Chapter 4

Discussion

The standard technique for isolation of closed circular DNA was dependent on the property of closed circular DNA. That was showed a different position of DNA under Cesium Chloride-Ethidium Bromide buoyant density gradient. Normally, it was separated to two positions of DNA, the upper position was linear DNA and the lower position was closed circular DNA because the closed circular DNA-EtBr complex was greater buoyant density than linear DNA-EtBr complex. Since all animal nuclear DNA was linear form, it could be separated to upper position, while the mtDNA which was closed circular form and could be separated to lower position (Radloff et al., 1967). For Macrobrachium rosenbergii, the CsCl-EtBr gradient method can separate the DNA into two bands. After both bands of DNA were tested with some restriction enzyme digestions and analyzed on agarose gel electrophoresis, fragment patterns obtained with the following enzymes: Ava 1, Bam H1 and Eco R1, were different among two DNA bands when digested with the same restriction enzyme (show in Fig 8). The upper band DNA did not show restriction fragment patterns but the lower band DNA appeared restriction fragment patterns. The lower band DNA was consequently identical to the mtDNA of M. rosenbergii because the mtDNA in animal was closed circular form, strikingly uniform in size and structure that it

was digested with appropriate restriction enzymes usually resulting a definded set of homogeneous fragments which gave sharp bands on gel electrophoresis. The nuclear DNA was heterogeneous form that it was revealed a smear band (Darley-Usmar et al., 1987). This data suggested that the chromosomal DNA was nearly buoyant density to mtDNA and the fixed angle rotor did not contribute good resolution for separating. Therefore the mtDNA was contaminated with smear band.

All animal mtDNA was resemble to many bacterial plasmids in structure so that rapid alkaline extraction method developed for the isolation of bacterial plasmids could also be applicable to mtDNA isolation from animal tissue (Palva, 1985). This method showed that the DNA obtained from each individual prawn was low yield. By phenol extraction method, the high background of contaminating chromosomal DNA was appeared. Such contamination could occur easily in crude mitochondria preparation because of this prawn had high number of chromosome about 114-115 (Malecha, 1979). The purified mitochondria preparation by discontinuous sucrose gradient prior to mtDNA extraction can reduce chromosomal DNA contamination (Tapper et al., 1983). An attempt to purify mitochondria from individual prawn and extract mtDNA by phenol or rapid alkaline extraction, the mtDNA obtained was not sufficient in quantity for further use. In addition, the determination for restriction fragment length polymorphism in mtDNA of individual prawn had some difficulties since chromosomal DNA produced complex pattern that interfered the analysis. This problem can be overcomed by the technique of molecular hybridization with specific probe of mtDNA.

The purified mitochondria was prepared from 15-20 prawns and mtDNA was extracted by phenol or rapid alkaline extraction.

The mtDNA obtained from both extraction methods were appeared to be less contaminated with chromosomal DNA and were identical to mtDNA from lower band of CsCl-EtBr gradient method with some restriction fragment patterns from Ava 1, Bam H1 and Eco R1 digestion. The phenol extraction was suitable for mtDNA preparation because this method obtained higher yield than rapid alkaline extraction, of which a large amount of DNA was loss in preparation.

The mtDNA obtained may be limited by its low yield for using as a specific mtDNA probe, the recombinant DNA technique may be employed to clone mtDNA in a plasmid vector. The recombinant DNA which had mtDNA fragment insertion was selected and purified. This recombinant DNA in large quantities can be used as a useful probe in the screening of restriction fragment length polymorphism in individual prawn.

The vector pUC12 was chosen for cloning because it had a small size, 2.7 kb, which could give higher efficiency in transformation and the B-galactosidase enzyme activity contained in this plasmid could be assayed by using x-gal. Some 6 nucleotide recognition enzymes were introduce to digest mtDNA and they gave large fragments, for example Bam H1 generated about 16 and 4.5 kb, which were very difficult to ligate with vector pUC12 and transform into host cell. For restriction enzyme that recognized 4 nucleotide, the Sau 3A1 restriction enzyme was selected for digestion of mtDNA prawn in the cloning system because it generated small fragments about 0.2-2.0 kb. and its restriction site was compatible to tetranucleotide of Bam H1 digested vector pUC12. The recombinant DNA was selected and used as a probe to detect restriction fragment length polymorphism in prawn mtDNA on criteria that the selected one must give the strong signal of inserted fragments with mtDNA probe to ensure

that insertion was mtDNA fragments. Since, the mtDNA probe had some degree of chromosomal DNA contamination, the weak signal of inserted fragments were possible to be the signal of chromosomal DNA fragments. In order to detect mtDNA fragment variation in individual prawn, the mtDNA was extracted from crude mitochondria preparations of individual prawn and digested with restriction enzyme Sau 3A1 for generating the mtDNA fragments to the same cloning system so that the inserted fragment in recombinant DNA can itself fragment hybridize.

The recombinant DNA No 1 which had inserted mtDNA fragment about 1.1 kb revealed genetic variation in Macrobrachium rosenbergii by restriction fragment length polymorphism in mtDNA. This can distinguish these prawns to two geographic populations. The prawns from Kung Kam Thong Farm and Bangpakong River showed highly repetitive hybridization fragment at 1.1 kb but Kraburi River's prawns showed at 0.7 kb (Fig 24,25). The prawns from Kung Kam Thong Farm were genetically similar to Bangpakong River. The reason was that the earlier generations of offsprings in this farm were produced from Bangpakong River's broodstocks and after that they were inbred producing offsprings in later generations. However, it might be possible the other mtDNA fragments were different because aquaculture conditions may induce genetic variation between domestic stocks and wild type stocks (Sbordoni et al., 1987).

In natural populations, the finding that the prawns from Bangpakong and Kraburi River exhibited two highly distinctive mtDNA genotypes which was showed size fragment differentiation These were correlated with geographic distribution having different local environments. The Bangpakong River discharged into the Upper Gulf of Thailand at Chachoengsao province but Kraburi River discharged into Andaman Sea at Ranong province and

these two rivers were not interconnection. These two types of mtDNA should be clearly distinguished two geographic population prawns. The geographical distribution of species has important consequence for the distribution of genetic variation among the populations by diversifying selection resulting from heterogeneous environments (Hedgecock, 1987). It had been revealed low level of gene differentiation in these prawns between Bangpakong River and Songkha Lake by isoenzyme patterns (Therakulkiat, 2524). The mtDNA was a suitable molecule for determining genetic variation and population genetics because the base substitutions of mtDNA has been showed to develop rapid evolution which appeared to be 5-10 times faster than nuclear DNA (Brown et al., 1979). So mtDNA was expected high degree of genetic differentiation more than isoenzyme patterns, of which was nuclear DNA product.

Size fragment variation in mtDNA has been observed in many organisms. The variation was resulted from either addition, deletion and point mutation, gain or loss of particular restriction sites. To explain the high rate of mtDNA variation, the possibility of a mutation rate due to the editing function of the mtDNA replication complex may be inefficient or lacking to repair other types of mtDNA damage that was absence of an enzymatic function, the excision and repair of thymine dimer (Clayton et al., 1974; Lansman and Clayton, 1975) and the mtDNA has a higher turnover rate than nuclear DNA in tissue (Rabinowitz and Swift, 1970), thus providing more rounds of replication during which errors could be generated. The another factor could contribute to a high chance of fixation which could be a result of lower function constraints on the mtDNA. If a given mitochondria gene was inactive, the organism might survive because the organism is polyploid for mitochondria genes; each

cell contains many mitochondrias, comprising at least one copy of the mitochondria genome (Bogenhagen and Clayton, 1974). A mutation inactivating a given gene in one genome might therefore have little or no affect in the fitness of the organism. If some mutant mtDNA did exist in an ovum, the ratio of mutant mtDNA to wild type mtDNA should vary very greatly in the primodial germ cells, originated from the ovum by repeated segmentation, some having a greatly increased proportion of mutant mtDNA. After passing through generations the progeny of these cells probably become to contain only the mutant mtDNA. Then once this homogeneity of the mutant mtDNA in the ovum has been established, it should be maintained by the strictly maternal inheritance. Thus a heterogeneous populations of wild type and mutant mtDNA in individual animal should finally be segregated. As the results from repeated mutations followed by repeated segmentation of mtDNA during evolution, it should enhance in a species to form the various mtDNA types that can be distinguished (Hayashi, 1979).

The total mtDNA molecules from two geographic populations were hybridized with recombinant DNA No 1 revealing no difference between these geographic populations. However, this data suggested that the inserted fragment in this recombinant DNA was a portion of fragment in mtDNA genome because the mtDNA of animal is homogeneous in size and structure and had high copy number which was contrast to the heterogeneous property of nuclear DNA. Moreover, recombinant DNA No 1 was revealed highly sequence repetitive with total mtDNA genomes in autoradiogram (Fig 23).

Variations in size fragment of mtDNA among individuals of the same geographic population. The Bangpakong River populations were also demonstrated a single band at about 1.1 kb. However, some individuals appeared different lower intensity band (Fig

27,28). It was indicated that the mtDNA in these samples were heteroplasmic. The Bangpakong River populations may have two types of mtDNA molecules which have difference in size fragment, one type was hybridization fragment at 1.1 kb. and the another type was smaller than 1.1 kb. Some individuals only possess one mtDNA type, whereas others, carrying both types in different proportion, were heteroplasmic. The occurrence of heteroplasmic genotype in individual has also been observed in some organisms, Drosophila mauritiana (Fauron and Wolstonholme, 1980), Holstein cow maternal lineage (Olivo et al., 1983), two species of Cnemidophorus lizards (Densmore et al., 1985), bowfin fishs and two species of treefrogs (Bermingham et al., 1986). The mitochondria heteroplasmic genotypes existed in an animal can be explained by random segregation of mitochondria genome during oogenesis of female. In many species mitochondria heredity is strictly maternal and heteroplasmy so that genetic variation can only be created by mutations affecting the female germ line. After the occurrence of a mutation, a heteroplasmic state must exist. Then mtDNA molecule segregates randomly at each cell division of oogenesis. If parental mtDNA molecules are transmitted in a random distribution, any mutant may become established as the major mitochondria genotype in a progeny, given a sufficient number of generations (Hauswirth et al., 1982; Solignac et al., 1983). Prawns from Kraburi River was likely to exhibit genetic variation among individuals. Although, most samples from this geographic population showed the same major hybridization fragment, the smallest hybridization fragment in lower intensity has been observed with size difference (Fig 29,30).

It will be of interest for future studies to increase the number of samples to determine the constitution of a single

breeding population or many discrete populations among in each geographic population. Especially the Bangpakong River was interconnected with other geographic areas, Ta Chin, Ma Kong and Chao Phaya River.

For old inbreeding, the degree of mtDNA variation among individuals was relatively low due to a single female breeding population (Ferris _et al_., 1982). One potential importance for genetic improvement in aquaculture was to decrease inbreeding in population by interracial hybridization. The interesting examples of interracial hybridization in fishes are the hybridization between the two racial stocks of rainbow trout (Menasveta, 1961) and the hybridization between the Chinese and European races of the common carp (Moav, 1976). In these two case studies the offsprings exceled their parent stocks.

The study of mtDNA molecules will lead to real advances in the understanding of genetic variation in _Macrobrachium rosenbergii_ which are widely geographic distribution. Besides, information of genetic variation can be applied to the population genetics, stock identifications and species migration for fishery management (Thompson and Contin, 1980; Lamb and Avise, 1986), biology in each population for genetic improvement in aquaculture (Hedgecock, 1977; Malecha, 1987). This technique of using the cloned fragments mtDNA as a probe, will allow the study of mtDNA variation in other marine organisms where high yields of mtDNA uncontaminated with nuclear DNA cannot easily be obtained.