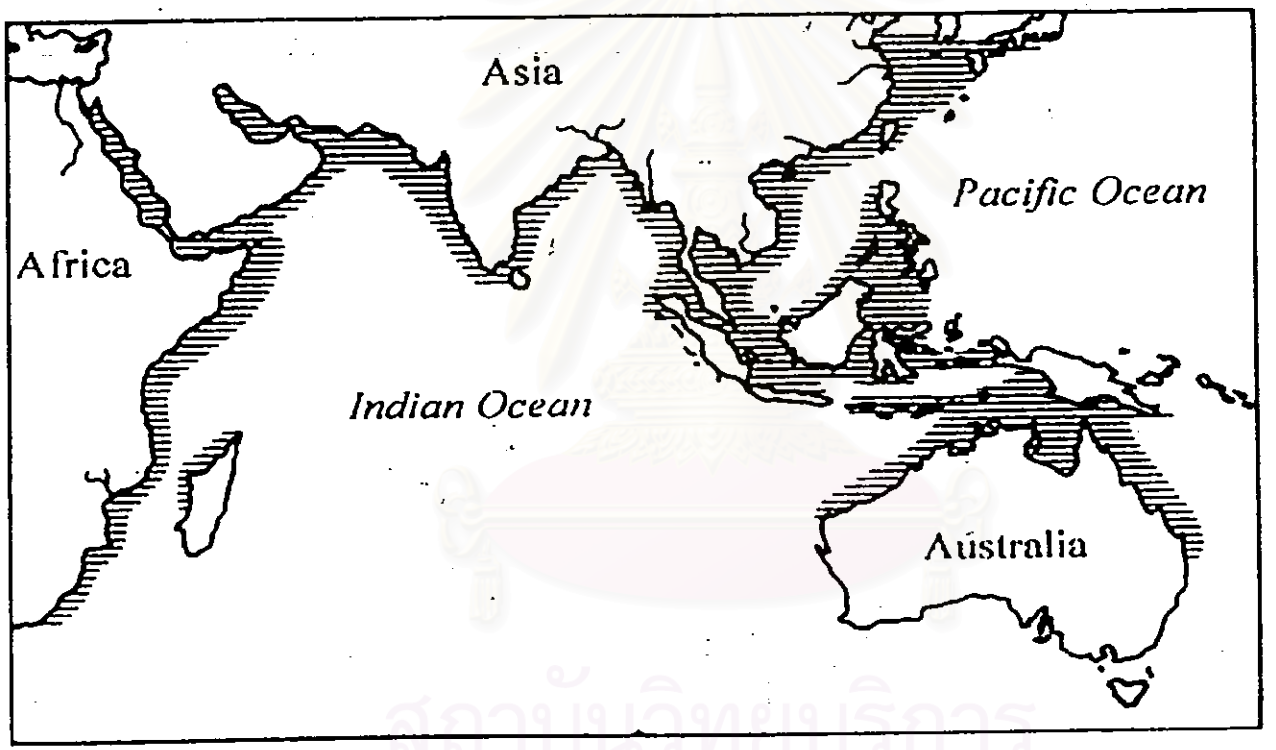

Figure 1.1 Geographic distribution of *P. monodon* in Indo-West Pacific region (Grey et al., 1983)

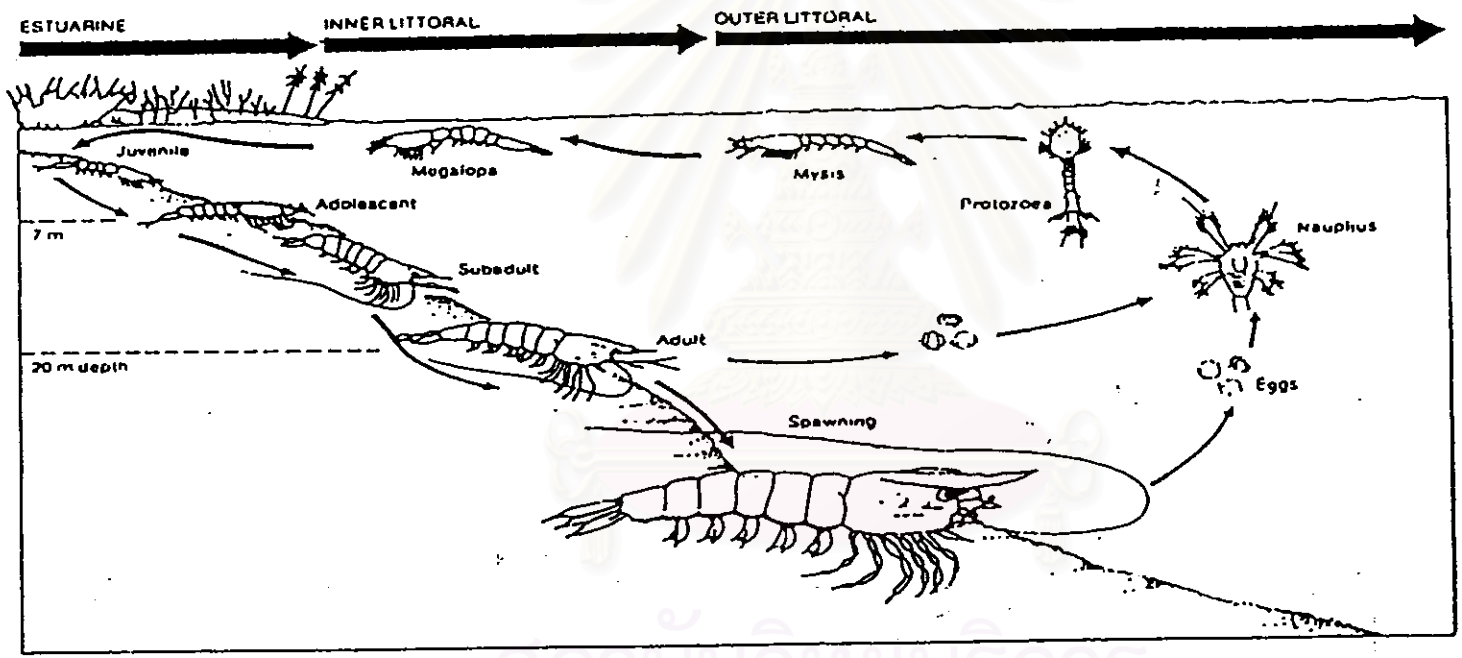

Figure 1.2 The life cycle of the giant tiger prawn, *P. monodon*, with stages in different habitats (Motoh, 1981)

factor, the origin of the founder population. This, in coupling with the escapes from the farms, can cause irretrievable loss of regional genetic diversity of a particular population. To manage this species properly, a genetic database on geographically different populations are required. This can be carried out by using several methods such as morphology, allozymes and DNA-based approaches. However, genetic markers developed from DNA are increasingly employed for practical fisheries management in several species.

Morphometric variation of *P. monodon*

Morephometric studies of *Penaeus* shrimps are limited. Most of which were from Hester (1983) and Goswami et al., (1986). Generally, morphometric markers used are influenced not only genetically but also environmentally. The shrimp can be divided into the thorax and abdomen (Figure 1.3). The thorax (or head) is covered by a single, immobile carapace which protects internal organs and supports muscle origin. The rostrum, extending beyond the tip of the antennular peduncle, is sigmoidal in shape and has 6-8 dorsal and 2-4 ventral teeth, mostly 7 and 3, respectively. The carapace is carinated with the adrostral carina, almost reaching to or not as far as the epigastric or first tooth. The gastro-orbital carina occupies the posterior one-third to one half distance between the post-orbital marine of the carapace and the hepatic spine. However, some characters for instance, differences of booty color can not unambiguously used because the colors in *Penaeus* species are controlled by the balance between pigment concentration and dispersing hormones which are strongly influenced by environmental stimuli. Therefore, this technique has not been widely used for determination of variation in *Penaeus* shrimp. (Lester et al., 1992).

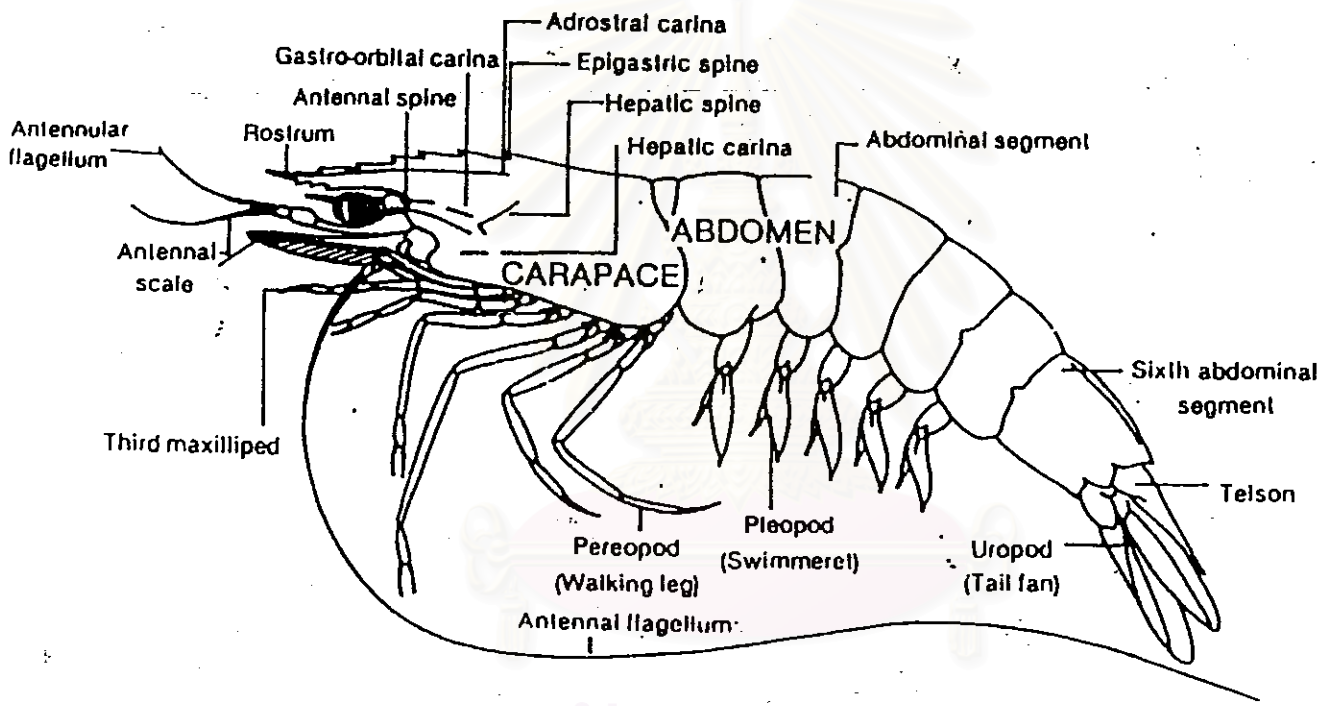

Fig. 1.3 Lateral view of *P. monodon* showing important parts.

Genetic markers

Genetic markers are extremely important for various genetic studies, for instance, species or stock identification, inter or intraspecific phylogeny, hybridization and phylogeography. Moreover, genetic markers can also be employed in breeding programes for determination of preparatory strains required for aquaculture purposes. Genetic markers can also be used in the development of genetic improvement programs. Once the correlations between genetic markers and qualitative trait loci (QTL) are identified (e.g. through genome mapping), such markers can be employed as the "marker assisted selection" (MAS) (Moore et al., 1997). Many genetic markers are useful for genetic studies. These include protein (allozymes) and DNA (variable number of tandem repeat or VNTR located in the nuclear genome, mitochondria DNA which is the maternal DNA)

1. Allozyme

Isozymes are functionally similar forms of enzymes composing of protein subunits produced by different gene loci or by different alleles at the same locus where as allozymes are variants of enzymes representing different allelic states of the same locus (Patricia et al., 1998). Electrophoretic migration of allozymes on a supporting medium depends on the net charges of the investigated polypeptide chains (Nei, 1987). The protein bands can be visualized by specific histochemical stain of the enzymes. Allozymes have been predominantly used for population genetic studies and identification of genetic markers in various animals until being replaced with DNA - based approaches at present. The advantages of allozyme approach due mainly to its cost-effective, less tedious and less time consuming. Therefore, large numbers of samples can be processed within a limiting period of time. Theoretically, it is a technique of choice to start with species whose have not been studied before. This technique is based on changing of electrostatical charges in investigated proteins. However, sixteen

of the common amino acids are electrostatically neutral. Consequently, nonsynonymous mutations from one neutral amino acid to other neutral base dose not alter the total charge of the invertigated polypeptide chain, resulting in an inability to detect such substitutions. Because of the redundancy of the genetic code and the fact that not every amino acid replacement leads to a charge difference, only 30% of all nucleotide substitutions result in polymorphic fragment patterns. Moreover, large amounts and several types of tissues are required, therefore killing of the investigated samples are unaviodable. Other limitations are the low number of alleles per locus reflecting the lower sensitively for detection in comparison to DNA techniques, detection of enzymes (or protein) where histochemical stains are not available and difficulties in interpretation of the results from polyploids. The preveous allozyme analyses indicated low levels of genetic diversity in *P. monodon* reflecting its in ability to be employed for wider applications. *P. monodon* have been found to express low levels of protein variation relative to other organisms. This may explain in part why the genetic structure of *P. monodon* has not been investigated in greater detail. *P. monodon* populations in Australia over a wide geographical range has demonstrated highly significant differences in gene frequencies between the west-coast population and those on the northen and eastern coastlines (Benzie et al., 1992).

2. Mitochondrial DNA

The animal mitochondrial DNA (mt DNA) is about 15.0-19.0 kb. The evolutionary rate of this genome is approximately 5-10 times faster than that of single copy nuclear DNA (Brown et al., 1997). Maternal inheritance and apparent haploidy of mtDNA are of central importance. There are about 10^5 mitochondria in a mammalian egg, and about 50 in the midpiece of the sperm. If the sperm contributes no mitochondria to the subsequent generation, and the mtDNA in the egg is homogeneous, then mtDNA will be transmitted as a haploid genome, and only within matrilines.

This interpretation depend on complete homoplasmy (no intraindividual variation in mtDNA, For species where there are sex-biased dispersal patterns such that females tend to be philopatric, a comparison with variatin in nuclear DNA would show much greater differentiation in the maternally transmitted genome (mtDNA) (Hoelzel and Dover. 1991). Mitochondria DNA is a haploid and non-recombinant molecule reflecting, only cne type of mt DNA in an individual. The mt DNA is suitable for population genetic studies because of its small size, rapid evolution and maternal inheritance (Billington & Hebert, 1991; Hallerman & Beckman, 1988). These properties allow several applications, for instance determination of genetic stock structure, investigation of female gene flow, and evaluation of hybrid animals in the natural hybrid zones. A primary restriction map of *P. monodon* has showed the most commonly observed haplotype. Seven hexameric restriction endonucleases has used. This restriction map, containing 18 cleavage sites, will serve as a basis for studies of resriction fragment length polymorphism using hybridisation and PCR (Klinbunga et al., 1995). Past of the mitochondrial 16 s-ribosomal RNA gene of *Penaeus notialis* and *Penaeus schmitti* has been amplified and sequenced. The comparition of sequences reveals an 11% nucleotide divergence between the two species, the secondary structure appears well conserved in spite of nucleotide divergence due to numerous substitutions and additions/deletions (Machado et al., 1992).

3. Variable numbers of tandem repent (VNTR)

VNTR is noncoding DNA and typically classified to be highly repetitive DNA. It can be further divided, based on the degree of repetition and the relative location of the elements of the basic repeated unit to satellites, minisatellites and microsatellits as illustrated (Krawczak and Schmidtke. 1994).

3.1 Satellites

Satellite DNA is very highly repetitive and usually occur at a few genomic loci in heterochromatin. It is composed of between 1,000 and more than 100,000 copies per locus. The length of each repeat may be from two to several thousand base pairs reflecting a cluster of between 100 and 5,000 kb in length. The centromeric clusters of alpha repeats are typical examples of classical satellite DNA (Brown et al., 1995)

3.2 Minisatellites

Minisatellites consist of DNA repeats of 9 to 100 base pairs in length and show a lower degree of repetition compared to satellite DNA (usually from two to several hundred time at a locus). Tendemly repetitive sequences has shown multiallelic resulting from variation of repeated number. Due to its functionless, minisatellite DNA has extremely high evolutionary rate making this DNA suitable for determination of individuality and parentage. Jeffreys (1985) developed hypervariable minisatellite probes derived from a tandem repetitive segment of the myoglobin intron in humans. These probes hybridize to conserved core sequences scattered along the human genome. Each minisatellite probe has different length, for instance, probe 33.15 is repeats of a 16 bp. The hybridization patterns of hypervariable probes distinguished all individuals except monozygote twins. Segregation of the hybridized bands are inherited in a Mendelian fashion.

Subsequently, these hypervariable minisatellite probes, particularly 33.6 and 33.15, have been applied for determination of genetic identity (or DNA fingerprint analysis) of several animal species. Multi-locus DNA fingerprinting patterns of salmonid fish were investigated by the ability of probe 33.6 and 33.15 to cross-hybridze to the Atlantic salmon (*Salm solar*), brown trout (*S. trutta*) and rainbow trout (*Onchorynchus mykiss*) (Taggart & Ferguson, 1990). Galvin et al.

(1995a) examined intraspecific genetic variation the Atlantic whiting (*Merlangius merlangus L.*) using Mmer-AMP2 locus. The observed heterozygosity of *M. merlangus* from this study was 94% indicating high genetic polymorphisms in this species.

The disadvantage of DNA fingerprints resulted from minisatellite probes is that complex multilocus patterns (about 20 or more bands) is generated. Moreover, it is difficult to determine a specific locus to which the band belongs and, more importantly, an allele of a particular locus cannot be determined. Due to the inability to dissociate heterozygotes from homozygotes, fingerprint data from minisatellite markers is regarded as one of the dominant markers. Basically, levels of variation resulted from minisatellites are too high for meaningful results for determination of genetic population subdivision of almost all of animal population. However, this DNA marker is potentially useful when analysis of kinship, individuality and parentage are required

3.3 Microsatellites

Microsatellites or simple sequence repeats (SSRs) consist of tendemly repeated core sequence that often vary in the repeat numbers and are flanked by conserved DNA sequences. Microsatellites contain short motifs (usually 1-6 nucleotides per repeat) and repeat up to about 100 times. Microsatellites are relatively highly variable but tend to have fever alleles than do minisatellites making them useful for genetic studies in many circumstances for example, highly polymorphic loci may be used for parental analysis whereas the loci having lower number of alleles can be used to investigate intraspecific subdivisions or in a few cases, at interspecific levels, (Krawczak and Schmidtlee, 1994; Mavghan et al., 1994; O'Reilly and Wright, 1995). Microsatellites are abundant, widespread distributed throughout the chromosome and are highly polymorphic in eukaryotic

genomes therefore, they are probably the most effective marker for mapping of the genome (Tautz, 1989).

Polymorphism at microsatellite loci was firstly demonstrated by Tautz (1989) and Weber & May (1989). Microsatellite arrays are generally short, 20 to 300 bp. These ranges are well within the capabilities to be amplified using conventional PCR. Generally, each of microsatellite DNA is flanked in a unique sequence. As a result, locus specific primers can be developed complementary to such flanking regions. The microsatellite DNA of individual can be amplified through the PCR. After electrophoretically fractionated, the sizes of microsatellite alleles at a particular locus can be estimated by compared to the DNA standard.

In human, the most common repeats are dinucleotide (GT/CA)n, (GA/CT) and (TA/AT)n, and a trinucleotide (CAG/GTC)n repeats (Tautz and Renz, 1984). Although tri- and tetranucleotide microsatellites have been isolated, dinucleotides especially the CA repeats are much easier to be isolated due to the abundance of dimeric microsatellite loci in the human genome.

It was found that microsatellites in human oncogenes are not stable. Moreover, the frequency of the (CAG)n microsatellites are greater than $(GT)_n$ and $(TATA)_n$. Interestingly, the number of repeats for the same loci are different tumours. Characterization of microsatellites in honey bee and bumble bee illustrated that the CT repeat found in both taxa were more abundant than the GT repeat. It was also reported that enriched library could elevate the number of recombinant clones containing the (CA)n by approximately 50 fold (Estoup et al., 1992; 1993). The microsatellites were classified as perfect, imperfect, and compound repeats. The polymorphic nature of perfect microsatellite loci have potential utility as genetic markers for population, breeding and evolutionary studies (Brooker A.L. et al., 1994). The application of three novel microsatellite VNTR loci to population differentiaion in Atlantic salmon (*Salmo salar*). Allele

frequencies, degree of polymorphism and heterozygosity has estimated for five populations from Nova Scotia, Canada and from Europe. These Atlantic salmon primers also amplify presumable homologous loci in nine other salmonid species. The polymorphic microsatellites loci reported here demonstrate great potential as genetic markers in population, breeding, and evolutionary studies (McConnell et al., 1995).

Garcia et al (1995) has developed microsatellite marker in the sea bass (*Dicentrachus labrax*). To investigate the genetic diversity of *D. labrax*, forty-two individuals wild-caught and their microsatellite genotypes was then examined. Thirty-six of which were genotypes, an heterozygotes corresponding to observed heterozygosity of 86%.

In the European flat oyster (*Ostrea edulis*), dinucleotide microsatellites were much more abundant than tri and tetramicrosatellites. The most common dinucleotide motif was (GA/TC)n followed by (AC/GT)n repeats. The former represented 65% of total dimeric microsatellites while the latter represented 38.5% of such DNA. Genetically inherited fashion of these DNA markers was examined and followed Mendel' law, (Naciri et al., 1995).

In teleosts, dinucleotide microsatellites, particularly $(GT)_n$ and $(GA)_n$, are highly abundant. These loci displayed high degrees of polymorphisms reflected by high heterozygosity of investigated taxa.

Bluegill microsatellites are similar to those of other fish, strengthening previous reporrts of significant differences in the organization of microsatellites between teleosts and mammals. The allele frequencies, the degree of polymorphism, and heterozygosities has estimated using bluegill from Lake Opinicon, Ontario. The predominant category of bluegill $(GT)_n$ microsatellites arre the perfect repeats, while the most common size-class including all categories contains sequences with lengths of 10-12 repeats (Colbourne et al., 1995).

Approx. 7% of the genome of *P. vannamei* consists of tandem repetitions of a sequence element containing one copy of a 162-168 bp sequence and variable numbers of a pentanucleotide sequence (Bagshaw and Buckholt. 1997).

The genomic library screening indicated that $(GT)_n$ microsatellites are more abundant than $(CT)_n$ in *P. monodon* genome. The predominant categories found in *P. monodon* microsatellites are imperfect repeats for both $(GT)_n$ and $(CT)_n$. The results of study demonstrate the presence of highly polymorphic microsatellite markers in *P. monodon*. The markers have been useful in population studies and parental determination in *P. monodon* (Tassanakajon et al.,1998)

At present, microsatellite markers is of interest for genetical fisheries management of various economically importan aquatic species including *P. monodon*. The reason for this is that they are versatile for various applications in providing information on genetic variation lavels, distribution of the marker alleles, mating system, stock identification especially for mixed stock fisheries which seems to be the case in *P. monodon*. Nonetheless, the most important step is to develop a technique to determine the genetic variation level in wild *P. monodon*.

Microsatellites were chosen because their highly polymorphic mature previously reported in various species (Carvalho and Pitcher, 1995). Furthermore, their co-dominantly segregated fashion of microsatellite is valuable for selective breeding programmes as homo-and heterozygotes can be detected (Wolfus et al., 1997).

The objective of this thesis was to isolate and to characterize dinucleotide microsatellites DNA in *P. monodon*. The many researchs about microsatellites have found dinucleotide microsatellites more than other microsatellites. Although, this thesis used dinucleotide microsatellites such as $(CT)_{15}$ and $(GT)_{15}$

oligonucleotide probes to detect the dinucleotide microsatellite loci. The flanking regions and dinucleotide microsatellites were identified by sequencing. The primer for PCR were designed by flanking regions. The optimization of PCR conditions were used to find the stutter bands of each locus. The stutter bands showed number of alleles per locus. It is facile to scorable bands because it shows a few bands. This method is not use to much of DNA, so the shrimp can alive. This genetic marker has the polymorphism of alleles used for identification parentage of progeny, selective breeding programe, making broodstock in order to maintain line purity, genetic typing of broodstocks, monitoring levels of inbreeding and the levels of gene flow among population.