2. LITERATURE REVIEWS

1. The systematics of *Penaeus*

Farfante (1969) separated the close thelycum American *Penaeus* spp. and raised these four groups to subgeneric status, close thylecum species *Penaeus* (*Penaeus*), *P. monodon* group (with an hepatic carina); *Penaeus* (*Fenneropenaeus*), *P. indicus* group (without an hepatic carina); *Penaeus* (*Litopenaeus*), open thylecum types; *Penaeus* (*Melicertus*), grooved prawns. Subsequent authors have separated the American grooved prawns into *Penaeus* (*Farfantepenaeus*) and *P. japonicus* into *Penaeus* (*Marsupenaeus*)

The systematics of the white prawn has been reported at GenBank database the website http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi

There are 4 species of white prawn belonging to subgenus *Fenneropenaeus* in Indo-West Pacific (Figure 1), including Thailand: *Penaeus* (*Fenneropenaeus*) *merguiensis*, *Penaeus* (*Fenneropenaeus*) *silasi*, *Penaeus* (*Fenneropenaeus*) *indicus* and *Penaeus* (*Fenneropenaeus*) *penicillatus*. The common names of these *Fenneropenaeus* are shown in Table1.

Figure 1

Table 1. The new scientific names and FAO names of the closely related species *P. merguiensis*, *P. silasi*, *P. indicus* and *P. penicillatus* (GenBank).

Old scientific name	New scientific name	FAO name
Penaeus merguiensis	*Fenneropenaeus merguiensis*	Banana prawn
Penaeus silasi	*Fenneropenaeus silasi*	False white prawn
Penaeus indicus	*Fenneropenaeus indicus*	Indian white prawn
Penaeus penicillatus	*Fenneropenaeus penicillatus*	Red-tail prawn

They are classified as: Kingdom, Eukaryota; Subkingdom, Metazoa; Phylum, Arthropoda; Subphylum, Crustacea; Class, Malacostraca; Subclass, Eumalacostraca; Superorder, Eucarida; Order, Decapod; Suborder, Dendrobranchiata; Infraorder, Penaeidea; Superfamily, Penaeoidea; Family, Penaeidae; Genus, Penaeus; Subgenus, Fenneropenaeus.

2. Subgenus *Fenneropenaeus*

There are four species in subgenus *Fenneropenaeus* found in Indo-Western Pacific but the generic name of *Fenneropenaeus* species is called *Penaeus* throughout this thesis. *P. merguiensis* is also in this subgenus. It is often confused with *P. silasi*, *P. indicus* or *P. penicillatus* (Carpenter and Niem, 1998).

2.1 *P. merguiensis* De Man, 1888

It is distributed in the Indo-West Pacific from the Arabian Sea to the South China Sea and Fiji (Carpenter and Niem, 1998). It lives on sand and mud, from the coastline and river mouths to depths of about 55 meters, usually less than 20 meters. It prefers turbid water. Sometimes it forms very dense schools and good catches are often linked with heavy rainfall. It is economically one of the most important prawn species in this area. From 1990 to 1995, the reported annual catch of *P. merguiensis* in the Western Central Pacific ranged from 44,303 to 52,087 tons. About 4/5 of this catch was around Indonesia. Trawls, fish corrals, pocket netting, beach seining, cast nets, and artisanal gear were the means of fishing. *P. merguiensis* also plays an important role in pond culture in Thailand (1,814 t in 1995), Malaysia (66 t in 1995 and Indonesia (24,610 t in 1995) (FAO Aquaculture Statistics, 1995). It is marketed both fresh and frozen form, consumed locally and exported.

2.2 *P. silasi* Muthu and Motoh, 1979

It is found on muddy bottoms in shallow waters to a depth of about 36 meters. Trawls and artisanal gear are used to catch it. It is an abundant species in the markets of Singapore and of commercial importance. It is marketed both fresh and frozen, mainly for local consumption. It is distributed in the Indo-West Pacific, so far only reported from Thailand (Andaman Sea and Gulf of Thailand), Indonesia, Malaysia and Singapore. Since this prawn can be easily confused with *P. indicus, P.*

merguiensis and *P. penicillatus*, its actual distribution is likely to be wider in the Indo-Malay region (Carpenter and Niem, 1998).

2.3 *P. indicus* H. Milne Edwards, 1837

It is found on sandy and muddy bottoms from the coastline to a depth of about 90 meters. Trawls, fish corrals, gill nets, beach seines and artisanal gear are used to catch it. It is also a suitable candidate for the prawn pond industry. It is an abundant species in the markets of the Philippines, Singapore and Australia. However, as this prawn is often confused with *P. silasi*, its reported abundance from Thailand to Indonesia remains uncertain. In the Philippines, it is often mixed and sold together with *P. merguiensis*. It is marketed both fresh and frozen form, consumed locally and exported. It is widely distributed in the Indo-West Pacific from the eastern coast of Africa to the Red Sea, Japan and Australia (Carpenter and Niem, 1998).

2.4 *P. penicillatus* Alcock, 1905

It is found on soft bottoms from the coastline to a depth of about 90 meters. It is caught with trawls, scoop nets, seines and artisanal gear. It is marketed both fresh and frozen form. It has been reported to be common in Malaysia, but can be easily confused with *P. indicus*, *P. merguiensis* and *P. silasi*, and is probably not so common. It is distributed in the Indo-West Pacific from Pakistan to Taiwan and Indonesia (Carpenter and Niem, 1998).

In Thai waters, there are two domestic species of the white prawn, *P. merguiensis* and *P. indicus* (http://www.nicaonline.com, 2002). The former species is found in the majority. Other reports about the white prawn in Thai water still were confused. In 1988, Leelapiyanart reported that *P. penicillatus* is not found in Thai peninsula waters, however, there are only three white prawn species found in Thai water, *P. merguiensis*, *P. silasi* and *P. penicillatus* (Chaitiamvong and Supongpan, 1992). These two latter reports are contradictory about the existence of *P. penicillatus* and *P. indicus*. *P. penicillatus* was not found in Leelapiyanart (1988) report while *P. indicus* was not found in Chaitiamvong and Supongpan (1992). This confused is a reason for this work to find out. In addition, the research in India (Muthu and Rao, 1973) and Malaysia (Chong and Sasekumar, 1982) has indicated that juvenile identification by morphological characters is limited. Nevertheless, Chong and Sasekumar (1982) could be distinguished the adults of *P. merguiensis*, *P. indicus* and *P. penicillatus* on the basis of morphological characters.

3. Systematics by Morphology

For the first 200 years of biological study, physical (anatomical) similarities were the basis for most constructions of phylogenies and classification. Early biologists relied on anatomic similarities, fossil similarities, and embryological similarities (all of which were anatomic) to determine which organisms were closely and which distantly related.

Grey et al. (1983) distinguished between *P. merguiensis* and *P. indicus* by using the rostrum and the distance between the gastro-orbital ridge and the hepatic spine.

They produced a guide with keys and colored photographs, but is restricted to the commoner Australian species.

Dall et al. (1990) used features similar to Grey et al. (1983), adding the third maxilliped in adult males. This character can identify *P. merguiensis*, *P. silasi, P. indicus* and *P. penicillatus*. In 1992, Chaitiamvong and Supongpan used features similar to Dall et al. (1990) to identify *P. merguiensis*, *P. silasi* and *P. penicillatus* in Thai waters. Carpenter and Niem (1998) reported that *P. merguiensis* is frequently confused with *P. indicus*, *P. silasi* and *P. penicillatus*. However, the third maxilliped in adult male still use to identify them.

Morphological features of systematic importance in *Fenneropenaeus* species include; the rostrum (Figure 2); the external sexual organs or the petasma in males, or the thylecum in females and the third maxillipeds. The summary taxonomic keys of these closed species are shown in Table 2.

3.1 The Rostrum

The rostrum is one of the features used to distinguish between these closely related species (Grey et al., 1983; Carpenter and Niem, 1998). The rostrum in *P. merguiensis* (Figure 3A) is horizontal and the rostral crest becomes very high and broadly triangular in large specimens (more defined in females). In *P. silasi* (Figure 3B), the tip of the rostrum is horizontal. The rostral crest is slightly to moderately elevated in juveniles and adult males but high and broadly triangular in large females. In *P. indicus* (Figure 3C), the rostrum is slightly curved at the tip and is sigmoidal in shape. The rostral crest is generally slightly elevated in large specimens with the crest

in adult females slightly higher than in males. In *P. penicillatus* (Figure 3D), the tip of the rostrum is horizontal. The rostral crest is generally slightly elevated in juveniles and adult males, but is moderately high in large females.

3.2 The Distance between The Gastro-orbital Ridge and The Hepatic Spine

The distance between the gastro-orbital ridge and the hepatic spine (Figure 3) is one of the features used to classify the closed white prawn species (Grey et al., 1983). In *P. merguiensis*, the gastro-orbital crest varies from distinct to nearly absent (in some specimens from the Philippines to Australia), and extends over the middle third of the posterior 2/3 of the distance between the hepatic spine and the orbital margin (Figure 3A). In *P. silasi*, the gastro-orbital crest is distinct and extends over the posterior 3/5 to 2/3 of the distance between the hepatic spine and the orbital margin (Figure 3B). In *P. indicus*, the gastro-orbital crest is distinct and extends over the posterior 3/5 to 2/3 of the distance between the hepatic spine and the orbital margin (Figure 3C). In *P. penicillatus*, the gastro-orbital crest is distinct, occupying 1/2 to 1/3 the distance between the hepatic spine and the orbital margin (Figure 3D).

Figure 2.

Table 2

Figure3

3.3 The Petasma (sexual organ) in Males

In the male the principal structure consists of the endopod (Figure 4), modified to form a tubular organ, the petasma. In the immature prawn the endopods are two simple, elongate, flattened structure, which elaborate as the prawn matures and become linked by a series of minute hook-like setae along the mid-line. The petasma is a simple, open, pod-like structure in *Penaeus* spp., but may be more elaborate and rigid in other genera. As the details of its structure usually differ considerably from species to species, it is used as a principal taxonomic feature (Dall et al., 1990).

About the sixth post-larval stage, the sperm ducts begin to differentiate and a depression appear on the protopod of the first pleopods (Figure 4). Soon afterwards the endopods begins to differentiate into the petasma. In *Penaeus* sp., petasma is pod-like and flexible with thin median lobes. It is usually with the small-thickened distal protuberances and forming a posterior tube-like projection. The lateral lobes are usually with thickened distal rounded margins.

The petasma in males have been to identify the white prawn (Grey et al., 1983; Carpenter and Niem, 1998). The distomedian projections are short and not reaching distal margin of costa in *P. merguiensis* (Figure 5A), are slightly bent and not overhanging distal margin of costa in *P. silasi* (Figure 5B), are strongly curved and overhang distal margin of costa in *P. indicus* (Figure 5C) and are slightly bent and not reaching distal margin of costa in *P. penicillatus* (Figure 5D).

Figure 4

Figure 5.

3.4 The Thylecum (sexual organ) in Females

The seminal receptacle of the female is the thelycum, which consist of modified sternal plates of the seventh and eighth thoracic somites. The details of the structure are unique to each species and are used extensively for specific identifications. Thyleca range from simple to complex open depression (open thyleca), to paired pouches, often with single or double covers (closed thyleca). There is usually a single and prominent median structure between the coxae of the fourth pereiopods (Figure 4).

Thelycum with anterior plate, between the coxae of the fourth pereiopods, is variable in shape and smaller than the posterior part of the thelycum.

In the thelycum in females (Grey et al., 1983; Carpenter and Niem, 1998) of *P. merguiensis* (Figure 6A), the anterior process is slightly rounded, concave and obscured by hairs, and the posterior process is elongated and inserted between the anterior part of the lateral plates. In *P. silasi*, the anterior process is slightly rounded and concave, generally distinct and not obscured by hairs, and the posterior process is elongated and inserted between the anterior part of the lateral plates (Figure 6B). In *P. indicus*, the anterior process is slightly rounded and convex, the posterior process elongated and inserted between the anterior part of the lateral plates (Figure 6C). In *P. penicillatus*, the anterior process is slightly rounded and obscured by hairs, and the posterior process is elongated and inserted between the anterior part of the lateral plates (Figure 6D).

Figure 6.

3.5 The Third Maxilliped

The maxillipeds (Figure 7) are thoracic appendages 1-3 and become more leg-like posteriorly. The third maxillipeds are used extensively for grooming.

The ratio between the distal and second segment in the third maxilliped is used to differentiate these close species (Chong and Sasekumar, 1982; Capenter and Niem, 1998). The distal segment of the third maxilliped is only about half as long as the second segment with a dense tuft of short hairs (slightly shorter than the distal segment) at its tip in *P. merguiensis* (Figure 8A). The distal segment of the third maxilliped is only about half as long as the second segment with a dense tuft of long hairs (the same length as the distal segment) at its tip in *P. indicus* (Figure 8B). The distal segment of the third maxilliped is much longer than the second segment with a dense tuft of long hairs (as long as the distal segment) at its tip in *P. penicillatus* (Figure 8C). The distal segment of the third maxilliped is about as long as the second segment with only a rudimentary tuft of hairs at its tip in *P. silasi* (Figure 8D).

Figure 7.

Figure 8.

4. Morphometric measurements

The morphometric characters are used to identify *P. merguiensis*, *P. indicus* and *P. penicillatus* (Chong and Sasekumar, 1982; Pendrey et al., 1999). The ratio of L_1: $L_{1,2}$. L_1 is the distance from anterior-most dorsal rostral tooth (the first tooth) to the tip of the rostrum. $L_{1,2}$ is the distance between first and second dorsal rostral teeth. The characters are used to separate them more clearly (Pendrey et al., 1999).

4.1 The ratio of L_1: $L_{1,2}$

Chong and Sasekumar (1982) and Pendrey et al. (1999) separated the juvenile *P. merguiensis* from the juvenile *P. indicus* by the ratio of L_1: $L_{1,2}$ (Figure 9). The ratio of L_1: $L_{1,2}$ of *P. merguiensis* and *P. indicus* is shown in Table 3.

Table 3. The L_1: $L_{1,2}$ ratio between *P. merguiensis* and *P. indicus* (Pendrey et al., 1999).

Species / Character	*P. merguiensis*	*P. indicus*
L_1:$L_{1,2}$	0.5-1.644	2-3.98

Figure 9

5. Molecular Systematic

Molecular systematics uses the molecular data to make inferences about population processes and phylogeny and in doing so creates a substantial comparative database for specific genes or proteins. Studies of molecular evolution uses these data to evaluate rates, processes, and constraints on molecular change through time (reviewed by Kimura, 1983; Li and Graur, 1991). The results of molecular evolutionary studies can then provide for more informed use of molecular markers in population genetics and phylogenetic analyses (Baldwin et al., 1998; Funk, 1999; Gasser et al., 1999; Cruickshank, 2002). The molecular genetic comparisons can provide a valuable framework for re-examination of the systematic relationships of Penaeid prawn (Baldwin et al., 1998; Gusmão et al., 2000; Hualkasin et al., 2003).

6. Molecular marker

The advent of molecular techniques in the mid 1960's enabled evolutionary researchers to first detect genetic variation in proteins (Margoliash 1963). A variety of molecular markers, including mini- and microsatellites DNA, different kinds of restriction fragments, PCR-markers and DNA sequences are available today for studying ecology and evolution (Hillis et al., 1996).

6.1 The Classes of Molecular Markers

Because normal protein or DNA molecules are used to score the genetic material, molecular markers are phenotypically neutral. This is a significant advantage

compared to traditional phenotypic markers. There are two kinds of molecular markers: Protein and DNA.

6.1.1 Protein Marker and General Principles

The relationship between the morphological phenotype (how the organism appears) and the genotype (the precise genetic information encoded by its DNA) is not easily discerned because of the major effects of environment in the expression of morphology. However, in the mid-1960s, new and powerful sources of evidence have been provided by biochemistry. The amino acid sequences of proteins or at the genes encoding those proteins were similar or different. A technique called allozyme electrophoresis (Harris, 1966; Lewontin and Hubby, 1966) became available that increased the ability to infer genotype from an examination of phenotype. Numerous inheritance studies demonstrated the direct relationship of allozyme phenotype to genotype.

Allozyme electrophoresis is an analytical technique that examines variation among that class of protein that controls much of cell metabolism-bidirectional catalytic enzymes, which are the molecules that convert one substrate to another without them being affected. Protein coding genes have two reasons that make them a preparation of choice for geneticists. First, an important proportion of these genes is polymorphic that is exist in the form of one or more alleles. Second, the alleles of protein coding genes are generally codominant (both alleles are expressed in heterozygous organism). This phenomenon allows us to relate the observed phenotypes to a given genotype.

The term of isozymes refers to all of the different forms of an enzyme that perform the same catalytic function (e.g., all of the forms of MDH). These include forms encoded at the same locus as well as those encoded at multiple loci. Enzymes (isozymes) coded by multiple loci are often differentially expressed among different tissue types (e.g., of the five lactate dehydrogenase loci (*LDH*, 1.1.1.27) in salmonids. The term of allozyme, on the other hand, refers to only those genetically different forms of an enzyme that are encoded at the same locus. The data on single-locus genetic variation obtained from allozyme variation study (Pasteur et al., 1988).

Isozyme - a molecular marker system based on the staining of proteins with identical function, but different electrophoretic mobilities. The technique is based on the principal that allelic variation exists from many different proteins. For example, alleles of malic dehydrogenase would both perform the correct enzymatic function, but the electrophoretic mobility of the two may differ. Therefore, two alleles would not migrate to the same location in a starch gel.

The procedures to identify isozyme variation is simple. A crude protein extract is made from some tissue sources. The extracts are next separated by electrophoresis in a starch gel or other supporter (cellulose polyacetate). The gel is then placed in a solution that contains reagents required for the enzymatic activity of the enzyme you are monitoring. In addition, the solution contains a dye that the enzyme can catalyze into a color reagent that stains the protein. In this manner allelic variants of the protein can be visualized in a gel.

Proteins are composed of amino acids joined by covalent peptide bonds to form polypeptides. These sequences, or primary structures, are genetically

determined. Each of the 20 amino acids has a unique side chain, characterized by its shape, size and charge. The side chains of five of these amino acids are either basic, thus positively charged (NH_3^+; lysine, arginine and histidine) or acidic and negatively charged (COO^-; aspartic acid and glutamic acid). Charged side chains are responsible for the movement of the proteins through the matrix during electrophoresis. The net charge of each protein varies with pH; at a low pH the amino acid group become positively charged, and at high pH the carboxyl groups become negatively charged. Most proteins have a point at which the effects of positive and negative charge are equal, the isoelectric point. Isoelectric proteins do not move in an electric field because they are attracted to neither the (positive) anode nor the (negative) cathode (Hillis et al., 1996).

Uncharged amino acids are either non-polar (hydrophobic) or polar. These amino acids can become hydrogen-bonded to one another resulting in folding (beta-structure) or helical (alpha helix) configurations, termed secondary structure. Depending on the primary and secondary structure, the molecule usually undergoes additional folding to form its tertiary structure. The shape and size of a protein also may have an effect on protein migration, depending on the pore size of the electrophoresis matrix. To some extent the shape of a particular protein is determined by the relative charges of adjacent amino acids because of the effect of like charges repelling and different charges attracting. Finally, many proteins contain more than one polypeptide chain (subunit) bound together by hydrogen bonds, van der Waals forces, ionic bonds, disulfide bonds and/or hydrophobic interactions. Proteins having

more than one polypeptide (multimeric) have a quaternary structure (Darnell et al., 1986).

Some forms of electrophoresis separate proteins on the basis of net protein charge, shape as measured by radius, strength of the electric field, and viscosity of the suspension medium. Under appropriate condition, the rate of movement increases with net charge and strength of the electric field and decreases with the size of the molecule.

All electrophoretic techniques consist of an electric power supply, a support matrix (cellulose acetate gel or strips, starch gel, etc.) and ionic buffers. Electric current is applied to opposite ends of the suspension medium via the ionic buffers. Molecules (e.g., proteins) having a net positively charge (cations) migrate to the cathode, and negatively charged proteins (anions) migrate to the anode. Following electrophoresis, the proteins may be visualized by a number of different methods, the most frequently used being specific histochemical staining. After electrophoresing a protein sample in the gel matrix, the individual proteins are selectively stained. Most of the stains provide a specific substrate for the enzyme, allow it to catalyze the particular reaction involved, and then develop a dye that can be visualized in normal light or by fluorescent under UV light. Thus, from the hundreds or thousands of enzymes in the crude extract, proteins with the same substrate utilization can be identified.

Protein electrophoresis is currently the only technique that allows us to study simultaneously several genes in the same individual, rapidly and relatively cheap. Electrophoresis technique allows between 20 and 30 individuals to be tested at

the same time. Large inter- or intra- specific sample can therefore be directly compared.

In each organism, the number of isozymes with a given catalytic activity is generally small, and only a few colored bands appear on the gel. Each band corresponds to a different protein, the synthesis of which is controlled by one, two or several genes. The enzymatic phenotypes observed on the gel can therefore be interpreted in terms of genotypes, that is, in terms of genes and their alleles.

The amino acid sequences of proteins are changed by mutations in the encoding DNA locus. Such mutations may alter shape and net charge, as well as catalytic efficiency and stability (Shaw, 1965). The most basic assumption that evolutionary biologists make in using isozyme data is that changes in the mobility of enzymes in an electric field reflect changes in the encoding DNA sequence. Thus if the banding patterns of two individuals differ, it is assumed that these differences are genetically based and heritable (see Matson, 1984).

6.1.2 Protein Marker and Genetic Diversity

Protein electrophoretic assays also reveal various aspects of gene expression that may be under genetic control. Because gene expression patterns themselves are products of the evolutionary process, they too can be informative as phylogenetic markers. The single-locus genetic data used to answer many basic biological questions about fish sparid species (Alarcon and Alvarez, 1999).

The various studies of protein marker: taxonomy (Pinto et al., 1992), the analysis of speciation and race formation in *Rhagoletis* fruit flies (Feder et al., 1988).

Redfield et al. (1980), using electrophoresis of a range of tissue proteins, found that the heterozygosity of a number of tropical decapod Crustacea was very low, the lowest being *P. merguiensis*.

Mulley and Latter (1980) analysed 37 genetic loci in 13 Australian penaeid species from two genera and confirmed that their genetic diversity was comparable with that of *P. merguiensis*.

Saetun (Project, 1997) compared the isozyme pattern of the white prawn by using isozyme electrophoresis Malate dehydrogenase (MDH), 6-Phosphogluconate dehydrogenase (6-PGDH) and Isocitrate dehydrogenase (ISCH). Moreover, Limcharearn (Project, 1997) identified the banana prawn by isozymes on polyacrylamide gel electrophoresis (discontinuous), Alcohol dehydrogenase (ADH); Glucose-6-phosphate dehydrogenase (G-6-PDH); Succinate dehydrogenase (SCDH); Lactate dehydrogenase (LDH); Glutamate dehydrogenase (GDH), Aspartate dehydrogenase (ADH), Glucose-6-phosphate isomerase, Aldehyde oxidase and hexokinase. However, they could not be separated the white prawn clearly.

Genetic variation was electrophoretically examined in populations of banana prawn from three locations along the coast of Thailand (Chonburi, Surat Thani and Satun) to determine the extent of genetic isolation (Sodsuk and Sodsuk, 1999). Five polymorphic enzymes (*ALAT*, Alanine aminotransferase; *GPI*, Glucose-6-phosphate isomerase; *IDHP*, Isocitrate dehydrogenase ($NADP^+$); *MPI*, Mannose-6-phosphate isomerase and *PGM-1*, Phosphoglucomutase) were applied to the genetic distance. MDH enzyme was monomorphic in their study. They concluded that there

was the differentiation of populations between the Andaman Sea and the Gulf of Thailand and also the differentiation among the populations in the Gulf of Thailand.

Pendrey et al. (1999) separated the large and juvenile prawn of *P. indicus* and *P. merguiensis* clearly by MDH isozyme pattern. They reported that the distance from the first tooth to the end of the rostrum was much longer for *P. indicus* than for *P. merguiensis*, and the ratio of L_1: $L_{1,\ 2}$ was higher for *P. indicus* (3.16) than *P. merguiensis* (1.56).

6.2 DNA Marker

DNA marker is obtained from the nuclear and organelle DNA.

6.2.1 Nuclear DNA (nDNA)

The DNA marker, which is developed from nuclear genome, called nuclear DNA marker. Genes residing within the nucleus are generally described as nuclear genes provided by Mendelian inheritance. The progeny of a mating to display Mendelian segregration for parental characters defines the Mendelian inheritance. The nuclear genes utilize segregation on the meiotic and mitotic spindles to distribute replicas to gametes or to daughter cells, respectively. In addition, the nuclear genes are expressed by means of cytoplasmic protein synthesis.

In molecular systematics, there has been a focus on repeated nuclear genes that are highly conserved. For example, the cluster of tandemly repeated rRNA genes includes some highly conserved regions (typically within the genes themselves), and several variable regions (including the spacers between genes, Intron

Transcribed Spacer). Microsatellite DNA was develop to obtain the population genetic data by the number of tandem dinucleotide repeats that tend to vary from individual to individual. RAPD (Random Amplified Polymorphism DNA) marker obtains from a large set of short and random oligonucleotide. By screening a large number of primer pairs it is possible, by chance, to find some that produce useful products. Single-copy nDNA sequences have been compared among species (e.g., ADH among *Drosophila*; Langley et al., 1981). However, the vast complexity of the nuclear genome, many nuclear genes should be useful for inferring higher-order phylogenetic relationships. The best example of the broad utility of nuclear genes is the ribosomal DNA repeat unit, which has intervening sequences that vary within and between populations and coding sequences that are so highly conserved as to be useful for comparison among widely divergent taxa (Mindell and Honeycutt, 1990).

6.2.2 Organelle DNA

Genes not residing with the nucleus are generally described as extranuclear. They are transcribed and translated in the same organelle compartment in which they reside. An organelle genome codes for some, but not all, of the proteins needed to perpetuate the organelles. The others are coded in the nucleus, expressed via the cytoplasmic protein synthetic apparatus, and imported into the organelles. In effect, the organelle genome comprise a length of DNA that has been physically sequestered in a defined part of the cell, and is accordingly subject to its own form of expression and regulation. Mitochondria and chloroplast both posses DNA genome

that code for all of the RNA species and for some of the proteins involved in the function of organelles.

The uniparental inheritance has seen in higher animals. The uniparental inheritance occurs when the genotype of only one parent is inherited and that of the other parent is permanently lost. In less extreme example, the progeny of one parental genotype exceed those of the other genotype. Usually, it is the mother whose genotype is preferentially (or solely) inherited. This effect is sometimes described as maternal inheritance. Maternal inheritance can be predicted by supposing that the mitochondria are contributed entirely by the ovum and not at all by the sperm. Thus the mitochondrial genes are derived exclusively from the mother; and in males they are discarded each generation.

Since the only organelle shown to possed DNA in higher animal cells is the mitochondrion, probably it is the sole extranuclear residence of genetic material.

The root of interest in animal mtDNA research is about interspecific and intraspecific phylogeny studies because of its cytoplasmically housed genome, phylogenetically favorable properties of maternal transmission, extensive intraspecific variation and usual absence of intermolecular genetic recombination (Hillis et al., 1996).

Animal mtDNA (*P. monodon*) is closed circular molecule (Figure 10), typically 16 kb in length, and composed of about 37 genes coding for 22 tRNAs, 2rRNAs, and 13 mRNAs specifying proteins involved in electron transport and oxidative phosphorylation (Wallace, 1986). A "control region" of about 1-kb initiates replication and transcription. Gene arrangement in mtDNA appears general stable,

although difference in gene order do distinguish some higher animal taxa (Desjardins and Morais, 1990; Okimoto et al., 1992; Pääbo et al., 1991). Usually, there are several copies of the genome in the individual organelle. Since there are multiple organelle per cell, there is a large number of organelle genome per cell. There are several hundreds mitochondia per cell and each mitochondrion has multiple copies of the DNA.

The general mode of animal mtDNA evolution, much also is understood (see the reviews in Avise and Lansman, 1983; Avise et al., 1987a; Birley and Croft, 1986; Harrison, 1989; Moritz et al., 1987; Wilson et al., 1985). Mitochondrial DNA normally evolves rapidly at the sequence level, no doubt due in part to a lack of known repair mechanisms for mutations that occur during replication (Wilson et al., 1985). In addition, a low fidelity of the DNA-replication process and a high concentration of mutagens (e.g., superoxide radicals, O^{-}_{2}) result from the metabolic functions performed by the mitochondria. Although addition/deletion changes in mtDNA are not rare, most differences between sequences reflect point mutations, with a strong initial bias for transition over transversions (Aquadro and Greenberg, 1983; Brown and Simpson, 1982; Brown et al., 1982; Greenberg et al., 1983). Most important for genetic marker purposes, mtDNA is transmitted predominantly through maternal lines in most species (Avise and Vrijenhoek, 1987; Dawid and Blackler, 1972; Giles et al., 1980; Gyllensten et al., 1985a; Hutchison et al., 1974).

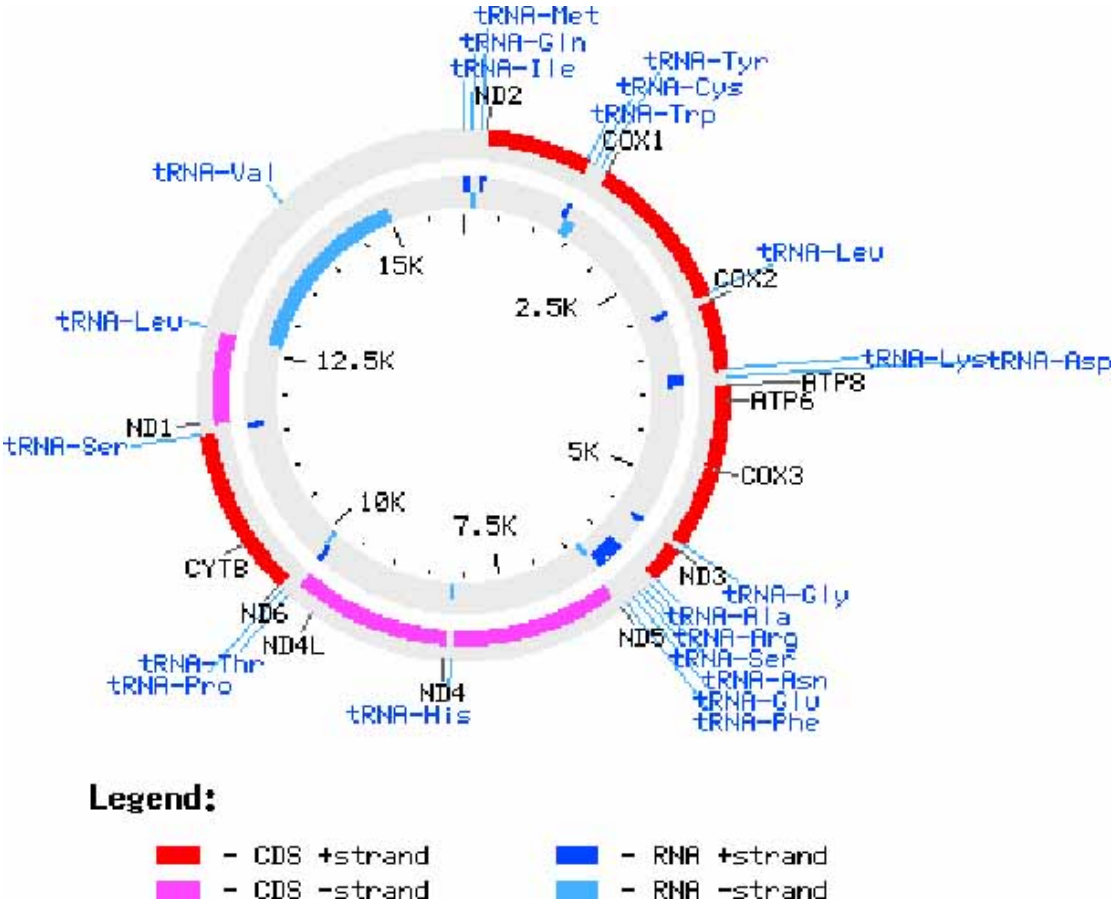

Figure 10. The mitochondrial genome of *Penaeus monodon* (retrieved from GenBank database).

7. Polymerase Chain reaction (PCR)

The polymerase chain reaction has also been used to amplify specific part of the mtDNA in vitro. PCR has increased the scope of material that can be used for DNA studies. Small amounts of nuclear DNA and mtDNA as a source of investigation, as specific primers will only amplify from select, known regions of DNA in stringent

PCR reaction. The primers employed to initiate the PCR process are short sequences (about 20-30 nucleotides long) that exhibit high sequence similarity to regions flanking the target sequence.

The PCR technique involves three steps (Figure 11): (a) denaturation of double strand DNA by heating; (b) annealing of the primers to the flanking region to be amplified; and (c) primer extension, the strands complementary to the region between the flanking primers are synthesized under the DNA polymerase enzyme. The double stranded products are cycled repeatedly through step (a) - (c). In each round of denaturation, annealing and extension, the target sequence is roughly doubled in the reaction mixture, so that after 20 or more rounds the product assumes overwhelming preponderance and can be sequenced directly as purified DNA spanning the primers. The amplification primers for PCR also can be employed as sequencing primer, allowing a direct coupling of these approaches. The PCR and sequencing procedures are becoming increasingly automated and can be carried out with commercially available temperature cycles coupled to a sequencing apparatus.

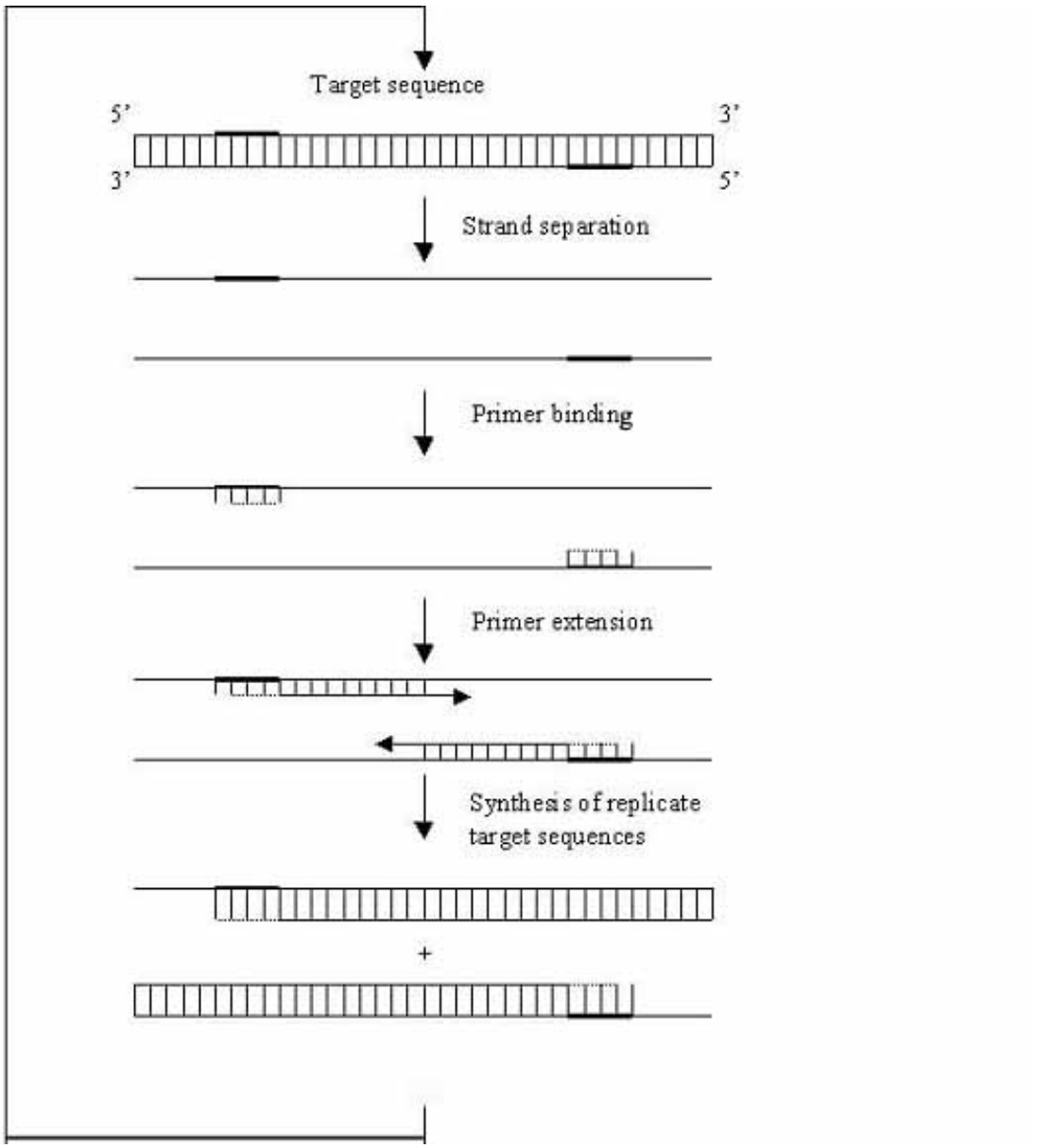

Figure 11. The amplification of a particular DNA sequence by the polymerase chain reaction (PCR).

The existence of full sequences of mtDNAs from several phyla has encouraged the development of a suite of so-called universal primers for this genome (e.g.,

Kocher et al., 1989). These primers allow access to the mitochondrial genomes of species otherwise unknown to molecular biology, and encourage the sequencing and comparison of homologous genes of closely related species and population within species. Universal primers have been developed from conserved sequences developed from conserved regions flanking those of interest to phylogenetic/ evolutionary and population studies (Simon et al., 1994). Such primers allow the amplification of the same region from a diverse range of taxa facilitating parallel research studies in new organisms. Universal PCR primers have been described to amplify across different sections of the mtDNA.

The sets of universal primer have been widely used for ribosomal genes (12S and 16S) and protein-coding genes. The ribosomal primers are highly conserved, yet span a region that includes enough variation to be phylogenetically useful at the species level and below. Among protein-coding genes in mtDNA, there is a wide range of level of conservation. Some proteins are so variable that it is difficult to align homologous amino acids (e.g., ATPase 6, 8). Others are so highly conserved that it may be difficult to detect any amino acid change among genera (e.g., cytochrome oxidase I). As expected from the neutral theory, when four folded degenerate sites are examined in these proteins, there is no relationship between rate of silent substitution and degree of amino acid conservation (Kessing, 1991). Thus even the most highly conserved gene (at the amino acid level) has as great a rate of silent changes as does the most variable gene. This makes the design of universal primer easier for highly conserved genes. Of course, amino acid evolution is faster in less conserved genes,

and for phylogenetic reconstruction this type of data often be the most informative (Hillis et al., 1996).

The mtDNA sequences that have received the most attention are the genes for ribosomal RNA (12S and 16S), cytochrome oxidase I and II and cytochrome b as well as the control region, however other regions are proving to be useful as well. Sequence comparison of selected regions of animal mtDNA, including the mtDNA ribosomal gene are useful for inferring phylogenetic relationships of species whose divergences are more result than those accessible using nuclear-encoded rRNA (Moritz et al., 1987)

The relatively variable regions within the rRNA gene make them useful for examining relationships within more closely related group such as mollusk (Ghiselin, 1988), arthropod (Hancock et al., 1988; Abele et al., 1989; Wheeler, 1989; Kim and Abele, 1990; Spears et al., 1992).

8. Mitochondrial DNA gene

8.1 Ribosomal RNA gene

Ribosomal RNA genes have been studies greatly because of their critical role in protein assembly (Brimacombe et al., 1990, Noller et al, 1990). As a result of their universal occurrence, sequence and structure conservation, and abundance, RNA genes have been used for phylogenetic analyses of a wide range of species. In addition, they also provided divergence levels, including the deepest branches of the tree of life (reviewed in Mindell and Honeycutt, 1990; Hillis and Dixon, 1991; Hamby and Zimmer, 1992). Many rRNA gene copies in the genome appear to evolve

in concert i.e. a substitution in one quickly spreads to the other copies (Hancock et al, 1988; reviewed in Hillis and Dixon, 1991). In animal, there are only two mitochondrial rRNA subunit genes and they are smaller, exist in only one copy per genome, and contain no spacer regions. The rate of evolution of rRNA genes varies considerably along the length of the molecule (Hillis and Dixon, 1991; Simon, 1991).

There are two ribosomal RNA gene in mitochondrial genome, 12S rRNA and 16S rRNA.

12S rRNA has been widely used to study the phylogenetic relationships among different levels of taxa such as families (Alves-Gomes et al., 1995; Douzery and Catzeflis, 1995; Ledje and Arnason, 1996), genera (Gatesy et al., 1997; Murphy and Collier, 1997) and species (Murphy and Collier, 1996; Halanych and Robinson, 1997). 12S rRNA primers amplify the gene for the small subunit ribosomal RNA in mitochondria. Like most ribosomal genes, it is fairly conserved among taxa, but there are regions of high sequence substitution. Overall, it seems to evolve at about the same rate as the average for the rest of the mitochondrial genome (Simon et al., 1990). The region amplified by the primers is short, about 410 bp, but it has been useful in phylogenetic studies of families, genera and even species within a genus (e.g., Gillespie et al., 1994)

16S rRNA is the large subunit ribosomal RNA gene in mtDNA. This gene is fairly conserved in sequence and secondary structure, and seems to evolve more slowly than the mitochondrial genome as a whole. Because the amplified fragment of this primers is larger than the 12S rRNA fragment (about 550 bp compare to 400 bp), it is slightly more useful in prawn phylogenetic reconstruction (Machado et al., 1993;

Maggioni et al., 2001; Arena et al., 2003; Bruyn et al., 2004). Also, there is enough variation in some species to be useful in population level studies.

Moritz et al. (1987) reported that the sequence comparisons of the mtDNA ribosomal genes are useful for inferring phylogenetic relationships of species.

Kocher et al. (1989) first published 12S rRNA primers. The 12S primers tend to work for most animal phyla. New primers can be designed to walk through the 3' part of the 12S gene. Simon et al. (1994) made the 12Sai and 12Sbi primers for insects and these primers also work for most crustaceans. This gene region has been useful in phylogenetic studies of families, genera, and even species within a genus (e.g., Gillespie et al., 1994)

Xiong and Kocher (1993) used sequence data from the 3'-half of the 16S to investigate relationships in a complex of five morphologically indistinguishable species of black flies recognizable only by chromosomal analysis. The rRNA sequences have been used to infer phylogenies across a very broad spectrum, from studies among life lineages to relationships among closely related species and populations (Hillis and Dixon, 1991).

Machado et al. (1993) compared part of the mitochondrial 16S ribosomal RNA gene of two species of prawn (*Penaeus notialis* and *Penaeus schmitti*). The sequence comparison revealed 11% nucleotide divergence between the two species.

Rungsrithum (Thesis, 1999) amplified and sequenced 12S and 16S regions. Two samples, SREE1 (strongly identify as *P. merguiensis*) and STWA6 (strongly identify as *P. indicus*), were strongly identified by using the morphological features. The nucleotide divergence between SREE1 and STWA6 of 12S and 16S data was

about 3 and 2.2%, respectively. Because no sequences of 12S and 16S were reported in the GenBank database and our percent divergence was low, it was not enough evidence to guarantee that STWA6 was really *P. indicus*.

8.2 Cytochrome oxidase subunit I (COI)

Cytochrome oxidase subunit I (COI) is the terminal catalyst in the mitochondrial respiratory chain and is involved in electron transport and proton translocation across the membrane (Saraste, 1990; Gennis, 1992). Its amino acid sequence is highly conserved across phyla, making it easy to align sequences to one another, and making it possible to design useful universal primers. Because it is so highly conserved, amino acid substitution are rare within species, but silent changes are just as common as other mtDNA genes with lower constraints on amino acid sequence (Kessing, 1991).

COI gene is the mitochondrial marker often used for evolutionary study because (1) it is the largest of the three mitochondrial-encoded cytochrome oxidase subunits (Clary and Wolstenholme, 1985; Beard et al., 1993) and (2) the protein sequence contains highly conserved functional domains and variable regions (Seraste, 1990; Gennis, 1992). COI sequence variations were applied to classify the species and study population genetic and phylogenetic (Baldwin et al., 1998; Lessios et al., 1999; Cruickshank, 2002; Linton et al., 2002).

9. Molecular data on phylogenetic studies

Molecular phylogeny is the study of evolutionary relationships among organisms by using techniques of molecular biology.

Since the late 1950's, various techniques have been developed in molecular biology, and this started the widely use of molecular data in phylogenetic studies. In particular, in the 1960's and 1970's the study of molecular phylogeny by using protein sequence data progressed enormously (Murata et al., 1985; Livingstone and Barton, 1993). Less expensive and more convenient methods, such as protein electrophoresis, DNA-DNA hybridization, and immunological methods, though less accurate than protein sequencing, were greatly used to study the phylogenetic relationships among populations or closely related species. The application of these methods also speeded up the development of measures of genetic distance and tree-making methods (see Fitch and Margoliash, 1967; Nei, 1975; Felsenstein, 1988)

The rapid collection of DNA sequence data since the late 1970's has already had a violent contact on molecular phylogeny. DNA sequence data are not only more copious, but also easier to analyze than protein sequence data. Thus they have been used, on the one hand, to infer the phylogenetic relationships among such closely related species such as human and apes (Goodman, 1963). On the other hand, to study very ancient evolutionary occurrences, such as the origin of the mitochondria and chloroplast and the divergence of phyla and kingdoms (Woese, 1987). DNA sequencing is likely to resolve many of the long-standing problems in phylogenetic studies such as the evolutionary relation ships among bacteria and unicellualer eukaryotes (Sogin et al., 1986, 1989). Indeed, molecular data have proved so powerful

in the study of evolutionary history that we may eventually be able to reconstruct a fairly complete phylogeny of the major groups of the organisms.

However, it should not desert traditional means of evolutionary inquiry, such as morphology, anatomy, physiology, and paleontology. Rather, different approaches provide complementary data. The taxonomy is based mainly on morphological and anatomical data and the paleontological information is the only data that can provide a time frame for evolutionary study.

10. Phylogenetic tree construction

There are three methods for construct into a phylogenetic tree, the distance method, the parsimony method and the maximum likelihood method. In this study, the distance method was used to construct our phylogenetic tree.

The distance method is the estimation of the true evolutionary distance, which reflects the actual mean number of changes per site that have occurred between a pair of sequences since their divergence from a common ancestor. The evolutionary distance between each pair of taxa is then equal to the sum of the length of each branch lying on the path between the members of each pair.

The distance is transformed by sequence data. The most common method of summarizing the relationship between two sequences is by their fractional (or percentage) similarity or dissimilarity. The sequence dissimilarity is equal to the number of aligned sequence positions containing non-identical residues (bases or amino acid) divided by the number of sequence positions compared

A general framework for describing distance measured uses a divergence matrix to represent the relative frequencies of each nucleotide (or amino acid) pair in a given pairwise comparison of two sequences.

The distance for Kimura's (1980) two–parameter model is calculated from the proportions of transition-type differences and transversion type differences. The proportion of transitions and transversions is estimated separately for each pair of taxa.

11. Phylogenetic studies in prawn

Palumbi and Benzie (1991) discovered high levels of molecular divergence between *Penaeus stylirostris*, *P. vannamei*, *P. esculentus* and *Metapenaeus endaevori*, as revealed by partial sequencing of 12S and COI mtDNA regions.

Machado et al. (1993) compared partial sequence of the mitochondrial 16S rRNA gene of *Penaeus notialis* and *Penaeus schmitti*. They revealed 11% nucleotide divergence between the two species. This fragment was compared with *Artemia salina* and *Drosophila yakuba* indicated high nucleotide divergence due to numerous substitutions and insertions/deletions. Sequences have been obtained from six individuals of *P. notialis* belonging to the same population, their comparison showed a 0.7% nucleotide diversity. They concluded that the 16S-rRNA gene appeared as a good marker for speciation studies in Crustacea.

Baldwin et al. (1998) studied the evolutionary relationships among 13 species of prawn genus *Penaeus.* Analysed of partial sequence of COI gene revealed the high

genetic divergence between species (8-24%). The observed relationships are concordant with biogeographic boundaries across the tropical range of *Penaeus*.

Shank et al. (1999) studied the evolutionary history of deep-sea prawn (Caridae: bresiliidae) inhabiting deep-sea hydrothermal vent and hydrocarbon seep environments using the mitochondrial Cytochrome Oxidase subunit I (COI) gene. Phylogenetic analyses recovered three distinct clades consistent with higher level taxonomy based on morphology. The phylogenetic results suggested that *Chorocaris* was paraphyletic and that *Mirocaris fortunata* and *M. keldyshi* might not be genetically distinct. The evolutionary relationships of vent-endemic prawn species did not appear to be correlated either with their extant biogeographic distribution or with the history of sea floor spreading.

Gusmão et al. (2000) compared species of Southwestern Atlantic penaeids (*Penaeus subtilis*, *Penaeus paulensis*, *P. brasiliensis* and *P. schmitti*) and estimated their phylogenetic relationships using allozymes and mitochondrial (COI) gene. Four main Brazilian penaeid species were discriminated and the new species of *Penaeus* were detected. This new species corresponded to one of the described morphotypes of *P. subtilis*. Their data also support the conclusion of Baldwin et al. (1998) that the subgenus *Farfantepenaeus* was polyphyletic.

Maggioni et al. (2001) compared the partial sequences of 16S rRNA mitochondrial gene obtained from 10 penaeid prawn species (*Farfantepenaeus paulensis, F. brasiliensis*, *F. subtilis*, *F. duorarum*, *F. aztecus*, *Litopenaeus schmitti*, *L. setiferus*, and *Xiphopenaeus kroyeri* from the western Atlantic and L. vannamei and L. stylirostris from the eastern Pacific. The phylogeny resulting from the 16S partial

sequences showed that these species form two well-supported monophyletic clades, consistent with the two genera proposed in a recent systematic review of the suborder Dendrobranchiata.

Arena et al. (2003) revealed the genetic relationship between *Litopenaeus setiferus* (L.) and *L. schmitti* (Burkenroad) determined by 16S mitochondrial sequences and enzymatic analysis. Using eigth polymorphic enzymes, the genetic distance between the two species was 0.165. The genetic distance between *L. setiferus* populations was 0.0057 and between *L. schmitti* populations was 0.0034. The homology percentage of 16S fragments was compared with those from six different prawn species. Ninety-seven percent of identity was found by analysis of a 409 bp of 16S mitochondrial DNA. The genetic distance was confirmed the classification proposed by Pérez-Farfante and Kensley (1997).

12. Population genetic studies in crustaceans

Meyran et al. (1997) used the 376 base segment of cytochrome oxidase subunit I (COI) to determine the extent of genetic differentiation among six species of the genus *Gammarus* (Crustacea, Amphipoda) common in France (*G. fossarum*, *G. pulex*, *G. lacustris*, *G. wautieri*, *G. roeseli*, *G. marinus*). Twenty-three different populations, 104 specimens were compared to examine their taxonomic status and their phylogenetic relationships. The strong sequence variability found at the interspecific level, generally marked by morphological or ecological similarities, was consistent with that observed for other crustaceans and supports existing species classifications.

A high level of genetic variation was observed at the intraspecific level, suggesting that the COI gene will be powerful marker for amphipod population biology.

In 1999, the resurrected genus *Lepidophthalmus* defines a group of estuarine burrowing prawn which, within the western Atlantic, were until recently treated as a single panmictic taxon comprising the species *L. jamaicense.* Individuals were collected from fourteen Gulf of Mexico, two Caribbean and one eastern Pacific localities and assayed them by allozyme electrophoresis for 19 presumptive loci. Polymorphic alleles were evaluated by principal components analysis. Allozyme data were summarized as *F*-statistics and used to calculate pairwise genetic distances between populations. Low heterozygosity within populations may be attributable to a semi-permanent fossorial adult habitat. Populations of *Lepidophthalmus* from the Gulf of Mexico resolve into three distinct genetic lineages whose pairwise allozyme-based distances are comparable to separate but congeneric taxa in other invertebrate groups. The most widespread lineage, which constituted all samples taken from the northern through northwestern Gulf, was identified as *L. louisianensis.* Two other lineages ("a" and "b") occurred sympatrically in a restricted area of the southwestern Gulf of Mexico. All three of these endemic Gulf lineages are allozymically distinct from the sampled Caribbean and eastern Pacific congeners, including *Lepidophthalmus jamaicense* and *L.* nr. *bocourti,* but their taxonomic status remained to be resolved. This report was the first account of sympatric distribution for any such genetically distinct lineages of the genus *Lepidophthalmus.*

Klinbunga et al. (1999) analysed the intraspecific popuation structure of the giant tiger prawn (*Penaeus monodon*) collected from 5 geographic locales (Phangnga,

Satun, and Trang from the west and Chumphon and Trad from the east of peninsular Thailand) using randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). This method indicated that genetic population differentiation does exist in this species ($P<0.0001$). Large genetic differences were observed between each of the Andaman Sea and Trad populations confirming genetic subdivision of *P. monodon* in Thailand.

A total of 88 genotypes from 3 polymorphic RAPD primers (UBC268, UBC273 and UBC 299) were observed. The distribution pattern of RAPD genotypes of *P. monodon* from Chumphon was similar to that of Phangnga, Satun and Trang but different from that of Trad. Within each region, the Andaman *P. monodon* is genetically homogeneous but significant geographic heterogeneity was found between Chumphon and Trad ($P< 0.0001$). Between region comparisons surprisingly revealed homogeneity between *P. monodon* from Chumphon and that of Phangnga, Satun and Trang while Trad was found to be reproductively isolated from the other populations. This circumstance is unlikely to occur naturally and opens the possibility that the anomalous gene pool found in Chumphon may have resulted from transplantation and/or farming activities of *P. monodon* (Klinbunga et al., 1999).

Hurwood et al. (2003) study the population structure in freshwater prawn (*Paratya australiensis*), in the Conondale Range, south-eastern Queensland with two subcatchments each within two river drainages sampled, by analysing a partial fragment of the mitochondrial COI gene. The allozyme study revealed a high degree of population structure, with the data interpreted as reflecting a pattern of restricted contemporary gene flow, primarily between streams within subcatchments. High

levels of differentiation occurred between all subcatchments. The mtDNA data largely conflicted with the hypothesis of restricted gene flow indicated that contemporary dispersal was highly unlikely, even between streams within subcatchments, with many sites fixed for unique mtDNA haplotypes. The level of divergence between the Stony Creek subcatchment and all other sampling sites indicated that it had been isolated for approximatedly 2-3 million years, while low levels of divergence were detected across the Conondale range between the Kilcoy and Booloumba Creek subcatchments. The sharing of alleles at certain allozyme loci between all subcatchments was likely to be the result of ancestral retention and possibly because of the effect of balancing selection.

Bruyn et al. (2004) analysed the phylogenetic relationships of 18 wild populations of the giant freshwater prawn, *Macrobrachium rosenbergii*, utilising a fragment of 16S rRNA mitochondrial gene, identified two major reciprocally monophyletic clades either side of a well known biogeographic barrier, Huxley's line. The level of divergence between the two clades (maximun 6.2%) far exceeds divergence levels within either clade (maximum 0.9%), and did not concord with geographical distance among sites. Eastern and western *M. rosenbergii* clades have probably been separated since Miocene times. Within clade diversity appears to have been shaped by dispersal events influenced by eustatic change.

13. PCR-RFLP

Molecular phylogeny is the study of evolutionary relationships among organisms by using techniques of molecular biology. When suitably large products of PCR

amplification are purified using commercially available kits and digested with endonucleases to reveal polymorphisms in an amplified product (amplicon) it is termed PCR-RFLP or cleaved amplification product (CAP). This method can be considered precursors to sequencing and prove useful in population analysis (Dowling et al., 1990; Wilding et al., 1999), differentiation the species (Schroeder et al, 2003).

Base substitutions or insertion/deletion (indel) events have often been detected using restriction endonucleases (REs). For RE analysis, fragments of identical mobility tend to be homologous for sequences from closely related individuals and perhaps even from most intraspecific comparison (depending on the rate of evolution of the DNA sequence in question). However, the likelihood of convergence- that is two samples having fragment of the same size but produced by different cleavage sites-increase as sequences become more different (Upholt, 1977).

The application of mtDNA RFLPs to phylogenetic analysis of congeneric species has been reviewed extensively (Wilson et al., 1985; Birley and Croft, 1986; Avise, 1986; Moritz et al., 1987; Avise, 1994). In general, the approach has proven useful for resolving relationships of closely related species.

Closely related animal mtDNAs can be compared using 4 bp recognizing REs (e.g., Brown, 1980; Moritz, 1991; Dowling and Childs, 1992; Dowling and Brown, 1993) but beyond ~2% sequence divergence it becomes too difficult to identify individual gains or losses of cleavage sites (see Hillis et al., 1992). In addition mtDNAs are best analyzed by mapping cleavage sites for 5- or 6- bp-recognizing REs (Dowling and Brown, 1993).

Restriction fragment length polymorphisms in polymerase chain reaction amplified fragments (PCR-RFLP) of mitochondrial DNA were used to differentiate species of New World screwworms (Diptera: Calliphoridae). Twenty-seven restriction enzymes were screened on five regions of mtDNA. Eleven restriction fragment length patterns diferentiated New World screwworm, *Cochliomyia hominivorax* (Coquerel), from secondary screwworm, *Cochliomyia macellaria* (F.). Five restriction frangment length patterns were polymorphic in *C. hominivorax* while all fragment patterns were fixed in *C. macellaria*. Diagnostic restriction fragment length patterns were used to characterize field samples and laboratory strains. The PCR-RFLP technique is flexible with regard to developmental stage of the sample and method of preservation. They were able to characterize specimens of all life stages from egg to adult including larvae preserved in alcohol and pinned adults. PCR-RFLP is rapid and inexpensive, enabling specimens to be characterized within 24 h for less than $2.50 (Taylor et al., 1996).

Siludjai et al. (1999) studied the genetic diversity and mtDNA patterns of a domesticated stock of the giant tiger prawn (*Penaeus monodon*) by restriction analysis of 16S rDNA and intergenic cytochrome oxidase subunit I –II (COI-COII). The results indicated that the diversity level of F_1 and the wild stock was comparable, thus eliminating the possibility of including an inbred founder population in their breeding programme.

Wolf et al. (1999) differentiated between several types of sturgeon caviar by PCR-RFLP. The PCR products of a region of mitochondrial genome (462 bp of cytochrome b) were cut with different restriction endonucleases (REs) resulting in species specific

restriction fragment length polymorphism (RFLP). This method was suitable to distinguish between 10 species of *Acipenser* and *Huso* originating from Europe and Asia.

Wolf et al. (2000) used the PCR-RFLP method to identify fish species such as salmon, cod, goosefish, bass, mackerel, tuna and tonguefish. The PCR amplification product of a specific part of the mitochondrial genome (cytochrome b) was cut with different restriction endonucleases resulting in species specific marker. This method is suitable for differentiating between, for example, Atlantic and several Pacific salmon species.

In this study, the morphological and morphometric characters, including MDH isozyme, was the preliminary method used to identify the species. Then the mitochondrial DNA was used to identify the samples that could not determine species and the samples, which could determine species in order to resolve the unidentified samples by morphological and isozymatic methods. So the morphological and isozymatic method was only a pre-screening for species identification, and ambiguous samples were left for resolution following the phylogenetic study.