# CHAPTER I

# INTRODUCTION

## 1.1 General introduction

The giant tiger shrimp, *Penaeus monodon* has dominated production of farmed shrimp along with the Pacific white shrimp (*Litopenaeus vannamei*) and is one of the most economically important penaeid species in South East Asia. Farming of *P. monodon* has achieved a considerable economic and social importance in the region, constituting a significant source of income and employment.

In Thailand, *P. monodon* had been intensively cultured for more than two decades and had contributed approximately 60% of the total cultivated shrimp production. The reasons for this are supported by several factors including the appropriate farming areas without serious disturbing from typhoons or cyclone, small variable of seawater during seasons, and ideal soils for pond construction. Culture of *P. monodon* had increased a national revenue, therefore *P. monodon* was, until recently, the most economically important cultured species in Thailand.

Marine shrimp farms and hatcheries are located along the coastal areas of Thailand where Nakorn Sri Thammarat and Surat Thani in Peninsular Thailand are the major parts of shrimp cultivation. In addition, Chanthaburi (eastern Thailand), Samut Sakhon and Samut Songkhran (central region) also significantly contribute on the country production. The intensive farming system has resulted in consistent production of marine shrimp of Thailand. Thailand has been regarded as the leading shrimp producer of cultivated shrimp for over a decade (Table 1.1)

The production of *P. monodon* is largely constrained by the current dependency on wild-caught broodstock which varies in both quanlity and quantity. Recently, the farming of *P. monodon* in the region has significantly declined and its production is replaced by the farming of the introduced Pacific white shrimp, *Litopenaeus vannamei*. (Table 1.2) This is due to two major problems in *P. monodon* culture. Firstly, captive breeding of *P. monodon* is difficult, so its farming still relies mainly on wild

**Table 1.1** Total shrimp production (metric tons) from the aquaculture sector during 2000 – 2005 in the regian

| Country | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 |
|---|---|---|---|---|---|---|
| Thailand | 290,000 | 280,000 | 250,000 | 350,000 | 360,000 | 360,000 |
| Indonesia | 110,000 | 90,000 | 102,000 | 168,000 | 180,000 | 230,000 |
| China | 200,000 | 300,000 | 280,000 | 400,000 | 350,000 | 280,000 |
| India | 85,000 | 80,000 | 125,000 | 100,250 | 100,000 | 100,000 |
| Vietnam | 75,000 | 95,000 | 85,000 | 110,000 | 160,000 | 115,000 |
| Malaysia | 17,000 | 20,000 | 24,000 | 280,000 | 280,000 | 320,000 |
| Philippines | 30,000 | 20,000 | 30,000 | 30,000 | 35,000 | 35,000 |
| Total | 807,000 | 885,000 | 896,000 | 1,186,250 | 1,213,000 | 1,152,000 |

(Source: World shrimp farming, 2004)

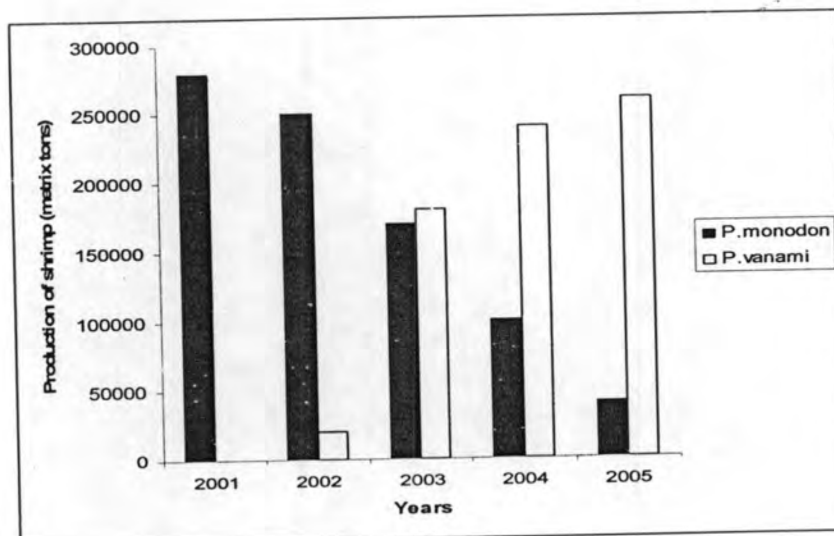


**Figure 1.1** A diagrame of production of *P.monodon* and *P.vanami* between 2001-2005 (Thai Society of Shrimp)

**Table 1.2** Giant Tiger Shrimp Export from Thailand between 2002 – 2005

| Month | 2002 | | 2003 | | 2004 | | 2005 | |
|---|---|---|---|---|---|---|---|---|
| | Quantity (MT) | Value (MB) | Quantity (MT) | Value (MB) | Quantity (MT) | Value (MB) | Quantity (MT) | Value (MB) |
| Jan | 11,345 | 3,894.80 | 13,360 | 4,671.66 | 11,746 | 3,145.25 | 5,625 | 1,510.76 |
| Feb | 10,821 | 3,763.43 | 11,453 | 3,916.07 | 12,606 | 3,356.08 | 4,193 | 1,098.32 |
| Mar | 12,578 | 4,260.60 | 11,594 | 3,890.99 | 4,610 | 1,332.69 | 4,537 | 1,248.03 |
| Apr | 12,308 | 4,026.19 | 11,230 | 3,895.05 | 5,782 | 1,785.85 | 3,603 | 968.76 |
| May | 14,655 | 4,946.44 | 12,594 | 4,120.20 | 7,082 | 1,937.04 | 4,313 | 1,194.83 |
| Jun | 15,545 | 5,468.02 | 12,446 | 4,089.82 | 8,414 | 2,780.45 | 6,623 | 1,749.64 |
| Jul | 14,285 | 5,019.06 | 14,055 | 4,563.97 | 9,902 | 3,142.45 | 6,857 | 1,885.88 |
| Aug | 17,295 | 5,741.69 | 15,731 | 5,036.36 | 8,327 | 2,595.77 | 6,678 | 1,870.81 |
| Sep | 19,808 | 7,196.31 | 17,988 | 5,611.11 | 10,498 | 3,358.83 | 7,339 | 1,946.05 |
| Oct | 21,265 | 8,041.29 | 18,057 | 5,358.97 | 10,853 | 3,321.91 | 6,864 | 1,857.45 |
| Nov | 18,939 | 6,982.56 | 11,857 | 3,454.28 | 9,797 | 2,997.04 | 6,618 | 1,817.32 |
| Dec | 12,206 | 4,485.87 | 10,832 | 2,974.41 | 8,904 | 2,458.51 | 6,162 | 1,605.64 |
| Total | 181,050 | 63,826.26 | 161,197 | 51,582.89 | 108,521 | 32,211.87 | 69,412 | 18,753.49 |

Source: Office of Agricultural Economics, Ministry of Agriculture and Cooperatives, 2006

broodstock. The annual production was reduced from approximately 200,000 MT to 69,412 MT in 2005 (Table 1.2) and about 30,000 MT in 2006.

### 1.2 Taxonomy of *P. monodon*

The giant tiger shrimp is taxonomically classified as a member of Phylum Artrhpoda; Subphylum Crustacea; Class Malacostraca; Subclass Eumalacostraca; Order Decapoda; Suborder Natantia; Infraorder Penaeidea; Superfamily Penaeoidea; Family Penaeidae, Rafinesque, 1985; Genus Penaeus, Fabricius, 1798 and Subgenus *Penaeus*. The scientific name of shrimp is *Penaeus monodon* (Fabricius, 1798) where the English common name is giant tiger shrimp or black tiger prawn (Bailey-Brook and Moss, 1992).

The external morphology of *P. monodon* and sex characteristics of male (petasma) and female (thelycum) are illustrated in Figure 1.2.

### 1.3 Ovarian development of *P. monodon*

In penaeid shrimp, the major part of ovaries is found within the cephalothorax area. The ovaries are paired, but partially fused in the cephalothoracic region, and consist of a number of lateral lobes. The intensity of the ovarian shadow is due to the different density of the ovaries and the pigmentation of the egg mass. The ovarian development of penaeid shrimp are classified to four different stages; underdeveloped stage (Stage I), developing or early vitellogenic stage (Stage II), nearly ripe or late vitellogenic stage (Stage III) and ripe or mature stage (Stage IV; Figure 1.3).

In an undeveloped stage, the ovaries either do not cast any shadow or a thin opaque line is seen along the length of the tail. At this point the ovaries are composed of a connective tissue capsule surrounding a soft vascular area containing oogonia, and accessory cells (called follicle or nurse cells, Figure 1.3A). The internal wall of the ovary capsule is lined with epithelial cells (called the germinal epithelium). Once the female is sexually mature, the germinal epithelium will produce oogonia by mitosis division throughout the reproductive life of the females.

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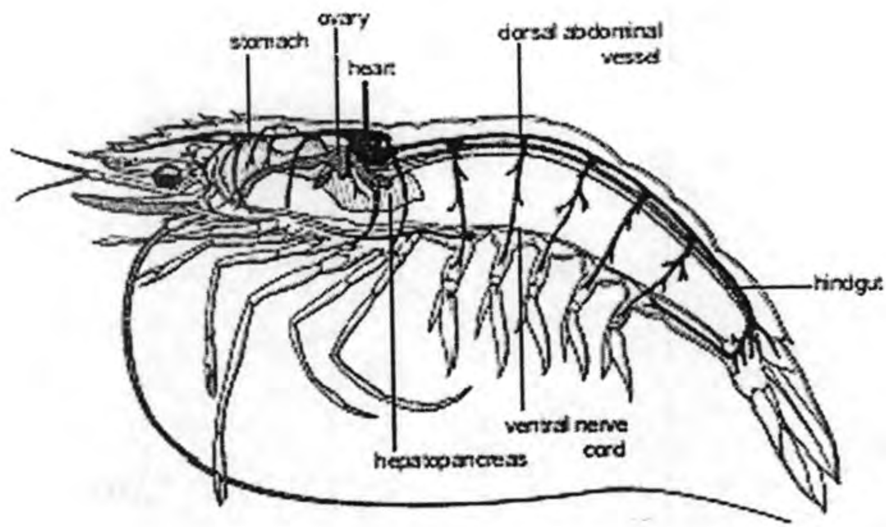
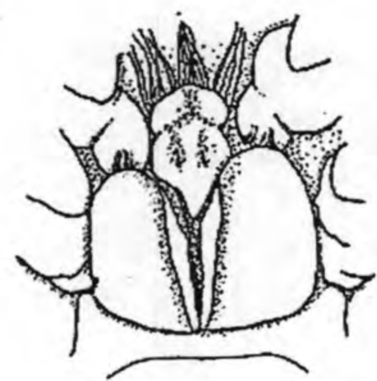



A.

B.



C.

**Figure. 1.2** External morphology of *P. monodon* (A). Sexes of juveniles and broodstok of penaeid shrimp can be externally differentiated by petasma of male (B) and thelycum of female (C)

The eggs develop from oogonia in an area known as the zone of proliferation. As the oogonia develop they increase in size and enter the first stage of meiotic division and henceforth are irreversibly destined to become haploid, with only one set of maternal chromosomes. At this point, although the developing eggs are increasing in size (Figure 1.3B), they are not as yet producing yolk, and are known as previtellogenic oocytes. At this stage the ovaries can be visualized with a light beam as a large centrally located opaque rope-like structure, and classified as the stage 2.

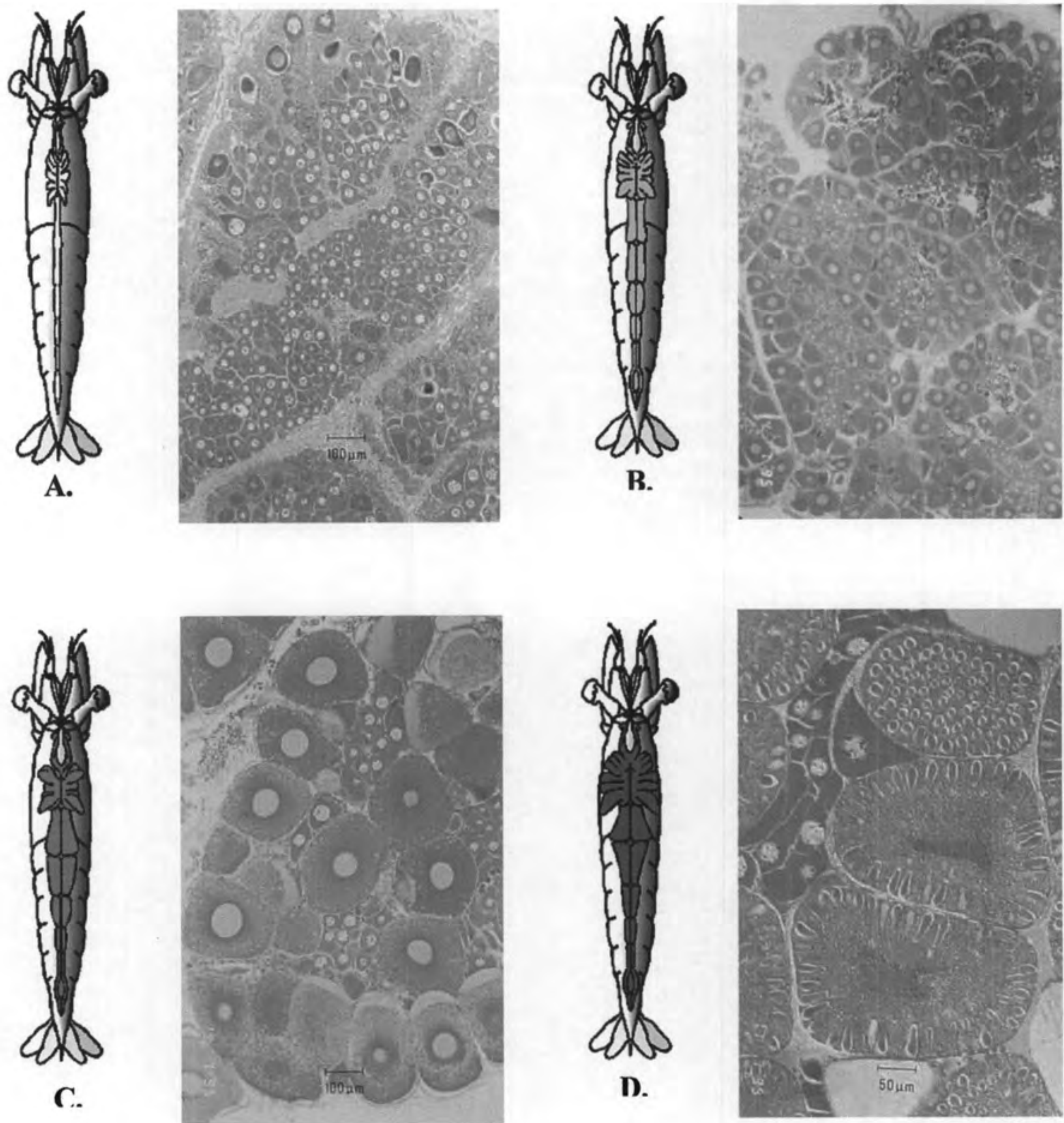



**Figure 1.3** Different ovarian development stages of *P. monodon.* **Panel A.**, the underdeveloped ovaries (Stage I), **B.**, the developing stage (Stage II), **C.**, the nearly ripe stage (Stage III) and **D.**, the ripe stage (Stage IV) (www.aims.gov.au/.../mdef/images/fig01-4a.gif).

As the oocytes develop, they migrate out towards the margins of the ovarian lobes in preparation for ovulation. During this migration, follicle cells are attached to the periphery of each oocyte. It is believed that the follicle cells produce the yolk that is internal in the oocytes in a process called vitellogenesis. As vitellogenesis proceeds, oocytes mature synchronously as yolk accumulates and develop a characteristic dark green colour as a result of deposition of carotenoid pigments. It is the carotenoid pigmentation that mainly causes the dark ovarian shadow during illumination of the female by the torchlight. The female is now in the stage 3 (Figure 1.3C).

By the end of vitellogenesis, the eggs develop cortical granules filled with a jelly-like substance destined to form part of the egg shell membrane after ovulation. At this time the shadow cast by the ovaries is large, resulting in a very distinct dark thick region extending the length of the abdomen, with an enlarged bulbous region directly behind the carapace, called the saddle. The saddle may not be as apparent in some broodstock. The female is now in a pre-spawning state and is regarded as the stage 4.

Penaeid shrimp are classified into two groups; open thelycum and closed thelycum species. The final phase of maturation, spawning, mating and their interrelationships differ significantly between the groups. The giant tiger shrimp, *P. monodon* is one of the closed thelycum species. Typically, mature males mate by insert in their spermatophore into the soft thelycum of newly molted immature females. Final maturation with germinal vesicle breakdown (GVBD) immediately precedes spawning in a closed thelycum species (Yano, 1988). Two phases are involved: the appearance of ripe ova. and germinal vesicle breakdown (GVBD) in preparation for fertilization after spawning (Figure 1.4).

Ovulation occurs when nuclei, shrunken during the late prematuration phase, have migrated to the peripheral cytoplasm of the oocytes. In the late phase of the maturation cycle, meiotic metaphase is arrested and remains visible just beneath the cytoplasmic membrane of the oocyte, indicating that GVBD is completed after ovulation.

Inmmediately after release from the female gonopore, the mature eggs, still in metaphase (for example in *Marsu. japonicus*), are fertilized by sperm released into the

seawater from the spermatophore held in the thelycum. Once begun, spawning is continuous, females releasing batches of eggs from the ripe ovaries and sperm from the spermatophore into the seawater, where fertilization takes place. Therefore, female shrimp have to repeat the process of molting, mating, and sexual maturation in order to achieve several spawnings during their life.

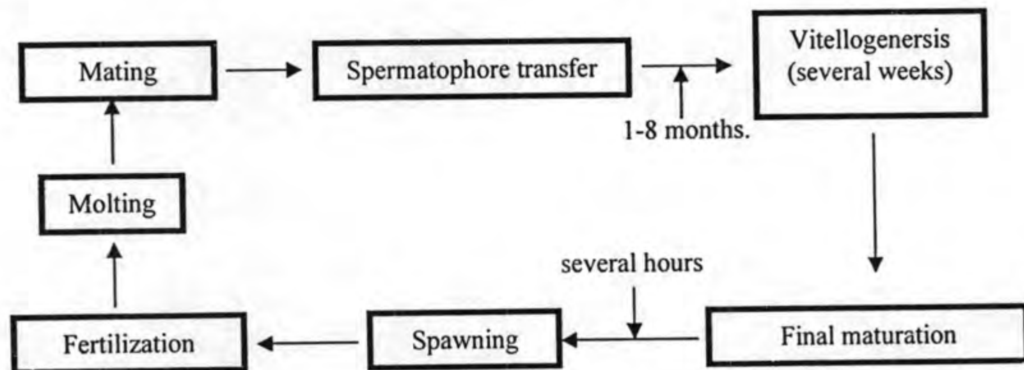



**Figure 1.4** Reproductive cycle of the close-thelycum penaeid shrimp.

### 1.4 Hormonal manipulation

Biological and physiological processes (growth, reproduction, body colour, and metabolism etc.) are hormonal controlled (Figure 1.5). Knowledge from shrimp endocrinology is necessary to develop the hormonal manipulation techniques in shrimp

Eyestalk hormones play the important role for regulating several physiological mechanisms and unilateral eyestalk ablation is practically used for induction of ovarian development and oviposition. The technique gives predictable peaks of

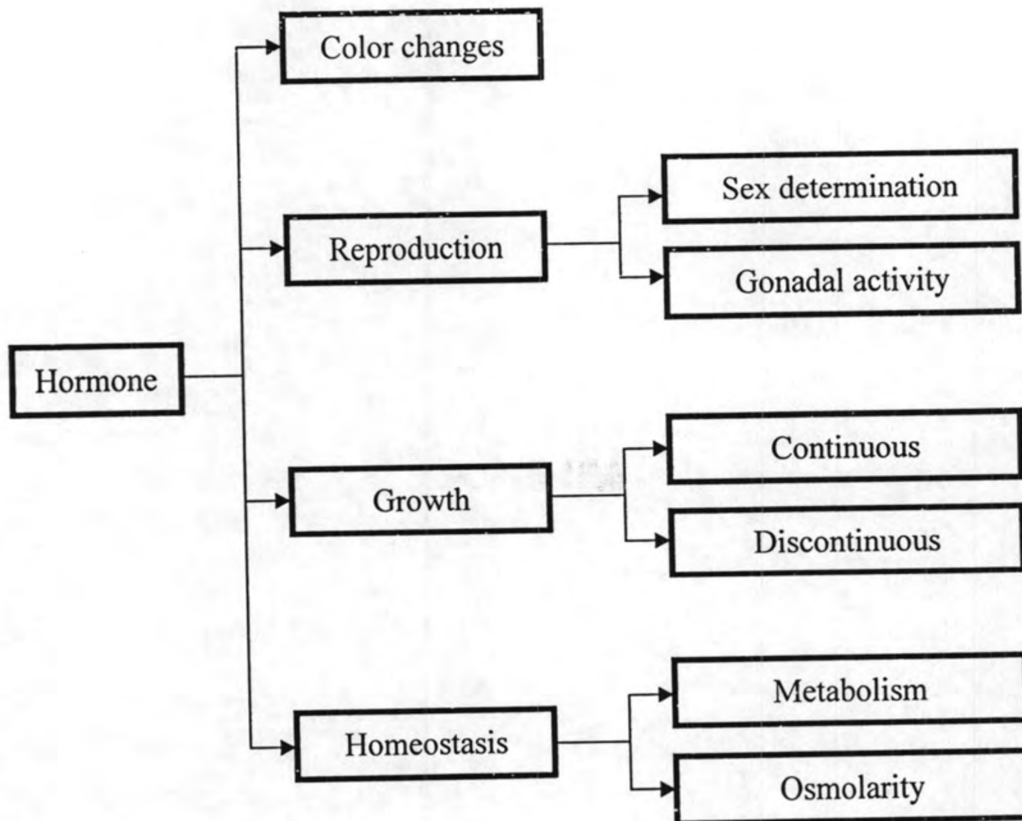



**Figure 1.5** Diagram illustrating the hormonal controls of physioiogical processes of penaeid shrimp.

maturation and spawning but many associated problems, which leads to an eventual loss in egg quanlity and causes high mortality and death (Benzie, 1998). Predictable maturation and spawning in captive shrimp without the use of eyestalk ablation is a long–term goal for the industry (Quackenbush, 1991).

Crustacean hyperglycemic hormone (CHH) is a member of a structurally related peptide family, which also includes molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH) and mandibular organ-inhibiting hormone (MOIH), it is the most abundant peptide in the eyestalk of crustacean (Chang et al., 1990) This hormone not only plays its major role in controlling the glucose level in the haemolymph, but is also significant to other processes such as ecdysteroid synthesis and ovarian maturation. Multiple forms of CHH have been reported. CHH has been isolated from several crustaceans such as crabs, (Kegel et al., 1989; Chung et al., 1998), lobster (Tensen et al., 1991), crayfishes (Kegel et al., 1991; Huberman et al., 1993), shrimp (Sithigorngul et al., 1999) as well as isopod (Martin et al., 1984).

The growth in crustaceans is not continuous because of the rigid exoskeleton. It is often shed to allow periodic growth. Molting is controlled by a complex interplay of hormones (Figure 1.6), in particular, the negative regulation of molt-inhibiting hormone (MIH) from the X-organ/sinus gland (XO/SG) complex which suppresses the synthesis or secretion of molting hormones (ecdysteroids) from the Y-organ (Fig 1.7).

MIH is classified as a member of the crustacean hyperglycemic hormone (CHH) family has been shown to inhibit the synthesis of the molting hormone, ecdysone, which release from the Y-organ of decapod crustacean keeps the animal in the intermolt stage that dominates its molting cycle. MIH is thus one of the major keys in mediating growth and reproduction.

Udomkit et al. (1999) reported the full length nucleotide and deduced amino acid sequences of Pem-CMG peptide, a member of crustacean CHH/MIH/GIH peptide family, in *P. monodon* by RACE-PCR technique. The two fragments constitute a combined cDNA length of 593 bp with a 77 bp overlapping region were identified. Sequence analysis reveals the presence of a 384 bp open reading frame (ORF). An ORF encoding Pem-CMG peptide from a cDNA of a single pair of the eyestalks was cloned. The deduced amino acid sequence of Pem-CMG peptide of *P. monodon* was compared with those in the CHH/MIH/GIH family from several crustaceans. Pem-CMG possessed a significant degree of homology to the peptides in this family (30% - 50% identity). The Pem-CMG peptide contains six conserved

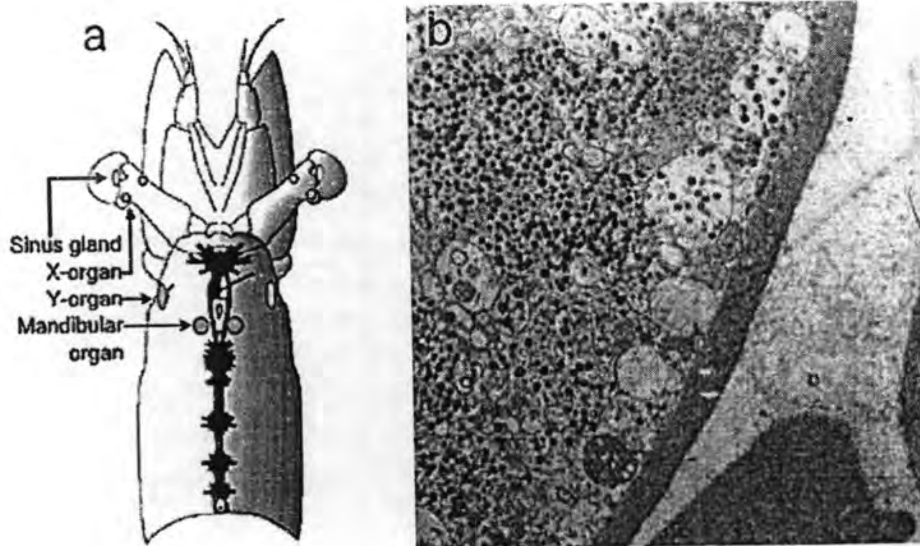



**Figure 1.6.** (a) A schematic diagram illustrating the major endocrine organs in shrimp. The sinus gland is composed of the terminals from neurons which have their cell bodies in the X-organ and brain. (b) Electron microscopy section (8500X) of the sinus gland demonstrating hormone filled vesicles (dark circles) which are fused and released their contents into the blood. (www.aims.gov.au/.../mdef/images/fig01-4a.gif)

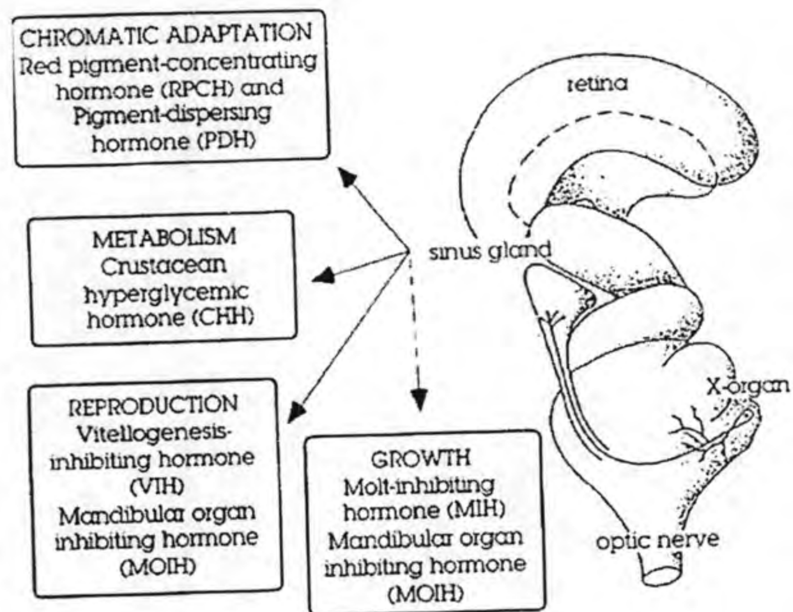



**Figure 1.7** Localization and hormones that control several systems from the sinus gland/X-organ complex of *P. monodon*. (Okumura, 2004)

cysteine residues which were found located at identical positions in all other members of CHH/MIH/GIH family. Most of the conserved amino acid residues were found in the N-terminal region whereas less homology was found in the C-terminal area.

Yodmuang et al. (2004) isolated cDNA encoding two types of *MIH*, *Pem-MIH1* and *Pem-MIH2* of *P. monodon* direct PCR amplification and PCR-based genome walking strategy. *Pem-MIH1* cDNA contained a 318 bp ORF encoded for a translated product containing 28 amino acids of the signal peptide and a putative mature *Pem-MIH* of 77 amino acids. *Pem-MIH1* and *Pem-MIH2* genes have the same structures. The interruption of the three exons by the two introns occurs at the same positions in both genes. RT-PCR was used to detect the expression of *Pem-MIH1* and *Pem-MIH2* in several tissues of *P. monodon* and found that *Pem-MIH1* was abundantly detected in the eyestalk and thoracic ganglia, whereas no transcript was present in the heart. A lower expression level was detected in the gill and the muscle, *Pem-MIH2* was detected only in the eyestalk but not in other tissues. A three dimensional structure of *Pem-MIH* is illustrated by Figure 1.8C.

The recombinant *Pem-MIH1* was expressed in *Pichia pastoris* as a secreted protein. Pem-MIH1 exhibited the ability to extend molting duration of *P. monodon* from 11.8 days to 16.3 days suggesting that Pem-MIH1 is responsible for molt-inhibiting function in this shrimp.

The late phase of ovarian maturation to form mature oocytes is called vitellogenesis. The process comprises the synthesis or accumulation or both of yolk proteins. The major component is vitellin (Vn) derived from a vitellogenin (VTG) precursor that are synthesized extra ovarian tissues or in the ovaries.

Penhouse (1943 and 1944 cited in Huberman, 2000) described that unilateral eyestalk ablation has been used to accelerate ovarian maturation and spawning in different shrimp species used as broodstock in aquaculture. The effect has been attributed to the presence of gonad inhibiting hormone, GIH (or vitellogenin inhibiting hormone, VIH) in the X- organ-sinus gland complex.

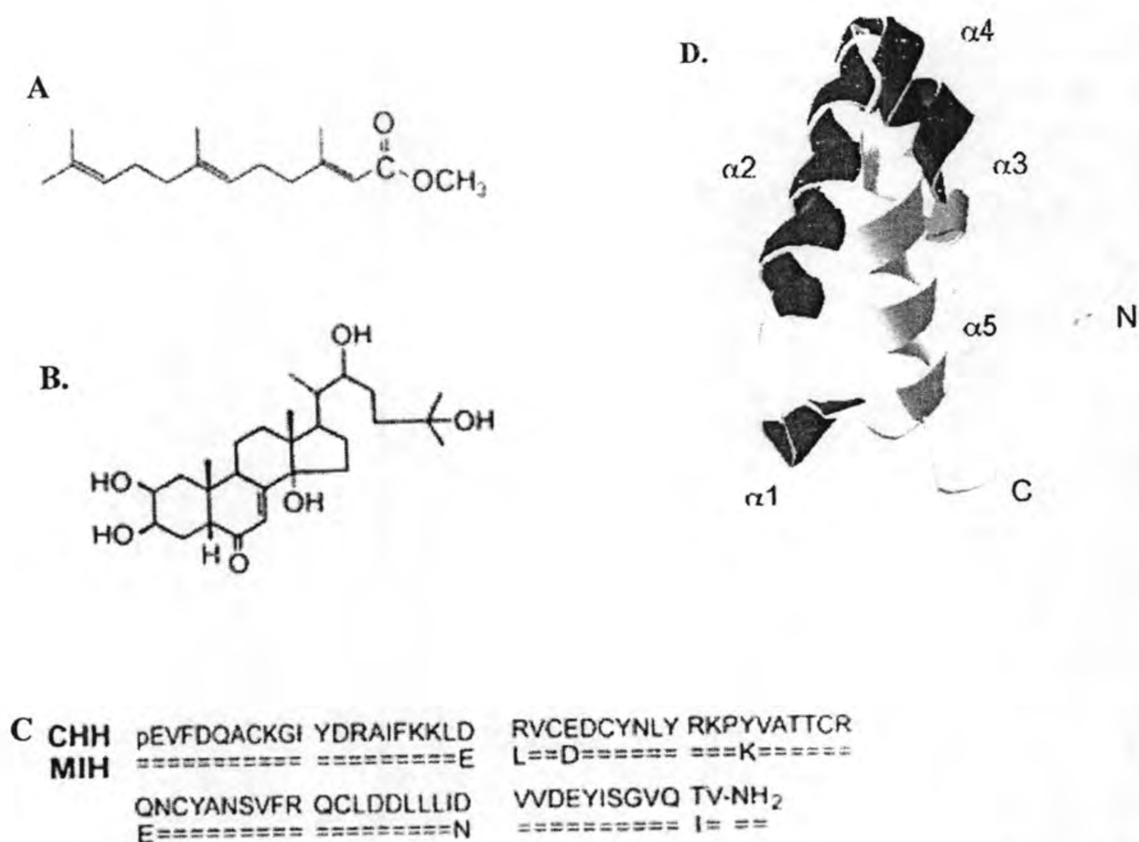



**Figure 1.8** The structure of methylfarnesoate (MF; panel A), ecdysteroid (panel B), deduced amino acid sequence of CHH and MIH (panel C) and the ribbon model of the homology-modeled structure of *Pem-MIH1* showing the *N* and *C* termini as well as the five helical domains (panel D) (www.aims.gov.au/.../mdef/images/fig01-4a.gif)

Identification and characterization of GIH is less successful than CHH and MIH. GIH was only reported in lobsters. Two isoforms of the GIH were isolated and sequenced by Soyez et al. (1991) from the sinus gland of the lobster *H. americanus*. Both consisted of 77 residues and MW of 9.135 Kda. GIH has not identified in penaeid shrimp.

Subsequently, Edomi et al., (2002) reported the identification, *in vitro* expression and localization of gonad-inhibiting hormone of the Norway lobster (*Nephrops norvegicus*). Recently, Ohira et al., (2006) reported that vitellogenin

ihhibiting hormone (VIH) was successfully cloned and characterized in *H. americanus*. Nevertheless, molecular mechanisms of this peptide hormone are still not understood.

Gonad stimulating hormones, GSH (or vitellogenin stimulating hormone, VSH), believed to be secreted by the supraesophageal and thoracic ganglia has been proposed to have the opposite effects of GIH (stimulates the gonadal maturation) of shrimp. However, this hormone has not been identified and characterized in any shrimp.

Ecdysteroids are known as the molting hormones in crustacean and insects and, in crustacean, the inactive forms are secreted and converted to 20-hydroxyecdysone by the Y-organ. Ecdysteriods stimulate vitellogenesis in some insects. However, the levels of ecdysteriods in hemolymph of *M. rosenbergii* were not related to vitellogenesis and showed no distinct relation to the molt cycle suggesting that ecdysteroids are not involved in vitellogenesis in the giant freshwater prawn (Okumura and Aida, 2000).

MF is structurally related to the juvenile hormone and synthesized by mandibular organ (MO). MF has been implicated in the regulation of crustacean development and reproduction in conjunction with eyestalk molt inhibiting hormones and ecdysteroids. The presence of MF in the medium for culturing of ovarian tissue *in vitro* of *L. vannamei* resulted in a significant increase in sizes of oocytes. In contrast, MF inhibits ovarian development in the tadpole shrimp, *Troops longicaudatus* (Tsukimura et al. 2006).

Laufer et al. (1998) reported stimulating effects of MF on ovarian maturation in the red swamp crayfish *Procambarus clarkii* in three different trials of MF administration. After 30 days of treatment, the ovaries of pre-reproductive females were 2- to 10-fold larger and were in the later stages of vitellogenesis than those of the controls and they were further along in maturation. Similar and statistically significant results were observed in a second 30-day trial, which was begun during the middle of the vitellogenic cycle. The control ovaries were white or yellowish colored but ovaries from the MF-fed groups were larger and showed a dose response for

which the 1-μg MF group had tan colored ovaries. A group receiving the higher dose of 2 μg MF had dark brown to black colored ovaries.

Farnesoic acid *O*-methyltransfcrase (*FAMeT*) catalyzes the methylation of farnesoic acid (FA) in the terminal step of MF synthesis. A schematic diagram of regulatory mechanism of vitellogenersis in shrimp is shown in Figure 1.9.

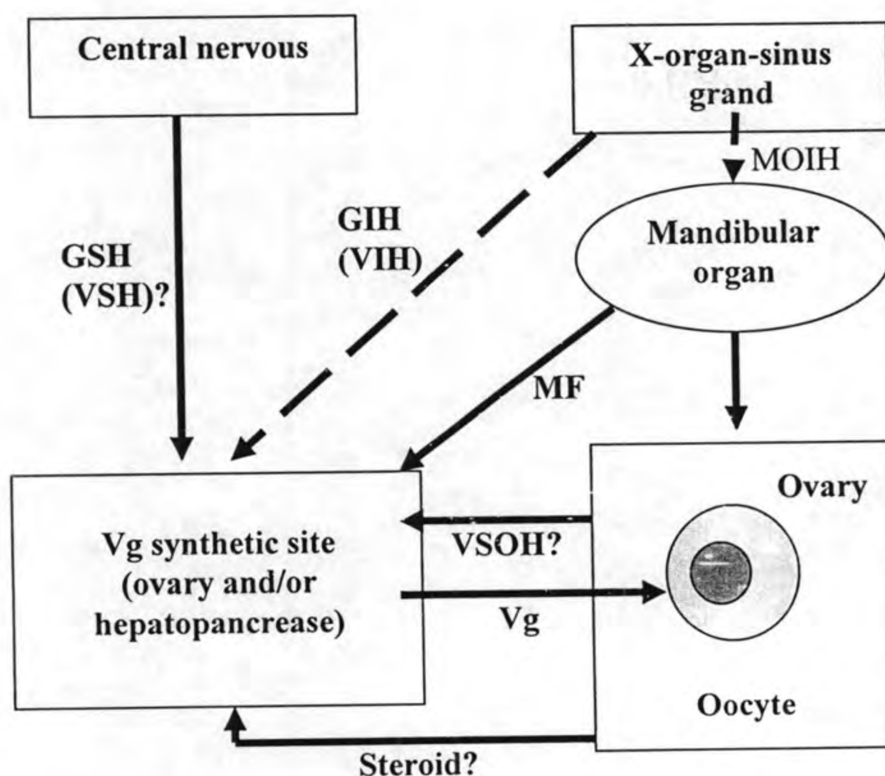



**Figure 1.9** Schematic diagram of the endocrine control of vitellogenesis in shrimp (Okumura, 2004) GIH: gonad inhibiting hormone, GSH: gonad stimulating hormone, MF: methyl farnesoate, MOIH: mandibular organ inhibiting hormone, Vg: vitellogenin, VIH: vitellogenesis-inhibiting hormone, VSH: vitellogenesis stimulating hormone

Biogenic amines (e.g serotonin or 5-HT, epinephrine and dopamine) and peptide neuroregulators are known to modulate the release of neuropeptide hormones from the sinus gland. Injections of serotonin and dopamine antagonist, spiperone (25 $\mu g\ g^{-1}$ body weight + 1.5 or 5 $\mu g\ g^{-1}$ body weight) induced ovarian maturation and spawning in wild *L. stylirostris* and pond reared *L. vannamei* (Alfaro et al., 2004).

Meeratana et al. (2006) investigated effects of serotonin on ovarian development in *M. rosenbergii*. Adult female prawns at the ovarian stage I (spent) were injected with 5-HT at 1, 5, 10, 20 and 50 $\mu g\ g^{-1}$ body weight (BW) intramuscularly on days 0, 5 and 10, and sacrificed on day 15. The low-dose, especially at 1 $\mu g\ g^{-1}$ body weight caused prawns to exhibit a significant increase in gonadosomatic index ($5.79 \pm 0.09\%$) as compared to the control (1.49%). The ovaries of most of these prawns could develop to stage IV (mature) and contained synchronously mature oocytes while most of the control ovaries remained at stage I and II. The medium- and high-dose treated prawns exhibited ovaries that could reach stages III and IV and contained various types of oocytes of different maturity. Pretreatment with 5-HT receptor antagonist, cyproheptadine (CYP), at 10 $\mu g\ g^{-1}$ body weight before 5-HT injection significantly suppressed the effect of 5-HT. Intramuscular injection of the culture medium of thoracic ganglion preincubated with 1 $\mu g\ ml^{-1}$ of 5-HT into CYP-pretreated prawns resulted in the increase of ovarian index about 5-6 times greater than in the control. The ovaries of most prawn could develop up to stage IV and contained synchronously developed vitellogenic and mature oocytes.

Effects of exogenous 5-HT on the reproductive performance of *P. monodon* was examined. 5-HT solution was injected into domesticated *P. monodon* broodstock at 50 $\mu g\ g^{-1}$ body weight and ovarian maturation and spawning were recorded. The levels of 5HT in ovaries were measured by ELISA. The 5-HT-injected *P. monodon* developed ovarian maturation and spawning rate at the level comparable to that of unilateral eyestalk-ablated shrimp. Hatching rate and the amount of nauplii produced per spawner were also significantly higher in the 5-HT-injected shrimp, compared to the eyestalk-ablated shrimp. 5-HT-positive reactions were found in the follicular cells of previtellogenic oocytes, in the cytoplasm of early vitellogenic oocytes and on the cell membrane and cytoplasm of late vitellogenic oocytes. 5-HT in the ovary was

present at 3.53 ± 0.26 ng/mg protein level in previtellogenic stage and increased to 17.03 ± 0.57 ng/mg protein level in the mature stage of ovaries (Wongpraset et al., 2006).

Recently, a putative 5-HT receptor from the ovary of *P. monodon* was cloned and characterized. It consisted of 2291 nucleotides, encoding a putative 5-HT1Pem receptor protein of 591 amino acids. The putative 5-HT1Pem receptor is expressed in all tissues examined and is constitutively expressed in the ovary during ovarian maturation and the spent phase. Polyclonal antibodies against the third intracellular loop (i3 loop) of the 5-HT receptor showed that the 5-HT1Pem receptor protein was expressed in the trabeculae of ovarian stages 1 and 2 but on the cortical rods and surrounding the oocyte membrane of stages 3 and 4 (Ongvarrasopone et al., 2006).

## 1.5 Molecular technique used for isolation and characterization of genes in this thesis

### 1.5.1 PCR

The introduction of the polymerase chain reaction (PCR) by Mullis et al. (1987) has opened a new approach for molecular genetic studies. This method is technique for enzymatically replicating DNA without using a living organism, such as *E. coli* or yeast and is a method using specific DNA sequences by the two oligonucleotide primers, usually 18 - 27 nucleotides in length. Million copies of the target DNA sequence can be synthesized from the low amount of starting DNA template within a few hours.

The PCR reaction components are composed of DNA template, a pair of primers for the target sequence, dNTPs (dATP, dCTP, dGTP and dTTP), PCR buffer and heat-stable DNA polymerase (usually *Taq* polymerase). The amplification reaction typically consists of three steps; denaturation of double stranded DNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal temperature, and extension of the annealed primers by heat-stable DNA polymerase. The cycle is repeated for 30 - 40 times (Figure 1.10). The amplification product is determined by agarose or polyacrylamide gel electrophoresis.

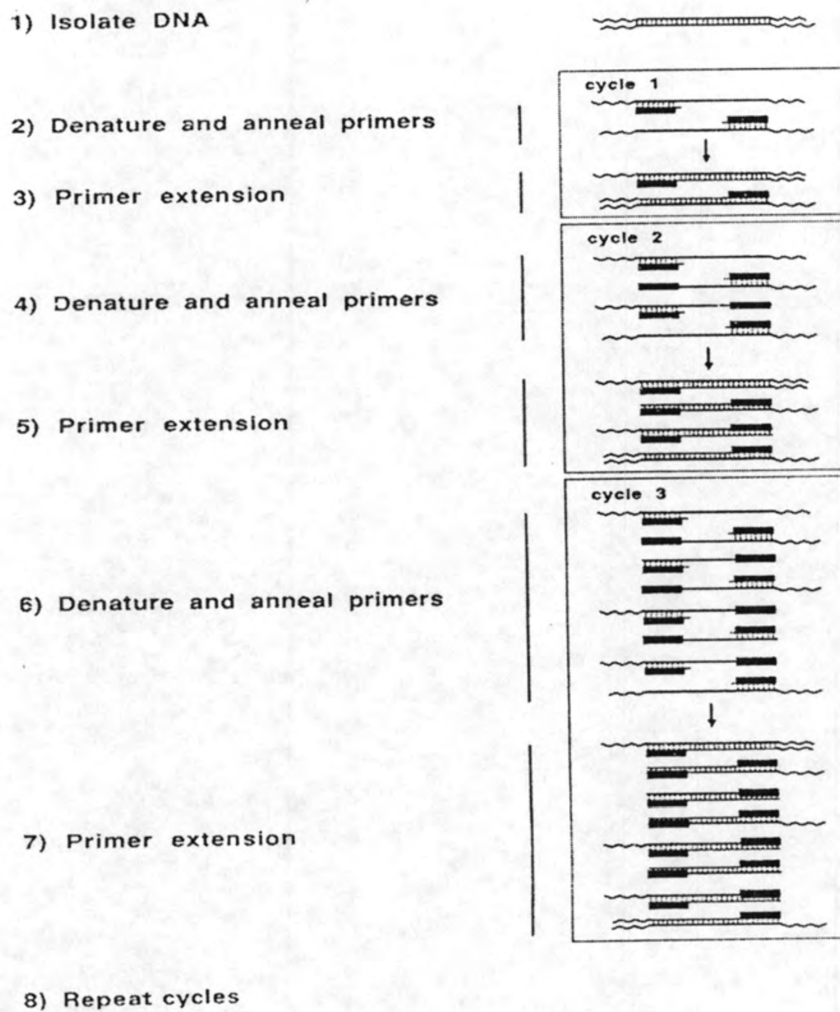



**Figure 1.10** General illustration of the polymerase chain reaction (PCR) for amplification of the target DNA.

### 1.5.2. Expression Sequence Tag (EST) analysis.

ESTs are large-scale single-pass sequences of randomly picked clones from a cDNA library usually constructed from mRNA at a particular developmental stage and/or tissue. This method has been widely employed for discovering novel and uniquely expressed genes, and for characterizing the gene expression profiles of several tissues.

The general principles for construction of a cDNA library (Figure 1.11) begin with purification of the target mRNA that is reverestranscribed to the first-strand cDNA. This step is catalyzed by reverse transcriptase using the oligo (dT) primer as the synthesizing primer. The second-strand DNA is then copied from the first-strand cDNA using *E. coli* DNA polymerase I. The double-strand cDNA is ligated to adapter and subsequently to an appropriate vector using T4 DNA ligase. The recombinant vector-cDNA molecules are packaged (λ vector) *in vitro* and transfected to the appropriate host. If a plasmid is used recombinant plasmid is transformed into *E. coli* host cells to generate a cDNA library.

ESTs can be sequenced from either 5′ or 3′ ends of cloned cDNA. The 3′ end of the cloned insert is usually marked by the poly A stretch which is often problematic for thermostable polymerase sequencing, and sequencing through poly T can reduce the length and quality of the subsequent sequence. Nevertheless, 3′ UTR usually exhibit high polymorphism and is a promising location for SNP identification. The 5′ ESTs have the advantage of being more likely to include some of the open reading frame (ORF) of the cDNA and thus facilitate identification of the encoded product.

EST sequences are used as the tag to homology search through the sequence data in the GenBank (Altschl et al., 1997). The Blast*N* program uses nucleotide sequence to compare against the NCBI nucleotide database whereas the BLAST*X* uses the translated protein products to compare against the NCBI protein database in all possible 6 reading frames. Sequences are considered to be significantly matched when the possibility value (E-value) is less than $10^{-4}$ and the match length is > 100 nucleotides for Blast*N* and a match length is > 10 amino acid residues for Blast*X*, respectively, (Anderson and Brass, 1998).

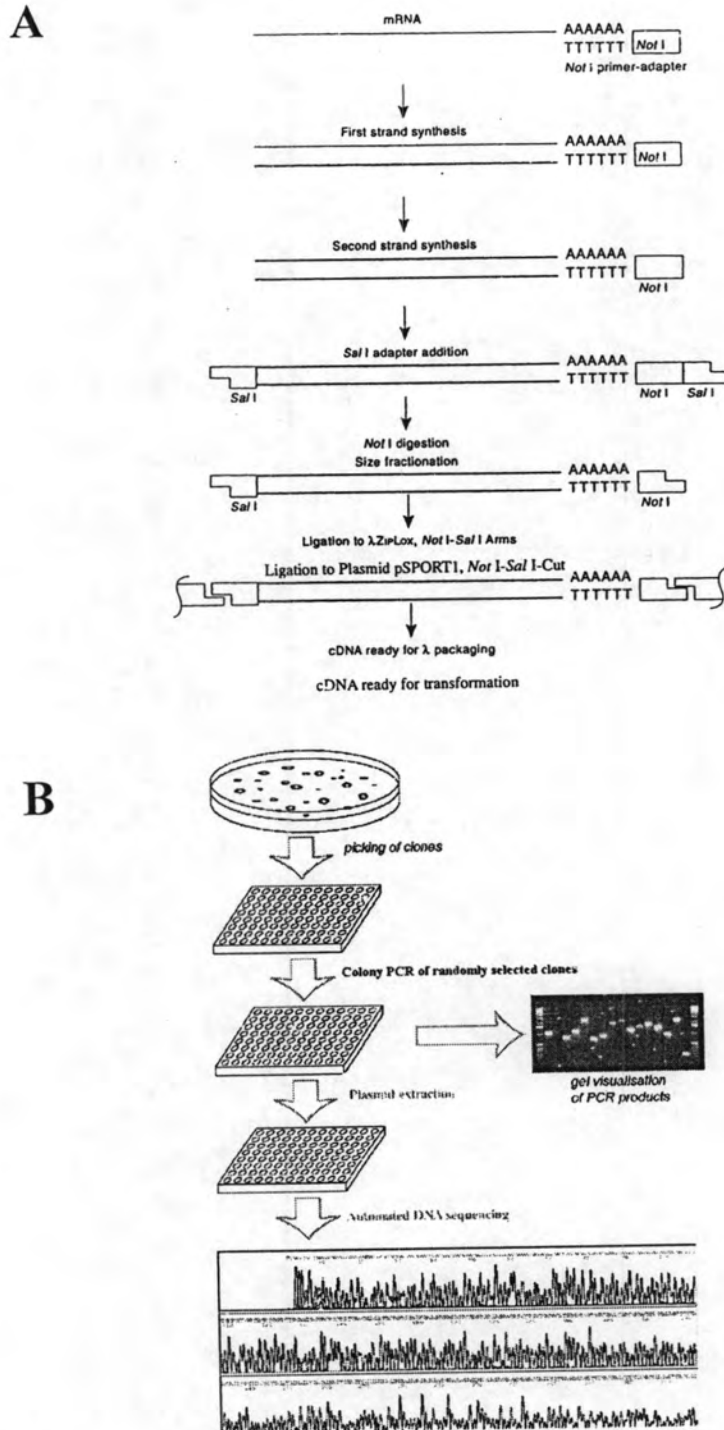



**Figure 1.11** Overview for construction of cDNA inserts (A) and automated DNA sequencing (single-pass) of randomly selected cDNA clones (the entire process simply called EST analysis).

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EST analysis is an important tool for several applications. They have mainly applied for rapid gene discovery of genes, comparative genomics and functional genomics in various organisms. After characterization and annotation, cDNA or designed oligonucleotides of transcripts can be further used for microarray analysis. Construction of genetic linkage maps and/or physical maps of interesting species can be carried out by development and sequencing of EST-derived markers using genomic DNA of species under investigation (Liu and Cordes, 2004).

### 1.5.3 Suppression subtractive hybridization (SSH)

Suppression subtractive hybridization is a powerful technique to compare two populations of mRNA to obtain clones of genes that are expressed (or differential expressed) in one population but not in the other. Although there are several different methods, a CLONTECH PCR select cDNA subtraction method is convenient for generation of differentially expressed sequences (Fig. 1.12).

First, cDNA is synthesized from two types of mRNA populations and cDNA that contains specific (differentially expressed) transcripts is regarded as where the tester, and the reference cDNA is regarded as the driver. The tester and driver are digested with *Rsa* I. The tester cDNA is divided to two portions, and each is ligated with different cDNA adaptor. The ends of adaptor do not have a phosphate group, therefore only one strand of each adaptor attachs to the $5'$ ends of cDNA. The two adaptors have stretches of identical sequences to allow annealing of the PCR primer once recessed ends have been filled in.

Tester and driver cDNAs are hybridized. In the first hybridization, an excess of the driver is added to each tester. The samples are heat-denatured and allow to anneal Differential expressed transcripts are then enriched. During the second hybridization, template for PCR amplification is generated from differentially expressed transcripts. Two rounds of suppression PCR was carried out and only differentially expressed transcripts are amplified exponentially. Subtractive cDNA products are then cloned into the T-vector. Positive clones are characterized by hybridization or sequencing

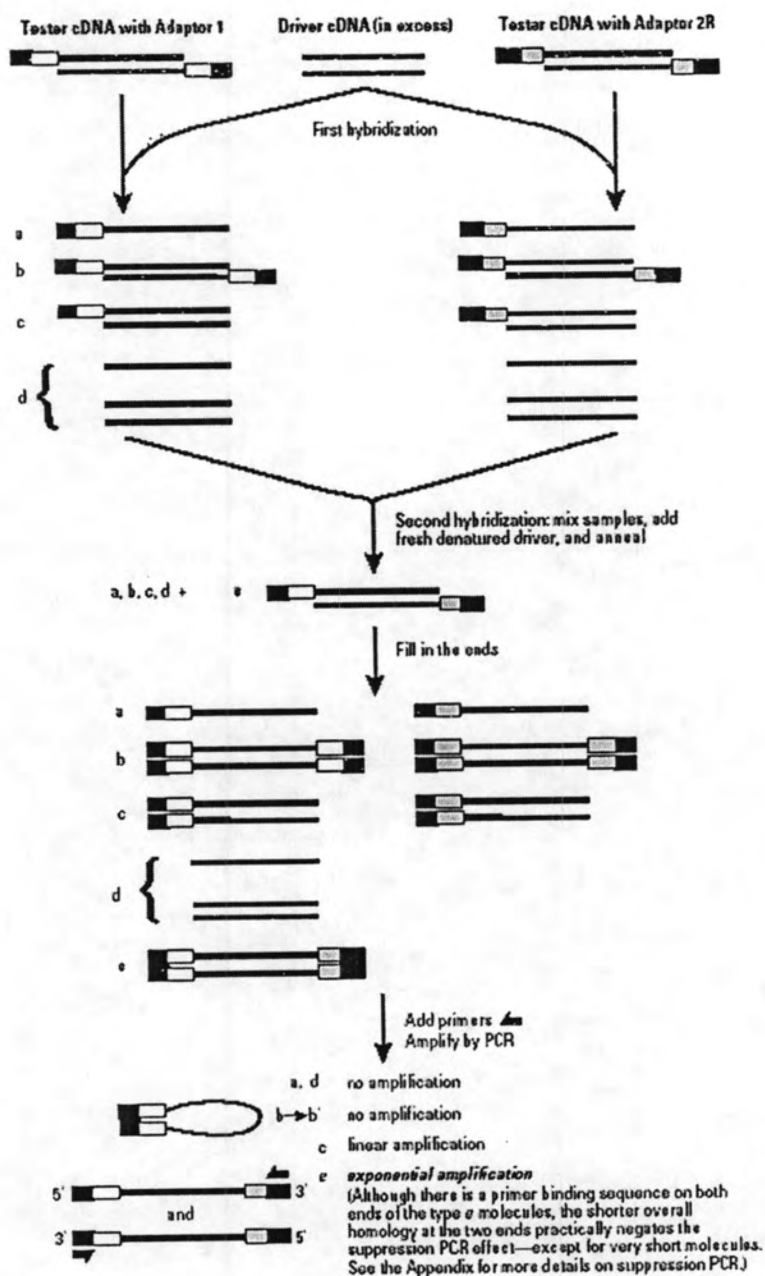


**Figure 1.12** Overview of the Clontech PCR-Select$^{TM}$ procedure. The cDNA in which specific transcripts are to be found is called "tester" and the reference cDNA is called "driver".

### 1.5.4 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a comparable method of conventional PCR but the first strand cDNA template rather than genomic DNA was used as the template in the amplification reaction. (Figure 1.13). It is a direct method for examination of gene expression of known sequence transcripts in the target species. The template for RT-PCR can be the first stranded cDNA synthesized from total RNA or poly $A^+$ RNA. Reverse transcription of total RNA can be performed with oligo(dT) or random primers using a reverse transcriptase The product is then subjected to the second strand synthesis using a gene-specific forward primer.

RT-PCR can also be used to identify homologues of interesting genes by using degenerate primers and/or conserved gene-specific primers from the original species and the first strand cDNA of the interesting species is used as the template. The amplified product is further characterized by cloning and sequencing.

Semi-quantitative RT-PCR is an quantitative approach where the target genes and the internal control (e.g. a housekeeping gene) were separately or simultaneously amplified using the same template. The internal control (such as *β-actin*; *elongation factor*, *EF-1α* or *G3PDH*) is used under the assumption that those coding genes are transcribed constantly and independently from the extracellular environment stimuli and that their transcripts are reverse transcribed with the same efficiency as the product of interesting transcript.

### 1.5. 5 Single strand conformational polymorphism (SSCP) analysis

Single-strand conformational polymorphism (SSCP) analysis was originally described by Orita et al (1989). SSCP is one of the most widely used for the detection of mutations and variation of the DNA (deletions, insertions and single nucleotide polymorphism, SNP). The amplified PCR product (usually less than 400 bp in length) is denatured and loaded into a low cross-link non-denaturing polyacrylamide gel (with or without glycerol supplementation). The principle of this technique relies on different mobility due to differential folding of the single strand DNA (Figure 1.14).

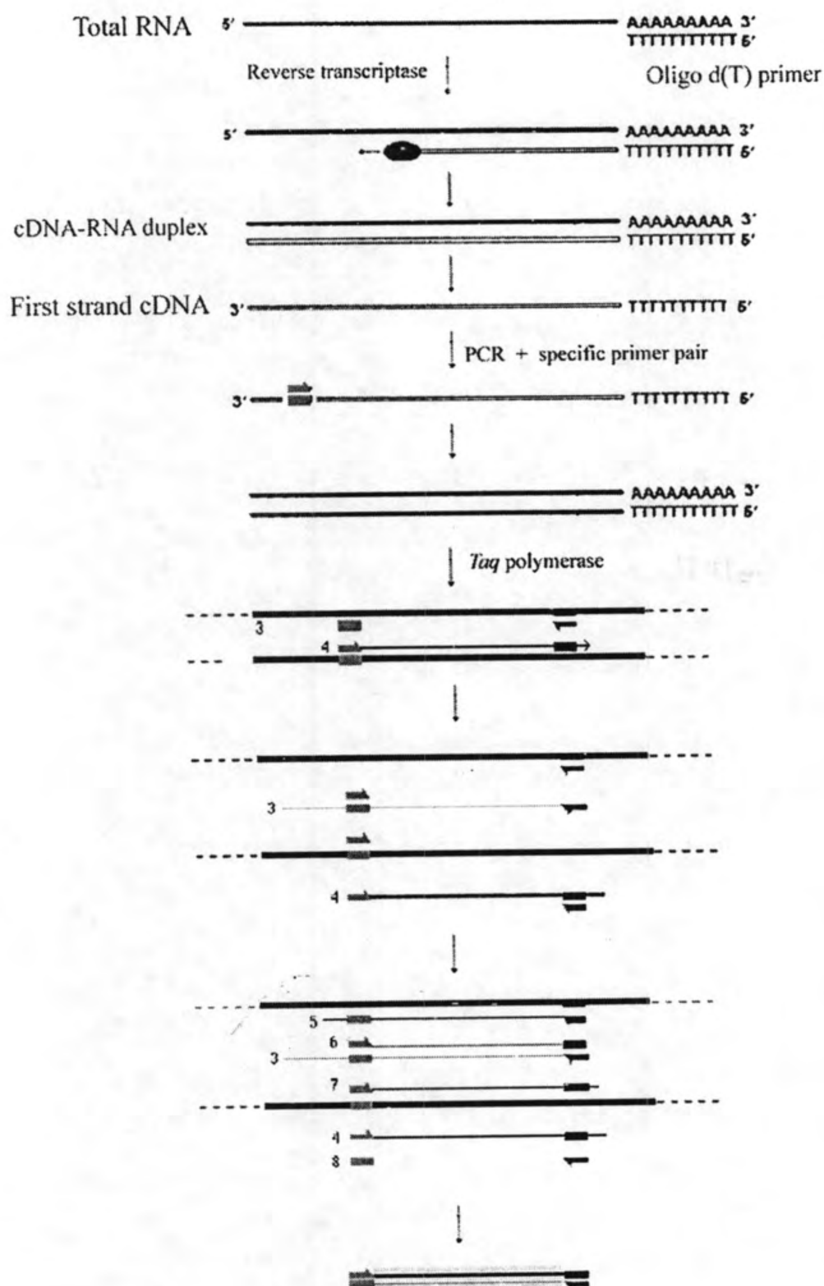


**Figure 1.13** Overall concepts of RT-PCR. During the first strand cDNA synthesis, an oligo d(T) (or random primers) primer anneals and extends from sites present within mRNA. The second strand cDNA synthesis primed by the 18 – 25 base specific primer proceeds during a single round of DNA synthesis catalyzed by thermostable DNA polymerase (e.g. *Taq* polymerase).

The major advantage of SSCP is that many individual PCR products can be screened for variability analysis simultaneously. Heteroduplexes can occasionally resolve from homoduplexes and give additional information on the presence of variants. Therefore, SSCP is regarded as one of the potential techniques that can be used to detect low polymorphism in various species prior to confirmation of the results by nucleotide sequencing. The other advantage of SSCP is that small PCR amplicons are required. This small sizes of PCR products are relative easy to amplify.

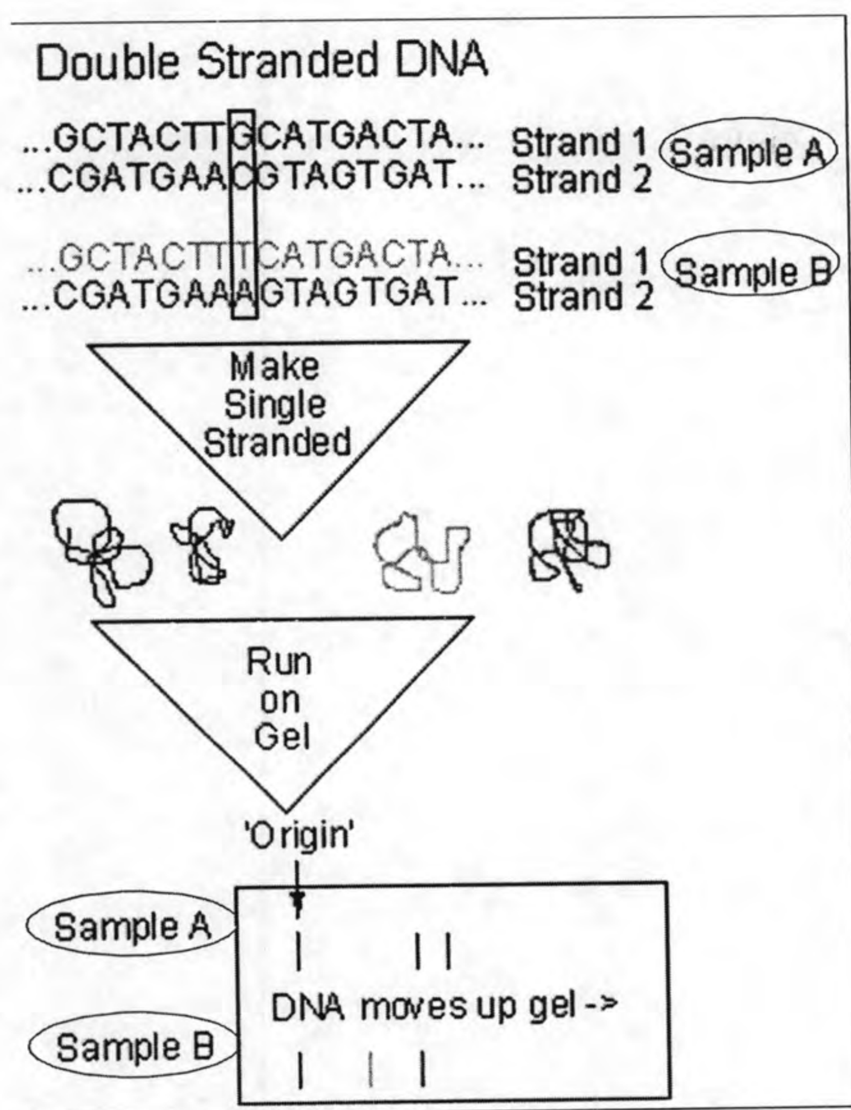


**Figure 1.14** A schematic diagram illustrating principles of SSCP analysis.

Nevertheless, the disadvantages of SSCP are reproducibility of the technique because SSCP patterns are affected by temperature and degree of cross-linking. Additionally, multiallelic patterns of some nuclear DNA markers may make the SSCP patterns complicated for precise estimation of allele frequencies precisely.

### 1.5.6 Rapid amplification of cDNA ends-polymerase chain reaction RACE-PCR

RACE-PCR is the common approach used for isolation of the full length of characterized cDNA. Using SMART (**S**witching **M**echanism **A**t 5′ end of **R**NA **T**ranscript) technology, terminal transferase activity of Powerscript Reverse Transcriptase (RT) adds 3 - 5 nucleotides (predominantly dC) to the 3′ end of the first-strand cDNA. This activity is harnessed by the SMART oligonucleotides whose terminal stretch of dG can anneal to the dC-rich cDNA tail and serve as an extended template for reverse transcriptase. A complete cDNA copy of original mRNA is synthesized with the additional SMART sequence at the end (Fig. 1.6).

The first strand cDNA of 5′ and 3′ RACE is synthesized using a modified oligo (dT) primers and serve as the template for RACE PCR reactions. Gene specific primers (GSPs) are designed from interested gene for 5′- RACE PCR (antisense primer) and 3′-RACE PCR (sense primer) and used with the universal primer (UPM) that recognize the SMART sequence. RACE products are characterized. Finally, the full length cDNA is constructed.

A

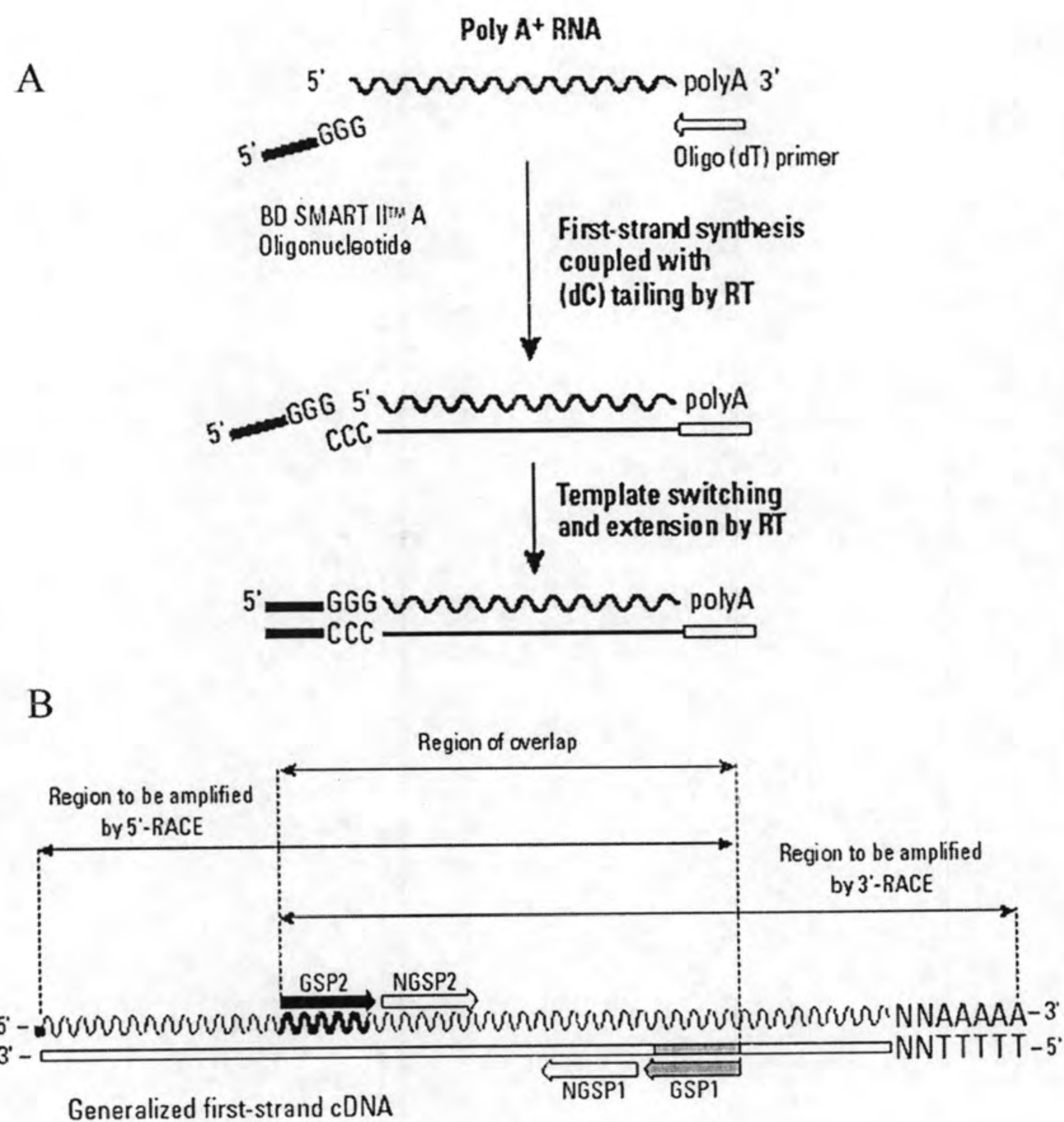


**Figure 1.15** Overview of the SMART$^{TM}$ RACE cDNA Amplification Kit.

A. Mechanism of SMART cDNA synthesis. First strand synthesis is primed using a modified oligo (dT) primer. After reverse transcriptase reaches the end of the mRNA template, it added several dC residues. The SMART II A Oligonucleotide annels to the tail of the cDNA and serves as an extended template for PowerScriptRT.

B. Relationships of gene-specific primers to the cDNA template. This diagram shows a generalized first strand cDNA template.

### 1.6 The importance for domestication of *P. monodon*

Farming of *P. monodon* presently relies almost entirely on wild-caught broodstock for the seed supply because breeding of *P. monodon* in captivity is extremely difficult. This open reproductive cycle and reliance on wild stocks of *P. monodon* results in heavy exploitation of female broodstock from wild populations.

The lack of high quality wild and/or domesticated broodstock of *P. monodon* has possibly caused an occurrence of a large portion of stunted shrimps at the harvest time (3-5 g rather than approximately 25 g body weight at 4 month cultivation period). As a result, the farmed production of *P. monodon* has significantly decreased since the last few years.

Progress in genetic and biotechnology researches in penaeid shrimps have been slow because a lack of knowledge on fundamental aspects of their biology (Benzie, 1998). A research concerning domestication of *P. monodon* is being carried out in Thailand by production of high quality pond-reared *P. monodon* broodstock. Subsequently, it is expected that selective breeding programmes of *P. monodon* will be the key to provide shrimps having commercially desired phenotypes (e.g. high growth rate and/or disease resistance) and to produce *P. monodon* stocks with the ability to induce high quality egg development in domesticated females without the irreversible side-effects caused by a typical eyestalk ablation technique (Lyons and Li, 2002).

Despite the success of the farmed production, the shrimp industry has encountered several problems including environmental degradation, outbreaks of diseases, and shortages of high quality broodstock. The white shrimp (*Litopenaeus vannamei*) was then introduced into the country and initially contributed on the cultured production of Thailand significantly. However, the price of *L. vannamei* is quite low and broodstock used relies almost entirely on genetic improved stocks brought from different sources. In addition, the labor costs in Thailand are higher than other countries (e.g. Vietnam and China) preventing the advantage of competition for the world market. In contrast, the market of premium-sized *P. monodon* is still open. Applications of the knowledge for genetic selection and biotechnology of *P. monodon* should be studied and practically implemented to fulfill that purpose.

Determining the relative effect that male and female broodstock quality is having on reproductive performance, particularly for key parameters such as hatching rate, will enhance the rate at which improvements reproductive performance of domesticated stocks can be achieved. Previous studies assessing the reproductive performance of reciprocally crossed wild and pond-reared broodstock found that the wild females outperformed domesticated females in terms of maturation, spawning and total egg production in *P. monodon* (Menasveta et al., 1993)

Although phenotypic improvement can be accomplished through conventional breeding programmes, knowledge from genome studies and molecular markers linked to important traits (marker-assisted selection, MAS) can also be directly applied to improve artificial selection processes more efficiently.

**1.7 Genes involved ovarian development and maturation in crustacean**

Different biotechnological approaches have been applied to induce female maturation of domesticated *P. monodon* but results are inconsistent owing to limited knowledge on genetic and hormonal control of *P. monodonn* reproduction. An initial step toward understanding molecular mechanisms of ovarian (and oocyte) maturation and sex differentiation in *P. monodon* is the identification and characterization of sex-specific/differential expression genes in ovaries of this economically important species.

Cyclin B is a ubiquitous regulatory protein that controls eukaryotic cell division cycle at the checkpoint from G2 to M-phase in combination with the catalytic subunit, Cdc2 kinase (also called cdk1) to form cyclin B-Cdc2 kinase complex, termed the M-phase Promoting Factor (Kishimoto, 1988; Nurse, 1990; Masui, 1992; Yamashita, 1998; Pines, 1999).

The M-phase Promoting Factor is also known as a maturation promoting factor (MPF) present in oocytes cytoplasm under the induction of the maturation inducing factor at the final stages of oocyte maturation (Nagahama, 1987). Progesterone has been shown to function as MIH in frogs and higher vertebrates, whereas in fish 17α,20β-dihydroxy-pregnen-3-one (17α,20β -DP) is the MIH. The activation of MPF leads to M-phase entry and progression of oocytes to meiosis maturation including chromosome condensation, germinal vesicle breakdown

(GVBD) and spindle formation (Masui and Markert, 1971; Mailer, 1990; Nurse, 1990).

Basu et al. (2004) reported that 17α,20β-dihydroxy-pregnen-3-one (17α,20β - DP), the maturation inducing hormone (MIH), induced complete germinal vesicle breakdown (GVBD) of oocytes of the freashwater perch *Anabas testudineus* at 21 h. An unusual cyclin, p30 cyclin B, has been identified in the oocyte extract using both monoclonal and polyclonal antibodies. Surprisingly, Cdc2 could not be identified, even though a Northern blot with *Cdc2* cDNA demonstrated expression of the gene. Purification of MPF through an immunoaffinity column followed by SDS-PAGE showed three proteins, Cdc2, cyclin B, and a 20 kDa fragment, indicating earlier failure in immunodetection may be due to the interference by this fragment. In uninduced oocytes, p30 cyclin B was present, and its expression was increased by MIH. MIH increased p30 cyclin B accumulation at 3 h, a high level which was maintained between 9 and 21 h, but an effective increase in GVBD and H1 kinase activation could only be observed between 15 and 21 h. This delay in active MPF formation was found to be related to the activation of Cdc25, phosphorylation of which was detected at 12 h, and a substantial increase occurred during 15–18 h. Sodium orthovanadate, a tyrosine phosphatase inhibitor, inhibited H1 kinase activity and GVBD, suggesting the requirement of Cdc25 activity in MPF activation. The results show occurrence of pre-MPF in uninduced oocytes and its conversion to active MPF requires dephosphorylation by Cdc25, the existence of which has not yet been shown in fish.

Recently, *cyclin B* was identified in ovaries of *M. japonicus*. Three *cyclin B* transcripts (2.4, 1.9 and 1.7 kb) which share an identical ORF of 1203 bp encoding a putative peptide of 401 amino acids with different length of 3' UTR, coexisted in ovaries. Quantitative real-time RT-PCR revealed that the short transcript (1.7 kb) was the most abundance, followed by the long (2.4 kb) and the medium (1.9 kb), and the three forms of these transcripts displayed different expression profiles during oogenesis (Qiu and Yamano, 2005).

Additionally, differential expression of *cathepsin C* (*dipeptidyl peptidase*) during the final stages of oocyte maturation of *M. japonicus* was also reported (Qiu *et*

*al*., 2005b). This suggested that various transcripts possess multifunctions and might perform different roles during oogenesis of *M. japonicus*.

Homologues of *peritrophin* (also *called cortical rod protein*, *CRP* and *shrimp ovarian peritrohpin*, *SOP*) and *thrombospondin* (*TSP*) are abundantly expressed in ovaries of penaeid shrimp (Leelatanawit et al., 2004). Khayat et al. found that two peritrophin-like cDNAs (*SOP*) were highly expressed during oogenesis in the green tiger shrimp (*P. semisulcatus*). They had peritrophin-A-like domains marked with six-cysteine residues. SOPs are major proteins in the ovaries and are extruded from the egg cortical crypts to form a protective layer around eggs immediately after spawning (Avarre et al., 2001; Khayat et al., 2001). Synthesis of *peritrophin* in ovaries of *P. semisulcatus* is inhibited by crustacean hyperglycemic hormone (CHH) purified from the sinus gland extract of *M. japonicus* (Avarre *et al*., 2001).

*Peritrophin* displays a non-differentially transcribed pattern between different stages of ovarian stages of *M. japonicus* (Okumura *et al*., 2006). The recombinant *peritrophin*-like protein of *Fenneropenaeus merguiensis* has the activity of binding Gram-negative bacteria and strong binding activity to chitin suggesting that it may also play an important role in the immune defense mechanisms (Du *et al*., 2006).

A *TSP* homologue (1060 amino acids) in invertebrates was firstly identified in *Drosophila melanogaster* (Adolph, 2001). Recently, complete sequences of three closely related *TSP* homologues encoding the major cortical rod proteins of 1114, 1032, and 991 amino acids (accession numbers AB121209, AB121210, and AB121211) were isolated and characterized in *M. japonicus* (Yamano *et al*., 2004). *MjTSP* protein levels dramatically increased after eyestalk ablation (Okumura *et al*., 2006).

Recently, major proteins in cortical rods have been purified and characterized in penaeid shrimps. One is SOP (29–35 kDa and 33–36 kDa) in *P. semisulcatus* (Khayat et al., 2001) or the so called cortical rod protein (CRP, 28.6 kDa and 30.5 kDa) in *M. japonicus* (Kim et al., 2004, 2005), and the other is *M. japonicus* thrombospondin (MjTSP, 130 kDa, 140 kDa, and 150 kDa, Yamano et al., 2003, 2004). The mRNAs of SOP, CRP, and MjTSP are expressed in the previtellogenic oocytes, and the proteins of SOP, CRP, and MjTSP are synthesized and accumulated in the vitellogenic oocytes as yolk materials. These proteins are first scattered

throughout the ooplasm and are then localized in cortical rods during the cortical rod formation.

Effects of removal of the X-organ-sinus gland complex on VTG, CRP, and MjTSP synthesis in *M. japonicus* were examined (Okumura et al., 2006). Immature females were bilaterally eyestalk-ablated. The synthesis process of *VTG*, *CRP*, and *MjTSP* was determined at both the transcriptional level (mRNA) and the translational level (protein) during the induced early stages of vitellogenesis which are the critical stages to start the synthesis of VTG, CRP, and MjTSP proteins. The synthesis process was monitored over a 7-day period after the ablation. The ovarian weight and hemolymph VTG levels increased in the ablated females. On two days after the eyestalk ablation (Day 2), all the sampled female *M. japonicus* had ovaries at the endogenous vitellogenic stage and GSI increased significantly ($1.27 \pm 0.23$, $P<0.05$) On Days 5 and 7, all the sampled female prawns had ovaries at the exogenous vitellogenic stage which contained the exogenous vitellogenic oocytes and GSI further increased due to yolk accumulation in the oocytes ($1.47 \pm 0.51$ on Day 5, $2.35 \pm 0.75$ on Day 7). Hemolymph VTG levels became significantly higher during Days 2 - 7 than on Day 0 The VTG mRNA levels in the ovary increased significantly during Days 1 – 7. The results indicated that VTG mRNA levels in the ovary increased concomitantly with vitellin accumulation in the ovary after eyestalk ablation. On the other hand, the CRP and MjTSP protein levels in the ovary increased after eyestalk ablation, whereas the CRP and MjTSP mRNA levels in the ovary did not change concomitantly. The results suggest that the regulatory mechanism of gene expression by eyestalk hormones is different between VTG (transcriptional control) and CRP-MjTSP (translational control).

One difficulty in identifying compounds that stimulate crustacean reproduction is the lack of adequate biological markers for reproduction. Vitellogenin is female-specifically expressed and can easily be purified and characterized. As a result, it was popularly applied to follow reproductive maturation of various animals. However, a problem with using the presence of yolk proteins as indicators of reproduction is that their presence in tissues does not clearly distinguish between synthesis, storage, or degradation.

To provide an insight into molecular aspects governing reproductive processes of *P. monodon* for future functional genomic studies, normal and suppression subtractive hybridization (SSH) cDNA libraries of ovaries were constructed. EST from the established libraries was sequenced (Leelatanawit et al., 2004; Prechaphol et al., 2007). A large number of transcripts were identified. Several transcripts can be used as the responsive indicators for reproduction at the present stages but their involvement for ovarian and oocyte maturation and/or differentiation of sexes in *P. monodon* need to be further investigated.

In this thesis, expression of a large number of gene homologues in ovaries and testes of *P. monodon* juveniles and brood stock was examined. The full length cDNA of *adipose differentiation related peptide*, *asparaginyl-tRNA synthetase*, *aspartate amino transferase, dolichil diphosphooligosaccharide-aminotransferase*, *endothelial cell growth factor 1*, *female sterile*, *nuclear autogenic sperm protein*, *3-oxoacid CoA transferase* and the major part of the ORF of *ovarian lipoprotein receptor* were isolated for the first time in *P. monodon*. Expression of these genes upon stimulating by serotonin was examined in juvenile *P. monodon* females.

### 1.8 The objective of this thesis

The objectives of this thesis were identification and characterization of gene involved with ovarian development of *P. monodon* and examination of expression profiles of various genes under normal serotonin (5-HT) – induced condition. Morever, the full length cDNA exhibiting specific or preferential expression patterns in ovaries of *P. monodon* were also identified and characterized.