GENETIC POPULATION STRUCTURE OF *PENAEUS MONODON* USING ALLOZYME AND MITOCHONDRIAL DNA ANALYSIS

A Thesis presented for the degree of Doctor of Philosophy to the University of Stirling

by

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TO MY PARENTS AND OUD

DECLARATION

I declare that this thesis has been composed by myself. The work described in this thesis is the result of my own research and has neither been submitted nor accepted for any other degree.

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ABSTRACT

The giant tiger prawn (*Penaeus monodon*) was collected from 11 different sites throughout its range (Kenya→Indo china→Philippines). Individuals were analyzed for allozyme (46 loci) and mtDNA restriction fragment length polymorphisms (RFLPs) using 5 restriction enzymes. The average heterozygosity calculated from the allozyme data H = 0.027 (0.020-0.032) was similars to other studies on decapod crustacean. In total 10 polymorphic (p 0.99) loci were observed several of these (*AAT-1**, *ALAT**, *GPI**, *IDHP**, *MDH-1**, *MPI** and *PGM**) contributing to significant differences in allele and genotype frequencies between populations. No significant differences were observed between samples collected at the same site in different years and no significant differences in HWE were observed within any single population. Genetic difference between all populations F_{st}=0.384 was high but was much smaller for SE Asian populations alone F_{st}=0.014. The allozyme data suggested that the *P. monodon* populations structure could be divided into 4 main groups: Kenya, Philippines, Andaman Sea, and South China Sea.

The mtDNA genome was estimated to be 15.76±.57 kb. Only four enzymes could be reliable used (*Bam*HI, *Eco*RV, *Pvu*II and *Sac*I) which gave 12 mtDNA haplotypes (Kenya population not included). Significant differences between haplotype frequencies were observed between the Andaman Sea and South China Sea populations. Nucleotide divergence data suggested two main clonal forms of mtDNA were present in SE Asian populations. These clones appear to have diverged gene 0.1-0.7 million years ago. Clone A was more

common in Andaman Sea population whereas Clone B was more common in South China Sea.

Both techniques suggested that *P. monodon* can be subdivided into at least 3 isolated groups: Kenya, Andaman Sea, and South China Sea populations. This pattern of isolation we see today may be a relic of past separation during the last glaciation. Which has been modified by recolonization of new areas as the sea levels rose and present day patterns of gene flow caused by prevailing current patterns.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

During the last decade, marine penaeids have assumed major significance in aquaculture. This is because of a strong market demand, with the highest prices coming in the international markets. Japan and the United States of America are the two major consumer markets that have caused the explosive growth of this industry. Certainly such demand has stimulated both governments and the private sector to promote investment in research and development of marine penaeids throughout the world. FAO aquaculture statistics (FAO, 1992) also show that marine penaeid production increased rapidly from 218,000 mt in 1985 to 719,000 mt in 1991. There are around 20 species of marine penaeid which are commercially important in terms of culture, i.e. banana prawn (*Penaeus merguiensis*), fleshy prawn (*P. chinensis*), giant tiger prawn (*P. monodon*), kuruma prawn (*P. japonicus*), white shrimp (*P. indicus, P. occidentalis, P. schmitti, P. setiferus, P. vannamei*), blue shrimp (*P. stylirostris*), greasyback shrimp (*Metapenaeus bennettae*) and greentail shrimp (*M. ensis*) etc.

Within the marine penaeid farming industry, giant tiger prawn *P. monodon* Fabricius 1978 is dominant (61% of the total production in 1994) (Aquaculture Digest, 1995). Since 1970, fisheries statistics indicate that *P. monodon* represent the largest production of cultured marine penaeid (FAO, 1992), and the yield has

increased very quickly from 46,672 t in 1984 to 447,130 t in 1994 (Aquaculture Digest, 1995; FAO, 1992). Apud *et al.* (1983) and Dore and Frimodt (1987) said that *P. monodon* is the biggest of the penaeid group, reaching up to 330 mm in body length or 500-600 g in body weight, and is of commercial importance in marine penaeid markets. Among the characteristics that make it an ideal aquaculture species are high survival rates of up to 90% in grow-out ponds and fast growth rates with sizes of up to 45 grams after 4-5 months in low density culture, although normal harvest size ranges from 15-40 individuals per kilogram (25-67 g). As mentioned by Apud *et al.* (1983), *P. monodon* can survive a wide range of temperature and salinity levels though it grows faster in low salinity (10-25 ppt). In addition it can tolerate being out of water for short periods of time making it easy to manage, harvest and transport.

Originally, *P. monodon* was one of the marine penaeid species produced in extensive traditional marine penaeid culture and in polyculture with milkfish (Apud *et al.*, 1983; Saisithi, 1989; Chen, 1990). Because the fry are easily captured from the wild, fish farmers bought the fry collected by rural people. The juveniles were easily identified and usually hide among weeds and grass in estuaries. The fishermen simply place bundles of grass in estuaries to attract the fry and scoop them out with dipnets. In 1968, intensive monoculture of the *P. monodon* was made possible with the first success in artificial propagation of *P. monodon* larvae. In the beginning, the industry was dependant on the capture of wild gravid females as broodstock. Shortage of female brooders in Taiwan quickly pushed the price up to US$ 1,900/kg (Chen, 1990). The Taiwanese thus started importing brood *P. monodon* from the Philippines, Malaysia, Thailand,

and Indonesia. In 1977, there was first notable success in inducing maturation in female *P. monodon* by eyestalk ablation (Chen, 1977). This made the female spawn in 3-4 days and can be repeat spawned after eyestalk ablation between 2-4 times before her death. This development of hatchery technology reduced the price of broodstock and made millions of seed available and started the boom in intensive *P. monodon* culture.

More recently, cultured females have been matured and spawned in captivity by eyestalk ablation within 7-12 months (Aquacop, 1977; Primavera, 1977). But Chotikun *et.al.* (1987) and Chen (1990) mentioned that females in captivity do not grow to a large size and have a lower fecundity than those in the wild. Also *P. monodon* are normally marketed long before sexual maturity and broodstock are not available from most commercial farms therefore no one encounters ripe females during rearing. Wild females from capture fisheries are still the dominant source of *P. monodon* brooders. However, eyestalk ablation seems to be effective with most large (a minimum of 80 g but preferably much larger) and healthy females (Chen, 1990). Seed Production Team (1984) showed that after eyestalk ablation, most large females (120-200 g) spawned within one week and 72% of these became gravid, whereas small females (100-130 g) did not spawn until 21-30 days and of these only 58% became gravid.

Since the explosive growth of intensive monoculture of *P. monodon* in Taiwan, in 1985 the prices of *P. monodon* climbed from US$ 3.20/kg to US$ 8.00/kg and Taiwan could not supply the Japanese market with *P. monodon* for year-round consumption, due to the cold season. This encouraged other tropical

countries to apply for a large amount of investment to produce *P. monodon* to supply Japan throughout the year (Kongkeo, 1989; Chen, 1990). The Southeast Asia countries, such as Indonesia, Malaysia, Thailand and the Philippines have ideal conditions for the development of marine penaeid industry. For instance, these countries have optimal geography, climate and other physical factors, such as; availability of broodstock; experienced and enthusiastic farmers willing to invest; plentiful raw materials and technology for feed production; also transport and cold storage facilities.

In 1987, prawn culture in Taiwan crashed due to disease problems with more disastrous harvests in 1988 and 1989 (Chen, 1990). Since then, Japan has increased *P.monodon* orders from other Southeast Asia countries and overall *P. monodon* production has shown a tremendous increase. For instance, *P. monodon* production by culture in Thailand has increased from 170 t in 1984 to 93,500 t in 1990, and had reached 214,750 t in 1994 (Aquaculture Digest, 1991 and 1995; FAO, 1992). The most recent official figures for SE Asian area in 1994 showed the production share between the main countries. Approximately 356,850 t about 79.8% of world *P. monodon* production came from SE Asia, i.e. Thailand (213,750 t), Indonesia (85,000 t) and the Philippines (24,000 t) (Aquaculture Digest, 1995). Southeast Asia is therefore the main centre of *P. monodon* farming in the world. It is also the centre of geographic distribution and the main fishing grounds for *P. monodon* (Solis, 1988; Dall *et al.*, 1990).

Because of the growth of *P. monodon* culture, demand for live broodstock is very high. Since *P. monodon* farming industry in SE Asian countries relies

almost solely on the capture of broodstock from the wild, even countries such as Thailand and Indonesia, need to import broodstock from other countries to meet the demand. The supply of broodstock is available from both the Gulf of Thailand and the Andaman Sea but preferences may exist for broodstock from a particular area. In Thailand, farmers (pers. comm, 1993) prefer *P. monodon* larvae from Andaman Sea brooders rather than those from the Gulf of Thailand. They are said to have larger offspring, lower mortality, better morphology and culture characteristics. At present nobody knows whether this preference is based on environmental or genetic differences influencing culture performance of these different populations. High demand, availability of preferred broodstock and larvae from certain areas stimulates the movement of broodstock both within and between countries and can lead to overfishing in some areas. The decrease in wild *P. monodon* production has resulted in a government policy to increase *P. monodon* production by releasing hatchery reared *P. monodon* into areas containing depleted natural populations. For instance, Thailand has released larvae of *P. monodon*, mainly from Andaman sea sources, into the Gulf of Thailand at the rate of about 30 millions larvae per year (Thai DOF, 1993), as well as the escapes from *P. monodon* hatcheries and growout ponds. This will lead to stock mixing and break down of any natural populations structures that exist as mentioned by Ryman (1981). The recognition of reproductively isolated and genetically differentiated subpopulations within a species is important for management of many commercially exploited species. The concept that each reproductively isolated population is the unit of management is now central in assisting fisheries biologists to optimise harvest in many species, notably in Pacific salmon hatcheries population (Larkin, 1981). Lester (1979) also stated

that different gene pools should be treated separately in research as well as management policy.

There has been little attempt to understand the population dynamics of *P. monodon* within and between SE Asian countries. In the past, fishery management has been largely interested with the abundance and size of the species no concerned and little attention has been directed toward an understanding of the genetics of its populations (Allendorf *et al.*, 1987). In the long term, fishery management is the conservation of existing resources to ensure a sustainable yield. This concept known as *stock concept* has dominated fisheries management for almost 50 years (Gulland, 1983; Carvalho and Hauser, 1994). The focal idea is to define the patterns of recruitment and mortality and harvest only extra yield. The fisheries that do not exceed this will not compromise the natural genetic stock. Failures to achieve this goal by fishery management have resulted in a large number of commercial fisheries having collapsed in the present century (Clark, 1976).

Population genetics studies have already greatly assisted in the management of fish stocks in both the aquaculture and fisheries sector. The application of biochemical genetic techniques known as protein electrophoresis has revolutionized studies on the population genetics of marine and estuarine invertebrates (see Gooch, 1975; Battaglia and Beardmore, 1978; Levinton, 1980 and 1982; Burton and Feldman, 1982). The technique of gel electrophoresis coupled with histochemical staining of specific enzymes or proteins has proved a simple but powerful tool for examining genetic variation in natural populations

and has been used to analyze the structure of natural populations for more than two decades (Lewontin and Hubby, 1966). Electrophoretic techniques have become crucial tools in the estimation of the level of molecular variation in particularly in aquatic organisms. In general, the allele frequency data obtained from electrophoretic studies can be examined to deduce the breeding structure or the relative reproductive isolation of a particular species. Such knowledge contains important information for the management of wild stocks and the initial selection and long-term management of potential stocks for aquaculture. The allozyme data can be used in a wide range of different ways: unique allozymic genotypes can be used as genetic tags for wild and cultured stocks, enabling interactions between different strains to be assessed, e.g. restocking. Allozymes are useful for species identification and monitoring hybridization in wild and farmed stocks. Overall levels of genetic variation enable the effects of inbreeding caused by poor management or selection programmes to be monitored, identifying potential problems in longterm viability. Allozymes are now also important in many genetic studies, as fish generally have few visible markers, particularly genomic manipulations in which the fate of the paternal or maternal genome are of some consequence (Seeb and Miller, 1990).

Recently, restriction endonuclease analysis of mitochondrial DNA (mtDNA) has also been employed at the species level to help determine population structure (Avise and Lansman, 1983; Avise, 1985). This technique is especially useful with closely related recently isolated populations (Brown *et al.*, 1979). It is believed that this method or related methodology, which directly, reflects differences at the DNA level, will most closely approach the ideal method

for quantifying genetic difference among population.

The purpose of the present study is to use a combination of electrophoretic techniques on proteins and restriction endonuclease analysis of mtDNA to determine the population breeding structure of *P. monodon* in Southeast Asia. This study will attempt to show the population breeding structure of this organism and should also detect the levels of reproductive isolation and genetic variation of the species, this work will or assist in making basic decisions on the management of the farmed and wild resource.

1.2 MOLECULAR GENETIC ANALYSIS OF POPULATIONS

Population genetics is concerned how Mendel's laws and other genetic principles apply to entire populations of organisms. Traditional methods of distinguishing fish stocks are limited to long-term breeding studies of a few morphological characters such as number of scales in a lateral series or relative body depth, or behavioral variation (Allendorf *et al.*, 1987; Smith, 1990). In the 1950s, there were two major discovery which were to have important implications for molecular genetics resulting in rapid developments in the identification and analysis of individual genes. First, Watson and Crick (1953) deduced the structure of the DNA molecule, which explained the direct relationship between genes and protein. Second, the initial improvement of electrophoresis techniques for protein separation (Smithies, 1955) and the histochemical staining of specific enzymes (Hunter and Markert, 1957) enabled for the first time a large number of

different gene products to be studied so reasonable estimates could be made of the levels of genetic variation in natural populations. Early studies on the population genetics of *Drosophila* (Lewontin and Hubby, 1966) and humans (Harris, 1966) suggested that much higher levels of variation exist throughout all classes of organisms than had been expected from the earlier studies using visible markers.

The relative simplicity of electrophoretic techniques led to an explosion of activity and revealed genetic variation within and among populations of animal and plant species. In fisheries studies, protein electrophoresis has been used as a primary tool to identify population structure in various fish species. The fairly rapid procedure of histochemical staining technology enabled large samples to be screened for over 100 enzyme loci simultaneously (Richardson *et al.*, 1986; Wishard-Seeb and Gunderson, 1988; Morizot and Schmidt, 1990). This technique still remains at this moment the fastest and most economical method for surveys of variation at a large number of genes. However, there are many cases in which electrophoresis fails to identify any genetic differentiation between populations or individuals (Utter, 1981; Grant, 1984). Lewontin (1974) estimated that variation at the proteinis is only about 30% of the variation present at the DNA level of the gene because of the various limitations of the technique.

Recent major advances in analysis of nucleic acids in the past decade have lead to the widespread study of mitochondrial DNA (mtDNA) variation of animals, especially for population and evolutionary studies. MtDNA because of its small size, relatively rapid rate of evolutionary change, and maternal haploid

inheritance make it appropriate for examining population history and structure within and between conspecific populations (Wilson *et al.*,1985; Avise *et al.*, 1987; Moritz *et al.*,1987). MtDNA is more rapidly evolving than chromosomal DNA and because we can look directly at mtDNA variation it is easier to observe. Moreover, it is easy to purify compared with other forms of DNA (Brown, 1983). Ferris and Berg (1987) also stated that, of all existing procedures, restriction endonuclease analysis of mtDNA most closely approaches the ideal method for quantifying genetic differences among populations. Therefore the use of both allozyme and Restriction Fragment Length Polymorphism (RFLPs) in mtDNA techniques enhances the detection of variability in population genetic studies.

1.2.1 Allozyme Electrophoresis

Electrophoresis is a powerful yet relatively simple technique that separates proteins according to their net charge and size in an electrical field. Ferguson (1980) and Hartl (1988) described it as '*the most widely used procedure for revealing genetic variation in enzymes and other proteins*'. In addition, the genetic interpretation of gel phenotypes is straightforward, and enables the researcher to examine many independent genes in individuals and populations.

The major applications of electrophoretic techniques in fisheries research are stock identification, population analysis, and species boundary determination. These will be used in analysis of population structure (e.g. geographic variation, mating systems, heterozygosity, and individual relatedness) which is perhaps the

most fundamental piece of information required for species management (Baverstock and Moritz, 1990).

1.2.1.1 Genetic relationship between gene & protein

The **Gene** is the basic unit of inheritance, by which hereditary characteristics are transmitted from parent to offspring during the reproductive process (Hartl, 1987; Lawrence, 1989); in humans traits such as hair colour, eye colour, skin colour, height, weight, and various aspects of behaviour are all hereditary characterictics. A single gene consists of a length of **deoxyribonucleic acid** or **DNA**. DNA, a double helix consists of two long strands wound together as first proposed by Watson and Crick (1953), is the genetic material found in almost all organisms. The genetic code carried in each DNA strand is composed of a sequence of nucleotide, i.e. purines (adenine and guanine) and pyrimidines (cytosine and thymine), abbreviated as A,G,C and T. Hartl (1980) and King and Stansfield (1985) described that the nucleotide in the two strands have a specific connection, where one strand carries an A, the other strand must carry a T, where one strand carries a G, the other must carry a C. Therefore, any change in nucleotide sequence in DNA molecule alters the genetics information in different forms of the same gene different forms of the same gene are called **alleles**.

Proteins are large molecules composed of one or more **polypeptide chains**, each polypeptide subunit composed of a linear chain of up to 20 different **amino acids** covalently connected by **peptide bonds** (King and Stansfield, 1985; Utter *et al.*, 1987). It has been found that each amino acid is coded by three different

combinations of the nucleotide called a **codon**. Therefore, the base sequence of DNA has a direct relationship to the structure of proteins because polypeptide chains that form proteins are coded by groups of codons or gene codes where protein synthesis occurs.

1.2.1.2 Protein electrophoresis

Protein electrophoresis is the movement of charged particles under the influence of an electric field (Ferguson, 1980; Richardson *et al.*, 1986). Since the side chains of amino acids are either acidic (COO^-; aspartic acid and glutamic acid) or basic (NH_3^+; lysine, arginine, and histidine) groups, the proteins migration through the gel is therefore determined by their amino acids composition; molecular weight, conformation, and net charge (Ferguson, 1980; Utter *et al.*, 1987; Murphy *et al.*, 1990).

Most proteins studied in electrophoresis are enzymes because it is easy to develop histochemical staining procedures to visualize the activity of specific enzymes as first published by Hunter and Markert (1957). Their position on the gel is detected directly by adding histochemical stains for specific enzymes following electrophoresis. The stains containing a specific substrate for the enzymes, the catalytic action of the enzyme is then linked into a pathway of chemicals or other enzymes which result in a visualization of the position of the enzyme in the gel. There are now over 100 of histochemical stain recipes used in human and in fish research, i.e. Brewer (1970), Shaw and Prasad (1970), Harris and Hopkinson (1976), Siciliano and Shaw (1976), Richardson *et al.*

(1986), Shaklee and Keenan (1986), Aebersold *et al.* (1987), Benson and Smith (1989), Morizot and Schmidt (1990), and Murphy *et al.* (1990). When an enzyme in an individual has an amino acid substitution that cause a difference in size, shape, net charge, or possibly catalytic activity and stability (Shaw, 1965), then the enzyme may have an altered electrophoretic mobility. The banding pattern observed will vary depending on the quaternary structure of the enzyme and whether it is present as a single or a multi locus for that enzyme. Electrophoresis with histochemical staining technique can therefore be used to detect a proportion of alleles that result in differences in electrophoretic mobility of any enzyme.

A. Allele frequencies

Enzymes that can be detected by electrophoretic techniques have two general forms, **isozymes** and **allozymes**. Markert and Moller (1959) defined isozymes as '*the different molecular forms in which proteins may exist with the same enzymatic specificity*'. These multiple forms of an enzyme which are produced by different gene loci have different properties such as optimum pH or isoelectric point and therefore have bands of different electrophoretic mobility (Markert and Moller 1959; Markert, 1983). Allozymes, a subset of isozymes, are enzymes that have different electrophoretic mobility as a result of allelic variation at the same locus (Prakash *et al.*, 1969). It is generally only allozyme data that is used in either systematic studies (Avise, 1974) or phylogenetic analysis (Swofford and Olsen, 1990).

In order to study genetic variation between populations, it is necessary to

measure allozyme variation in terms of allele frequency. **Allele frequency** defined by King and Stansfield (1985) is the proportion of particular allele at the locus. However, the allele frequency in a sample of individuals is only an estimate of the true allele frequency in the whole population. But, the estimate will be close to the true frequency if the sample is sufficiently large; at lease 100 or more individuals (Richardson *et al.*, 1986; Hartl, 1988).

B. Polymorphism

Polymorphism is defined as '*the existence of different forms of individuals within a species or a population*' (Lawrence, 1989). By calculating allele frequency, technically a locus is said to be **polymorphic** if the most common allele has a frequency of less than 0.95 (Richardson *et al.*, 1986; Hartl, 1988) or 0.99 (Ferguson, 1980). Conversely, a **monomorphic** locus is one that is not polymorphic, and **rare alleles** defined by Hartl (1988) are alleles with frequencies of less than 0.005, and usually one or two individuals per thousand are heterozygous for rare alleles at any locus.

1.2.1.3 Limitations of protein electrophoresis

In nearly all organisms, with the exception of some viruses, the genetic information is encoded in deoxyribonucleic acid (DNA) as a sequence of nucleotide bases. The detection of genetic variation by gel electrophoresis and histochemical staining of proteins is an indirect estimate of changes in the sequence. Consequently, the technique has limitations: the genetic code has redundancy and all base substitutions do not necessarily result in changes of

amino acids sequence in the polypeptide chain; 16 of the 20 common amino acids are electrostatically neutral, so some base substitutions that result in a different amino acid being attached to the polypeptide chain will not result in a charge differences and will therefore not be detected on the gel. It has been estimated that standard electrophoretic techniques detect only about one-third of base substitutions in DNA (Lewontin, 1974). A third limitation of the technique is that the protein molecule can be modified by post-translational changes, such as the banding of small molecules, that can alter the mobility of the protein through the gel but that do not have a direct genetic basis. Finally, electrophoretic techniques are used to estimate genetic variation in genes that are transcribed and translated into proteins. This class of genes involves only about 10% of the total genome, so the variation detected at protein gene loci may not be representative of the genome as a whole (Smith, 1990).

It is also important to establish that differences in electrophoretic mobility are not caused by non-genetic factors such as the conditions and length of storage of the samples. Prolonged storage may result in shadow bands or conformational isozymes which can cause problems in recording genetic information from phenotypes on gels (Utter *et al.*, 1987). Although repeating runs and using fresh samples as controls can solve many of these problems.

1.2.2 Mitochondrial DNA Restriction Fragment Length Polymorphism

In the last few years advances in techniques permit the analysis of

population genetics and systematics by examining variation at the DNA level. Of these techniques, the examination of mitochondrial DNA variation has been widely used. The analysis of RFLPs of mtDNA has shown that there is extensive intraspecific variation in most species (Wilson *et al*, 1985; Avise *et al*, 1986; Saunders *et al*, 1986). It is therefore important to understand the structure of mtDNA and methodology of mtDNA study.

1.2.2.1 Molecular properties & transmission genetics

The nucleus is not the only organelle in eukaryotic cells that retains genetic information. Mitochondria also contain their own DNA which is essential to their function. What are the features of mtDNA that make it a powerful tool for studying the genetic structure of populations? First, the mitochondrial genome consists of a single, duplex, closed-circular DNA molecule. The mitochondrial genome is comparatively small ranging in size from 16,000 to 19,000 bp; the mammalian mitochondrial DNA (mtDNA) has a molecular weight of about 11×10^6 d, 10^{-5} times less than that the size of the nuclear genome (Avise, 1985; King and Stansfield, 1985). The mtDNA is usually present as a number of identical copies. Generally there are about 5-10 identical copies per organelle making it rather easy to purify (Brown, 1983). Second, mtDNA is generally homoplasmic within an individual (Avise, 1985; Billington and Hebert, 1991), only a single mtDNA nucleotide sequence exists within an individual, that is, all the molecules are identical in an organism so any tissue can be used as a source. Third, mtDNA is maternally inherited and nonrecombining (Hutchinson *et al.*, 1974; Lansman *et al.*, 1983; Gyllensten, 1985). It is transmitted intact from the

female parent to progeny through the cytoplasm of the egg without being altered by recombination or meiotic segregation. Because only females transmit their mitochondria to their progeny, mtDNA genotypes exhibit a evolutionary history of asexual (matriarchal) transmission. Thus, if there is asymmetrical dispersal by sex, and males disperse during spawning, mtDNA might reveal more genetic divergence than would be expected from biparental studies such as allozymes or nuclear RFLPs (Bermingham, 1990; Billington and Hebert, 1991). Finally, all animals have mtDNA which is remarkably conservative in size, function and organization across most animal taxa studied (Brown, 1983; Moritz *et al.*, 1987). Considering the conserved characteristics of gene order in mitochondrial genome, it might be assumed that the primary sequence would also be well conserved. In contrast, evolution at the nucleotide sequence level of mtDNA is rapid, approximately 5-10 times faster than single-copy nuclear DNA (Brown *et al.*, 1979).

The resolution capability of mtDNA studies appears to be enhanced by these characteristics. Thus, in general, conspecific population and related species show greater differentiation of mitochondrial than nuclear genes. For example, Sanger (1986) as cited by Ovendon (1990) found no allozyme genetic differences between western and northern Victorian populations of the river blackish (*Gadopsis marmoratus*) but Ovendon *et al.* (1988) detected sequence diversity of mtDNA from two populations of about 6.4%. These make mtDNA a useful molecule for studies of population genetics and evolution.

1.2.2.2 Mitochondrial DNA isolation

Mitochondrial DNA is usually obtained from liver or gonadal tissues because of their mitochondrial enrichment but any other tissue may be used (Chapman and Brown, 1990; Billington and Hebert, 1991). MtDNA may be isolated either from fresh or frozen tissues. For fresh tissue, nuclei are first removed from homogenized tissue by low speed centrifugation, mitochondria are pelleted by high speed centrifugation, and then mtDNA is released by membrane lysis. With rapid isolation methods (Maniatis *et al.*, 1982; Chapman and Powers, 1984), mtDNA is recovered by phenol extraction followed by ethanol precipitation. Alternatively, mtDNA can be purified using Caesium chloride density gradient centrifugation (Lansman *et al.*, 1981). For frozen tissue, total nucleic acids are extracted: tissues are powdered in liquid nitrogen, lysed with Sodium dodecyl sulphate (SDS), centrifuged to remove debris, extracted with phenol, and nucleic acids collected by alcohol precipitation (Gross-Bellard *et al.*, 1972; Ausubel *et al.*, 1987; Sambrook *et al.*, 1989).

1.2.2.3 Restriction endonuclease analysis of mitochondrial DNA

Restriction endonucleases (REs) are enzyme that cleave duplex DNA at a constant position within a specific recognition sequence, typically 4-6 base pairs (bp) long. Over 400 REs have been isolated from bacteria and at least 85 different recognition sequences characterized (Fuchs and Blakesley, 1983; Roberts, 1984). To date, the study of mtDNA variation is based upon digestion of the molecule using these REs. The fragments produced are then separated

according to their molecular weight by gel electrophoresis, agarose or polyacrylamide gels.

The method of visualization of fragments is dictated by the purity and amount of mtDNA. For purified mtDNA from fresh tissue, there are three primary methods for visualization of fragment pattern. First, fragments can be directly identified by ethidium bromide (EtBr) staining. The EtBr detection limit is normally about 1-5 ng of DNA in a band (Sharp *et al.*, 1973 as cited by Chapman and Brown, 1989)). However the photographic method was developed and this increases detection to 100 pg or less (Chapman and Brown, 1989). Second, silver staining of DNA fragments is reported to be more sensitive, allowing detection of 10-100 pg amounts of DNA (Guillemette and Lewis, 1983). Third, sensitivity can be increased by end-labelling with radionucleotides followed by autoradiography (Brown, 1980). This method is also highly sensitive, 100-500 pg of DNA can be visualized. For crude mtDNA or total nucleic acids, Southern-blotting methods (Southern, 1975) are appropriate because of decreasing sample preparation time and the possibility of utilizing frozen or limited tissue. This transfer hybridization is reportedly the most sensitive of the methods. With this system the amount of DNA required per digested is 100 times less than needed for direct visualization by EtBr staining, allowing detection of picogram quantities of a fragment (Southern, 1975).

1.2.3 Population Genetics

Population genetics is the study of the genetic composition of populations.

Such studies are essential to help us properly understand evolution. Fundamentally, evolution is the result of progressive change in the adaptation of organisms to their environment. Such studies help us understand and predict the effects of such genetic phenomena as segregation, recombination and mutation; at the same time, taking into account such ecological and evolutionary factors as population size, pattern of mating, geographic distribution of individuals, migration, and natural selection (Hartl, 1980). Ideally, population genetics tries to describe the types and frequencies of genes in a population, to explain how the population's genetic composition came to be the way it is, and to predict how the population would change as a result of natural selection or as a result of artificial selection applied by a plant or animal breeder.

1.2.3.1 Scope of population genetics

The word "*population*" in general usage refers to a group of organisms belonging to the same species, but in population genetics, it does not usually stand for an entire species. Hartl (1988) states that it refers instead to a group of individuals of the same species living within a sufficiently restricted geographical area that any member of the population can potentially mate with any other member. Geographical structure, a physical barrier such as mountains, oceans, rivers, often separates different populations of the same species. Members of a species are rarely distributed homogeneously in space: biological or behavioral characteristic often result in some sort of clumping or aggregation, some schooling, flocking, herding, or colony formation (Hartl, 1980). This separation of two populations originally of the same species may led to the

isolation of populations so that they are unable to interbreed and eventually a new species may be formed.

The total population of a species may be split up into a group of subpopulations. A **subpopulation** can be defined as '*a reproductive community of individuals who share a common gene pool*' or as '*a self-sustaining genetic unit of population*' (Dobzhansky, 1950 and Anon, 1976 as cited by Richardson *et al.*, 1986). Subpopulation is sometimes referred to as **local population, Mendelian population** or **deme**, and has been defined as the smallest collective unit of a population (Krebs, 1972). Then a subpopulation could be considered as the fundamental unit of a population, usually referred to simply as a population. In population genetics, the focal point is on the local interbreeding unit in which the genetic and biological potential of different parts of the unit are essentially similar, and in which there is sufficient movement between members within the geographic area of the population to confirm cohesion.

The first step in understanding the population biology of a species is the study of population structure which is perhaps the most fundamental piece of information for a species that requires to be managed. Population structure of animals has traditionally been studied by using mark-release-capture methods, e.g. Blower *et al.* (1981). However, Murphy *et al.* (1990) said that this technique has several limitations, and recently, a molecular genetics approach has been increasingly used to analyze the population structure of many species. There are essentially three principle models of population structure which can result in differentiation of genetic patterns within and between geographic localities

(Richardson *et al.*, 1986; May and Krueger, 1990). These are, first, the panmictic model: when random mating occurs, the entire population consists of a single panmictic unit (free interchange). Second, the stepping-stone or island or discrete subpopulation models: the population consists of a series of small subpopulation, each mostly isolated from each other (no interchange between subpopulation). Third, the isolation-by-distance model: consists of a continuous population, but organisms exchange genes only with geographical distribution in an undifferentiated environment (local interchange only). The first step in understanding the population biology of a specific species is to consider which model best describes the population structure.

1.2.3.2 Theoretical population genetics

In 1908, the mathematical relationship of genotypic frequencies was proposed independently by the English mathematician G. H. Hardy and a German physician W. Weinberg, and has become known as **Hardy-Weinberg Law** (Hardy, 1908; Weinberg, 1908). The law stating that genotype frequencies of a diploid species will remain constant from generation to generation in an infinitely large, interbreeding population in which mating is at random and there is no selection, migration, or mutation (Ferguson, 1980; Richardson *et al.*, 1986; Hartl, 1988). If the allele frequency of one of the alleles at a locus is p and that of the other is q then the frequencies of the two homozygotes and heterozygote are given by p^2, q^2 and $2pq$ respectively.

The genetic hypothesis can then be established by breeding experiments.

If breeding tests are impossible, genetic interpretations may be checked by calculating allele frequencies and comparing the observed genotype frequencies with the Hardy-Weinberg expectations (Li, 1976; Ferguson, 1980). Therefore, where the frequency distribution of genotypes are significant different from those expected from the allele frequencies, then an imbalance of one or more of the above assumptions exists. Richardson *et al.* (1986) and Smith (1990) suggested that imbalances can be the result of stock mixing, natural selection, or drift in small populations, mutation also but any effects are likely to be small.

1.2.3.3 Genetic variation

The genetic constitution of an individual is called its **genotype**. The physical expression of a genotype is called the **phenotype**. However, the phenotype is the result of the interaction of the environment and the genotype of that particular organism (Richardson *et al.*, 1986) and two individuals having the same genotype can nevertheless have different phenotypes because of differences in the environment. In natural populations, different phenotypes among the individuals can be found in most traits. Population study must cope with the measurement of this phenotypic diversity which is caused by differences in the genotypes among individuals.

Because the processes for the formation of gametes are complicated, e.g. cell division, chromosome and DNA replication. Utter *et al.* (1987) mentioned that misparings of bases occur and occasionally lead to amino acid substitutions in proteins, e.g. a base substitution of TTC for TTA results in an amino acid

substitution of phenylalanine for leucine in a polypeptide chain. These changes in the DNA are **mutations** and randomness of mutants means that isolated populations accumulate different mutation over time which are the origins of genetic variation in living organisms. Therefore, studies of genetic variation are useful to understand the ancestral history of a group of species.

There are a variety of statistics for measuring the amount of genetic variation in populations and species. The simplest characters are the alleles and genotype frequencies and heterozygosity. However the latter is the most informative measures which are calculated or expected frequencies of heterozygotes (Ferguson, 1980). **Heterozygosity** can be denoted for a single locus (***H***) or as an average over several loci (***H***) which can calculate as demonstrated in Appendix 4. Another commonly used measure is the proportion of polymorphic loci (***P***), but this one does not take into account allele frequencies at a locus. Ferguson (1980) mentioned that the other measures are the average number of alleles per locus, and the effective number of alleles per locus.

1.2.4 Data Analysis

1.2.4.1 Allozyme data analysis

This particular population genetic study will characterize genetic variation within the species *P. monodon* throughout much of its natural range. From data collected, a variety of analyses are examined to define the population structure of the species. Electrophoretic data are collected as genotypes and converted to

allele frequencies then analysed as described below.

A. Genotypic distribution

In the analysis of allozyme data it is assumed that each sample set consists of a set of independent genes taken from a single panmictic population (single interbreeding group of individuals). For testing that a single panmictic population is sampled, the data must be compared to an optimal theory ***"Hardy-Weinberg Equilibrium"***. Richardson *et al.* (1986) described that there are two ways to test the assumption. Firstly, the numbers of each genotype in the sample can be compared with that expected in a randomly mating population with a particular allele frequencies. Secondly, the variation in allele frequencies observed between replicate sample sets can be examined.

A mixed population consisting of individuals from two different groups with different alleles frequencies will have a different distribution of phenotypes from that expected under Hardy-Weinberg Equilibrium conditions (Richardson *et al.*, 1986; Utter *et al.*, 1987). Richardson *et al. (1986)* explained that because the populations may consist of small groups of relatives (i.e. inbreeding) or larger independent subpopulation that were geographically mixed at the time of sampling (i.e. Wahlund Effect). In both cases there will be a deficiency in the total number of heterozygotes observed compared with that expected.

A comparison between the observed marker of genotype and the marker expected from Hardy-Weinberg Equilibrium can be tested for statistical significance, using a χ^2 test for goodness-of-fit or the log likelihood χ^2 test (G-

test) if these differences are small (Ferguson, 1980; Utter *et al.*, 1987; Hartl, 1988). This suggests that the population is in Hardy-Weinberg Equilibrium and can be used for inter-area comparisons, but if significant heterogeneity in genotype frequencies are found within the population then the structuring present in that area should be studied and understood (Richardson *et al.*, 1986).

B. Genetic differentiation

The characterization of genetic variation within and between populations enables estimates of association between pairs of genes in samples from different populations to be made. There are two methods for deducing the population structures.

B1. Chi-squared test of population heterogeneity of allelic & genotypic frequencies.

Testing the null hypothesis that the allelic and genotypic frequencies in different samples are not significantly different can be done by measuring the divergence of an observed value from the expected value at a single locus or over all loci. An examination for heterogeneity of allelic frequencies among populations can be deduced using the chi-squared test of homogeneity (Siegel, 1956) and genic contingency chi-square test of Workman and Niswander (1970). Heterogeneity in genotypic frequencies can be carried out using the G-test (Sokal and Rohlf, (1981). Ferguson (1980) and May and Krueger (1990) suggested that G-values derived from independent comparisons can be pooled together and retested. This pooling and retesting will show whether geographic clustering of the population retain the differentiation when all populations are tested at one

time. Moreover, Nass chi-square analysis (Nass, 1959) and sequential Bonferroni technique (Holm, 1979; Rice, 1989) can be used to increase statistical power.

B2. *F*-statistics

F-statistics are estimated by comparing the genotypic array of the population surveyed with that expected from a random mating population. This was proposed by Wright (1965, 1978) and Nei (1973, 1975, 1977) and the use of this statistic was reviewed by Hartl and Clark (1989). This is done by measuring the ratio of the difference between the observed heterozygosity to that expected under Hardy-Weinberg equilibrium.

F-statistics are useful for surveying population structure because there are three distinct levels of subdivision measured: within populations (F_{IS}), between subpopulation (F_{ST}), and the total populations (F_{IT}). May & Krueger (1990) suggested that F_{IT} values are seldom used since any type of departure from a single panmictic population will lead to a significant F_{IT}. If the sampled population consists of subsamples from different locations, F_{IS} values will show the departures from Hardy-Weinberg by measuring the amount of heterozygote deficiency or excess observed in that sample. F_{ST} values help us to understand the degree of population differentiation within species. These tools are often used by population biologists because they can easily be associated with the inbreeding coefficient which increases the homozygosity in a population (Wright, 1921).

To date, there are two ways of estimating the *F*-statistics, one that computes unbiased estimators of gene diversity components, developed by Nei

(Nei, 1973, 1977; Nei and Chesser, 1983), and the other with variance components developed by Cockerham (Cockerham, 1969, 1973; Weir and Cockerham, 1984). Goudet *et al.* (1994) commented that both groups of estimators give the same result for F_{IS} when sample sizes are equal, but Weir & Cockerham's F_{IS} deals with the problem of unequal sample sizes by weighting the F_{IS} obtained from each sample by its sample size.

C. Genetic distance

To quantify levels of genetic distance between pairs of populations, a large number of measures based on polymorphic loci have been proposed (Wright, 1978). The commonly used measures recently reviewed and demonstrated by Swofford and Olsen (1990) are those of Nei (1972, 1978) and Rogers (1972).

C1. Nei's distance

The most widely used genetic distance was that of Nei (1972, 1978). The measure is derived from the probability that two alleles, one drawn from each population unit being compared, are the same. However, Nei's distances (in either their original form or as modified by Hillis, 1984) are nonmetric in that they frequently violate the triangle inequality as reviewed by Swofford and Olsen (1990). Weir (1990) also stated that Nei's distance is appropriate for long-term evolution when population diverge because of drift and mutation.

C2. Rogers' distance

Rogers' distance, a simple and easily interpretable geometric basis, is the Euclidean distance over all loci between the allele frequency vectors for each

locus of the two populations being compared. In principle, it is equivalent to Mahalanobis' distance for morphological characters so that the relative distance between population units calculated from morphological and from allelic data can be compared (Richardson *et al.*, 1986). However, Rogers' coefficient shares with Nei's the undesirable property of being too heavily influenced by within-taxon heterozygosity (Wright, 1978; Hillis, 1984); the distance between two taxa that are fixed for multiple alleles exceeds that between two taxa in which one or both are heteroallelic but have no alleles in common.

1.2.4.2 MtDNA data analysis

The variability in fragment patterns resulting from mtDNA digestion profiles is interpreted as genetic variation at the nucleotide level. Thus, the differentiation of fragments pattern constitute the raw data in mtDNA population surveys. Considering all restriction enzymes study, each individual can be summarized as a composite letter code that described its observed mtDNA genotype. Individual samples that share a composite mtDNA genotype, often called **haplotype**, therefore are said to be a member of the same maternal clone. There are two characters for analyzing population structure, i.e., physical maps of restriction sites and fragment data (Nei and Li, 1979; Nei and Tajima, 1981; Dowling *et al.*, 1990). Restriction site characters are preferred to restriction fragment characters because fragment data provide redundant information. However, Bermingham (1990) suggested that working with conspecific populations and very closely related species, either class of data provide very similar estimates of DNA sequence divergence and phylogenetic relationship.

A. Genetic variation

For intrapopulational variation, a measure of genetic variation at the nucleon level called *nucleon* or *haplotypic diversity* can be estimated from the frequency of the different haplotypes (Nei and Tajima, 1981). This is equivalent to heterozygosity (h) or gene diversity used in protein polymorphism studies. In mtDNA studies, however this measure is not appropriate (Nei and Li, 1979) because mtDNA contains many genes and therefore the gene diversity would be close to 1 in many populations. Nei (1987) also commented that nucleon size generally increases as the sample size increase and varies greatly with the gene studied, therefore they cannot be used as a general measure. The more appropriate measure is *nucleotide diversity* (π), a measure of genetic variation within population at the nucleotide level which can be estimated from shared fragment or restriction site data (Nei and Li, 1979).

B. Genetic differentiation

To determine significant difference between populations using haplotype frequencies, Nass (1959) chi-square analysis with small expectations can analyse both individual and multiple mtDNA genotype frequencies. To judge the minimum significant level a minimum table-wide significance value can calculated using the sequential Bonferroni technique (Holm, 1979; Rice, 1989). A chi-square test can also determine the significant differentiation between populations using the frequencies of haplotypes in each population. However, Ovendon (1990) suggested that the number of haplotypes found in mtDNA study is often large and the distribution of individuals between the haplotypes is often skewed. By using small sample size, this can invalidate the χ^2 analysis. As

proposed by Roff and Bentzen (1989), this problem can be overcome by comparing the original χ^2 value with that estimated from 100-10,000 Monte Carlo repeated randomizations of the original matrix. To quantify genetic differences between pairs of population, Nei's unbiased genetic distance (Nei, 1978) and Rogers' distance (Rogers, 1972) can be calculated on individual mtDNA genotypes and mtDNA haplotype frequencies. Unweighed pair-group arithmetic average (UPGMA) clustering method (Sneath and Sokal, 1973) for genetic distance can be constructed using the BIOSYS-1 computer program (Swofford and Selander, 1989).

The most useful statistic for estimating sample diversity is a study of genetic polymorphism at the DNA level called *nucleotide diversity*. There are two different ways to determine nucleotide sequence diversity. Firstly is the average of the proportion of different nucleotide between two sequences (p) (Upholt, 1977), secondly is the average of the number of nucleotide substitutions between two sequences (d) using fragment data (Nei and Li, 1979) or site data (Nei and Tajima, 1981). However, in most eukaryotic genes, Nei and Jin (1989) suggested that d is nearly the same as p, and d is more appropriate for evolutionary studies than is p. All genetic distance values can be carried out using REAP, the restriction enzyme analysis package version 4.1 (McElroy *et al.*, 1993).

Where different populations have distinct mtDNA haplotypes, the number of nucleotide substitutions possessed by each haplotype can determine the evolutionary relationship between them (Nei, 1987). Phylogeny dendrograms can

be constructed from the presence or absence of restriction sites or fragments using the computer packages (PHYLIP, version 3.5, Felsenstein, 1995; PAUP, version 2.4, Swofford, 1990). If the differences between populations appeared as genotype frequency differences, estimation of nucleotide divergence over all pairwise comparisons can be estimated by considering the effect of polymorphism following Nei and Tajima (1981). Clustering dendrogram can then be constructed from matrices of numerical similarity (nucleotide divergence) between populations using the UPGMA clustering method computed by PHYLIP: phylogeny inference package version 3.5 (Felsenstein, 1995).

1.3 BIOLOGY OF *PENAEUS MONODON*

The economic importance of *Penaeus monodon* to the economies of SE Asia countries has been described. Some of the potential problems of its widespread culture have also been mentioned. Despite their economic importance in capture fisheries and aquaculture we know relatively little about the biology and population genetics of Penaeids in general. From our limited knowledge on the biology and studies in *P. monodon* and other Penaeids can we get any information on the possible population structure in this species?

The term shrimps and prawns are common English names used synonymously because no systematic distinction can be made between the two common names (Wickins, 1976; Holthuis, 1980). The terms commonly used in different countries, marine species of penaeid are called 'shrimp' in the U.S.A.,

'prawns' in Australia, India, and South Africa, while either term may be used in Japan. In Great Britain small carideans (e.g., *Pandalus montagui*) are called shrimp, larger species are called prawns (Wickins, 1976). In general there is a great deal of confusion when using common names to describe shrimps and prawns. Here I will use marine penaeid as the generic term and call *P. monodon* by its scientific name.

1.3.1 Morphology

The external morphology of *P. monodon* has been described by a number of workers from various countries (Villaluz and Arriola, 1938; Holthuis, 1949; Kubo, 1949; Racek, 1955, 1957 and 1972; Hall, 1956, 1961 and 1962; Dall, 1957; Cheung, 1960; Motoh, 1981 and 1986; Bate, 1888; Solis, 1988).

The description of *P. monodon* is as follows (Fig. 1.1). They are laterally compressed, elongate, with a well-developed abdomen adapted for swimming. The body is smooth and glabrous. The head (five somites) and thorax (eight somites) are fused into a cephalothorax, which is completely covered by the carapace. The rostrum, extending beyond the tip of the antennular peduncle, is sigmoidal in shape, and has 6-8 dorsal and 2-4 ventral teeth, mostly 7 and 3 respectively. The carapace is carinate with the adrostral carina, almost reaching to or not as far as the epigastric or first tooth. The gastro-orbital carina occupies the posterior one-third to one-half distance between the post-orbital margin of the carapace and the hepatic spine. The hepatic carina is prominent and slightly curved, extending behind the junction with antennal spine. The antennular

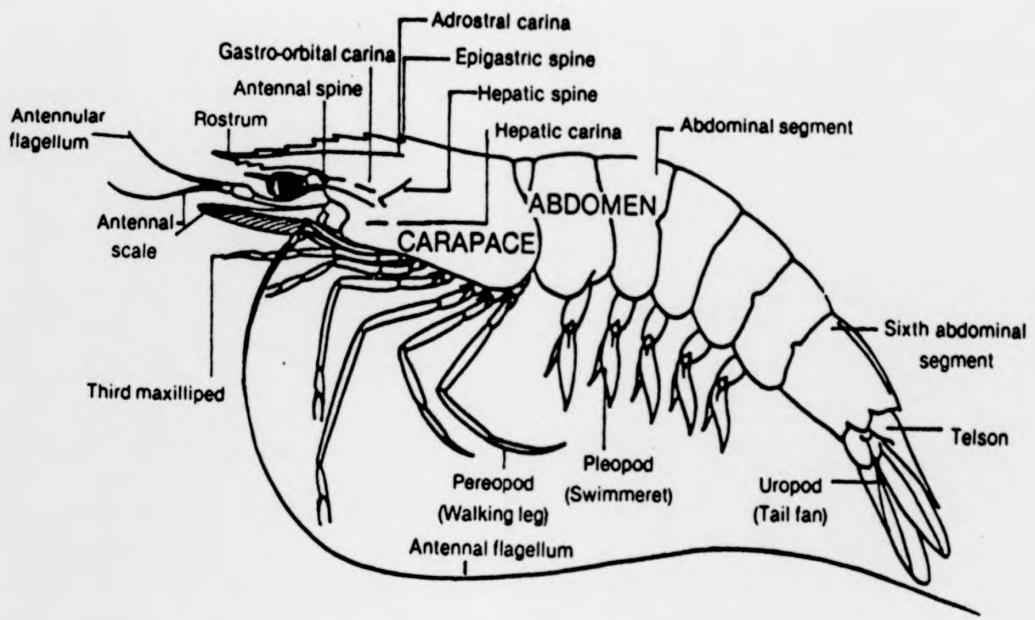

Fig. 1.1 Lateral view of *P. monodon* showing important parts (after Motoh, 1981)

flagellum is sub-equal or slightly longer than the peduncle. Exopods are present on the 1st and 4th pereopods but absent on the 5th. The abdomen is carinate dorsally from the anterior one-third of the 4th, to the posterior end of the 6th, somites. The telson has deep median groove, without dorso-lateral spines.

The colour in life has the following features: carapace and abdomen is transversely banded with red and white. The antennae are greyish brown, the pereopods and pleopods are brown with red fringing setae. In shallow brackish waters or when kept in ponds, the colour changes to dark brown and often blackish (Motoh, 1981). Within *Penaeus spp.*, Dall *et al.* (1990) mentioned that the species are mostly easy to identify and many have distinctive colouring which is constant in the adults. However, within the genus, the species can most easily be separated by presence or absence of a hepatic carina (Dall *et al.*, 1990)

1.3.2 Taxonomy

The genus *Penaeus* Fabricius (1798) was placed on the Official List of Generic Names in Zoology as Name No. 498 upon the discovery and description of *P. monodon* by John Christ Fabricius in 1798 (Mohamed, 1970). There are so many synonyms and in the older literature it is often confused with *P. semisulcatus*. With the revision of the specific name monodon by Holthius, the two species have become stabilized and the name *P. monodon* is now generally accepted (Hall, 1961; Mohamed, 1970; Motoh, 1981). These two species can easily separate by adrostral carina extending behind the epigastric tooth and postrostral carina distinctly grooved in *P. semisulcatus*, but adrostral carina

reaching to or not as far as the epigastric tooth and postrostral carina not grooved in *P. monodon* (Dall *et al.*, 1990). No subspecies are currently recognized for this species and *P. monodon manillensis* (Villaluz and Arriola, 1983) proved to be based on an abnormal specimen of *P. semisulcatus* (Mohamed, 1970; Motoh, 1981).

1.3.3 Classification

The taxonomic definition of giant tiger prawn based on Mohamed (1970) is as follows:

Phylum Arthropoda
Class Crustacea
Subclass Malacostraca
Order Decapoda
Suborder Natantia
Infraorder Penaeidea
Superfamily Penaeoidea
Family Penaeidae Rafinesque, 1815
Genus *Penaeus* Fabricius, 1798
Subgenus *Penaeus*
Species *monodon*

Scientific name:

Penaeus (Penaeus) monodon Fabricius, 1798.

Synonyms:

Penaeus carinatus Dana, 1852

Penaeus tahitensis Heller, 1862

Penaeus semisulcatus exsulcatus Hilgendorf, 1897

Penaeus coerulius Stebbing, 1905

Penaeus bubulus Kubo, 1949

Penaeus monodon monodon Burkenroad, 1959

FAO Names:

English: Giant tiger prawn

French: Crevette géante tigrée

Spanish: Camarón tigre gigante

Local Names:

Australia: Jumbo tiger prawn; Giant tiger prawn; Blue tiger prawn; Leader prawn; Panda prawn

Burma: Jar-pazun; Pazun-kya

Cambodia: Bangkear

Germany: Bärenschiffskielgarnele

Hongkong: Ghost prawn; Gwai ha

India: Jinga (Bombay region); Kara chemmeen (Kerara); Yera (Madras); Bagda chingri (Calcutta)

Indonesia: Udang windo; Udang pantjet

Japan: Ushi-ebi

Kenya: Kamba (large sizes); Kamba ndogo (small sizes)

Pakistan: kalri (word also used for other species)

Philippines: Sugpo; Jumbo tiger shrimp

South and East Africa: Tiger prawn

Taiwan: Grass shrimp

Thailand: Kung kula-dum

Vietnam: Tôm sú

1.3.4 Zoogeography

The *P. monodon* is a tropical shallow-water marine decapod crustacean, trawled over mud or sand bottom to 110 m (Grey *et al.*, 1983). The fry, juvenile, and adolescent stages occupy shore areas and mangrove estuaries occasionally entering rivers, while most of the adults inhabit deeper waters down to 162 m (Racek, 1957). Dall *et al.* (1990) said that the Penaeidae are predominantly tropical stenotherms with a minimum temperature about 15 °C. Temperatures below this caused by increasing latitude or cold current, are probably the main barriers to distribution. Motoh (1986) showed that in the Philippines the vertical distribution of this species was from the surface in brackish water rivers down to about 160 m offshore, where the range of water temperature and salinity was between 22-34 °C, and 4-35 ‰, respectively. But in ponds, the range of the physio-chemical conditions is generally greater than those under natural conditions. In Thailand, Kungvankij *et al.* (1973a) surveyed the distribution and abundance of *P. monodon* in the Andaman Sea showed that it was the dominant species in sandy bottom conditions at a depth of 35-40 m. Promsakha (1980) reported that *P. monodon* in the Gulf of Thailand have breeding grounds at a depth of 26-50 m, 11-15 miles from the shorelines, and where water salinity is higher than 31 ppt.

The species is widely distributed throughout the greater part of the Indo-West Pacific region (Fig. 1.2): South Africa, Tanzania, Kenya, Somalia, Madagascar, Saudi Arabia, Oman, Pakistan, India, Bangladesh, Sri Lanka, Indonesia, Thailand, Malaysia, Singapore, Philippines, Hongkong, Taiwan, Korea,

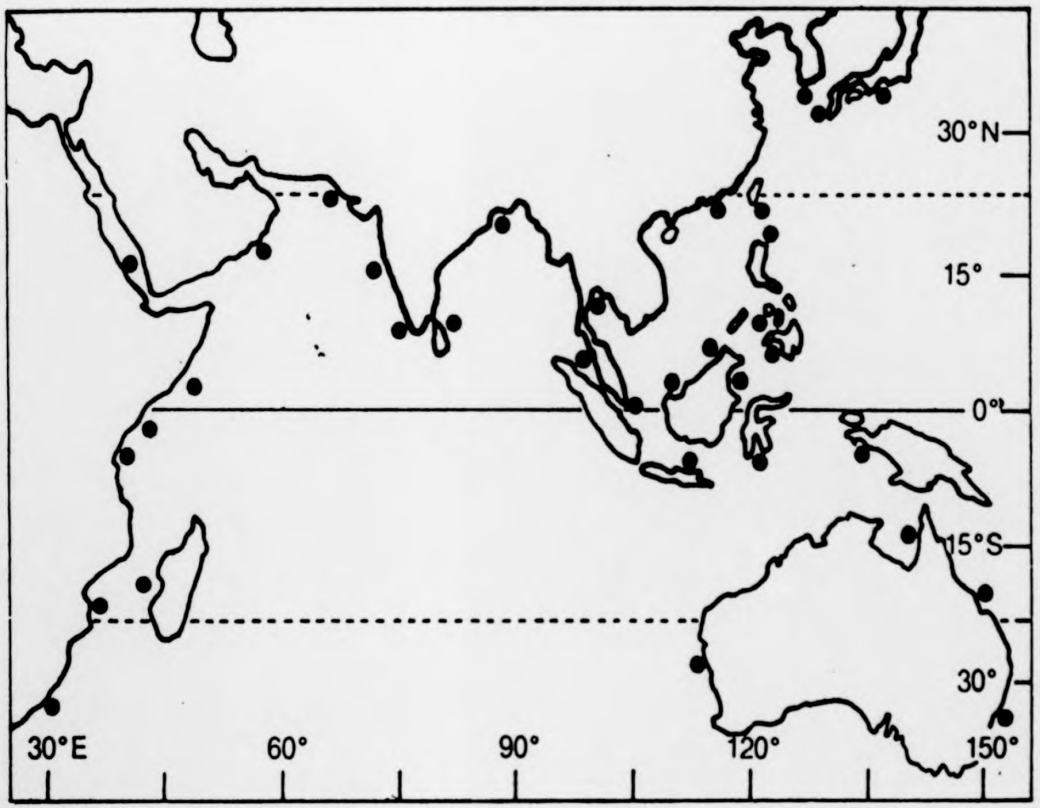

Fig. 1.2 Geographic distribution of *P. monodon* in Indo-West Pacific Region.

Japan, Australia, and Papua New Guinea (Racek, 1955 and 1972; Holthuis and Rosa, 1965; Motoh, 1981 and 1985). Dall *et al.* (1990) stated that, normally, they are widely distributed within the Indo-West Pacific region. The short larval life of the Penaeidae also restricts the range. The cold water on the westcoasts of both North and South America, and the eastern Pacific deep ocean act as an effective barrier to their dispersal, the distribution is therefore restrics this species to the Indo-West Pacific region.

In general, *P. monodon* is distributed from 30°E to 155°E in longitude and from 35°N to 25°N in latitude (Solis, 1988). However, the main fishing grounds are mostly located in tropical countries, particularly Malaysia, Indonesia, the Philippines, India, Bangladesh and Thailand.

1.3.5 Life History

The typical penaeid life cycle can involve both estuarine and oceanic phases the differences are dependent on whether the species has a predominantly estuarinal and inshore or offshore habitat and whether they are demersal or pelagic in behaviour (Dall *et al.*, 1990). Another difference is that the eggs of different species are either demersal or pelagic after release by the female. In addition, Kutkuhn (1966) has point out that the environment causes the main modifications in the life histories. In general, development of *P. monodon* can be divided into three stages: planktonic larval (nauplius, protozoea, mysis, and post larval stages), juvenile and adult stages.

During the spawning season, adult *P. monodon* undertake seasonal spawning migrations away from estuaries and tidal lakes to various depths on the continental shelf where copulation occurs. During copulation the male deposites a spermatophore which contains the spermatozoa in the female thelygum long before spawning (Solis, 1988). Normally, spawning of *P. monodon* takes place offshore at night at about 18-36 m deep. During spawning, the eggs are extruded from the paired genital pored at the same time as spermatozoa from the thelygum and eggs are fertilized in the water by the movements of pleopods (Motoh,1986).

The spawning of *P. monodon* is all year round, but there appears to be peaks of activity. In the Philippines there seems to be two peaks in a year: February-March and October-November, but these can vary from year to year (Motoh, 1981). In Singapore, Hall (1962) reported a peak from February to April. Rajyalakshmi *et al.* (1985) reported a peak in larvae from October through April. Corresponding to the post monsoon stability in the water movement and the increasing salinity of the Orissa Coast in India. In Taiwan Su and Liao (1986) reported the main spawning adults from June to December. In Thailand, Kungvankij *et al.* (1973b) reported two peak spawning seasons in the Andaman Sea; February and August, while Promsakha (1980) recorded two peaks in January and August in the Gulf of Thailand.

After hatching, the planktonic stages migrates inshore, and the postlarvae settle on their preferred "*nursing grounds*". The postlarvae settle in mangrove areas, muddy estuaries and may migrate up river for up to 85 km, where the salinity can be very low. The juveniles are euryhaline and can tolerate the low

salinities common in estuaries (Dall *et al.*, 1990). When they are about half adult length, the juveniles leave the estuary and migrate offshore until they mature and spawn.

1.4 POPULATION GENETICS STUDY IN MARINE SPECIES

1.4.1 Allozyme Studies

1.4.1.1 Genetic variation among natural populations in crustaceans

The first comprehensive population genetic studies in crustacean was on the polychromatism of copepods (*Tisbe reticulata*) and isopods (*Sphaeroma serratum* and *Jaera albifrons*) by Bocquet 1951, Bocquet *et al.* 1951, and Bocquet 1953 as cited by Hedgecock (1986). These and subsequent work on the genetics of colour and morphology in the Crustacea assisted the syntheses of evolutionary theory. However, by the 1970s, the externally visible polymorphism of crustacean were set aside for biochemical genetic variation and allozyme studies which have continued to proliferate since that time.

In the mid 1960s, since the first papers reported the use of electrophoretic techniques to investigate genetic variation in *Drosophila* (Lewontin and Hubby, 1966) and in humans (Harris, 1966), a large number of different crustaceans have been analyzed. The majorities of these studies have used Cladocera and other small species instead of Decapoda because they were easier to manage and breed.

In the surveys on both plants and animals, Selander (1976) found that about 25 to 50 percent of loci have allozyme polymorphism (P = 0.25 to 0.50), and an average individual is heterozygous at 5 to 15 percent of its loci ($\overline{H}$ = 0.05 to 0.15). Invertebrates have the highest level of genetic variation (P = 0.50, $\overline{H}$ = 0.15), whereas vertebrates tend to be at the lowest level (P = 0.25, $\overline{H}$ = 0.05).

In 97 crustaceans species surveyed for variation at 12 to 43 loci by Hedgecock *et al.* (1982), 30.5% of the loci in the average population are polymorphic and 7.3% of the loci in an average individual are heterozygous (P = 0.305, $\overline{H}$ = 0.073). The survey also showed among-taxa variation of genetic variation in the crustaceans. Kruskal-Wallis non-parametric analysis of variance on H and P over 10 major taxonomic groups showed highly significant (p<0.001) among-taxa variation. Although unequal within-taxa variation, it is manifest that Diplostraca, Copepoda, Cirripedia, and Euphauseacea have much higher levels of variation ($P \geq 0.38$, $\overline{H} \geq 0.12$) than the remaining forms ($P \geq 0.28$, $\overline{H} \geq 0.07$). Hedgecock (1982) inferred that a general trend for higher levels of genetic variation corresponded with smaller body size or low mobility.

The amount of genetic variation observed in a number of Decapods is summarised in Table 1.1. Compared with other animals, these values are rather low (Nelson and Hedgecock, 1980; Hedgecock *et al.*, 1982); in both freshwater penaeid (P = 0.10-0.22, $\overline{H}$ = 0.039-0.063, Hedgecock *et al.*, 1979) and marine penaeid (P = 0.22-0.26, $\overline{H}$ = 0.019-0.038, Lester, 1979; Redfield *et al.*, 1980; Mulley and Latter, 1981ab; Lester, 1983 ; Sbordoni *et al.*, 1987). The typical life cycle of marine penaeid includes both estuarine and off-shore phases, plus

seasonal migration to various depths on the continental shelf. Therefore, it is possible that the low level of variation is ascribed to "*bottleneck effect*" or small population size. In addition, Mulley and Latter (1980) proposed that the widespread nature of these penaeid in various different habitats has caused the selective elimination of mutational variation, and thus accounts for the low levels of heterozygosity.

Table 1.1 Summary of Genetic allozyme variation in economically important Decapoda (after Hedgcock, 1986).

Taxonomic group	Number of species	No.of loci per species	*P*	$\overline{H}$
Penaeidae				
Metapenaeus	6	32.0	0.22	0.019 (0.013-0.026)
Penaeus	14	28.9	0.26	0.038 (0.022-0.058)
Palaemonidae				
Palaemon	8	19.8	0.16	0.062 (0.042-0.086)
Palaemonetes	3	21.3	0.22	0.063 (0.060-0.067)
Macrobrachium	6	19.3	0.10	0.039 (0.022-0.060)
Nephropidae				
Astacidae				
Palinuridae	12	26.4	0.19	0.045 (0.035-0.056)
Cancridae				
Portunidae				
Xanthidae	5	30.8	0.30	0.064 (0.028-0.111)

In decapods, the variation also varies significantly among genera and families as shown in Table 1.1. Within the Panaeidae, *Metapenaeus* has significantly fewer alleles and lower average heterozygosity than *Penaeus*. Within the Palaemonidae, *Macrobrachium* appears to have significantly lower average variation than *Palaemon* and *Palaemonetes* (Chow and Fujio, 1985). In contrast to penaeid, crayfish and lobster (nephropidae, astacidae, and palinuridae) seem to be a much more homogeneous group, as heterozygosity averaged over three families are no greater than those within an individual genera of penaeid. Finally, the five brachyurans species have heterozygosities ranging from 0.013 to 0.128 for *Cancer magister* and *Callinectes arcuatus* (Hedgecock, 1986) which is almost identical to average value ($\overline{H}$ = 0.038) of Hedgecock (1982).

The distribution of genetic variation in crustaceans was similar to studies on other species. *FBALD**, *GPI**, *IDDH**, *MPI**, *PEPC**, and *PGM**, tended to be more polymorphic than *AMY**, *GLUDH**, *G3PDH**, *LDH** , *PT**, and *SOD**. 17% of the average crustacean population were polymorphic at *GPI**. Further, comparision of observed with expected genotypic proportions in populations of all decapod groups was under Hardy-Weinberg equilibrium. Hedgecock (1986) inferred that mating among conspecific adult decapods in natural populations is sufficiently random and that progeny groups are sufficiently well mixed to satisfy Hardy-Weinberg equilibrium.

1.4.1.2 Geographic differentiation among conspecific crustacean populations

The geographic distribution of a species has important consequences for

the distribution of genetic variation among the populations. Diversifying selection resulting from spatially heterogeneous environments can cause divergence of gene frequencies among populations. We should therefore consider the evidence of genetic divergence among natural conspecific populations.

In branchiopods, the use of electrophoretic technique to examine geographically isolated populations of the brine shrimps, *Artemia franciscana* and *A. tunisiana*, has shown significant intrapopulation genetic differences (Abreu-Grobois, 1986). The North American species, *A. franciscana*, exhibit significant population subdivisions (F_{ST} = 0.24, p<0.01), while the Old World species, *A. tunisiana*, does not (F_{ST} = 0.12, n.s.). In addition, there are electrophoretic studies of subdivision of *A. franciscana*, showing strong ecological isolation of populations inhabiting waters of different ionic compositions (Vanhaecke *et al.*, 1984), different temperature and salinity tolerance among populations (Bowen *et al.*, 1985), and interstrain differences in reproductive traits (Browne, 1980 and 1983).

In decapods, particularly marine species with pelagic larvae, direct studies of deme sizes, migration rates and individual reproductive successes are quite difficult to estimate especially with comparison to topography, hydrography, ocean and tidal current patterns or life history data. However, Burton (1983) suggests that many marine species, including decapods, are subdivided despite the potential for dispersal by pelagic larval and gene flow.

In palaemonid, geographical differentiation has been more consistently reported, owing to the greater isolation among the freshwater or estuarine habitats occupied by this group. Firstly, Mulley and Latter (1980) reported evidence of population subdivision in the grass shrimp, *Palaemonetes pugio*, in the vicinity of Galveston Bay, Texas. The divergence among populations was divided into small populations in landlocked ponds by random genetic drift. For European species of *Palaemon*, Berglund and Lagercrantz (1983) reported highly significant divergences of allelic frequencies at almost all loci among five localities from Arcachon, France, to Fiskebäckskil, Sweden.

In some freshwater penaeid species, the population subdivision relates to a shortened larval period or to low larval salinity tolerance, so that dispersal between estuaries is restricted, such as the differentiation of *Macrobrachium rosenbergii* populations between the distinct faunas of continental Southeast Asia and Pacific oceanic islands, and between Sri Lanka and the Indian subcontinent (Hedgecock *et al.*, 1979). Chow and Fujio (1985) show interesting differences in population subdivision among Japanese species of *Palaemon* and *Macrobrachium*. *M. nipponese* larvae have a lower salinity tolerance than their congenerics. The species is substantially subdivided with $F_{ST} = 0.204$ among all habitats: 0.123 among four river populations, and 0.216 among four lake and pond populations. Four other palaemonid species they studied appear to be genetically fairly homogeneous.

In American lobster *Homarus americanus*, between the Gulf of St. Lawrence and Massachusetts, Tracey *et al.* (1975) found that the average genetic

similarity for 44 loci among population is high but there was evidence of segregation at the *ME* locus in offshore populations. Burton (1983) also points out slight but significant differences in allele frequencies at other loci in the lobster study.

For spiny lobsters *Panulirus argus*, and brachyuran crabs, gene frequencies are rather homogeneous over broad geographic ranges (Hedgecock, 1986). Slight differences in gene frequencies and in the distribution of rare alleles are reported by Menzies and Kerrigan (1978) among Caribbean populations of *P. argus*. Also, Nelson and Hedgecock 1980) found no evidence for differentiation among populations of the Dungeness crab *Cancer magister*.

In allozyme studies of marine penacid, there is little or no geographic differentiation in gene frequencies. Mattoccia *et al.* (1987) reports slight (F_{ST} = 0.04) but significant differences in allelic frequencies among populations of *Penaeus kerathurus* from the Mediterranean Sea, primarily at the *MPI**, *PGM** and *PHI** loci. In addition, Lester (1979) reports homogeneity of allelic frequencies in samples of *P. aztecus, P. duorarum* and *P. setiferus* collected from throughout the Gulf of Mexico. Moreover, comparison of *P. stylirostris* from Ecuador and the Gulf of California reveal no statistically significant differences in allelic frequencies at the *GPI**, *PGM**, and *PGDH** loci (Lester, 1983).

Mulley and Latter (1981ab) studied geographic differentiation in commercially important penaeid of northern and eastern Australia and found that the level of genetic differentiation corresponds with life history parameters.

Species with limited potential for gene flow, such as *Metapenaeus bennettae*, which is restricted to estuaries and tidal lakes and is rare in oceanic waters show significant differences in allele frequencies between samples collected off the eastern cost of Australia (Mulley and Latter, 1981a). Other species such as *P. latisulcatus* and *M. endeavouri*, which occur over wide sea areas but have a discontinuous distribution due to a lack of suitable habitat, likewise show genetic differentiation between regions (Mulley and Latter, 1981b). In contrast, species such as *P. plebejus*, which makes extensive oceanic migrations show little genetic differentiation between Mooloolaba in Queensland and Lakes Entrance in Victoria (Mulley and Latter, 1981a). Richardson (1982) investigation of stock structure of *P. latisulcatus* in South Australia failed to find variation over a limited geographical range, but did uncover temporal variation in the genetic composition of populations.

For *P. monodon*, Mulley and Latter (1980) detected three polymorphic loci at *ALP-1**, *PGM** and *LDH** with a mean heterozygosity of 0.008±0.005. Ko *et al.* (1983) reported mean heterozygosity of 0.013 over 29 enzyme loci and found three polymorphic loci. Tam and Chu (1993) found four polymorphic loci (*GPI**, *PGM-1**, *LAP** and *TR**) with mean heterozygosity of 0.040±0.022. Benzie *et al.* (1993) observed eight polymorphic loci at *GPI**, *LGG**, *LT-1**, *MDH-1**, *MDH-2**, *MPI**, *PGDH** and *PGM** with mean heterozygosity of 0.05 calculated from the eight polymorphic loci. They reported highly significant difference in gene frequencies between populations on the west coast and those on the northern and eastern coastlines of Australia. These differences suggest that gene flow in recent times has not sufficient to eliminate genetic differences caused by isolation during

the last glacial of these population over 14000-17000 years ago (Benzie *et al.*, 1993).

1.4.2 Mitochondrial DNA Studies

On the basis of mtDNA genetic diversity in populations of terrestrial and freshwater species, it was predicted that the mitochondrial genome could be ideal for classifying geographic structuring of marine populations and might therefore be useful for fishery management (Avise 1985; Ferris and Berg, 1987). However, previous surveys showed that marine species seem to have less mtDNA diversity than terrestrial and freshwater species and data also suggest that some marine species may show little or no mtDNA differentiation over large geographic areas (Avise, 1985). The relative lack of intraspecific population structure in most marine species is related to the physical environment and their life history-generally high dispersal capabilities. Waldmann and Wirgin (1994) said that the low levels of mtDNA sequence divergence among marine species is probably the result of bottleneck effects. This is because effective population size (N_e) of the the mtDNA genome is effectively one fourth that of the bisexually inherited nuclear genome, making it more sensitive to erosive population bottlenecks (Billington and Hebert, 1991). Even though, Ovendon (1990) suggested that marine species may have a slower rate of mtDNA evolution and in some species may maintain more frequent family-specific mortality. However, Avise (1985) indicated that mtDNA analysis for marine species has been biased because the analysis has been carried out only on the species that were found to have no or low level of allozyme variation. Recently, there have been several studies

showing high intraspecific mtDNA genetic variation. Marine species therefore may be more structured than has previously been thought.

Patterns of mtDNA variation have been examined in many marine species. In aquatic invertebrates, Skibinski (1994) concluded that the most widely studied groups appear to be bivalve mollusc, echinoderms, and arthropods, particularly crustacea. While most of the echinoderms papers are studies about molecular evolution, the bivalve and crustacean papers are studies on population genetics.

For mollusc, Edwards and Skibinski (1987) surveyed mtDNA variation within the hybrid zone of the mussels *Mytilus edulis* and *M. galloprovincialis* along the coastline of south-western England. Although no distinct mtDNA haplotypes were found, χ^2 test showed significant differences in mtDNA genotype frequencies between mussels from *M. edulis* populations (Padstow and Bude) and *M. galloprovincialis* population (Swansea). A clear genetic subdivision for mollusc has been recorded in the American oyster *Crassotrea virginica* (Reeb and Avise, 1990). 212 individuals were collected from 14 continuous locations on the eastern coast of the United States from the Canadian border to Texas, with 13 restriction enzymes all 82 haplotypes were distinguished into two distinct groups, one on Atlantic coasts and the other within the Gulf of Mexico, with a breakpoint on the coast of Florida. The mean sequence divergence between the two distinct haplotypes was 2.6%. However, no genetic variation was found in mtDNA restriction fragments of blacklip abalone *Haliotis rubra* collected from the coastline of Tasmania. Despite the limited of larval dispersal of this species, Barrett (1989) as cited by Ovendon (1990) concluded that the amount of gene

flow may be large enough to homogenize mtDNA of the species.

In echinoderms, Vawter and Brown (1986) and Palumbi and Wilson (1990) both studied intraspecific mtDNA variation in the North American sea urchin (*Strongylocentrotus purpuratus*) and the estimation of mtDNA sequence divergence were 0.99% and 0.48% respectively. Palumbi and Wilson (1990) found four clades among 38 individuals but no subdivision of mtDNA haplotypes between Atlantic and Pacific populations. However, they did find significant genetic differences between Atlantic and Pacific populations with total intraspecific divergence of 1%.

For arthropods, Saunders *et al.* (1986) detected spectacular sequence variation of mtDNA in horseshoe crab *Limulus polyphemus*. Three haplotypes were observed only in the northern populations (Georgia to New Hampshire) and seven haplotypes were observed only in the southern (Cape Canaveral to Panama City). This dramatic difference (p = 2%) was a surprise, because the horseshoe crab is continuously distributed along the coastline and has free-swimming larvae with no obvious barriers to gene flow. Saunders *et al.* (1986) commented that this genetic break point corresponds to a transition area between warm-temperate and tropical marine faunas as seen in the American oyster paper (Reeb and Avise , 1990).

Within crustacea, sequence variation of mtDNA were found in many species. Crease *et al.* (1990) and Bucklin and Kann (1991) found highly subdivided species of cladoceran *Daphnia pulex* and copepod *Calanus pacificus*

within 100 km. In decapods, Komm *et al.* (1982) and McLean *et al.* (1983) found distinctive restriction haplotypes for spiny lobster (*Panulirus argus*) between populations separated by 110 km and 280 km respectively. But these studies used low numbers of animal (24 and 24) and restriction enzymes (2 and 6). Geographic subdivision were found in five species of rock lobster *Jesus verreauxi, J. novaehollandiae, J. edwardsii, J. lalandii* and *J. tristani* using mtDNA (Brasher *et al.*, 1992ab). The long life of phyllosoma larvae and prolonged pelagic stage of *J. spp.* lead to genetic similarity between Australian and New Zealand populations. However, intraspecific mtDNA nucleotide sequence diversity was generally high (0.33-0.99%) between these populations, suggesting restrictions to gene flow across the Tasmanian Sea.

For *P. monodon*, Benzie *et al.* (1993) and Bouchon *et al.* (1994) both detected genetic divergence of mtDNA between populations. Bouchon *et al.* (1994) digested total mtDNA of *P. monodon* with twelve restriction enzyme; nine of them were 6-base cutters:*Bam*HI, *Bgl*II, *Cla*I, *Eco*RV, *Pst*I, *Pvu*II, *Sac*I, *Stu*I and *Sty*I, two were 4-base cutters: *Hha*I and *Msp*I, and one a 7-base cutter: *Eco*O109. Three restriction enzymes exhibited polymorphism between populations, i.e. *Bgl*II, *Hha*I and *Pst*I with high intraspecific mtDNA sequence divergence between the Fiji strain and the Australia/Malaysia strains of 1.68%. These three strains were obtained from laboratory stock. Though they may represent different natural population the small population sizes used in the laboratory over a number of years appears to have resulted in inbreeding and the loss of all intrastrain variation. However, Benzie *et al.* (1993) observed four polymorphic enzymes, i.e. *Bam*HI, *Eco*RV, *Sac*I and *Eco*O109, and they did find

a significant difference between *P. monodon* wild populations from east and west Australian coasts (χ^2=7.23, d.f.=2, P<0.05) but not among the east coast populations (χ^2=1.0, d.f.=2, P>0.5) which appeared to confirm their earlier finding using Allozymes.

1.5 THE AIMS OF THE STUDY

The biology suggests that dispersal is possible but that it may be restricted by spawning ground and nursery habitat and is likely to be controlled by the local hydrological conditions pertaining at any site. From allozyme studies, decapods generally have low levels of heterozygosity but a reasonable number of loci, possible 8-10, could give good information on the breeding structure of *P. monodon* in the present study. MtDNA analysis, although there have been few studies in decapods should give complementary information on the structure past and present of the species. In the present study, samples have been collected over the species range but will concentrate on the Malay Peninsula. To ensure that there was no temporal variation samples were collected twice with an interval of 1 year between samplings. All populations were analysed for both allozyme and mtDNA. The aims of the present study are as follows:

1.5.1 To determine the population breeding structure of *P. monodon* in Southeast Asia and Kenya.

1.5.2 To determine the levels of genetic differentiation within and between populations.

1.5.3 To investigate the genetic relationships among geographically isolated populations of *P. monodon* between the Andaman Sea and the Gulf of Thailand.

1.5.4 To compare the level of allozyme variation and restriction fragment length polymorphisms (RFLPs) of mtDNA.

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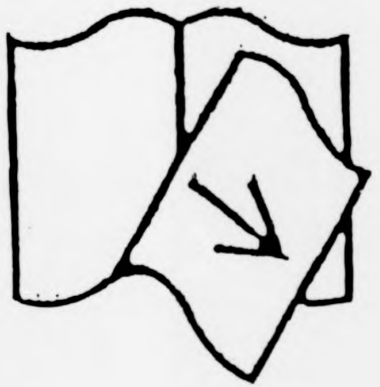


variation in specific enzymes in this studies. 70 enzyme systems were tested in 8 buffer systems. The buffers which gave the best separation and resolution were then selected for population studies. To visualize isozyme banding patterns specific enzyme histochemical staining recipes of Harris and Hopkinson (1965), Shaw and Prasad (1970), Redfield and Salini (1980), Richardson *et al.* (1986), Morizot and Schmidt (1990) and Murphy *et al.* (1990) were used with minor modifications (see Appendix 1).

2.2.3.1 Preparation of starch gel

A starch gel slab (18x15x0.55 cm) was prepared from 11-12% w/v hydrolysed potato starch (Sigma No. S-4501). The weighed dry starch was added to the appropriate electrophoresis gel buffer in an Erlenmeyer flask it was then immediately swirled over a Bunsen burner to make a uniform suspension and heated. After the solution was gently boiled for about 2 min it was degassed using a water aspirator to remove air bubbles, poured into a gel mould then covered with a glass plate. The hot gel was allowed to cool at room temperature for at least 2 h or more usually overnight before being used.

2.2.3.2 Sample loading

The cooled starch gel was removed from the mould, blotted dry with tissue paper and the slab was cut into two by a cut being made parallel and approximately 4 cm from the one of the longest sides edge of the gel. The two pieces of the slab were then separated ready for the sample wicks to be applied

to the cut edge of the longer piece of gel. Samples were prepared simply by the freeze thawing of the particular tissue being analysed, same tissues were ground with a plastic mortar that would fit into the Eppendorf tube. Filter paper sample wicks (Whatman 1) of 2x6 mm being placed directly against the tissue so that it absorbed any cytoplasm released after thawing from the damaged tissue. The wicks were then placed against the cut edge of the gel slice. To ensure the best possible resolution the gel and the samples were maintained at 4°C. With approximately 1 mm separating paper wicks, up to 30 samples can be applied to an 18x15 cm gel.

In order to monitor the progress of the electrophoresis run, the first and the last wicks were soaked in 0.1% bromophenol blue tracking dye and inserted about 3-4 mm from the edge of the gel mould. This dye has a large negative charge and migrates anodally through the gel ahead of all proteins. Once all samples had been loaded, the gel was put back into the gel mould and tighten with a small spacer. To standardize mobility, tiger prawn control samples were loaded on each gel for comparison of the banding patterns of unknown samples. All polymorphic individuals were rerun side by side to confirm any differences in mobility.

2.2.3.3 Electrophoresis

There were 4 electrophoresis buffers that gave good resolution in tiger prawn, namely TBE pH 8.5, TCE pH 7.0, TCB pH 8.6, and CTC pH 8.0. Buffer preparation and running condition are listed in Appendix 1. The gel mould with

sample wicks was placed in a Shandon electrophoresis tray containing the appropriate buffer and the circuit was completed using soft-cotton-cloths. To prevent excessive dehydration, each gel was covered with a piece of polythene sheet. Then the gel was subjected to a DC power supply and run in a refrigerator at 4°C using 200-250 V for 4-5 h. The paper wicks were removed after 15-20 minutes because the wick can prevent current flow across the gel and reduce the resolution of bands (Aebersold *et al.*, 1987).

2.2.3.4 Gel slicing

After completion of the electrophoresis run, the gel was removed from the bath and the mould discarded and then trimmed to remove desiccated edges and unnecessary portions of the gel. It was then dried by blotting it on tissue paper. The gel was sliced horizontally into 1.5 mm slabs on a Shandon slicing table. Up to 3 slabs can be cut from 5.5 mm thickness gel. Each slab was put into a separate staining tray, the top slice was inverted as staining resolution is much better on a cut surface.

2.2.3.5 Enzyme histochemical staining

To stain each gel slab, the enzyme stains were applied either as agar overlays or as solutions depending on enzyme system and staining recipes (listed in Appendix 1). For histochemical staining, dry chemicals were weighted first, then dissolved in an appropriate pH staining buffer (Appendix 1) for a particular enzyme and then the liquid cofactors were added. In the agar overlays technique,

the staining solution was mixed with equal volume of 2% agar at 50-60°C and immediately poured over the gel surface. The temperature of agar should not too high as it will denature linking enzymes in stains. After the agar was set, most the staining slabs were kept at 37°C in a dark incubator to speed up enzyme reaction and limit exposure to light for some of the dyes. Positive staining can be seen as violet or black colour. For enzymes detected with ultraviolet light, enzyme activity was visualized under long-wave (365 nm) UV light. Incubation times vary depending on the enzyme, therefore the enzyme stains must be continuously monitored to prevent overstaining.

2.2.3.6 Preservation of stained gels

The gels could be preserved as a permanent record using a number of techniques which depended on the type of stain. The correct fixing solution and method for preservation of each stain are shown in Appendix 1. Stains which are in the gel slab can be preserved by using acid-alcohol or glycerol fixing solutions. The agar overlay is peeled off and the gel sealed in plastic bag and kept at 4°C or can be wrapped in cellophane sheet making transparent gel (Numachi, 1980) and kept at room temperature. Murphy *et al.* (1990) recommended that agar overlay stains can be fixed and dried on filter paper (e.g., 3 mm), then pressed flat and wrapped in plastic for safe handling because the agar will retain dangerous chemicals for years. The overlays are stored in the dark at room temperature to prevent the fading of the staining by light.

2.2.4 Interpretation of Electrophoretic Patterns

Enzyme names, abbreviation and enzyme number followed that recommended by the International Union of Biochemistry's Nomenclature Committee (IUBNC, 1984; Shaklee, *et al.*, 1990), in which loci are identified by italic abbreviations reflecting the name of enzyme or protein, e.g. *MDH** for Malate dehydrogenase. For multilocus isozymes, when there is more than one locus coding for that enzyme, these isozymes were identified by hyphenated Arabic number, e.g. *MDH-1**, *MDH-2**; the numbers are assigned sequentially in relation to the electrophoretic mobilities of homomeric isozymes, starting with the slowest or least anodal subunit. Variant alleles were also identified according to their relative electrophoretic mobilities and the mobility of the most common allele in the species designed as 100.

2.2.5 Data Analysis

Estimates of gene frequencies, basic statistical tests and clustering procedures were done by using the BIOSYS-1 computer program (Swofford and Selander, 1989) and calculating formulas are summarised in Appendix 2. All polymorphic loci either before or after combining were tested for conformity to Hardy-Weinberg equilibrium model of alleles in population by using chi-square goodness-of-fit test. However exact significance probabilities (analogous to Fisher's exact test for 2 x 2 contingency tables) were calculated, avoiding the difficulties encountered in using the chi-square distribution for small sample (Vithayasai, 1973). In addition, the log likelihood χ^2 test (G-test) were also

calculated and preferred if the expected values are small (Ferguson, 1980).

Tests for homogeneity among the samples were carried out between years at same site, adjacent paired localities, paired comparison, and simultaneously among all locations. Genetic differentiation were estimated using both allele and genotype frequencies. Genic contingency chi-square test for a difference in allele frequencies (Workman and Niswander, 1970) and Nass chi-square analysis with small expectations for heterogeneity in genotype frequencies (Nass, 1959) were performed to test significant difference between populations. In addition, the significance values used were corrected using the sequential Bonferroni technique (Holm, 1979; Rice, 1989) to increase statistical power.

F-statistics (Wright, 1965, 1978; Nei 1973, 1977) were also computed using the BIOSYS-1 computer program (Swofford and Selander, 1989) to partition genetic differentiation within population (F_{IS}) and between population (F_{ST}). A chi-square test was then used to evaluate the significance of F_{IS} and F_{ST} values (Li and Horvitz, 1953; Workman and Niswander, 1970; Waple, 1987) as shown in Appendix 2 .

To quantify genetic differences or similarities between pairs of population, Nei's genetic distance (Nei 1972, 1978) and Rogers' distance (Rogers, 1972) were calculated based on all loci studied. These genetic distance can be visualized by using the unweighed pair-group arithemetric average (UPGMA) clustering method (Sneath and Sokal, 1973) to produce a genetic distance dendrogram.

2.3 RESULTS

2.3.1 Genetic Interpretation of Allozyme Banding Patterns

From the 70 different enzymes that were tested, a total of 32 enzymes could be reliably stained which resulted in the identification of 46 putative loci being scored in *P. monodon*, of which 10 loci were consistently polymorphic at 99% level, namely, *AAT-1**, *AAT-2**, *ALAT**, *ESD**, *GPI**, *IDHP**, *MDH-1**, *MDH-2**, *MPI** and *PGM**. The banding patterns observed at the polymorphic loci were consistent with the expected quaternary structure of the particular enzyme. The protein structure of all enzymes investigated, the number of encoding loci, and the tissues specific to enzyme loci are shown in Table 2.2. All 46 loci examined were detected in the anodal zone of the gel.

ASPARTATE AMINOTRANSFERASE (AAT)

[GLUTAMIC-OXALOACETIC TRANSAMINASE (GOT)]

Two loci, *AAT-1** and *AAT-2**, were observed in abdominal muscle and pleopod in *P. monodon* sampled in the study. Mulley and Latter (1980) only identified 1 locus in Australian *P. monodon* but it is generally assumed most higher invertebrates and vertebrates express two loci: a cytoplasmic and a mitochondrial form (Hedgecock *et al.*, 1982). Mattoccia *et al.* (1987) also observed two loci in *P. kerathurus*.

Both loci were variable but heterozygotes usually appeared as elongated bands rather than discrete 3 banded heterozygotes. However, the fast heterozygote for *AAT-2** [which the enzyme structure has been reported as dimeric in fish

Table 2.2 Enzyme loci observed, subunit structure, the buffer system, and the tissue sources for each locus in *Penaeus monodon*

Enzyme	Abbrev.	EC No.	Subunit structure	Buffer system	Locus	Tissue
Aspartate aminotransferase	**AAT**	2.6.1.1	Dimer	TBE,TCB	*AAT-1**	Pl,M
					*AAT-2**	Pl,M
Acid phosphatase	**ACP**	3.1.3.2	Monomer, Dimer	TBE,CTC	*ACP-1**	P
					*ACP-2**	P
Adenosine deaminase	**ADA**	3.5.4.4	Monomer	TBE	*ADA-1**	P,M
					*ADA-2**	Pl,H,M
Adenylate kinase	**AK**	2.7.4.3	Monomer	TBE	*AK**	Pl,M
Alanine aminotransferase	**ALAT**	2.6.1.2	Dimer	TBE	*ALAT**	Pl,M
Alkaline phosphatase	**ALP**	3.1.3.1	Monomer, Dimer	TCB	*ALP-1**	P
					*ALP-2**	P
Aldehyde oxidase	**AO**	1.2.3.1	Dimer	TCE	*AO-1**	Pl,P
					*AO-2**	Pl
Arginine kinase	**ARK**	2.7.3.3	Dimer	TBE	*ARK**	Pl

Table 2.2 (continued)

Enzyme	Abbrev.	EC No.	Subunit structure	Buffer system	Locus	Tissue
Esterase	**EST**	3.1.1.-	Monomer	TBE	*EST-1**	P
					*EST-2**	Pl
					*EST-3**	Pl,P
Esterase-D	**ESD**	3.1.-.-	Dimer	TBE	*ESTD**	Pl
Fructose-bisphosphate aldolase	**FBALD**	4.1.2.13	Tetramer	TCE	*FBALD**	Pl,M
Fumarate hydratase	**FH**	4.2.1.2	Tetramer	TCE	*FH**	Pl,M,H
Glyceraldehyde-3-phosphatase dehy.	**GAPDH**	1.2.1.12	Tetramer	TBE	*GAPDH**	Pl,M
Glutamate dehydrogenase	**GLUDH**	1.4.1.-	Hexamer	TBE	*GLUDH**	Pl,M
Glyceral-3-phosphate dehydrogenase	**G3PDH**	1.1.1.8	Dimer	TCE	*G3PDH**	Pl,M
Glucose-6-phosphate dehydrogenase	**G6PDH**	1.1.1.49	Dimer	TCE	*G6PDH**	Pl,M
Glucose-6-phosphate isomerase	**GPI**	5.3.1.9	Dimer	TBE	*GPI**	Pl,M,P

Table 2.2 (continued)

Enzyme	Abbrev.	EC No.	Subunit structure	Buffer system	Locus	Tissue
Hexokinase	**HK**	2.7.1.1	Monomer	TCE	*HK-1**	Pl,M,H,P
					*HK-2**	Pl,H
L-Iditol dehydrogenase	**IDDH**	1.1.1.14	Tetramer	TBE	*IDDH**	Pl,M
Isocitrate dehydrogenase ($NADP^+$)	**IDHP**	1.1.1.42	Dimer	TCE,CTC	*IDHP**	Pl,H
L-Lactate dehydrogenase	**LDH**	1.1.1.27	Tetramer	TCE	*LDH-1**	Pl,E
					*LDH-2**	Pl,H
Malate dehydrogenase	**MDH**	1.1.1.37	Dimer	TCE	*MDH-1**	Pl,H,M
					*MDH-2**	Pl,H,M
Malic enzyme ($NADP^+$)	**MEP**	1.1.1.40	Tetramer	TBE	*MEP**	Pl,H
Mannose-6-phosphate isomerase	**MPI**	5.3.1.8	Monomer	TBE	*MPI**	Pl,H,E
Octanol dehydrogenase	**ODH**	1.1.1.73	Dimer	TBE	*ODH**	Pl,M

Table 2.2 (continued)

Enzyme	Abbrev.	EC No.	Subunit structure	Buffer system	Locus	Tissue
Peptidase-C	**PEPC**	3.4.-.-	Monomer	TBE	*PEPC-1**	P
					*PEPC-2**	P
					*PEPC-3**	P,Pl
Phosphogluconate dehydrogenase	**PGDH**	1.1.1.44	Dimer	TBE	*PGDH**	Pl,M,P
Phosphoglucomutase	**PGM**	5.4.2.2	Monomer	TCE	*PGM**	Pl,M,H
Pyruvate kinase	**PK**	2.7.1.40	Tetramer	TBE	*PK-1**	Pl,M
					*PK-2**	Pl,M
Pyrroline dehydrogenase	**PYDH**	1.5.1.12	Subunit	TCE	*PYDH**	Pl,M
Superoxide dismutase	**SOD**	1.15.1.1	Dimer, Tetramer	TBE	*SOD-1**	Pl,M
					*SOD-2**	Pl,M
Xanthine dehydrogenase	**XDH**	1.2.3.2	Monomer, Dimer	TBE	*XDH**	P

NOTE: M = muscle, P = hepatopancreas, Pl = Pleopod

(Aebersold *et al.*, 1987; Morizot and Schmidt, 1990)] was clearly seen as a 3 banded heterozygote in the Philippines population containing the dimeric structure of the enzyme in this species.

Three alleles were observed in both *AAT-1** (**150, *100, *89*) and *AAT-2** (**115, *100, *78*) (Fig. 2.2). Because of low resolution of this enzyme on the gel, other studies on Penaeids have not reported so many alleles or confirmed the dimeric structure (Mulley and Latter, 1980; Mulley, 1981ab; Richardson, 1982; Sbordoni *et al.*, 1986; Lavery and Staples, 1990). These studies have recorded these loci as being monomeric or only having rare alleles.

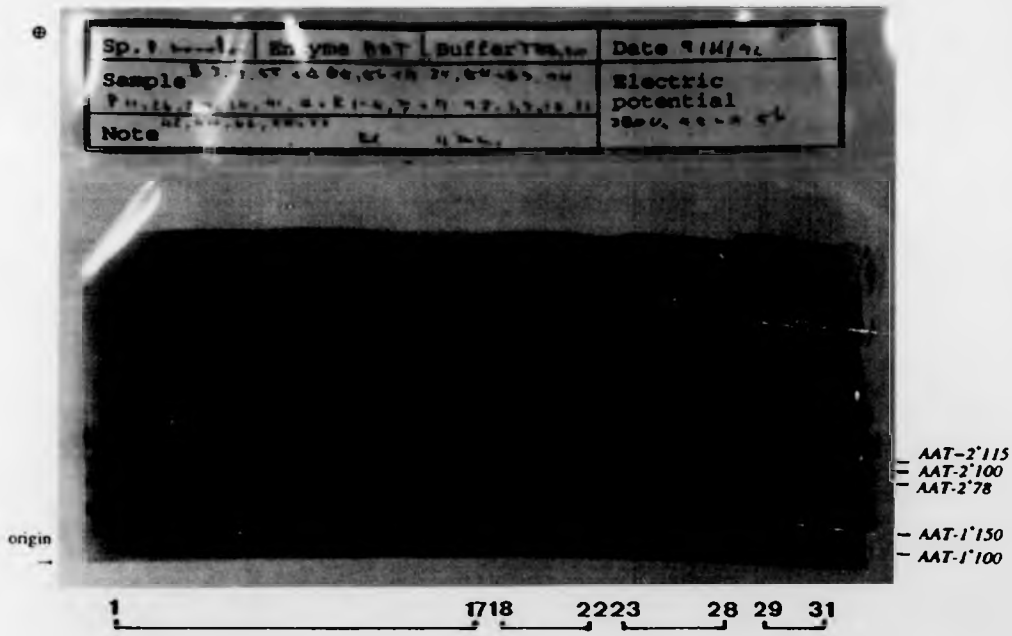


Fig. 2.2 Zymogram exhibiting variation at *AAT* in pleopod extracts from *P. monodon*: genotype *AAT-1*150/150* is at lanes 18-22, genotype *AAT-1*100/100* is at lanes 1-17, 23-31, genotype *AAT-2*115/100* is at lanes 11, 14, 24-25, 29, 31, genotype *AAT-2*100/100* is at lanes 1-10, 12, 15, 17-23, 26-28, 30, genotype *AAT-2*100/78* is at lanes 13, 16.

ACID PHOSPHATE (ACP)

Two loci, *ACP-1** and *ACP-2**, were detected. Two loci were also found in *P. kerathurus, and P. stylirostris* (Lester, 1983; Mattoccia *et al.*, 1986) and in *Metapenaeus bennettae* and *M. dalli* (Salini and Moore, 1985) but only one locus was found in *P. monodon* and other penaeid species by Mulley and Latter (1980) and Sbordoni *et. al.* (1986). This enzyme was detected in the hepatopancreas as in previous studies on penaeid (Mulley and Latter, 1980; Redfield and Salini, 1980). No variation was observed for this enzyme in this or previous studies on *P. monodon* (Mulley and Latter, 1980; Benzie *et al.*, 1992).

ADENOSINE DEAMINASE (ADA)

Two monomorphic loci, *ADA-1** and *ADA-2**, were observed for this enzymes. Two monomorphic loci were detected in post larva and juvenile stage but three loci in adults of *P. esculentus* and *P. semisulcatus* (Lavery and Staples, 1990). Three loci were also detected in *P. japonicus* (Sbordoni *et. al.*, 1986a and 1986b). Both loci in this study were observed in abdominal muscle, whereas *ADA-1** was tissue specific in pleopod muscle and heart, and *ADA-2** in hepatopancreas.

ADENYLATE KINASE (AK)

A single monomorphic locus was resolved for *AK** as was the case for most of the penaeid which have been analysed. Only in *M. endeavouri* (Mulley and Latter, 1981b) have any variant alleles been observed in a tropical Australian population, suggesting low levels of variation in this enzyme. The enzyme was observed in pleopod and abdominal muscle which was consistent with the reports of Mulley and Latter (1980) and Redfield and Salini (1980).

ALANINE AMINOTRANSFERASE (ALAT)

[GLUTAMIC-PYRUVATE TRANSAMINASE (GPT)]

Activity reflecting a single dimeric *ALAT** was detected. This locus was strongly and clearly observed in pleopod tissue. This locus was found to be highly polymorphic in this species as shown in Table 2.3 A, 2.3 B, 2.3 C. In other species of penaeid this enzyme was observed to be monomorphic (Richardson, 1982; Salini and Moore, 1985; Lavery and Staples, 1990). In this study, three alleles, **100*, **68*, **52*, were found throughout the populations in SE Asia. The African population was fixed for the **68* allele.

ALKALINE PHOSPHATASE (ALP)

Two loci, *ALP-1** and *ALP-2**, were observed in hepatopancreas. In this study the resolution was variable, some putative heterozygotes were observed at both *ALP-1** and *ALP-2** but were not scored as repeatability was low. Mulley and Latter (1980) also observed two loci and found no variation at *ALP-1** and a few heterozygotes at *ALP-2**. Benzie *et al.* (1992) observed no variation at these loci in Australian *P. monodon* populations.

ALDEHYDE OXIDASE (AO)

Two monomorphic loci, *AO-1** and *AO-2**, were detected in *P. monodon*. Both loci were scored in pleopod muscle, whereas only *AO-2** was active in hepatopancreas. The observation of two invariant loci in this species is consistent with the studies on other *Penaeus spp.* (Lester, 1983; Sbordoni *et al.*, 1986a and 1986b), but only a single locus was found in thirteen marine penaeid species studied by Mulley and Latter (1980).

ARGININE KINASE (ARK)

A single monomorphic locus *ARK** was scored in pleopod tissue. A single monomorphic locus is consistently resolved in penaeid; *P. esculentus, P. semisulcatus* (Salini and Moore, 1985), *M. bennettae, M. dalli* (Lavery and Staples, 1990). Shaklee and Keenan (1986) also suggested that this locus is only present in invertebrates.

ESTERASE (EST)

α-Naphthyl acetate was used as the enzyme substrate in the staining mixture. Three different loci, *EST-1**, *EST-2** and *EST-3**, were scored for this enzyme. *EST-1** and *EST-2** were tissues specific in hepatopancreas, whereas *EST-3** was detected in hepatopancreas and pleopod. All three loci were monomorphic. Three EST loci have been consistently recorded in *P. monodon* and in other marine penaeid (Mulley and Latter, 1980).

ESTERASE-D (ESD, ESTD)

A single polymorphic locus *ESD** with 4 alleles, **105,* **100,* **89* and **75*, was scored for this enzyme in pleopod. The polymorphic patterns appeared only as an elongated band for the slower heterozygote but three bands separated for faster heterozygote. The three-banded heterozygotes confirm the dimeric structure of this enzyme found in other species (Harris and Hopkinson, 1965; Richardson *et al.*, 1986).

FRUCTOSE-BIPHOSPHATE ALDOLASE (FBALD, ALDO)

A single anodal monomorphic locus was resolved for *FBALD** in *P. monodon*.

One monomorphic locus is consistently resolved in pleopod in this and other species (Mulley and Latter, 1980; Redfield and Salini, 1980; Benzie *et al.*, 1992).

FUMARATE HYDRATASE (FH)

[FUMARASE (FUM)]

Only one monomorphic locus, *FH** was scored in pleopod, heart and hepatopancreas. In pleopod the enzyme often appeared as a triple-banded pattern. Richardson *et al.* (1986) commented that this is a complex locus and often resolves extra cathodal sub-bands. This enzyme is also usually observed as a single monomorphic locus in other penaeids (Mulley and Latter, 1980; Lavery and Staples, 1990; Benzie *et al.*, 1992).

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH, GAPD)

Only a single locus was observed in pleopod and abdominal muscle for *GAPDH**. The detection of one GAPDH loci with no variation in *P. monodon* is consistent with the findings of Mulley and Latter (1980) and Benzie *et al.* (1992).

GLUTAMATE DEHYDROGENASE (GLUDH, GLUD)

Activity reflecting only a single locus *GDH** was scored. The enzyme which was detectable in pleopod, heart and abdominal muscle was monomorphic. These observation have been confirmed by other allozyme studies in *P. monodon* and other penaeid (Mulley and Latter, 1980; Lavery and Staples, 1990; Benzie *et al.*, 1992).

GLYCEROL-3-PHOSPHATE DEHYDROGENASE (G3PDH, GPD)

Only a single monomorphic locus was observed in pleopod and abdominal muscle for *G3PDH** in all populations. This is consistent the results from other species (Mulley and Latter, 1980; Benzie *et al.*, 1992).

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH, G6PD)

Activity reflecting a single monomorphic *G6PDH** locus was detected in pleopod and abdominal muscle as in other studies on *P. monodon* (Mulley and Latter, 1980; Benzie *et al.*, 1992). In other penaeid studies, *G6PDH** has been observed as both a monomorphic (Sbordoni *et al.*, 1986 and 1987; Lavery and Staples, 1990) and polymorphic locus (Mattoccia *et al.*, 1987).

GLUCOSE-6-PHOSPHATE ISOMERASE (GPI)

A single polymorphic *GPI** locus was observed which expressed strongly and quickly in pleopod, abdominal muscle and hepatopancreas (zymograms needed to read within 20 min). The *GPI** locus was polymorphic in almost all populations. This was similar to the results of Benzie *et al.* (1992) in Australian *P. monodon*, *GPI** is also highly polymorphic in other species of penaeid (Mulley and Latter, 1980; Richardson, 1982; Lester, 1983; Salini and Moore, 1985; Lavery and Staples, 1990) and in many other species of invertebrate (Hoelzel, 1992).

There were five variant alleles, ^{*133}, ^{*125}, ^{*100}, ^{*78} and ^{*60}, in this study which agreed with the findings of Benzie *et al.* (1992) as shown in Fig. 2.3. *GPI** heterozygotes were triple banded confirming the dimeric structure of this enzyme

in penaeid as recorded by Mulley and Latter (1980), Richardson (1982), Salini and Moore (1985), Salini (1987) and Lavery and Staples (1990).

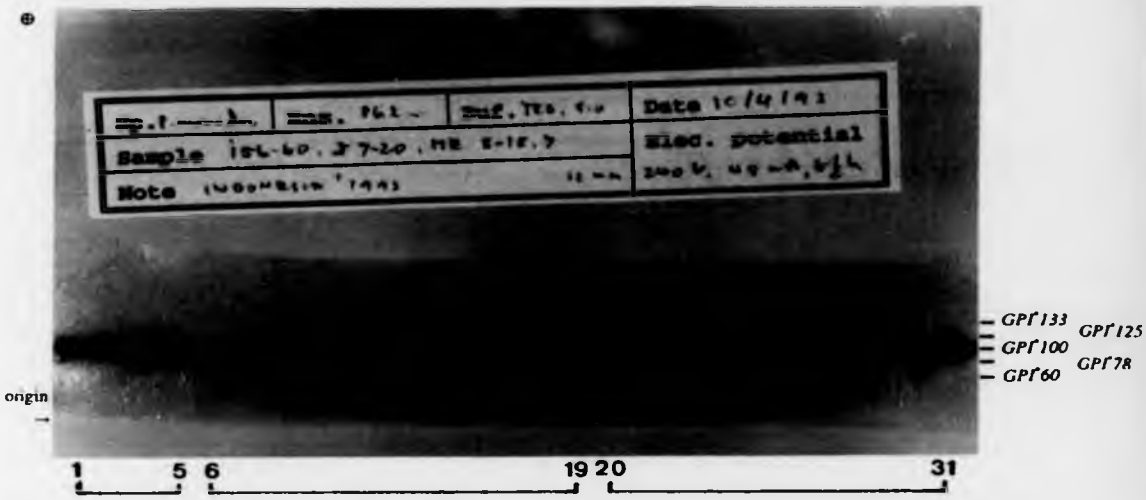


Fig. 2.3 Zymogram exhibiting variation at *GPI* in pleopod extracts from *P. monodon*: genotype *GPI***135/100* is at lanes 21, genotype *GPI***125/100* is at lanes 8, 9, genotype *GPI***100/100* is at lanes 1-6, 11, 14-20, 22-24, 28, 29, 31, genotype *GPI***100/78* is at lanes 7, 13, 25-27, 30, genotype *GPI***100/60* is at lanes 10, 12.

HEXOKINASE (HK)

Two anodal loci, *HK-1** and *HK-2**, were detected in *P. monodon*. *HK-1** was observed in pleopod and heart, but *HK-2** was detected in almost all tissues, i.e. pleopod, abdominal muscle, heart, hepatopancreas. Lester (1979 and 1983) and Salini and Moore (1985) also reported two loci in a number of penaeid species, however Mulley and Latter (1980) only scored one locus.

HK-2* appeared to be variable with double-banded heterozygotes, but, the patterns were not consistent and genotype frequencies in all populations were not in Hardy-Weinberg equilibrium because of a deficit of heterozygotes. Because of this it was decided to treat it as an invariant locus.

L-IDITOL DEHYDROGENASE (IDDH)

[SORBITOL DEHYDROGENASE(SDH, SORD)]

Activity reflecting only a single locus *IDDH** was scored. The enzyme which was detectable in pleopod and abdominal muscle was monomorphic in all populations. A similar finding was made in the allozyme studies on *P. monodon* by Mulley and Latter (1980) and Benzie *et al.* (1992).

ISOCITRATE DEHYDROGENASE(NADP$^+$) (IDHP, IDHI)

A single polymorphic locus was resolved for *IDHP** in *P. monodon*. The products were expressed as very sharp bands and with a strong staining intensity in pleopod muscle and heart. Some workers (Mulley and Latter, 1980; Richardson *et al.*, 1986) described a single variant locus in most of the penaeid species they analysed whereas Salini and Moore (1985) and Mattoccia *et al.* (1986) found it to be polymorphic in *M. bennettae, M. dalli* and *P. kerathurus*.

In this study 3 variant alleles, ^{*100}, ^{*90} and ^{*80}, were observed (Fig. 2.4). All heterozygotes exhibited a three-banded pattern in a 1:2:1 ratio of staining intensity, as expected from the dimeric structure of this enzyme (Harris and Hopkinson, 1976).

Fig. 2.4 Zymogram exhibiting variation at *IDHP* in pleopod extracts from *P. monodon*: genotype *IDHP*100/100* is at lanes 1, 3-4, 8-9, 11-13, 19-22, 26, 28, 32, genotype *IDHP*100/90* is at lanes 2, 5-7, 15-18, 23-25, 27, 29-31, genotype *IDHP*90/90* is at lanes 14.

L-LACTATE DEHYDROGENASE (LDH)

Two loci, *LDH-1** and *LDH-2**, were scored in pleopod muscle, whereas *LDH-1** and *LDH-2** had tissue specific activity in eye and hepatopancreas respectively. Two loci were also found in *P. kerathurus, P. stylirostris* and *P. japonicus* (Mattoccia *et al.*, 1986; Sbordini *et al.*, 1986a and 1986b), but only one in *P. monodon* and other penaeid species (Mulley and Latter, 1980; Lavery and Staples, 1990). No variants were found in either locus in this study. This agreed with the observation of Benzie *et al.* (1992) in this species, whereas Mulley and Latter (1980) thought it was polymorphic in the Australian populations they analysed.

MALATE DEHYDROGENASE (MDH)

Two loci, *MDH-1** and *MDH-2**, were scored in this study (Fig. 2.5) which is consistent with other studies on *P. monodon* (Benzie *et al.*, 1992) and in other penaeid species studied (Mulley and Latter, 1980; Mulley, 1981a and 1981b; Richardson, 1982; Lester, 1983; Salini, 1983; Sbordoni *et al.*, 1986a and 1986b; Lavery and Staples, 1990). Both loci were polymorphic and expressed in pleopod, abdominal muscle and heart. MDH has been reported as dimeric in nearly all vertebrates and invertebrates (Richardson *et al.*, 1986; Aebersold *et al.*, 1987). Heterozygote appeared as a deeper band, three bands not being clearly visualized under these conditions of staining.

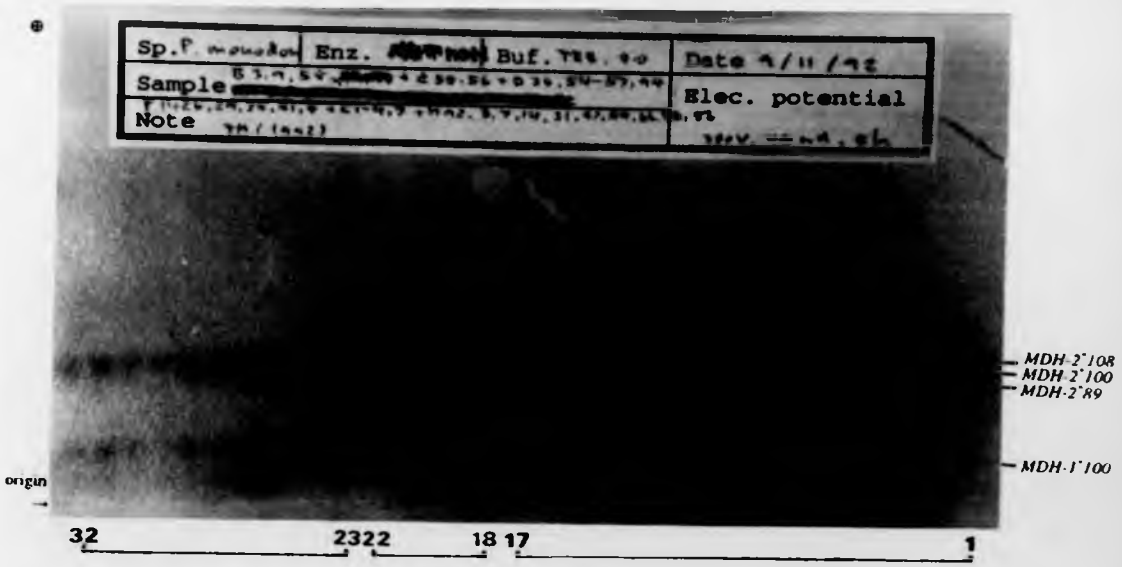


Fig. 2.5 Zymogram exhibiting variation at *MDH* in pleopod extracts from *P. monodon*: genotype *MDH-1*100/100* is at lanes 1-32, genotype *MDH-2*108/100* is at lanes 12, genotype *MDH-2*100/100* is at lanes 1-11, 13-17, 23-32, genotype *MDH-2*89/89* is at lanes 18-22 (start from right to left).

*MDH-1** had 2 alleles (**115, **100*) and *MDH-2** 3 alleles (**108, **100, **89*) as shown in Fig. 2.5. This was the same as the observations of Benzie *et al.* (1992) in *P. monodon*. In contrast, Mulley and Latter (1980) found no variation at either locus.

MALIC ENZYME(NADP+) (MEP, ME)

A single anodal monomorphic locus was resolved for *MEP** in *P. monodon* and this is consistent with other studies on this species and other penaeid (Mulley and Latter, 1980; Lavery and Staples, 1990; Benzie *et al.*, 1992). This loci was observed in pleopod and heart in line with other observations in penaeid (Mulley and Latter, 1980; Redfield and Salini, 1980).

MANNOSE-6-PHOSPHATE ISOMERASE (MPI)

A single *MPI** locus was observed in pleopod, heart and eye. *MPI** was polymorphic in almost all populations studied. *MPI** is known to be polymorphic in Australian *P. monodon* populations (Benzie *et al.*, 1992), and in many other species of penaeid (Mulley and Latter, 1980; Lester, 1983; Mattoccia *et al.*, 1987; Salini, 1987).

There were five alleles, **115, **110, **100, **93* and **86*, observed in this study as shown in Fig. 2.6 which is identical with the findings of Benzie *et al.* (1992) in Australian populations of this species. *MPI** heterozygotes showed a double banded pattern confirming the monomeric structure of this enzyme.

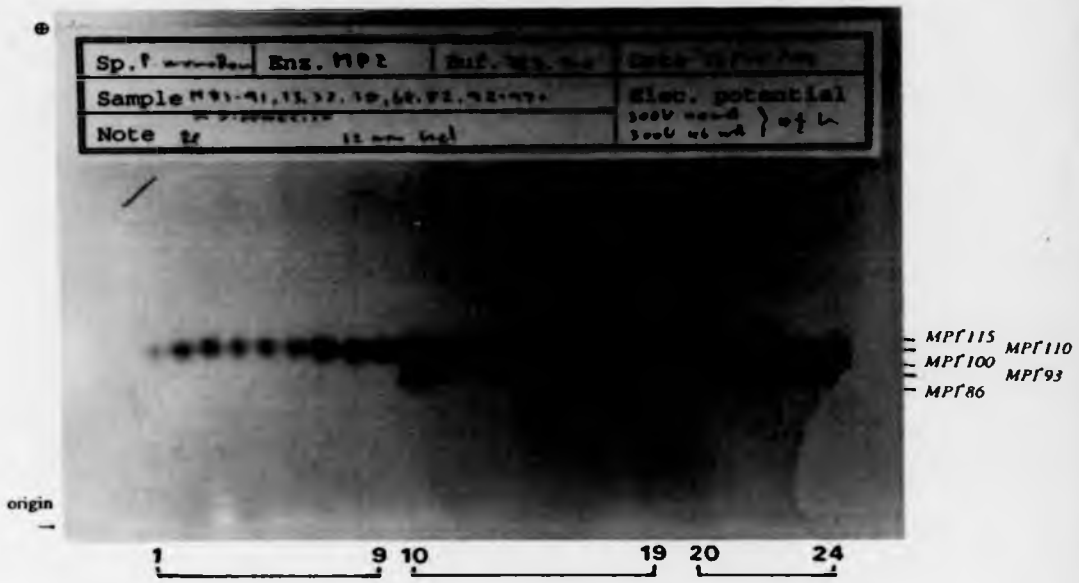


Fig. 2.6 Zymogram exhibiting variation at *MPI* in pleopod extracts from *P. monodon*: genotype *MPI*115/93* is at lanes 22, genotype *MPI*110/100* is at lanes 13, 20, genotype *MPI*110/93* is at lanes 23-24, genotype *MPI*100/100* is at lanes 1-9, 12, 14-19, genotype *MPI*100/93* is at lanes 11, 21, genotype *MPI*100/86* is at lanes 10.

OCTANOL DEHYDROGENASE (ODH)

A single monomorphic locus for *ODH** was scored in pleopod and abdominal muscle. Similarly, one locus is consistently resolved in other studies on penaeid (Mulley and Latter, 1980; Salini and Moore, 1985; Lavery and Staples, 1990). In this study, ODH appears to be analogous to ADH this was also the case in the previous studies of Mulley and Latter (1980) and Redfield and Salini (1980) in other penaeid species.

PEPTIDASE-C (PEPC)

Glycerol-L-leucine was used as the enzyme substrate in the staining mixture. Three presumed loci, *PEPC-1**, *PEPC-2** and *PEPC-3**, were scored in this enzyme. All products were detected in hepatopancrease, whereas *PEPC-1** was only active in pleopod muscle. All three loci were monomorphic confirming the reports in other penaeid studies (Mulley and Latter, 1980). However, *PEPC-1** looked to be polymorphic but the resolution was inadequate under the electrophoresis condition used. *PEP-1** was shown to be polymorphic in the study of Benzie *et al.* (1992) in *P. monodon* by using cellulose acetate gels.

PHOSPHOGLUCONATE DEHYDROGENASE (PGDH, PGD)

A single monomorphic locus was resolved for *PGDH**. The products were observed in pleopod, abdominal muscle and hepatopancreas. The observation of a single locus agreed with the studies in other penaeid (Mulley and Latter, 1980; Lester, 1983; Lavery and Staples, 1990; Benzie *et al.*, 1992). Benzie *et al.* (1992) observed marginal levels of polymorphism at variant alleles, *PGDH***106*, **92*, in two Australian populations.

PHOSPHOGLUCOMUTASE (PGM)

Activity reflecting a single monomeric *PGM** locus was scored. This enzyme stained very quickly and gave sharp resolution in pleopod, abdominal muscle and heart. *PGM** was highly polymorphic in this species , a total of 7 alleles (**113*, **108*, **105*, **100*, **95*, **90* and **85*) were observed (Table 2.3 A, 2. 3 B, 2.3 C, Fig. 2.7). These results agreed with the studies of Mulley and Latter (1980) and Benzie *et al.* (1992) in *P. monodon*.

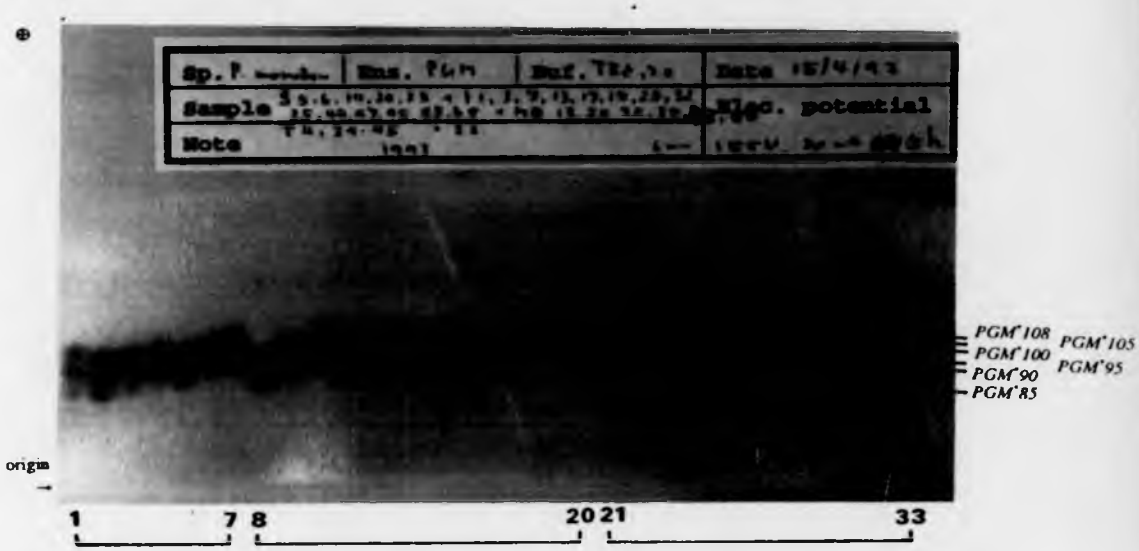


Fig. 2.7 Zymogram exhibiting variation at *PGM* in pleopod extracts from *P. monodon*: genotype *PGM*108/100* is at lanes 7, 18, 22, 25, genotype *PGM*105/100* is at lanes 10, 17, 19, genotype *PGM*100/100* is at lanes 4, 6, 13, 21, 23, 27-33, genotype *PGM*100/95* is at lanes 1, 5, 20, genotype *PGM*100/90* is at lanes 2, 3, 9, 11, 12, 14-16, 26, genotype *PGM*100/85* is at lanes 24, genotype *PGM*100/85* is at lanes 8.

In this study, PGM allozymes always produced double-banded homozygotes and either three or four-banded heterozygotes. These additional anodal sub-bands were observed in other studies on *P. stylirostris, P. setiferus* (Lester and Cook, 1987), and *M. bennettae* (Salini, 1987) and are also commonly found in vertebrates (Richardson *et al.*, 1986; Morizot and Schmidt, 1990).

PYRUVATE KINASE (PK)

Two monomorphic loci, *PK-1** and *PK-2**, were observed for this enzyme. Both loci appeared in pleopod and abdominal muscle. Only one monomorphic locus

was detected in *P. esculentus*, *P. semisulcatus*, *M. bennettae*, and *M. dalli* (Salini and Moore, 1985; Lavery and Staples, 1990).

1-PYRROLINE DEHYDROGENASE (PYDH)

Only a single monomorphic *PYDH** locus was observed in pleopod and abdominal muscle in all populations studied. The detection of one PYDH locus with no variation in *P. monodon* is consistent with the findings of Mulley and Latter (1980) and Benzie *et al.* (1992) .

SUPEROXIDE DISMUTASE (SOD)

Two monomorphic loci, *SOD-1** and *SOD-2**, were scored in pleopod and abdominal muscle. In contrast to this study, Mulley and Latter (1980) and Lavery and Staples (1990) observed only one locus in penaeid. However Richardson *et al.* (1986) and Morizot and Schmidt (1990) suggested that there are two loci for this enzyme a cytoplasmic and a mitochondrial form encoded by separate gene loci.

XANTHINE DEHYDROGENASE (XDH)

Activity reflecting a single monomorphic *XDH** locus was scored in hepatopancrease confirming the observations in other penaeid prawn species (Mulley and Latter, 1980; Redfield and Salini, 1980; Mattoccia *et al.*, 1986; Sbordini *et al.*, 1986 and 1987).

2.3.2 Allele Frequencies

The allele frequencies for the polymorphic loci are presented in three Tables. Table 2.3 A is the allele frequencies observed in each individual sample obtained for the temporal stability study of populations in Thailand. Table 2.3 B, includes the combined samples after testing for temporal stability in Thai populations and the other geographically remote sample sites. Table 2.3 C is the allele frequencies of the pooled samples after testing for homogeneity. The dendrograms showing variation in allele frequencies of *ALAT**, *GPI**, *IDHP** and *MPI** are also illustrated in Fig. 2.8, 2.9, 2.10 and 2.11 respectively. A total of 10 loci were shown to be polymorphic at the 99% level and 5 at the 95% level. All polymorphic loci generally shared the same common allele in all populations excluding *ALAT*68* in the Philippines, and *ALAT*68* and *MPI*110* in the Kenyan population. The Kenya population was fixed for unique alleles at *AAT-1*150* and *MDH-2*89*.

2.3.3 Hardy-Weinberg Equilibrium

Test for goodness of fit to Hardy-Weinberg equilibrium were undertaken on genotype frequencies at each polymorphic locus in each sample collected. A total of 132 analyses including Fisher's exact test and G-test for small expected values were completed. Hardy-Weinberg test for goodness-of-fit were also performed on combined samples after testing for temporal (see Table 2.3 A) and spatial (see Table 2.3 C) homogeneity. This work and tests resulted in 2 significant deviation from expectations.

Table 2.3 A Allele frequencies at ten polymorphic loci in four populations and year of collection of *P. monodon* in Thailand for temporal stability study.

	Population								
	Trat			Surat		Phuket		Satun	
Locus	1991	1992	1993	1991	1992	1991	1993	1992	1993
*AAT-1**									
a 150	-	-	-	-	-	-	-	-	-
b 100	1.000	.995	1.000	.994	.995	.991	.990	.990	.990
c 89	-	.005	-	.006	.005	.009	.010	.010	.010
*AAT-2**									
a 115	.009	.005	-	.006	.005	.009	.010	.010	.010
b 100	.991	.995	1.000	.994	.995	.991	.990	.990	.990
c 78	-	-	-	-	-	-	-	-	-
*ALAT**									
a 100	.605	.484	.544	.656	.615	.612	.643	.640	.540
b 68	.386	.505	.444	.339	.345	.353	.337	.320	.430
c 52	.009	.011	.011	.006	.040	.034	.020	.040	.030
*ESD**									
a 105	.009	-	-	-	.005	-	-	-	-
b 100	.964	.984	.967	.956	.980	.957	.980	.970	.980
c 89	.023	.005	-	.011	.010	.034	.015	.025	.010
d 75	.005	.011	.033	.033	.005	.009	.005	.005	.010
*GPI**									
a 133	-	-	-	-	-	-	-	-	-
b 125	.009	-	.011	-	.005	.017	.010	.010	.010
c 100	.905	.898	.856	.917	.925	.862	.832	.880	.860
d 78	.082	.097	.133	.061	.065	.121	.143	.110	.130
e 60	.005	.005	-	.022	.005	-	.015	-	-
*IDHP**									
a 100	.682	.731	.756	.733	.805	.655	.694	.685	.710
b 90	.318	.269	.244	.267	.195	.345	.306	.315	.280
c 80	-	-	-	-	-	-	-	-	.010
*MDH-1**									
a 115	.005	-	.011	-	-	-	-	-	.010
b 100	.995	1.000	.989	1.000	1.000	1.000	1.000	1.000	.990
*MDH-2**									
a 108	-	-	-	-	-	-	-	.005	.010
b 100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.995	.990
c 89	-	-	-	-	-	-	-	-	-
*MPI**									
a 115	-	-	-	-	-	-	-	-	-
b 110	.018	.005	-	.006	-	.026	.036	.015	.010
c 100	.968	.995	1.000	.983	.990	.966	.954	.975	.990
d 93	.014	-	-	.011	.010	.009	.005	.010	-
e 86	-	-	-	-	-	-	.005	-	-
*PGM**									
a 113	-	.005	-	-	-	-	-	-	-
b 108	.009	.005	-	-	.005	.009	.010	.005	.010
c 105	.018	.022	-	-	.015	.009	.031	.030	.000
d 100	.932	.957	.989	.967	.925	.940	.929	.955	.950
e 95	-	-	.011	-	.010	-	-	-	.020
f 90	.036	.011	-	.028	.045	.034	.031	.010	.020
g 85	.005	-	-	.006	-	.009	-	-	-
(n)	110	93	45	90	100	58	98	100	50

Table 2.3 B Allele frequencies at ten polymorphic loci in nine populations of *P. monodon*: 1=Trat, 2=Surat, 3=Phuket, 4=Satun, 5=Kedah, 6=Dungun, 7=Aceh 8=South Java, 9=North Java.

	Population								
Locus	1	2	3	4	5	6	7	8	9
*AAT-1**									
a 150	-	-	-	-	-	-	-	-	-
b 100	.996	.995	.990	.990	.990	1.000	.980	1.000	1.000
c 89	.004	.005	.010	.010	.010	-	.020	-	-
*AAT-2**									
a 115	.006	.005	.010	.010	.020	.022	.010	.008	-
b 100	.994	.995	.990	.990	.980	.978	.990	.992	1.000
c 78	-	-	-	-	-	-	-	-	-
*ALAT**									
a 100	.548	.634	.631	.607	.582	.578	.610	.608	.550
b 68	.442	.342	.343	.357	.408	.389	.360	.367	.400
d 52	.010	.024	.026	.037	.010	.033	.030	.025	.050
*ESD**									
a 105	.004	.003	-	-	-	.011	-	-	-
b 100	.972	.968	.971	.973	.969	.933	.980	.983	1.000
c 89	.012	.011	.022	.020	.020	.033	.020	.017	-
d 75	.012	.018	.006	.007	.010	.022	-	-	-
*GPI**									
a 133	-	-	-	-	-	-	.010	-	-
b 125	.006	.003	.013	.010	-	.011	.010	.008	.050
c 100	.893	.921	.843	.873	.908	.967	.820	.933	.850
d 78	.097	.063	.135	.117	.092	.022	.160	.058	.050
e 60	.004	.013	.010	-	-	-	-	-	.050
*IDHP**									
a 100	.714	.771	.679	.693	.755	.889	.530	.775	.750
b 90	.286	.229	.321	.303	.245	.111	.470	.225	.250
c 80	-	-	-	.003	-	-	-	-	-
*MDH-1**									
a 115	.004	-	-	.003	-	-	-	-	-
b 100	.996	1.000	1.000	.997	1.000	1.000	1.000	1.000	1.000
*MDH-2**									
a 108	-	-	-	.007	-	-	.010	-	-
b 100	1.000	1.000	1.000	.993	1.000	1.000	.990	1.000	1.000
c 89	-	-	-	-	-	-	-	-	-
*MPI**									
a 115	-	-	-	-	-	-	-	-	-
b 110	.010	.003	.032	.013	.000	.011	-	.008	-
c 100	.984	.987	.958	.980	.980	.989	.990	.967	1.000
d 93	.006	.011	.006	.007	.010	-	.010	.017	-
e 86	-	-	.003	-	.010	-	-	.008	-
*PGM**									
a 113	.002	-	-	-	-	-	-	-	-
b 108	.006	.003	.010	.007	.020	-	.020	.017	-
c 105	.016	.008	.022	.020	.031	.022	-	.033	-
d 100	.952	.945	.933	.953	.878	.933	.960	.883	1.000
e 95	.002	.005	-	.007	.010	-	.010	-	-
f 90	.020	.037	.032	.013	.051	.044	-	.067	-
g 85	.002	.003	.003	-	.010	-	.010	-	-
(n)	248	190	156	150	49	45	50	60	20

Table 2.3 C Allele frequencies at ten polymorphic loci in six populations of *P. monodon*: 1=Trat, 2=Surat & Dungun 3= Andaman Sea, 4= Java Sea, 5=Philippines, 6=kENYA.

	Population					
Locus	1	2	3	4	5	6
*AAT-1**						
a 150	-	-	-	-	-	1.000
b 100	.996	.996	.989	1.000	.993	-
c 89	.004	.004	.011	-	.007	-
*AAT-2**						
a 115	.006	.009	.011	.006	.015	-
b 100	.994	.991	.989	.994	.971	1.000
c 78	-	-	-	-	.015	-
*ALAT**						
a 100	.548	.623	.614	.594	.441	-
b 68	.442	.351	.358	.375	.544	1.000
c 52	.010	.026	.028	.031	.015	-
*ESD**						
a 105	.004	.004	-	-	-	-
b 100	.972	.962	.973	.988	.941	1.000
c 89	.012	.015	.021	.013	.044	-
d 75	.012	.019	.006	-	.015	-
*GPI**						
a 133	-	-	.001	-	-	-
b 125	.006	.004	.010	.019	-	.011
c 100	.893	.930	.859	.913	1.000	.973
d 78	.097	.055	.126	.056	-	.016
e 60	.004	.011	.004	.013	-	-
*IDHP**						
a 100	.714	.794	.675	.769	.853	.863
b 90	.286	.206	.323	.231	.147	.137
c 80	-	-	.001	-	-	-
*MDH-1**						
a 115	.004	-	.001	-	-	-
b 100	.996	1.000	.999	1.000	1.000	1.000
*MDH-2**						
a 108	-	-	.004	-	.007	-
b 100	1.000	1.000	.996	1.000	.993	-
c 89	-	-	-	-	-	1.000
*MPI**						
a 115	-	-	-	-	-	.033
b 110	.010	.004	.017	.006	-	.445
c 100	.984	.987	.973	.975	.993	.346
d 93	.006	.009	.007	.013	.007	.176
e 86	-	-	.002	.006	-	-
*PGM**						
a 113	.002	-	-	-	-	-
b 108	.006	.002	.011	.013	.022	-
c 105	.016	.011	.020	.025	.000	-
d 100	.952	.943	.937	.913	.934	1.000
e 95	.002	.004	.005	-	-	-
f 90	.020	.038	.023	.050	.044	-
g 85	.002	.002	.004	-	-	-
(n)	248	235	405	80	68	91

Fig. 2.8 Dendrograms illustrating the variation in allele frequencies at *ALAT** in SE Asian and East African *P. monodon*.

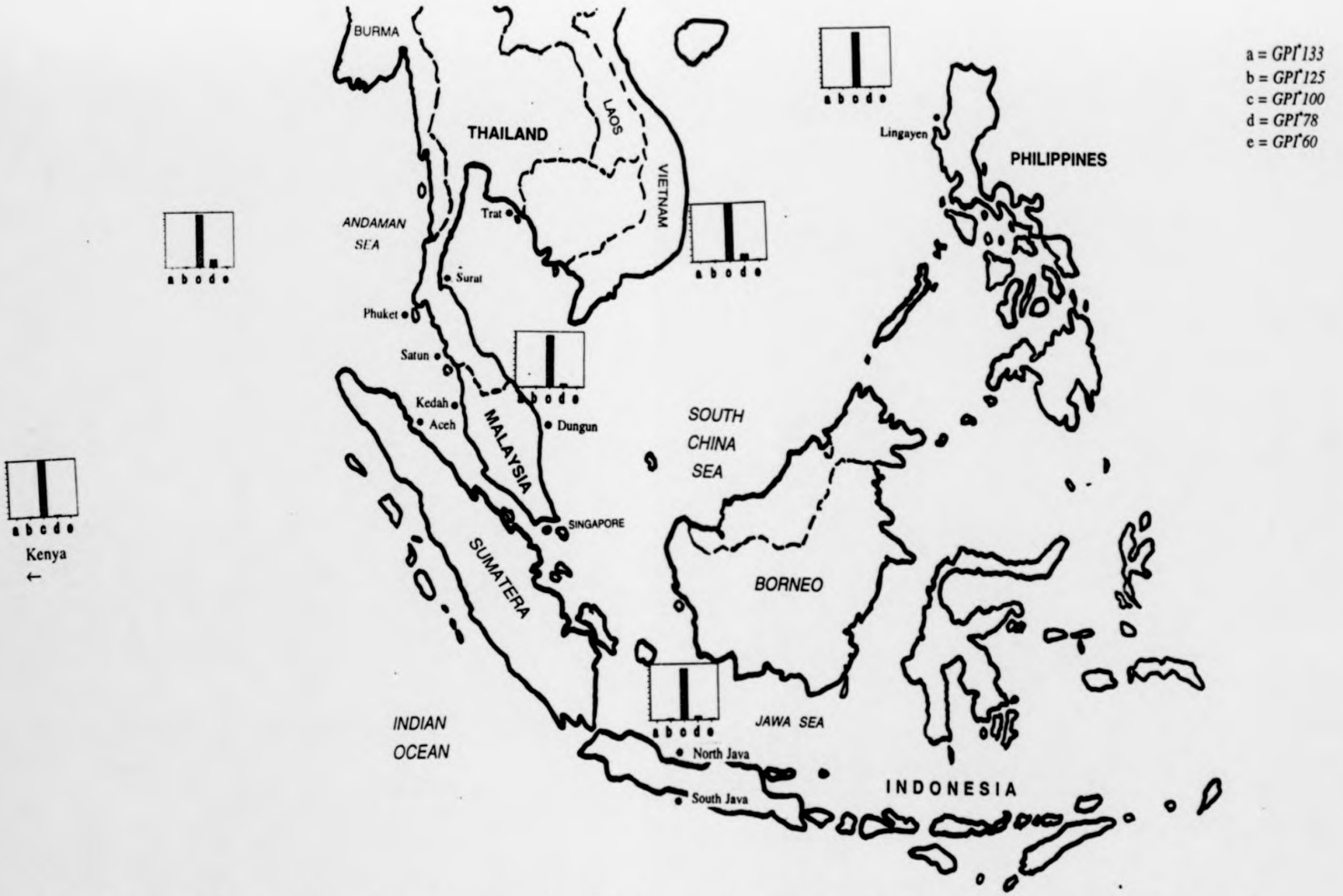


Fig. 2.9 Dendrograms illustrating the variation in allele frequencies at *GPI** in SE Asian and East African *P. monodon*.

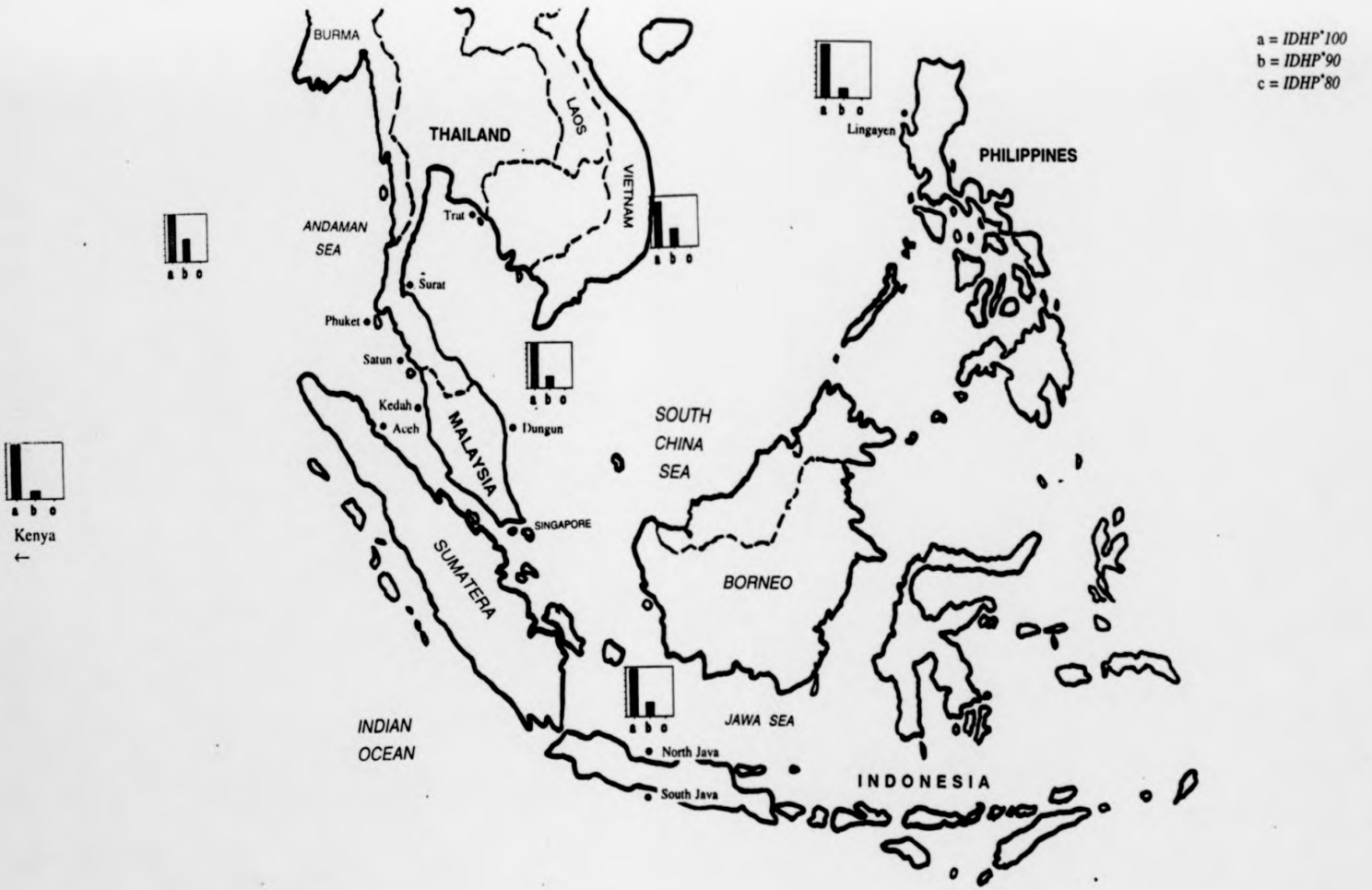


Fig. 2.10 Dendrograms illustrating the variation in allele frequencies at *IDHP** in SE Asian and East African *P. monodon*.

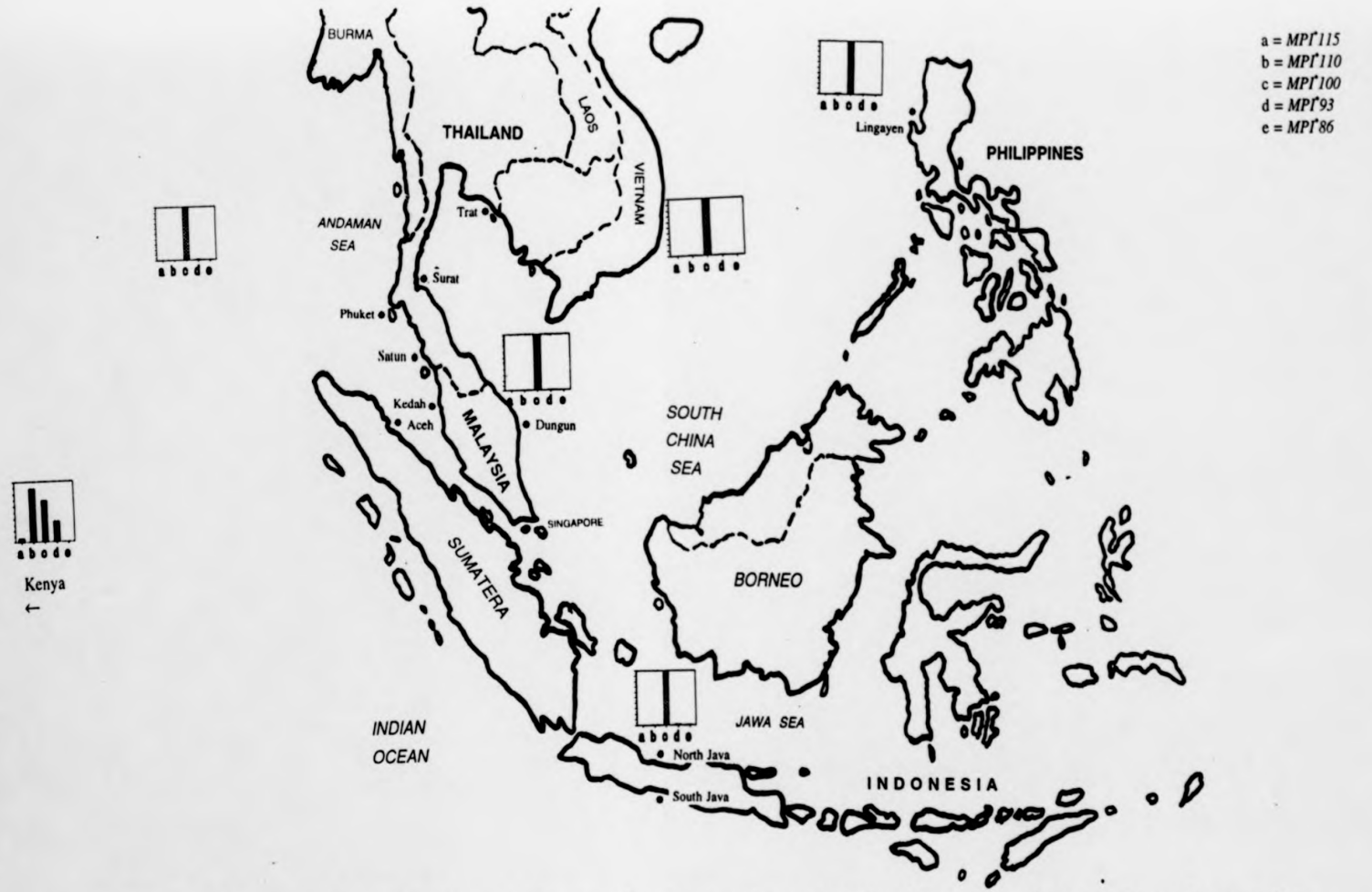


Fig. 2.11 Dendrograms illustrating the variation in allele frequencies at *MPI** in SE Asian and East African *P. monodon*.

The results showed that in general the genotype frequencies in all populations, both individually and in realistic combinations were in Hardy-Weinberg equilibrium. This suggests that these populations or groupings fulfil the basic assumption which underlay the Hardy-Weinberg Law: large randomly mating population with little or no migration etc.

2.3.4 Genetic Variability

The number of rare alleles, mean number of alleles per locus, percentage of loci polymorphic and mean heterozygosity over all 46 loci are listed in Table 2.4 for all populations. The data showed very little difference between the eleven populations surveyed apart from the North Java and Kenyan populations, where mean number of alleles per locus and the percentage of loci polymorphic are rather low. The number of rare alleles was also high within all of the Thai populations. The proportion of loci polymorphic was generally low with an average of 15.8% and 7.5% at the frequency of common allele <0.99 and <0.95 respectively, but at its lowest in the North Java (6.5% and 6.5%) and Kenyan populations (6.5% and 4.3%). Average heterozygosity over all loci for the eleven populations is 0.027 (0.020-0.032), however the Philippines and Kenyan populations have the lowest values of heterozygosity of 0.020 and 0.021 respectively.

Table 2.4 Genetic variability at all 46 loci observed in eleven populations of *P. monodon*
1=Trat, 2=Surat, 3=Phuket, 4=Satun, 5=Kedah, 6= Dungun, 7=Aceh, 8=South Java, 9=North Java, 10=Philippines, 11=Kenya

	Population										
	1	2	3	4	5	6	7	8	9	10	11
Number of rare alleles (less 1%)	11	9	5	7	0	0	0	2	0	0	0
Mean number of alleles per locus	1.4 (.2)	1.4 (.2)	1.4 (.1)	1.4 (.1)	1.3 (.1)	1.3 (.1)	1.3 (.1)	1.3 (.1)	1.1 (.1)	1.3 (.1)	1.1 (.1)
Polymorphic loci (%)											
.99	19.6	17.4	17.4	21.7	17.4	15.2	19.6	15.2	6.5	17.4	6.5
.95	6.5	8.7	8.7	6.5	8.7	8.7	6.5	8.7	6.5	8.7	4.3
Mean heterozygosity											
Direct count	.030	.025	.032	.031	.031	.024	.032	.026	.025	.020	.021
Expected	.029	.026	.033	.030	.031	.024	.033	.028	.026	.024	.021
Sample size	248	190	156	150	49	45	50	60	20	68	91

2.3.5 Genetic Differentiation

2.3.5.1 Between-year comparisons

No significant differences in allele or genotype frequency were observed between year classes in any population at any locus (Appendix 2). The probability levels obtained for allele and genotype frequencies in the Gulf population were for Trat (P=0.105) and Surat Thani (P=0.139) and in the Andaman Sea were for Phuket (P=0.929) and Satun (P=0.436). The lower probability levels at Trat and Surat possibly suggesting less stability in the Gulf populations.

The stability of allele frequencies over time is one of the essential assumptions of the Hardy-Weinberg principle, and is expected in natural populations as long as all the underlying assumptions of the analysis are meet. The mechanism of Mendelian inheritance therefore will keep the allele frequencies constant and preserve genetic variation (Hartl, 1988). In the few other studies in which temporal stability was tested in penaeid species other workers also found no evidence of fluctuating allele or genotype frequencies, Salini (1987) studied *M. bennettae* in 1982 and 1983 at Moreton Bay and Lake Macquarie, east coast Australia, and Mulley and Latter (1981b) studied *P. latisulcatus* in 1975 and 1976 in the Gulf of Carpentaria, Exmouth Gulf, and Gulf of St Vincent, Tropical Australia.

2.3.5.2 Population comparisons

A. Within-population differentiation

Considering *F*-statistics (Table 2.5), even though a significant value of F_{IS} (F_{IS}=0.061; χ^2_3=8.39, P<0.05) was found at *ALAT** in the 6 populations analysis, the mean F_{IS} values were not significant (F_{IS}=0.028). Allele frequencies of polymorphic loci of all populations studied also showed no significant differences from Hardy-Weinberg expectations as described earlier, indicating little within-population structuring [The data can be carried out at population level].

B. Between-population differentiation

Since genotype frequencies were stable over time in all four Thai populations, data from multiple collections at the same site were therefore combined for the macrogeographical analyses. Contingency chi-square and Nass chi-square tests between pairs of adjacent populations were analyzed for the existence of discrete stocks. When two adjacent localities did not exhibit significant differences, suggesting panmixia, the collections were then combined and another round of chi-square testing between adjacent populations carried out. This process was continued until all geographically sensible tests were performed. As was mentioned earlier combined data sets were all retested for goodness-of-fit to Hardy-Weinberg equilibrium but no significant differences were observed.

The testing for adjacent pairwise comparisons showed that the Andaman Sea sample at Phuket, Satun and Kedah appeared homogeneous at all loci and data were therefore pooled. Aceh was shown to be significantly different at

Table 2.5 Summary of F_{st} and Wright's F_{st} at all loci.

A. 4 populations (Trat, Surat & Dungun, Andaman Sea, Pooled Java)

Locus	F(IS)	F(IT)	F(ST)	F-Wright
AAT-1	-.008$^{n.s.}$	-.005	.003$^{n.s.}$	.002
AAT-2	-.009$^{n.s.}$	-.008	.001$^{n.s.}$	.000
ALAT	.006$^{n.s.}$	.010	.004***	.001
ESD	-.023$^{n.s.}$	-.020	.003*	.001
GPI	-.061$^{n.s.}$	-.052	.009***	.006
IDHP	.036$^{n.s.}$	.046	.011***	.008
MDH-1	-.003$^{n.s.}$	-.001	.002$^{n.s.}$	.000
MDH-2	-.004$^{n.s.}$	-.001	.003$^{n.s.}$	.002
MPI	-.016$^{n.s.}$	-.015	.002$^{n.s.}$	.000
PGM	.041$^{n.s.}$	.044	.003**	.000
Mean	.006$^{n.s.}$	.013	.007***	.004

B. 5 SE Asia populations (Trat, Surat & Dungun, Andaman Sea, Pooled Java, Philippines)

Locus	F(IS)	F(IT)	F(ST)	F-Wright
AAT-1	-.008$^{n.s.}$	-.005	.003$^{n.s.}$	.000
AAT-2	-.015$^{n.s.}$	-.010	.005***	.001
ALAT	.061$^{n.s.}$	.079	.019***	.015
ESD	-.032$^{n.s.}$	-.025	.007***	.003
GPI	-.061$^{n.s.}$	-.032	.027***	.024
IDHP	.056$^{n.s.}$	.076	.021***	.018
MDH-1	-.003$^{n.s.}$	-.001	.002$^{n.s.}$	.000
MDH-2	-.006$^{n.s.}$	-.002	.004$^{n.s.}$	.000
MPI	-.016$^{n.s.}$	-.013	.003$^{n.s.}$	.000
PGM	.021$^{n.s.}$	.025	.003*	.000
Mean	.033$^{n.s.}$	.050	.018***	.014

C. 6 all pooled populations (Trat, Surat & Dungun, Andaman Sea, Pooled Java, Philippines, Kenya)

Locus	F(IS)	F(IT)	F(ST)	F-Wright
AAT-1	-.008$^{n.s.}$	.969	.969***	.969
AAT-2	-.015$^{n.s.}$	-.008	.007***	.002
ALAT	.061*	.243	.194***	.191
ESD	-.032$^{n.s.}$	-.021	.011***	.007
GPI	-.058$^{n.s.}$	-.026	.031***	.028
IDHP	.052$^{n.s.}$	.078	.027***	.023
MDH-1	-.003$^{n.s.}$	-.001	.003$^{n.s.}$	.001
MDH-2	-.006$^{n.s.}$	.987	.987***	.987
MPI	-.015$^{n.s.}$	.379	.388***	.384
PGM	.021$^{n.s.}$	.032	.011***	.007
Mean	.028$^{n.s.}$	.401	.384***	.382

NOTE: n.s. = non significant difference
* = $P < 0.05$
** = $P < 0.01$
*** = $P < 0.001$

*IDHP** ($\chi^2_{1,97}$=7.18, *P*=0.030) from the pooled sample but this became non-significant after correction (the sequential Bonferroni test), as was the test for combined loci data (χ^2_{22}=33.0, *P*=0.062). The Aceh population was therefore added to the Andaman Sea pool. Similarly in the Indonesian populations, North and South Java populations, showed significant difference at *GPI** ($\chi^2_{3,47}$=10.63, *P*=0.021), but were not significantly different after correction and for total polymorphic loci (χ^2_{14}=16.70, *P*=0.273). These result implied genetic homogeneity both within the Andaman sea and within the North and South Java samples.

The Gulf of Thailand and the East Malaysian peninsula samples were compared and no significant differences were observed between Surat and Dungun for the total polymorphic loci (χ^2_{18}=23.50, *P*=0.170) but *IDHP** was significant ($\chi^2_{2,02}$=6.66, *P*=0.040) for the individual locus comparisons. However this disappeared after correction and so they were combined. Trat however remained significantly different from the east coast samples for the ten loci comparison and at *ALAT** ($\chi^2_{4,46}$=21.45, *P*<0.001) suggesting reproductive isolation across the Gulf of Thailand.

The combining of the populations eventually resulted in 6 population groupings, namely: the Andaman sea, Trat, Surat and Dungun, North and South Java, Philippines and Kenya. The simultaneous contingency chi-square test of allele frequencies for the six localities over all polymorphic loci exhibited highly significant differences (χ^2_{140}=6079.49, *P*<0.0000) as shown in Appendix 2 [with seven loci showing significant differences: *AAT-1** (*P*=0.0000), *AAT-2**

(P=0.0002), *ALAT** (P=0.0000), *GPI** (P=0.0000), *IDHP** (P=0.0000), *MDH-2** (P=0.0000) and *MPI** (P=0.0000)]. Mean F_{ST} (F_{ST}=0.384; χ^2_{365}=63019.59, P<0.0001) and Wright's F_{ST} (F_{ST}=0.382; χ^2_{365}=62855.04, P<0.0001) also revealed highly significant interpopulation differentiation as shown in Table 2.5. F_{ST} values above 0.25 (Wright, 1978) indicate very great genetic differentiation confirming clear reproductive isolation within the sampled geographic range.

When calculating only within SE Asian countries excluding Kenya for five pooled populations, the test also showed significant differences (χ^2_{100}=211.47, P<0.0000), but only four truly polymorphic loci exhibited significant differences: *AAT-2** (P=0.0002), *ALAT** (P=0.0002), *GPI** (P=0.0000), *IDHP** (P=0.0000). Mean F_{ST} (F_{ST}=0.018; χ^2_{276}=2573.42, P<0.0001) and Wright's F_{ST} (F_{ST}=0.014; χ^2_{276}=2001.55, P<0.0001) also revealed significant differences, indicating reproductive isolation within SE Asia. When the Philippine population was excluded, the result also exhibited significant difference (χ^2_{72}=120.36, P<0.001), but only at two loci: *GPI** (P=0.0000), *IDHP** (P=0.0000). Mean F_{ST} (F_{ST}=0.007; χ^2_{207}=935.09, P<0.0001) and Wright's F_{ST} (F_{ST}=0.004; χ^2_{207}=534.34, P<0.0001) also demonstrated significant differences but a low level of genetic differentiation within the more western sites countries (Thailand, Malaysia and Indonesia).

Pairwise comparisons at each locus between each pooled population (Table 2.6 and Appendix 2) gave essentially the same results as the simultaneous test. Kenya was significantly different from other populations because of fixed variant alleles at *AAT-1*150, ALAT*68* and *MDH-2*89*, higher allele frequencies at *MPI*110* and *IDHP*100* and because it was fixed for the *PGM*100* allele. The

Table 2.6 Pairwise comparisons in genotype frequencies of six *P. monodon* populations

Single locus significant differences are below the diagonal, and *p*-values for multiple locus comparison are above diagonal

	Trat	Surat & Dungun	Andaman Sea	Java	Philippines	Kenya
Trat	-	0.015	0.045	0.166	0.000	0.000
Surat & Dungun	*ALAT** (0.000)	-	0.000	0.52	0.000	0.000
Andaman Sea	*ALAT** (0.004)	*GPI** (0.000) *IDHP** (0.000)	-	0.228	0.000	0.000
Java	n.s.	n.s.	n.s.	-	0.002	0.000
Philippines	*GPI** (0.002) *IDHP** (0.002)	*ALAT** (0.000) *GPI** (0.007)	*AAT-2** (0.005) *ALAT** (0.000) *GPI** (0.003) *IDHP** (0.002)	*GPI** (0.002)	-	0.000
Kenya	*AAT-1**(0.000) *ALAT** (0.000) *GPI** (0.004) *IDHP** (0.000) *MDH-2**(0.000) *MPI** (0.000)	*AAT-1**(0.000) *ALAT** (0.000) *MDH-2**(0.000) *MPI** (0.000)	*AAT-1** (0.000) *ALAT** (0.000) *GPI** (0.002) *IDHP** (0.000) *MDH-2** (0.000) *MPI** (0.000)	*AAT-1**(0.000) *ALAT** (0.000) *MDH-2**(0.000) *MPI** (0.000) *PGM** (0.000)	*AAT-1**(0.000) *ALAT** (0.000) *ESD** (0.004) *MDH-2**(0.000) *MPI** (0.000) *PGM** (0.002)	

Philippines was significantly different from other populations by having the variant allele *AAT-2*78*, and higher frequencies of the *ALAT*68* and *IDHP*100* allele. The Philippines population was significantly different from other populations at a number of polymorphic loci in pairwise comparisons: Trat at *GPI** ($\chi^2_{4.31}$=17.58, *P*=0.0020) and *IDHP** ($\chi^2_{2.03}$=13.70, *P*=0.0015), the pooled Surat and Dungun at *ALAT** ($\chi^2_{4.21}$=22.68, *P*=0.0000) and *GPI** ($\chi^2_{2.30}$=11.08, *P*=0.0070), the pooled North and South Java at *GPI** ($\chi^2_{4.30}$=18.70, *P*=0.0015), and the pooled Andaman Sea at *AAT-2** ($\chi^2_{1.54}$=9.33, *P*=0.0050), *ALAT** ($\chi^2_{3.84}$=23.59, *P*=0.0000), *GPI** ($\chi^2_{4.25}$=17.01, *P*=0.0030) and *IDHP** ($\chi^2_{2.23}$=13.99, *P*=0.0015). These significant differences of individual loci suggest that the Philippine population is much more isolated from the Andaman Sea populations than those from Trat, Surat Thani, East Malaysia and Indonesia.

Within the more western sites populations, the comparisons were heterogeneous because of differences in gene frequencies. Trat was significantly different from the pooled Andaman sea population at all polymorphic loci (χ^2_{24}=36.90, *P*=0.044) and at *ALAT** ($\chi^2_{4.26}$=15.98, *P*=0.0040), and the pooled Surat and Dungun at all polymorphic loci (χ^2_{20}=36.13, *P*=0.0148) and at *ALAT** ($\chi^2_{4.46}$=21.21, *P*=0.0005). The pooled Andaman sea population was also significantly different from pooled Surat and Dungun at all polymorphic loci (χ^2_{23}=66.90, *P*<0.0001), and at *GPI** ($\chi^2_{8.52}$=31.83, *P*<0.0001) and *IDHP** ($\chi^2_{4.22}$=27.95, *P*<0.0000). However, the pooled North and South Java was not significantly different from any of these populations although at present there is no obvious reason to combine them with one or other of these populations because of the degree of geographic isolation.

2.3.6 Genetic Distance & UPGMA Clustering Dendrogram

Nei's (Nei 1972, 1978) and Rogers' (Rogers, 1972) genetic distances between the six pooled populations are shown in Table 2.7. All estimated values were high between Kenya and all other populations, rather low between the Philippines and the other SE Asia countries and very low between the remaining populations. This relationship was visualized using UPGMA cluster analysis. Both genetic distance dendrograms (Fig 2.12 A. and B.) showed that the Kenyan and Philippine populations, which are geographically remote, are clearly distinct from the SE Asia population. The various genetic distance measure resulted in very similar dendrograms. The only unexpected finding was the clustering of Trat with the Andaman Sea samples rather than the East Malaysia and Java samples.

2.4 DISCUSSION

The present allozyme study on *P. monodon* demonstrates geographic sub-structuring of the breeding populations over the large zoogeographical range of this species, both between the East African coast (Kenya) and SE Asia, and within the SE Asia region. This contrasts with previous earlier observations that *P. monodon* is genetically homogeneous over large parts of its range (Ko *et al.*, 1983) and between close geographic populations in Australia (Mulley and Latter, 1980).

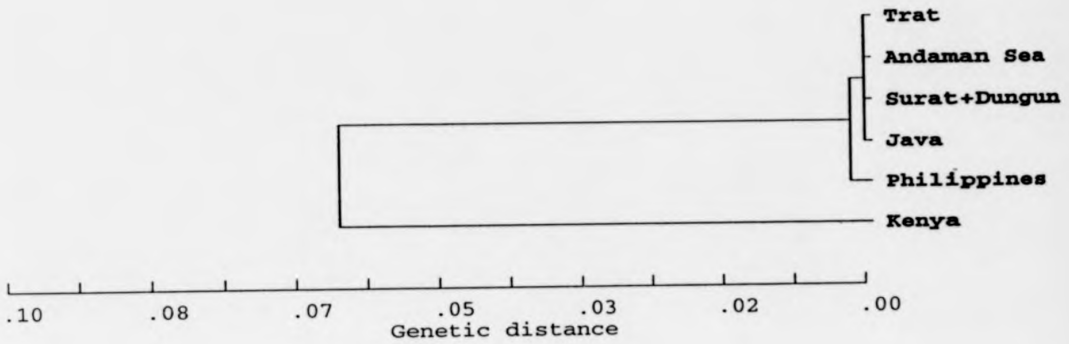
Table 2.7 Average genetics distances between six *P. monodon* populations

Nei's (1978) unbiased genetic distance is below the diagonal
Rogers' (1972) genetic distance is above the diagonal

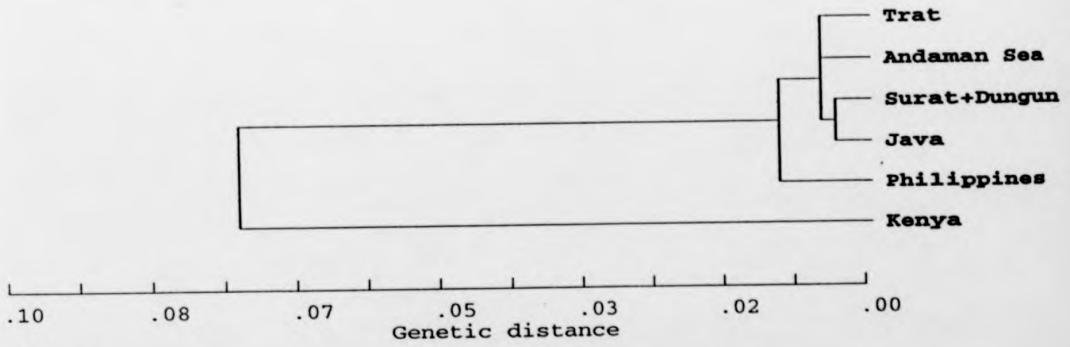
Population	1	2	3	4	5	6
1 Trat	****	.004	.005	.005	.010	.074
2 Andaman Sea	.000	****	.006	.005	.013	.077
3 Surat & Dungun	.000	.000	****	.003	.008	.074
4 Java	.000	.000	.000	****	.009	.074
5 Philippines	.001	.002	.001	.001	****	.069
6 Kenya	.061	.063	.063	.062	.057	****

Fig. 2.12 Dendrogram showing the genetic relationship among six populations of *P. monodon*. The software package BIOSYS-1 (Swafford and Selander, 1989) was used to produce the dendrogram.

A. Coefficient used: Nei (1978) unbiased genetic distance



B. Coefficient used: Rogers (1972) genetic distance



The findings of this study are more similar to those of Benzie *et al.* (1992) on Australian *P. monodon* populations, which showed significant heterogeneity between the populations which were separated by 100's rather than 1000's of kilometres. They also believed that much of the heterogeneity they saw today is a relic of an earlier separation of the populations during the last glacial epoch when sea levels were much lower than today (Fig. 2.13). The differences remaining after a rise in sea level even after several thousand years of potential gene flow.

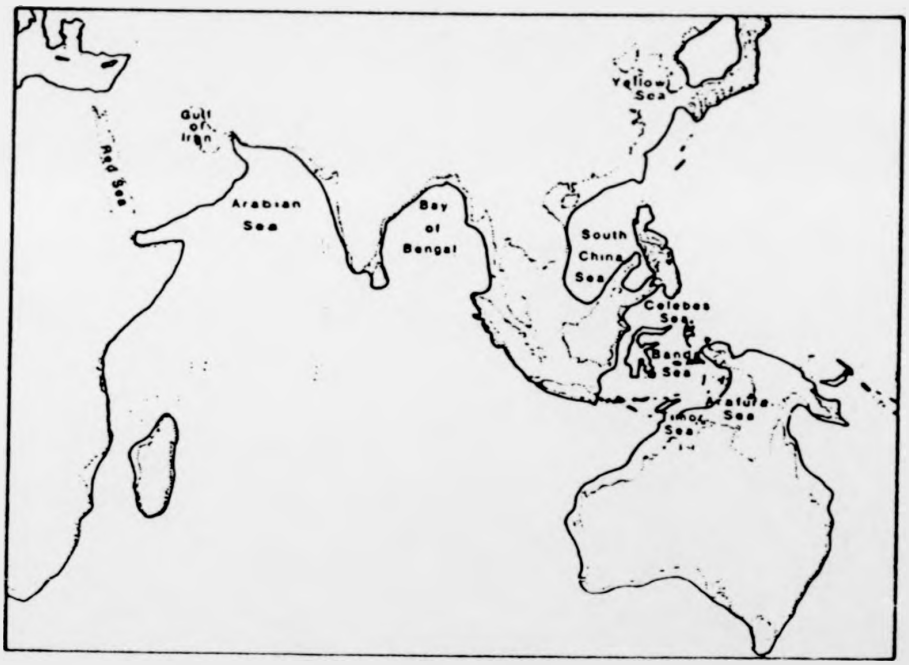


Fig. 2.13 Maximum extent of shorelines in the Indo-West Pacific during the Quaternary Ice Ace, with sea levels 135 m below present levels (after Dall *et al.*, 1990)

The studies of penaeids have reported either no observable differences (Proctor *et al.*, 1974; Marvin and Caillouet, 1976; Lester, 1979) or significant geographical variation among isolated populations of a number of species (Mulley and Latter, 1981a and 1981b; Richardson, 1982; Mattoccia *et al.*, 1987; Salini, 1987). The level of biochemical genetic differentiation of penaeids appears to be correlated with life history traits particularly larval dispersal. Species with limited gene flow, such as *M. bennettae* which is restricted entirely within estuaries and tidal lakes exhibited significant differences in gene frequencies between east-coast of Australia populations (Mulley and Latter, 1981a; Salini, 1987). Other species, such as *M. macleayi* in which the adults generally spawn in the sea but the postlarvae return to estuaries or estuarine-like environments have similar patterns of genetic differentiation (Mulley and Latter, 1981a). These offshore spawning species however have some degree of mixing as larvae do not necessarily return to the nursery grounds of their parents. In contrast, *P. plebejus* undertakes extensive breeding migration into deepsea water, and whose larvae develop in inshore waters with relatively high salinity showed no genetic differentiation in Australian populations (Mulley and Latter, 1981a). These three penaeid groups fall into the life cycle classifications Type 1, 2 and 3, respectively of Dall *et al.* (1990). However, Dall *et al.* (1990) stressed that there is overlap between them. The species *P. latisulcatus* and *M. endeavori* which are classified Type 3 were found to show significant differences among widely separated populations (Mulley and Latter, 1981b). Richardson (1982) also identified genetic differences in *P. latisulcatus* within the Gulf of St Vincent, confirming that subpopulation of this species exist. *P. latisulcatus* and *P. endeavori* although spawning offshore over wide sea areas can have a discontinuous dispersal

because their postlarvae only inhabit inshore substrates such as sandy muds, sands and coralline rubble (Grey *et al.*, 1983). In contrast, the relation between genetic differentiation of populations and life history type may be ambiguous (Mulley and Latter, 1981a). Species with a Type 2 life history which might be expected to show some differentiation such as *P. merguiensis* and *M. ensis* but did not (Mulley and Latter, 1980), this may be because of the small samples sizes, limited geographic range and relatively small number of polymorphic loci used by these workers. Among six Mediterranean populations of *P. kerathurus* which has a Type 2 life history (Mattoccia *et al.*, 1987) reproductively isolated population could be delineated within hundreds of kilometres.

The level of population sub-structuring is dependent on life cycle characteristics and is more prominent in species with limited larval dispersal. However it is clear that even in species with long-lived planktonic larval stage, genetic differentiation can occur. The geographical range of the population being dependent on hydrological characteristics of the area or behavioral or substrate preferences shown by the animal. How can the substructuring observed in this study of *P. monodon* be explained. *P. monodon* is a Type 2 species in which the mature adults migrate offshore to spawn, the larvae are planktonic for 20 days, the post larvae will then migrate towards the shore and will enter shallow water or mangroves (Motoh, 1986). The main source of dispersal in this species is the larval stage drift with the prevailing current and possible movements of the juveniles and adults with the current. Therefore different direction of prevailing current associated with greater dispersal against current possibly reducing dispersal result in population sub-structuring.

In general, the character of the genetic variation observed in *P. monodon* populations during this study corresponds to that recorded for several other penaeid species. Nearly all populations have the same common allele and a number of less common or rare variants. Tests of genotype frequencies, both temporal and spatial predictions, agreed with Hardy-Weinberg equilibrium indicating that mating among conspecific adult *P. monodon* in these natural populations is random and that progeny groups are sufficiently well mixed. The mean heterozygosity ($\overline{H}$) in *P. monodon* over all 46 loci in this study is 0.027 (0.020-0.032). This result is similar to that reported by Hedgecock (1986) of 0.038 (0.022-0.058) over 29 loci for 14 *Penaeus spp.* but higher than that reported for 7 Indo-Pacific species of *Penaeus* with an average of 0.018 (0.006-0.033) at 40 loci surveyed by Mulley & Latter (1980). Comparing these results to others on *P. monodon*, $\overline{H}$ is lower than Australian *P. monodon* population, Benzie *et al.* (1992) reported mean heterozygosity values of $\overline{H} = 0.05$, based on 8 polymorphic loci, but higher than those found on a wide range of other populations, Ko *et al.* (1983) reported values ranging from 0-0.023 with an average of 0.013 over 29 loci and Mulley & Latter (1980) reported $\overline{H} = 0.008$ over 40 loci. Hedgecock (1986) mentioned that any heterozygosity value is heavily dependent upon the loci studied and sample size. Benzie *et al.* (1992) have calculated heterozygosity from only 8 polymorphic loci, Ko *et al.* (1983) and Mulley and Latter (1980) used sample size of 5-32 and 12 individuals respectively. This study used large sample sizes, large number of loci, geographically isolated populations and hopefully gives a good overall estimate of overall genetic variation in this species. The levels observed are conservative as several loci which are potentially variable have been omitted until better

resolution can be obtained. Nevertheless, penaeid prawns protein heterozygosity are among the lowest recorded among any animals (Mulley and Latter, 1980; Redfield *et al.*, 1980; Ko *et al.*, 1984; Salini and Moore, 1985; Tam and Chu, 1993).

Within the 10 populations studied, the Kenya and the Philippines populations have lower levels of genetic variability, both populations have heterozygosities of about $\overline{H} = 0.02$. These populations are on the edge of the species range and are possibly suffering from reduced gene flow and founder effect. Ferguson (1980) commented that the *founder effect* is a special type of genetic drift where a small number of founder individuals of a new population represent only a limited part of the parental population. Such populations can sometimes only receive a non representative proportion of the species variation and can often be fixed for alleles that are rare in other parts of the species range. The lower genetic variability in such populations will take a long time to recover towards the species average, at least one million years (Nei, 1976). A significant reduction in heterozygosity due to bottleneck effects is well documented in hatchery stocks, Sbordoni *et al.* (1986) found 60% reduction of average heterozygosity in artificial populations of *P. japonicus* with small numbers of breeding animals.

Mulley and Latter (1980) suggested selective elimination of mutational variation at the majority of loci as the most possible explanation for the low levels of heterozygosity they observed in penaeids. Dall *et. al.*, (1990) stated that Mulley and Latter (1980) hypothesis it is extremely unlike to occur in a species

with high fecundity and a geographic distribution throughout the Indo-West Pacific Region. It being more likely that non-lethal mutations would rapidly accumulate throughout the species range (Dall *et al.*, 1990). The most likely reason for the low average heterozygosity is the limited gene flow within these populations that restricts the interchange of individuals between populations. In this study both peripheral populations not only showed reduce $\overline{H}$, the Philippines population showed an alternate rare allele (*AAT-2*78*) and Kenyan population showed alternate fixed alleles (*AAT-1*150*, *MDH-2*89*). Glaciation had a major effect in subdividing Penaeid populations and subsequent rises in sea level around 15000 years ago resulted in the repopulation of some areas and possible 'founder effects' in many of these new populations. Although mixing and recombination occurred in some areas, some isolated stocks still remain at the periphery of the species range. As these events occurred relatively recently, levels of $\overline{H}$ have not been able to recover. Present day hydrographic features have enabled some populations to rise, as in the South China Sea, but there is a limited gene flow from the Andaman Sea to the South China Sea and to the Philippines. The centre of distribution is in fact the centre of mixing as *P. monodon* moved in from the Java Sea to both the Andaman Sea and the South China Sea. Phenomena like higher levels of heterozygocity and possession of rarer alleles are more apparent at the centre of mixing than in peripheral areas.

The Kenyan, east African population has a lower mean number of alleles per locus and mean heterozygosity than the other populations studied. It has different common alleles at *ALAT*68* and *MPI*110*, and loci also fixed for alternate alleles at *AAT-1*150*, and *MDH-2*89* indicating this peripheral

population was well isolated geographically and reproductively from the other populations studied as revealed by all statistical analysis and shown by the distance UPGMA dendrogram. There is no well-defined geographic barrier between the East African coast and Indo-Malaysian sub-region. Today the Kenyan and SE Asia groups of prawns are separated by more than 7,000 kilometres therefore geographic distance could be the main barrier to gene exchange today. Dall *et al.*, (1990) stated that in the Bay of Bengal there is a prevailing clockwise circulation and also in the Arabian Sea both with a eastward current. These unfavourable current could limit the westward gene flow movement of some species. In addition, the hypersaline inshore waters of the Arabian Sea could also act as a barrier to isolate populations. However, it was not possible to collect samples from intervening populations but it would be interesting to see whether there was a cline of variation or a rapid drop associate with the various hydrological features.

The divergence of protein molecules is time-dependent and related to evolutionary time known as molecular clock hypothesis (Fitch, 1976; Wilson *et al.*, 1977; Thorpe, 1982). However, this is only probably true if large number of enzymes are used so possible differences in evolutionary rates between enzymes are evened out. The evolutionary clock predicts that amino acid substitution is approximately constant and therefore the accumulated number of substitutions is approximately proportional to time elapsed (Thorpe, 1982). Such clocks may be calibrated in real time of divergence by comparison to the fossil record. From fossil record, *Penaese* is the oldest extant genus of the penaeidae which has been found in Jurassic shales (Dall *et al.*, 1990) and became more common in the

Cretaceous Period (Glaessner, 1969). However, based on I values between *Penaeus* and *Metapenaeus*, Dall *et al.*(1990) and Tam and Chu (1993) estimated the time of divergence, t = 3.9-4.7 x 10^6 years, which is consistent with evidence from palaeogeography that the two genera diverged in the late Pliocene Epoch (Tertiary Period). Within the penaeid, high I values between closely related species, such as *P. merguiensis, P. penicillatus, P. monodon* and *P. semisulcatus* (Mulley and Latter, 1980; Salini and Moore, 1987; Dall *et al.*, 1990; Tam and Chu, 1993) suggested an estimated time of divergence, t = less than 2.0 x 10^6 years, suggesting that many *Penaeus* species could have arisen during the last glacial epoch. Using Nei's (1975) estimated rate of gene substitution per year, $\alpha = 10^{-7}$, the average genetic distance (D) between Kenyan and Asia populations = 0.06 for this study. We therefore calculated from the assumption that $t = D/2\alpha$ (Nei, 1975; Dall *et al.*, 1990), the separation between these populations would have occured about 0.3 million years late in the last glacial epoch. Philippines exhibited genetic distance with the Andaman Sea and the other South China Sea populations D = 0.02 and 0.01 respectively. Therefore the Philippines may divert from the Andaman Sea and the other South China Sea at around 0.1 and 0.05 million years ago also in the last glacial epoch.

Dall *et al.* (1990) stressed the most important effect on gene flow in the past was the creation of land barriers as sea level dropped dramatically altered penaeid distribution. There was a separation of the Indian Ocean and the Indo-pacific areas in the Quaternary Glacial Period by the Malaysian land mass and deep water south of Sumatra (see Fig. 2.13) The land barrier between Malaysia, Sumatra, Borneo and Java therefore effectively cut off the penaeid populations

to the east from those in the Indian Ocean. As a matter of fact we have to postulate that there was either low species diversity pre-glacially in the western region, or a major reduction in diversity during the glacial period. This can be seen by the species diversity in the two areas, there are 25 endemic species in the Indo-Malaysian sub-region, 3 in the Arabian Sea and only 1 in East African. Moreover, there is a constant decrease in species abundance from Malaysia through the Bay of Bengal to South Africa. As the land barrier disappeared with rising sea levels, migration of species to the west began, giving the species gradient seen today (Dall *et al.*, 1990). *P. monodon* it is essentially a solitary species which burrows during the day and only feeds at night so fishing pressure is lower compared to shoalling or aggregating species such as *P. merguiensis* reduced. It can survive a wide range of temperature and salinity levels (Apud *et al.*, 1983). Coupled with larval dispersal behavior therefore *P. monodon* is widely distibuted throughout the Indo-West Pacific region.

Within SE Asia groups, there was a separation of the Philippines population from the others as shown by genetic distance and UPGMA Clustering Dendrogram. The *AAT-2*78* allele is present at 0.15 but is not observed in any other populations. The Philippines populations also has a different common alleles at *ALAT*68* and a lower level of heterozygosity than the other populations. The 2,000-3,000 kilometres distance between the Philippines and the other ASEAN countries, suggests that the isolation-by-distance model is one explanation for the differentiation between these two groups. Richardson *et al.* (1986) stated that the isolation-by-distance model will have an independent effect at each locus, as there is no underlying subpopulation structure limited gene flow

at particular geographical locations. So different loci showed significant differences in pairwise comparisons between the Philippines and the others populations (see Table 2.6). Once again island barriers have limited gene flow and the distribution of marine penaeid between Malaysia, Sumatra, Borneo, Java and the South China Sea. Dall *et al.* (1990) showed that there was a separation of these two areas in the Quaternary Glacial Period by the land mass of Malaysia and the Philippines (see Fig. 2.13). Benzie *et al.* (1992) also detected highly significant differences in gene frequencies of *P. monodon* between adjacent population. He commented that at maximal lowering of sea level 14,000-17,000 years ago, Australia and New Guinea were connected by dry land from Cape York to Melville Island. Therefore populations on the western coast were probably isolated from those on the eastern coast for several thousand years and evidence of this restricted gene flow between these populations is still present today. It is possible that Philippine populations may have more in common with Australian populations than with other ASEAN populations because of these sea level changes and the prevailing oceanic currents of that period.

The pairwise comparisons resulted in apparently homogeneous populations being combined into 3 larger but genetically distinct groups (the Andaman Sea, Surat & Dungun, and Java populations), despite some of the populations which were grouped together being separated by many 100's of Km. These three areas are subject to strong wind driven and strong surface current throughout the year particularly during the monsoons which cover the main spawning periods. This combined with the spawning migrations by the adults to water dept of 160 m in the Philippines (Motoh, 1986), 26-50 m in the Gulf of Thailand (Promsakha and

Lohsawadkun, 1988), and 35-40 m in Andaman Sea (Kungvankij *et al.*, 1973a), many miles offshore would encourage substantial larval dispersal in these areas.

It is possible that the Indonesian, Malaysian and Thai populations of *P. monodon* probably originated from the Java populations refugia after the last glacial epoch. It can be seen that there was no significant difference, both at individual loci and over all polymorphic loci, between Java populations compared to Trat, Pooled Surat & Dungun and Andaman Sea populations (see Table 2.6). Considering the map of the Quaternary Ice Age and assuming earlier that there was either a low species diversity in the western region or a reduction in diversity during the Ice Age, as sea levels rose, *P. monodon* migrated to the Indian Ocean and South China Sea. In this case the statistical differences arose from founder effects and subsequent reduction in gene flow between these areas. The reproductive isolation of populations in the Andaman Sea and South China Sea was confirmed by significant differences of pairwise comparisons between Andaman Sea populations against Trat and pooled Surat Thani and Dungun. An alternative explanation, is that we must hypothesize that *P. monodon* was present in all areas, or nearby all areas, where the sea remained each having their own allele frequencies depending on population structure present. As sea levels rose so populations recombined, mixed, and repopulated. Some populations have possibly been isolated longer and have not mixed with other populations and may be genetically very distinct, eg. Kenya, Philippines. The Andaman Sea population remaining relatively isolated from the South China Sea by the restricted and continuous NW current flow in the straits of Malacca.

Within the region of the East Malaysian Peninsula, Trat was significantly different from pooled Surat Thani & Dungun. Thai 3-year catch statistics (Promsakha, 1980; Thubthimsang, 1978-1980) and data from major fishing gear statistics (Viphasiri, 1984) showed that the breeding grounds of *P. monodon* were found at depths of 26-50 metres and where water salinity was higher than 31 ppt. Adult *P. monodon* were most abundant in two areas, one is between Ranong and Trat, the other is between Chumporn and Surat Thani (Fig. 2.14), therefore there are likely to be two sub populations of the species in the Gulf of Thailand. The *P. monodon* in the Gulf of Thailand bred throughout the year (Promsakha, 1980; Promsakha and Lohsawadkun, 1988), with the higher percentages of ripe females being bimodal during May to December and January to April (Fig. 2.15). Promsakha and Lohsawadkun (1988) also concluded that The Gulf of Thailand is just a small body of water oceanographically speaking. With the complicated and uncertain patterns of surface current circulation (Fig. 2.16), all marine penaeid were believed to be a single population. They also concluded that their relative abundance in some area as revealed by earlier investigations and perhaps can be attributed to the inshore feature which influence the survival of juveniles such as suitable nursing grounds and the prevailing oceanic current. Moreover, surface winds and surface current (Dale, 1956; Open University, 1989) demonstrated high movement within South China Sea through the Java in either direction suggesting the mixture of dispersal *P. monodon* larvae between these area. As the Java populations showed no genetic significant different with Trat and pooled Surat and Dungun therefore these five populations, i.e. Trat, Surat, Dungun, North and South Java, trend to be same population.

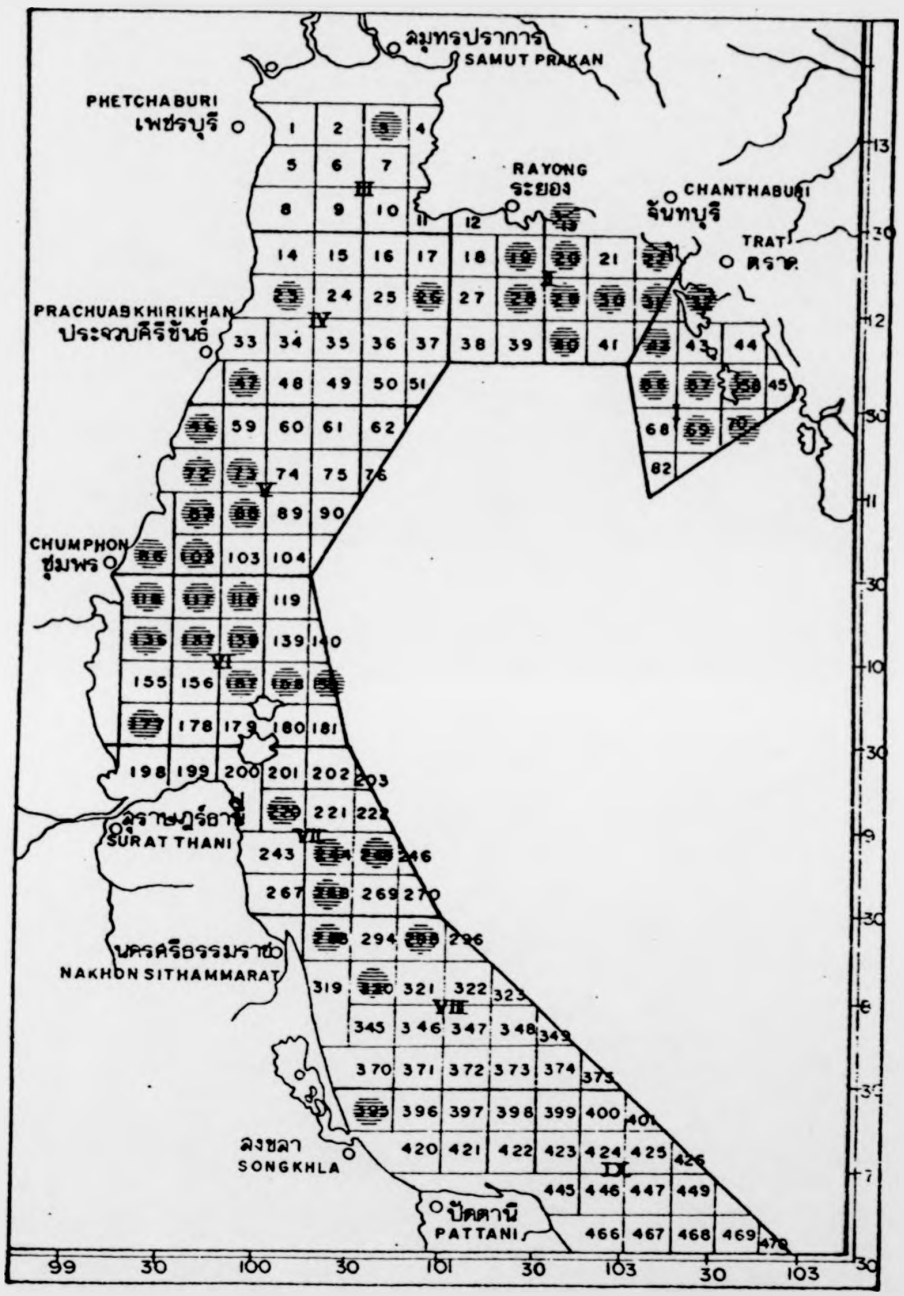


Fig. 2.14 Distribution area of *P. monodon* surveyed by research vessel, in 1977-1979 (after Promsakha, 1980).

Fig. 2.15 Monthly variation in percentage of female P. monodon caught in the Gulf of Thailand in 1977-1979 (after Promsakha, 1980)

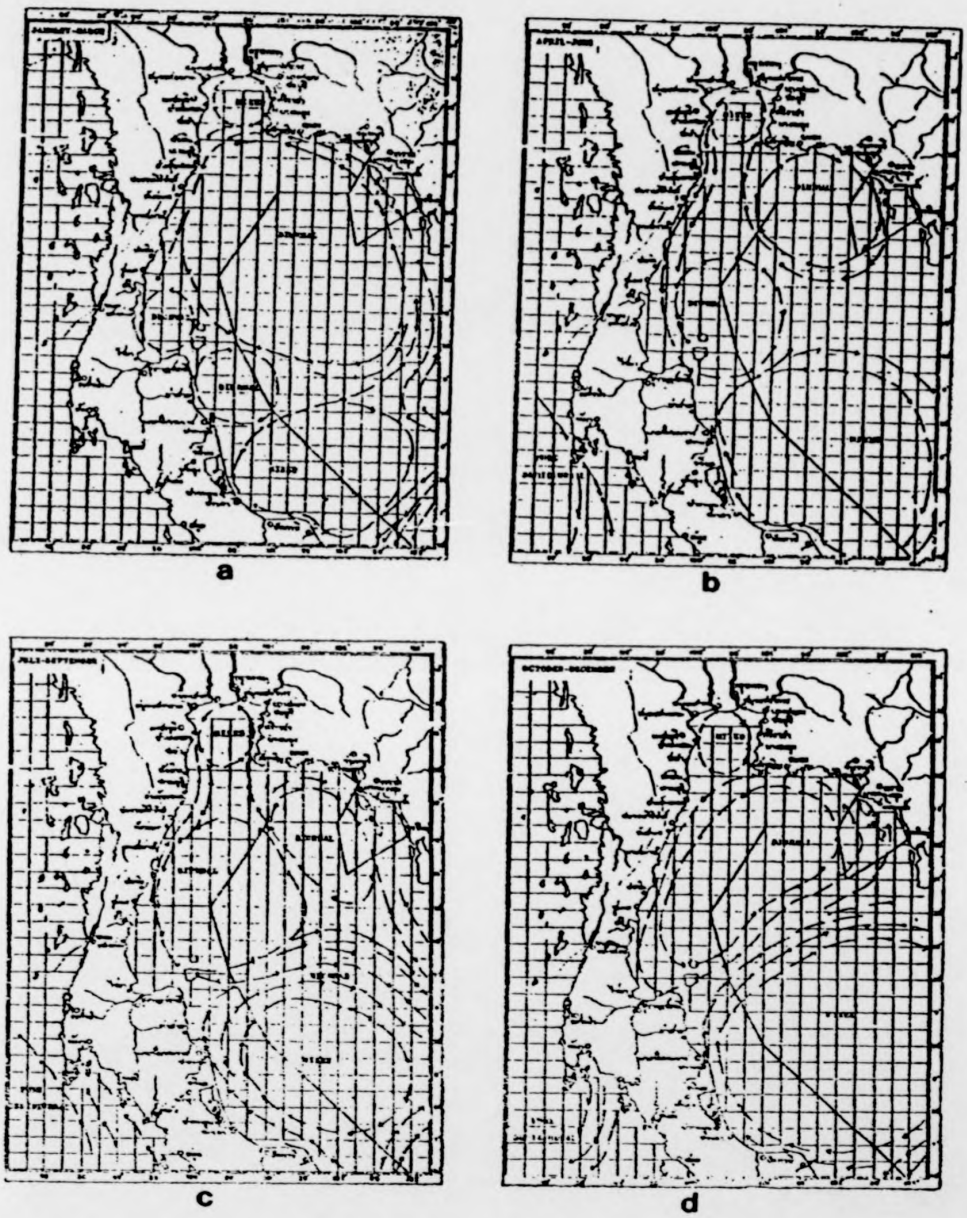


Fig. 2.16 Surface current circulation in the Gulf of Thailand: a. January-March, b. April-June, c. July-September, d. October-December. (from Promsakha and Lohsawadkun, 1988).

Another major factor which might effect levels of genetic differentiation in the Gulf is the programme of release of *P. monodon* larvae to enhance the natural stocks as well as the continuing chronic and sometimes acute escapes from farms. The majority of these *P.monodon* coming from Andaman Sea sources therefore it may have an effect on the Gulf of Thailand populations.

In conclusion, population structures of *P. monodon* in the present study appear to be 4 populations, namely, Kenyan population, the Philippines population, Andaman Sea population and East Malaysian population.

CHAPTER 3

ANALYSIS OF GENETIC VARIATION USING MtDNA RFLPs IN *P. MONODON*

3.1 INTRODUCTION

Over the past decade it has been recognized that fisheries management of aquatic organism populations requires information on stock structure so that genetically divergent populations can be preserved. In the early 1970s allozyme analysis became the basic tool for the quantitative assessment of fish population structure and the application of this information to the problems of stock identification (Allendorf *et al.*, 1987). Both natural populations (Lester, 1979; Mattoccia *et al.*, 1987) and hatchery stocks (Allendorf & Phelps, 1980; Ryman & Ståhl, 1980) were analyzed using allozyme data, however the value of such analyses has been constrained because of the low number ofmarker polymorphic loci. Recently, the development of molecular genetic techniques has greatly increased our ability to detect genetic markers by examining variation at the DNA level. Of these techniques, the examination of mtDNA variation as a tool for population genetics and fisheries management has been widely accepted (Avise, 1985; Ferris & Berg, 1987; Billington & Hebert, 1991; Ovendon, 1991). The intraspecific mtDNA variation of marine species has now been widely studied

both in invertebrates (Komm *et al.*, 1982; McLean *et al.*, 1983; Saunder *et al.*, 1986; Palumbi & Wilson, 1990) and vertebrates (Avise *et al.*, 1987, 1989; Wirgin *et al.*, 1989).

Mitochondria, organelles in the cytoplasm of eukaryotic cells, contain small amount of a circular DNA duplex and there are generally about 5-10 identical copies per organelle. The mitochondrial genome is comparatively small (16,000-19,000 nucleotides or 16-19 kilobase pairs); the mammalian mitochondrial DNA (mtDNA) has a molecular weight of about $11x10^6$ d, 10^{-5} times less than that the size of the nuclear genome (King and Stansfield, 1985). The resolution capability of mtDNA for population genetics studies appears to be enhanced by the rapid rate of base substitution in this type of DNA. The rate of base substitution estimated for some vertebrates appears to be 5-10 times faster than that of single-copy nuclear DNA (Brown, *et al.*, 1979). These properties make mtDNA a useful molecule for studies of population genetics and evolution. Thus, in general, conspecific populations and related species show greater differentiation of mitochondrial than nuclear genes (Saunders *et al.*, 1986; Ward, *et al.*, 1989, Bernatchez and Dodson, 1990; Reeb and Avise, 1990).

From the allozyme chapter it can be seen that levels of protein variation are low and although they suggest population subdivisions more markers are needed to see whether smaller scale isolation is present. The main objective of this chapter has been to analyse mtDNA restriction patterns in ten populations of *P. monodon*. The basic strategy for the mtDNA analysis was to extract total nucleic acids, digest the compound with restriction endonucleases, use Southern

transfer, radiolabeled probes and DNA-DNA hybridization and identify the mtDNA fragments with X-ray film. The data was then analysed to determine the levels of genetic variability within and between populations.

3.2 MATERIALS & METHODS

3.2.1 Samples

The mtDNA was extracted from *P. monodon* in 10 of the sample sites used in the allozyme analysis (Table 3.1). Two sites Trat and Satun were also sampled in different years to assess temporal stability of mtDNA variation. The Kenya sample could not be used because of difficulties encountered with DNA from this older material.

Table 3.1 Sample locations, year and number of individuals of *P. monodon* collected for mitochondrial DNA analysis

	Collection sites	Year of collection	Number of samples
1	Trat (Gulf of Thailand)	'92, '93	8, 8
2	Surat Thani (Gulf of Thailand)	'92	8
3	Phuket (Andaman Sea, Thailand)	'92	8
4	Satun (Andaman Sea, Thailand)	'92, '93	7, 8
5	Kedah (W. Pen. Malaysia)	'92	6
6	Dungun (E. Pen. Malaysia)	'92	10
7	Aceh (Sumatra, Indonesia)	'92	8
8	South Java Site (Indonesia)	'93	6
9	North Java Site (Indonesia)	'93	8
10	Lingayen (West Philippines)	'92	8

3.2.2 DNA Isolation

There are three basic methods for the isolation of mtDNA, i.e., density gradient ultracentrifugation using isopycnic caesium chloride gradients (Lansman *et al.*, 1981; Hauswirth *et al.*, 1987), phenol extraction of partially purified mitochondria (Chapman & Powers, 1984; Palva & Palva, 1985; Jones *et al.*, 1988), and total nucleic acid extractions (Gross-Bellard *et al.*, 1972; Ausuble *et al.*, 1987; Sambrook *et al.*, 1989). The first two methods obtain highly purified mtDNA but require large fresh tissue samples. Because the tissue samples of *P. monodon* were already frozen this study used total nucleic acids for analysis. This is because tissue stored for long periods at -70°C will have linearized mtDNA and damaged mitochondria which are impossible to isolate by the former two methods. Tegelström (1992) commented that mtDNA preparations heavily contaminated with nuclear DNA will inhibit some visualization methods.

3.2.2.1 Tissue selection

Total nucleic acids were extracted from liver, eggs, abdominal muscle and pleopod muscle of *P. monodon*. The quantity of total DNA was checked using ethidium bromide staining.

3.2.2.2 Extraction method

In this study mtDNA fragments in total DNA were visualized by hybridization with radiolabelled mtDNA. This DNA-DNA hybridization using

radiolabelled probes to identify restriction fragments requires mtDNA samples in a fairly pure condition for probes as contaminating nuclear DNA will also label and obscure the mtDNA bands (Chapman & Brown, 1991). This study therefore used two methods of mtDNA extraction. First, purified mtDNA was obtained from fresh *P. monodon* using a phenol extraction procedure. Second, total nucleic acids were extracted from frozen samples.

A. Mitochondrial DNA extraction

The purification of mtDNA for the radiolabelled probes followed the phenol extraction of partially purified mitochondria recommended by Chapman & Powers (1984) with slight modifications as shown in Appendix 3. This rapid method relies on size differences between mitochondria and nuclei. The degree of homogenization is critical and tissues should be subjected to only one or two passes with minimal force through the homogenizer (Chapman & Brown, 1990). Chapman & Powers (1984) commented that the tissue can be overground by additional strokes leading to nuclear DNA contamination and loss of mitochondria. In the present study, 360 mg of pleopod tissue was homogenized with a glass Teflon homogenizer in 1 ml of 7.5% sucrose-TEK buffer containing 50 mM Tris, pH 7.5, EDTA 10 mM, 1.5% KCL and 7.5% sucrose. Differential centrifugation using an underlayer of 1.1 M sucrose-TEK solution was followed by two low-speed (1,000g for 10 min) and two high-speed centrifugations (18,000g for 1 h and 30 min). The mitochondrial pellet was resuspended in TEK solution and lysed by 1% Non-idet. This detergent will not lyse nuclear membranes at this concentration (Chapman & Brown, 1990). Intact nuclei were centrifuged, the supernatant was drawn off and was given a phenol extraction and

ethanol precipitation. The mtDNA precipitate was dried under vacuum and resuspended in TE buffer and frozen at -20°C.

B. Total nucleic acid extraction

High molecular weight DNA of *P. monodon* was extracted by following Klinbunga (pers com) which was modification from Ausubel *et al.* (1993), Sambrook *et al.* (1989) and Hillis *et al.* (1990) (complete procedure in Appendix 3). Tissues were powdered in liquid nitrogen with a mortar and pestle, then about 300-400 mg of ground tissues was homogenized with a glass Teflon homogenizer in 1 ml TEN buffer (100mM Tris, pH 8.0, 100mM EDTA and 250 mM NaCL), and then lysed and deproteinized by adding 1% SDS, and 0.2 mg/ml proteinase K, mixing thoroughly and incubating at 55°C for 2 h. Afterwards, the solution was centrifuged to remove debris and was extracted 3 times with phenol and 3 times with chloroform:Isoamyl alcohol and centrifuged at 10,000g for 10 min after each extraction. The clear aqueous phase was adjusted to a salt concentration of 2.0 M by adding 0.5 M NaCL and the DNA precipitated with 2 volumes of ice-cold 99.99% ethanol. The precipitate was then dried under vacuum and resuspended in TE buffer. Carbohydrate and RNA contaminants were digested using α-amylase buffer and DNase-free RNase A and incubated at 55°C for 1 h. The solution was reprocessed and organic solvents and salt were removed by centrifugation of the aqueous layer through Millepore Ultrafree-MC filters. The precipitate was then dried under vacuum and resuspended in TE buffer and stored at 4°C.

3.2.2.3 DNA concentration determination

Before further analysis, it is important to determine the quantity and quality of DNA. This present study used two methods for detection, DNA was determined by UV absorbance with a spectrophotometer and mtDNA was determined by comparison with known DNA standards.

A. Spectrophotometer Concentration and purity of samples was checked in a spectrophotometer by taking readings at wavelengths of 260 and 280 nm and using the formula below.

$$DNA\ conc.\ (\mu g/ml) = \left(\frac{500\ \mu l}{x\ \mu l}\right)\left(\frac{50\ \mu g}{ml}\right) OD\ (260,280)$$

Where x is the volume of DNA used.

An optical density (OD) of 1 corresponds to approximately 50 µg/ml for double-stranded DNA, 40 µg/ml for single-stranded DNA (Maniatis *et al.*, 1982; Hillis *et al.*, 1990). The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate for the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} of 1.8 and 2.0, respectively. If there is contamination with protein and/or phenol the ratio will be less than 1.8. If there is contamination with RNA the ratio will be more than 2.

B. Ethidium bromide Fluorescent Quantification If there is not sufficient DNA (<250 ng/ml) or the DNA is heavily contaminated with other UV-absorbing substances the use of a spectrophotometer is not recommended. The concentration of mtDNA was therefore rapidly estimated using ethidium bromide staining following agarose gel electrophoresis. The amount of fluorescence emitted by ethidium bromide is proportional to the total mass of DNA. The quantity of DNA in samples was estimated by comparison against a λ*Hind*III standard marker. Maniatis *et al.* (1982) commented that as little as 1-5 ng of DNA can be detected by this method.

3.2.3 Digestion of DNA & MtDNA by Restriction Enzymes

The restriction enzymes used are listed in Table 3.2. Five hexanucleotide restriction enzyme (*Bam*HI, *Eco*RV, *Hind*III, *Pvu*II, *Sac*I) were chosen for this study because they had previously revealed haplotype variability in a range of penaeid including *P. monodon* (Benzie *et al.*, 1993). Total nucleic acid was digested according to the supplier's directions (Pharmacia Ltd) using 2-4 units of enzyme per 1 ng DNA (complete procedure in Appendix 3). Digestions were incubated at 37°C for 4-24 h in 50 µl reaction mixtures containing 1-2x restriction buffer, 0.1% BSA and 2mM spermidine. After digestion, the restricted DNA was purified by extraction once with phenol and once with chloroform:Isoamyl alcohol, then the DNA was precipitated with ethanol and resuspended in 10 µl TE buffer.

Table 3.2 List of restriction enzymes used and reaction conditions

Enzyme	Recognition sequence	Rxn. temp.[a]	Inact. temp.[b]	Isoschizomers	Comments
*Bam*HI	G↓GATCC	37	60	*Bst*I	Star activity
*Eco*RV	GAT↓ATC	37	100		
*Hind*III	A↓AGCTT	37	90		
*Pvu*II	CAG↓CTG	37	95		
*Sac*I	GAGCT↓C	37	60	*Sst*I	

NOTE:

[a]Reaction temperature (°C) at which the reaction should be performed.
[b]Inactivation temperature (°C) at which the enzyme is inactivated after 15 min of incubation.

Purified mtDNA of *P. monodon* was also digested using six-base sequence recognition endonuclease to identity 1 or 2 fragments that might be used as a mtDNA probe (Appendix 3). In general, probes prepared from one species may be useful in others because sequence homology among mtDNA from different species allows heterologous probes to hybridize. However Chapman & Brown (1990) suggested that conspecific probes are more efficient than heterologous molecules.

3.2.4 Separation of DNA & mtDNA Fragments

3.2.4.1 Agarose gel electrophoresis

A. DNA fragments separation

DNA fragments were separated on 0.8-1% agarose gels using the TBE buffer system (Maniatis *et al.*, 1982). Agarose gels are preferred when using six-base enzymes because they improve the accuracy of fragment size estimation over the rang from 300 to 20,000 base pairs (Dowling *et al.*, 1990). Ultra pure agarose (Gibco BRL) was mixed, with TBE buffers and ethidium bromide (Appendix 3). Bacteriophage λ DNA digested with *Hind*III was included in each gel as a molecular weight standard which had seven bands ranging in size from 400 to 21,000 base pairs (Murray & Murray, 1975 as cited by Lansman *et al.*, 1981). This marker was in TE buffer which is similar in ionic strength to the sample buffers. Thus, samples with loading dye were run on horizontal agarose gels at a voltage of 5 V/cm at room temperature.

B. MtDNA fragments separation

Purified mtDNA from *P. monodon* which was digested by restriction enzyme was run in a low melting point agarose gel at 4°C. By viewing on a UV transilluminator (302 nm), the mtDNA concentration was calculated by comparison with λ*Hind*III marker, the fragment from the gel was cut out with a minimum of excess gel, washed with sterile dH_2O, and stored at -20°C.

3.2.4.2 Estimation of fragment sizes

Fragment sizes of mtDNA were estimated from their mobilities relative to those of fragments of known sizes. After electrophoresis, the gel was photographed with a ruler next to the size marker to allow fragment sizes to be determined from the final autoradiograph. However rough measurements can be taken directly from the negative of the gel photograph. By using a local 2 × 3 point method (Sealey and Southern, 1990), a plot of mobility (M) against molecular weight or size (L) will give a curved line and fragment sizes can be calculated using the formula below:

$$L = k_1/(M-M_0)+k_2$$

where:

$$M_0 = \frac{M_3-M_1((L_1-L_2)/(L_2-L_3) \times (M_3-M_2)/(M_2-M_1))}{1-((L_1-L_2)/(L_2-L_3) \times (M_3-M_2)/(M_2-M_1))}$$

$$k_1 = \frac{L_1-L_2}{1/(M_1-M_0)-1/(M_2-M_0)}$$

$$k_2 = L_1-k_1/(M_1-M_0)$$

NOTE:

L = molecular weight or size of unknown
M = mobility of unknown molecular weight
M_0 = empirical correction factor determined by imposing the condition that the three lines joining the three points all have the same slope
L_1, L_2, L_3 = three molecular weight standards which most closely to the unknown molecular weight of mobilities M
M_1, M_2, M_3 = mobilities of L_1, L_2, L_3

3.2.5 Methods for the Visualization of mtDNA Fragments

There are two ways to detect mtDNA fragments, i.e., fluorochromic staining and radiolabelling techniques. The easiest way is by staining with ethidium bromide but it is a relatively insensitive technique for fragment visualization and can only be used when large amounts of purified mtDNA are available and there is no need to detect very small fragments (Dowling *et al.*, 1990). The isotopic technique offers a much higher sensitivity than ethidium bromide staining and is suitable for use with small samples of mtDNA. The present study therefore used both techniques as appropriate to detect mtDNA fragments.

3.2.5.1 Fluorochromic staining (Ethidium bromide)

Mitochondrial DNA was detected in agarose gels by staining with ethidium bromide (EtBr) which was included in the gel mix at concentration of 0.5 µl/ml. The mtDNA is directly visualized using this UV fluorescing dye which binds to the DNA molecule. A photographic technique that increases the sensitivity of ethidium bromide staining by 10 times has been described by Chapman & Brown (1989). The gel is placed on a UV transilluminator (302 nm) and photographed at an exposure of f 5.6 for about 25 sec with a Polaroid direct screen instant camera with Kodak 22A Wratten filter (deep orange) and Polaroid black & white Type 665 film (high resolution negative, 50 ASA).

3.2.5.2 Radiolabelling techniques (Hybridization with mtDNA probes)

Identification of nucleotide sequences by DNA/DNA hybridization and transfer techniques was first described by Southern (1975). This isotopic method consists of four basic steps following separation of digested DNA after agarose gel electrophoresis: (1) transfer of the DNA from the gel onto a filter, (2) labelling the mtDNA probes, making it single stranded, (3) hybridization and washing the filter, and (4) autoradiography (complete procedure in Appendix 3).

A. Southern transfer (transfer of DNA to nylon membrane) DNA fragments were vacuum blotted under alkaline conditions (Appendix 3) as recommend by Reed & Mann (1985) using the VacuGene XL Vacuum blotting System (Pharmacia). First, DNA fragments were depurinated using acid conditions to break down large fragments into small pieces for more efficient, transfer until the bromophenol blue in the gel turns yellow. Second, DNA fragments were transferred to nylon membrane (Hybond-N+, Amersham, Inc) using an NaOH transfer technique, rinsed with SSC, dried and kept in Whatman 3MM paper at room temperature.

B. Labelling of mtDNA probe *P. monodon* mtDNA fragments were prepared as radioactive probes for use in hybridization experiments by random priming (Feinberg & Vogelstein, 1983). Prior to being labelled, purified mtDNA from *P. monodon* was cut by *Eco*RV digestion and separated by electrophoresis through low-melting-point agarose gel. The probe was radiolabelled to high specific activity with dATPα^{32}P (ICN) using Boehringer Mannheim Biochemical

random primed DNA labelling kit. First, probes were denatured by boiling and mixed with hexanucleotides, DNA polymerase I (Klenow enzyme labelling grade), and all four dNTPs (dATP, dCTP, dGTP, dTTP), one or more of which may be radiolabelled. Later, the mixture was incubated at room temperature for 4-24 h where the hexanucletides bind to the probe and act as primer for DNA synthesis.

C. Hybridization Hybridization was carried out using a modified technique of the procedure described by Reed and Mann (1985). The filter was prewashed in SSPE buffer and prehybridized in solution with carrier DNA (calf thymus DNA) by incubation at 60°C for 4-12 h. The radiolabelled probe was boiled for 10 min, chilled quickly on ice, and added to the hybridization solution, then incubated at 60°C for 4-24 h. The hybridization mixture was decanted and the filter washed, and sealed in plastic bag.

D. Autoradiography The radioactivity was determined by using a Geiger counter (Mini-Monitor Scintillation Probes) to estimate the time for autoradiography. The filter was exposed to nylon membrane (Hyperfilm™-MP; High performance autography film, Amersham) in a cassette with one or more intensifying screens. The cassette was stored at -70°C for 1-2 days depended on radioactivity and number of intensifying screens. The cassette was then removed from the freezer, allowed to defrost for 30 min , and then the film was developed (Kodak D19 developer) and fixed.

3.2.6 Data Analysis

For mtDNA analysis, the composite restriction fragment patterns of each individual can be defined as a mtDNA haplotype. To determine intrapopulational variation, an average number of nucleotide differences per fragment between two sequences or ***nucleotide diversity*** (π) can then be estimated according to Nei and Li (1979). The nucleotide diversity can be estimated by first measuring the similarity between haplotype patterns:

$$S_{ij} = 2n_{ij}/(n_i + n_j)$$

where n_i and n_j are the numbers of restriction fragments in the ith and jth haplotypes, and n_{ij} is the number of shared fragments.

$$\pi = (-\ln S_{ij})/r$$

where r is the number of base pairs recognized by the restriction enzyme.

In the present study, the differences between populations also appeared as genotype frequency differences. To verify genetic differentiation between population, individual and combined mtDNA genotype frequencies and haplotype frequencies were tested using the method of Nass (1959) for chi-square analysis with small expectations. To judge the significance level a minimum table-wide significance value was calculated using the sequential Bonferroni technique (Holm, 1979; Rice, 1989). A chi-square test (Siegel, 1956) can also determine

the significant differentiation between populations using the frequencies of haplotypes in each population. However, Ovendon (1990) suggested that the number of haplotypes found in mtDNA studies are often large and the distribution of individuals between the haplotypes is often skewed. By using small sample size, this can invalidate the χ^2 analysis. For the present study, this problem was overcome by comparing the original χ^2 value with that estimated from 1000 Monte Carlo repeated randomizations of the original matrix. This method, which was presented by Roff & Bentzen (1989), was performed using REAP, the restriction enzyme analysis package version 4.1 (McElroy *et al.*, 1993).

To determine the evolutionary relationship between haplotypes, ***nucleotide substitutions (d)*** was estimated from the number of shared fragments (Nei & Lei, 1979; Nei, 1987, eq. 5.55). We first measure the expected proportion of shared DNA fragments (F) between haplotype patterns:

$$F = 2m_{xy}/(m_x+m_y)$$

where m_x and m_y are the numbers of restriction fragments in the xth and yth haplotypes, and m_{xy} is the number of shared fragments. Then we estimate G by the following iterative formula.

$$G = [F(3-2G_1)]^{1/4}$$

The iteration is repeated until $G = G_1$. An initial trial value is $G_1 = F^{1/4}$. Once G

is obtained, d can be estimated by

$$d = -(2/r)\log_e G.$$

Phylogenies were then constructed from a matrix of the average number of nucleotide substitutions between haplotypes using UPGMA clustering method computed by PHYLIP: phylogeny inference package version 3.5 (Felsenstein, 1995). To quantify genetic differences between pairs of population, Nei's unbiased genetic distance (Nei, 1978) and Rogers' distance (Rogers, 1972) were calculated based on individual mtDNA genotypes and mtDNA haplotypes frequencies using BIOSYS-1 computer program (Swofford & Selander, 1981).

Nucleotide divergence between all pairs of populations were also calculated, d_A between population X and Y can be estimated by following Nei and Tajima (1981) and Nei (1978, eq. 10.21).

$$d_A = d_{XY} - 0.5(d_X + d_Y)$$

Unweighed pair-group arithmetic average (UPGMA) clustering method (Sneath and Sokol, 1973) for Nei's and Rogers' genetic distance were constructed using BIOSYS-1 computer program (Swofford & Selander, 1981) and the nucleotide divergence trees were constructed using PHYLIP: phylogeny inference package version 3.5 (Felsenstein, 1995).

3.3 RESULTS

3.3.1 Isolation, Purification and Digestion of DNA and mtDNA

3.3.1.1 DNA

As in the allozyme study, pleopod was the best tissue, as it gave a good yield, low contamination and less smearing of bands than other tissue (Fig 3.1). Pleopods were thus used for all experiments. Pleopods can be dissected from live *P. monodon*-a biopsy technique which allows the determination of mtDNA variation without the need to sacrifice the individual, which is similar, but yields much higher levels than blood mtDNA analysis in fish (Billington & Hebert, 1990). From one pleopod, about 230 mg of tissue (100-120 g *P. monodon*), about 70 µg DNA (≈ 0.3 mg DNA per gram starting tissue) can be extracted. By using 1,2,5, and 10 µg DNA for each restriction enzymes digestion, it was shown that as little as 1 µg DNA could be seen on negative film (Fig 3.2), however for clarity 10 µg DNA per digestion were used in the present study. One pleopod should enable up to 7 restriction enzyme reactions to be analyzed.

Partial digestion experiments showed that total nucleic acid can be completely digested within 1-24 hours as shown in Fig 3.3. However, for convenience and for complete digestion, the incubation was processed overnight at 37° C for up to 24 h.

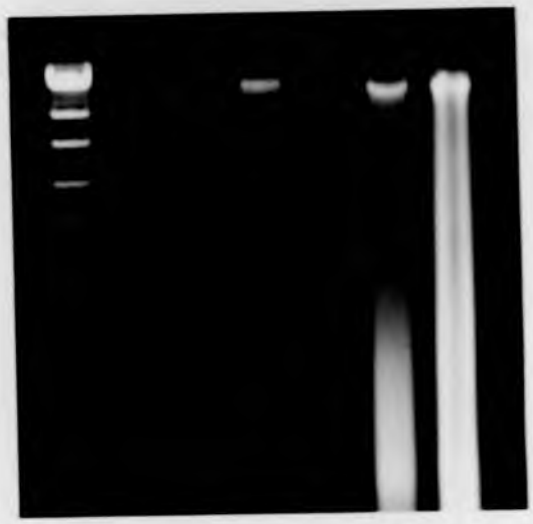


Fig. 3.1 Comparison of purified total DNA between tissue samples of *P. monodon*: lanes 2-4 are pleopod, lanes 5-6 are hepatopancrease and lane 1 is λ*Hind*III marker.

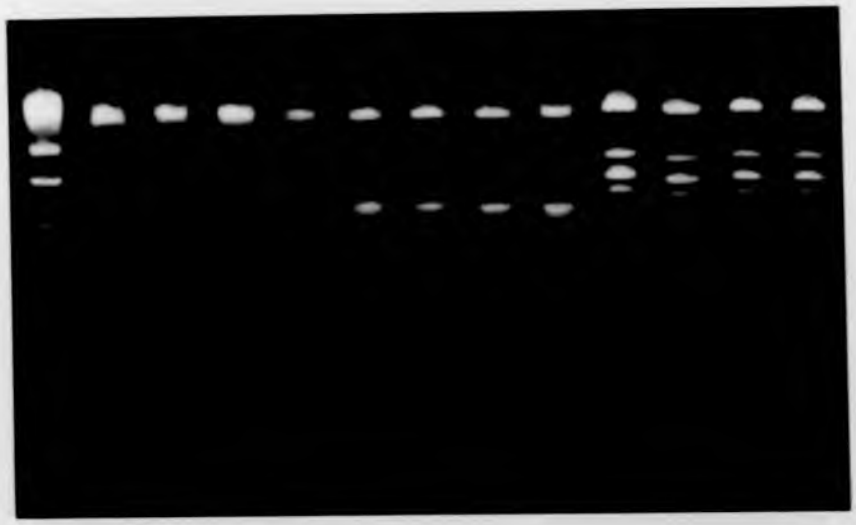


Fig. 3.2 REs digestion experiment of total DNA extracted from *P. monodon* pleopod: *Apa*I restriction fragments are in lanes 2-4, *Pvu*II restriction fragments are in lanes 6-9, *Eco*RI restriction fragments are in lanes 10-13 with DNA of 1,2,5,10 μg respectively. Lane 1 is λ*Hind*III marker.

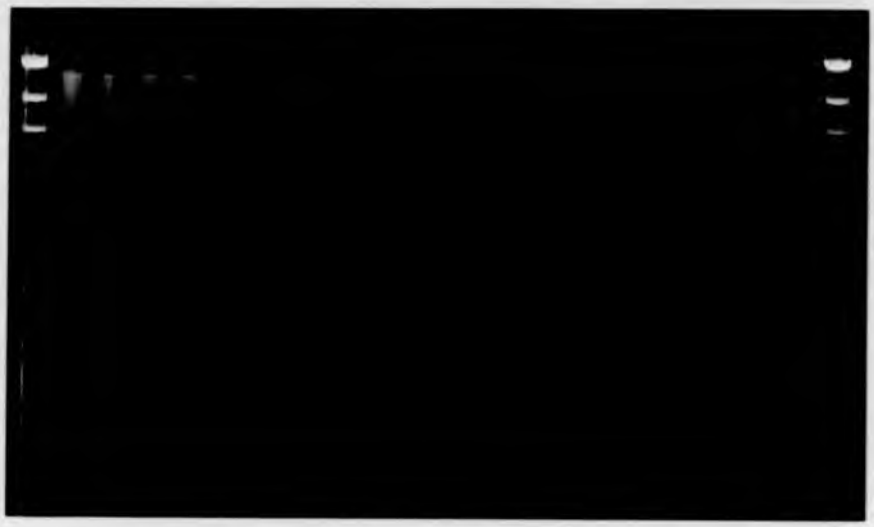


Fig. 3.3 Partial digestion experiment of total DNA extracted from *P. monodon* pleopod, *Bam*HI digestion fragments sample 1 (lanes 2-6), sample 2 (lanes 7-11), and *Eco*RV digestion fragments sample 1 (lanes 12-16) sample 2 (lanes 17-21) with incubation time of 0.5, 1, 2, 4 and 24 h respectively. Lanes 1 and 22 are λ*Hind*II marker.

3.3.1.2 MtDNA

MtDNA from pleopod of live *P. monodon* was extracted. Using phenol extraction, yields of about 100 ng per gram starting tissue were obtained. The purity of mtDNA was tested by agarose gel electrophoresis. No nuclear DNA contamination was detectable in ethidium bromide staining. Then mtDNA was digested with *Eco*RV (as this enzyme gave only one fragment), run in a low melting point agarose gel, cut and stored at -20°C ready to be used as the radiolabelled probe.

3.3.2 Mitochondrial DNA Restriction Patterns of *P. monodon*

3.3.2.1 Visualization

Using 10 µg of total nucleic acid extracted from frozen *P. monodon* pleopod, mtDNA patterns restricted with six-base pair restriction enzyme can be seen on the negative of Polaroid type 665 film using ethidium bromide staining. The patterns were verified as mtDNA by probing Southern transfers of the gels with ^{32}P-labelled purified mtDNA, then compared to the bands observed in the restriction patterns obtained from purified mtDNA with the same enzymes.

Fluorochromic staining and radiolabelling of samples from the same individuals were compared. Fig. 3.4 A, 3.5 A, 3.6 A, 3.7 A, 3.8 A shows the ethidium bromide stained gel of *Bam*HI, *Eco*RV, *Pvu*II, *Sac*I, and *Hind*III restriction fragments respectively, and Fig. 3.4 B, 3.5 B, 3.6 B, 3.7 B, 3.8 B shows an autoradiograph of a DNA/DNA hybridization gel of *Bam*HI, *Eco*RV, *Pvu*II, *Sac*I and *Hind*III restriction fragments respectively. Both the ethidium bromide stained gel and the DNA/DNA hybridization autoradiograph of *Eco*RV, *Hind*III, and *Pvu*II exhibited corresponding patterns, but there were extra bands (non mtDNA fragments) in both *Bam*HI and *Sac*I in the ethidium bromide staining. As mtDNA banding patterns can be seen using ethidium bromide, samples with clear bands can be viewed directly from Polaroid picture and negative film.

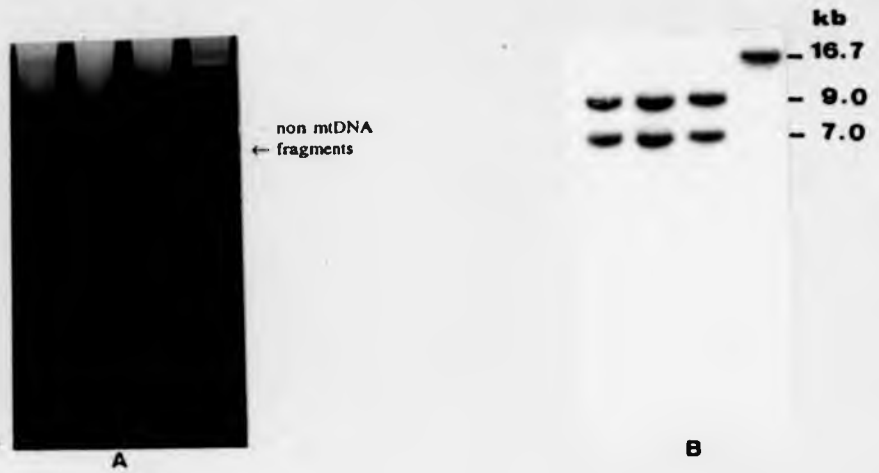


Fig. 3.4 Comparison of *Bam*HI digestion fragments of *P. monodon*, A. Ethidium-bromide stained gel, B. DNA/DNA hybridization autoradiograph.

Fig. 3.5 Comparison of *Eco*RV digestion fragments of *P. monodon*, A. Ethidium-bromide stained gel, B. DNA/DNA hybridization autoradiograph.

Fig. 3.6 Comparison of *Pvu*II digestion fragments of *P. monodon*, A. Ethidium-bromide stained gel, B.DNA/DNA hybridization autoradiograph.

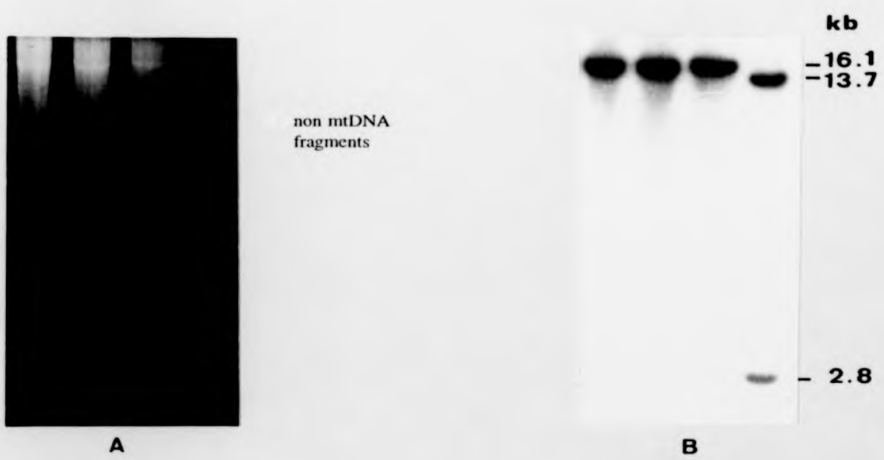


Fig. 3.7 Comparison of *Sac*I digestion fragments of *P. monodon*, A. Ethidium-bromide stained gel, B. DNA/DNA hybridization autoradiograph.

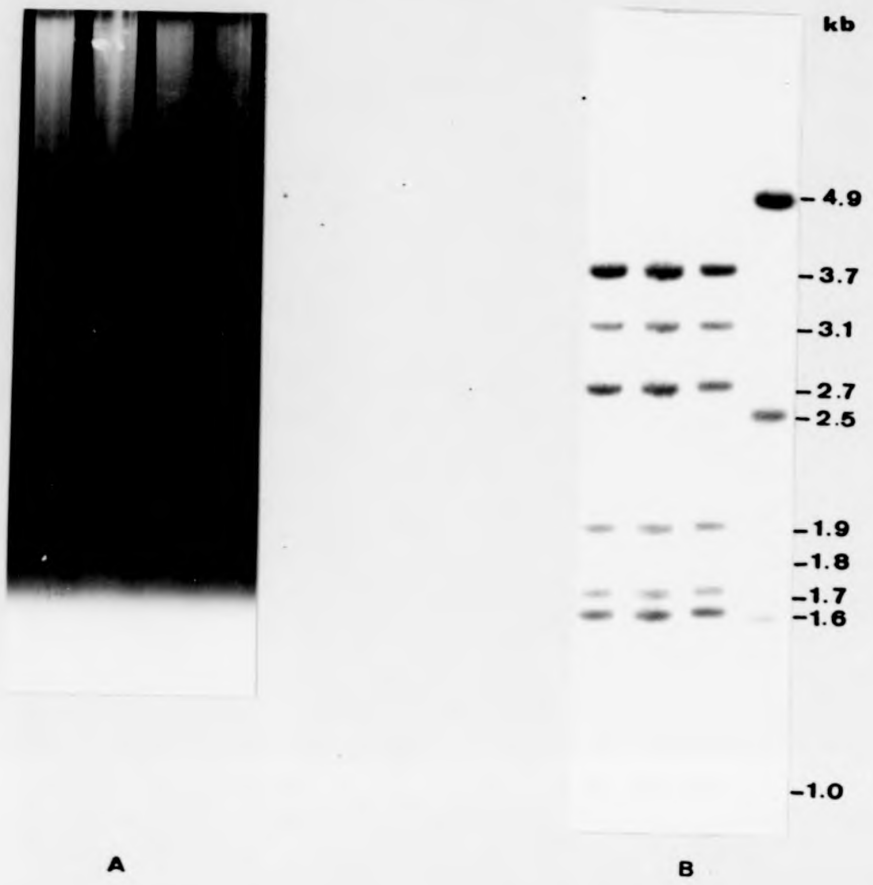


Fig. 3.8 Comparison of *Hind*III digestion fragments of *P. monodon*, A. Ethidium-bromide stained gel, B. DNA/DNA hybridization autoradiograph.

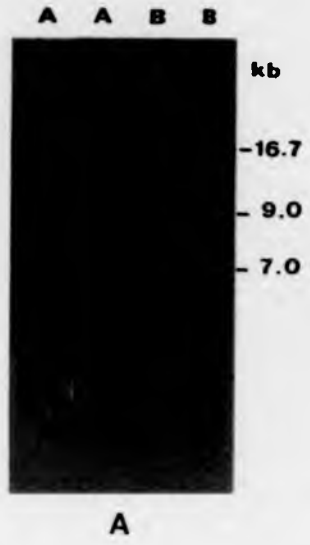
3.3.2.2 Fragment patterns

Restriction morphs, the fragment patterns of *P. monodon* mtDNA digested with each of five 6bp restriction enzyme namely, *Bam*HI, *Eco*RV, *Pvu*II, *Sac*I and *Hind*III are presented in Fig. 3.9, a total of 31 fragments were observed. Size estimates for *P. monodon* mtDNA fragments obtained by digestion with five restriction enzyme are shown in Table 3.3. The total molecular size of the *P. monodon* mtDNA genome was estimated to be about 15.76±0.57 kb. All five restriction enzyme fragment patterns were found to be polymorphic in all 10 locations studied.

***Bam*HI**

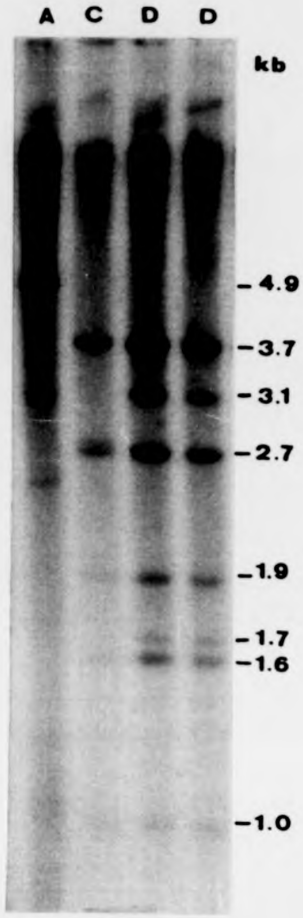
Two genotypes of *Bam*HI were found in *P. monodon*. *Bam*HI genotype A appeared to have a single cut site, 16.7 kb, while *Bam*HI genotype B produced two fragments, 9.0 kb and 7.0 kb. These two patterns were consistent with the results of Benzie *et al.* (1993) for the same species. The Australian populations had a size of 14.6 kb for genotype A and 9.2 kb & 7.1 kb for genotype B. In contrast, Bouchon (1994) found only genotype B, 9.35 kb & 7.10 kb, in laboratory strains of Malaysian, Australian and Fijian *P. monodon* populations. The change from *Bam*HI genotype A to *Bam*HI genotype B can be most easily explained by the addition of a single new cut site. Some samples showed incomplete digestion and had three bands patterns, mixture of genotype A and B, for the present study these were interpreted as genotype B (see Fig 3.9 A).

Fig. 3.9 DNA/DNA hybridization autoradiograph showed restriction fragment patterns of *P. monodon* mtDNA.



A. MtDNA fragment patterns digested with *Bam*HI.
B. MtDNA fragment patterns digested with *Eco*RV.

C. MtDNA fragment patterns digested with *Pvu*II.
D. MtDNA fragment patterns digested with *Sac*I.



E

E. MtDNA fragment patterns digested with *Hind*III.

Table 3.3 Fragment sizes (in kilobased pairs) for each restriction profile produced by five enzymes used in this study (Dashes indicate fragments missing from a specific restriction profile). Networks of evolution relationships among the profiles also shown, where arrows indicate direction of site loss (not necessarily direction of evolution). Letters not connected by arrows apparently differ by two or more restriction site changes.

Enzyme	Restriction profile			
***Bam*HI**	**A**	**B**		
	16.7	—		
	—	9.0		
	—	7.0		
Σ	**16.7**	**16.0**		
	B → A			
***Eco*RV**	**A**	**B**	**C**	
	16.6	—	—	
	—	10.9	—	
	—	—	9.4	
	—	—	5.8	
	—	4.6	—	
Σ	**16.6**	**15.5**	**15.2**	
		B → A ↑ **C**		
***Pvu*II**	**A**	**B**	**C**	**D**
	15.5	—	—	—
	—	—	8.0	8.0
	—	7.6	7.6	—
	—	6.4	—	—
	—	—	—	4.7
	—	—	—	2.6
	—	1.5	—	—
Σ	**15.5**	**15.5**	**15.6**	**15.3**
		D ↔ C → A ↑ **B**		

Table 3.3 (continued)

Enzyme	Restriction profile					
***Hind*III**	**A**	**B**	**C**	**D**	**E**	**F**
	4.9x2	4.9x2	—	—	—	4.9
	—	—	—	—	4.0	—
	—	—	3.7	3.7	3.7	—
	3.1	—	—	3.1	3.1	3.1
	—	—	2.7x2	2.7	—	2.7
	2.5	2.5	—	—	—	—
	—	—	1.9	1.9	1.9	—
	—	1.8	—	—	—	—
	—	—	1.7	1.7	1.7	1.7
	—	1.6	1.6	1.6	1.6	1.6
	—	—	1.0	1.0	1.0	1.0
Σ	**15.4**	**15.7**	**15.2**	**15.7**	**17.0**	**15.0**
			B → A ↑ **F**			
			C → D → E			
***Sac*I**	**A**	**B**	**C**			
	16.1	—	—			
	—	13.7	—			
	—	—	11.1			
	—	—	. 4.1			
	—	2.8	—			
Σ	**16.1**	**16.5**	**15.2**			
		B → A ↑ **C**				

***Eco*RV**

Three restriction morphs were observed in *P. monodon*. *Eco*RV genotype A had a single band while both *Eco*RV genotype B and C were double banded but with different molecular weights. The single morph was replaced by 10.9 kb and 4.6 kb fragments for *Eco*RV genotype B and by 9.4 kb and 5.8 kb fragments for *Eco*RV genotype C. Genotype A, 17.5 kb, and C, 9.8 kb & 6.0 kb, were consistently found in Australian *P. monodon* populations (Benzie *et al.*, 1993) but only genotype A, 15.75 kb, was found in laboratory strains of Malaysian, Australian and Fijian populations of *P. monodon* by Bouchon (1994).

***Hind*III**

Six polymorphic genotypes of *Hind*III were detected in this species. *Hind*III genotype A produced four fragments, 4.9, 4.9, 3.1 & 2.5 kb. The two bands of the same size and mobility at 4.9 kb appearing as a more intense band on the gel. Fragments 3.1 kb of *Hind*III genotype B was replaced by fragments 1.8 and 1.6 kb. *Hind*III genotype F had 6 fragments, 4.9, 3.1, 2.7, 1.7, 1.6 & 1.0 kb. *Hind*III genotype A could be converted to *Hind*III genotype F by the replacement of 2.7 & 1.7 kb for a single band of 4.9 kb, and 1.6 & 1.0 kb for a single band of 2.5 kb. *Hind*III genotypes C, D & E all showed 7 fragments. They shared the same fragments at 3.7, 1.9, 1.7, 1.6 & 1.0 kb but genotype C had a presumed double band of 2.7 kb, genotype D was 3.1 & 2.7 kb bands and genotype E had 4.1 & 3.1 kp bands.

In the present study many samples digested with the enzyme *Hind*III were undigested or incompletely digested. This may have been due to the samples not

being adequately purified or being contaminated with RNA. The remaining extracted pleopod DNA samples were inadequate to undertake further digestion, therefore *Hind*III was not used for mtDNA analysis.

***Pvu*II**

Four genotypes for *Pvu*II were detected in this species. *Pvu*II genotype A produced one 15.5 kp fragment, *Pvu*II genotype B had 3 fragments of 7.6, 6.4 & 1.5 (*Pvu*II genotype B only appeared to have two fragments of 7.6 & 6.4 kb but actually has three fragments, 1.5 kb was detected by Klinbunga (pers comm), C had two fragments of 8.0 & 7.6 kp, and *Pvu*II genotype D showed three fragments of 8.0, 4.7 and 2.6 kb. However, Bouchon (1994) found only genotype C, 8.6 kp & 7.8 kp, in laboratory strains of Malaysian, Australian and Fijian *P. monodon* populations. *Pvu*II genotype A could be changed to *Pvu*II genotype C by the addition of a single cut site and *Pvu*II genotype C could be changed to *Pvu*II genotype D by the replacement of 4.7 & 2.6 kb for a single band of 7.6 kb. The *Pvu*II genotype C could be changed to *Pvu*II genotype B by the replacement of 6.4 & 1.5 kb for 8.0 kb.

***Sac*I**

Three genotypes of *Sac*I were scored in *P. monodon*. *Sac*I genotype A appeared to have a single cut site while *Sac*I genotype B and C showed two fragments of 13.1 & 2.8 kb and 11.1 & 4.1 kb respectively. Patterns A and B were consistently observed in Australian *P. monodon* with a molecular weight of 17.6 kb and 14.5 & 2.9 kb respectively (Benzie *et al.*, 1993). On the other hand, Bouchon (1994) detected only genotype A (15.45 kp), in laboratory strains of

Malaysian, Australian and Fijian *P. monodon* populations. The change from *Sac*I genotype A to *Sac*I genotype B or C could be explained by the addition of a single new cut site in each case.

3.3.2.3 Mitochondrial DNA individual genotypes

Mitochondrial DNA genotype frequencies of four restriction enzymes (*Bam*HI, *Eco*RV, *Pvu*II and *Sac*I) for 10 separate SE Asian populations are presented in Table 3.4 and Fig 3.10 A, B, C and D.

*Bam*HI

Genotype A was generally more common in the western populations. Genotype B was more common in the eastern populations apart from Surat, and was monomorphic in Kedah population.

*Eco*RV

In general all populations had the same common genotype A, and were monomorphic in Kedah and Dungun populations.

*Pvu*II

*Pvu*II showed the same results as *Bam*HI with common genotype A in the western populations, and common genotype C in the eastern populations apart from Surat.

*Sac*I

Genotype A was generally more common in the eastern populations apart from Kedah. Genotype B was common in the western populations apart from Trat and Surat.

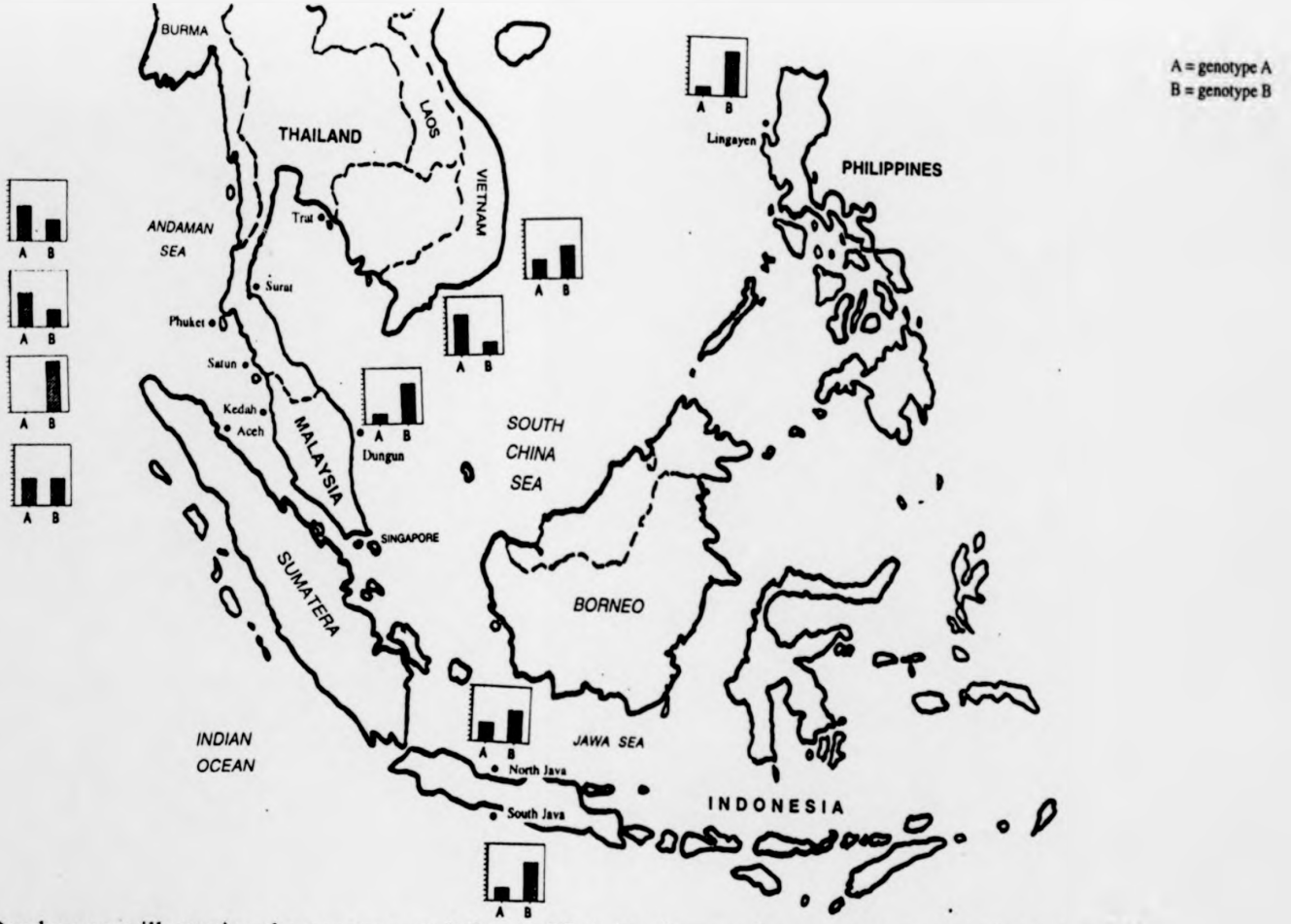


Fig. 3.10 A. Dendrograms illustrating the genotype variation in SE Asian *P. monodon* populations of individual mtDNA genotype frequencies at *Bam*HI.

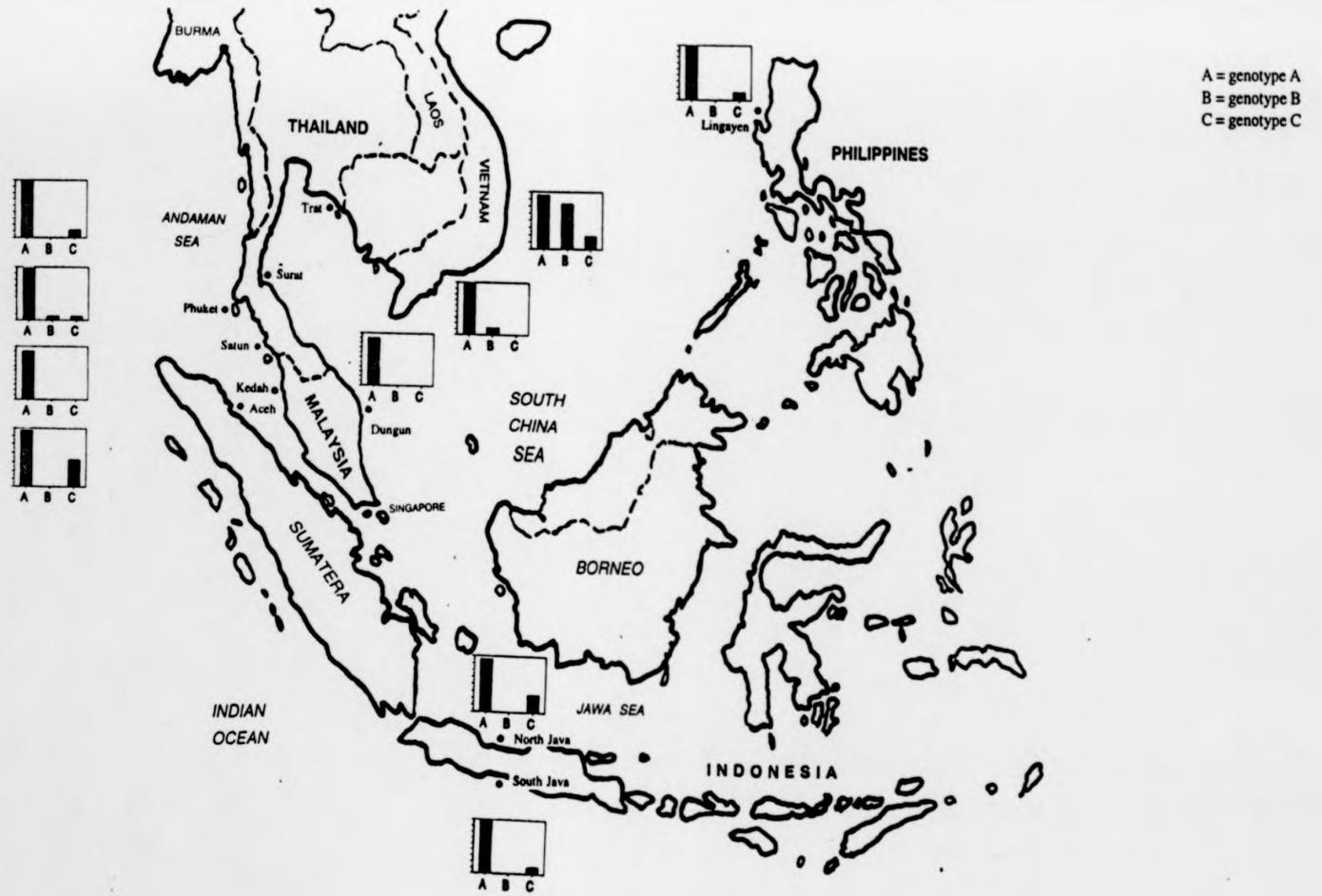


Fig. 3.10 B. Dendrograms illustrating the genotype variation in SE Asian *P. monodon* populations of individual mtDNA genotype frequencies at *Eco*RV.

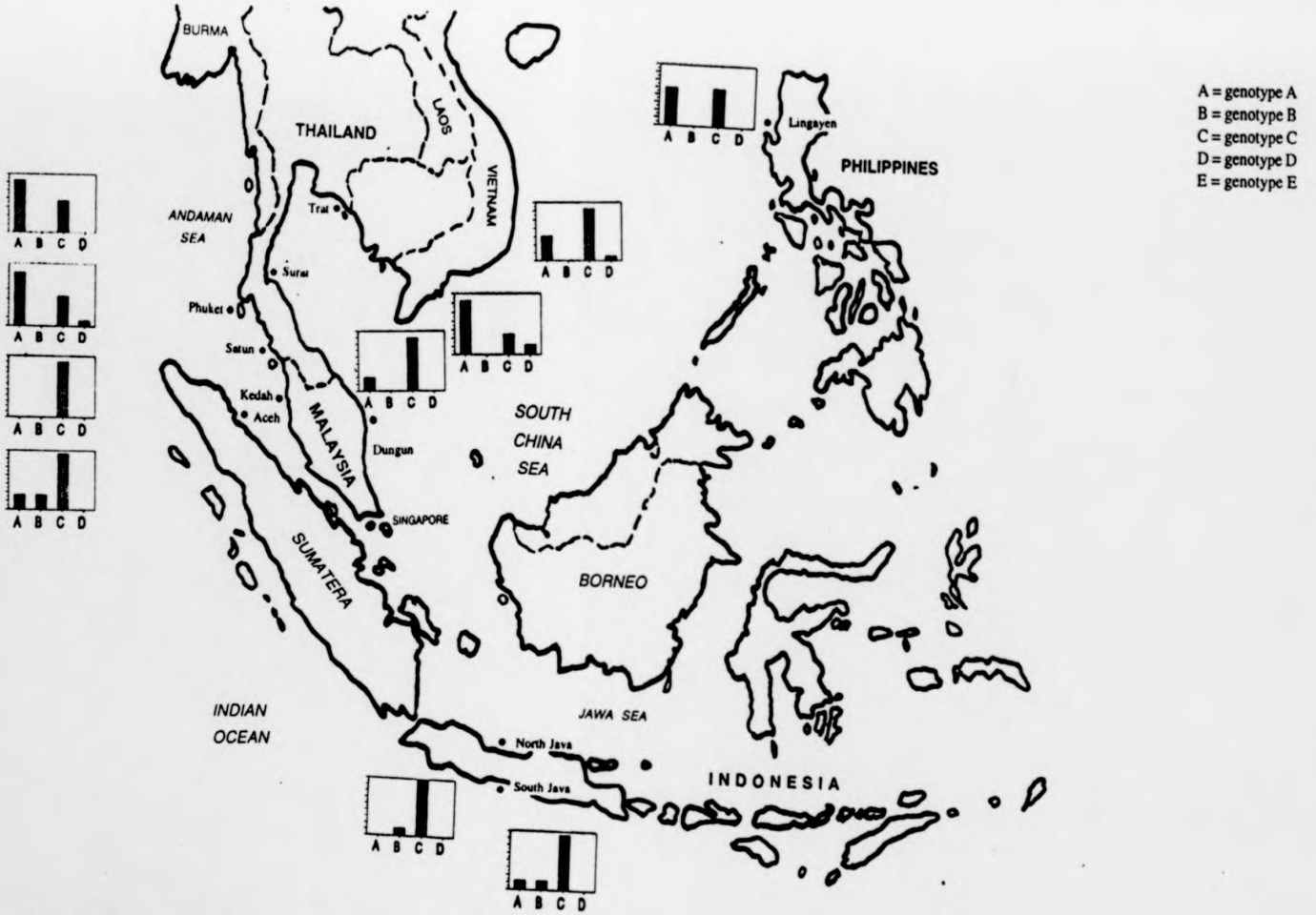


Fig. 3.10 C. Dendrograms illustrating the genotype variation in SE Asian *P. monodon* populations of individual mtDNA genotype frequencies at *Pvu*II.

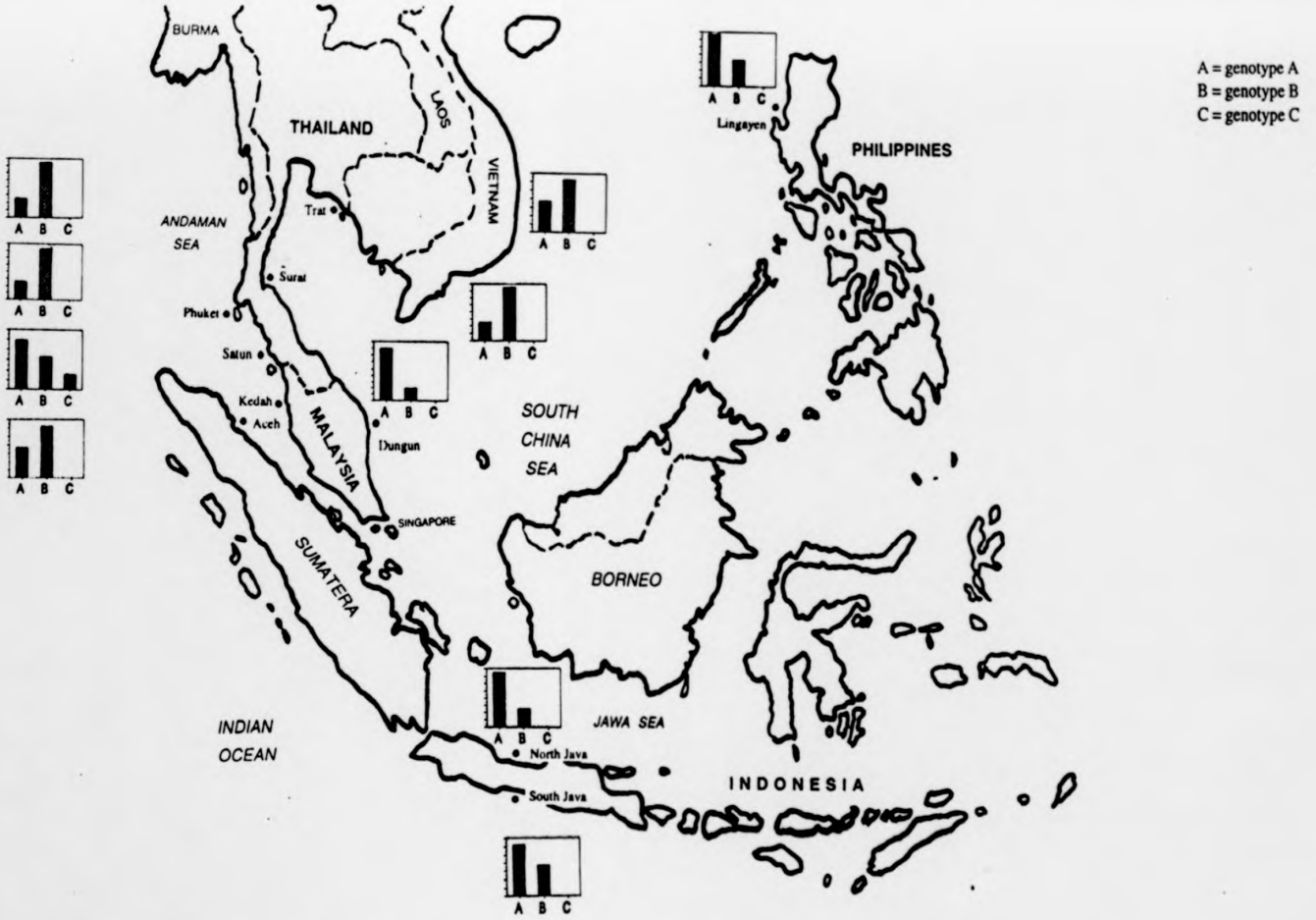


Fig. 3.10 D. Dendrograms illustrating the genotype variation in SE Asian *P. monodon* populations of individual mtDNA genotype frequencies at *Sac*I.

Table 3.4 Frequencies of individual mtDNA genotype in 10 populations of *P. monodon*: 1=Trat, 2=Surat, 3=Phuket, 4=Satun, 5=Kedah, 6=Dungun, 7=Aceh, 8=South Java, 9=North Java, 10=Philippines

	Population									
Locus	1	2	3	4	5	6	7	8	9	10
*Bam*HI										
A	.375	.750	.625	.667	-	.200	.500	.167	.250	.375
B	.625	.250	.375	.333	1.000	.800	.500	.833	.750	.625
*Eco*RV										
A	.750	.875	.875	.867	1.000	1.000	.875	.667	.875	.750
B	.063	.125	-	.067	-	-	-	-	-	-
C	.188	-	.125	.067	-	-	.125	.333	.125	.250
*Pvu*II										
A	.313	.625	.625	.600	-	.200	.500	.167	.125	-
B	-	-	-	-	-	-	-	.167	.125	.125
C	.625	.250	.375	.333	1.000	.800	.500	.667	.750	.875
D	.063	.125	-	.067	-	-	-	-	-	-
*Sac*I										
A	.375	.250	.250	.267	.500	.800	.375	.667	.625	.750
B	.625	.750	.750	.733	.333	.200	.625	.333	.375	.250
C	-	-	-	-	.167	-	-	-	-	-
n	16	8	8	15	6	10	8	6	8	8

3.3.2.4 Mitochondrial DNA haplotypes

Twelve different mtDNA haplotypes were resolved among ten populations using the four restriction enzymes, *Bam*HI, *Eco*RV, *Pvu*II and *Sac*I. Details of mtDNA haplotypes, composite mtDNA genotypes and number of samples for each collection location were summarised in Table 3.5 and Table 3.6. Descriptions of mtDNA haplotype and frequencies of mtDNA haplotypes for twelve locations before pooling were summarised in Table 3.7 A and 3.8 A, and ten populations after pooling were summarised in Table 3.7 B, 3.8 B and Fig. 3.11. There were two common mtDNA

haplotypes. Haplotype I designated AAAB found in 32 individuals and was the common haplotype in Surat, Phuket, Satun and Aceh populations. Haplotype VII designated BACA was found in 40 individuals and was the common haplotype in Trat, Kedah, Dungun, North Java, South Java and the Philippines populations. However, Kedah and the Philippines populations did not have haplotype I and the Satun 1992 population did not have haplotype VII. Haplotype IV and XII appeared in all Thai populations either side of the Peninsula but were not found in the geographically close populations in Malaysia. The only other occurrence was a haplotype II, V and VI in the Philippines population, haplotype III, X and XI in North and South Java populations, and haplotype IX in Kedah population.

Table 3.5 Mitochondrial DNA haplotype demonstrated in *P. monodon* using four endonucleases

Haplotypes	Restriction morphs			
	*Bam*HI	*Eco*RV	*Pvu*II	*Sac*I
I	A	A	A	B
II	A	A	C	A
III	A	A	C	B
IV	A	B	D	B
V	A	C	C	B
VI	B	A	B	A
VII	B	A	C	A
VIII	B	A	C	B
IX	B	A	C	C
X	B	C	B	B
XI	B	C	C	A
XII	B	C	C	B

Table 3.6 The mtDNA haplotype, composite mtDNA genotypes (*Bam*HI, *Eco*RV, *Pvu*II, and *Sac*I) of each collection location of *P. monodon*

Collection location	Year	mtDNA haplotype	Composite mtDNA genotypes	No. of samples
1 Trat (Thailand)	1992	I	AAAB	2
		IV	ABDB	1
		VII	BACA	2
		VIII	BACB	1
		XII	BCCB	2
	1993	I	AAAB	3
		VII	BACA	4
		XII	BCCB	1
2 Surat (Thailand)	1992	I	AAAB	5
		IV	ABDB	1
		VII	BACA	2
3 Phuket (Thailand)	1992	I	AAAB	5
		VII	BACA	2
		XII	BCCB	1
4 Satun (Thailand)	1992	I	AAAB	6
		XII	BCCB	1
	1993	I	AAAB	3
		IV	ABDB	1
		VII	BACA	4
5 Kedah (Malaysia)	1992	VII	BACA	3
		VIII	BACB	2
		IX	BACC	1
6 Dungun (Malaysia)	1992	I	AAAB	2
		VII	BACA	8
7 Aceh (Indonesia)	1993	I	AAAB	4
		VII	BACA	3
		XII	BCCB	1
8 S. Java (Indonesia)	1993	I	AAAB	1
		VII	BACA	3
		X	BCBB	1
		XI	BCCA	1
9 N. Java (Indonesia)	1993	I	AAAB	1
		III	AACB	1
		VII	BACA	5
		X	BCBB	1
10 Lingayen (Philippines)	1992	II	AACA	1
		V	ACCB	2
		VI	BABA	1
		VII	BACA	4

Table 3.7 Descriptions of the mtDNA haplotypes observed in *P. monodon*

A. Twelve locations before pooling

		Trat		Sura	Phuk	Satun		Keda	Dung	Aceh	SJa	NJa	Ling
		92	93	92	92	92	93	92	92	93	93	93	92
I	AAAB	2	3	5	5	6	3	-	2	4	1	1	-
II	AACA	-	-	-	-	-	-	-	-	-	-	-	1
III	AACB	-	-	-	-	-	-	-	-	-	-	1	-
IV	ABDB	1	-	1	-	-	1	-	-	-	-	-	-
V	ACCB	-	-	-	-	-	-	-	-	-	-	-	2
VI	BABA	-	-	-	-	-	-	-	-	-	-	-	1
VII	BACA	2	4	2	2	-	4	3	8	3	3	5	4
VIII	BACB	1	-	-	-	-	-	2	-	-	-	-	-
IX	BACC	-	-	-	-	-	-	1	-	-	-	-	-
X	BCBB	-	-	-	-	-	-	-	-	-	1	1	-
XI	BCCA	-	-	-	-	-	-	-	-	-	1	-	-
XII	BCCB	2	1	-	1	1	-	-	-	1	-	-	-
n		8	8	8	8	7	8	6	10	8	8	6	8

B. Ten populations after pooling

		Trat	Sura	Phuk	Satu	Keda	Dung	Aceh	SJa	NJa	Lin	Total
I	AAAB	5	5	5	9	-	2	4	1	1	-	32
II	AACA	-	-	-	-	-	-	-	-	-	1	1
III	AACB	-	-	-	-	-	-	-	-	1	-	1
IV	ABDB	1	1	-	1	-	-	-	-	-	-	3
V	ACCB	-	-	-	-	-	-	-	-	-	2	2
VI	BABA	-	-	-	-	-	-	-	-	-	1	1
VII	BACA	6	2	2	4	3	8	3	3	5	4	40
VIII	BACB	1	-	-	-	2	-	-	-	-	-	3
IX	BACC	-	-	-	-	1	-	-	-	-	-	1
X	BCBB	-	-	-	-	-	-	-	1	1	-	2
XI	BCCA	-	-	-	-	-	-	-	1	-	-	1
XII	BCCB	3	-	1	1	-	-	1	-	-	-	6
n		16	8	8	15	6	10	8	8	6	8	93

C. Ten populations for 2 clone haplotypes

	Trat	Sura	Phuk	Satu	Keda	Dung	Aceh	SJav	NJav	Ling	Total
A	6	6	5	10	-	2	4	1	2	3	39
B	10	2	3	5	6	8	4	5	6	5	54
n	16	8	8	15	6	10	8	8	6	8	93

Table 3.8 Frequencies of mtDNA haplotypes observed in *P. monodon*

A. Twelve locations before pooling

		Trat		Sura	Phuk	Satun		Keda	Dung	Aceh	SJa	NJa	Ling
		92	93	92	92	92	93	92	92	93	93	93	92
I	AAAB	.250	.375	.625	.625	.857	.375	-	.200	.500	.167	.125	-
II	AACA	-	-	-	-	-	-	-	-	-	-	-	.125
III	AACB	-	-	-	-	-	-	-	-	-	-	.125	-
IV	ABDB	.125	-	.125	-	-	.125	-	-	-	-	-	-
V	ACCB	-	-	-	-	-	-	-	-	-	-	-	.250
VI	BABA	-	-	-	-	-	-	-	-	-	-	-	.125
VII	BACA	.250	.500	.250	.250	-	.500	.500	.800	.375	.500	.625	.500
VIII	BACB	.125	-	-	-	-	-	.333	-	-	-	-	-
IX	BACC	-	-	-	-	-	-	.167	-	-	-	-	-
X	BCBB	-	-	-	-	-	-	-	-	-	.167	.625	-
XI	BCCA	-	-	-	-	-	-	-	-	-	.167	-	-
XII	BCCB	.250	.125	-	.125	.143	-	-	-	.125	-	-	-
n	8	8	8	8	7	8	6	10	8	6	8	8	

B. Ten populations after pooling

		Trat	Sura	Phuk	Satu	Keda	Dung	Aceh	SJa	NJa	Lin
I	AAAB	.313	.625	.625	.600	-	.200	.500	.167	.125	-
II	AACA	-	-	-	-	-	-	-	-	-	.125
III	AACB	-	-	-	-	-	-	-	-	.125	-
IV	ABDB	.062	.125	-	.067	-	-	-	-	-	-
V	ACCB	-	-	-	-	-	-	-	-	-	.250
VI	BABA	-	-	-	-	-	-	-	-	-	.125
VII	BACA	.375	.250	.250	.267	.500	.800	.375	.500	.625	.500
VIII	BACB	.062	-	-	-	.333	-	-	-	-	-
IX	BACC	-	-	-	-	.167	-	-	-	-	-
X	BCBB	-	-	-	-	-	-	-	.167	.125	-
XI	BCCA	-	-	-	-	-	-	-	.167	-	-
XII	BCCB	.188	-	.125	.067	-	-	.125	-	-	-
n		16	8	8	15	6	10	8	6	8	8

C. Ten populations for 2 clone haplotypes

	Trat	Sura	Phuk	Satu	Keda	Dung	Aceh	SJa	NJa	Lin
A	.375	.750	.625	.667	-	.200	.500	.167	.250	.375
B	.625	.250	.375	.333	1.00	.800	.500	.833	.750	.625
n	16	8	8	15	6	10	8	6	8	8

Fig. 3.11 Dendrograms illustrating the mtDNA haplotype variation in SE Asian *P. monodon* populations.

3.3.3 Mitochondrial DNA Variation

Intrapopulation mtDNA heterogeneity (*nucleotide diversity* (π) within population) corresponded to the number of shared fragments in each population. The values were slightly different between populations (Table 3.9), the lowest was in Kedah (0.011490) and the highest in South Java (0.065232) with an average of 0.0466. The Kedah population was the only one that showed obviously low nucleotide diversity because of the low level of mtDNA polymorphism (see Table 3.4.)

Table 3.9 MtDNA variation within population of *P. monodon*.

Collection sites	Nucleotide diversity
1 Trat	0.052896
2 Surat Thani	0.054663
3 Phuket	0.052824
4 Satun	0.052740
5 Kedah	0.011490
6 Dungun	0.037638
7 Aceh	0.059004
8 South Java	0.065232
9 North Java	0.041167
10 Philippines	0.038119
Average	0.046577 ±0.000023

3.3.4 Mitochondrial DNA Differentiation

The present study, the differences between populations are presented as genotype frequency differences therefore Nass chi-square statistical analyses were used to test individual and combined mtDNA genotype frequencies, and mtDNA haplotype frequencies differences. MtDNA haplotype frequencies were also analysed using Monte Carlo simulation techniques.

3.3.4.1 Between year

Samples from 2 populations in Thailand (Trat & Satun) were tested for temporal stability of mtDNA genotypes between the years 1992 and 1993. In the Trat population, all chi-square tests showed no significant difference between years (see Appendix 4). The Satun population exhibited significant differences at *Sac*I (P=0.03) and mtDNA haplotype frequencies (P=0.018). However, after correcting (the sequential Bonferroni test) there were no significant differences. Combined mtDNA genotype frequencies (P=0.220) and MtDNA haplotype frequencies using Monte Carlo simulation (P=0.05) also showed no significant differences. The result suggested stability of mtDNA genotype frequencies over time therefore the data from multiple collections at the same site were pooled for further analysis.

3.3.4.2 Between population

Pairwise comparisons showed 6 significant differences at individual mtDNA genotype frequencies as summarised in Table 3.10 A. The Philippines exhibited significant difference from Surat, Phuket and Satun at *Pvu*II. Kedah showed significant differences from Surat, Satun at *Bam*HI and *Pvu*II. Dungun was significant differences from Satun at *Sac*I.

9 significant differences were detected at combined mtDNA genotype frequencies as shown in Table 3.10 A. There were significant differences between the Philippines and Surat (P=0.020), Phuket (P=0.000) and Satun (P=0.030), and between Kedah and Surat (P=0.007) and Satun (P=0.017) as in the individual mtDNA genotype frequencies analyses. But significant differences between Dungun and Trat (P=0.007), Surat (P=0.009) and Phuket (P=0.048), and between Phuket and Kedah (P=0.030) were also observed. The combined mtDNA genotype frequencies between Dungun and Satun did not pick up the significant difference (P=0.070).

Pairwise comparisons of haplotype mtDNA frequencies, using both Nass chi-square analyses and Monte Carlo simulation techniques, gave very similar results apart from Phuket v.s. N Java and Dungun v.s. Philippines as summarised in Table 3.10 B. The Philippines population exhibited significantly different from Trat (P=0.025), Surat (P=0.016), Phuket (P=0.010), Satun (P=0.006), Aceh (P=0.042), and Dungun (P=0.014) but not Kedah (P=0.117), South Java (P=0.168), and North Java (P=0.194).

Table 3.10 Pairwise comparisons in mtDNA genotype and mtDNA haplotype frequencies in ten *P. monodon* populations.

A. Nass'chi-square analysis of individual mtDNA genotype is below the diagonal with *p*-values, and *p*-values for combined mtDNA genotype is above the diagonal.

Population	1	2	3	4	5	6	7	8	9	10
1 TRAT	*****	0.550	0.800	0.700	0.320	0.007*	0.610	0.180	0.160	0.600
2 SURAT	n.s.	*****	0.730	0.870	0.007*	0.009*	0.550	0.070	0.140	0.020*
3 PHUKET	n.s.	n.s.	*****	0.840	0.030*	0.048*	0.809	0.280	0.330	0.000*
4 SATUN	n.s.	n.s.	n.s.	*****	0.017*	0.070	0.850	0.090	0.130	0.030*
5 KEDAH	n.s.	*Bam*HI (0.006) *Pvu*II (0.005)	n.s.	*Bam*HI (0.006) *Pvu*II (0.015)	*****	0.720	0.160	0.680	0.110	0.600
6 DUNGUN	n.s.	n.s.	n.s.	*Sac*I (0.009)	n.s.	*****	0.420	0.700	0.330	0.650
7 ACEH	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*****	0.650	0.130	0.620
8 S JAVA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*****	0.820	0.550
9 N JAVA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*****	0.560
10 LINGAYEN	n.s.	*Pvu*II (0.001)	*Pvu*II (0.006)	*Pvu*II (0.003)	n.s.	n.s.	n.s.	n.s.	n.s.	*****

* = significant difference

B. *p*-values of mtDNA haplotype using Nass' chi-square analysis is below the diagonal, and using Monte Carlo simulation technique is above the diagonal.

Population	1	2	3	4	5	6	7	8	9	10
1 TRAT	*****	0.500	0.743	0.585	0.080	0.162	0.809	0.150	0.175	0.025*
2 SURAT	0.500	*****	0.753	0.876	0.012*	0.030*	0.405	0.074	0.049	0.016*
3 PHUKET	0.750	0.800	*****	0.900	0.019*	0.033*	0.607	0.090	0.040*	0.010*
4 SATUN	0.550	0.950	0.940	*****	0.006*	0.029*	0.808	0.168	0.016*	0.006*
5 KEDAH	0.120	0.018*	0.020*	0.008*	*****	0.042*	0.049	0.290	0.089	0.117
6 DUNGUN	0.240	0.028*	0.030*	0.018*	0.040*	*****	0.094	0.331	0.260	0.014*
7 ACEH	0.900	0.700	0.900	0.920	0.045*	0.130	*****	0.298	0.089	0.042*
8 S JAVA	0.290	0.180	0.180	0.100	0.270	0.320	0.380	*****	0.750	0.168
9 N JAVA	0.170	0.049	0.060	0.030*	0.210	0.540	0.200	0.900	*****	0.194
10 PHILIPPINES	0.019*	0.013*	0.008*	0.004*	0.190	0.050	0.004*	0.200	0.200	*****

* = significant difference

Indonesian populations, North and South of Java showed no evidence of mtDNA genetic differentiation (P=0.750). The North Java, but not the South Java, was significantly different from Surat (P=0.049), Phuket (P=0.040) and Satun (P=0.016) populations but no others.

Within the Andaman Sea populations, Phuket, Satun and Aceh appeared to be genetically homogeneous. But the Malaysian west coast population of Kedah appeared to be different to the adjacent Thai Andaman Sea populations [Phuket (P=0.019), Satun (P=0.006)], Aceh population (P=0.049), and the east coast Malaysian population of Dungun (P=0.042). Likewise Dungun is different to the adjacent Thai population of Surat (P=0.030) and the Andaman sea group [Phuket (P=0.033), Satun (P=0.029)].

The Gulf of Thailand population of Trat exhibited no significant differences with other populations apart from Philippines (P=0.025). Surat appeared to be significantly different from Kedah (P=0.012), North Java (P=0.049), and Philippines (P=0.016). Dungun also showed significant difference with Phuket (P=0.033), Satun (p=0.029), Kedah (P=0.042), and Philippines (P=0.014).

The results from the mtDNA individual genotypes, mtDNA haplotypes and all chi-square analyses of mtDNA restriction enzyme frequencies, appears to be separated *P. monodon* into two groups, i.e. Phuket, Satun, Aceh and Surat & Trat, Kedah, Dungun, South Java, North Java and Philippines. Moreover, the data suggested reproductive isolation of the Philippines population from Malaysian

populations, and the Andaman sea populations. Therefore there is evidence of geographic structuring of the breeding population between the Andaman Sea populations and South China Sea populations. Within this there appears to be two geographically aberrant populations-Surat which tends to group with the Andaman Sea and Kedah which appears to group with the South China Sea.

3.3.5 Genetic distance & UPGMA clustering dendrogram

Nei's and Rogers' genetic distances based on individual mtDNA genotype and mtDNA haplotype frequencies between all pairwise comparisons are presented in Table 3.11 A and B, and Fig. 3.12 A1, A2 and B1, B2 respectively. The genetic distance data gave essential very similar results. There were two main grouping which included all Thai populations, Aceh, and the other which included both Malaysian populations and the remaining South China Sea populations. These results were unexpected as they grouped populations either side of the peninsular together, e.g. Surat and Trat with Andaman Sea sites and Kedah with South China Sea sites.

Nucleotide divergence between all pairwise comparisons of ten populations were also calculated as summarised in Table 3.12 and Fig. 3.13. Average percentage of divergence was 0.80%, the highest range was 3.79% between South Java and Surat. This gave almost on identical dendrogram to that generated by Nei's and Rogers' genetic distances.

Table 3.11 Genetic distance values calculated from mtDNA genotype frequencies between ten populations of *P. monodon*.

A. Nei's (1978) unbiased genetic distance using individual mtDNA genotype is below the diagonal, and mtDNA haplotype is above the diagonal.

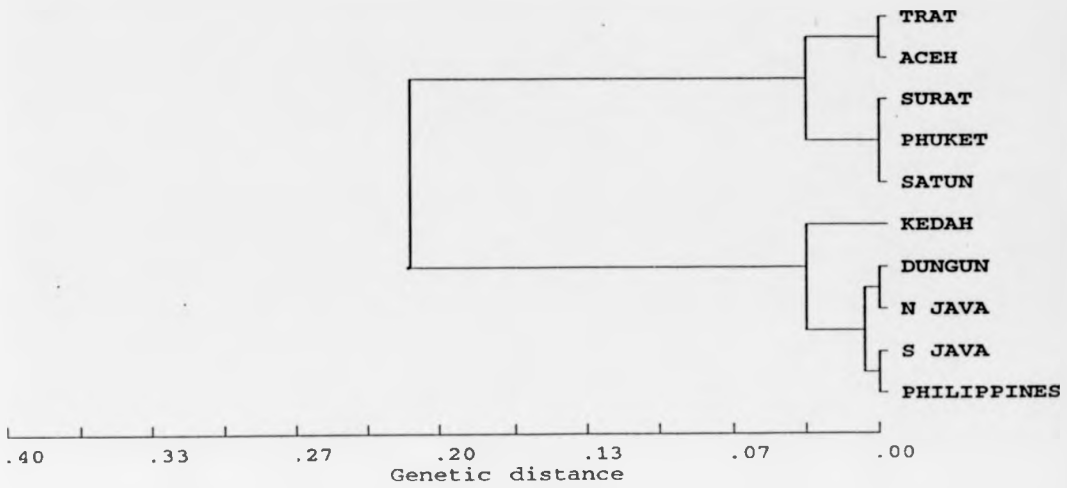
Population	1	2	3	4	5	6	7	8	9	10
1 TRAT	****	.035	.023	.025	.069	.060	.000	.021	.041	.074
2 SURAT	.103	****	.000	.000	.206	.153	.000	.094	.138	.193
3 PHUKET	.040	.000	****	.000	.206	.153	.000	.094	.138	.193
4 SATUN	.062	.000	.000	****	.194	.142	.000	.086	.127	.182
5 KEDAH	.098	.480	.340	.380	****	.062	.128	.041	.040	.052
6 DUNGUN	.079	.343	.248	.274	.029	****	.074	.015	.001	.049
7 ACEH	.000	.014	.000	.000	.176	.109	****	.037	.064	.116
8 S JAVA	.031	.355	.226	.266	.032	.008	.091	****	.000	.029
9 N JAVA	.018	.271	.174	.203	.013	.000	.056	.000	****	.027
10 PHILIPPINES	.068	.366	.262	.290	.057	.015	.122	.000	.000	****

B. Rogers' (1972) genetic distance using individual mtDNA genotype is below the diagonal, and mtDNA haplotype is above the diagonal.

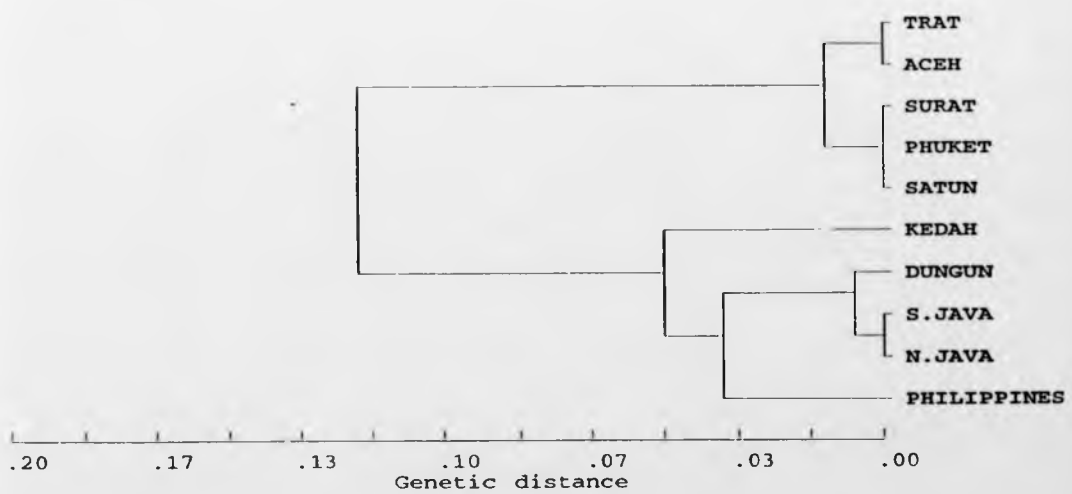
Population	1	2	3	4	5	6	7	8	9	10
1 TRAT	****	.140	.125	.119	.178	.172	.077	.130	.147	.177
2 SURAT	.253	****	.063	.033	.276	.250	.088	.207	.234	.265
3 PHUKET	.192	.094	****	.033	.276	.250	.063	.207	.234	.265
4 SATUN	.202	.059	.045	****	.265	.238	.061	.196	.222	.254
5 KEDAH	.300	.484	.435	.442	****	.183	.229	.167	.159	.171
6 DUNGUN	.245	.431	.381	.388	.165	****	.189	.135	.092	.167
7 ACEH	.100	.179	.094	.121	.345	.288	****	.157	.177	.217
8 S JAVA	.198	.438	.371	.387	.239	.163	.281	****	.088	.149
9 N JAVA	.168	.379	.300	.324	.185	.115	.208	.101	****	.140
10 PHILIPPINES	.184	.432	.362	.379	.243	.163	.269	.141	.125	****

Fig. 3.12 Dendrogram of ten populations of *P. monodon* derived by a UPGMA cluster analysis of genetic distance values. The software package BIOSYS-1 (Swafford and Selander, 1989) was used to produce the dendrogram.

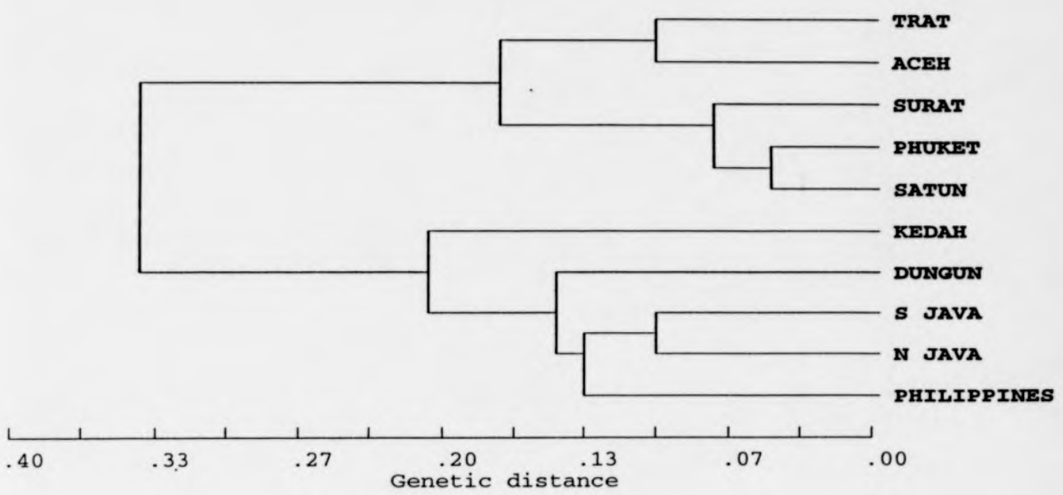
A1. Individual mtDNA genotype used Nei (1978) unbiased genetic distance



A2. MtDNA haplotype used Nei (1978) unbiased genetic distance



B1. Individual mtDNA genotype used Rogers (1972) genetic distance



B2. MtDNA haplotype used Rogers (1972) genetic distance

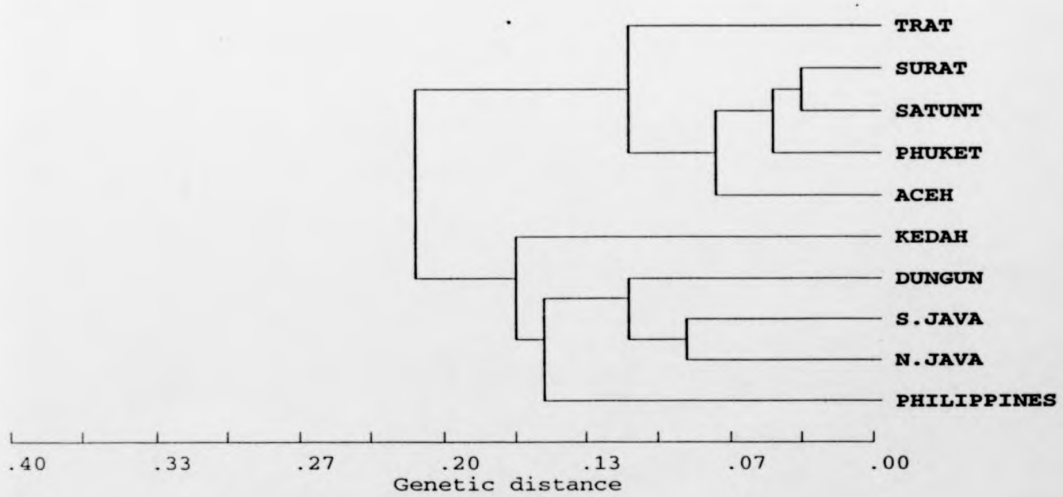
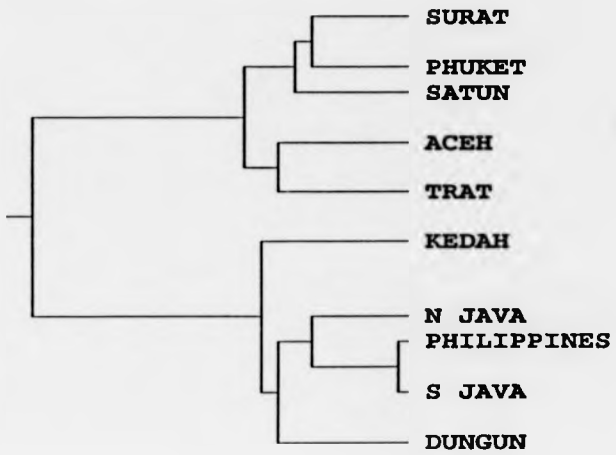


Table 3.12 Nucleotice divergence between ten populations of *P. monodon* calculated from 12 haplotypes.

Population	1	2	3	4	5	6	7	8	9
1 TRAT	*******								
2 SURAT	0.00396								
3 PHUKET	0.00044	0.00000							
4 SATUN	0.00251	0.00000	0.00000						
5 KEDAH	0.00910	0.03451	0.02619	0.02938					
6 DUNGUN	0.00460	0.02543	0.01801	0.02107	0.00000				
7 ACEH	0.00000	0.00000	0.00000	0.00000	0.01340	0.00698			
8 S JAVA	0.00466	0.03788	0.02972	0.03226	0.00000	0.00000	0.01479		
9 N JAVA	0.00052	0.01921	0.01230	0.01528	0.00000	0.00000	0.00261	0.00000	
10 Philippines	0.00471	0.02685	0.01679	0.02125	0.00030	0.00000	0.00596	0.00000	0.00000

Note: Average 0.00801
Max 0.03788

Fig. 3.13 Dendrogram of ten populations of *P. monodon* derived by a UPGMA cluster analysis of nucleotide divergence from 12 haplotypes. The software package PHYLIP (Felsenstein, 1995) was used to produce the dendrogram.



Considering both the genetic distance and nucleotide divergence and comparing this to the chi-square analysis gives a similar pattern of divergence Two groups, i.e. Phuket, Satun, Surat, Trat and Aceh group & Kedah, Dungun, South Java, North Java and the Philippines group.

To consider the evolutionary relationship, nucleotide substitutions (d) between haplotype were estimated (Table 3.13). Maximum mtDNA nucleotide divergence within *P. monodon* species was $d = 0.350$ between haplotype I and XI, and nucleotide divergence between the two common haplotypes I and VII was $d = 0.106$. To visualize the correlation between these twelve haplotypes, the UPGMA clustering dendrogram of nucleotide substitutions was computed and displayed in Fig. 3.14. The tree was divided into two groups, the first group included haplotypes I, II, III, IV and V, another group comprised haplotypes VI, VII, VIII, IX, X, XI and XII. The haplotype data of each group were therefore pooled together; i.e., clone A represented Haplotype I, II, III, IV & V, and clone B represented Haplotype VI, VII, VIII, IX, X, XI & XII. Descriptions of clonal haplotype and haplotype frequencies of ten populations were presented in Table 3.7 C and 3.8 C respectively. A dendrogram of the clonal haplotypes of each population is shown Fig. 3.15. It can be seen that clone A was more common in the Andaman groups apart from the Kedah population, and clone B was more dominant in the South China Sea groups apart from the Surat population.

Nucleotide divergence between all pairwise comparisons of ten populations was reanalysed as summarised in Table 3.14. The UPGMA clustering dendrogram of clonal haplotype (Fig. 3.16) did show two branches but with

different combinations. The first group consists of Phuket, Satun, Aceh and Surat, second group consists of Trat, Philippines, South Java, North Java, Dungun and Kedah. The dendrogram produced a different outcome from earlier procedures, the Trat population was moved to South China Sea group and Surat population was more isolated from the Andaman Sea populations.

In conclusion, *P. monodon* within SE Asian could generally be divided into two distinct groups: Andaman Sea and South China Sea. However, the Thai populations, Trat and Surat tended to group with the Andaman sea populations and Kedah an Andaman Sea population appeared to group with South China Sea populations. These results are discussed and possible explanations for this results.

Table 3.13 Nucleotide substitution between 12 haplotypes of *P. monodon*.

Haplotype	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
I	*******										
II	0.05441										
III	0.01814	0.01814									
IV	0.04549	0.07112	0.03245								
V	0.04057	0.04057	0.014884	0.03663							
VI	0.11174	0.04057	0.071122	0.35000	0.12226						
VII	0.10586	0.01814	0.040568	0.12226	0.07112	0.01488					
VIII	0.04057	0.04057	0.014884	0.05441	0.03245	0.03245	0.01488				
IX	0.11174	0.04057	0.045495	0.12701	0.07592	0.03245	0.01488	0.01920			
X	0.07592	0.12226	0.054414	0.08864	0.02708	0.02708	0.05441	0.02708	0.05848		
XI	0.35000	0.04057	0.071122	0.12701	0.03245	0.03245	0.01488	0.03245	0.03245	0.02708	
XII	0.07112	0.07112	0.032453	0.05848	0.01262	0.05441	0.03245	0.01262	0.03663	0.010956	0.01262

Table 3.14 Nucleotice divergence between ten populations of *P. monodon* calculated from 2 clone haplotypes

Population	1	2	3	4	5	6	7	8	9
1 Trat	*******								
2 Surat	0.01040								
3 Phuket	0.00142	0.00000							
4 Satun	0.00567	0.00000	0.00000						
5 Kedah	0.01323	0.05671	0.03781	0.04537					
6 Dungun	0.00000	0.02730	0.01369	0.01949	0.00235				
7 Aceh	0.00000	0.00000	0.00000	0.00000	0.02268	0.00386			
8 S Java	0.00000	0.03024	0.01575	0.02184	0.00000	0.00000	0.00504		
9 N Java	0.00000	0.02079	0.00851	0.01386	0.00378	0.00000	0.00000	0.00000	
10 Philippines	0.00000	0.00851	0.00000	0.00378	0.01134	0.00000	0.00000	0.00000	0.00000

Fig. 3.14 Phylogenic relationship of 12 haplotypes derived by UPGMA cluster analysis of base substitution. The software package PHYLIP (Felsenstein, 1995) was used to produce the dendrogram.

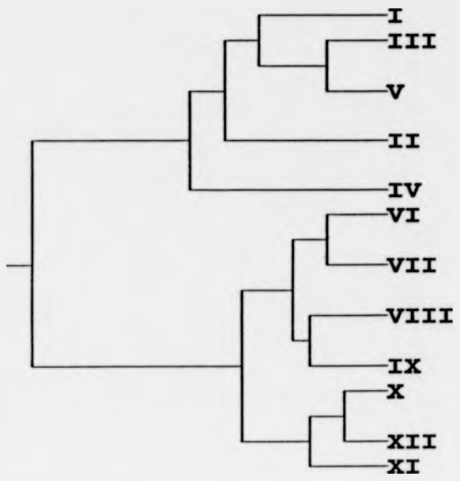
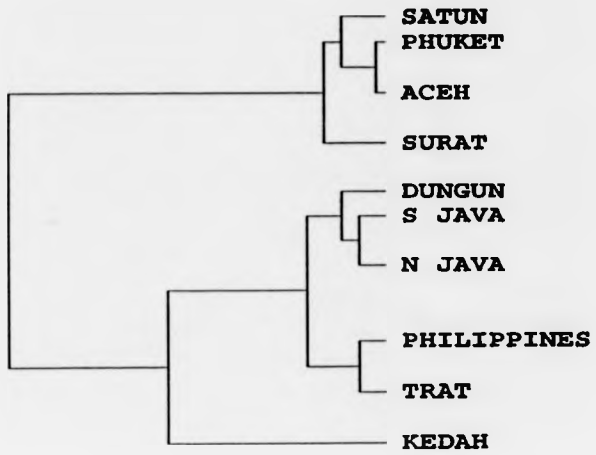


Fig. 3.15 Dendrograms illustrating 2 clonal haplotype variation in SE Asian *P. monodon* populations.

Fig. 3.16 Dendrogram of ten populations of *P. monodon* derived by a UPGMA cluster analysis of nucleotide divergence from 2 clone haplotypes. The software package PHYLIP (Felsenstein, 1995) was used to produce the dendrogram.



3.4. DISCUSSION

Using ultracentrifugation on CsCL-ethidium bromide density gradients, a time consuming method for the isolation of mtDNA, Benzie *et al.* (1993) obtained a yield of 10 digests from *P. monodon* fresh samples (4 digests from muscle and 6 digests from fresh mature oocytes), but this reducing to 1-2 digests from frozen muscle. Lansman *et al.* (1981) reported a yield of 3 µg per gram wet weight tissues of adult mouse (*Peromyscus maniculatus*). The liver, heart and kidneys of the rodents weight about 2 g can be digested and give about 10 reactions. Chapman and Brown (1990) summarised that most labs claim yields of 0.1 to 1.0 µg per gram tissue from caesium gradients. Using a simplified procedure, Powel and Zuniga (1983) obtained mtDNA from 75 mg of fresh tissues of mixed sex *Drosophila pseudoobscura* (50 adult) to perform only one digestion. Bouchon *et al.* (1994) detected an average mtDNA of 7 µg/g from frozen ovaries of *P. monodon* which provided theoretically enough DNA for 17 digests per animal. But because of storage problems during transport the yield was reduced to 10 digests per sample. In the present study, frozen samples were used for allozyme analysis and the difficulties of transportation and storage meant that muscle, hepatopancreas and ovaries were highly contaminated with nuclear DNA. We therefore extracted total DNA from pleopod and detected mtDNA fragments using radiolabelling techniques. However, the method gave high resolution, one pleopod yielding up to 7 digests. In addition, by using pleopod as a tissues sample we need not sacrifice the animal being studied.

The radiolabelling technique used in this study is one of the best methods

routinely used to detect small DNA fragments (Feinberg and Vogelstein, 1983; Chapman and Brown, 1990). However, the use of photographic methods as an alternatives to radiolabelling has several advantages such as speed, reduced expense and increased safety as recommended by Chapman and Brown (1989). The smallest fragments they could detect was 1.2 kb. Benzie *et al.* (1993) and Bouchon *et al.* (1994) reported the smallest fragments they could detect on ethidium bromide stained agarose gel of purified mtDNA digestion patterns of *P. monodon* was 1 kb. Ethidium bromide staining for the present study which can be seen on negative of Polaroid film was consistent with autoradiograph of of DNA/DNA hybridization gel. The smallest fragments routinely detected was the 2.6 kb of *Pvu*II but 1.5 kb fragment was not seen using ethidium bromide. However because we knew the mtDNA patterns of animal being studied we therefore could used ethidium bromide staining for this mtDNA RFLPs study.

The size of the mtDNA genome of *P. monodon* was shown to be comparable with other invertebrate animals. There was no evidence of size variation among the mitochondrial genomes surveyed. The molecular size of about 16 kb pairs (15.76±0.57) is consistent with mtDNA studied in the other marine crustacea: *Panulirus argus* (Komm *et al.*, 1982; McLean *et al.*, 1983), *Artemia salina* (Batuecas *et al.*, 1988), *Jasus spp.* (Brasher *et al.*, 1992), *Penaeus notialis* and *P. schmitti* (Monnerot *et al.*, 1992). Within the same species, the size of *P. monodon* mtDNA is consistent with the studies of Bouchon *et al.* (1994) of 15.83±0.16 kb in laboratory strains of Fijian, Malaysian and Australian populations. Benzie *et al.* (1993) unexplained restriction morph had a total mtDNA of 17.7 kb in three Australian populations.

The fragment patterns of all five enzyme (*Bam*HI, *Eco*RV, *Hind*III, *Pvu*II and *Sac*I) of *P. monodon* mainly agreed with the studies of the same species (Benzie *et al.*, 1993; Bouchon *et al.*, 1994). Bouchon *et al.* (1994) digested total mtDNA of *P. monodon* with twelve restriction enzymes; nine of them were 6-base cutters: *Bam*HI, *Bgl*II, *Cla*I, *Eco*RV, *Pst*I, *Pvu*II, *Sac*I, *Stu*I and *Sty*I, two were 4-base cutters: *Hha*I and *Msp*I, and one a 7-base cutter: *Eco*O109. Only three restriction enzymes exhibited polymorphism, i.e. *Bgl*II, *Hha*I and *Pst*I. Benzie *et al.* (1993) observed four polymorphic enzymes, i.e. *Bam*HI, *Eco*RV, *Sac*I and *Eco*O109, three of these giving similar results in the present study.

MtDNA differentiation between year classes was not observed between population studies of *P. monodon* (Trat and Satun). The result demonstrated that there was stability of mtDNA both in the Gulf of Thailand and the Andaman Sea confirming the results of the allozyme study. For mtDNA genotypes differentiation, the distinct break points in the mtDNA genotype by having several mutation steps and clearly identifying a phylogenetic split were observed (e.g. Lansman *et al.*, 1983; Saunders *et al.*, 1986). Even though such a clear cut difference this has not been detected in the present study a preponderance of certain haplotypes in a given area was observed. Haplotype I, AAAB, which was common in Andaman Sea groups might be ancestral of this area. Haplotype VII, BACA, the common haplotype in South China Sea groups could be derived from this area. These two populations might be separated by Malaysian land mass barrier since Quaternary Glacial Period as same as the explanation in allozyme chapter. After the rising of sea level the species was combined exhibiting two common haplotypes in both areas. However there is strong current between these

two areas (Dale, 1956; Open University, 1989) the species can not recolonize therefore the result showed evidence of geographic structuring of *P. monodon* breeding population between these two areas.

Mitochondrial DNA analysis has been widely applied to population genetic studies, however there have been few investigation of penaeid prawn species. Previous surveys showed that marine species appeared to have lower mtDNA sequence diversity (lower than 1.0%) than terrestrial and freshwater species (Ovendon, 1990). Data also suggested that some marine species may show little or no mtDNA differentiation over large geographic areas (Avise, 1985). In the present study, the observation of mtDNA variation in *P. monodon* was below average (divergence $\approx$ 0.80%), the maximum value of divergence (3.79%) was between Surat and South Java. The average divergence between two separate groups (Trat, Surat, Phuket Satun and Aceh group, & Kedah, Dungun, South Java, North Java and Lingayen group) is also high at 1.80%. The result suggest that there is more mtDNA variation in *P. monodon* than has been thought.

Analysis of mtDNA has been used to find out a species history. Moritz *et al.* (1987) mentioned that mtDNA diversity within species is correlated with the time since a maternal ancestor was last shared which may correspond to a reduction in numbers or to the origin of the species at some time in the past. Brown *et al.* (1982) estimated the rate of base substitution of mammalian mtDNA $\lambda = 10^{-8}$ per site per year. Based on the assumption that the base substitution rate of *P. monodon* is not substantially different from that of mammalian mtDNA. From assumption that $T = d_A/2\lambda$ (Nei, 1987), the nucleotide substitution of 0.0822

between the two main groups of *P. monodon* haplotype (group A comprise of haplotype I, II, III, IV & V and group B comprise of haplotype VI, VII, VIII, IX, X, XI & XII, see Table 3.13 and Fig. 3.14), could have been generated from a common haplotype some 4.11 million years ago. With the average nucleotide divergence between Andaman Sea and South China Sea groups $d_A = 0.018$, the time since divergence between these populations would be about 0.9 million years ago. *P. monodon* population between these two areas therefore could be separated in the late Ice age after the separation of the two main haplotypes. This result supported by Nei (1987) that the time of gene splitting is usually much earlier than the time of population splitting.

Benzie *et al.* (1993) and Bouchon *et al.* (1994) both detected mtDNA genetic difference between *P. monodon* populations. Benzie *et al.* (1993) using mtDNA genotype frequencies reported a significant difference between *P. monodon* wild populations from east and west Australian coasts (χ^2=7.23, d.f.=2, P<0.05) but not among the east coast populations (χ^2=1.0, d.f.=2, P>0.5). Bouchon *et al.* (1994) detected high intraspecific mtDNA sequence divergence between the Fijian strain and the Australia/Malaysia strains of 1.68%. These three strains were obtained from laboratory stock. Though they may represent a natural population but not a real sample size, there was no variation within any population. However, Bouchon *et al.* (1994) concluded that geographic structuring of the breeding population of *P. monodon* within the Indo-West Pacific zoogeographical region appears to be separated into three groups: Indian Ocean, West Pacific and Melanesian Archipelago.

In the present study, clustering dendrograms using single and multiple mtDNA genotype frequencies of Nei's and Rogers' distance, and nucleotide divergence have corresponding identified two separate groups: Trat, Surat, Phuket Satun and Aceh group & Kedah, Dungun, South Java, North Java and Lingayen (see Fig. 3.12 A & B, Fig. 3.13). The haplotype divergence and phylogeny dendrograms suggest *P. monodon* within SE Asia may be divided into two genetically distinct groups: Andanan Sea group (Phuket, Satun, Aceh, Trat and Surat) & South China Sea group (Dungun, Kedah, South Java, North Java and Philippines). The clustering dendrogram using the 2 clonal haplotypes puts Kedah in the South China Sea group, and Surat in the Andaman Sea group. From the clonal genotype frequencies it can be seen that clone A is dominant in the Andaman Sea area except Kedah, and clone B is dominant in the South China area except for Surat. Why should these three populations (Trat, Surat and Kedah) break a general pattern of geographic isolation. First, sample sizes in this study are small and the potential number of genotypes in any population is quite high, diversity about 4.67%. This could result in sampling error so that some genotypes may be missed, e.g. Satun 92-93 samples. Larger sample sizes would be required to ensure that all possible haplotypes are obtained from each site. Second, the populations being analysed are disturbed and may not be typical of the area. This is unlikely to be the case for Kedah as this is a relatively underdeveloped area and little or no prawn farming. However Surat is the center of prawn farming in the Gulf and local population may well contain many farm escapees and recruits from enhancements. Many of the broodstock used in this area come from the Andaman Sea. Third, selection operating to maintain these differences, some haplotypes may be selective control. Finally, the majority of

P. monodon that are imported are females and any effects of farming are more likely to show up in the maternally inherited mtDNA than at the allozyme level.

The conclusion of the present study that the Andaman Sea populations and South China Sea populations of *P. monodon* could be effectively genetically isolated has been found by Benzie *et al. (1993)* in Australia and postulated by Bouchon *et al.* (1994) in their study. As mention in chapter 2 the differentiation today is depended on the former separation of *P. monodon* population since the Quaternary Glacial Period (Dall *et al.*, 1990). Because sea levels were lower than today, the Malaysian land mass acted as reproductive barrier giving a very different pattern of gene flow than we see today. Other examples of reproductive barriers caused by the formation of land bridges were either by falling of sea levels (Bassian Isthmus; Galloway and Kemp, 1981) or rising of continents (Isthmus of Panama; Vawter *et al.*, 1980). The clones A and B appear to have been separated about $5x10^6$ year ago would this correspond with the above assumption. As the fact that many marine fishes and invertebrates have immense dispersal capabilities at some stages of life cycle (Burton, 1983; Avise, 1985; Ovendon 1990). Subsequently, by the tremendous dispersal ability of *P. monodon* within two areas the present study therefore showed highly polymorphic of mtDNA genotypes in all populations. However, it can be seen that gene flow has been insufficient to produce genetic homogeneity in *P. monodon*, genetic differentiation between two areas therefore still exist.

CHAPTER 4

GENERAL DISCUSSION

&

CONCLUSION

The allozyme data suggested that there were at least 3 partially isolated populations of *P. monodon* in SE Asia, i.e. Phuket, Satun, Kedah and Aceh within Andaman Sea area; North Java and South Java; Surat Thani and Dungun in the east of Malaysian Peninsula. The Trat population does show significant differences from the East Malaysian populations but the hydrological information suggests that may be it should be part of the same grouping. Certainly the Northern part of the Gulf of Thailand is isolated from the main monsoonal wind driven sytems, and populations entrained within this gyre may remain isolated from others in the South China Sea.

The Gulf of Thailand is the origin of the Thai prawn farming industry and its natural fisheries have been heavily exploited in recent years. This has undoubtedly had an effect on the population structure of the local prawn species. In response to the demands of fishermen the Department of Fisheries has extensively restocked the Gulf of Thailand with larval prawns, over 30 millions per year. Many of these broodstock werecollected from the relatively healthy Andaman Sea populations. The rapid and uncontrolled expansion of the prawn

farming industry and the often turbulent weather patterns in this part of the world have resulted in long term chronic escapes and catastrophic releases associated with floods etc. It would be impossible to imagine that this level of gene transfer has not had some impact on the observed levels of genetic differentiation in particularly on the Surat population. The Trat area because of its isolation and the ability of farmers still to obtain broodstock from the gulf area to the east (Cambodia), may be less effected by transfers compared to the western shore of the Gulf; even though the current patterns in this area would suggest that mixing in inevitable.

It is a pity that we do not have examples of prawns from the Andaman Sea and Gulf of Thailand prior to the farming activities. This material may show marker alleles or RFLPs characteristic of these areas which might enable us to estimate the possible levels of introgression. The overall picture is that of an Andaman Sea population isolated from the South China Sea by the Malacca straits. Today most of the gene flow is probably from the Andaman Sea to the Gulf of Thailand because of restocking and farming. The South China Sea populations appear to be fairly homogeneous from Java to the Gulf of Thailand. This is not surprising because of the strong reversible wind generated currents through this area which could result in considerable dispersion from any spawning area in any direction depending on the time of spawning.

The Kenya and the Philippine populations appear to be well separated from the other populations studied here on the basis of their genetic differences which is probably related to the historic isolation patterns associated with sea

level changes during the last glaciation and their geographic remoteness to Thailand, Malaysia and Indonesia.

The mtDNA data divides the *P. monodon* populations studied into two main groups: the Andaman Sea and the South China Sea populations. The differences between these areas were probably more obvious using this technique as shown by the various dendrograms. The small sample sizes make some of the individual enzyme data suspect but the discovery of two main clonal lineages A & B and their dominant haplotypes AAAB (I) & BACA (VII) and their predominance in the two sea areas is very clear. Once again we have the problem of a disturbed population structure but presumably this is mostly in one direction from Andaman Sea to South China Sea. Looking at the distribution of these two clones, in all Andaman Sea populations apart from Kedah the A clone predominates and in all South China Sea populations apart from Surat the B clone predominates even in the Philippines. The estimated time of divergence between these clones is about 0.1-0.2 million years b.p. would suggest that the population had been isolated into two main groups one in the west and the other in the east and what we see today is a result of secondary mixing between these isolates as sea levels rose and new areas where recolonized. The level of mixing being determined by the prevailing current patterns or the order in which the stocks began to combine. Today the Andaman Sea and South China Sea are joined by the Malacca straits which is narrow and has a NW current pattern throughout the year. So any mixing will be from South to North.

The more clear cut differences we see using mtDNA is related to the maternal clonal inheritance. The original clones remaining intact and not being recombined as in the case of the nuclear genome. It would be interesting to obtain more populations from the west to see whether the A clone becomes more frequent. It is a pity that the Kenya samples could not be used as this might have given some indication of the extent of any secondary mixing. The finding of Benzie *et al.* (1993) in Australian penaeid and those in this study suggest that sea level changes associated with glaciation have had as much effect on population divergence.

The transfer of stocks between areas without consideration of the genetic attributes of the donor stock and indigenous populations in the areas of introduction could be harmful. Hybridization between stocks has occasionally been proposed as a means of increasing genetic variation and production characteristics of natural populations (Ihssen, 1976; Ovendon, 1990). In 1978, Moav *et al.* proposed for "*genetic improvement*" of wild fish populations which had suffered from "*genetic deterioration*" could be improved then by breeding with pollution-resistant genotypes. But most geneticists think that it is a model for the loss of genetic diversity, as Nelson & Soulé (1986) stressed that if there exist coadapted groups of genes within each subpopulation, combination may result in the breakup of the adaptedness of each subpopulation to its environment. This can be seen in a case in which minnows introduced to hybridize with bait (*Gala orcutti*) and seemed to supplant an apparently less well adapted native chub (*Gala mojavensis*) (Hubbs, 1955 cited by Waldman and Wirgin, 1994). Ovendon *et al.* (1988) also commented that introduced *Gadopsis marmoratus* in southern

Tasmania has complicated the resolution of evolution in the species. Examining stocked populations from known origins, Billington *et al.* (1992) confirmed that such populations show similar proportions of mtDNA haplotypes as their source populations. The genetic implications of introduced fishes on native species have been well documented by Ferguson (1990). He concluded that the introduction of alien fish can destroy the genetics and adaptation of local populations

The present results suggest the giant tiger prawn *P. monodon* may have a more structured population than has been thought. It is cleare that geographic structuring of the breeding populations of *P. monodon* exists in the wild. As yet no comparative testing on the genetic differences between these stocks has been undertake. The present preference for Andaman Sea prawns for aquaculture may be because of environmentally induced traits such as size and fecundity but this study clearly shows that we have two different stocks which have a reasonable large degree of isolation. Commercial preference may well be related to underlying genetic differences. Such genetic stocks are the fundamental reproductive units of species and require fisheries management policies taking this population structuring into account. There is a conflict in the use and management of natural resources leading to overexploitation of wild stocks, especially for broodstock, and a trade which results the movement of *P. monodon* broodstock and larvae all over SE Asia. This complex problem requires concerted efforts from intergovernment organizations to define and implement policies on the conservation of these natural resources. The management of *P. monodon* should be based on each population and therefore should be harvested and treated separately in research as well as management policy. Ryder *et al.*

(1981) also suggested that restoration of the former ecosystem should be the aim of rehabilitation, not the development of stocks that can survive under the current degraded environment. The management practices on fish populations can be examined by the use of genetically marked fish. A number of studies have begun assessment of allozyme variation and mtDNA variation and found variation between both wild and hatcheries stocks in allozyme studies (Sbordoni *et al.*, 1987) and mtDNA studies (Gyllensten and Wilson, 1987; Palva and Palva, 1987; Grewe and Hebert, 1988; Hynes *et al.*, 1989; Palva *et al.*, 1989; Danzmann *et al.*, 1993; Ferguson *et al.*, 1993).

In the present study although we found low level of allozyme variation the data showed evidence of geographic structuring of the *P. monodon* breeding population. Unfortunately no stock diagnostic allozyme markers are available in the species which limits its usefulness in stock assessment, pedigreeing and selection programs. However, this technique is practically suited to population studies: it is relatively easy and inexpensive technique, it is a fairly rapid procedure to perform on a large scale, and a number of loci can be screened simultaneously. Allozyme electrophoresis therefore remains an important tool and becomes the first method for studying and understanding population genetic structure of such a species, but not the technique of choice for prawns because of the low levels of variation. In the mtDNA study, there was no occurrence of distinct mtDNA genotypes population structure in *P. monodon*. This might be the case for the species as there is no obvious barrier within the area being studied and their high larval dispersal capabilities making the species homogeneous over large geographic areas. Detection of dominant haplotypes in

Andaman Sea and South China Sea populations suggested mtDNA divergence between two areas. It may be possible by using larger sample sizes and more enzymes to detect greater structure with this species. Moreover, there are new DNA-level research techniques with greater impact for detecting polymorphisms, i.e. DNA fingerprinting (Jefferies *et al.*, 1985) and PCR (polymerase chain reaction) technique (Saiki *et al.*, 1988). The former reveals enormous variability in a wide range of organisms but it is difficult to determine which bands in a DNA fingerprint are allelic (Park and Moran, 1994). Therefore we can not evaluate Hardy-Weinberg equilibrium making it less powerful than allozymes detection for breeding structure studies. Lynch (1989) also commented that multiple-site VNTRs (variable number tandem repeat) cannot be used to determine degree of relatedness beyond parent-offspring, and cannot be used to measure the level of inbreeding. The PCR technique for enzymatic amplification of specific DNA sequences has a number of advantages: it is very rapid, it can utilize tiny quantities of tissue (Beckenbach, 1991; Park and Moran, 1994). However, PCR assays require sequence information (primers) which is presently not available in *P. monodon* and takes time to produce. However, it appears that the high levels of genetic variability that can be detected particularly using microsatellite VNTR'S may be a very powerful tool for resolving population structure and even monitoring the structure in farmed populations.

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APPENDIX 1

ELECTROPHORESIS BUFFERS & BIOCHEMICAL STAINING RECIPES

1. Electrophoresis Buffers

CTC: Continuous tris-citrate buffer, pH 8.0

Electrode buffer:

Tris 0.25 M	30.3	g/l
Citric acid 0.075 M	15.8	g/l

Mix: Adjust pH with 4N NaOH

Gel buffer: Dilute electrode buffer 1 : 25

Mix: 8.8 ml electrode buffer + 211.2 ml dH_2O up to 220 ml

Electrophoresis: 150 V, 30 mA, 4 h

TBE: Tris-borate-EDTA, pH 8.5 (Ferguson,1984)

Elecctrode buffer:

Tris 0.5 M	60.6	g/l
Boric acid 0.25 M	15.0	g/l
EDTA (Na_2 salt) 0.016 M	6.0	g/l

Mix: adjust pH with HCL

Gel buffer: Dilute electrode buffer 1 : 10

Mix: 22 ml electrode buffer + 198 ml dH_2O up to 220 ml

Electrophoresis: 180-200 V, 30-32 mA, 5-5½ h

TCB: Tris-citrate-borate, pH 8.6 (Ferguson,1984)
(Lithium-borate/Tris-citrate)

Electrode buffer, pH 8.6

Boric acid 0.3 M	18.6	g/l
LiOH 0.1 M	4.2	g/l

Mix: adjust pH with NaOH, HCL

Gel buffer, pH 8.6

Tris 0.076 M	9.2	g/l
Citric acid 0.005 M	1.1	g/l
Electrode buffer 5.3%	53.0	ml/l
(Boric acid 0.015 M, LiOH 0.005M)		

Electrophoresis: 150 V, 45 mA, 4 h

TCE: Tris-citrate-EDTA, pH 7.0 (Hillis 1988)

Electrode buffer:

Tris 0.135 M	16.4	g/l
EDTA (Na_2 salt) 0.0013 M	0.4	g/l
Citric acid 0.045 M	9.5	g/l

Gel buffer: Dilute electrode buffer 1 : 14

Mix: 15.7 ml electrode buffer + 204.3 ml dH_2O up to 220 ml

Electrophoresis: 150-170 V, 28-30 mA, 5-5½ h

2. Staining Recipes

Aspartate aminotransferase (AAT) **EC 2.6.1.1**
[glutamic-oxaloacetic transaminase (GOT)]

Substrate	20 ml
Pyridoxal-5-phosphate	1 mg
Fast Blue BB salt	50 mg
2% Agar	20 ml

Substrate:

0.2 M Tris-HCL, pH 8.0	20 ml
L-aspartic acid	80 mg
α-ketoglutaric acid	40 mg

Readjust the pH to 8.0 with NaOH, store at 4°C

Fix: Glycerine
Comments: Rapid stain, final pH 8.0 is critical to the success of the stain

Acid phosphatase (ACP) **EC 3.1.3.2**

α-Naphthyl â phosphate	80 mg
Polyvinylpyrrolidone	400 mg
Fast blue BB salt or fast garnet GBC salt	80 mg
0.2 Na-acetate buffer or PO_4-citric â, pH 5.5	40 ml
Distilled water	40 ml

Substrate: Dissolved α-naphthyl â phosphate in 1 ml acetone
Fix: Acid-alcohol
Comments: Pre-soak gel 1 h in stain buffer before staining

Adenosine Deaminase (ADA) **EC 3.5.4.4**

Adenosine	40 mg
MTT or NBT	5 mg
PMS	1 mg
Xanthine oxidase	0.2 u
Nucleoside phosphorylase	1 u
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

Adenylate kinase (AK) **EC 2.7.4.3**

Glucose	80 mg
ADP	20 mg
NADP	5 mg
$MgCL_2$	40 mg
MTT or NBT	5 mg
PMS	1 mg
G6PDH	15 u
Hexokinase	20 u
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

Alanine aminotransferase (ALAT) **EC 2.6.1.2**
[Glutamic-pyruvate transaminase (GPT)]

L-Alanine or DL-alanine	400 mg
α-Ketoglutaric â	40 mg
NADH	10 mg
Lactate dehydrogenase	200 u
0.2 M Tris-HCL, pH 7.5	20 ml
2% Agar	20 ml

Comments: UV stain, rapid staining

Alkaline phosphate (ALP) **EC 3.1.3.1**

β-Naphthyl â phosphate (Na_2 salt)	40 mg
Fast blue RR salt	40 mg
$MgSO_4.7H_2O$	90 mg
Distilled water	80 ml

Fix: Acid-alcohol
Comments: Some **ALP** require manganese ions for activity, using $MnCl_2$ instead of $MgSO_4$

Aldehyde oxidsase (AO) **EC 1.2.3.1**

Benzaldehyde or acetaldehyde	1 ml
NAD	10 mg
MTT	5 mg
PMS	1 mg
0.2 M Tris-HCL, pH 8.5	20 ml
2% Agar	20 ml

Fix: Glycerine

Arginine kinase (ARK) **EC 2.7.3.3**

Phospho-L-arginine	15 mg
Glucose	40 mg
ADP	15 mg
NADP	5 mg
MTT or NBT	5 mg
PMS	1 mg
G6PDH	10 u
Hexokinase	30 u
0.2 M Tris-HCL, pH 7.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

Esterase (EST) **EC 3.1.1.-**

Substrate	2 ml
Fast blue RR salt	50 mg
0.1 M P_4O, pH 6.5-7.0 or 0.2 T-HCL,7.0	40 ml
Distilled water	40 ml

Substrate: Prepare 1% α-napthyl acetate or β- in 50% acetone
Fix: Acid-alcohol
Comments: 1.Incubate gel sliceat at room temperature, dark is not required.
2.Other substrates are α-napthyl propionate & α-napthyl butyrate

Esterase-D (ESD,ESDT) **EC 3.1.-.-**

4-Methylumbelliferyl acetate	2 mg
0.1 M P_4O, pH 7.0 or 0.2 T-HCL,7.0	20 ml
2% Agar	20 ml

Substrate: Dissolved substrate in 1 ml acetone and 1 ml dH_2O
Comments: Incubate at 37°C and view under longwave (365 nm) UV light.

Fructose-biphosphate aldolase (FBALD,ALDO) **EC 4.1.2.13**

Fructose-1,6-diphosphate	200 mg
NAD	15 mg
MTT or NBT	5 mg
PMS	1 mg
G3PDH	40 u
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

Fumarate hydratase (FH) **EC 4.2.1.2**
[Fumarase (FUM)]

Sodium fumarate (fumaric â)	60 mg
NAD	10 mg
MTT	5 mg
PMS	1 mg
Malic dehydrogenase	50 u
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

Glyceraldehyde-3-phosphate deh. (GAPDH) **EC 1.2.1.12**

Substrate	5 ml
NAD	15 mg
MTT or NBT	5 mg
PMS	1 mg
0.2 M Tris-HCL, pH 7.0	15 ml
2% Agar	20 ml

Substrate: Dissolved 150 mg fructose-1,6-di. in 5 ml stain buffer add aldolase 20 u. Incubate at 37°C for 30 min then add to stain mix)
Fix: Acid-alcohol

Glutamate dehydrogenase (GLUDH,GLUD) **EC 1.4.1.3**

L-Glutamic â (Na_2 salt)	1 gm
NAD	10 mg
MTT or NBT	5 mg
PMS	1 mg
0.2 M Tris-HCL, pH 8.5	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

Glyceral-3-phosphate dehydrogenase (G3PDH) **EC 1.1.1.8**

DL-α-Glycerophosphate	150 mg
NAD	15 mg
MTT or NBT	5 mg
PMS	1 mg
0.2 M Tris-HCL, pH 8.5	20 ml
2% Agar	20 ml

Fix: Acid-alcohol
Comments: Incubate for 1 h before adding PMS

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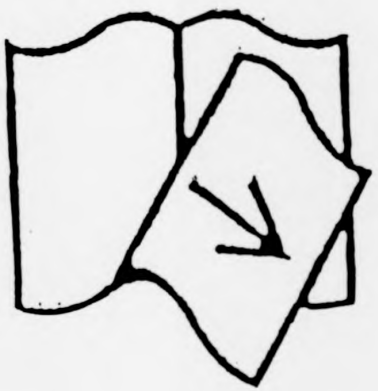

Iditol dehydrogenase (IDDH,SDH) **EC 1.1.1.14**

D-Sorbitol	400 mg
NAD	10 mg
MTT	5 mg
PMS	1 mg
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Glycerine

Isocitrate dehydrogrnase(NADP+) (IDHP) **EC 1.1.1.42**

Isocitric â (Na_2 salt)	100 mg
NADP	5 mg
$MgCL_2$	60 mg
MTT or NBT	5 mg
PMS	1 mg
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

L-Lactate dehydrogenase (LDH) **EC 1.1.1.27**

Substrate (lactate solution)	4 ml
NAD	10 mg
MTT or NBT	5 mg
PMS	1 mg
0.2 M Tris-HCL, pH 7.0	16 ml
2% Agar	20 ml

Substrate: Dissolve 10.6 ml of 85% DL-lactic â in 100 ml H_2O, adjust pH to 7.0 with LiOH

Fix: Acid-alcohol

Malate dehydrogenase (MDH) **EC 1.1.1.37**

Substrate (DL-malate solution)	4 ml
NAD	10 mg
MTT or NBT	5 mg
PMS	1 mg
0.2 M Tris-HCL, pH 8.5	20 ml
2% Agar	20 ml

Substrate: Dissolve 13.4 g of DL-malic â in 100 ml H_2O, adjust pH to 7.0 with NaOH
Fix: Acid-alcohol

Malic enzyme(NADP+) (MEP,ME) **EC 1.1.1.40**

DL-Malate solution	4 ml
NADP	5 mg
$MgCL_2$	40 ul
MTT or NBT	5 ml
PMS	1 ml
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol
Comments: Check **MDH**, eliminated by adding 20 mg oxaloacetic â

Mannose-6-phosphate isomeras (MPI) **EC 5.3.1.8**

Mannose-6-phosphate	30 mg
NADP	5 mg
$MgCL_2$	20 ug
MTT or NBT	5 mg
PMS	1 mg
G6PDH	10 u
Glucose-6-phosphate isomerase	12 u
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol
Comments: **LDH** may appear as faint bands, suppressed by adding 50 mg pyruvic â.

Octanol dehydrogenase (ODH) **EC 1.1.1.73**

Octanol	2 ml
NAD	10 mg
MTT	5 mg
PMS	1 mg
0.2 M Tris-HCL, PH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol
Comments: In penaeid prawn, **ODH** is the same product as **ADH**

Peptidase-C (PEPC) **EC 3.4.-.-**

Peptide substrate	20 mg
Peroxidase	2 mg
L-Amino â oxidase or snake venome	4 mg
3-Amino-9-ethyl carbazole (dissolve in DMSO) or O-dianisidine (dissolve in acetone)	5 mg
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Substrate: Glycyl-L-leucine or glycyl-leucine
Fix: Glycerine

Phosphogluconate dehydrogenase (PGDH) **EC 1.1.1.44**

6-Phosphogluconic (Na_3 salt)	40 mg
NADP	10 mg
$MgCL_2$	40 ug
MTT or NBT	5 g
PMS	1 g
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

Phosphoglucomutase (PGM) **EC 5.4.2.2**

Glucose-1-phosphate (Na_2 salt)	40 mg
NADP	5 mg
$MgCL_2$	40 ug
MTT or NBT	5 mg
PMS	1 mg
G6PDH	3 u
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

Pyruvate kinase (PK) **EC 2.7.1.40**

Fructose-1,6-diphosphate (Na_2 salt)	20 mg
Phospho(enol)pyruvate (Na_2 salt)	20 mg
ADP	25 mg
NADH	20 mg
KCL	100 mg
$MgCL_2$	60 ml
Lactate dehydrogenase	100 u
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

Pyrroline dehydrogenase (PYDH) **EC 1.5.1.12**

L-Pyroglutamic â	20 mg
NAD	10 mg
MTT	5 mg
PMS	1 mg
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Acid-alcohol

Superoxide dismutase (SOD) **EC 1.15.1.1**

MTT or NBT	5 mg
PMS	1 mg
0.2 M Tris-HCL, pH 8.0, 8.5	20 ml
2% Agar	20 ml

Fix: Acid-alcohol
Comments: Incubate gel slice exposed to light at room temp or 37°C

Xanthine dehydrogenase (XDH) **EC 1.1.1.204**

Hypoxanthin	150 mg
NAD	10 mg
MTT	5 mg
PMS	1 ml
0.2 M Tris-HCL, pH 8.0	20 ml
2% Agar	20 ml

Fix: Glycerine

3. Staining Buffers

Tris-HCL, 0.2 M, pH 7.0-8.5

Trietholamine	12.1 g/l

Mix: Adjust pH with concentrated HCL

Phosphate buffer, 0.1 M, pH 7.0-7.5

$Na_2HPO_4.12H_2O$, 0.1 M (pH 8.9)	12.9 g/l
$NaH_2PO_4.2H_2O$, 0.1 M (pH 4.2)	7.6 g/l

Mix: Mix by adding Na_2HPO_4 to bring pH to 7.0-7.5

Phosphate-citric acid, 0.05 M, pH 5.5

Citric â (monohydrate) 10.5 g/l

Mix: Adjust pH with 0.1 M Na_2HPO_4

Na-acetate, 0.05 M, pH 5.5

Sodium acetate.$5H_2O$ 6.8 g/l

Mix: Adjust pH with 1N HCL (≈15 ml)

4. Stock Solutions

Reagents and cofactors

MTT (5 mg/ml)

MTT (tetrazolium salt) 500 mg/100 ml

Mix: Dissolved MTT in dH_2O and store in dark bottle at 4°C

PMS (1 mg/ml)

Phenazine methosulphate 100 mg/100 ml

Mix: Dissolved PMS in dH_2O and store in dark bottle at 4°C

NAD (10 mg/ml)

NAD (Nicotinamide adenine dinucleotide) 1 g/100 ml

Mix: Dissolved NAD in dH_2O and store at 4°C

NADP (5 mg/ml)

NADP (Nicotinamide adenine dinucleotide phosphate)	500	mg/100 ml

Mix: Dissolved NADP in dH_2O and store at 4°C

$MgCL_2$ (20 mg/ml)

$MgCl_2$ (anhydrous)	2	g/100 ml

Mix: Dissolved $MgCl_2$ in dH_2O and store at 4°C

DL-Isocitric â (50 mg/ml)

DL-Isocitric â	5 g
0.2 M Tris-HCL, pH 8.0	100 ml

Mix: Dissolved DL-isocitric â in Tris-HCL and store at 4°C

Hypoxanthine solution (50 mg/ml)

Hypoxanthine	5 g
0.1 M KOH	50 ml
0.2 M Tris-HCL, pH 8.0	50 ml

Mix: Adjust pH to 8.0 with NaOH and store at 4°C

Substrates solution

AAT substrate solution, pH 8.0

L-Aspartic â	400 mg
α-Ketoglutaric â	200 mg
0.2 M Tris-HCL, pH 8.0	100 ml

Mix: Adjust pH with 4N NaOH

DL-Malate sloution, pH 8.5 (60 mg/ml)

DL-Malic â 6 g/100 ml

Mix: Dissolved DL-malic â in dH_2O, adjust pH with 4N NaOH

DL-Lactate solution, pH 7.0 (1 M)

85 % DL-Lactic â 10.6 ml/100 ml

Mix: Dissolved DL-Lactic â in H_2O, adjust pH with LiOH

Fumerate solution, pH 8.0 (50 mg/ml)

Fumeric â 5 g/100 ml
Mix: Dissolved fumalic â in dH_2O, adjust pH with 4N NaOH

Linking enzyme

G_6PDH solution (1 u./5 ul)

D-Glucose-6-phosphate 1000 u/5 ml

Mix: Dissolved G_6PDH in dH_2O and store at 4°C

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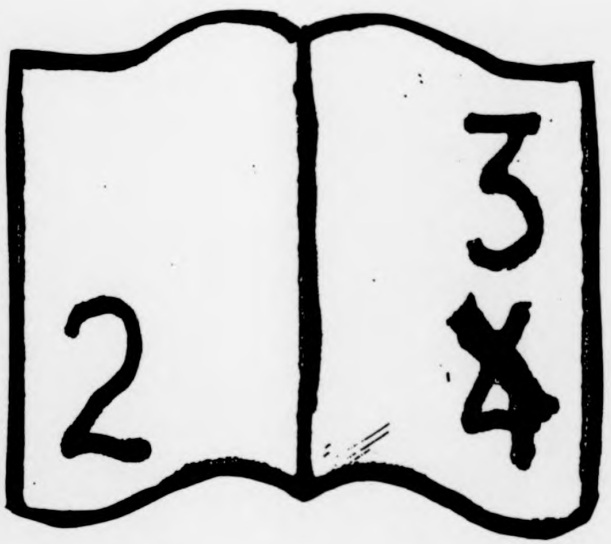

APPENDIX 2

ALLOZYME ANALYSIS

1. Calculating Formula Employed For Allozyme Analysis

1.1 Allele frequency (*p*)

$$p = \frac{2H_o + H_e}{2N}$$

where H_o = number of homozygotes for that allele
H_e = number of heterozygotes for that allele
N = number of individuals examination

1.2 Hardy-Weinberg distribution

A. Chi-squared value (χ^2) for goodness of fit

$$\chi^2 = \sum \frac{(Obs - Exp)^2}{Exp}$$

'Obs' = observed genotype frequencies
'Exp' = expected genotype frequencies

B. The log likelihood χ^2 test (G-test) for small values.

$$G = 2\Sigma Obs \ln\left(\frac{Obs}{Exp}\right)$$

The important point for calculation of degree of freedom when examining genotypic values is that the number of independent genotypes is less than N-1 and the number of degrees of freedom can be calculated as

$$\tfrac{1}{2}(n^2 - n)$$

1.3 Expected heterozygosity (unbiased estimate of Nei, 1978)

For a single locus, an unbiased estimate of heterozygosity is given by

$$H = 1 - \Sigma x_i^2$$

whereas the corresponding unbiased estimate of $\overline{H}$ averaged over all loci is

$$\overline{H} = \Sigma(1 - \Sigma x_i^2)/n$$

where p_i is the frequency of the ith allele at a locus in a sample from the population, h_k the value of h for the kth locus, and r the total number of loci investigated. The sample size n may be vary from locus to locus.

$$S.E. = \sqrt{Var}$$

$$Var = \Sigma (h_k - \hat{H})^2/r(r - 1)$$

1.4 F-statistics, F_{ST} (the variance component estimation of Wright, 1978)

F_{ST} = (Actual variance)(Limiting variance)$^{-1}$

Actual variance = Total variance - Sampling variance

Total variance = $r^{-1} \Sigma(p - p)^2$

Sampling variance = $r^{-1} \Sigma(2n)^{-1}p(1 - p)$

Limiting variance = $p(1 - p)$

where p is the frequency of an allele at a locus in a population, p the averaged allelic frequency over all populations at that allele and that locus, n the number of individuals sampled for that locus in that population, and r the number of populations.

NOTE: Negative variance components are sometimes obtained using Wright's (1978) procedure. Resulting from the assumption for computational purposes, the estimated total variance is partitioned orthogonally into estimated variance components. Thus if the sum of a part of the estimated variance components exceeds the estimated total variance, the remaining estimated variance components take on negative values. The program BIOSYS-1 counted these values as zero for the next step of calculation.

The significant of F_{IS} and F_{ST} values test by using chi-square (Workman and Niswander, 1970; Waples, 1987)

Test of F_{IS}

$$\chi^2 = N(F_{IS})^2(k-1)$$

$$\text{d.f.} = (k(k-1))/2$$

where N = total Number of individuals sampled
k = the number of alleles at the locus

Test of F_{ST}

$$\chi^2 = 2NF_{ST}(k-1)$$

$$\text{d.f.} = (k-1)(s-1)$$

where s = Number of population sampled

1.5 Genetic identity and distance

A. Nei's coefficient of genetic identity (I) between two taxa (Nei, 1972, 1978)

$$I = \frac{\Sigma x_i y_i}{\sqrt{(\Sigma x_i^2 \Sigma y_i^2)}}$$

where x_i and y_i are the frequencies of the ith allele in population X and Y respectively.

The mean genetic identity is the mean over all loci study

$$\overline{I} = \frac{I_{xy}}{\sqrt{(I_x I_y)}}$$

Where I_{xy}, I_x and I_y are the arithmetic means, over all loci of $\Sigma\, x_i y_i$, $\Sigma\, x_i^2$ and $\Sigma\, y_i^2$ respectively.

The genetic distance (D) is estimated by

$$D = -\ln I$$

B. Rogers' genetic distance (Roger, 1972)

$$D_R = (1/L)\sum_L \sqrt{\Sigma(x_i - y_i)^2/2}$$

L = number of loci investigate

2. Pairwise Contingency Chi-Square Analysis

TRAT 91, 92 & 93

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.462	2	.79355
AAT-2	2	.900	2	.63750
ALAT	3	5.954	4	.20261
ESD	4	10.813	6	.09433
GPI	4	4.234	6	.64502
IDHP	2	2.144	2	.34227
MDH-1	2	1.890	2	.38859
MPI	3	6.612	4	.15786
PGM	7	18.518	12	.10085
(Totals)		51.529	40	.10464

SURAT 91 & 92

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.006	1	.94041
AAT-2	2	.006	1	.94041
ALAT	3	5.002	2	.08202
ESD	4	5.098	3	.16475
GPI	4	3.065	3	.38165
IDHP	2	2.756	1	.09687
MPI	3	1.126	2	.56936
PGM	6	7.448	5	.18940
(Totals)		24.507	18	.13910

PHUKET 91 & 92

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.019	1	.88986
AAT-2	2	.019	1	.88986
ALAT	3	.732	2	.69360
ESD	3	1.374	2	.50313
GPI	4	2.403	3	.49306
IDHP	2	.501	1	.47894
MPI	4	.963	3	.81015
PGM	5	3.323	4	.50528
(Totals)		9.335	17	.92918

SATUN 92 & 93

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.000	1	1.00000
AAT-2	2	.000	1	1.00000
ALAT	3	3.542	2	.17013
ESD	3	1.007	2	.60446
GPI	3	.259	2	.87848
IDHP	3	2.329	2	.31203
MDH-1	2	.252	1	.61589
MDH-2	2	2.007	1	.15661
MPI	3	1.140	2	.56544
PGM	5	7.752	4	.10111
(Totals)		18.288	18	.43683

SURAT-DUNGUN

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.476	1	.49037
AAT-2	2	2.480	1	.11529
ALAT	3	1.091	2	.57962
ESD	4	3.919	3	.27035
GPI	4	4.781	3	.18857
IDHP	2	6.660	2.02	.04000
MPI	3	2.177	2	.33669
PGM	6	2.471	5	.78081
(Totals)		23.564	18	.16985

SOUTH JAVA-NORTH JAVA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-2	2	.335	1	.56248
ALAT	3	.861	2	.65024
ESD	2	.675	1	.41127
GPI	4	10.630	3.47	.02100
IDHP	2	.012	1	.91382
MPI	4	1.368	3	.71317
PGM	4	4.402	3	.22119
(Totals)		16.696	14	.27275

PHUKET-SATUN-KEDAH

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.004	2	.99813
AAT-2	2	.855	2	.65222
ALAT	3	3.198	4	.52519
ESD	3	.210	4	.99486
GPI	4	6.694	6	.35009
IDHP	3	3.393	4	.49440
MDH-1	2	1.369	2	.50444
MDH-2	2	2.741	2	.25397
MPI	4	7.966	6	.24058
PGM	6	12.076	10	.27999
(Totals)		38.505	42	.62516

POOLED ANDAMAN SEA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.824	3	.84379
AAT-2	2	.879	3	.83038
ALAT	3	3.196	6	.78384
ESD	3	.949	6	.98748
GPI	5	15.966	12	.19278
IDHP	3	14.796	6	.02190
MDH-1	2	1.702	3	.63646
MDH-2	2	3.312	3	.34593
MPI	4	11.437	9	.24694
PGM	6	19.187	15	.20538
(Totals)		72.249	66	.27919

6 ALL POOLED POPULATIONS

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	3	2258.755	10	.00000*
AAT-2	3	34.513	10	.00015*
ALAT	3	281.885	10	.00000*
ESD	4	25.817	15	.04000
GPI	5	63.246	20	.00000*
IDHP	3	51.801	10	.00000*
MDH-1	2	3.992	5	.55057
MDH-2	3	2260.065	10	.00000*
MPI	5	1061.093	20	.00000*
PGM	7	38.325	30	.14159
(Totals)		6079.492	140	.00000

5 SE ASEAN POPULATIONS

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	4.371	4	.35814
AAT-2	3	30.020	8	.00021*
ALAT	3	29.639	8	.00024*
ESD	4	18.431	12	.10323
GPI	5	47.511	16	.00006*
IDHP	3	35.473	8	.00002*
MDH-1	2	3.428	4	.48899
MDH-2	2	5.575	4	.23320
MPI	4	12.459	12	.40955
PGM	7	24.563	24	.42981
(Totals)		211.470	100	.00000

4 ASEAN POPULATIONS

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	4.390	3	.22229
AAT-2	2	1.052	3	.78858
ALAT	3	15.083	6	.01962
ESD	4	12.605	9	.18130
GPI	5	28.859	12	.00413*
IDHP	3	23.788	6	.00057*
MDH-1	2	3.006	3	.39076
MDH-2	2	4.177	3	.24299
MPI	4	10.025	9	.34843
PGM	7	17.374	18	.49756
(Totals)		120.360	72	.00031

TRAT-SURAT & DUNGUN

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.003	1	.95677
AAT-2	2	.203	1	.65201
ALAT	3	21.210	4.46	.00050*
ESD	4	.941	3	.81542
GPI	4	7.373	3	.06092
IDHP	2	9.080	2.03	.01500
MDH-1	2	1.899	1	.16818
MPI	3	1.335	2	.51303
PGM	7	5.535	6	.47727
(Totals)		36.129	20	.01485

TRAT-ANDAMAN SEA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	1.846	1	.17428
AAT-2	2	.866	1	.35203
ALAT	3	15.980	4.26	.00400*
ESD	4	5.929	3	.11511
GPI	5	3.837	4	.42846
IDHP	3	2.644	2	.26666
MDH-1	2	1.051	1	.30537
MDH-2	2	1.841	1	.17480
MPI	4	2.443	3	.48573
PGM	7	3.903	6	.68979
(Totals)		36.897	24	.04481

TRAT-POOLED JAVA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.647	1	.42114
AAT-2	2	.001	1	.97701
ALAT	3	5.261	2	.07206
ESD	4	2.613	3	.45523
GPI	4	5.893	3	.11692
IDHP	2	1.847	1	.17412
MDH-1	2	.647	1	.42114
MPI	4	3.964	3	.26533
PGM	7	6.271	6	.39356
(Totals)		27.144	21	.16613

TRAT-PHILIPPINES

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.029	1	.86502
AAT-2	3	8.600	2.05	.01527
ALAT	3	4.964	2	.08356
ESD	4	6.473	3	.09073
GPI	4	17.580	4.13	.00200*
IDHP	2	13.700	2.02	.00150*
MDH-1	2	.550	1	.45827
MDH-2	2	3.653	1	.05597
MPI	3	1.408	2	.49459
PGM	7	8.353	6	.21335
(Totals)		60.512	22	.00002

TRAT-KENYA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	3	678.000	2	.00000*
AAT-2	2	1.106	1	.29301
ALAT	3	171.852	2	.00000*
ESD	4	5.245	3	.15469
GPI	4	13.503	3	.00367*
IDHP	2	15.907	1	.00007*
MDH-1	2	.736	1	.39093
MDH-2	3	678.000	2	.00000*
MPI	4	355.923	3	.00000*
PGM	7	9.130	6	.16642
(Totals)		1251.402	22	.00000

SURAT & DUNGUN-ANDAMAN SEA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	1.641	1	.20022
AAT-2	2	.200	1	.65467
ALAT	3	.176	2	.91568
ESD	4	10.490	3.62	.02500*
GPI	5	31.830	8.52	.00000*
IDHP	3	27.950	4.22	.00003*
MDH-1	2	.581	1	.44604
MDH-2	2	1.745	1	.18653
MPI	4	5.309	3	.15053
PGM	6	7.120	5	.21184
(Totals)		66.902	23	.00000

SURAT & DUNGUN-POOLED JAVA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.683	1	.40854
AAT-2	2	.077	1	.78076
ALAT	3	.507	2	.77625
ESD	4	3.875	3	.27533
GPI	4	3.238	3	.35643
IDHP	2	.441	1	.50676
MPI	4	3.255	3	.35397
PGM	6	5.953	5	.31083
(Totals)		18.028	19	.52059

SURAT & DUNGUN-PHILIPPINES

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	.205	1	.65034
AAT-2	3	7.785	2.11	.02400*
ALAT	3	22.676	4.21	.00002*
ESD	4	4.939	3	.17631
GPI	4	11.079	2.30	.00700*
IDHP	2	2.383	1	.12268
MDH-2	2	3.462	1	.06281
MPI	3	.599	2	.74120
PGM	6	8.762	5	.11896
(Totals)		54.331	20	.00005

SURAT & DUNGUN-KENYA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	3	652.000	2	.00000*
AAT-2	2	1.559	1	.21188
ALAT	3	221.918	2	.00000*
ESD	4	7.168	3	.06673
GPI	4	7.609	3	.05482
IDHP	2	4.109	1	.04266*
MDH-2	3	652.000	2	.00000*
MPI	4	348.970	3	.00000*
PGM	6	10.907	5	.05326
(Totals)		1254.239	20	.00000

ANDAMAN SEA-POOLED JAVA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	1.794	1	.18039
AAT-2	2	.309	1	.57805
ALAT	3	.230	2	.89115
ESD	3	1.509	2	.47036
GPI	5	9.235	4	.05548
IDHP	3	5.572	2	.06168
MDH-1	2	.198	1	.65657
MDH-2	2	.594	1	.44071
MPI	4	2.090	3	.55388
PGM	6	5.052	5	.40958
(Totals)		26.584	22	.22745

ANDAMAN SEA-PHILIPPINES

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	0.157	1	.69175
AAT-2	3	9.327	1.54	.00500*
ALAT	3	23.586	3.84	.00000*
ESD	3	3.829	2	.14742
GPI	5	17.010	4.25	.00300*
IDHP	3	13.990	2.23	.00150*
MDH-1	2	.168	1	.68184
MDH-2	2	.368	1	.54395
MPI	4	2.733	3	.43459
PGM	6	6.844	5	.23249
(Totals)		82.694	23	.00000

ANDAMAN SEA-KENYA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	3	992.000	2	.00000*
AAT-2	2	2.041	1	.15314
ALAT	3	245.561	2	.00000*
ESD	3	5.055	2	.07984
GPI	5	19.680	5.36	.00200*
IDHP	3	25.362	2	.00000*
MDH-1	2	.225	1	.63533
MDH-2	3	992.000	2	.00000*
MPI	5	489.184	4	.00000*
PGM	6	12.080	5	.03370
(Totals)		1792.084	24	.00000

POOLED JAVA - PHILIPPINES

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	1.180	1	.27726
AAT-2	3	2.912	2	.23321
ALAT	3	10.110	4.69	.06000
ESD	3	5.235	2	.07297
GPI	4	18.697	4.30	.00150*
IDHP	2	3.351	1	.06715
MDH-2	2	1.180	1	.27726
MPI	4	1.915	3	.59014
PGM	4	3.888	3	.27385
(Totals)		40.916	18	.00156

POOLED JAVA-KENYA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	2	342.000	1	.00000*
AAT-2	2	1.141	1	.28548
ALAT	3	160.754	2	.00000*
ESD	2	2.288	1	.13034
GPI	4	6.788	3	.07897
IDHP	2	5.057	1	.02453*
MDH-2	2	342.000	1	.00000*
MPI	5	150.219	4	.00000*
PGM	4	29.980	7.54	.00015*
(Totals)		684.852	16	.00000

PHILIPPINES-KENYA

Locus	No. of alleles	Chi-square	D.F.	P
AAT-1	3	318.000	2	.00000*
AAT-2	3	5.421	2	.06650
ALAT	3	107.227	2	.00000*
ESD	3	10.982	2	.00412*
GPI	3	3.796	2	.14987
IDHP	2	.060	1	.80614
MDH-2	3	992.000	2	.00000*
MPI	4	138.548	3	.00000*
PGM	3	12.395	2	.00203
(Totals)		597.772	17	.00000

NOTE: * = significant difference

APPENDIX 3

MTDNA RFLPS PROCEDURE

1. Preparation of Total DNA from Frozen Tissue

Tissue: Frozen pleopod of *P. monodon*

Materials:

Liquid nitrogen
Digestion buffer
-TEN buffer, pH 8.0 (350 µl)
-1 % sodium dodecyl sulfate (40 µl 10% SDS)
-0.2 mg/ml proteinase K (8 ul 10 mg/ml proteinase K)
Equilibrated phenol
Chloroform:Isoamyl alcohol = 24:1
99.99% ethanol
70 % ethanol
TE buffer, pH 8.0
α-amylase buffer, pH 8.0

Cell preparation:

1. Quickly grind tissue to a very fine powder in liquid nitrogen with a mortar and pestle and always keep tissue frozen.
2. Rapidly transfer powder tissue to glass-Teflon homogenizer containing TEN and proteinase K solution (400 µl digestion buffer per 150 mg tissue), then homogenize two times.
3. Immediately add 10 %SDS to a concentration of 1% and homogenize one more time. There should be no clumps and the samples will be viscous.

Cell lysis & digestion:

1. Incubate the samples at 55°c for 2 h in tightly capped tubes. Mix occasionally during the incubation to keep the tissue suspended. After incubation the samples should be relatively clear.
2. Centrifuge at 12,000g for 10 min to remove cell debris, then transfer aqueous phase to sterile tube.

Sample weight (mg)	Digestion buffer (µl)				TE (µl)
	Volume	TEN	prot. K	10% SDS	
< 150	400	350	8	40	60
150-300	800	700	16	80	100
300-450	1200	1050	24	120	150
450-600	1600	1400	32	160	200

Extraction of nucleic acids:

1. Extract the samples with an equal volume of equilibrated phenol by mixing gently but thoroughly until the emulsion forms and incubate at room temperature for 5 min. If the phase separate, gentle mix again.
2. Centrifuge at 10,000g for 10 min at room temperature. If the organic and aqueous are not well-separate, centrifuge again for longer time and higher speed.
3. Carefully remove the aqueous phase layer with wide bore micropipette tip and transfer to new tube. Be careful not to disturb the cellular debris or the interphases. Discard the interphase and organic phase.
4. Reextract the aqueous phase with phenol two times or until no protein is visible at the interphase.
5. Extract the aqueous phase three times with equal volume of C:IAA and centrifuge at 10,000g for 10 min to remove traces of phenol.
6. Transfer the aqueous phase to a fresh tube.

Purification of DNA:

1. Estimate the volume of the DNA solution.
2. Adjust salt concentration to 2.0 M by addition of 5.0 M NaCL, mix well.
3. Add exactly 2 volumes of ice-cold 99.99% ethanol and mix gently. The DNA should immediately form a stringy precipitate. Leave the ethanolic solution on ice to allow the precipitate of DNA to form for 15-30 min. Recover DNA by centrifugation at 12,000g for 10-15 min. If low concentration of DNA, store the samples at -25°C for 1-2 h for complete precipitation of DNA and more extensive centrifugation may be required (may be leave overnight at -25°C).
4. Remove the supernatant with wide bore tip. Rinse the pellet twice with 70% ethanol and recentrifuge at 12,000g for 2 min or 5,000g at 3 min. Take care not to disturb the pellet of nucleic acids.
5. Decant ethanol and dry the pellet under vacuum for 10 min (take care not to disturb the pellet of nucleic acids.

Carbohydrate & RNA digestion:

1. **Carbohydrate digestion**, resuspend DNA in α-amylase buffer, then add α-amylase to a concentration of 25 µl/ml and incubate at 37°C for 2 h.
2. **RNA digestion**, add RNaseA 100 µg/ml and incubate for 1 h.
3. Extract the solution once with phenol and once with C:IAA.
4. Remove organic solvents and salt from the DNA; dialyse the aqueous phase through ultra free-ML by centrifugation at 5,000g for 25 min.
5. Adjust salt concentration, precipitate, wash and dry (May be kept in ethanol at –25°C.
6. Dry under vacuum for 10 min.
7. Resuspend DNA in TE buffer until dissolved at 37°C for 1-2 h. Rinse the walls of the tube with the buffer to dissolve all of nucleic acids.
8. Store the samples at 4°C. The DNA can be stored in definitely in the presence of ethanol at -25°C.

Quantitation of DNA and RNA

Two methods are widely used to measure the amount of DNA and RNA.

1. **Ethidium bromide fluorescent quantification of the amount of double-strand DNA.** The DNA is monitored by directed visualization the UV-induced fluorescence emitted by ethidium bromide molecules intercalated into the DNA. Because the amount of fluorescence is proportional to the total mass of DNA, the quantity of DNA therefore can be estimated by comparing with a standard marker.
2. **Spectrophotometric determination of DNA and RNA**. Quantity of DNA and RNA was measured at wavelengths of 260 nm and 280 nm. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate for the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} of 1.8 and 2.0, respectively. If there is contamination with protein or phenol, the ratio will be less than the values. If there is contamination with RNA, the ratio will be more than 2.

NOTE:

1. Genomic DNA used for hybridization studies must be pure and free from contaminant such as glycerol, protein, metallic ions, and other impurities.
2. To achieve the best recovery, the organic phase and interphase may be back-extracted.
3. Over pelleting the DNA will make resuspension difficult. Difficulties arise when redissolving buffers contain $MgCl_2$ or $NaCl_2$. It is therefore preferable to dissolve the DNA in small volume of buffer and adjust the composition later.
4. For RNA digestion, after carbohydrate digestion add 100 µg/ml DNase-free RNase (1 µl of 10 µg/µl stock per 100 µl DNA solution) and incubating 1 h further at 37°C.

2. Preparation of MtDNA from Fresh Tissue

Tissue: Pleopod of *P. monodon*

Materials:

TEK buffer, pH 7.5
7.5% Sucrose-TEK (0.25 M)
37.5% Sucrose-TEK (1.1 M)
10% Non-idet TEK

Cell preparation :

1. Dissect and clean pleopod in TEK and 7.5% sucrose-TEK buffer.
2. Squeeze out muscle tissue from pleopod, keep the tissue cool on ice.
3. Cut tissue into pieces and weight.

Mitochondrial isolation :

1. Homogenize the tissue-two strokes in glass-Teflon homogenizer containing 7.5% sucrose-TEK buffer (0.36 g/ ml), separate homogenized solution into two parts, homogenize one stroke each and leave to make cells swell and precipitate.
2. Transfer the homogenate to 50 ml polypropylene tube.
3. Underlay the solution with 37.5% sucrose-TEK buffer by passing a long stem Pasteur pipette through the homogenate.
4. Centrifuge at 1,000g (2,800 RPM) for 10 min at 4°C.
5. Draw off the supernatant into another 50 ml centrifuge tube, be careful of interphase and repeat step 3-4.
6. Draw off the supernatant from the second low speed run and the mitochondria are pelleted by centrifugation at 18,000g (12,600 RPM) for 1 h at 4°C.
7. Draw off the supernatant and discard. Resuspend the mitochondrial pellet in 10 ml TEK buffer and centrifuge at 18,000g for 30 min.

mtDNA isolation:

1. Resuspend the mitochondrial pellet in TEK solution (1-2 µl/mg starting tissue).
2. Make the solution up to 1% non-idet by adding a 10% non-idet TEK and mix gently. The supernatant will clear almost immediately, but leave on ice for 10 min for complete lysis.
3. Centrifuge at 12,000g for 10 min to remove debris including intact nucleic.

4. Transfer the supernatant to sterile tube (The solution may be kept at -70°C.
5. Extract three times with an equal volume of equilibrated phenol, centrifuge at 10,000g for 10 min each.
6. When the aqueous is clear and not milky, the phenol is removed three times with C:IAA (24:1).
7. Draw off the supernatant, adjust salt concentration (3 M CH_3COONa, pH 5.2) and add two volumes of ice-cold 99.99% ethanol.
8. Store the sample at -25°C for 2 h to ensure complete precipitation of nucleic acids or at -70° for 30 min.
9. Pellet nucleic acids by centrifugation at 12,000g for 15 min.
10. Wash the pellet twice with 500 µl of 70% ethanol to remove traces of phenol and salt, do not disturb the pellet. Decant ethanol and dry the pellet under vacuum for 10 min.
11. Resuspend the pelleted mtDNA in 100 µl TE buffer per gram starting tissue (1 µl/10-20 mg).
12. Keep the sample at -25°C.

NOTE:

1. Use loose fitting glass-Teflon homogenizer type and try to generate little or no suction. The tissue should be subjected to only pass with minimal force.
2. If a clear pellet does not form, wash and pellet mitochondria three times.
3. Overgrinding the tissue by additional strokes leads to nuclear DNA contamination and loss of mtDNA.
4. Addition of 1% non-idet lyses mitochondrial membranes but not nuclear membranes.

3. Agarose Gel Electrophiresis

Materials:

Agarose
Ethidium bromide (10 µl/ml)
10x TE Buffer, pH 7.0
Gel-loading dye
Gel mould
Electrophoresis tray

Preparation of agarose gel:

1. Prepare gel mould by taping on opposite sides of horizontal gel unit and inserting on the comb to form the wells.
2. Prepare agarose gel by adding 0.8% of powder agarose to a measured quantity of electrophoresis buffer.
3. Boil agarose with intermittent swirling until it dissolves.
4. Leave the solution until cool at 50-55°C, then add 10 µl/ml stock ethidium bromide to a final concentration of 0.5 µl/ml.
5. Pour warm agarose into the mould. Let the gel completely set (30-45 min at room temp), then carefully remove the comb and tape. The gel is now ready to use or may be kept at 4°C for at least 1 day.

Volume (ml)	Agarose (g)	10x TEB (ml)	H_2O (ml)	EB (µl)
77	0.62	7.7	69.3	3.85
200	1.60	20.0	180	10.0

Loading samples:

1. Put gel mould into electrophoresis tray, then add 1x TEB buffer until cover the gel to a dept of about 1 mm.
2. Mix the sample with 1/5 volume of gel-loading buffer, centrifuge at 4,000g for 2 min.
3. Load samples into well.

Content	77 ml gel			200 ml gel	
	tDNA	mtDNA	λHindIII 1μg/5μl	tDNA	λHindIII 1μl/5μl
TE buffer	18	-	15	8.5	5
DNA	2	20	5	1.5	10
Loading dye	5	5	5	4	4
Total (ml)	25	25	25	14	14

Electrophoresis:

To obtain maximum resolution of DNA fragments, gel should be run at no more than 5 V/cm (77 ml gel is running at 100 V, 50 mA for 2.5-3 h, 200 ml gel is running at 120 V, 120 mA for 4 h or 35 V, 38-40 mA for 16 h.)

Monitoring:

The DNA fragments is monitored by directed visualization under UV transilluminator.

4. Digestion DNA With Restriction Endonuclease

tDNA:

1. Set reaction in 100 µl and use 10 µg DNA.
2. Restriction enzyme:DNA = 4:1
3. BSA 0.1%, add 1 µl/100 µl of 10 mg/ml stock solution.
4. Spermidine 2 mM, add 5 µl/100 µl of 40 mM stock solution.
5. Incubate at 37°C for 4-16 h.
6. Extract once with Phenol and once with C:IAA, dry and resuspend in 10 µl TE.

	*Bam*HI	*Eco*RV	*Hind*III	*Pvu*II	*Sac*I
H_2O	52	57.3	61	61.5	67.5
RE buffer	20	20	10	10+5NaCL	10
Spermidine	10	5	10	5	5
BSA	1	1	1	1	1
DNA	15	15	15	15	15
RE enzyme	2	1.7	3	2.5	1.5

mtDNA:

1. Set reaction to 20-40 µl and incubate at 37°C for 4-16 h.
2. Stop reaction by addition of 0.5 M EDTA to a concentration of 10 nM, then boil at 65°C for 10-15 min to reduce quatity.

Content	Pleopod	Hepatopancleaus
H_2O	-	5.5
RE buffer	8	4
mtDNA	31.5	10
*Eco*RV	0.5	0.5
Total (µl)	40	20

NOTE:

1. Restriction enzyme
   -*Bam*HI = 1:4 = 2 µl (20 u./µl, 2x RE buffer)
   -*Eco*RV = 1:2 = 1.7 µl (11.5 u./µl, 1x RE buffer)
   -*Hind*III = 1:4.5 = 3 µl (15 u./µl, 1x RE buffer)
   -*Pvu*II = 1:2 = 2.5 µl (8 u./µl, 2x RE buffer + 50 M NaCL)
   -*Sac*I = 1:2.5 = 1.5 µl (15 u./µl, 1x RE buffer)
2. REs are heat sensitive, then store at -25°C and work on ice.
3. Enzyme are stored in 50% glycerol to prevent denaturation by freezing. The glycerol can affect REs activity if present at greater than 5% of the final reaction mixture therefore the volume of REs should be less than 10% of the total mixture.

5. Preparation of λ*Hind*III Marker

Content	0.5 μg/5 μl	1 μg/5 μl
undigested λ*Hind*III	29* (10 μg)	58* (20 μg)
H_2O	53	22
RE buffer	10	10
Spermidine	5	5
BSA	1	1
*Hind*III	2 (30 u.)	4 (60 u.)
Total (μl)	100	100

Procedure:

1. Mix the solution, then incubate at 37°C for 4-36 h.
2. Extract once with phenol and once with C:IAA
3. Precipitate with ice-cold 99.99% ethanol, kept at -20°C 2 h for complete precipitation.
4. Recover by centrifugation at 12,000g for 10 min.
5. Wash twice with 70% ethanol and dry under vacuum for 10 min.
6. Resuspend in 100 ml TE buffer for 1-1.5 h.

NOTE:

* depends on concentration of λHindIII stock solution.
Restriction enzyme
-*Hind*III = 1:3 = 2 μl (15 u./μl, 2x RE buffer)

6. Nucleic Acids Transfer

Methodology:

Alkaline vacuum transfer of DNA by using vacuum blotting system

Solution:

Depurination solution	0.2 N HCl
Alkaline buffer	1 M NaOH
Washing solution	2x SSC

Procedure:

1. **Setting up**, clean and wet vacuum unit and connect the pump.
2. Pre-treat the nylon membrane (Amersham's Hybond-N+; positively charged nylon) in dH_2O, place on the porous screen by using clip.
3. Place the plastic mask on the membrane (≈5 mm overlap).
4. Place on the frame and tighten the locking clamps, then check the suction pump.
5. Wet the membrane, put the gel on membrane by starting with one of the edges, slid the gel onto the membrane. Avoid entrapping air bubbles by adding dH_2O under the gel
6. Switch on the pump.
7. **Depurination**, immediately pour 50 ml of 0.2 N HCl onto the gel, leave until the bromphenol blue turn yellow (≈25 min). Wipe the gel with a gloved finger and remove the excess liquid by using pipette.
8. **Transfer**, immediately pour 1 litre of 1 M NaOH onto the gel to cover it to about twice its depth, then transfer for 1.5 h.
9. Remove transfer solution.
10. Turn off the pump, mark well positions.
11. Remove the gel, take note.
12. Wash the filter in 20x SCC for 10 min to eliminate agarose.
13. Air dry for 30 min, then keep in 3MM filter paper at room temp.

NOTE:

1. Acid depurinates DNA by breaking large fragments into small pieces for more efficient transfer.
2. Nylon membrane can be rehybridized many times.

7. Recovery of MtDNA Fragments from L.M.A.

1. Prepare a gel of low-melting-point agarose by heating in 1x electrophoresis buffer to 70°C, cool to 37°C then add ethidium bromide to a final concentration of 0.5 µg/ml. Pour the gel and leave to cool.
2. Add loading dye into mtDNA samples, load samples and carry out electrophoresis at 4°C at 100 V for 3 h or until the marker runs through the end of the gel.
3. Quickly view under UV light and cut out the desired fragment(s) from the gel with a minimum of excess agarose by using new scalpel blade. Put the agarose slice into a pre-weighted microcentrifuge tube and weight.
4. Wash twice with sterile dH_2O and leave at 4°C for 20 min or store the gel at -25°C until using.
5. Add sterile dH_2O 3 ml per gram of gel slice.
6. Heat at 65°C for 2-5 min to melt the gel, mix well.
7. Store immediately at -25°C.
8. Calculate the mtDNA concentration.
   -weight of gel slice 0.0395 gm
   sterile dH_2O 118 µl
   total volume 158 µl
   -Comparison of mtDNA with intensity of λ*Hind*III marker
   -Concentration of mtDNA ≈ 2.5 times of 1 µl marker
   = 2%50x2.5x1000 = 100 ng
   -Using 10 ng mtDNA per filter
   = 158x10/100 = 15.8 µl

8. Random Primed DNA Labelling

Reaction:

1. Add the following chemicals step by step into a clean microcentrifuge tube.

-dH_2O to total of	50 µl
-dCTP	2.5 µl
-dGTP	2.5 µl
-dTTP	2.5 µl
-Reaction mixture (10x)	5.0 µl
-Denature mtDNA (10 ng)	x µl
-Labelled nucleotide (^{32}P, dATP = 20 µCi)	y µl
-Klenow fragment	1 µl

2. Mix gently, then centrifuge briefly to collect the reaction mix at the bottom.
3. Proceed at room temp for 4-24 h.

Measurement of radioactivity (% incorporation):

1. Dilute radiolabelled DNA 2% with 0.2 M EDTA, mix well.
2. Make note on Whatman DE-81 paper for washed and unwashed, then fold up.
3. Spot 5 µl of the solution onto the centre of the paper.
4. Wash with 0.5 M, pH 6.8 PO_4 for 2 times, 5 min per wash.
5. Dry the filters under a lamp for 10 min.
6. Count radioactivity using Geiger'counter.
7. Calculate %incorporation by comparison washed and unwashed filter.

NOTE:

1. The procedure may be used for labelling between 10 ng to 1 µl of DNA.
2. In general, 1 µCi of nucleotide per ng of input DNA will yield probes labelled to approximately $1x10^9$ dpm/µg.
3. For 10 ng of mtDNA, 20 µCi of ^{32}P is required, e.g., the reference date of ^{32}P is 1/5/93, from *the Decay curve*, it can be estimated the concentration of ^{32}P by comparison to reference concentration.
   -4 days before = 1.22 times, 20 µCi = 2µl/1.22 = 1.6 µl
   -4 days after = 0.85 times, 20 µCi = 2µl/0.85 = 2.4 µl
4. Prepare denatured DNA by using "DNA/agarose" for labelling as follows.
   -Heat in water bath at 95-100°C for 7 min.
   -Incubate in water bath at 37°C for 10 min, then centrifuge briefly
   -use immediately.
5. Denatured DNA from 3 times agarose gel should not be more than 25 µl.

9. Hybridization for DNA Probing

Stock solution:

20x SSPE
20X SSC
20% SDS
10% BLOTTO
50% Dextran Sulfate
10 mg/ml carrier DNA (calf thymus DNA)

Prewet filter:

Wet nylon membrane with 1.5x SSPE, then place in hybridization tube and warm at 65°C.

Prewashing:

Prewash with 0.5x SSPE/0.1% SDS at 65°C for 1h.

Prehybridization:

1. Prepare prehybridization as follow.

-dH_2O	7.75 ml
-20x SSPE	0.75 ml (1.5X)
-20% SDS	0.50 ml (1.0%)
-10% BLOTTO	0.50 ml (0.5%)
-10 mg/ml denatured carrier DNA*	0.50 ml (0.5 mg/ml)

* The carrier DNA must be denatured immediately before adding to the hybridization solution by heating at 100°C for 5 min then cool on ice immediately. Denatured DNA may be stored at -25°C for up to 1 week.

2. Place the nylon membrane into hybridization tube (with the sample DNA sideup), then add prehybridization solution and carry on at 60°C for 4-24 h.

Hybridization:

1. Prepare fresh hybridizationsolution and pre-warm at 50°C.

-dH_2O	6.25 ml
-20x SSPE	0.75 ml (1.5X)
-50% Dextran sulfate	2.00 ml (10%)
-20% SDS	0.50 ml (1.0%)
-10% BLOTTO	0.50 ml (0.5%)
-Denatured labelled probe*	

* Immediately before use, DNA probe is boiled for 10 min and cool immediately on ice. Mix with pre-warmed hybridization solution.

2. Pour off the prehybridization.
3. Add pre-warmed hybridization solution.
4. Hybridize at 60°C for 4-24 h.

Washing:

1. At the completion of hybridization, rince out the hybridization solution.
2. Wash at 50°C in the following solution (50 ml per membrane).

-2x SSPE	= rince twice
-2x SSPE/0.1% SDS	= 10 min, 2 times
-0.5x SSPE/0.1% SDS	= 10 min, 20 min
-0.1x SSPE/0.1% SDS	= 10 min

Autoradiography:

1. After final wash, cut the filter to 20x18 cm, remove as much excess liquid as possible but do not let the filter dry out as this makes it difficult to rehybridize, then wrap in plastic and seal.
2. The radioactivity of filter is determined to estimate the time for autoradiography.

50 cps	=	overnight
15-20 cps	=	1 day
10-14 cps	=	2 day

3. The filter is expose to X-ray film (Hyperfilm™-MP, Amersham) in an X-ray cassette with an intensifying screen.
4. Place the cassette at -70°C (Membrane containing moderate or high levels of radioactivity can be exposed at room temperature without a screen to give improved resolution).
5. Remove the cassette from the freezer, allow 30 min for them to defrost before developing.
6. Develope and fix the film with Kodak X-ray film developer and fixer solution.

10. Stock Solutions for MtDNA Analysis

α-amylase buffer (50 mM tris-HCL pH 8.0, 10 mM EDTA, 10 mM NaCL)

Tris	4.61 g/l
EDTA	0.58 g/l
NaCL	3.72 g/l

:Dissolve Tris, EDTA and NaCL in dH_2O and adjust pH with concentrated HCL. Dispense into aliqouts and sterilize by autoclaving.

0.5 M EDTA, pH 8.0

Disodium ethylene diamine tetraacetate 186.1 g/l

:Add EDTA into dH_2O and adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets and EDTA will not dissolve until pH is adjusted to approximately 8.0). Dispense into aliquots and sterilize by autoclaving.

Ethidium bromide (10 mg/ml)

Ethidium bromide 1 g/100 ml

:Add ethidium bromide in dH_2O. Stir on magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminium foil or transfer the solution to a dark bottle and store at room temperature.

1 N HCL

HCL 87.3 ml/l

:Add concentrated HCL in dH_2O and adjust the volume to 1 liter.

10% Non-idet TEK

Non-idet P-40 10 ml/100 ml

:Add non-idet in TEK buffer and sterilize through sterile bottle by filtration.

10% SDS, pH 7.2-7.4

Sodium dodecyl sulfate 100 g/l

:Dissolve 100 g of electrophoresis-grade SDS and heat to 68°C to assist dissolution. Adjust pH by adding a few drop of 1/20 concentrated HCL and sterilize through sterile bottle by filtration.

3 M Sodium acetate, pH 5.2

$CH_3COONa \cdot 3H_2O$ 408.1 g/l

:Dissolve sodium acetate$\cdot H_2O$ in dH_2O and adjust pH to 5.2 with glacial acetic acid. Dispense into aliqouts and sterilize by autoclaving.

5 M NaCL

NaCL 292.2 g/l

:Dissolve NaCL in dH_2O and sterilize by autoclaving.

20x SSC, pH 7.0

3 M NaCL 175.3 g/l

0.3 M $Na_3C_6H_5O_7 \cdot 2H_2O$ 88.2 g/l

:Dissolve NaCL and trisodium citrate$\cdot 2H_2O$ in dH_2O and adjust pH to 7.0 with a few drop of HCL. Dispense into aliqouts and sterilize by autoclaving.

20x SSPE, pH 7.4

3 M NaCL 175.3 g/l

0.2 M $NaH_2PO_4 \cdot H_2O$ 88.2 g/l

0.02 M EDTA

:Dissolve NaCL, sodium phosphate and EDTA in dH_2O and adjust pH to 7.4 with NaOH (~6.5 ml of a 10 N solution). Dispense into aliqouts and sterilize by autoclaving.

1 M Tris

Tris 121.1 g/l

:Dissolve Tris base in dH_2O and adjust the pH to the desired value by adding concentrated HCL. Allow the solution to cool to room temperature before making the final adjustments to the pH and sterilize by autoclaving.

10x TBE buffer (0.89, 0.89, 0.01 M)

Tris 108 g/l

Boric acid 55 g/l

0.5 M EDTA, pH 8.0 40 ml/l

:Dissolve Tris, boric acid and EDTA in dH_2O and sterilize by autoclaving.

TE buffer (10, 1 mM, pH 8.0)

Tris 1.21 g/l

EDTA 0.37 g/l

:Dissolve Tris and EDTA in dH_2O and adjust pH with concentrated HCL. Dispense into aliqouts and sterilize by autoclaving.

TEN buffer (100, 100, 250 mM, pH 8.0)

Tris 12.11 g/l

EDTA 37.22 g/l

NaCL 14.61 g/l

:Dissolve Tris, EDTA and NaCL in dH_2O and adjust pH with concentrated HCL. Dispense into aliqouts and sterilize by autoclaving.

TEK buffer (50, 10 mM, 1.5%, pH 7.5)

Tris 6.06 g/l

EDTA 3.72 g/l

KCL 15.00 g/l

:Dissolve Tris, EDTA and KCL in dH_2O and adjust pH with concentrated HCL. Dispense into aliqouts and sterilize by autoclaving.

0.25 M Sucrose-TEK buffer

Sucrose 75 g/l

:Dissolve sucrose in TEK solution and sterilize by autoclaving.

1.1 M Sucrose-TEK buffer

Sucrose 376.53 g/l

:Dissolve sucrose in TEK solution and sterilize by autoclaving.

Proteinase K (10 mg/ml)

Proteinase K 10 mg/ml

:Dissolve Proteinase K in dH_2O and stored at -20°C.

RNase A (DNase-free)
Rnase 10 mg/ml
:Dissolve pancreatic RNase in TE, boil for 15 min on water bath, cool at room temperature, and store at -20°C.

10% BLOTTO
Nonfat powdered milk 10 g/100 ml
:Dissolve nonfat powder milk in sterile dH_2O and store in sterile bottle at 4°C.

50% Dextran sulfate
Dextran sulfate 50 g/100 ml
:Dissolve dextran sulfate in sterile dH_2O and store in sterile bottle at 4°C.

Calf thymus DNA
Calf thymus DNA 1 g/100 ml
:Dissolve 1 g of calf thymus DNA (Sigma type III sodium salt) in 100 ml of 0.4 M NaOH. Stir on magnetic stirrer overnight at room temperature. Boil on water bath for 45 min to shear the DNA, then chill on ice and neutralize with glacial acetic acid (pH 4-7). Centrifuge to remove debris, then add two volumes of ice cold 99.99% ethanol, place at -20°C for 1 h, and collect the DNA by centrifugation. Wash the pellet with 70% ethanol, vacuum dry for 10 min, and dissolve in 50 ml of TE buffer, pH 7.5. Determine concentration of DNA and dilute to 10 mg/ml. Store at -20°C in 1 ml aliquots.

Equilibrated phenol
Redistillation phenol 500 g
:Melt redistillation phenol at 68°C in fume cupboard, add equal volum of 1 M Tris, pH 8.0, shake well and leave 4-16 h for absorbtion. Remove aqueous layer and repeat the extractions. Remove aqueous layer, add equal volume of 0.1 M Tris, pH 8.0 and 0.2% β-mercaptoethanol, shake well and remove aqueous. Repeat the extractions until the pH of aqueous phase is >7.8 (≈ 3 times). Store at 4°C under 0.1 M Tris, pH 8.0 for periods of up to 1 month.

chloroform:Isoamyl alcohol (24:1)
:mix 24 ml of chloroform with 1 ml of isoamyl alcohol, then store under 0.1 M Tris, pH 8.0, in dark bottle at 4°C. The chloroform denatures proteins and facilitates the separation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction.

APPENDIX 4

MITOCHONDRIAL DNA ANALYSIS

Pairwiwe Analysis between Ten Populations

Trat 92-93

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.000	1	1.000
ECOR5	3	3.333	2	0.189
PVU2	3	2.400	2	0.301
SAC1	2	2.133	1	0.144
(Totals)		7.867	6	0.248
Multiple mtDNA		7.28	9.7	0.680
Monte Carlo simulation				0.430

Satun 92-93

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	2.220	1.11	0.160
ECOR5	3	4.038	2	0.133
PVU2	3	7.690	4.39	0.140
SAC1	2	5.230	1.17	0.030
(Totals)		14.710	11.21	0.220
Multiple mtDNA		22	10.16	0.018
Monte Carlo simulation				0.050

Trat-Surat

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	3.070	1.07	0.090
ECOR5	3	3.711	2	0.156
PVU2	3	6.000	2	0.049
SAC1	2	0.750	1	0.386
(Totals)		9.86	10.9	0.550
Multiple mtDNA		5.89	6.55	0.500
Monte Carlo simulation				0.500

Trat-Phuket

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	2.685	1	0.101
ECOR5	3	1.461	2	0.482
PVU2	3	4.731	2	0.094
SAC1	2	.750	1	0.386
(Totals)		9.627	6	0.141
Multiple mtDNA		4.76	7.57	0.750
Monte Carlo simulation				0.743

Trat-Satun

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	2.630	1.03	0.150
ECOR5	3	2.018	2	0.365
PVU2	3	5.560	2	0.062
SAC1	2	0.832	1	0.362
(Totals)		8.220	10.9	0.700
Multiple mtDNA		5.96	7.01	0.550
Monte Carlo simulation				0.585

Trat-Kedah

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	3.340	1.13	0.080
ECOR5	3	3.667	2	0.160
PVU2	3	6.188	2	0.045
SAC1	3	4.890	2.92	0.180
(Totals)		15.260	13.39	0.320
Multiple mtDNA		13.50	8.27	0.120
Monte Carlo simulation				0.080

Trat-Dungun

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	1.769	1	0.183
ECOR5	3	5.909	2	0.052
PVU2	3	2.373	2	0.305
SAC1	2	4.510	1.05	0.040
(Totals)		25.180	10.42	0.007
Multiple mtDNA		11.50	8.73	0.240
Monte Carlo simulation				0.162

Trat-Aceh

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.686	1	0.408
ECOR5	3	1.461	2	0.482
PVU2	3	2.286	2	0.319
SAC1	2	0.000	1	1.000
(Totals)		4.432	6	0.618
Multiple mtDNA		2.94	7.56	0.900
Monte Carlo simulation				0.809

Trat-S Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	1.746	1	0.186
ECOR5	3	1.650	2	0.438
PVU2	4	6.788	3	0.079
SAC1	2	2.994	1	0.084
(Totals)		13.178	7	0.068
Multiple mtDNA		14.66	11.94	0.290
Monte Carlo simulation				0.150

Trat-N Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.750	1	0.386
ECOR5	3	1.461	2	0.482
PVU2	4	6.750	3	0.080
SAC1	2	2.685	1	0.101
(Totals)		11.646	7	0.113
Multiple mtDNA		19.54	15.33	0.170
Monte Carlo simulation				0.175

Trat-Lingayen

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.000	1	1.000
ECOR5	3	1.200	2	0.549
PVU2	4	12.020	6.88	0.100
SAC1	2	3.070	1.07	0.900
(Totals)		12.950	14.40	0.600
Multiple mtDNA		31.19	17.26	0.019
Monte Carlo simulation				0.025

Surat-Phuket

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.582	1	0.446
ECOR5	3	4.000	2	0.135
PVU2	3	2.400	2	0.301
SAC1	2	0.000	1	1.000
(Totals)		6.982	6	0.323
Multiple mtDNA		6.500	10.4	0.800
Monte Carlo simulation				0.753

Surat-Satun

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.342	1	0.559
ECOR5	3	1.476	2	0.478
PVU2	3	0.657	2	0.720
SAC1	2	0.015	1	0.902
(Totals)		2.490	6	0.869
Multiple mtDNA		1.290	5.39	0.950
Monte Carlo simulation				0.876

Surat-Kedah

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	7.940	1.09	0.006*
ECOR5	2	1.615	1	0.204
PVU2	3	15.600	4.27	0.005*
SAC1	3	5.950	2	0.051
(Totals)		32.540	15.27	0.007
Multiple mtDNA		22.01	10.42	0.018
Monte Carlo simulation				0.012

Surat-Dungun

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	5.460	1.06	0.020
ECOR5	2	2.647	1	0.104
PVU2	3	11.340	4.19	0.030
SAC1	2	5.460	1.06	0.020
(Totals)		25.900	11.70	0.009
Multiple mtDNA		11.340	4.19	0.028
Monte Carlo simulation				0.030

Surat-Aceh

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	2.133	1	0.144
ECOR5	3	4.000	2	0.135
PVU2	3	3.556	2	0.169
SAC1	2	0.582	1	0.446
(Totals)		10.271	6	0.114
Multiple mtDNA		7.14	9.89	0.700
Monte Carlo simulation				0.405

Surat-S Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	4.670	1.08	0.040
ECOR5	3	10.480	6.26	0.120
PVU2	4	14.550	9.12	0.120
SAC1	2	2.450	1.09	0.140
(Totals)		26.612	17.09	0.070
Multiple mtDNA		20.89	15.79	0.180
Monte Carlo simulation				0.074

Surat-N Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	3.980	1.06	0.049
ECOR5	3	4.000	2	0.135
PVU2	4	7.430	2.41	0.035
SAC1	2	2.290	1.07	0.150
(Totals)		19.605	13.80	0.140
Multiple mtDNA		27.57	16.92	0.049
Monte Carlo simulation				0.049

Surat-Lingayen

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	2.290	1.07	0.150
ECOR5	3	6.154	2	0.046
PVU2	4	30.210	9.89	0.001*
SAC1	2	3.980	1.06	0.049
(Totals)		32.300	17.14	0.020
Multiple mtDNA		33.33	16.67	0.013
Monte Carlo simulation				0.016

Phuket-Satun

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.080	1	0.777
ECOR5	3	1.476	2	0.478
PVU2	3	1.129	2	0.568
SAC1	2	0.015	1	0.902
(Totals)		2.700	6	0.845
Multiple mtDNA		1.29	5.39	0.940
Monte Carlo simulation				0.900

Phuket-Kedah

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	6.020	1.11	0.018
ECOR5	2	1.615	1	0.204
PVU2	2	11.667	1.11	0.018
SAC1	3	5.950	2	0.051
(Totals)		23.060	12.16	0.030
Multiple mtDNA		22.01	10.42	0.020
Monte Carlo simulation				0.019

Phuket-Dungun

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	3.420	1.07	0.019
ECOR5	2	2.647	1	0.104
PVU2	2	3.420	1.07	0.019
SAC1	2	5.460	1.06	0.022
(Totals)		17.150	9	0.048
Multiple mtDNA		11.34	4.19	0.030
Monte Carlo simulation				0.033

Phuket-Aceh

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.508	1	0.476
ECOR5	2	0.000	1	1.000
PVU2	2	0.508	1	0.476
SAC1	2	0.582	1	0.446
(Totals)		1.598	4	0.809
Multiple mtDNA		0.400	2.73	0.900
Monte Carlo simulation				0.607

Phuket-S Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	5.882	1	0.015
ECOR5	2	1.768	1	0.184
PVU2	3	7.194	2	0.027
SAC1	2	4.861	1	0.027
(Totals)		12.970	10.73	0.280
Multiple mtDNA		20.89	15.80	0.180
Monte Carlo simulation				0.090

Phuket-N Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	2.290	1.07	0.140
ECOR5	2	0.000	1	1.000
PVU2	3	9.460	2	0.070
SAC1	2	4.571	1	0.032
(Totals)		12.410	10.92	0.330
Multiple mtDNA		27.57	16.91	0.060
Monte Carlo simulation				0.040

Phuket-Lingayen

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	2.000	1	0.157
ECOR5	2	0.821	1	0.365
PVU2	3	15.850	4.45	0.006*
SAC1	2	3.980	1.06	0.049
(Totals)		17.040	10.65	0.000
Multiple mtDNA		33.33	16.67	0.008
Monte Carlo simulation				0.010

Satun-Kedah

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	8.020	1.10	0.006*
ECOR5	3	1.768	2	0.413
PVU2	3	11.210	3.06	0.015*
SAC1	3	6.320	3.08	0.110
(Totals)		29.150	14.42	0.017
Multiple mtDNA		24.03	10.01	0.008
Monte Carlo simulation				0.006

Satun-Dungun

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	5.270	1.05	0.022
ECOR5	3	2.899	2	0.235
PVU2	3	10.010	3.89	0.035
SAC1	2	6.880	1.05	0.009*
(Totals)		28.970	13.95	0.070
Multiple mtDNA		18.54	8.19	0.018
Monte Carlo simulation				0.029

Satun-Aceh

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	1.217	1	0.270
ECOR5	3	1.476	2	0.478
PVU2	3	1.992	2	0.369
SAC1	2	0.578	1	0.447
(Totals)		5.263	6	0.511
Multiple mtDNA		1.76	5.34	0.920
Monte Carlo simulation				0.808

Satun-S Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	4.510	1.10	0.045
ECOR5	3	5.490	2	0.064
PVU2	4	9.790	5.41	0.110
SAC1	2	3.070	1.11	0.090
(Totals)		21.170	13.70	0.090
Multiple mtDNA		17.44	11.18	0.100
Monte Carlo simulation				0.168

Satun-N Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	3.700	1.07	0.070
ECOR5	3	1.476	2	0.478
PVU2	4	15.620	6.99	0.030
SAC1	2	2.890	1.07	0.100
(Totals)		21.210	15.11	0.130
Multiple mtDNA		28.14	15.92	0.030
Monte Carlo simulation				0.016

Satun-Lingayen

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	3.612	1	0.057
ECOR5	3	3.927	2	0.140
PVU2	4	22.650	7	0.003*
SAC1	2	5.070	1.07	0.030
(Totals)		27.660	14.81	0.030
Multiple mtDNA		38.84	17.17	0.004
Monte Carlo simulation				0.006

Kedah-Dungun

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	2.743	1	0.098
PVU2	2	2.743	1	0.098
SAC1	3	4.848	2	0.088
(Totals)		7.380	10.16	0.720
Multiple mtDNA		14.27	6.83	0.040
Monte Carlo simulation				0.042

Kedah-Aceh

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	4.560	1.17	0.045
ECOR5	2	1.615	1	0.204
PVU2	2	4.560	1.17	0.045
SAC1	3	4.083	2	0.130
(Totals)		16.999	12.30	0.160
Multiple mtDNA		19.28	10.55	0.045
Monte Carlo simulation				0.049

Kedah-S Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	2.182	1	0.140
ECOR5	2	3.670	1.67	0.120
PVU2	3	4.800	2	0.091
SAC1	3	2.286	2	0.319
(Totals)		15.320	20	0.680
Multiple mtDNA		37.13	33.75	0.270
Monte Carlo simulation				0.290

Kedah-N Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	3.500	1	0.061
ECOR5	2	1.615	1	0.204
PVU2	3	3.500	2	0.174
SAC1	3	2.888	2	0.236
(Totals)		11.503	6	0.074
Multiple mtDNA		37.15	31.53	0.210
Monte Carlo simulation				0.089

Kedah-Lingayen

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	3.447	1.29	0.080
ECOR5	2	3.500	1	0.061
PVU2	2	1.615	1	0.204
SAC1	3	3.500	2	0.174
(Totals)		11.633	13.20	0.600
Multiple mtDNA		24.38	18.75	0.190
Monte Carlo simulation				0.117

Dungun-Aceh

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	3.600	1	0.058
ECOR5	2	2.647	1	0.104
PVU2	2	3.600	1	0.058
SAC1	2	3.420	1.07	0.080
(Totals)		9.430	9.11	0.420
Multiple mtDNA		7.57	4.26	0.130
Monte Carlo simulation				0.094

Dungun-S Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.055	1	0.815
ECOR5	2	5.839	1.63	0.035
PVU2	3	3.556	2	0.170
SAC1	2	.711	1	0.399
(Totals)		8.310	11.30	0.700
Multiple mtDNA		10.86	9.03	0.320
Monte Carlo simulation				0.331

Dungun-N Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.129	1	0.720
ECOR5	2	2.647	1	0.104
PVU2	3	2.829	2	0.243
SAC1	2	1.357	1	0.244
(Totals)		6.961	5	0.223
Multiple mtDNA		9.96	11.14	0.540
Monte Carlo simulation				0.260

Dungun-Lingayen

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	1.357	1	0.244
ECOR5	2	4.650	1.75	0.080
PVU2	3	5.760	2	0.056
SAC1	2	.129	1	0.720
(Totals)		9.250	11.66	0.650
Multiple mtDNA		22.52	13.25	0.050
Monte Carlo simulation				0.080

Aceh-S Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	3.319	1	0.068
ECOR5	2	1.768	1	0.184
PVU2	3	5.133	2	0.077
SAC1	2	2.333	1	0.127
(Totals)		8.310	10.78	0.650
Multiple mtDNA		16.90	15.79	0.380
Monte Carlo simulation				0.298

Aceh-N Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	2.133	1	0.144
ECOR5	2	0.000	1	1.000
PVU2	3	6.400	2	0.041
SAC1	2	2.000	1	0.157
(Totals)		10.533	5	0.061
Multiple mtDNA		21.42	17.24	0.200
Monte Carlo simulation				0.089

Aceh-Lingayen

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.508	1	0.476
ECOR5	2	0.821	1	0.365
PVU2	3	12.810	4.7	0.020
SAC1	2	2.291	1.07	0.150
(Totals)		8.580	10.76	0.620
Multiple mtDNA		27.55	11.30	0.004
Monte Carlo simulation				0.042

S Java-N Java

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.283	1	0.595
ECOR5	2	1.768	1	0.184
PVU2	3	0.233	2	0.890
SAC1	2	0.052	1	0.820
(Totals)		2.336	5	0.801
Multiple mtDNA		6.54	12.46	0.900
Monte Carlo simulation				0.750

S Java-Lingayen

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	1.458	1	0.227
ECOR5	2	0.233	1	0.630
PVU2	3	3.129	2	0.209
SAC1	2	0.233	1	0.629
(Totals)		5.054	5	0.409
Multiple mtDNA		46.02	42.48	0.200
Monte Carlo simulation				0.168

N Java-Lingayen

Locus	No. of alleles	Nass Chi-square	D.F.	P
Individual mtDNA				
BAMH1	2	0.582	1	0.446
ECOR5	2	0.821	1	0.365
PVU2	3	2.154	2	0.341
SAC1	2	0.582	1	0.446
(Totals)		4.138	5	0.530
Multiple mtDNA		57.64	51.88	0.200
Monte Carlo simulation				0.194

* = significant difference

Glossary

Allele One or more alternative forms of a gene, each possessing a unique **nucleotide** sequence and affecting the structure and/or function of a single product (RNA and/or protein).

Allozymes Enzymes differing in electrophoretic mobility as a result of allelic differences at a single gene (cf. **Isozyme**).

Autoradiograph Image on an X-ray film created by radioactive or chemiluminescent labelled DNA fragments.

Base pairs (bp) A single pair of complementary nucleotides from opposite strands of the DNA double helix. The number of base pairs is used as a measure of length of a double-stranded DNA.

Bottleneck Fluctuations in allelic frequencies when a large population passes through a contracted stage and then expands again with an altered genetic composition as a consequence of genetic drift.

Effective population size (N_e) The number of individuals in an ideal, randomly breeding population with a 1/1 sex ratio that would have the same rate of heterozygosity decrease as the actual population under consideration.

Electrophoresis The separation of macromolecules (e.g. enzymes or DNA) in the presence of an electric current. In molecular genetics, differences in charge, size or shape (i.e. differences in electrophoretic mobility) of the macromolecules are used to estimate genetic differentiation.

Gene flow The movement of genes into or out of a population by interbreeding, or by migration and interbreeding.

Genetic distance A measure of the number of allelic substitutions per gene that have occured during the separate evolution of two populations or species.

Genetic drift Variation in allele frequency from one generation to another due to chance flutuations. It is generally greater in populations with small **effective populatin size** and high inbreeding.

Genetic marker A genetically inherited variant from which the genotype can be inferred from the phenotype as identified during genetic screening.

Haplotype Nucleotide sequence of an individual's mtDNA genes charcterized by **restriction fragment length polymorphisms (RFLPs)** or direct sequencing. It is the multilocus analogue of an allele.

Locus A physical position of a gene on a chromosome. *plu.* loci

Recombination The occurence of progeny with gene combinations other than those found in their parents, caused by the independent assortment of chromosomes and gametes in a sexual species or by crossing-over between chromosomes.

Restriction enzyme An enzyme that cleaves double-stranded DNA. Type I are not sequence specific; typeII cleave DNA at a specific sequence of nucleotides known as **restriction** or **recognition sites**.

Restriction fragment length polymorphism (RFLPs) Variations occuring within a species in the length of DNA fragments generated by a specific **restriction enzyme**. Such variation is generated either by base substitutions that cause a gain or loss of sites, or by insertion/deletion mutations that change the length of fragments independent of **restriction site** change.

Restriction site A specific sequence of nucleotide bases which is recognized by a **restriction enzyme**. The enzyme will cleave both DNA strands at a specific location within that sequence. Variation in the present and absence of restriction sites among individuals generates **Restriction fragment length polymorphism (RFLPs)**.