CHAPTER 1

INTRODUCTION

1.1 Taxonomy of *Penaeus monodon*

In this thesis, the definition of *P. monodon* is used according to the definition of Food and Agriculture Organization (FAO) convention. Because of the absence of systematic basic of distinction, the term "shrimp" or prawn are common English names used synonymously.

The taxonomic definition of black tiger shrimp is as follow:

Phylum Arthropoda

Class Crustacea

Subclass Malacostraca

Order Decapoda

Suborder Natantia

Infraorder Penaeid

Family Penaeidae Rafinesque,1815

Genus *Penaeus* Fabricius, 1798

Subgenus *Penaeus*

Species *monodon*

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

Common name : black tiger shrimp or giant tiger shrimp (or prawn)

FAO name : giant tiger shrimp (or prawn)

It has also four synonyms :

Penaeus carinatus Dana, 1852

P. caeruleus Stebbings, 1903

P. monodon var, *manilensis* Villaluz and Arriola, 1938

P. bubulus Kubo,1949

1.2 Morphology

Externally the shrimp can be basically divided into two parts, thorax (head) and abdomen (Fig. 1.1), covered by a single, immobile carapace which protects the internal organs and supports muscle origins. The rostrum, extending beyond the tip of the antennular peduncle, is sigmoidal in shape, has 7-8 dorsal and 3-4 ventral teeth and curves down very slightly. Rostral ridge lacks a distinct groove behind it, and the hepatic ridge is long and curved. Telson has a groove but does not has lateral spines. The eye stalks and eyes, sensory antennies and antennae arise rostrally. The walking legs (or pereiopods) are thoracic appendages. Gills are formed sac-like outgrowths of the base of the walking legs and situated in branchial chambers on either side of the thorax. The carapace extends laterally covering the gills completely. The abdomen has the obvious segmentation of invertebrates. A pair of swimming legs (or pleopods) arise from each of the six abdominal segments. A tail-fan containing a telson, which bears the anus, and two uropods attached to the last (6^{th}) abdominal segments. The cuticle consists of chitin and protein derivatives where calcium carbonate and calcium phosphate are deposited. Pigments are deposited in the cuticle for colour appearance by pigment cells (chromatophores) in the hypodermis. Parts of the digestive tract are lined by chitinous cuticle. For *P. monodon*, carapace and abdomen have black bands giving a tiger-striped appearance to this species. Pleoplods may appear red. *P. monodon* is the largest of the commercial species reaching 330 mm or more in length (Bailey-Brook and Mass, 1992; Anderson, 1993).

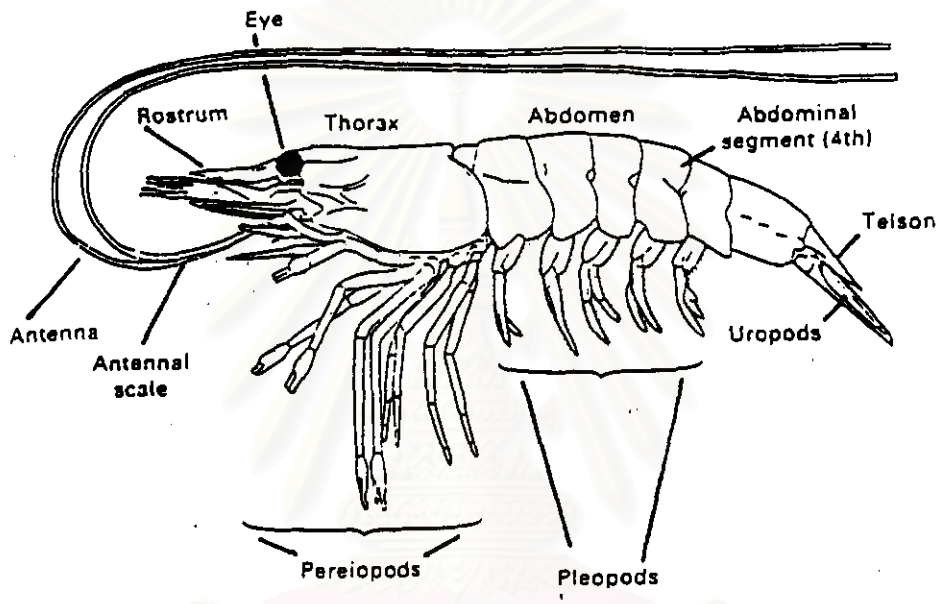

Fig. 1.1 External anatomy of *P. monodon* (Bailey-Brook and Mass, 1992).

1.3 Life Cycle

The penaeid life cycle includes several distinct stages found in a variety of habitat. Juveniles often prefer brackish water of estuaries and coastal wetlands, while adults are usually found off-shore at higher salinities and greater depths of 18.3-27.4 metres for spawning. Larval stages inhabit plankton-rich surface waters off-shore with an on-shore migration as they develop (Fig. 1.2).

The development of penaeid shrimps is complex. Larvae hatching from the fertilized eggs pass through a series of moults and metamorphic stages before reaching adult-like juveniles. Juveniles continue growing and moulting as they develop into mature adults.

Upon mating, fertilized eggs sink to the bottom, and hatching of the first stage, the nauplius, occurs about 12 hours later. The hatching stage is complete within 24 hours. The planktonic larvae remain offshore for about 3 weeks and develop through five naupliar, three potozoeal, and two or three mysis substages depending on actual species, each representing a moult. Following the mysis III stage, the larvae moult through several postmysis or post-larvae (PL) stages. The post-larvae have all the appendages and organs seen in adult shrimps. Post-larvae migrate shoreward and settle in nursery areas closed to the shore or in estuaries, where they grow quickly to juvenile and sub-adults, tolerating variable physico-chemical environments. Sub-adults migrate back to the sea where they finally mature and are ready to mate and spawn. The life span of penaeid shrimps are rarely longer than two years (Provenzano, 1985; Bailey-Brook and Moss, 1992; Anderson, 1993).

1.4 Shrimp Cultured Production of Thailand

Southest Asia and Oceania are areas of great significance in world shrimp

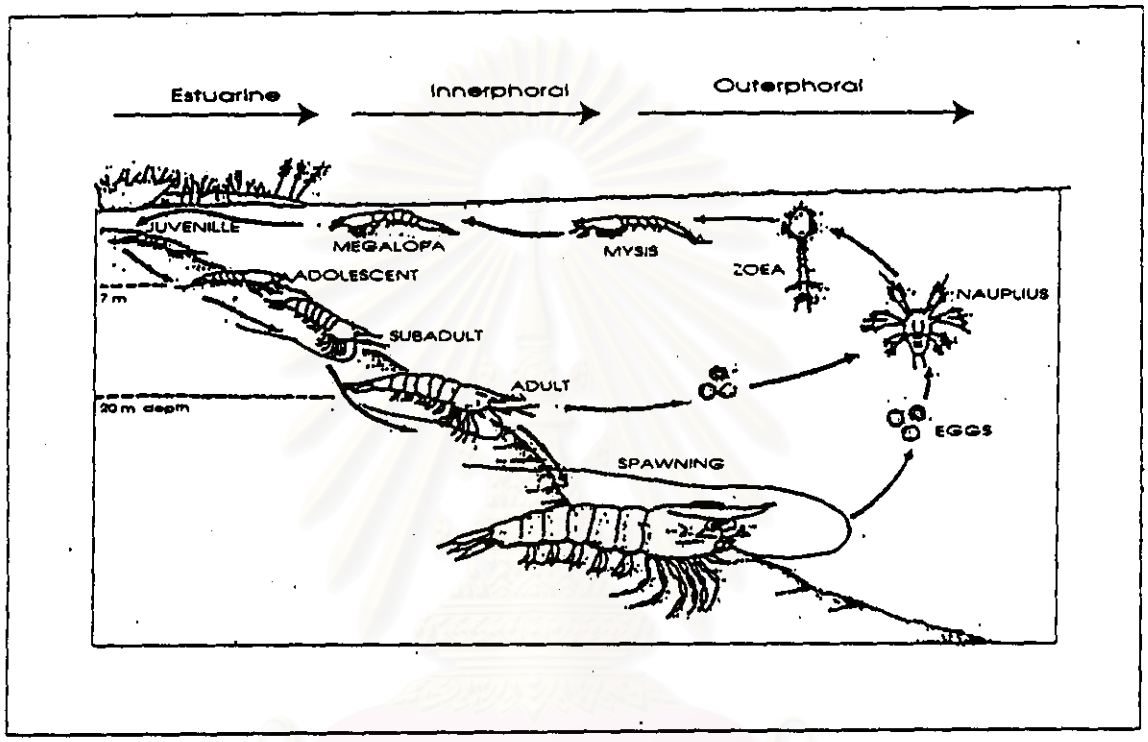

Fig. 1.2 Life cycle of penaeid shrimps (Bailey-Brook and Mass, 1992).

culture production. The export of cultured shrimp and prawn provides substantial economic benefit. Shrimps are the most valuable fisheries, particular in terms of value foreign exchange earning, because of a strong market demand and the highest prices in the international market.

One important economic benefit of Thailand exportation is shrimp production. Most of all are cultured shrimps from farming along coastal areas of the country. In Southern Thailand, the number of shrimp farms have increased since 1985, shrimp farms are located in provinces along the west and east coasts, including Prachuap Kiri Khan, Surat Thani, Nakorn Sri Thammarat, Songkhla, Satun and Trang. In the east, shrimp farms are mostly located in Angsila Distic and along the Gulf of Thailand (Asian Shrimp News, 2 nd Quarter, 1991).

There are several species of shrimps in Thailand and black tiger shrimp (*Penaeus monodon*) ranks first as the major important marine product, providing an annual income of over ten billions baht. From 1994 to 1996, Thailand was the first country in world culture shrimp production as shown in Table 1.1. The total area of intensive shrimp cultivation in 1991 was expanded up to 200,000 rai. About 60 % of this was in the central and east and the remaining 40 % was in the south. Intensive and super-intensive culture are at high risk of economic failure. Disease outbreaks cause decreasing of cultured shrimp production. During 1992 and 1994, yellow head and white spot diseases began to have impact in Thailand's cultured shrimp production. The most severe problem was observed at the eariy quarter of 1996 in which production decreased by 10 % and 9 % compared to that in 1994 and 1995, respectively (Table 1.2).

1.5 Disease of *P. monodon* in Thailand

Because of increasing shrimp farms and lack of the proper knowledge involving shrimp biology, farm management and control of diseases, the problems of

Table 1.1 World culture 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
Others	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 Thailand' s quarterly shrimp harvest

Quarter	1994	1995		1996	
	MT	MT	% Change	MT	% Change
1st	51,000	46,500	-9	30,000	-35
2nd	58,000	60,000	+3	50,000	-17
3rd	75,000	70,000	-7	80,000	+14
4th	66,000	48,500	-27	45,000	-7
Total	**250,000**	**225,000**	**-10**	**205,000**	**-9**

Source : Asian Shrimp News, 4th Quarter 1996

diseases become more serious in the shrimp industry. Of the infectious diseases, bacterial, fungal, protozoa and viral diseases are all found to cause the loss in cultured *P. monodon* production. Bacterial infections caused by *Vibrio* spp. are the most common diseases. The signs of vibriosis in shrimp are light or dark-brown focal lesions and necrosis of appendage tips. A number of antibiotics and antibacterial substances have been treated and reported to be effective in controlling vibriosis in this species. Fungal diseases of *P. nonodon* are commonly observed in zoea and mysis as well as in the grow-out stages. These problems can be practically eliminated by proper management, i.e. improvement of the water quality and keeping the pond bottom in a hygienic condition. Infection by protozoan is commonly encountered in the grow-out ponds. Formalin treatment at 15-25 ppm for 30 minutes to 1 hour with large amount of water exchange can decrease the infection without adverse side-effects. Among known diseases of *P. monodon*, viral infections have proved to be the most serious diseases at present (Subasinghe and Shariff, 1994).

As in 1992, some of important viruses has been reported in Asian and worldwide shrimp cultured farms. Therefore, different types of diseases and viruses have been named, depending on the countries as summarized in Tables 1.3 (Kasornchandra and Boonyaratpalin, 1996). In Thailand, Philippines and Australia, several species of viruses; white spot baculovirus (WSV) , yellow-head virus (YHV) , and penaeid haemocytic rod-shaped virus (PHRV), have been recently isolated from infected shrimps under intensive aquaculture. These viruses have caused and continued to pose significant problems for the shrimp industry. The Southeast Asia and Oceania shrimp industry is continually faced danger of viral diseases because of the practice of large-scale import and export of shrimp larvae among countries in order to supply sufficient seed for shrimp farming operations.

Table 1.3 Viruses in Penaeid shrimp cultivation with major economic impact

A. viruses in endoderm-derived (hepatopancreas & midgut cecum) tissue tropism. (1) Hepatopancreatic parvo-like virus (HPV) (2) *P. monodon*-type baculovirus (MBV) and type-C baculovirus (3) Baculovirus penaei (BP) (4) Baculovirus midgut gland necrosis virus (BMNV)
B. viruses with systemic ectoderm- and mesoderm-derived tissue tropism (5) Infected hypodermal and hematopoietic necrosis virus (IHHNV) (6) Yellow-head virus (YHV) (7) White spot virus or systemic ectodermal and mesodermal baculovirus (SEMBV)

1.5.1 Yellow-Head Disease

In early 1992, an outbreak of yellow-head disease of cultured *P. monodon* in Southern Thailand was first found at Pakpanang District, Nakorn Sri Thammarat and had further spread to shrimp farms around Songkhla Lake. In late 1992, a yellow-head disease caused extensive losses in the cultivation of *P. monodon* in Southern Thailand (Asian Shrimp News, 2 nd Quarter, 1992).

This disease is named after the clinical occurrence. It affects the shrimps at the grow-out stage of 5-35 days after stocking whose size between 5-15 grams. During infection, affected shrimps show a reduction in food consumption, tend to move to the water surface near the edge of the pond and remain motionless. The body of infected shrimps will show light-yellow coloration due to the pale color of hepatopancreas and gills (Limsuwan, 1991) (Fig. 1.3). The mortality may reach as high as 100 % of affected populations within 3-5 days from on set of disease. Other pathogenic agents, i.e. bacteria, parasites or fungi, were not found in the affected shrimp.

The virus was then named yellow-head virus (YHV) and was discovered by examination of the ultra thin sections of infected shrimps and disease-induced shrimps using electron microscopy. From the initial discovery of Boonyaratpalin et al. in 1993, the virus was considered to be a granulosis-like DNA virus, but later research by Wongteerasupaya et al. (1995 a) showed that this virus was actually an RNA virus. Based on morphology of negative stained virions by transmission electronmicroscopy (TEM) and RNA content, YHV resembles rhabdoviruses, coronaviruses (single-strand plus RNA), or paramixovirus (single-strand minus RNA) rather than baculoviruses which had been reported in lymphoid organs of *P. monodon* from Australia (Spann et al., 1995). From the microscopic views, the mature virus particles are rod-shaped ranging from 150-170 nm in length and 40-50 nm in width. Cross section of the complete virions showed an electron dense

(a) (b)

Fig. 1.3 The YHV syndrome of infected *P. monodon*

(a) : Normal *P. monodon*

(b) : YHV-infected *P. monodon*.

nuclear core of 20 to 30 nm in diameter surrounded by a trilaminar viral envelope (Fig. 1.4). In addition, no drugs, chemicals or prevention methods can be used to control this virus permanently. Therefore, the yellow-head disease is still epidemic for shrimp cultivations.

1.5.2 White Spot Disease

Recently, another serious disease outbreak known as white spot disease or red disease with white patches has emerged among shrimp cultures in Asian countries. It was first reported from farmed specimens of *P. japonicus*, *P. monodon* and *P. penicillatus* in Taiwan in 1992 and was founded in China in 1993 (Table 1.4). In late 1994, the disease has spread throughout Asian countries, such as Japan, Indonesia, Thailand, Malaysia, India and Bangladesh (Wongteerasupaya et al., 1996). In Southern Thailand, the white spot outbreaks were found in the same area where yellow-head disease was found. The disease had caused the heavily decreasing of shrimp production.

White spot virus (WSV), a causative agent of white spot disease, is a virus among all presently known penaeid viruses for its combined wide host species range, ability to cause widespread acute epizootics within 2-7 days during which mortality rates range from 10 % - 70 % and finally up to 100 % with massive systemic pathology. Shrimp infected with WSV shows rapid reduction in food consumption followed by general reddish coloration together with broken antenae and circumscribed whitish spots of 1-2 nm or more in diameter in the cuticle or shell. The whitish spots occur first in the carapace and fifth-sixth abdominal segments and spread throughout the entire body shell (Takahashi et al., 1994). The white spots appear to be abdominal deposits of calcium salts by the cuticular epidermis. Because of pink to reddish-brown coloration of dying shrimp, this disease is also called "red disease". The WSV infected shrimps are showed in Fig.

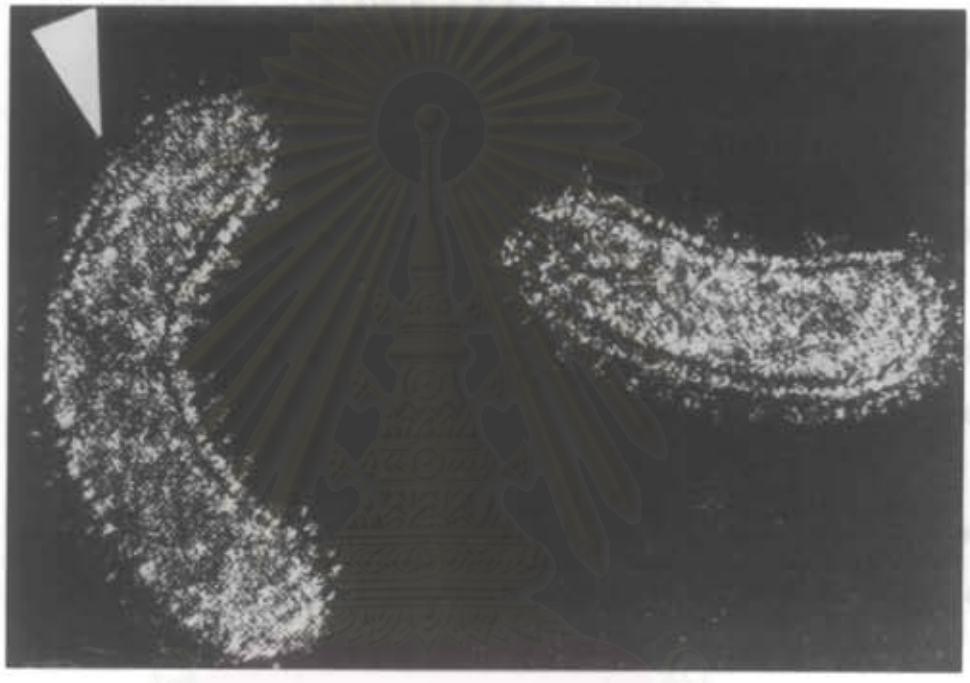

Fig. 1.4 Negatively stained YHV virions from hemolymph of *P. monodon* using TEM (Tongchuea, 1996).

Table 1.4 Summary of the findings in recent SEMBV shrimp disease outbreaks in Asia

Country	Species	Clinical signs
China	*P. monodon* *P. chinensis*	Mass mortalities, 45 d, reddish body, white spots on inside surface of carapace
Japan	*P. japonicus*	Mass mortalities, 3-5 g, reddish body, white spots on inside surface of carapace
Taiwan	*P. monodon* *P. japonicus* *P. penicillatus*	Mass mortalities, white spots on the carapace, appendages and the inside surface of the body, reddish coloration
Indonesia	*P. monodon* *P. indicus*	Mass mortalities, 30-40 d, pale body with white spots or patches on the inside of carapace and shell
Thailand	*P. monodon* *P. merguiensis*	Mass mortalities, 30-70 d, reddish body with white patches on inside surface of carapace and shell
Malaysia	*P. monodon*	Mass mortalities, 40-75 d, reddish body with white patches or spots on inside surface of carapace and shell
India	*P. monodon* *P. indicus*	Mass mortalities, 50-70 d, reddish body, white patches or spots on inside surface of carapace and shell

Source : Asian Shrimp News, 3 rd Quarter 1996

1.5.

Characterization of the virions was first discovered in Thailand from experimentally infected shrimp of *P. monodon* in 1995 (Wongteerasupaya et al., 1995b). The result from electron microscopic study and nucleic acid content indicated that this virus was a new non-occluded baculovirus consisting of double-stranded DNA of approximately 168 kb in size. It was named PmNOBII (the second non-occluded baculovirus reported in *P. monodon*) or SEMBV (systemic ectodermal and mesodermal baculovirus). Similar studies were described for WSV infected *P. monodon* in Taiwan where the causative virus was named PmNOBIII (Chou et al. 1995; Wang et al., 1995).

Transmission electronmicroscopy (TEM) of negatively stained virions from hemolymph samples (Fig. 1.6) reveal that intact virions are cylindrical to elliptical or obovate whose size are 121±9 nm in width at the widest point and 276±26 nm in length, excluding the multifilament appendages. The appendages are attached to the end of virions (Wongteerasupaya et al., 1996). Based on morphology, size, site of virion assembly, cellular pathology and nucleic acid content, WSV (or SEMBV) is classified to be type C virus in subfamily *Nudibaculovirinae* (Francki et al., 1991), a member of family *Baculovirolidae* (Huger and Krieg, 1991).

1.6 Application of Molecular Techniques in Fisheries and Aquacultures

The basic principle of all molecular marker approaches is to employ inherited, discrete and stable markers to identify genotypes at different taxonomic levels (individuals, populations or species). Such genetic data can provide information on the levels and distribution patterns of genetic variability in related to mating patterns, life history, population size, migration and evolution. The use of molecular techniques has increased dramatically over the past several years.

In the past, traditional methods such as comparative anatomy, morphology

Fig. 1.5 SEMBV-infected *P. monodon* with broken antennae and circumscribed whitish spots or patches in the carapace.

Fig. 1.6 TEM of negatively stained SEMBV virions. This virions were purified by urografin density centrifugation from hemolymph samples (Tongchuea, 1996).

(a) : Low magnification of 5 fully enveloped virions showing multifibrillar appendages

(b) : Higher magnification of 4 enveloped virions with multifibrillar appendages

(c) : Unevenloped nucleocapsids

and physiology had been used to evaluate genetic variation but these methods are not sufficient for genetic variation studies of several species. During the past decade, molecular techniques have been increasely employed. The term "molecular markers" was called for this development which are based on polymorphisms in protein or DNA molecules.

1.6.1 Protein Studies

Proteins are composed of one or more polypeptides, which are chains of amino acid. Because of the different charges of site chains of amino acids, protein studies take advantage of the fact that non-denatured proteins with different net charges migrate at different rates through the starch gel (or acrylamide gels or other supporting media such as cellulose acetate) under electric field.

In 1950s, protein electrophoresis supplied a new source of markers and permitted individuals to be identified as homozygotes or heterozygotes at a given locus (Hunter and Markert, 1957). Over the last 25 years, protein markers had been the main technique used for describing the genetic structure of natural populations (Lewonthin, 1991). The potential of using protein markers were widely recognized. First, genetic variation at a large number of nuclear loci could be studied with relatively easy, fast and cost-effective. Second, gene duplication resulting from ancestral tetraploidy and increasing of the number of loci could be examined with this technique as in salmoind genome reported by Allendorf and Thorgaard (1984). Third, the genetic basis for variation of protein loci could often be inferred directly from electrophoretic patterns because of the codominant expression of the enzyme loci. Forth, it was relatively easy for different laboratories to examine the same loci and used identical allelic designations such that the data sets from different laboratories can be combined.

Electrophoresis technique of allozyme and isozyme were used to examine

genetic variation in fisheries and aquaculture. Due to the differences of encoded loci, the term "allozyme" is related to different allelic forms of nuclear-encoded enzymes, whereas "isozyme" is related to different loci. Isozymes are functionally similar but separable form of enzymes, encoded by more than one locus (Markert and Moller, 1959). Isozymes have played a pre-eminent role in population studies of aquatic species for more than three decades.

Several studies had found that genetic variation in enzyme (or protein) loci was relatively low. In penaeid shrimps, examination of isozyme and allozyme variation indicated that both isozyme and allozyme showed low levels of genetic variation (Hedgecock, 1977; Lester, 1983; Sunden, 1991). In 1994, Garcia et al. examined allozyme variability and the results indicated a few polymorphism in cultured *P. vannamei.* In 1996, Sodsuk reported low level of genetic differentiation in Thai *P. monodon* using 46 allozyme loci.

The studies described above indicated the weakness of using enzyme (protein) as markers. This is because the information in the codons is degenerated such that there are usually several possible codons for a given amino acid. Therefore, a protein may be the product of a coding gene with fairly substantial changes variation in the DNA sequences but limited variation in the amino acid composition of the protein. From this reason, the enzyme (protein) markers can be used to examine only a specific set of genes within the total genome and cannot detect genetic variations that do not affect the amino acid sequences of a protein. Silent substitutions in the codons or in the noncoding regions cannot be detected with this technique. The other drawback of this technique is that it requires the protection of enzymes activities. Thus, the samples must be treated with care and stored under proper condition (Scopes, 1987; Acquaah, 1992).

1.6.2 DNA Studies

DNA is the basic genetic material found in cells of all organisms. It can be regained from both living and dead tissues. In many cases, only nanograms of DNA are needed for analysis when it is amplified by PCR (polymerase chain reaction). The molecule is quite stable as recognizable sequences remain intact for hundreds of millions of years (Cano et al., 1993).

1.6.2.1 Animal Mitochondrial DNA

One of the well studied genome in animals is mitochondrial DNA (mtDNA). MtDNA is the second major class of DNA found in mitochondria. The mitochondrial genome is a closed circular, double-stranded DNA molecule ranging in size from 15.7-19.5 kb, and is generally composed of 37 genes coding for 22 tRNAs, 2 rRNAs and 13 mRNAs specifying proteins involved in electron transport and oxidative phosphorylation (Lynch and Jarrell, 1993).

Mitochondrial DNA is regarded as a single locus because of the absence of genetic recombination. The small genome size and high copy number of mtDNA make it easy to be isolated. This unique features of mitochondrial DNA molecules have become a well-established and valuable tool for many applications in population biology (Moritz et al., 1987; Avise et al., 1989; Cruzan et al., 1993).

Animal mtDNA are commonly used to study genetic variation within and among species (Dowling et al., 1990). In addition, various studies have shown that animal mitochondrial DNA is widely employed in evolutionary studies because the rate of sequence evolution is greater than or equal to that of nuclear DNA (Hauswirth and Laipis, 1982; Cann and Wilson, 1983; Wu and Li, 1985; Boulding et al., 1993; Yang et al., 1994).

Nevertheless, the mitochondrial genome possesses a small non-coding

region known as the displacement loop region (D-Loop), an origin of replication for the mitochondrial genome, that is much more variable than the remaining genes of vertebrate mitochondrial genome. Therefore it is a very useful marker for the study of divergent populations or species (Upholt and Dawid, 1977; Hauswirth et al., 1984; Zullo et al., 1991; Hoelzel et al., 1994).

From the properties of mtDNA as described above, it is suitable for stock identification and evolution. However, there have been very few reports about mtDNA diversity and transmission in penaeid shrimps. In 1997, Alcivar-Warren et al. used RFLPs to show mtDNA haplotypes in offspring of viral infected *P. vannamei*. Restriction fragment length polymorphism (RFLPs) of the mitochondrial genome (Bouchon et al., 1994) and gene sequence data of the COI and/or 16S ribosomal genes (Machado et al., 1993) showed a surprising degree of genetic differences among species.

1.6.2.2 Nuclear DNA studies

Nuclear genomes are much larger than mtDNA, ranging from $< 10^6$ nucleotide bases in some bacteria to $>10^{11}$ in some plants. Nuclear DNA (nDNA) can be classified in several manners according to its function, structure, or location, etc. Many of these categories overlap, and a particular segment of nDNA may fall into several categories (Park and Moran, 1995) (Fig. 1.7).

Nuclear DNA contains both unique single copy and repetitive regions. Single-copy regions generally code for particular gene products. Non-repetitive DNA or single-copy DNA is a DNA sequence that is present only once in the haploid genome. Approximately 70 % of the mammalian genome contains repetitive DNA (Alberts et al., 1983). Repetitive DNA consists of a core sequence that is repeated in varying degrees. They may be made up of coding segments such as ribosomal RNA (rRNA) genes, or non-coding tandemly repeated units.

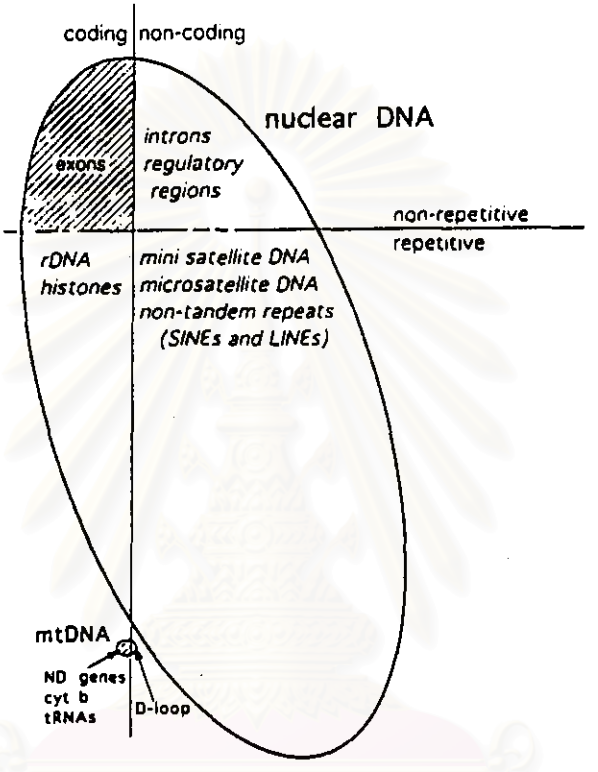

Fig. 1.7 Representation of DNA categories.

Satellite DNA is one of the repetitive DNA that contains tandemly repeated short nucleotide sequences (Britten and Kohne, 1968). The repeat units may be of tandem repeat (VNTR) loci, have shown high variation level giving rise to multiple alleles at high frequency (Nakamura et al., 1987; Kuhnkein, 1990).

Simple tandem-repetitive regions of DNA (or minisatellites) contains repeat units from ten to a hundred nucleotides long (Jeffreys et al., 1985) and are highly polymorphic (Taggart and Furguson, 1990). Polymorphisms of minisatellite loci are detected by cutting genomic DNA with a tetrameric restriction enzyme, separating the fragments by agarose gel electrophoresis, Southern blotting to nylon membrane, and hybridizing to a repeat-sequence probe to identify fragment length difference arising from variation in the repeat numbers (Jeffreys et al.,1985a; Burke 1989). Scoring of the band patterns with numbers of resolvable bands, even for technically excellent autoradiographs, are difficult (Piper and Parker, 1992). Therefore, minisatellite loci cause technical difficulties especially for comparisons of results from different gels.

The other type of repetitive DNA is microsatellites. Microsatellites consist of short motifs (usually 1-6 unit nucleotides per repeat) that repeat up to about 100 times. Microsatellite DNA has recently applied to study population genetic structure and pedigree analysis because a single locus may posses as many as 30 -50 alleles providing extremely high genetic polymorphism (Amos et al., 1993). Microsatellite DNA markers need to be developed from a species under investigation. Known primers are not likely to amplify the same locus across related taxa unless the microsatellite region is flanked by highly conserved sequences where priming sites are located (Fitzsimmons et al., 1995). Microsatellite loci are analyzed by amplifying the target region using PCR followed by polyacrylamide gel electrophoresis allowing resolution of alleles of different size.

Tassanakajon et al. (1998) isolated and characterized microsatellite markers in *P. monodon*. They found two micrasatellite loci, CUPmo18 and CUPmo386, that

were successfully used in population studies and parental determination in *P. monodon*. The number of markers were not sufficient to be used for genome mapping. Microsatellites isolated from penaeid shrimps are large in size (100 repeats or more), and have degenerate ends making difficulties for designation of primers (Moore et al., 1997).

1.7 The Polymerase Chain Reaction (PCR) and Randomly Amplified Polymorphic DNA (RAPD) Techniques

1.7.1 PCR

The isolation of thermal stable DNA polymerase (*Taq* polymerase) from the hot spring bacteria *Thermus aquatica* led to an efficient mean of amplifying short fragments of DNA by polymerase chain reaction (PCR) using automated thermal cyclers. The PCR permits in *vitro* selective amplification of a particular DNA region. The DNA region to be amplified must be flanked by regions of known sequences to which the oligonucleotide primers are annealed (Ehrlich, 1989; Rolfs et al., 1992). The reaction components required are single-strand DNA template, deoxynucleotide triphosphate (dNTPs) and a DNA polymerase enzyme.

Synthetic oligonucleotide primers, usually 20-30 base long, are complementary to the ends of the flanking regions of DNA template. The primers are combined with a small amount of genomic DNA in nanogram quality, free deoxynucleotides, a reaction buffer, and *Taq* DNA polymerase. A new DNA strand, complementary to the template can then be enzymatically synthesized under appropriate conditions. During a series of heating and cooling cycle, single-strand DNA templates are generated by heat-denaturation. The two primers can then anneal to their complementary sequences on either side of the DNA template during cooling step. Therefore, PCR allows DNA analysis to be performed from small

amount of DNA samples. The amount of target DNA doubles in each cycle and microgram quantities of DNA target can be easily obtained.

The commonly used reaction buffers in PCR containing Mg^{2+}, monovalent cations and some co-factors. The co-factors may help stabilizing the enzyme, influence the enzyme processivity and/or DNA melting temperature (T_m) (Saiki, 1988). The PCR itself requires three thermal steps: (1) denaturation of double-strand DNA at 92-96 °C (2) annealing of the primers to the complementary sites of the template at 45-72 °C and (3) extension of the primer from the 3' end by successive additions of dNTPs. DNA strand extension occurs at 72 °C. A cycle comprise of denaturation, annealing and extension steps; each step is defined by a fixed period of time. The replication in such cycles then leads to the amplification of DNA (see Fig 1.8).

The major difficulty of PCR is that it requires target DNA sequence information for the design of amplification primers, thereby often limiting its usefulness. The time and cost of obtaining the sequence information are prohibitive for many applications. For most uses of anywhere from one to a few hundred nucleotides long, and the variable numbers of PCR, one must determine the sequences of regions flanking at a given locus, and this can entail considerable effort when working with a new species. The use of "randomly chosen primers", described under RAPD below, does allow one to identify genetic markers relatively quickly in species for which extensive sequence information is not. available. The development of the RAPD-PCR technique can be used in rapid screening and sequencing of the inserts (Brown et al., 1993).

1.7.2 **Random Amplified Polymorphic DNA (RAPDs)**

Williams et al. (1990) and Welsh and McClelland (1990) have concurrently developed a PCR-based technique that employed a single, 10 base-long primer of

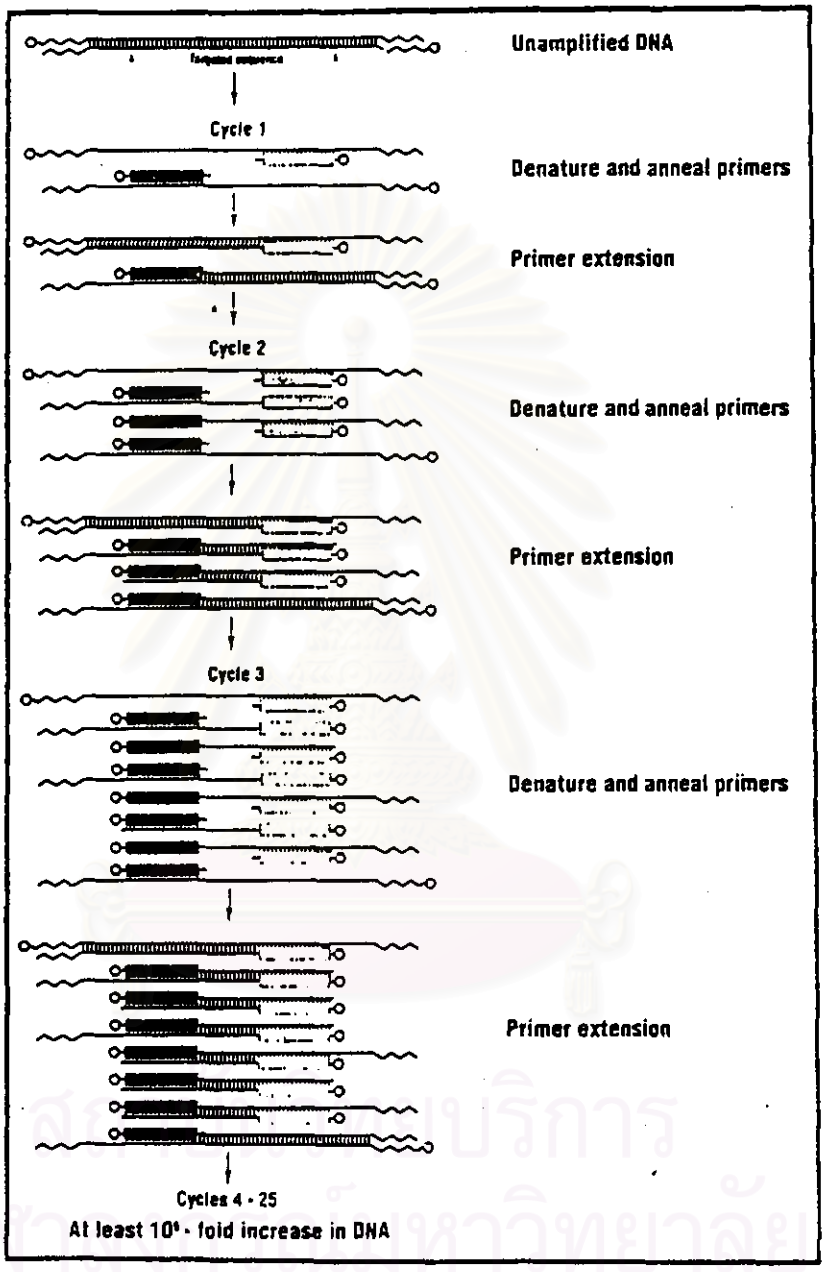

Fig. 1.8 Diagrammatic representation of the cycles of the polymerase chain reaction (PCR).

arbitrary DNA sequence with 50 -80 % G+C content. The primer anneals to DNA at one or more locations and the inverted regions are amplified. Some of the amplified regions are repetitive while others are single-copy DNA. Williams et al. (1990) and Welsh and McClelland (1990) suggested the term "random amplified polymorphic DNA" (RAPD) to describe this approach and demonstrated the use of RAPD markers for genome-linkage mapping in a variety of organisms. They also showed that the amplified fragments are Medelian inherited in the dominant fashion.

RAPD method is currently receiving a particular attention because of its extreme simplicity and require minimal amounts of genomic DNA (Brown et al. 1993). An arbitrary primed fingerprint is generated by subjecting a small amount of template DNA to PCR at relaxed stringency with the randomly selected PCR primer (with a few minor restrictions). A single decamer primer in a RAPD amplifies several bands from the genomic DNA of each individual duing to inverted primer annealing sites randomly located 50 to 300 bp apart (Johnson et al., 1994; Echt et al., 1992). Mutations in the primer annealing sites, e.g. insertions and deletions, can create dominant or, less frequentlly, codominant genetic variants. Divergence of even a fraction of a percent between two genomes often results in a different fingerprint pattern because a somewhat different set of sites in the genome have the best matches with the primer. PCR products that are shared between some individuals act as polymorphic markers. Each primer gives a different pattern of RAPD-PCR products. Polymorphism between individuals (or strains) is detected as difference between the pattern of DNA fragments amplified from the different DNAs using a given primer (Welsh et al., 1991; Micheli et al., 1994). Characterization of genomic DNA through identification and determination of such random polymorphic markers has proved a powerful application of DNA technology over the last decade. Therefore, the RAPD techniques has been widely used in molecular biology.

Analyses of RAPD markers do not require DNA cloning, or hybridization with

labeled DNA probes. The markers are simply scored from the ethidium bromide stained agarose gels following electrophoresis. RAPD technique is therefore, a simpler, less costly and less labor intensive than other DNA marker methodologies (Caetano-Anolles et al., 1991).

The RAPD technique has provided many useful markers that have been used to assist the breeding programs (Garcia and Benzie, 1995), to construct genetic maps (Faure et al., 1994; Das et al., 1996), and to identify cultivars (Echt et al., 1992; Novy et al., 1994). Some markers closely linked to important genes, e.g.various productivity traits and disease resistance (Ronald et al., 1992; Haley et al., 1993; Schachermayer, 1994). Other markers allowed the unequivocal identification of different lines or species and populations (Kambhampati et al., 1992; Castagnone-Sereno et al., 1994). The RAPD technique is also a tool for phylogenetic studies (Wei and Wang, 1995) and is being increasingly applied in the development of chromosome-, population-, species-, and genome-specific markers (Quiros et al., 1991; Wei and Wang, 1995).

In Penaeid shrimp, RAPD technique is used as a tool to identify genetic diversity. Garcia et al. (1994) evaluated the genetic diversity of cultured *P. vannamei* shrimp using three molecular genetic techniques, RFLPs, RAPD and allozyme variability. They reported that RAPD showed higher level of diversity among populations than RFLPs and allozyme variability. They also obtained a population-specific marker of individuals showing high growth. In 1995, Garcia and Benzie reported that RAPD markers will be useful in providing markers for breeding programs of *P. monodon*. They suggested that the levels of variation are likely to be adequate to obtain markers to assist selective breeding programs. Tassanakajon et al. (1997) showed a 950 bp specific RAPD marker found only in *P. monodon* originating from Satun-Trang provinces. They suggested that the RAPD marker can used as a potential population-specific marker for this species.

Approximately 2 years after serious viral epizootic caused by yellow-head and white spot viruses, good harvests of shrimp farms in spite of the presence of viral infections were reported. The infected shrimps, called " tolerance shrimps", were apparently healthy and grow normally. Although, the " tolerance hypothesis" had been recently proposed by Flegel (1997) to explain the different response to viral infection between normal and tolerance shrimps, questions were still remained because of the lack of knowledge on shrimp defense mechanisms.

The primary goal of this thesis was to obtain the genetic differentiation between the normal and viral tolerance *P. monodon* using basic molecular techniques. The information will lead to understanding of the mechanism of tolerance to viral infection and may help solving the viral problem in the shrimp industry.

The RAPD patterns of normal and viral tolerance *P. monodon* were compared. The DNA sequence of marker was determined. Specific primer pair was designed and used for PCR amplification of the shrimp genomes to differentiate between normal and viral tolerance *P. monodon*.