

**MICROSATELLITE DNA MARKER BASED MOLECULAR
ECOLOGICAL EVALUATION STUDIES IN
MACROBRACHIUM SPECIES**

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ECOLOGICAL EVALUATION STUDIES IN
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By

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CERTIFICATE

This is to certify that the thesis entitled “*Microsatellite DNA marker based molecular ecological evaluation studies in Macrobrachium species*” submitted by Mr. DIVU, D., I.D.No. DFK 601 in partial fulfillment of the requirement for the award of Ph.D. in fishery Microbiology of the Karnataka Veterinary, Animal & Fisheries Sciences University, Bidar is a record of bonafide research work carried out by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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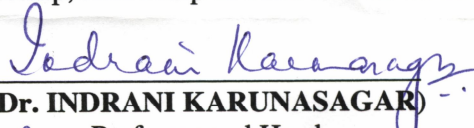
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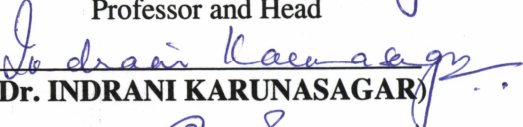
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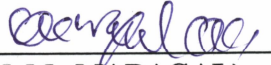
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I. INTRODUCTION

The sustainability of exploited populations is a primary goal in the management of natural resources. Due to the nature of the aquatic environment, exact estimates of population size, the geographical extent of populations and other important demographic parameters are almost impossible to obtain. Many important fisheries around the world continue to decline or have collapsed through over-fishing (Hilborn *et al.*, 2003). Indeed, current estimates indicate 29% of the world's fisheries have collapsed and are no longer economically viable, and these levels are predicted to rise (Worm *et al.*, 2006). One of the major issues associated with fisheries management is optimising yield without compromising ecosystem or population processes (Fogarty *et al.*, 1991; Caddy and Seijo 2005; Worm *et al.*, 2006). Population decline beyond a critical point may be detrimental for at least two reasons. First, allelic effects, whereby fitness declines with population size, may result in reproductive and recruitment failure and severely constrain recovery potential of a population (Levitan and Sewell, 1998). Second, over-fishing may lead to reduce genetic diversity in a population (Hauser *et al.*, 2002), and small populations will also be susceptible to further loss of genetic diversity through drift (Allendorf and Luikart, 2007).

In the absence of active intervention or enhancement, the recovery of collapsed fisheries will rely entirely on new recruitment. Understanding of the likely source and abundance of new recruits has therefore become a critical goal of fisheries science and management. For some fisheries, such as large finfish, direct tagging of individuals to understand emigration or immigration is possible (Davis and Stanley, 2002; Block *et al.*,

2005). However, for commercially important benthic invertebrate species such as shrimps and prawns the primary dispersal phase is usually a microscopic, pelagic larva, which is difficult to tag and impossible to track. For such species, the application of genetic techniques has proven critical for understanding connectivity among populations (Hellberg *et al.*, 2002).

The giant freshwater prawn (*Macrobrachium rosenbergii*) is one of the most economically important species of cultured crustaceans. Shrimp and prawn populations in their natural habitat around the world are threatened by a variety of pressures including habitat destruction, environmental contaminants, gene pool depletion and disease (Lester and Pante, 1992; Primavera *et al.*, 1993; Purdom *et al.*, 1994; Lightner, 1996b; Brock *et al.*, 1997). It is of international concern to have access to clean wild stocks with the highest genetic diversity and to establish policies for regulation of movement of wild and cultured stocks of shrimp and prawn intended for breeding purposes (Johnson, 1994). The movement of specific pathogen-free (SPF) prawn and shrimp stocks developed through captive breeding should also be regulated, as some of them could potentially be inbred and susceptible to viral diseases (Alcivar-Warren *et al.*, 1997; Wolfus *et al.*, 1997). *M. rosenbergii* has been imported to many other tropical and subtropical areas of the world and is the species most favoured for farming purposes. Many *Macrobrachium* species have been transferred from their natural location to other parts of the world, initially for the research purposes. *M. rosenbergii* remains the species most used for commercial farming and consequently is the one which has been introduced to many countries. Its first import occurred during 1965, from Malaysia to Hawaii for research purposes (Fujimura, 1966).

Some wild stocks of *M. rosenbergii* have been seen to decline rapidly in recent years; which is largely as a result of over harvesting, habitat loss and increased pollution, particularly

in South East Asia. Declines have been recorded in Bangladesh, India, Indonesia, Malaysia, Philippines and Thailand (New *et al.*, 2000). The species is now believed to be extinct in Singapore, largely as a result of pollution and loss of natural habitat (Ng, 1997). Wild stocks can provide immediate resource for addressing genetic diversity problems in cultured stocks due to inbreeding depression etc, and consequently require conservation. To protect natural resources and to maintain a sustainable prawn aquaculture industry, the key strategy is to prevent the loss of genetic diversity of wild prawns and to increase the genetic variability of cultured stocks (Alcivar-Warren, 1994). To this end, the patterns and extent of genetic diversity that are present in wild stocks need to be adequately documented so that we can identify which stocks may carry unique genetic attributes and prioritize conservation efforts. Gene diversity depends on the mutation rate of genes, the size and demographic history of the population in which these mutations occur, the time over which such diversity accumulates and biological factors such as selection. Sequence variation is the currency of genetics; the central aim of all genetics is to correlate specific molecular variation with phenotypic changes. The degree of diversity that can be maintained in a population is directly proportional to population size (Nei, 1987).

Molecular markers could provide a method to assist the selection of individuals in a prawn breeding programme, which would benefit in enhancing the domestication of species. Several molecular marker studies have demonstrated the ability to determine parentage, analyse pedigree and identify species (Herbinger *et al.*, 1995; Perez-Enriquez *et al.*, 1999; Sugaya *et al.*, 2002; Chan, 2003; Hara and Sekino, 2003; Jackson *et al.*, 2003; Castro *et al.*, 2004; McDonald *et al.*, 2004; Sekino *et al.*, 2004; Vandeputte *et al.*, 2004). The development of highly polymorphic genetic markers such as minisatellites and microsatellites had provided

an essential tool for identifying parentage relationships among individuals and obtaining pedigree information in aquaculture selection programs (O'Reilly and Wright, 1995; Ferguson and Danzmann, 1998).

Genetic variation is an important feature of populations both for short term fitness of the individuals and the long term survival of the population through allowing the adaptation to occur in changing environmental conditions (Erckmane and Sugunan., 2003). Identification and characterization of population units are imperative for fisheries management because efficient resource utilization can best be achieved when managing on the population level. The possible neglect of genetic diversity in fisheries management decisions might have been due to lack of tools for the determination of genetic variation and to placing emphasis on environmentally induced characters such as morphological or physical characters used for evaluating variation among populations (Dahle *et al.*,1997). Therefore effective molecular techniques should be developed to study the genetic variation among the stocks.

Against this background, the present study was carried out with following objectives;

1. To develop microsatellite DNA markers for giant freshwater prawn *M. rosenbergii*
2. To evaluate the microsatellite markers in *M. rosenbergii* population
3. Use these markers to study the molecular ecology of the species.
4. Cross species evaluation of microsatellite markers
5. Use of DNA microsatellites for the detection of some infectious viruses present in the asymptotic carrier individuals of *M. rosenbergii*

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Genetic studies are concerned with variation across a wide range of spatial and temporal scales from within individuals, among individuals within a population, among populations, to among species and higher level taxa. Different processes may operate at these different levels. Molecular genetics is principally concerned with the processes of development, mutation and the transfer of genes from one generation to the next. Population genetics refers to the distribution of genetic variation among individuals within a population, and among populations within a species.

Systematics is the study of relationships among species. Microevolution deals with changes seen within the species. Macroevolution is speciation and higher level divergence. The ideal marker to examine genetic variation within an individual requires a technique that can distinguish single base pair changes. Studies to find variants causing certain traits need markers spread throughout the genome. Species diagnosis would ideally use a marker that is invariant within a species but distinguishes it from all other species.

2.1. Difference between molecular and other markers

Markers are heritable variable traits that indicate the underlying genetic differences between individuals, populations, species or higher taxa. Non-molecular markers have some serious limitations; their inheritance can only be proven by controlled breeding trials i.e. non-mendelian inheritance; a trait in one group may not be present in other groups, making comparison between groups impossible; they may not reflect actual genetic variation due to phenotypic plasticity or the same character state may be the result of analogous traits. Morphological and ecological traits should not be disregarded since they reflect the results of

selection and there are examples of clear morphological and behavioural differentiation without genetic differentiation at neutral loci (Bensch *et al.*, 1999).

Although molecular markers have been available for several decades, their use has grown very rapidly in the last few years. The variety of methods available, including polymerase chain reaction (PCR) leads to the explosion of information in all areas of genetics in recent years. The application of genetic markers has allowed rapid progress in aquaculture investigations of parentage assignments, genetic variability and inbreeding, species and strain identification and the construction of high-resolution linkage maps for aquaculture species (Liu and Cordes, 2004). Molecular markers could provide a method to assist the selection of individuals in a prawn-breeding programme, which would facilitate the domestication of species. Several molecular marker studies have demonstrated the ability to determine parentage, analyze pedigree and identify species (Herbinger *et al.*, 1995; Perez-Enriquez *et al.*, 1999; Sugaya *et al.*, 2002; Chan, 2003; Hara and Sekino, 2003; Jackson *et al.*, 2003; Castro *et al.*, 2004; McDonald *et al.*, 2004; Sekino *et al.*, 2004; Vandeputte *et al.*, 2004). The development of highly polymorphic genetic markers such as minisatellites and microsatellites has provided an essential tool for identifying parentage relationships among individuals and obtaining pedigree information in aquaculture selection programs (O'Reilly and Wright, 1995; Ferguson and Danzmann, 1998).

2.2. Selection criteria for the marker of choice

Different molecular markers provide genetic information, which is suited to only a certain range of questions at a particular scale (Waycott, 1998; Shaw *et al.*, 1999; Sole *et al.*, 1999). A very fine scale marker can detect and quantify differences between individuals but it becomes useless when applied to members of different species. Different markers may reveal

different patterns because they detect variability at different scales (Raybould *et al.*, 1999). For example, many allozyme studies on marine fish have failed to detect any geographic structuring, although more rapidly evolving markers have in some cases revealed fine scale structure (Neigel, 1994). It is likely that each evolutionary or genetic process may have different effects on each type of marker. For example, the effective population size for mitochondrial DNA is quarter of that of nuclear markers; therefore any processes that are population size dependent will vary in their effect on mtDNA versus nuclear regions. Hence it is essential to choose a technique, which is appropriate to the question being asked (Yakubu *et al.*, 1999).

There is a range of marker types available. They all have different characteristics such as cost, ease of use and type of variation detected. There is no perfect marker that can be used to answer all questions. The choice of marker to use depends not only on considerations of cost and ease-of-use, but most importantly on the questions for which answers are being sought (Yakubu *et al.*, 1999). Levels of variation for any marker are in large part based on the rate and mode of mutation relative to the effects of genetic drift (Scribner *et al.*, 1994).

2.3. Population genetic models and processes

The ideal population in a classic model consist of diploid sexually reproducing individuals, infinite in size (no stochastic effects), with no selection, migration, mutation or overlapping generations and perform random mating (Weir, 1996). For this ideal population, the frequency of each allele remains constant over time and genotype frequencies in one generation can be predicted from allelic frequencies in the previous generation (Hardy-Weinberg Equilibrium). Population genetic models attempt to describe the effects of one or a few processes that violate the assumptions of an ideal population.

2.3.1. Genetic drift

In a population of finite size, allelic frequencies will vary from generation to generation as a result of random sampling of gametes. This process is called genetic drift. When the allele frequencies drift to zero, those alleles get extinct from the population. This shows that two populations originated from the same ancestral population, if completely isolated for a long period, by genetic drift alone to have different allelic frequencies and even have fixed allele differences. Gene flow will prevent the populations from drifting apart, provided there must not be a high degree of isolation (Hedrick, 2000).

In an expanding population, the probability of occurring any drift is greatly reduced, as there is less likelihood of loss of any alleles. On the other hand, a temporary dramatic decrease in population size, called bottleneck, greatly increases the probability of allele loss. Similarly the founder effect occurs when a new population is started by a very few individuals and thus has few alleles. The resultant low diversity can have critical implications if variation is low enough to prevent adaptations to new challenges (Hedrick, 2000).

2.3.2. Mutation and mating

Mutations may occur throughout the genome; however the rate of incorporation of mutations differs greatly in different regions of the genome. Coding regions are in general less variable than non-coding regions, presumably due to selection against changes that cause the protein less functional than the original version. Insertions and/or deletions in coding regions may cause frame shift mutations that can completely change the rest of the protein amino acid sequence. Some nucleotide substitutions, however, do not cause changes in amino acids due to

redundancy of the genetic code. These are called silent mutations or synonymous mutations (Di Rienzo *et al.*, 1994).

All genetic variation first originates from mutations. It is the occurrence and subsequent persistence and the spread of these mutations that creates the variation, which allows evolution to occur. Breeding systems range from asexual reproduction to strict sexual mating. Even among the strict sexually reproducing species assortative mating can occur, or if there is unrecognised sub-population structure within a sample this can lead to an apparent deviation from Hardy Weinberg Equilibrium (HWE) even though within the sub-populations there is random mating. This is called Wahlund effect and is revealed as an excess of homozygotes in a strictly sexual species. While collecting such samples care must be taken to ensure that individuals are from the same population and collected from the same location and at the same time (Hedrick, 2000).

2.3.3. Gene flow

Some individuals may leave the population and produce offspring in another population. The level of gene flow has significant consequences for the evolution of populations and thus species. This high gene flow allows the spread of new alleles and reduces the genetic drifting apart of populations. The process generally slows down the local adaptation, but there are circumstances where gene flow may enhance local adaptation. There are three distinct indirect methods for estimating gene flow from observed genetic variation; they are the estimation from F_{ST} analogs, private alleles and coalescent analyses (Slatkin and Maddison, 1989; Hudson *et al.*, 1992).

2.3.4. 1. Migration and gene flow

A migrant must disperse, breed and successfully pass on its genes in order to cause gene flow. A common rule of thumb is that gene flow greater than one individual per generation is sufficient to prevent genetic differentiation of two populations through random drift. As each gene may have different critical levels due to different levels of selection, and the gene flow will be sufficient to prevent fixation of alternative alleles. This does not imply that allelic frequencies will be the same in both the populations under consideration (Allendorf and Phelps, 1981).

Gene flow is a unifying force that prevents populations from diverging. Gene flow breaks down the geographical or other boundaries that could otherwise isolate populations. As a result of isolation between populations, and the consequent limitations in exchange of genes, we expect that populations will diverge by genetic drift or as a result of selection for alleles that adapt each population to its local niche (Engel *et al.*, 2004). But if gene flow occurs at a sufficiently high level, then otherwise isolated populations will not diverge genetically. Instead, they become united and evolve as a single evolutionary unit. Gene flow moves dominant or virulence alleles into new populations. Gene flow thus introduces new alleles that can displace old alleles, if they are better adapted to the current host. The population subdivision that results from genetic drift can be overcome by gene flow (Hedrick, 2000). If enough individuals are exchanged between two populations that are experiencing independent genetic drift, then the drifting populations become genetically linked and population subdivision will not occur.

2.3.4.2. Evaluation of migration and gene flow

Understanding migration and gene flow between populations is one of the most important aspects of population genetics, and is vital when investigating conservation genetics, hybridization, and disease genetics. Estimates of divergence between populations (e.g. using F_{ST} or similar statistics, or coalescent methods) are useful for understanding historical gene flow and relationships between populations, but newer methods attempt to quantify the level of recent migration (Wilson and Rannala, 2003) or identify the parents of particular individuals (Jones and Ardren, 2003) using more sophisticated algorithms. One way to determine recent migration and gene flow history is to perform assignment tests.

There are several types of assignment methods available to researchers. Assignment tests based on likelihood algorithms are the most popular as they can estimate population structure without a prior knowledge of population limits (Pritchard *et al.*, 2000; Corander *et al.*, 2003; Falush *et al.*, 2003; Manel *et al.*, 2005). Bayesian assignments tests have received the greatest attention over the past few years, with clustering-based methods by far the most widely used. Programs like STRUCTURE (Pritchard *et al.*, 2000), BAPS (Corander *et al.*, 2003), and STRUCTURAMA (Huelsenbeck and Andolfatto, 2007) can all assign individuals to a cluster based on a probabilistic model, each with slightly different underlying assumptions and different methods of searching parameter space. The assignment method developed by Rannala and Mountain (1997), implemented in either GeneClass2 (Piry *et al.*, 2004) or BayesAss+ (Wilson and Rannala, 2003), can be used to estimate the frequency of migrants within known populations. A major advantage of this method over other clustering assignment tests is that they provide a posterior probability of each individual's migration ancestry. These various methods can complement each other, with clustering assignment tests used to

determine the appropriate population substructuring followed by analyses using assignment methods to estimate recent migration between those demes.

2.3.5. Selection

Most genetic models assume that the genetic markers are selectively neutral. Genetic markers provide useful information because they are variable within and/or among populations or species. In order to explain this variation in terms of population genetic processes, it is necessary to make certain assumptions about how the variation arose.

The infinite allele model was originally developed for allozyme electrophoresis and restriction sites. It assumes that each new version of allele is equidistant from each other version (Tajima, 1996). Sequence data and restriction site analysis ideally use this model. These types of data can potentially reveal the phylogenetic history of the sequence. It cannot be used, when mutation rate varies substantially among sites (Tajima, 1996).

2. 4. Measures of diversity

Measures of diversity indicate the extent of genetic variation in a population. There is a large number of diversity measures applicable to different types of markers: percent of variable loci; average number of substitutions/site; heterozygosity, frequency of private alleles (Nei, 1987).

Hierarchical analysis finds the distribution of the total genetic variation between hierarchical levels. All F_{ST} analogs attempt to quantify the amount of total variation that is due to differences between populations. Most statistics that describe genetic differentiation from genetic markers (e.g., F-statistics) rely solely on allele identity information. This information is often used to infer phylogenetic relationships or to obtain indirect estimates of gene flow.

2.5. Molecular genetic markers and their utilities

The utility of molecular genetic markers extends beyond mapping and fingerprinting experiments into population genetics, where allele frequencies rather than individuals serve as the focus of study. Allele frequency data are useful for studying evolutionary relationships of closely related species or populations.

Microsatellite loci are the loci that vary in the number of repeats of a simple DNA sequence and are becoming commonly used in the analysis of natural populations. Microsatellite loci are often highly polymorphic and relatively easy to survey and hence offer a path for greater understanding of population structure. Microsatellite loci are typically characterized by high mutation rates and hence a high level of polymorphism. The mutation process, that causes preferentially stepwise changes in the number of repeats and thus allele size in a microsatellite locus (Arcot *et al.*, 1995, Zhu *et al.*, 2000). Hence, the difference in size between two different alleles might be informative. The larger the difference, the higher the number of mutation events (thus time lapse) is expected to have occurred since common ancestry. There is thus a “memory” of past mutation events (Levinson and Gutman, 1987).

Two alleles are homoplastic when they are ‘identical in state’ (IIS), though not ‘identical by descent’ (IBD). Homoplasy depends strongly on the model of molecular evolution considered. Among the most widely used models, both the stepwise mutation model (SMM) (Ohta and Kimura, 1973, Valdes *et al.*, 1993) and the K-alleles model (KAM) (Kimura, 1968) generate some homoplasy, while the infinite alleles model (IAM) (Crow and Kimura, 1964) does not. Homoplasy has important consequences in population genetics, because it affects how population structure may be interpreted. However, the consequences of

homoplasy on the parameters used to describe population structure are difficult to predict (Rousset, 1996).

As microsatellites may evolve in a stepwise fashion, and fit either the SMM or the two-phase model (TPM), homoplasy is expected at microsatellite loci (Freimer and Slatkin, 1996; Jarne and Lagoda, 1996). Variability at microsatellite loci is generally analyzed as size differences between electromorphs, i.e., stretches of amplified DNA at a particular locus migrating at the same speed in an electric field. Electromorphs are therefore IIS. However, it is sometimes possible to distinguish between electromorphs through sequencing, as a given electromorph may hide a set of different sequences (hereafter referred to as alleles). Part of homoplasy can therefore be uncovered, and this is referred to as size homoplasy. All the identical sequences are IIS but not necessarily IBD. Size homoplasy at microsatellite loci may also arise as a consequence of the variation in flanking regions, particularly among species (Grimaldi and Crouau-Roy, 1997).

2.6. Population management

Proper management of the population begins with the acquisition of the stock and this is a crucial step, whatever the goal may be. The acquisition of a stock is difficult and expensive and should be considered the single most important aspect of broodstock management because this act determines the maximum amount of genetic variance and also determines how much inbreeding will be produced via future matings (Tave, 1999). The genetic size of the acquired population (N_e) will have the greatest effect on the probability of success in the fish culture experience. A “founder effect” (small genetic size) would be dramatic since it will produce inbreeding and genetic drift.

2.7. Inbreeding

Inbreeding is a more serious problem in fish and prawn farming than it is with livestock. Fish and prawn are highly fecund and there is a great temptation to spawn as few fish as possible in order to save money and labour. If inbreeding occurs, it can reach levels that cause growth rate or other production phenotypes to decrease significantly (Kincaid, 1976, 1983; Gjerde *et al.*, 1983; Sbordoni *et al.*, 1986; Su *et al.*, 1996). In addition, the loss of genetic variation (genetic drift) can make future selective breeding programs ineffective. The selective breeding becomes important since genetics produces superior stocks of fish and raises the yearly yield significantly through selective breeding and crossbreeding programs (Bartley, 1998; Tave, 1999).

Constructing and maintaining pedigrees have proven difficult for both wild and captive fish and/or prawn populations. Parentage assignment using microsatellites has proven to be very useful in aquaculture. DNA microsatellites could be used for retrospective parental assignments and comparisons of viability of offspring with different parental origins without bias from unknown variations in laboratory systems or farm environments and, moreover, they make possible the estimation of relatedness between pairs of individuals with unknown origin (Estoup *et al.*, 1998a; Norris *et al.*, 2000; Jackson *et al.*, 2003; Sekino *et al.*, 2003; Borrell *et al.*, 2004).

The level of inbreeding of an individual is usually measured by the inbreeding coefficient ' f ' (the probability that two alleles at a locus are identical by descent in that individual, Gall, 1987). There is a close mathematical relationship between f and relatedness ' r '; thus for non-inbred individuals: $f = r_{FM}/2 + r_{MF}/2$ where f is the inbreeding coefficient of the offspring, r_{FM} is relatedness between the female and the male and r_{MF} is relatedness

between the male and the female (Wilson, 1980; Hartl and Clark, 1989; Liautard and Sundstrom, 2005).

Inbreeding is a main issue in evolutionary and conservation biology because of its detrimental effects on fitness and its consequences upon population viability (Frankham, 1995). Mating between relatives leads to a reduction in individual fitness known as inbreeding depression, which is thought to be the consequence of the increased homozygosity through identity by descent at all loci across the genome (Charlesworth and Charlesworth, 1987; Ballou *et al.*, 1995). Inbreeding depression could be the result of two different mechanisms. First, inbreeding can lead to the expression of recessive deleterious alleles when homozygosity increases (dominance hypothesis). Alternatively, heterozygotes may be fitter than either homozygote (overdominance hypothesis). Expression of deleterious recessive alleles is thought to be the main cause of inbreeding depression (Charlesworth and Charlesworth, 1999). Imperfect pedigree information may lead to miscalculations of the inbreeding coefficient (Marshall *et al.*, 2002; Markert *et al.*, 2004; Pemberton, 2004, 2008). A common problem both in captive and wild populations is that the assignment of paternity on the basis of behavioural observations (copulations) may lead to mistakes when females mate promiscuously; extra-pair copulations are more difficult to detect because they tend to be less conspicuous, leading to errors when constructing pedigrees unless paternity is determined by molecular methods (O'Connor *et al.*, 2006). Incomplete information may also make the pedigree inaccurate because unknown individuals are assumed to be unrelated (Marshall *et al.*, 2002).

Constructing pedigrees requires detailed information, which is not always available, and in these cases inbreeding coefficients cannot be calculated. An alternative approach has been developed to test for inbreeding depression indirectly. If inbreeding causes

heterozygosity depletion, then measuring heterozygosity levels with molecular markers, may provide a good indication of inbreeding levels. Currently, microsatellites are frequently the markers of choice to estimate heterozygosity and different measures have been developed. These include: multilocus heterozygosity (MLH) and standardized heterozygosity (sMLH) (Coltman *et al.*, 1999), internal relatedness (IR) (Amos *et al.*, 2001), and heterozygosity by locus (HL) (Aparicio *et al.*, 2006).

2.8. Polymorphic information content (PIC)

The PIC (Botstein *et al.*, 1980) is a metric which characterizes how informative a genetic marker is; it was defined for measuring the usefulness of a co-dominant genetic marker to identify the allele, transmitted by a heterozygote parent which is affected from a genetic disease, whose expression is from the dominant allele. PIC is always smaller than heterozygosity, as some crossings among heterozygous individuals may not be informative. For a locus with k alleles, PIC can be calculated as:

$$PIC = 2 \sum_{i=1}^{k-1} \sum_{j=i+1}^k p_i p_j (1 - p_i p_j)$$

where p_i is the population frequency of the i -th allele and k is the total number of alleles.

2.9. Genetic diversity and its effective maintenance

Genetic diversity is of great importance to the sustainability of populations (Hamrick *et al.*, 1991). Genetic variation within populations can be lost through genetic drift (Allendorf *et al.*, 1987), a process intensified when population size becomes small. Genetic variation among populations can be lost when previously restricted gene flow between populations is increased

by stocking or removal of natural barriers, causing differentiation between populations to be lost as a result of the homogenization of two previously distinct entities (Altukhov and Salmenkova, 1987; Campton, 1987; Utter, 2003).

The primary two procedures for maintaining genetic diversity have been the establishment of *ex situ* gene banks and maintenance of *in situ* natural genetic resources. However, conservation of genetic diversity through the use of gene banks has been hindered by many factors including limited facilities, expense, and difficulties involved in the preservation of female gametes. While storage of sperm has been relatively straightforward, cryopreservation of eggs has been difficult due to their large sizes (Tiersch *et al.*, 1994). It is believed that sustainability of natural genetic resources is fundamentally important for the preservation of genetic diversities.

Among the molecular markers available in population genetics, microsatellites emerged as those with finest resolution for labelling of populations and individuals, due to their high variability, abundance, neutrality, codominance and unambiguously scoring of alleles (Tautz, 1989; Weber and May, 1989). Microsatellite genotyping has proved to be a powerful tool for accurate genetic assessment of population differentiation and pedigree tracing of hatchery populations from various fishery animals (Norris *et al.*, 2000; Neff, 2001; Sugaya *et al.*, 2002; Li *et al.*, 2003; Yu and Guo, 2005).

2.10. Importance of microsatellite DNA as a molecular marker

Microsatellites are also called simple sequence repeats (SSR), short tandem repeats (STR), or simple sequence length polymorphisms (SSLP) but microsatellites and simple sequences repeats (SSR) are now the most widely accepted denomination. Though some can be found in transcription units (Perry and Bousquet, 1998), they have no known function and

appear to be solely maintained by their ability to replicate and expand in the genome within the limits established by the negative selection pressure of the fitness loss that they may cause (Charlesworth *et al.*, 1994, Amos and Rubinstzein, 1996). They display a selectively neutral behaviour, a co-dominant inheritance that allows discrimination of homo- and heterozygotic states in diploid organisms, have a frequent occurrence and an even distribution throughout the nuclear genome, and can also be found in the chloroplastic (Vendraming *et al.*, 1996, Van der Straete *et al.*, 2002) and mitochondrial genomes (Soranzo *et al.*, 1998). Their high length polymorphism, due to a different number of repeats within the microsatellite regions, can be easily and reproducibly detected via the polymerase chain reaction (PCR).

Microsatellites are powerful mapping tools due to their polymorphism, codominance and cross priming in different species and are also good candidates as anchor points within and across genetic maps. The use of these markers in fundamental research (genetic structure, gene flow studies, genome mapping and molecular phylogenetics) and applied research (marker-aided breeding, provenance testing, commercial certification) is now under way (Ellegren, 2000). Given the time and costs needed to identify such markers, potential cross-amplification of primers designed for a species between species and genera has to be extensively examined, although successful amplification tends to decrease with an increasing evolutionary distance.

SSRs are appropriate markers for describing patterns of intraspecific genetic diversity and assessing the evolutionary history of populations (Zhang and Hewitt, 2003) because they are usually highly polymorphic, multi-allelic, show codominant inheritance, and results can be analysed using straightforward statistics (Goldstein and Schlotterer, 1999). The use of SSR markers poses some problems in taxa exhibiting polyploidy, mainly because of the inability to

identify precisely the number of homologous alleles with these markers, and therefore to estimate other statistics such as heterozygosity (Trewick *et al.*, 2002; Wilmhoff *et al.*, 2003; Andreakis *et al.*, 2007; Huang *et al.*, 2008). Several methods have been proposed to render microsatellites suitable also in polyploid species (Samadi *et al.*, 1999; Espinoza and Noor, 2002; Bruvo *et al.*, 2004; Babaei *et al.*, 2007). In all these methods, individual alleles for each locus are scored in a binary matrix and similarity indices developed for dominant markers such as amplified fragment length polymorphisms (AFLP) and restriction fragment length polymorphisms (RFLP) are applied. Here we score each locus amplification products as in a dominant marker system, and we make use of appropriate computer programs and statistical descriptors developed for dominant markers. On the basis of frequencies gathered from microsatellite alleles and multilocus genotypes, we infer robust population-level statistics in this species despite the disparities encountered in its ploidy level.

2.11. Measures of the microsatellite variation

Microsatellite polymorphism is based on the short sequences that are repeated in tandem at one or more places in the genome. Each location in a chromosome that contains core repeats may have different number (n) of copies of the repeat (Schlotterer and Tautz, 1992). This means that at any particular location, a microsatellite repeat has multiple allele in the population, where each allele different in its value of number. With this approach, each genome yield a different fragment size due to the differing number of core repeat, and therefore yields a distinct allele in the gel electrophoresis.

Alleles in natural populations usually differ in frequency from one allele to the next. The allele frequency of a prescribed allele among a group of individuals is defined as the proportion of all allele at the locus that are of the prescribed type. The frequency of any

prescribed allele in a sample is therefore equal to twice the number of genotypes homozygous for the allele (because each homozygote carries two copies of the allele), plus the number of genotypes heterozygous for the allele (because each heterozygote carries one copy), divided by two times the total number of individuals in the sample (Shriver *et al.*, 1993).

The allele frequency in a population for diploid organism can be estimated the by equation below (Guo and Thompson, 1992). In generally, suppose that among ‘N’ individuals sampled from a population the numbers of ‘AA, Aa, and aa’ genotypes are ‘N_{AA}, N_{Aa}, and N_{aa}’ respectively. If p and q represent the allele frequency of ‘A and a’, respectively, with ‘p + q = 1’. The estimate of allele frequency in the population can be equated as

$$\text{Allele frequency} = \frac{2N_{AA} + N_{Aa}}{2N}$$

and the estimated sampling variance is

$$[\text{Var}(p)] = \frac{(p)(1-p)}{2N}$$

From the equation, N’AA’, N’aa’ were represented the number of homozygous at allele ‘A and a’, and N_{Aa} was represented the number of heterozygous, for such allele, N is the number of investigated individuals.

The genotype frequencies for a gene with two alleles can be deduced. Assume that genotype frequencies of AA, Aa and aa in the parental generation are D, H and R, respectively, where D+H+R = 1.0. The allele frequencies of A and a are given by

$$p = (2D+H)/2 = D + H/2 \text{ and } q = (2R + H)/2 = R + H/2$$

The new genotype frequencies are calculated as the sum of the cross products shown.

$$D' = D^2 + 2DH/2 + H^2/4 = (D + H/2)^2 = p^2$$

$$H' = 2DH/2 + 2 DR + H^2/2 + 2HR/2 = 2(D + H/2)(R + H/2) = 2pq$$

$$R' = H^2/4 + 2HR/2 + R^2 = (R + H/2)^2 = q^2$$

The new genotype frequencies P', Q' and R' simplify to :

$$AA : D' = p^2 \qquad Aa : H' = 2pq \qquad Aa : R' = q^2$$

a result known as the Hardy-Weinberg principle after Godfrey Hardy and Wilhelm Weinberg in 1908. The Hardy-Weinberg principle provides the foundation for many theoretical investigations in the population genetics. One of the most important implications emerges when we calculate the allele frequencies p' and q' of A and a. the allele frequencies of A and a are (Hartl, 2000)

$$p' = (2D' + H')/2 = (2p^2 + 2pq)/2 = p(p + q) = p$$

$$q' = (2R' + H')/2 = (2q^2 + 2pq)/2 = q(p + q) = q$$

Because the allele frequencies remain the same generation after generation, so do the genotype frequencies in the proportions p^2 , $2pq$ and q^2 , which are often called the Hardy-Weinberg equilibrium (HWE).

For Chi-square test (χ^2) for HWE, the mere fact that observed genotype frequencies may happen to fit HWE cannot be taken as evidence that all of the assumptions in the model are valid. The principle is not very sensitive to certain kinds of departures from the assumptions, particularly those pertaining to a very large population size with no migration, mutation, or selection. On the other hand, the relative insensitivity departures from its assumptions gives the principle some robustness, because it implies the HWE can be valid to a first approximation even when one or more of the assumptions is violated.

The usual test for goodness of fit of observed data to HWE is a chi-square test.

The test statistic is usually symbolized χ^2 , and under the hypothesis of HWE the χ^2 has approximately a chi-square distribution. The value of χ^2 is calculated as

$$\chi^2 = \frac{\sum (\text{obs} - \text{exp})^2}{\text{exp}}$$

For linkage disequilibrium, HWE, statistically, means that the alleles present at a locus are in random association with each other in the genotypes. It therefore may seem paradoxical that two gene, *A* and *B*, present in the same population may each obey HWE individually, yet the allele *A* and *B* can remain in nonrandom association in the gametes that from each generation. The measure of linkage disequilibrium have a predefined range is

$$p = D / \sqrt{p_1 p_2 q_1 q_2}$$

The correlation between linkage the *A* and *B* alleles present in gametes and can range from -1 to $+1$. This measure has the convenient feature that the χ^2 value for goodness of fit to the hypothesis that $D = 0$ is given by (Hartl, 2000).

$$\chi^2 = p^2 n$$

In *F* coefficients, Wright (1965) developed an approach to partitioning the genetic variation in a subdivided population that provides an obvious description of differentiation. This approach consists of three different *F* coefficients used to allocate the genetic variability to the total population level (T), subdivisions (S) and individuals (I). These three values, F_{ST} , F_{IT} , and F_{IS} are interrelated so that

$$F_{ST} = F_{IT} - F_{IS} / 1 - F_{IS}$$

F_{ST} is a measure of the genetic differentiation over subpopulations and is always positive. F_{IT} and F_{IS} are measures of the deviation for Hardy-Weinberg proportions within subpopulations and in the total population, respectively, where positive value indicate a deficiency of heterozygotes, and negative values indicate an excess of heterozygotes (Hedrick, 2000).

2.12. Parentage analysis

Parentage analyses can provide a finer scale assignment test than those that assign individuals to clusters based on genotypes. More information is needed however, and this can vary depending on the level of sampling possible and/or the prior knowledge of relationships (Jones and Ardren, 2003). Exclusion based parentage analyses are the simplest form of parentage assignment, but require the most *a priori* information (Jones and Ardren, 2003). Categorical allocation of parentage can be used when exclusion-based analyses fail to exclude all but one potential parent (Meagher and Thompson, 1986). Fractional allocation is similar to categorical allocation, but it is allowed to assign only a fraction of the offspring to a potential parent (Devlin *et al.*, 1988). Both the categorical and fractional methods use a likelihood-based approach where a log of the odds (LOD) score is used to statistically assign parentage. Both methods perform best with exhaustive or near-exhaustive sampling of all individuals. However, not only are exhaustive sampling difficult in highly mobile species, but completing this for multiple populations are prohibitively expensive and time-consuming in most systems. Even in botanical studies where the immobility of individuals makes complete sampling of reproducing individuals easier, there is often the issue of dormant individuals within the seed bank.

2.13. Cross species usage of microsatellite DNA markers

The widespread use of microsatellites has been limited by the fact that PCR primers require a high degree of homology to work, implying that novel species-specific markers would have to be isolated when starting the analysis of a new species (Steinkellner *et al.*, 1997). Success in the cross-species amplification of any DNA sequence is inversely related to the evolutionary distance between the two species (Steinkellner *et al.*, 1997). Hence, research on species relationships has increasingly focused on assessing the ability of SSR primers to amplify the same loci across different species and genera (Byrne *et al.*, 1996; Katzir *et al.*, 1996; Isagi and Suhandono, 1997; Smulders *et al.*, 1997; Steinkellner *et al.*, 1997; Witsenboer *et al.*, 1997). The development of which remains a costly and lengthy process despite the continuing improvements in its efficiency (Zane *et al.*, 2002). Transfer of primers (cross-species amplification) offered an alternative to *de novo* development in plants (Peakall *et al.*, 1998, Rossetto, 2001), with transfer rates ranging from 90% within subgenera to 35% within the family, and potential polymorphism from 78 to 58%, respectively (Rossetto, 2001).

2.14. Linkage disequilibrium (LD)

The pattern and extent of non-random associations among polymorphic markers distributed over the genome are related to the evolutionary rate of population structure for a species (Tishkoff *et al.*, 1996, 2001; Stephens *et al.*, 2001; Ardlie *et al.*, 2002; Weiss and Clark, 2002). One measure of such non-random associations, linkage disequilibrium (LD), is often affected by various evolutionary forces, such as selection, genetic drift, mutation, admixture, population structure, etc., which have operated in the population. For this reason, by estimating and testing for the extent and distribution of LD throughout the genome, the

evolution of population structure can be inferred (Tishkoff and Williams, 2002). LD analysis based on multiple markers can provide additional information about population structure by estimating the extent and distribution of non-random associations throughout the genome (Stephens *et al.*, 2001; Ardlie *et al.*, 2002; Dawson *et al.*, 2002). For a random-mating population, the LD between two markers decays with generation in a proportion depending on the recombination fraction between the markers (Lynch and Walsh, 1998). Thus, by comparing the rate of LD decay over genetic distances, the evolutionary history of a population can be inferred (Dawson *et al.*, 2002; Gabriel *et al.*, 2002; Tishkoff and Williams, 2002). Also, the rate of the LD decay as a function of generation has established a fundamental principle for the high-resolution mapping of complex traits in a population (Rafalski and Morgante, 2004).

2.15. Null alleles

Null alleles have posed recurring challenges for population genetics following the introduction and application of each new method of molecular assay (including serological typing, protein electrophoresis, RFLPs, and microsatellites). One of the earliest examples involved the human ABO blood group system, wherein the O allele is a null allele that produces no phenotype (*ie.*, is masked by the presence of the A or B alleles, which are codominant to each other). For the special ABO case, formulae have been developed for estimating the frequency of the O allele (Yasuda and Kimura, 1968) as well as for calculating the average paternity exclusion probability (Weir, 1996). By definition, a microsatellite null allele is any allele at a microsatellite locus that consistently fails to amplify to detected levels via the polymerase chain reaction (PCR).

Null alleles can also be generated via differential amplification of size-variant alleles (Wattier *et al.*, 1998). Due to the competitive nature of PCR, alleles of short length often amplify more efficiently than larger ones, such that only the smaller of two alleles might be detected from a heterozygous individual. Null alleles caused by differential amplification are sometimes termed ‘partial nulls’ because they can often be made visible by loading more samples or by adjusting contrast. A third source of null alleles involves PCR failure due to inconsistent DNA template quality or low template quantity. These problems are insidious because in some cases only one or a few loci (or alleles) fail to amplify, whereas others amplify with relative ease from the same DNA preparation (Gagneux *et al.*, 1997; Garcia de Leon *et al.*, 1998). When DNA template at a locus is poor in some specimens but not others, the poor samples may appear ‘homozygous’ rather than heterozygous for the null allele.

Apart from these primary causes of ‘bona fide’ null alleles, several population genetic phenomena might give the false impression that microsatellite null alleles are present in a given study. Biological factors such as Wahlund effect or inbreeding, for example, can cause significant heterozygote deficits relative to Hardy–Weinberg equilibrium (HWE) that might be misconstrued as evidence for null alleles (Chakraborty *et al.*, 1992). However, proper multilocus analysis can normally distinguish these causes because such population genetic factors should register more or less concordantly across loci, whereas the effects of null alleles are locus-specific.

2.16. Marker –assisted selection

Mapping disease-resistance genes is a challenge in many organisms, because often disease-resistance cannot be quantified for a given genotype. The identification and mapping of disease-resistance genes or quantitative trait loci (QTLs) may provide valuable information

and tools for marker-assisted selection. Marker-assisted selection is particularly useful for the development of disease resistant prawns because breeding decisions are sometimes made in the absence of disease-exposure (Guo, 2004). One of the prerequisite for QTL mapping is the availability of a large number of genetic markers. Two types of markers, microsatellites (MS) and amplified fragment length polymorphisms (AFLP), are commonly used for linkage and QTL mapping (Lewis *et al.*, 1990). MS markers are excellent markers for QTL mapping because of their high levels of polymorphism and co-dominant nature. MS are also expensive to develop and use (Brown *et al.*, 2000; Reece *et al.*, 2004).

Marker assisted selection (MAS) is based on the concept that, it is possible to infer the presence of a gene from the presence of a marker tightly linked to the gene. Strategies to find markers tightly linked to the target gene are similar to those that are used for fine-QTL mapping. Once a tight linkage is found between a molecular marker and a gene of interest, the inheritance of the gene can be traced in breeding programme (Chistiakov *et al.*, 2006). For a successful MAS programme, clearly identified and quantified phenotypes are a prerequisite. Microsatellite markers, which are linked to the target QTLs, are useful in the preliminary stages of parents for crossing and thereby subsequent genetic characterization of the progeny. There are not yet large-scale fish breeding programme using MAS. However, the current status of genetic information and advanced genomic tools available for rainbow trout, Atlantic salmon and channel catfish provide an opportunity for the successful application of the DNA marker-based technology to selective breeding in these species (Chistiakov *et al.*, 2006).

AFLPs are anonymous and dominant markers that are less transferable and informative than microsatellites, but they can be effectively used in backcrosses as co-dominant markers. Their poor transferability is compensated by the large number of markers that can be quickly

developed without prior knowledge of DNA sequences. AFLP markers have been widely used for QTL mapping and breeding in plants (Jin *et al.*, 1998; Goodwin *et al.*, 2003; Bai *et al.*, 1999; Hartl *et al.*, 1999; Altinkut *et al.*, 2003), as well as in aquatic animals (Jackson *et al.*, 1998; Palti *et al.*, 1999, 2001 and 2002; Streelman and Kocher, 2002; Shirak *et al.*, 2002; Cnaani *et al.*, 2003). AFLPs have been shown to be effective in linkage mapping in oysters (Yu and Guo, 2003; Li and Guo, 2004). Because of the nature of AFLP markers, markers segregating through the female cannot be mapped to the male map for comparison and consolidation.

Another requirement for QTL mapping is availability of reference families where QTLs are well defined and segregating. There is no highly inbred and disease resistant stock available for making reference crosses in the giant freshwater prawn. On the other hand, high levels of variability in the disease-resistant and wild stocks may provide sufficient segregation of disease-resistance QTLs. Another limitation is that resistance to some diseases can only be measured by survival. Disease-resistance QTLs can only be identified by markers, which show significant frequency shifts after disease-inflicted mortalities.

2.17. Importance of effective population size

The success of hatchery breeding programmes is related strongly to the effective population sizes that are used for founding new generations (Pante *et al.*, 2001; Koljonen *et al.*, 2002). The most widely used method in hatcheries traditionally has been to pick out a few individuals with favourable characteristics, and use those individuals as founders to produce thousands of offspring. This method involves a bottleneck phenomenon effect. Consequently, the hatchery produced broodstocks face the risk of inbreeding due to elevated levels of relatedness among individuals and, therefore, loss of genetic variation. Pante *et al.*, (2001)

showed that the genetic composition of broodstocks of rainbow trout, *Oncorhynchus mykiss*, varied depending on number of founders and the methods that were used. The size of the founding population is an important factor in the overall level of genetic variability over time. A small founding population means lower levels of genetic variation, which then decreases by drift over time. This reflects an important issue that must be considered in establishing hatcheries, namely founder history. If a few founder individuals are used to produce offspring and a few of these offspring are used later as founder individuals themselves, this will create an even narrower bottleneck compared to the situation where few founder individuals are used but are replaced continuously with wild-caught individuals. Basic population genetic theory predicts that heterozygosity decreases about 54 % after 15 generations with an effective population size of only 10 (using the relationship $H(t) = (1 - 1/2 * N_e)^t H(0)$, where H is heterozygosity at time t and zero (start)). Increasing effective population size to 30 individuals results in an expected decrease of heterozygosity of 22% after 15 generations, and with 50 individuals the reduction is only 14%. Thus, a rather moderate increase in effective population size at founding pays off quickly in terms of lower rate of loss of genetic variability.

2.18. Fitness difference between natural and hatchery reared populations

The impact of stocking on natural populations has been discussed widely and investigated in many ways; the studies done during the last decade have mostly been conducted using a genetic approach. A number of these studies have shown differences between wild- and hatchery-produced fish that could lower the fitness of wild individuals in the case of introgression (Reisenbichler and Rubin, 1999). The situation in hatcheries traditionally has been that a limited number of individuals with favourable characteristics have been used to produce thousands of offspring (Wedekind, 2002). One male can, for instance, be

used to fertilise a number of females in several generations, which inevitably leads to that same male fertilising his own offspring. This will of course strongly contribute to the depletion of genetic diversity and thereby to inbreeding. The genetically depleted individuals produced in hatcheries are then released into the wild. The effect of these individuals on wild populations can be numerous: depletion of genetic diversity, contamination of diseases and increased competition (Bohlin *et al.*, 2002; Heggenes *et al.*, 2002). As salmonid fishes are characterized by the existence of a number of local populations that exhibit a large degree of differentiation between them (Hindar *et al.*, 1991; Estoup *et al.*, 1998b), the genetic backgrounds of stocked-individuals are important to consider when released into the wild. The major reasons for loss of genetic variation in hatchery populations most often are bottlenecks and small effective population sizes, due to inappropriate mating designs. It is therefore of great importance that hatchery methods are optimised with regard to mating designs (number of breeders, relatedness of breeders, etc.) when it comes to preserving genetic variation and passing that variation on to the next generation.

2.19. Asymptomatic carrier organisms of shrimp virus

Many of the wild aquatic crustaceans such as pest crabs, mud crabs, pest prawns, insects, lobsters, shrimps, mantis shrimp and freshwater prawn harbour WSSV (Lo *et al.*, 1996; Otta *et al.*, 1999; Hossain *et al.*, 2001a, 2001b). These crustaceans act as reservoirs and may transmit the virus to healthy shrimps during culture (Wang *et al.*, 1998). Good management practices have been proposed as the only approach to significantly reduce the heavy mortality, as there is no cure for viral diseases. Till recently, diagnostic procedures for viral disease were largely dependent upon classical methods such as history, clinical signs and histological examinations that were often limited by low sensitivity (Durand *et al.*, 1996). This

has been overcome by the development of molecular methods for rapid disease diagnosis (Lightner and Redman, 1998). Apart from rapidity, molecular methods also allow the detection of asymptomatic carriers (Otta *et al.*, 1999; Nadala and Loh, 2000), which harbour low levels of virus load.

Many carrier animals that are common in prawn culture area transmit the virus to penaeid culture system with intake of water (Chakraborty *et al.*, 2002). Crustacean carriers, which enter prawn ponds, may transmit WSSV and when they die is likely to be eaten by shrimps due to their cannibalistic nature. Birds may mechanically transmit the virus between ponds by releasing captured shrimps over neighbouring ponds. Within the culture system WSSV is transmitted by cannibalism of moribund prawns and carcasses or via contaminated water (Chang *et al.*, 1996). MBV is transmitted by ingestion of free virus and cannibalism. It is also believed to be transmitted vertically from broodstock to offspring. HPV is transmitted vertically from brood stock to progeny and horizontal during the post larval stages (Brock and Lightner, 1990). In two studies on captured brood stock specimens (Flegel *et al.*, 1997), none showed characteristic histopathology of HPV. This suggests that the virus may not originate with the brood stock but with some other carrier in the cultivation system. Successful experimental infections by oral challenge in post larvae of the black tiger shrimp, *P. monodon* have recently been reported (Catap *et al.*, 2003). Catap and Travina (2005) have also reported successful horizontal transmission of HPV in *P. monodon* post larvae.

2.20. Important crustacean viral diseases of significance in India

The practise of transporting shrimp stocks between facilities and/or different geographic regions has resulted in the introduction of many known penaeid shrimp viruses to regions where they may not have previously existed. Whether or not these introduced viruses

have escaped the culture facilities to which they have been introduced and have become established in local wild penaeid stocks is not known. Evaluation of non-native penaeids by the rapidly growing shrimp culture industry is an essential component to the future growth and development of that industry. Prevention of such exotic pathogen introduction is dependent upon the use of quarantine, certification and inspection policies, and procedures that are supported by reliable diagnostic tests. Since there is no known treatment for viral diseases, the major strategy for disease management is avoidance. However, in practice this is very difficult. In Asia, the devastating mortalities caused by WSSV have led to development of strategies such as PCR screening of brood stock before spawning and PCR screening of larvae before stocking to avoid the entry of virus into aquaculture system. But, this has led to only partial success because WSSV has a broad host range (Lo *et al.*, 1996, Hossain *et al.*, 2001a, 2001b) and can survive in a number of carrier animals that can be a source of virus for the ponds. Further, WSSV survives in water up to 20 days. Hence, avoidance of pathogens can be done through selection of specific pathogen free broodstock, exclusion of carrier animals in culture systems, filtration and sanitisation of water before intake.

2.20.1. White spot syndrome virus (WSSV)

WSSV continues to be one of the most serious disease problems faced by the shrimp farming industry worldwide. This virus was first reported in 1982 in *Fenneropenaeus japonicus* cultured in northeastern Taiwan (Chou *et al.*, 1995). Since then, WSSV has caused mortalities and consequent serious damage to the shrimp industry. Considering its virulent nature, wide host range, wide geographic distribution, high mortality, catastrophic economic losses, WSSV has become the single most-dangerous virus to the

penaeid shrimp farming industry. WSSV is known to affect most commercially important species of penaeid shrimp including *Penaeus monodon*, *P. chinensis*, *P. merguensis*, *P. aztecus*, *P. stylirostris*, *P. vannamei*, *P. duorarum*, *P. setiferus*, *F. japonicus* and *F. indicus* (Lightner, 1996a). Wild marine shrimp such as *P. semisulcatus*, *Metapenaeus dobsoni*, *M. monoceros*, *M. elegans*, *Heterocarpus* sp., *Aristeus* sp., *Parapanaeopsis stylifera*, *Solenocera indica*, *Squilla mantis* and fresh water prawn species *M. rosenbergii* have also been found to harbor this virus (Lo *et al.*, 1996; Hossain *et al.*, 2001a; Chakraborty *et al.*, 2002). This virus has also been detected in many captured and cultured crustaceans and other arthropods including crabs (*Charybdis feriatus*, *C. annulata*, *C. lucifera*, *C. cruciata*, *Macrophthalmus sulcatus*, *Gelasimus marionis*, *Metapograpsus messor*, *Scylla serrata*, *Sesarma oceanica*, *Matuta planipes*, *Helice tridens*, *Pseudograpsus intermedius*), pest prawn *Acetes* sp., small palaemonid prawn, larvae of Ephydriidae insect and *Artemia* (Lo *et al.*, 1996, Maeda *et al.*, 1998, Otta *et al.*, 1999, Chen *et al.*, 2000, Hossain *et al.*, 2001a, 2001b; Chakraborty *et al.*, 2002).

Since WSSV can infect ovary, vertical transmission of the virus from brood stock to eggs has been demonstrated (Lo *et al.*, 1997) and presence of WSSV in wild brood stock by a number of investigators (Lo *et al.*, 1996, Otta *et al.*, 1999). Lightly infected brood stock (nested PCR positive) may produce either infected or uninfected larvae. PCR is being commonly used in Asia to screen *P. monodon* larvae before stocking in ponds and the risk of crop loss has been reported to be high when larvae positive for WSSV by non-nested PCR are stocked. Simultaneous presence of WSSV, with other viruses such as monodon baculovirus (MBV) and hepatopancreatic parvovirus (HPV) has been reported from India (Otta *et al.*, 2003, Umesha *et al.*, 2003).

2.20.2. Monodon baculo virus (MBV)

MBV is the first reported virus of *P. monodon* and the second virus of penaeid shrimp (Lightner and Redman, 1981). MBV has been identified and reported in *P. monodon*, *P. merguensis*, *P. semisulcatus*, *P. kerathurus*, *P. vannamei*, *P. esculentus*, *P. penicillatus*, *F. indicus*, *Metapenaeus ensis* (Johnson and Lightner, 1988, Lightner, 1988, Chen *et al.*, 1989a, Ramasamy *et al.*, 1995, Vijayan *et al.*, 1995, Karunasagar *et al.*, 1998b). The occurrence of MBV with WSSV and HPV in hatchery reared *P. monodon* post larvae was reported for the first time in India by Manivannan *et al.*, (2002). MBV is generally found in mixed infection with other pathogens including viruses (IHHNV, HPV, WSSV), bacteria (*Vibrio* spp, *Pseudomonas* spp), parasites (*Zoothamnium* spp, *Epistylis* spp.;(Anderson *et al.*, 1987, Lightner *et al.*, 1987, Chen *et al.*, 1989a, Manohar *et al.*, 1996, Karunasagar *et al.*, 1998a, Umesha *et al.*, 2003). Transmission of MBV occurs only horizontally through faecal oral route. MBV in the faecal matter of brood stock, infect eggs and larvae in hatcheries. The best way to eliminate MBV from hatchery is to identify carrier broodstock or to spawn females individually and discard contaminated batches of larvae (Flegel *et al.*, 1995a).

2.20.3. Hepatopancreatic parvo virus (HPV)

The HPV was first reported by Lightner and Redman (1985) in postlarvae of *Penaeus chinensis*. In Thailand, HPV in the black tiger shrimp, *P. monodon*, was first reported in 1992 by Flegel and Sriurairatana (1993, 1994). Presence of HPV in *P. monodon* postlarvae in India was reported by Manivannan *et al.*, (2002) and Umesha *et al.*, (2003). This virus infects several penaeid species and is widely distributed in many parts of the world, including Asia, Africa, Australia and north and South America (Paynter *et al.*, 1985, Colorni *et al.*, 1987, Brock and Lightner, 1990, Fulks and Main, 1992, Lightner and Redman, 1992, Lightner,

1996a, Pantoja and Lightner, 2000). A number of cultured and wild penaeids have been reported as hosts for HPV (Flegel *et al.*, 1992b, Bower *et al.*, 1994). The transmission of HPV is believed to be both vertical and horizontal (Lightner and Redman, 1992).

2.20.4. Infectious hypodermal and hematopoietic necrosis virus (IHHNV)

IHHNV was first detected in juvenile *Penaeus stylirostris* from Hawaii in 1981 (Lightner *et al.*, 1983). The virus has since been detected in a number of other penaeid species and from stocks around the world, including the Americas, Oceania and Asia (Lightner 1996a,b, Flegel, 1997b). IHHNV was detected earlier mainly by histological examination, but with the emergence of Polymerase chain reaction (PCR) this technique has been a handy tool in detection of this virus.

2.21. Macrobrachium rosenbergii

2.21.1. Habitat and biology of *M. rosenbergii*

Giant freshwater prawn species lives in tropical freshwater environments influenced by adjacent brackishwater areas. It is often found in extremely turbid conditions. Gravid females migrate downstream into estuaries, where eggs hatch as free-swimming larvae in brackishwater. After metamorphosis, post larvae assume a more benthic life style and begin to migrate upstream towards freshwater.

Larvae mostly consume zooplankton (mainly minute crustaceans), very small worms, and larval stages of other crustaceans. Postlarvae and adults are omnivorous, eating algae, aquatic plants, molluscs, aquatic insects, worms, and other crustaceans.

Males and females have different growth rates and males exhibit heterogenous individual growth (HIG); these are vitally important factors in grow-out management. Three distinct male morphotypes and a number of intermediary types exist. They are small male

(SM), orange claw males (OC), and blue claw males (BC). The normal male developmental pathway is SM → OC → BC. BC males have extremely long second pereopods; those of OC males are golden coloured; SM have small, slim, almost translucent claws. The type and behaviour of the males affects the growth rates of other prawns. The transition from rapidly growing OC to the slowly growing BC morphotype follows a "leapfrog" growth pattern. An OC metamorphoses into a BC only after it has become larger than the largest BC in its vicinity. The presence of this new BC male then delays the transition of the next OC to the BC morphotype, causing it to attain a larger size following its metamorphosis. BC males dominate OC males, regardless of their size, and suppress the growth of SM (New *et al.*, 2000).

2.21.2. History of the freshwater prawn culture

Although reared in captivity from time immemorial, modern farming of this species originated in the early 1960s when FAO expert Shao-Wen Ling, working in Malaysia, found that freshwater prawn (*M. rosenbergii*) larvae required brackish conditions for survival. This discovery led to larval rearing on an experimental basis. By 1972 the Hawaiian team led by Takuji Fujimura had developed mass rearing techniques for commercial-scale hatchery production of prawn post larvae (PL). This development spawned the first commercial farms in Hawaii and elsewhere. Both Thailand and Taiwan Province of China became pioneers in modern giant river prawn culture. The introduction of broodstock, initially from Hawaii and Thailand, into non-indigenous areas around the world began in the 1970s. Since then, giant river prawn culture has developed in every continent, particularly in Asia and the Americas (FAO, 2002). *M. rosenbergii* has been imported to many other tropical and subtropical areas of the world and is the species most favoured for farming purposes.

2.21.3. Broodstock management

When required for hatchery use, female broodstock are usually obtained from grow-out ponds but also sometimes from capture fisheries. Normally, "berried" (egg-carrying) females are only used once. Commercial farms in tropical regions do not normally maintain captive broodstock for breeding purposes but adults are over-wintered indoors in temperate regions in order to stock ponds with PL as early as possible in the short grow-out season. The typical male to female ratio in broodstock holding systems is 1-2 blue-clawed males or 2-3 orange-clawed males per 20 females, at a total stocking density of 1 prawn per 40 litres. Some seed (PL; juveniles) is obtained from the capture fishery where *M. rosenbergii* is indigenous, typically in the Indian sub-continent, but most is now hatchery reared (New *et al.*, 2000).

2.21.4. Economic importance and market trends

The development of freshwater prawn farming was inhibited in the past by its longer hatchery phase and lower grow-out productivity compared to marine shrimp. These constraints are now balanced by a number of positive factors concerning its sustainability and the development of a distinct and expanding market niche for freshwater prawns. The culture of *Macrobrachium* spp. is less likely to have a detrimental impact because freshwater prawns cannot be reared at densities as high as those commonly used in marine shrimp farming. Productivity is generally lower, management is less labour intensive, and the potential for the abuse or waste of resources is minimal, and (unlike the inland culture of marine shrimp) the grow-out of *Macrobrachium* does not make agricultural land saline. Specific negative effects of *M. rosenbergii* culture on the environment have yet to be documented. It is particularly well-suited to small long-term family businesses, can be practised by relatively unskilled

fishing and rural people, generates products which may be consumed by all social classes, and is amenable to integration with crop production (FAO, 2006).

Farmed production is rapidly expanding in Asia. Production in India and Thailand expanded by more than 50 percent per year between 1999 and 2002; this trend is expected to continue. There is also potential for expansion in Bangladesh, a traditional exporter from its capture fisheries. In total, the output of *M. rosenbergii* from aquaculture expanded during the decade 1993-2002 from 17000 tonnes to 195000 tonnes, an APR of 31 percent/yr. Further global expansion is difficult to predict, since it depends mainly on the volume of consumer demand. However, even if a very modest expansion of 10 percent/year occurs, global farmed production of *M. rosenbergii* will have significantly exceeded 400000 tonnes by 2010 (FAO, 2007). Initially in India scampi was a subsidiary crop obtained from fish farms and over the years farmers have converted the system to a wholly scampi based one after realizing the profits earned from farming this species. Despite being third in world production status, the farming technology needs further improvement in order to improve productivity and profitability of the farms. Unlike fish or marine prawn culture where management is relatively easy, scampi culture is difficult owing to the complex social set up, behaviour and heterogeneous growth patterns between sexes.

Both domestic and international markets for the giant freshwater prawn exist and are expanding. Peeled, mostly wild-caught *M. rosenbergii* have long been exported globally, but farmed shell-on (and normally head-off) freshwater prawns are also a familiar sight in the supermarkets of Europe now. India, Bangladesh, Viet Nam and Thailand export a significant proportion of their wild-caught and farmed prawns. Freshwater prawns are a distinct product from marine shrimp, which have their own favourable culinary characteristics (FAO, 2007).

MATERIALS AND METHODS

III. MATERIALS AND METHODS

3.1. Sample collection and storage

Samples of *Macrobrachium rosenbergii* were obtained during the period of October 2005 to January 2006 and September 2007 to November 2007 from the southern peninsular region of Indian subcontinent. The sampling sites were located in the 'Nethravathi' estuary in Mangalore, Karnataka state and Vembanadu Lake in Kerala state. These are the two states in southern part of India where giant freshwater prawn populations found to be naturally abundant in the fluvial and lacustrine ecosystems. Here they are found in both lotic and lentic habitat.

A total of 70 unrelated wild individual samples of *M. rosenbergii*, 42 from Kerala state and 28 from Karnataka state were used for microsatellite polymorphism analysis. Berried females, with eggs of different maturity stages and also spent animals dominated the samples. For the initial microsatellite detection and enrichment, total genomic DNA was extracted from the muscle tissue of *M. rosenbergii* which were kept at -80°C. In the case of studies involving characterization of microsatellite loci and population structure, DNA was extracted from the pleopods of live animals without sacrificing the sampled freshwater prawns. Live prawns were collected and maintained in the 'Live Fish Laboratory' of Department of Fishery Microbiology, College of Fisheries, Mangalore.

3.2. Development of microsatellite DNA markers for the giant freshwater prawn

M. rosenbergii

3.2.1. DNA extraction

Genomic DNA was extracted and purified according to Sambrook *et al.* (1989) with minor modifications. Tissue was ground into the form of a powder in the presence of liquid nitrogen with a mortar and pestle. After this the liquid nitrogen was allowed to evaporate off and each one gram powdered tissue was dispersed uniformly in 10 volumes of extraction buffer. The powdered tissues were allowed to spread over the surface of the extraction buffer and finally the material was allowed to submerge into the buffer completely. The solution was then incubated for 1h at 37°C. Proteinase K was added to a final concentration of 100µg/ml and mixed well. This was kept in a water bath for 3h at 50°C by swirling the viscous solution periodically. The solution was cooled to room temperature and an equal volume of equilibrated phenol was added to the solution. Then the two phases were mixed gently by turning the tube end to end for 10 min till an emulsion was formed. By centrifugation at 5000x g for 15 min at room temperature the two phases were separated. Using a wide-bore pipette, the top viscous aqueous phase was transferred to a clean centrifuge tube. This phenol extraction was repeated twice. After the third extraction with phenol the aqueous phase was transferred to a fresh centrifuge tube and 0.2 volumes of 10M-ammonium acetate and 2 volumes of ethanol at room temperature were added. The tube was swirled until the solution was mixed thoroughly. The DNA was collected by centrifugation at 5000x g for 5 min at room temperature. The DNA precipitate was washed with 70% ethanol. After washing ethanol was evaporated off and the genomic DNA was resuspended in 1xTE buffer (pH 8.0) and kept at 4°C in a refrigerator.

3.2.2. DNA concentration and quality determination

The extracted genomic DNA was loaded into 0.8% agarose gel. This 0.8% agarose gel is the medium for the electrophoresis separation and visualization of the quality of isolated DNA samples. After electrophoresis, the gel was stained by ethidium bromide dye and visualized under ultraviolet light. The standard DNA marker was also loaded into the gel for size estimation. The concentration of the extracted DNA was measured and estimated by ultraviolet absorption of the spectrophotometer at 260nm (OD₂₆₀). Extracted DNA absorbs UV light at the 260nm wavelength (OD₂₆₀), whereas protein absorbs UV light at 280nm wavelength (OD₂₈₀). Determining ratio of the absorbed OD at 260 nm over 280 nm can assess the purity of extracted DNA. If the ratio is lower than 1.5, the purity is poor which is due to the protein or other contamination in the extracted solution. However, if the ratio is greater than 1.5, the absorption is probably due to the nucleic acid. NanoDrop, ND-1000 spectrophotometer (JH BIO Innovations Pvt. Ltd, Bangalore) and Shimadzu UV-1601 spectrophotometer (USA) also used to ascertain the quality and quantity of extracted DNA.

The DNA concentration of each sample can be calculated by the assumption:

$$\text{DNA concentration (mg/ml)} = (\text{OD}_{260}) \times \text{dilution factor} \times 50 \text{ mg/ml}$$

$$\text{Purity of DNA} = \frac{\text{Optical density at 260 nm}}{\text{Optical density at 280 nm}}$$

3.2.3. Agarose gel electrophoresis

The agarose gels were prepared in 1x TAE buffer with appropriate percentage (w/v) of agarose and buffer. When the molten agarose had cooled to below 65°C, ethidium bromide was added to a final concentration of 0.5 µg/ml and the gel was cast. Ten micro liters of the DNA were mixed with 4 µl of 6x gel loading buffer and loaded into the wells. 1kb DNA ladder (Bangalore Genei, Bangalore) was used as a molecular weight marker. Electrophoresis was carried out at 100-120 volts and the bands were visualized using Gel doc (Herolab, Germany). The reagents required for agarose gel electrophoresis are mentioned in the appendix.

3.2.4. Restriction digestion

The genomic DNA was digested to sticky-end products using the restriction enzyme *Sau3AI*. 0.7 units of *Sau3AI* (Bangalore Genei, Bangalore) source were used for the digestion of 1µg of genomic DNA. The reagents used for restriction digestion were shown in table 1. The digestion mixture was incubated at 37°C for 2h and then heated at 65°C for 10 min. All these steps were performed in a thermocycler.

3.2.5. Adapter preparation

10µl each of oligoA (5'- GGC CAG AGA CCC CAA GCT TCG -3') and oligoB (5'- PO₄ – GAT CCG AAG CTT GGG GTC TCT GGC C -3'), which were having a concentration of 200pm/µl respectively, were mixed together by vortexing and then spinned briefly. The oligo mix was denatured by heating at 80°C for 5 min and renatured slowly at room temperature for 1h. Sixty µl of milliQ water was added to the mix to make a final

3.2.8. Purification of PCR products

The PCR products were purified to remove excess dNTP's, enzymes and primers using QIAquick PCR purification kit (QIAGEN, USA). Reagents supplied with the kit are buffer PB, buffer PE and buffer EB.

A 100 μ l of PCR product was mixed with 500 μ l of buffer PB and was applied to the QIAquick spin column and centrifuged to bind the DNA to the column. The column containing the DNA was washed using 750 μ l of the PE buffer and the bound DNA was eluted out using 50 μ l EB buffer (Fig.1).

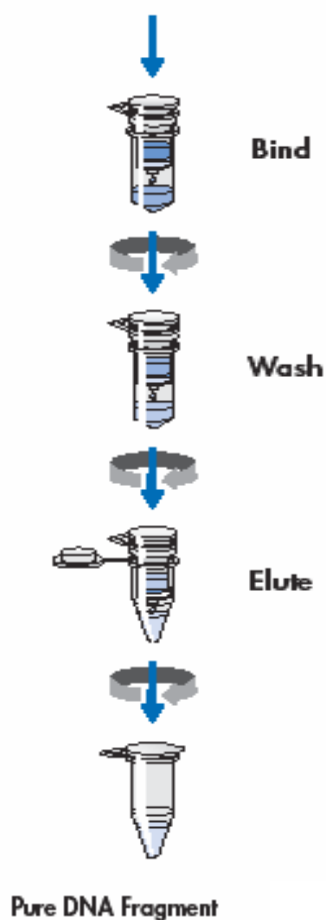


Fig.1. Procedure for purification of PCR products using QIAquick PCR

3.2.9. Hybridization

To identify and enrich PCR fragments containing microsatellite DNA, the PCR products were hybridized with biotinylated (CA)₁₅ or (AT)₁₅ probes (Biserve Biotechnologies Pvt. Ltd., Hyderabad). The reagents used for hybridization were listed in table 5 and 6. With the microsatellite probe, other reagents were mixed and heated at 95°C for 10 min and subsequently incubated at 55°C overnight for (CA)₁₅ and 50°C for (AT)₁₅ biotinylated microsatellite probes respectively. All these steps were performed in DNA Engine DYAD (MJ Research, USA) thermocycler.

3.2.10. Capture of microsatellite containing DNA fragments using streptavidin coated magnetic beads

After hybridization, genomic DNA fragments complementary to the microsatellite motifs were recovered by the method of magnetic bead capture. 100µl of streptavidin coated magnetic beads called Dyna beads (QIAGEN, USA) were mixed with 100µl of 1X washing buffer in a microcentrifuge tube. By keeping the tube in a magnetic rack the supernatant was carefully removed and discarded. This step was repeated twice. The magnetic beads were again mixed with 200µl of 2X washing buffer and resuspended. The hybridization solution was added into the same tube. The reagents used in the reaction were mentioned above. The reaction mixture was incubated at 42°C for 2h, mixing it in every 10 min interval. After the incubation, by keeping the tube in a magnetic rack, the supernatant was removed and discarded. To the remaining pellets, 200µl of S1 reagent was added and mixed well by pipetting. This was incubated at room temperature for 5 min and then supernatant was removed by keeping the tube on a magnetic rack for 1 min. In the remaining pellet 200µl of S2 reagent was added and mixed well by pipetting. The mixture was kept at 45°C for 5 min and

then the supernatant was removed and discarded by keeping over a magnetic rack. This step was performed twice. The remaining pellet was eluted with 120µl of 1x TE in two steps at 95°C for 10 min. The supernatant was collected in a new microcentrifuge tube and kept at 4°C. The composition of the reagents was mentioned in the appendix.

3.2.11. Microsatellite capture PCR

The products recovered by magnetic bead capture were amplified by PCR using the primer sequence binding to the adaptor possessed by the adapter. The reagents used in the PCR reaction mixture and thermocycling conditions were listed in table 7 and 8. The PCR amplified products were checked on a 2% agarose gel by electrophoresis.

3.2.12. Ligation of enriched DNA into the vector

The reagents used for the ligation were mentioned in the table 9. Tapping on the sides of the tubes gently mixed the reagents. Then incubated at room temperature for 5 min, and then proceeded with transformation. The DNA fragments, which possess the A-overhang site due to the terminal transferase activity of *Taq* DNA polymerase during the PCR reaction, were ligated to the T-overhang site of the plasmid pCR2.1 TOPO.10 (Invitrogen, USA).

3.2.13. Transformation protocol

The ligated plasmids were then transformed into electrocompetent *E. coli* cells. The competent cells were thawed on ice and then gently mixed with 2µl of ligation mixture. This was incubated on ice for 5 min and then transferred to a water bath at 42°C for 30 seconds. Then snap cooled on ice. SOC medium (250µl) was added and incubated at 37°C for 1 h with agitation. Then according to manufactures instruction aliquots (50µl, 100µl and 150µl) were spread on agar plates containing LB, ampicillin (100µg/ml) and X-gal (50 mg/ml) and incubated overnight at 37°C.

3.2.14. Screening the microsatellite positive clones by PCR

The clones were screened by Lac selection method by making use of β -galactosidase activity. Screening for β -galactosidase presence or absence involves a lactose analogue called X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), which is broken down by β -galactosidase to a product that is coloured 'blue'. The recombinants were identified by their inability to synthesize β -galactosidase, hence they produce 'white' colonies. After the overnight growth of the transformed cells on the plates, the plates kept at 4 °C for one h, so that false positive clones change color from white to blue. Positive clones, which were white in colour, picked up from the plates and put on other LB-ampicillin-X-gal plates with marked grids for further screening following the above, described method. The white clones once again selected from these plates and performed screening PCR.

The test cultures were grown overnight in 3 ml Luria Bertani broth (Hi Media, Mumbai). 50 μ l of this was suspended in 450 μ l of TE buffer (pH 8) and lysed at 95°C for 15 min, in hot bath, followed by snap cooling in ice. The cell debris was settled by centrifugation at 10,000 rpm for 5 min, and the supernatant was used for PCR amplification. Screening PCR was performed using respective unbiotinylated microsatellite probe and adapter sequences as the primers. The thermocycling conditions are listed in table 10.

3.2.15. Long-term storage of microsatellite positive clones

The microsatellite positive clones were grown overnight in LB-ampicillin media. Microsatellite positive clones (900 μ l each) were mixed with 900 μ l of glycerol broth in 2ml storage tubes. The transferred clones were immediately preserved viable at -80°C. The composition of glycerol broth used is mentioned in appendix.

3.2.16. M13 PCR

The insert sizes of the screening PCR positive clones were confirmed with M13 PCR using M13 universal primers. The reagents used for performing the PCR were listed in table 11 and the thermocycling conditions in the table 12. Analyses of the amplification products were achieved by electrophoresis of the PCR products on a 2% agarose gel. The clones, which were shown an insert size range between 200 and 1000bp, were selected for sequencing. The primers used for M13 are the following.

M13 Forward Primer sequence

5'- CTGGCCGTCGTTTAC -3'

M13 Reverse Primer sequence

5'- CAGGAAACAGCTATGAC - 3'

3.2.17. Plasmid DNA extraction

Reagents supplied with the kit are lysis solution, RNase solution, lysozyme, wash buffer concentrate, elution buffer and fast plasmid spin column assembly.

Young bacterial cultures at the logarithmic growth phase were used for plasmid DNA extraction. 1.5 ml of bacterial culture was centrifuged to sediment the cells to the bottom of the microfuge tube. The clear supernatant was discarded and to the pellet, 400 μ l of ice-cold complete lysis solution was added. The pellet was mixed thoroughly and incubated at room temperature for 3 min. Subsequently, the lysate was transferred to the spin column assembly and centrifuged at $13,000 \times g$ for 60 sec. Four hundred microliter of diluted wash buffer was added to the spin column and centrifuged at $13,000 \times g$ for 1 min. For elution, 50 μ l of elution buffer was added to the spin column placed in a collection tube and centrifuged at $13,000 \times g$.

The purified plasmid was collected in the collection tube and stored. The concentration of the purified plasmid DNA was determined using NanoDropTM ND-1000 spectrophotometer (JH BIO Innovations Pvt. Ltd, Bangalore) and Shimadzu UV-1601 spectrophotometer (USA).

3.2.18. DNA sequencing

Double –stranded sequencing was performed by using an automated ABI 3100 Genetic Analyzer that uses fluorescent label dye–terminators (Sanger’s sequencing method). Universal M13 forward and reverse primers are used for sequencing the vector and insert region. The sequencing was done at Bangalore Genei, Bangalore, and Bioserve Biotechnologies Pvt. Ltd, Hyderabad. The information obtained on the sequence was compared with all accessible sequence available in the NCBI (National Center for Biotechnology Information). The sequence homology was determined by using Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/wagtail.uovs.ac.za/BLAST>) (Altschul *et al.*, 1997). Multiple sequence alignment of microsatellite sequences was performed with ClustalW (<http://www.ebi.ac.uk/cgi-bin/clustalW>) software package (version 1.81) (Thompson *et al.*, 1994).

3.2.19. Design of oligonucleotide primers and standardization

Oligonucleotide primers complementary to the flanking regions of the repeats from the sequencing data were designed by the computer software primer3 (Rozen and Skaletsky, 2000) obtained from the web site <http://www-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi>. Several criteria have been taken into account for primer designing like a Guanine or Cytosine nucleotide at the 3’end and no primer dimer formation while annealing etc., so that

the primer sets have approximately equivalent annealing temperatures. The designed primers were manufactured in the Biosreve Biotechnologies Pvt.Ltd.Hydrabad.

Initially suitability of the designed primers were checked with the cell lysates of the respective clones which contains the particular insert sequences using which we designed the primers. Gradient PCR was performed to find out the accurate annealing temperature of the primers, so that they will yield single PCR product containing the intended microsatellite region. Further conformation was performed with genomic DNA of *M. rosenbergii*.

3.3. Analysis of microsatellite polymorphism and the molecular ecology of the species

3.3.1. Microsatellite loci amplification and scoring

The samples with good DNA quality were used for microsatellite analysis. Amplified products were electrophoresed in polyacrylamide gels and visualized by ethidium bromide staining method. All samples were run next to a molecular weight ladder to determine the size. To avoid error in scoring due to differences in the gels, a control DNA sample was included in each set of samples for each gel. To score stutter bands the pattern of stutter bands like number of base pairs separating the bands, number of bands and the arrangement of faint and intense bands were considered (O'Reilly and Wright, 1995). The patterns of stutter bands amplified by a particular set of primers were usually the same. In general, the strongest band was chosen for allele size determination (Tautz, 1989). The criteria used to determine the allele size were the first primary band, even if it is slightly less intensive than the second one. In most samples, a single set of stutter bands represents a single allele or homozygous state. The possibility of overlapped sets of two stutter bands, or heterozygous state, was analyzed by comparing their patterns with those of other samples. The additional relatively 'heavy' band below the first

allele was scored as the second allele. In samples where only one band or one set of stutter bands existed, the individual animals were assumed to be homozygous.

3.3.2. Analysis of microsatellite polymorphism and the molecular ecology of the species

The allele size of each microsatellite locus in population was determined using GelCompar II version 2.5 (Applied Maths, USA). Variation in microsatellite loci for the two wild populations of *M. rosenbergii* were estimated on the basis of number of alleles per locus, allele size range, frequency of the most common allele and unique alleles for each population at each locus using the program GENEPOP version 3.1 (Raymond and Rousset, 1995). The observed heterozygosity (H_o) and the expected heterozygosity (H_E) were estimated according to Beardmore *et al.* (1997) and Nei (1987) respectively using the software POPGENE version 1.31 (Yeh *et al.*, 1999). Genetic differentiation is the acquisition of allele frequencies that differ among populations (F_{ST}) was estimated by F-statistics program (Wright, 1951). Null alleles across the loci were detected using the software MICROCHECKER (Van Oosterhout *et al.*, 2003).

The FSTAT statistical package (2.93 version) (Goudet, 1995, 2001, 2002) was used to estimate the total variation in gene frequencies (F_{IT}) partitioned into components of variation occurring within (F_{IS}) and among (F_{ST}) samples for each locus following Weir and Cockerham (1984). Significance levels of F_{IS} were assessed through randomisation of alleles (1000 times) within samples for each broodstock. Pairwise F_{ST} values between samples and 'p' values were calculated (for significance levels of F_{ST} , multilocus genotypes were randomised between pairs of samples (1000 permutations), and the statistical significance was calculated after the Bonferroni correction for sample sizes (Rice, 1989; Goudet, 2001). The expected frequencies of null alleles were estimated using equation 4 of Brookfield (1996), $r = (H_E - H_o) / 1 + H_E$. The

F_{IS} describes the variance within individuals, relative to their sub-populations. The F_{ST} is the variance within sub-populations, relative to the expected variance in the total population, and F_{IT} shows the correlation of alleles within individuals over all populations. F_{ST} significant values for each microsatellite locus were determined by bootstrapping over samples.

We also performed Ohta's (1982) two-locus population subdivision analysis (D statistics). In this analysis, the variance in linkage disequilibrium is partitioned into the following components: D_{IS}^2 (variance of within-subpopulation disequilibrium), D_{ST}^2 (variance of the correlation of genes of the two loci of different gametes of one subpopulation relative to that of the total population), D'_{IS}^2 (variance of the correlation of genes of the two loci of one gamete in a subpopulation relative to that of the total population), D'_{ST}^2 (variance of the disequilibrium of the total population), and D_{IT}^2 (total variance of disequilibrium). The Ewens–Watterson test for neutrality (1,000 permutations per test) (Manly, 1985) was performed for each locus to detect possible effects of selection on inter population allele distribution.

3.4. Cross species evaluation of microsatellite markers

The following species were used for evaluating cross species amplification of the microsatellite DNA markers developed for *Macrobrachium rosenbergii*. *M. idella*, *Fenneropenaeus indicus*, *Penaeus monodon*, *Metapenaeus dobsoni*, *M. monoceros*, *M. affinis*, *Parapenaeopsis stylifera*, *Solenocera crassa*, *Heterocarpus woodmasoni*, *H. gibbosus*, *Aristeus alcocki*, *Seranopsis longipes*, *Solenocera choprai*, *S. hextii*, *Penaeus fissuroides*, *Charybdis hoplites*, *C. riversandersoni*, *Scylla serrata*, *Portunus pelagicus*, *P. sanguinolentus* and *Squilla mantis*. *M. idella* samples were collected from the backwaters of Kuttanadu region from Kerala state. The other species used in this study collected from both Mangalore

and Cochin coasts. The sample particulars used for cross species amplification experiment are mentioned in table: 13.

3.5. Use of DNA microsatellite markers for the detection of some infectious virus carrier state

3.5.1. Sample preparation

Fresh samples were used for the detection of viruses. The samples were directly used for the DNA extraction. The target organs like gills, cuticle, stomach, pleopods and hepatopancreas were excised and washed tissues with sterile distilled water before DNA extraction.

3.5.2. Detection of different viruses by polymerase chain reaction

Approximately 100 mg of individual organs like gills, hepatopancreas, gut, antenna, muscle and cuticle were removed aseptically from freshwater prawn samples. After washing the organs they were transferred to 1.5 ml sterile microcentrifuge tubes. 850µl of digestion buffer (10mM Tris HCl, pH 8.0; 0.1M EDTA, pH 8.0; 0.5% Triton x 100, 6M Guanidine Hydrochloride, 0.1M Sodium Acetate) was added and made as homogenates. The homogenate was incubated at room temperature (28°C) for 30 min. Following incubation, the mixture was centrifuged at 800xg for 5 min. The supernatant was transferred to a fresh microcentrifuge tube and equal volume of phenol: chloroform: Isoamylalcohol (25:24:1) was added and centrifuged at 1000xg for 5 min. The supernatant containing DNA was transferred to a fresh tube, precipitated by adding 3 volumes of cold absolute ethanol, centrifuged at 14000x g for 5 min. The DNA pellets were washed with 70% ethanol and vacuum dried. The pellet was dissolved in 100 µl of 1xTE buffer. Extracted DNA samples were stored at -20°C till used for further PCR analyses.

Nested PCR procedure was followed for the detection of WSSV, HPV, MBV and IHHNV in the freshwater prawn samples. PCR was carried out in 30 µl reaction mixture containing 2.0µl of template DNA, 1x assay buffer (10mM Tris-HCl pH 9.0, 1.5mM MgCl₂, 50mM KCl, 0.01% Gelatin), 100 mM each of the four dNTP's, 10 picomoles of forward and reverse primers and 0.9 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore). Thermocycling conditions for different viruses are given in the table 14. The PCR was performed for 30 cycles in a thermocycler (MJ Research, inc., USA). The primer sequence data used for the detection of above mentioned viruses are mentioned in table 15.

RESULTS AND DISCUSSION

IV. RESULTS

4.1. Genomic DNA extraction and isolation of microsatellite region in genomic DNA

For the initial microsatellite detection and enrichment, total genomic DNA was extracted from the muscle tissue of *Macrobrachium rosenbergii*. In the case of studies involving characterization of microsatellite loci and population structure, DNA was extracted from the pleopods of live animals without sacrificing the sampled freshwater prawns. The concentration and purity of genomic DNA was 449 µg/ml and 1.8 respectively and was considered suitable for further studies (Table: 16).

Presence of a continuous smear of DNA fragments in the agarose gel indicated the digestion of the DNA by the restriction enzyme (Fig: 2). Similarly a continuous smear in the agarose gel indicated successful ligation of the adapters in the digested DNA fragments and PCR amplification of those fragments (Fig: 3). The fragments hybridizing with the probes were captured using streptavidin coated magnetic beads. These were further enriched by PCR using primers binding to adapter sequences. The magnetic bead captured DNA fragments, which containing microsatellite repeats were electrophoresed on a 2% agarose gel. A bright smear indicated the success of hybridization (Fig: 4). Screening PCR was performed for the positive clones using the respective non-biotinylated microsatellite probes and adapter sequences as primers. Double bands in the 2% agarose gel were confirmatory of the respective microsatellite positive clones (Fig: 5). The insert size in every clone was confirmed by PCR using the M13 universal primers (Fig: 6). The clones showing the insert sizes ranging between 200 bp and 1000 bp were selected for sequencing.

4.2. DNA sequencing

From the sequencing results it was observed that the clone MRMA27 had 13 perfect repeats complementary to the (CA)₁₅ microsatellite motif. For clones MRMA8, MRMB1, MRMB7, MRMB10 and MRMB15 the repeat microsatellite motif was (GA)_n varying in their copy numbers. The microsatellite loci MRMA8 contained two perfect repeats (GA)₃₈ and (GA)₃₀ interspersed with a non-repeating region of 87 bases. The obtained sequences have been deposited in GenBank and accession numbers obtained. Among 27 clones that were sequenced, only 13 had perfect repeats with adequate flanking regions. The GenBank accession numbers, microsatellite locus names and repeat motifs are mentioned in table 17.

4.3. Standardization of microsatellite primers developed

The primer sets designed for the flanking regions of the microsatellite loci and primer annealing temperatures standardized through gradient PCR method are given in table 18.

4.4. Analysis of microsatellite DNA polymorphism in *M. rosenbergii* population

Among the thirteen microsatellite loci developed and used in this study, all were found to be highly polymorphic and showed considerable variation in the tested population samples of *M. rosenbergii* (Fig: 7–19). A total of 59 different alleles were found over all the microsatellite loci tested and the number of alleles per locus ranged from 3 to 9. The allelic size ranges from 75 to 422bp. The overall allelic frequencies of various microsatellite loci were in the range of 0.0143 to 0.7000. The average observed and expected heterozygosities ranged between 0.6785 ± 0.1883 and 0.6881 ± 0.1117 respectively (Table 19). The average observed and expected homozygosities ranges between 0.3215 ± 0.1883 and 0.3119 ± 0.1117 respectively (Table 19). The average expected Nei's heterozygosity (Nei, 1973) value for all

the tested microsatellite loci was 0.6770 ± 0.1106 (Table 19). The mean Shannon index values (Shannon and Weaver, 1949) estimated for all the microsatellite loci used were 1.2925 ± 0.3591 (Table 19). The number of genotypes estimated across all the microsatellite loci in both the tested populations of giant freshwater prawn found in the range of 1 to 46 genotypes. The locus MRMC2 exhibited the highest range of the number of genotypes (9 - 46) and the locus MRMB16 showed the least (1–17). The microsatellite loci MRMA27, MRMB1, MRMB16, MRMB25 and MRMB26 showed significant departures from Hardy-Weinberg Equilibrium probabilities after Bonferonii correction for sample sizes (Table 19). The mean polymorphic information content (PIC) values found to be in the range of 0.61 to 0.72. The F_{ST} values lies between 0.0449 and 0.6172 with a mean value of 0.2553 for all the microsatellite loci used in this study. The F_{IS} values lies between + 0.1878 and – 0.9134 with a mean value of – 0.3518 and the F_{IT} values showed a range between + 0.3780 to – 0.3417 with an average value of – 0.0067 respectively. The average N_m value found to be 0.7294 across all the marker loci used in this study (Table 20). The Ewens-Watterson test for neutrality indicates that the loci MRMB13, MRMB15, MRMB16, MRMB24, MRMB25, MRMB26 and MRMA27 with significant evidence for heterozygote deficiency in the populations tested and the other six microsatellite loci showed significant evidence for heterozygosity excess (Table 21). Other than the loci MRMB13, MRMB15, MRMB16, MRMB25 and MRMB1, remaining eight microsatellite marker loci used were found to be neutral. The Ohta's D-statistics results for population subdivision shows that $D'_{IS}{}^2$ value is greater than that of $D'_{ST}{}^2$ in the samples tested.

4.5. Use of the developed and standardized microsatellite DNA markers to study the molecular ecology of the species

4.5.1. Karnataka population samples of *M. rosenbergii*

All the thirteen microsatellite loci found to be highly polymorphic with the percentage polymorphic loci value of 100 %. The locus MRMB7 consist of 6 alleles and the locus MRMA27 consists of 4 alleles were the major ones. Other than these two loci rest all microsatellite loci showed two alleles each in the Karnataka population of freshwater prawn samples. The microsatellite locus MRMB7 showed the evidence for two rare alleles with a relative allele frequency of 0.07 and 0.11 respectively. None other loci showed the presence of any rare alleles with this population. The total number of alleles for all the thirteen microsatellite loci for Karnataka population was found to be 32 including the rare alleles.

The average heterozygosity values for all the thirteen microsatellite loci used in the Karnataka population was found to be 0.5055 ± 0.1285 . The mean observed heterozygosity value was 0.7302 ± 0.2056 and the expected heterozygosity value was 0.5101 ± 0.1393 respectively (Table 22). Four microsatellite loci out of thirteen showed a significant deviation from Hardy-Weinberg equilibrium ($p < 0.05$) in Karnataka population of freshwater prawn populations. The loci, which showed significant departures from HWE, are MRMB16, MRMB24, MRMB1 and MRMA27 respectively using Markov chain method (Table 23). The loci MRMA27 showed significant evidence for heterozygote deficiency in the Karnataka population tested and the other twelve microsatellite loci showed significant evidence for heterozygosity excess. None of the loci showed significant evidence for the presence of null alleles in this population. Other than the loci MRMB1 and MRMB16 remaining eleven microsatellite marker loci used were found to be neutral i.e. in the direction of directional selection, using the Ewens-Watterson neutrality test with 1000 simulated samples (Table 24). The mean F_{IS} and F_{IT} values estimated for the population was found to be -0.4987. The F_{ST} values found to be very nearer to zero for Karnataka population of *M. rosenbergii* (Table 25).

4.5.2. Kerala population samples of *M. rosenbergii*

Among the thirteen microsatellite loci used twelve were found to be highly polymorphic. The percentage polymorphism value calculated was 92.31 %. The microsatellite MRMB13 found to be monomorphic in Kerala population of *M. rosenbergii*. The total number of alleles found across all microsatellite loci used was 35. The allele frequencies showed a range between 0.0238 and 1.00. The highest number of alleles (5) was found at the locus MRMA27 and the lowest number (1) was observed at the locus MRMB13. The microsatellite loci MRMB7, MRMB16, MRMA27 and MRMA8 showed the evidence of the occurrence of rare alleles. The more number of rare alleles (3) were found at the locus MRMA27.

The average heterozygosity value across all the microsatellite loci was found to be 0.5238 ± 0.1943 . The mean observed and expected heterozygosities for all the loci were 0.6325 ± 0.3312 and 0.5373 ± 0.1987 respectively. The Nei's expected heterozygosity value for all the microsatellite loci in this study estimated as 0.5238 ± 0.1943 (Table 26).

The microsatellite loci MRMB11 and MRMB24 were found to be in Hardy-Weinberg equilibrium ($p < 0.05$) (Table 27). The loci MRMB26, MRMB24, MRMB16 and MRMA27 showed evidence for heterozygosity deficiency and the remaining other loci showed heterozygosity excess. The MICRO-CHECKER software results show that the loci MRMA27 and MRMB26 possess null alleles in this population (Table 28). The loci MRMB11 and MRMB25 loci found to be deviated from neutral status when compared to other microsatellite marker loci used in this study (Table 29). The Shannon information index value for this population across all marker loci used was found to be 0.8609 ± 0.3959 . The mean F_{IS} and F_{IT} values estimated for all marker loci for this population was found to be -0.2074. The F_{ST} values found to be very nearer to zero for Kerala population of *M. rosenbergii* (Table 30).

4.6. Cross species amplification of microsatellite markers

Cross species amplification was performed using different types of crustacean species including freshwater prawns, shrimps and crabs. The freshwater prawn *M. idella* showed considerable amplification of all the microsatellite regions with the microsatellite primers developed for *M. rosenbergii*. None other genera used in this study showed PCR amplification with the microsatellite primers developed in this study.

4.7. Prevalence of viruses in *Macrobrachium rosenbergii*

Thirty samples of freshwater prawn were analyzed for the presence of viruses by PCR. Of these, 12 samples were positive for WSSV, 20 samples were positive for HPV and 7 for MBV. None of the samples was positive for IHHNV. The results of individual samples are indicated in table 31. Three samples were positive for three viruses. 11 samples were positive for dual viruses, 7 for WSSV and HPV, 4 for MBV and HPV. Eleven samples were positive for single virus, 5 for WSSV alone, 6 for HPV alone. Among the different microsatellite loci tested only those loci with the repeat motifs (GA)₄₄, (GA)₃₆, (GA)₂₆, (GA)₁₀ and (CT)₁₇ were found to be amplifying in virus infected individual samples of *M. rosenbergii*.

V. DISCUSSION

5.1. Development of microsatellite DNA markers for *M. rosenbergii*

Among the molecular markers available in population genetics, microsatellites emerged as those with finest resolution for labeling of populations and individuals, due to their high variability, abundance, neutrality, codominance and unambiguously scoring of alleles (Weber and May, 1989; Tautz, 1989). Microsatellite genotyping has proved to be a powerful tool for accurate genetic assessment of population differentiation and pedigree tracing of hatchery populations from various fishery animals (Neff, 2001; Norris *et al.*, 2000; Yu and Guo, 2005; Sugaya *et al.*, 2002; Li *et al.*, 2003a).

Genetic studies on *M. rosenbergii* using DNA markers are very rare. A few reports on the development and characterization of a few microsatellite loci from *M. rosenbergii* (Chand *et al.*, 2005; Chareontawee *et al.*, 2006, 2007; Sudsuk and Sodsuk, 1998) are available. However no details are available on the genetic diversity of cultured and wild stocks in different regions or countries (Liu and Cordes, 2004; Norris *et al.*, 2000). For most efficient marker development, microsatellite enriched genomic DNA libraries are made (Ostrander *et al.*, 1992; Kijas *et al.*, 1994). We constructed a partial genomic library of *M. rosenbergii* using Sau3AI restriction enzyme. The rate of positive clones obtained was 94%. In this study, we have successfully amplified the microsatellite region using primers designed from the sequence information generated which was standardized for the identified microsatellite loci (Divu *et al.*, 2008a and 2008b). The BLAST results revealed the novelty of the microsatellite loci developed in this study since they did not show any similarity with the previously deposited GenBank sequences of *Macrobrachium* species and also with those deposited by Chand *et al.*, (2005) and Charoentawee *et al.*, (2006).

In the present study, we developed and used thirteen microsatellite markers to estimate the level of genetic diversity within two wild populations of giant freshwater prawn, and to compare the degree of genetic differentiation between them. In the last few years' microsatellites have become one of the most popular molecular markers used in many different fields. Usually microsatellite loci can be isolated *de novo* from the interested species or achieved from closely related species in which

microsatellites have been characterized well. However markers developed for a species may not be useful for others. So it is necessary to isolate microsatellites from most of the species for the first time due to the fact that microsatellites are usually found in noncoding regions where the nucleotide substitution rate is higher than that in coding regions.

Microsatellite or simple sequence repeats (SSRs) are tandemly repeated motifs of 1 to 6 bases found in all eukaryotic genomes to date (Coreno and Ginelli, 1967, Toth *et al.*, 2000). They are present in both coding and noncoding regions and are usually characterized by a high degree of length polymorphism (Bechman and Webber, 1992). Microsatellites have been widely employed in many fields soon after their first description (Litt and Luty, 1989; Tautz *et al.*, 1986) because of the high polymorphism and the relative ease of scoring. These are powerful genetic markers and extremely valuable tool for genome mapping in many organisms (Schuler *et al.*, 1996; Knapik *et al.*, 1998), for forensic DNA studies, paternity testing (Schulz and Reichert, 2002), population genetics and conservation or management of biological resources (Jarne and Lagoda, 1996).

The traditional methods of screening microsatellites from partial genomic libraries of the species of interest, is to screen several thousands of clones through colony hybridization with repeat containing probes (Rassmann *et al.*, 1991). The rate of positive clones obtained by means of this traditional method usually ranged from as high as 12% to as low as 0.04% (Zang *et al.*, 2002). We constructed (CA)_n and (GA)_n enriched libraries for *M. rosenbergii* using the microsatellite enrichment method. Genetic studies on *M. rosenbergii* using DNA markers like some reports on the development and characterization of a few microsatellite loci in “Eastern” form of *M. rosenbergii* are only available. No further data is available on the genetic diversity of cultured and wild stocks in different regions or countries (Liu and Cordes, 2004; Norris *et al.*, 2000).

For most efficient marker development, microsatellite enriched genomic DNA libraries are made (Ostrander *et al.*, 1992; Kijas *et al.*, 1994). Chand *et al.* (2005) and Charoentawee *et al.* (2006) used microsatellite enrichment technique to identify the microsatellite loci in the genome of *M.*

rosenbergii. We constructed a partial genomic library of *M. rosenbergii* using *Sau3AI* restriction enzyme. The rate of positive clones obtained was 94%. The microsatellite loci MRMA8 contained a total of 68 (GA) repeats in the form of 38 and 30 numbers of repeats respectively with a non-repetitive genomic region of 87 bases in between. The other described loci viz MRMA27, MRMB1, MRMB7, MRMB10 and MRMB15 contained perfect repeats of 13 (CA/GT), 44 (GA/GT), 26 (GA/GT), 35 (GA/GT) and 33(GA/GT) respectively (Divu *et al.*, 2008a, 2008b).

5.2. Analysis of microsatellite polymorphism in *M. rosenbergii* population and the molecular ecology of the species

In this study, we have successfully amplified the microsatellite region using primers designed from the sequence information generated and they were standardized for the identified microsatellite loci. All the thirteen microsatellite loci reported here showed considerable variation in the two populations of *Macrobrachium spp* examined. A mean of 53 different alleles were found over all the loci in both the populations and the number of alleles per locus ranged from 4 to 15. The allelic size varied in the range of 75 to 422 base pairs. The average allelic frequencies of various microsatellite loci lie in the range of 0.73 to 0.94. The average observed and expected heterozygosities ranged between 0.750 to 1.0 and 0.395 to 0.682 respectively. The extent of polymorphism observed in the present study was very high in the tune of 75 to 100 %. The average PIC value for all the thirteen microsatellite loci tested was found to be 0.88 indicating the very high level of polymorphism existing in them (Divu *et al.*, 2008a, 2008b).

Four loci in the population sample of scampi collected from Kerala showed the presence of rare alleles. The locus MRMA27 contained three rare alleles in this population. This emphasizes the genetic instability of this population in the wild environment. But only one locus in the scampi population

sample from Karnataka showed the evidence for single rare allele. A bottleneck effect may create a more devastating consequence for the Kerala population of *M. rosenbergii* collected from Vembanadu estuary than that for the Karnataka population collected from Nethravathi estuary.

The loci MRMA27, MRMB1, MRMB16 and MRMB24 showed significant departures from HWE after sequential Bonferoni corrections for sample sizes ($P < 0.005$) in Karnataka population of freshwater prawn samples. The MICROCHECKER software result reveals that none of the 13 microsatellite loci in this population show evidence for null alleles. Also there was no evidence for scoring error due to stuttering and large allele dropouts. A deviation from Hardy–Weinberg disequilibrium could have resulted from non-random mating inbreeding and /or natural selection. Excluding the loci MRMB11, MRMB13 and MRMB24, all other microsatellite loci showed significant departures from HWE after sequential Bonferoni correction in Kerala population of *M. rosenbergii*. The loci MRMA27 and MRMB26 were showing evidence for the presence of null alleles in this population. Since all the analyzed samples amplified, we are eliminating the chances of degraded DNA. Also we are not entirely confident that the non-amplified samples represent null allele homozygote only. Hence we are preferred to accept the results of Brookfield-1 equation conferring the chances that the non-amplified samples may be artifacts, null allele homozygotes or problems of PCR (Brookfield, 1996). In our survey, null alleles were confirmed after several repetitions of the assay, using the same conditions and also different annealing temperatures. About nonamplifying or null alleles from different studies, two facts emerge. First, these alleles are common; and, second, where flanking sequences were obtained for nonamplifying alleles, a mutation was found to have occurred in one of the priming sites (Pemberton *et al.*, 1995). The exclusion of even a few nonamplifying homozygotes can have dramatic effects on the interpretation of genotype frequency distributions and could lead to mistaken interpretations about the level of inbreeding in a population (Pemberton *et al.*, 1995).

If there is unrecognized sub-population structure within a sample this can lead to an apparent deviation from HWE even though within the sub-populations there is random mating. This may be another reason for the Kerala population samples scampi to show significant departures from HWE in majority of microsatellite loci used in this study (Divu *et al.*, 2008a, 2008b).

Chi-square test was performed to verify whether any linkage disequilibrium exists between the marker loci and the populations. It was found that no linkage disequilibrium observed for the same at 99% confidence levels. This suggests that the population under study is in linkage equilibrium and hence the loci segregate independently. This further reveals their potential usefulness as marker loci (Divu *et al.*, 2008a, 2008b).

The informativeness of the microsatellite loci should be determined by the allelic richness possessed by them. The allelic frequencies of all the above mentioned thirteen microsatellite loci showed an average range of 0.0143 to 0.7000 in both the population of the giant freshwater prawn. This higher range of allelic frequency values reveals the higher allelic richness carried by all the thirteen microsatellite loci tested in the present study (Divu *et al.*, 2008a, 2008b).

Heterozygosity is important to both natural and cultured populations because it provides a large spectrum of genotypes for adaptive response to changing conditions. Highly heterozygous individuals are far superior to less heterozygous individuals in many economically important characteristics like growth, fertility and disease resistance (Beardmore *et al.*, 1997). Heterozygosity is used as a general index of population diversity at genetic level and has drawn much attention from aquaculturists and ecologists. However, this important index is not perfect, especially in describing the range of genetic variation as suggested by Beardmore *et al.*, (1997) who surmise that it is necessary to distinguish between quantitative and qualitative differences in diversity especially when genes and alleles at low frequencies are being considered.

The observed heterozygosity range in this study was 0.28 to 1.0 and is similar to that reported by Charoentawee *et al.*, (2006). The overall heterozygosity was high (75 to 100%) in the present study

and was in agreement with that obtained by Brooker *et al.*, (2000) for *Penaeus mondon* (>90%) from Australia and Xu *et al.*, (2001) (94%) from Philippines. However the results differed from the average heterozygosity reported using the allozyme technique for crustaceans (7.3%) by Hedgecock *et al.*, (1982). The number of genotypes per locus ranges from 1 to 46, indicating that these two wild populations of *M. rosenbergii* retain very high genetic diversities. But the relative comparison of genotypes and heterozygosities between Karnataka and Kerala populations of *M. rosenbergii*, it is obvious that Kerala population shown higher genetic diversity than Karnataka population. The microsatellite loci MRMB10, MRMA8 and MRMC2 showed higher mean number of genotypes per locus (23.33, 23.33 and 22.66 respectively) and the loci MRMB7 and MRMA27 shown least number of genotypes per locus (8.75 and 6.00 respectively). The average observed heterozygosity (0.7302) was found to be significantly higher than that of expected heterozygosity in Karnataka population of *M. rosenbergii* (Divu *et al.*, 2008a, 2008b). Also the number of rare alleles found to be less (3) in this population. Populations that have undergone bottleneck are likely to have lost rare alleles, but may still contain substantial amount of heterozygosity (Nei *et al.*, 1975). Populations after a recent bottleneck should have significant heterozygosity excess compared to that based on the observed number of alleles. For Kerala population of *M. rosenbergii* the mean observed heterozygosity (0.6325) was not significantly very high with that of mean expected heterozygosity (0.5373). The total number of rare alleles found to be six in Kerala population individuals, which is significantly much higher than that of Karnataka population of freshwater prawn individuals used in this study.

The component of heterozygote deficiency implies a genetic differentiation, that is, in allele sizes and frequencies, within and between populations of a species. This fine scale differentiation within the species could be reflected in a substantial difference between the observed and expected heterozygosity. A combination of the presence of null alleles and fine-scale genetic differentiation may have been the cause of the heterozygote deficiency in Karnataka population of *M. rosenbergii*, but we could not quantify, using our data, the importance of each factor as a cause of the heterozygote deficit.

Genetic variation in a stock is the basis for genetic improvement; however, aquaculture practices can affect the genetic diversity of a species (Norris *et al.*, 1999). Generally, small effective population size and unmonitored selective breeding programs are the major causes for loss of genetic diversity in cultured species (Hansen *et al.*, 2001). Therefore, it is important to understand the distribution of the genetic diversity within a brood stock to ensure adequate management of the stock for selective breeding. The information on the genetic variability levels of giant freshwater prawn *M. rosenbergii* in the wild is scanty. The total number of alleles per all microsatellite loci in Karnataka and Kerala populations tested ranges from 32 - 36. The average number of alleles per thirteen microsatellite loci was 5.23. The genetic variability level can be depicted by the number of alleles per locus of the microsatellite markers (Arora and Bhatia, 2004). Both the tested populations possess higher genetic variability since they contain higher number of alleles across all the microsatellite marker loci used in the present study. This implies that both the tested populations of giant freshwater prawn from south India have their significant importance in commercial breeding operations (Divu *et al.*, 2008a, 2008b).

The microsatellite loci MRMB1 and MRMB16 in Karnataka scampi population samples and the loci MRMB11 and MRMB25 in Kerala scampi population samples found to be having non-neutral status. These four microsatellite loci showed lower observed homozygote allele frequencies when compared with the observed heterozygote allele frequencies in their respective population samples. Negative normalized deviates imply observed homozygosity values lower than expected homozygosity, in the direction of balancing selection when there is significant deviation from neutrality expectation. Significant positive values are in the direction of directional selection. Ewens–Watterson Neutrality test by Watterson (1977) showed that if the alternative hypothesis is that alleles are maintained by heterozygote advantage (with an equal fitness for all heterozygotes), the effect of such selection is to make allele frequencies more even than expected under neutrality. The likelihood ratio test for balancing selection can be reduced to a function of the population homozygosity (the probability that two alleles picked at random from a population are identical). In other words; homozygosity is a

sufficient statistic for testing the hypothesis of symmetric heterozygote advantage (which decreases homozygosity). Although it is unlikely that selection is so even across loci, and the situation is complicated by the fact that allele frequencies have to be estimated from samples, by comparing sample homozygosity to the distribution from Ewen's sampling formula, Watterson's homozygosity test is a powerful way of detecting unexpectedly even allele frequencies.

The $D'_{IS}{}^2$ value exceeds $D'_{ST}{}^2$ in the present analysis indicates that random drift is responsible for the variance in the frequency of allele combinations. According to Ohta (1982), these relations hold for unequal systematic disequilibria where selection favors for specific allele combinations only in few subsamples. The relationships $D'_{IS}{}^2 > D'_{ST}{}^2$ and $D_{ST}{}^2 > D_{IS}{}^2$ usually holds well when migration is limited in the population under analysis. But in our analysis the former part of the relation found to be true, but the latter part of the relationship was getting in the reverse order. This may be due to the reason that the individual samples collected from the Vembanadu estuary usually lacks the chances for effective migration due to the presence of a barrage constructed in the middle of the estuary for preventing the seawater ingress to the freshwaters during summer season. Also the values of $D_{ST}{}^2$, $D'_{IS}{}^2$ and $D_{IT}{}^2$ may become quite large when migration is limited (Ohta, 1982). Our analysis results are also emphasizing this observation, with the respective values of 0.1495, 0.1155 and 0.1565. The chances of epistatic natural selection acting on the total subdivided populations seems to be nil since the mean value of $D_{ST}{}^2 > D_{IS}{}^2$ ($0.1495 > 0.0056$).

The average Nm value estimated in this study with the help of thirteen microsatellite loci in two population samples of *M. rosenbergii* found to be significantly high (0.7294). This implies that an average of 7 individuals per every generation shows effective migration in the populations. A migrant must disperse, breed and successfully pass on its genes in order to cause gene flow. A common rule of thumb is that gene flow greater than one individual per generation is sufficient to prevent genetic differentiation of two populations through random drift. As each gene may have different critical levels

due to different levels of selection, and the gene flow will be sufficient to prevent fixation of alternative alleles. The effects of genetic drift can be overcome by gene flow. This does not imply that allelic frequencies will be the same in both the populations under consideration (Allendorf and Phelps, 1981).

F_{ST} measures the effect of population subdivision, which is the reduction in heterozygosity in a subpopulation due to genetic drift. F_{ST} is the most inclusive measure of population substructure and is most useful for examining the overall genetic divergence among subpopulations. It is also called coancestry coefficient or fixation index (Weir and Cockerham, 1984). F_{ST} is usually calculated for different genes, and then averaged across all the loci, and all the populations. Both the freshwater prawn populations analyzed in this study showed a F_{ST} value of zero. This indicates a panmixis condition in both the populations with no population subdivision with the occurrence of random mating and no genetic divergence within the population. Population subdivision results in the loss of genetic variation (measured by heterozygosity) within subpopulations due to their small population size and genetic drift acting within each one of them (Nei, 1973). This means that population subdivision would result in decreased observed heterozygosity relative to that expected heterozygosity under random mating as if the whole population was a single breeding unit.

F_{IS} is a measure of the deviation of genotypic frequencies from panmictic frequencies in terms of heterozygous deficiency or excess. It is what is known as the inbreeding coefficient (f), which is conventionally defined as the probability that two alleles in an individual are identical by descent (autozygous). It is the correlation between the uniting gametes relative to the gametes drawn at random from within a subpopulation averaged over all subpopulations. It shows the degree to which heterozygosity is reduced below the expectation (Wright, 1951). The value of F_{IS} ranges between -1 and +1. Both the tested populations of scampi showed high F_{IS} values nearer to -1. This indicates a heterozygote excess or outbreeding condition existing in these populations compared with the HWE expectations.

5.3. Cross species evaluation of microsatellite markers

The present work intended to determine the extent to which pairs of primers designed for the amplification of SSR loci in *Macrobrachium rosenbergii* can be used for assessing some other important species and genera. Cross-species amplification is very crucial to the use of SSRs for assessing the genetic variation within and among species is the ability of primers from one species to amplify homologous loci in related species (Kijas *et al.*, 1995). Cross-species use of microsatellite loci not only saves time and effort but also enables the setting up of genetic tools (Harr *et al.*, 1998). The cross amplification of some polymorphic microsatellites from domestic species, such as cattle, sheep, etc has already been succeeded (Engel *et al.*, 1996., Bonnet *et al.*, 2002). The utility of the *M. rosenbergii* microsatellite primers to produce PCR-amplified products across the genus and species was demonstrated using *M. idella* species, which used to be found along with *M. rosenbergii* in same aquatic niche. A decline of amplification success was observed with increase of genetic distance (Isagi and Suhandono, 1997; Steinkellner *et al.*, 1997; White and Powell, 1997; Witsenboer *et al.*, 1997). The species like shrimps, squilla and crabs those are most genetically distant from freshwater prawns, no amplification products for the microsatellite loci were detected. In species with low degrees of relationship, therefore, the same SSRs loci cannot be found. This result implies that the microsatellite loci developed and used in this study are exclusively amplifying successfully only in freshwater prawn species only. Hence these markers can also be used for product identification in processing industry to check adulteration with any other types of meat along with freshwater prawns. According to Smulders *et al.*, (1997), the lack of amplification of an allele in certain accessions can be the result of divergence in the sequences flanking the microsatellite, creating a null allele. The production of an undetectable amount of PCR product is another explanation given by Smulders *et al.*, (1997) and Lavi *et al.*, (1994). Nevertheless, these studies were carried out, using a detection method that is less sensitive than fluorescence.

5.4. Use of DNA microsatellite markers for the detection of some infectious viruses

carrier state

Macrobrachium rosenbergii is the most important and economically cultured palaemonid in the world and it is now farmed in large scale in different parts of the world including India. In 2002, the freshwater prawn production showed a significant increase, reaching an all time high of 20,000 tonnes in India. Infectious diseases caused by viruses and bacteria constitute the main barrier to the development and continuation of crustacean aquaculture, each cultivated species being sensitive to several types of pathogens. We observed a correlation between the prevalence of these viruses with certain microsatellite repeat regions identified and characterized in *M. rosenbergii* viz (GA)₄₄, (GA)₃₆, (GA)₂₆, (GA)₁₀ and (CT)₁₇. These markers could serve as a unique tool for marker-assisted selection since these are associated with disease phenotypes in scampi. There is no highly inbred and disease resistant stock available at present for making reference crosses in the fresh water prawns. On the other hand, high levels of variability in the disease-resistant and wild stocks may provide sufficient segregation of disease-resistance QTLs. Mapping disease-resistance genes is a challenge in many organisms, because often disease-resistance cannot be quantified for a given genotype. Prevailing low market price for shrimp and occurrence of white spot syndrome made the farmers to think for a viable alternate culture in low saline coastal areas. The only option available to the farmers is freshwater prawn *M. rosenbergii*. Unfortunately an area specific (Nellore, Andhra Pradesh) problem in the form of appendage deformities is affecting freshwater prawn production and its survival (Ravi Kumar *et al.*, 2004). Under these circumstances some percentage of farmers has considered either mixed culture of shrimp (*P. monodon*) with *M. rosenbergii* or by crop rotation between these two species as a viable alternative for their sustenance and economic viability. This situation invites the possibility of transmitting pathologically significant organisms from native to non-native hosts. The present study clearly indicates that the shrimps that were positive for different viruses by PCR reaction did not show any clinical signs of the diseases and there was no mortality. The results of this study also show that PCR is a useful tool for monitoring the health of animals in shrimp and prawn ponds. Highly sensitive

nested PCR test will be able to detect very low levels of infection (Belcher and Young, 1998). When PCR becomes positive by non-nested reaction, it is an indication of progression of infection to the status of disease.

Various microsatellite based pathogen screening strategies are developed in the field of fisheries; some important ones are the following. The genomic DNA of rainbow trout can be screened for detecting the loci which controls the activity of natural killer cells (NK- cells) (Zimmerman *et al.*, 2004) which linked to resistance to infectious hematopoietic necrosis virus (Palti *et al.*, 1999; Khoo *et al.*, 2004), ceratomyxosis (Nichols *et al.*, 2003) and pancreatic necrosis virus (Ozaki *et al.*, 2001). Polymorphic loci associated with resistance to infectious salmon anemia have been identified in Atlantic salmon (Grimholt *et al.*, 2003; Moen *et al.*, 2004). Resistance to iridovirus infection in the Red sea breams is reported to be associated with six microsatellite loci (Inami *et al.*, 2005).

As there is no cure for viral diseases, preventive measures have been proposed as the only way to significantly reduce the heavy mortality. Accurate and timely diagnosis helps significantly in effective management of a disease. Till recently diagnostic procedures for viral diseases were largely dependent upon classical methods such as history, clinical science and histological examination of moribund animals. Molecular methods have been developed for rapid disease diagnosis (Ligtner and Redman, 1998, Rai *et al.*, 2009). Apart from rapidity molecular methods allow the detection of asymptomatic carriers (Otta *et al.*, 1999; Nadala *et al.*, 2000), which harbour latent virus.

SUMMARY

VI. SUMMARY

The freshwater prawn, *Macrobrachium rosenbergii* is one of the widely cultured freshwater prawn species all over the world. Culture of this species has expanded rapidly not only within Asia but also in regions far remote from the natural distribution of the species and is now cultured in at least 43 countries across five continents. The application of genetic markers has allowed rapid progress in aquaculture investigations of parentage assignments, genetic variability and inbreeding. Microsatellite markers have been shown to be very useful for verifying pedigrees in prawn lines and pedigree tracing of hatchery populations. Genetic studies on *M. rosenbergii* using DNA markers are very rare. At the global level, India follows China and Thailand in freshwater prawn production and occupies the third position. The species are believed to be declining as a result of over-exploitation. So understanding the genetic diversity in wild stocks is important for developing sound conservation strategies. Populations with a high level of genetic variation have greater prospects in terms of higher growth rate, developmental stability, viability, fecundity and resistance to environmental stress and disease. Therefore knowledge of the genetic background of a species and its population structure is essential for success in any breeding management for the selection of genetically diverse brood stock.

The present research is aimed at identifying microsatellite loci in the genome of giant freshwater prawn, *M. rosenbergii* from India and understanding their usefulness to study genetic

diversity of wild populations. For most efficient marker development, microsatellite enriched genomic DNA libraries are made. In this study, we have successfully amplified the microsatellite region containing (GA) and (CT) repeats using PCR primers. The PCR fragments were purified and sequenced. From the sequence information generated microsatellite loci were identified. For the initial microsatellite detection and enrichment, total genomic DNA was extracted from the muscle tissue of *M. rosenbergii*. In the case of studies involving characterization of microsatellite loci and population structure, DNA was extracted from the pleopods of live animals without sacrificing the sampled freshwater prawns.

Thirteen well identifiable, highly polymorphic microsatellite marker loci in the 'western' form of *M. rosenbergii* genome have been developed. Among the thirteen microsatellite loci developed and used in this study, all were found to be highly polymorphic and showed considerable variation in the tested population samples of *M. rosenbergii*. A total of 59 different alleles were found over all the microsatellite loci tested and the number of alleles per locus ranged from 3 to 9. The average observed and expected heterozygosities ranged between 0.6785 ± 0.1883 and 0.6881 ± 0.1117 respectively. The number of genotypes estimated across all the microsatellite loci in both the tested populations of giant freshwater prawn found in the range of 21 to 35 genotypes. The locus MRMB1 exhibited the highest range of the number of genotypes (2 - 19) and the locus MRMB13 showed the least (3–10). None of the loci showed significant linkage disequilibrium for all pairs of loci. Both the major populations of giant freshwater prawn from south India tested have a very high genetic variability and thereby present significance in commercial breeding operations.

Cross species amplification was performed using different types of crustacean species including freshwater prawns, shrimps and crabs. The freshwater prawn *M. idella* showed considerable

amplification of all the microsatellite regions with the microsatellite primers developed for *M. rosenbergii*. We observed a correlation between the prevalence of these viruses with certain microsatellite repeat regions identified and characterized in *M. rosenbergii* viz (GA)₄₄, (GA)₃₆, (GA)₂₆, (GA)₁₀ and (CT)₁₇. These markers could serve as a unique tool for marker –assisted selection since these are associated with disease phenotypes in scampi.

The data generated in this study provide useful information on the genetic variation and differentiation in two major wild populations of *M. rosenbergii* from southern peninsular India. This information can be applied for future genetic improvement by selective breeding, and to design suitable management guidelines for these genetic materials. Availability of such data could be very useful for stock management, selective breeding programme and sustainable use of wild resources.

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VII. BIBLIOGRAPHY

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ABSTRACT

VIII. ABSTRACT

Thirteen well identifiable, highly polymorphic microsatellite marker loci in the ‘western’ form of *M. rosenbergii* genome have been developed. The utility of a codominant marker such as microsatellites for accurately assessing the occurrence of heterozygote genotypes in species with an outbreeding mode of reproduction is clear. High levels of intraspecific variation, which were detected in study, make these SSR markers suitable for studies of population differentiation of the species. This factor is essential for establishing the criteria for conservation of populations of a species under *in situ* and *ex situ* conditions. The microsatellite technique opens new perspective for studying the structure of closely related populations, population samples over a reduced geographic scale and less isolated populations. It is also a unique tool for tracking pedigrees in breeding programmes and marker – assisted selection. Availability of such data could be very useful for stock management, selective breeding programme and sustainable use of wild resources. Individual species with a high level of genetic variation have greater prospects in terms of higher growth rate, developmental stability, viability, fecundity and resistance to environmental stress and disease. Therefore Knowledge of the genetic background of a species and its population structure is essential for success in any breeding management and conservation program. The data generated in this study provide useful information on the genetic variation and differentiation in two major wild populations of *M. rosenbergii* from southern peninsular India. This information can be applied for future genetic improvement by selective breeding, and to design suitable management guidelines for these genetic materials.

APPENDIX

IX. APPENDIX

3.2.1. Reagents used for DNA extraction

3.2.1.1. SDS (10%)

SDS	10 g
MillQ water	100 ml

3.2.1.2. Extraction Buffer

10 mM Tris HCl, pH 8.0
0.1 M EDTA, pH 8.0
0.5 % SDS
20 µg/ml pancreatic RNase

3.2.1.3. Ammonium acetate (10 M)

Ammonium Acetate	- 770.8 g
Distilled water	- 1000 ml

The reagent was filter sterilized using 0.45µ filter and stored at room temperature.

3.2.1.4. Stock Proteinase K solution (Bangalore Genei, Bangalore)

Five milliliter of distilled water was added to 100 mg of proteinase K powder. The stock prepared would contain 20 mg / ml concentration and stored at -20°C.

3.2.1.5. Phenol equilibration (Sambrook *et al.*, 1989)

To melted phenol, equal volume of buffer was added (0.5 M Tris HCl, pH 8.0) and stirred using a magnetic stirrer for 15 minutes at room temperature. The upper aqueous phase was discarded and an equal volume of 0.1 M Tris HCl, pH 8.0 was added to phenol and stirred for further 15 minutes. This

process was repeated until a pH of 7.8 was obtained. Hydroxyquinoline was also added to a final concentration of 0.1%. This equilibrated phenol was used for DNA extraction.

3.2.1.6. TE buffer

Tris-HCl	10 mM
EDTA	1 mM
pH	8.0

3.2.3.1. Materials required for agarose gel electrophoresis

3.2.3.1.1. Agarose (HiMedia, Mumbai)

3.2. 3.1.2. TAE buffer (50X)

Composition

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH, 8.0)	100 ml

The solution was made up to a final volume of 1 liter using MilliQ water and sterilized by autoclaving at 121°C for 15 min. A 1 X TAE buffer for electrophoresis was prepared by diluting 20 ml of stock solution to 1 litre using MilliQ water.

3.2. 3.1.3. Gel loading buffer

Bromophenol blue	0.25 g
Sucrose	40 g
Distilled water	100 ml

3.2.3.1.4. Ethidium bromide

A stock solution of ethidium bromide was prepared by dissolving 5 mg of ethidium bromide (Sigma, USA) in 1 ml of distilled water. The solution was either added to agarose during preparation or to the staining medium (1 X TAE buffer) to yield a final concentration of 0.5 µg/ml. In the later case, the gel was allowed to stain in the staining solution for 20 min and destained in distilled water for 10 min to remove excess stain.

3.2.3.1.5. DNA ladders

3.2.3.1.5.1. λ-DNA molecular weight markers

λ-DNA completely digested with EcoRI and HindIII yielding 13 fragments of 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564 and 125 base pairs.

3.2.3.1.5.2. 100 bp DNA Ladder plus (Gene Ruler TM)

This marker had fragments of 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200 and 100 base pairs.

3.2.3.1.5.3. 1kb DNA Ladder (Gene Ruler TM)

This marker had fragments of 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250 base pairs.

3.9.1. Standard saline citrate solution (SSC, 20X)

Sodium chloride	175.4 g
Sodium citrate	88.2 g
Distilled water	1000 ml
pH	7.0

The ingredients were dissolved in 800 ml distilled water and pH adjusted to 7.0 with 10 N sodium hydroxide. The volume was made up to 1000 ml and stored at room temperature.

The 20 X SSC was used for preparing 1 X SSC and 2 X SSC.

3.9.2. S1 Reagent

Sodium dodecyl sulphate (SDS)	1.0 g
2X SSC	1000 ml

3.9.3. S2 Reagent

Sodium dodecyl sulphate (SDS)	1.0 g
1X SSC	1000 ml

3.12.1. Reagents used for transformation

3.12.1.1. Composition of SOC Media

Tryptone	2.0 g
Yeast extracts	0.5 g
1 M NaCl	1.0 ml
1 M KCl	0.25 ml
2 M MgCl ₂	1.0 ml
2 M Glucose	1.0 ml

3.12.1.2. X-Gal

Dissolve 100 mg 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) in 2 ml N, N'-dimethyl-formamide. Cover with aluminium foil and store at -20°C. The final concentration of X-Gal will be 50 mg/ ml.

3.12.1.3. Luria Bertani Medium (HiMedia, Mumbai)

Casein enzymic hydrolysate	10.0 g
Yeast extract	5.0 g
Sodium chloride	10.0 g
Agar agar	20.0 g
Distilled water	1000 ml
pH	7.5±0.2

The medium was autoclaved at 121°C for 15 min at 15 lb pressure and cooled to 50°C. Antibiotic solution was added to a desired concentration and poured into pre-sterilised plates. The antibiotic containing LB agar plates were stored in refrigerator for not more than a week.

3.16.1. Glycerol Broth

Glycerol (100%)	30ml
LB Broth	70 ml
Ampicillin	100µg/ml
pH	7.0

The medium was sterilized by autoclaving at 110° C for 15 min

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Table 1. Reagents used for Restriction Digestion

Materials	Quantity (µl)
MilliQ water	39.8
Buffer E	9
BSA	0.9
Genomic DNA	39
<i>Sau</i> 3AI	1.3
Total reaction volume	90

Table 2. Reagents used for Adapter ligation

Materials used	Quantity (µl)
MilliQ water	48.0
T4 DNA Ligase buffer	20.0
Restricted digested DNA	90.0
Adapter mix	2.0
T4 DNA ligase	40.0
Total reaction volume	200.0

Table 3. Reagents used in the PCR of adapter ligated DNA.

Materials used	Quantity (µl)
MilliQ water	18.0
10X Assay Buffer	2.5
dNTPs	2.0
Oligo.A	1.25
Adapter ligated DNA	1.0
Taq DNA Polymerase	0.25
Total reaction volume	25.0

Table 4. Thermocycling conditions for adapter ligated DNA

Steps	Temperature (°C)	Time
1. Initial denaturation	95	5 minutes
2. Denaturation	95	50 seconds
3. Primer Annealing	56	1 minutes
4. Primer Extension	72	2 minutes
Step2- 4 repeated 30 times		
5. Final extension	72	10 minutes

Table 5. Hybridization of adapter ligated DNA to biotinylated microsatellite (CA)₁₅ probe

Materials used	Quantity (µl)
PCR product	1.0
Oligo (CA) ₁₅	2.0
12X SSC and 0.1% SDS	50.0
MilliQ water	47.0
Total reaction volume	100.0
Incubation temperature	55°C

Table 6. Hybridization of adapter ligated DNA to biotinylated microsatellite (AT)15 probe

Materials used	Quantity (µl)
PCR product	1.0
Oligo (AT)15	2.0
12X SSC and 0.1% SDS	50.0
MilliQ water	47.0
Total reaction volume	100.0
Incubation temperature	50°C

Table 7. Reagents for the PCR following the microsatellite capture

Reagents used	Quantity (µl)
MilliQ water	29.0
10X Assay Buffer	5.0
dNTPs	4.0
Oligo.A	1.5
Adapter ligated captured DNA	10.0
Taq DNA Polymerase	0.5
Total reaction volume	50.0

Table 8. Thermocycling conditions for microsatellite enrichment

Steps	Temperature (°C)	Time
1.Initial denaturation	95	3 minutes
2. Denaturation	95	30 seconds
3. Primer annealing	60	30 seconds
4. Primer extension	72	30 seconds
Step 2- 4 repeated 5 times		
5. Denaturation	92	30 seconds
6.Primer annealing	60	30 seconds
7. Primer extension	72	55 seconds
Step 5- 7 repeated 30 times		
8.Final extension	72	30 minutes

Table 9. Reagents for the Ligation of microsatellite DNA into the vector

Materials used	Quantity (µl)
Fresh cleaned PCR product	4.0
Salt solution	1.0
TOPO vector	1.0
Total reaction volume	6.0

Table 10. Thermocycling conditions for microsatellite screening PCR

Steps	Temperature (°C)	Time
1.Initial delay	95	3 minutes
2. Denaturation	95	30 seconds
3. Primer annealing	60	30 seconds
4. Primer extension	72	30 seconds
Step 2- 4 repeated 5 times		
5. Denaturation	92	30 seconds
6.Primer annealing	60	30 seconds
7. Primer extension	72	55 seconds
Step 6- 8 repeated 30 times		
8.Final extension	72	30 minutes

Table 11. Reagents for M13 PCR.

Materials used	Quantity (µl)
MilliQ water	19.8
10X Assay Buffer	3.0
dNTPs	2.4
Forward Primer	2.0
Reverse Primer	2.0
Taq DNA Polymerase	0.3
Plasmid DNA	0.5
Total reaction volume	30.0

Table 12. Thermocycling conditions for M13 PCR

Steps	Temperature (°C)	Time
1. Initial denaturation	94	10 minutes
2. Denaturation	94	10 minutes
3. Primer Annealing	55	1 minutes
4. Primer Extension	72	1 minutes
Step 2-4 repeated 30 times		
5. Final extension	72	10 minutes

Table 13. Cross species tested for amplification of the microsatellite marker loci

Sl. No.	Species used	No. of samples
1	<i>Macrobrachium idella</i>	65
2	<i>Aristeus alcocki</i>	30
3	<i>Seranopsis longipes</i>	36
4	<i>Penaeus fissuroides</i>	30
5	<i>Fenneropenaeus indicus</i>	40
6	<i>Penaeus monodon</i>	35
7	<i>Metapenaeus dobsoni</i>	42
8	<i>M. monoceros</i>	32
9	<i>M. affinis</i>	38
10	<i>Parapenaeopsis styliifera</i>	36
11	<i>Heterocarpus woodmasoni</i>	30
12	<i>H. gibbosus</i>	40
13	<i>Seranopsis longipes</i>	35
14	<i>Solenocera choprai</i>	40
15	<i>S. hextii</i>	32
16	<i>Charybdis hoplites</i>	50
17	<i>Portunus pelagicus</i>	40
18	<i>Portunus sanguinolentus</i>	35
19	<i>Scylla serrata</i>	24
20	<i>Charybdis riversandersoni</i>	32
21	<i>Squilla mantis</i>	30

Table 15. PCR Primer sequence data for the detection of WSSV, HPV, MBV and IHNV

Virus	Primer	Sequence (5'-3')	Reference
WSSV 1 st step	IK 1	TGGCATGACAACGGCAGGAG	Otta <i>et al.</i> , 2003
	IK 2	GGCTTCTGAGATGAGGACGG	
WSSV 2 nd step	IK 3	TGTCATCGCCAGCACGTGTGC	
	IK 4	AGAGGTCGTCAGAGCCTAGTC	
HPV 1 st step	441F	GCA TTA CAA GAG CCA AGC AG	Phromjai <i>et al.</i> , 2001
	441R	ACA CTC AGC CTC TAC CTT GT	
HPV 2 nd step	HPVnF	ATA GAA CGC ATA GAA AAC GCT	Umesha <i>et al.</i> , 2003
	HPVnR	CAG CGA TTC ATT CCA GCG CCA CC	
MBV1 st step	1.4F	CGA TTC CAT ATC GGC CGA ATA	Belcher and Young, 1998
	1.4R	TTG GCA TGC ACT CCC TGA GAT	
MBV2 nd step	1.4NF	TCC AAT CGC GTC TGC GAT ACT	Otta <i>et al.</i> , 2003
	1.4NR	CGC TAA TGG GGC ACA AGT CTC	
IHNV 1 st step	648F	GAACGGCTTTCGTATTTTGG	Rai <i>et al.</i> , 2009
	648R	AGCGTAGGACTTGCCGATTA	
IHNV 2 nd step	309F	TCCAACACTTAGTCAAAACCAA	Tang <i>et al.</i> , 2007
	309R	TGTCTGCTACGATGATTATCCA	

Table 15: Thermocycling conditions for the detection of WSSV, HPV, MBV and IHHNV

Primers	<i>Cycling conditions</i>			No. of cycles	Product size
	Denaturation	Annealing	Extension		
IK1 and IK 2 (WSSV)	94°C for 30 s	55°C for 30 sec	72°C for 30 sec	30	486 bp
IK3 and IK4 (WSSV)	94°C for 30 s	55°C for 30 sec	72°C for 30 sec	30	310 bp
(HPV) 441F and 441R	94°C for 30 s	55°C for 30s	72°C for 30s	30	441bp
I step (MBV 1.4F and MBV 1.4R)	94°C for 30 s	65°C for 30s	72°C for 60s	40	533 bp
II step (MBV1.4 NF & MBV1.4 NR)	94°C for 30 s	60°C for 30s	72°C for 60s	35	361 bp
IHHNV309F IHHNV309R	95°C for 30 sec	55°C for 30 sec	72°C for 30 sec	30	309
IHHNV648F IHHNV648R	94°C for 30 sec	55°C for 30sec	72°C for 30sec	30	648

Table 16. Analysis for DNA purity

Quantity of sample used (µl)	Optical density at 260nm	Optical density at 280nm
2	0.0054	0.0017
4	0.0093	0.0035
6	0.0204	0.0117
8	0.0284	0.0179
Ratio (260/280)		1.82
Concentration of DNA (µg/ml)		449.06

Table 17: Microsatellite loci with their repeat motif and GenBank accession numbers

Sl. No.	GenBank accession No.	Locus name	Repeat motifs
1	DQ793215	MRMA8	(GA) ₆₈
2	DQ723216	MRMA27	(GT) ₁₃
3	EF515167	MRMB1	(GA) ₄₄
4	EF515168	MRMB10	(GA) ₃₅
5	EF515169	MRMB7	(GA) ₂₆
6	EF515170	MRMB11	(CT) ₃₀
7	EF515171	MRMB15	(GA) ₃₃
8	EF515172	MRMB16	(GA) ₃₆
9	EF515173	MRMB25	(CT) ₁₇
10	EF515174	MRMB27	(GA) ₃₁
11	EF515175	MRMC2	(GA) ₁₀
12	EU022582	MRMB24	(GA) ₂₈
13	EU022583	MRMB26	(GA) ₃₁
14	EU034628	MRMB13	(CT) ₁₆
15	EU812454	MRMBD28	(GA) ₃₁
16	EU812455	MRMB30	(GA) ₂₄
17	EU812456	MRMB31	(GA) ₃₀
18	EU812457	MRMB35	(GT) ₁₅ (GA) ₂₅
19	EU812458	MRMB36	(GA) ₁₉

Table 18. Microsatellite primer sequences with their PCR annealing temperatures

Microsatellite loci	GenBank Accession	Primer sequences	Annealing temperature
MRMA8	DQ793615	FP- TTGACTAGGCTTCGAACCC RP- AAACCGATTTCTGTCTTACGC	48°C
MRMA27	DQ793616	FP- TTAGGGTGTGGAGTAACAGG RP- TTCGCTGAATACGCGCATGAC	53°C
MRMB1	EF515167	FP- AGTAACAACAGTCAGGAAGAGT RP- TTGTCTGCATTCGGTAGTCTTT	46°C
MRMB7	EF515169	FP- ACTTCGGAACAAGGGATTAT RP- GAATCGAAAGCAGTCTCCTT	44°C
MRMB10	EF515168	FP- AGAGGCACTACAGAAGACCAA RP- ATCCTCAGGTCTCCCTTCGT	48°C
MRMB15	EF515171	FP- AAGAAGGGGTGAAATAAGAGAAAG RP- GATACGGAGATTACATAGTTGTAGG	46°C
MRMB11	EF515169	F: GACGCTGCCAAAAAGAAAAG R: ACCGTGCCATTAACCTTCCAA	44°C
MRMB13	EU034628	F: AGGTCGGCCACTTCATTACA R: CCCCTAAGGACCCAATGAAT	47°C
MRMB15	EF515171	F: GAAAGAGAATAAAGTGGGGAAGAA R: GGATGTTGATACATTAGAGGCATAGA	46°C
MRMB16	EF515172	F: CTCTTGACAACCCAAAATCA R: AATGGGCCACCTCTCCTTAT	45°C
MRMB24	EU022582	F: CACCCACCAATTTTATACGC R: TTTAGGTGCCCCAACATAGC	47°C
MRMB25	EF515173	F: CCGGTCCAAAGGAATACAGA R: GTGGGTGGTGTCCCTATGTT	48°C
MRMB26	EU022583	F: CGCATGATAGTGACGGAATG R: GAAGGAAGATAAAAATCAAGCA	48°C
MRMC2	EF515175	F: TTGGGTGCAGGAAGTCTTGT R: GGGTCTTGGGAGATTTCAGTG	46°C

Table19. Microsatellite marker loci analysis results for the *M. rosenbergii* samples.

Loci	Effective sample size	No of alleles	HWE p	H _O	H _E	ho	he	Nei's heterozy.	Shannon index	No. of genotypes
MRMB11	70	4	0.2343	0.7429	0.7019	0.2571	0.2981	0.6918	1.2742	8 – 31
MRMB13	42	3	0.0748	0.4762	0.6690	0.5238	0.3310	0.6531	1.0770	10 – 16
MRMB15	70	4	0.0284	0.6857	0.7255	0.3143	0.2745	0.7151	1.3152	9 – 27
MRMB16	70	6	0.0000*	0.7714	0.8137	0.2286	0.1863	0.8020	1.6528	1 – 17
MRMB24	48	3	0.0505	0.5833	0.6064	0.4167	0.3936	0.5938	0.9901	8 – 26
MRMB25	70	6	0.0000*	0.7429	0.8199	0.2571	0.1801	0.8082	1.7238	7 – 21
MRMC2	70	3	0.0284	0.6857	0.5130	0.3143	0.4870	0.5057	0.8697	9 – 46
MRMA8	70	3	0.0544	0.6857	0.6360	0.3143	0.3640	0.6269	1.0320	12 – 30
MRMA27	54	9	0.0013*	0.5185	0.8106	0.4815	0.1894	0.7956	1.8953	2 – 21
MRMB1	70	4	0.0001*	0.9429	0.7491	0.0571	0.2509	0.7384	1.3634	14 – 23
MRMB7	70	8	0.0046	1.0000	0.7797	0.0000	0.2203	0.7686	1.7355	2 – 28
MRMB10	68	3	0.0190	0.7059	0.6462	0.2941	0.3538	0.6367	1.0551	16 – 32
MRMB26	50	3	0.0006*	0.2800	0.4743	0.7200	0.5257	0.4648	0.8181	7 – 35

Ho: Observed Heterozygosity, H_E: Expected Heterozygosity, ho : Observed Homozygosity, he : Expected Homozygosity

* Indicates $p < 0.05$

Table 20. Summary of *F*-Statistics and gene flow for all loci

Locus	F_{IS}	F_{IT}	F_{ST}	N_m
MRMB11	-0.3419	-0.0619	0.2086	0.9485
MRMB13	-0.6250	0.3780	0.6172	0.1550
MRMB15	-0.5157	0.0626	0.3815	0.4053
MRMB16	-0.3810	-0.0207	0.2609	0.7082
MRMB24	-0.3858	-0.1318	0.1833	1.1140
MRMB25	-0.2544	0.0995	0.2821	0.6362
MRMC2	-0.5157	-0.3417	0.1148	1.9286
MRMA8	-0.4157	-0.0544	0.2552	0.7295
MRMA27	0.1878	0.3291	0.1740	1.1864
MRMB1	-0.9134	-0.2718	0.3354	0.4955
MRMB7	-0.3458	-0.2854	0.0449	5.3174
MRMB10	-0.5441	-0.0776	0.3021	0.5775
MRMB26	0.1727	0.2603	0.1060	2.1094
<u>Mean</u>	-0.3518	-0.0067	0.2553	0.7294

Table 21. The Ewens-Watterson test for neutrality for all microsatellite loci

Locus	k	Obs. F	Min. F	Max. F	Mean	SE	L95	U95
MRMB11	4	0.3082	0.2500	0.9180	0.5470	0.0257	0.3049	0.8902
MRMB13	3	0.3469	0.3333	0.9093	0.6218	0.0267	0.3639	0.9082
MRMB15	4	0.2849	0.2500	0.9180	0.5405	0.0278	0.3090	0.8902
MRMB16	6	0.1980	0.1667	0.8673	0.3991	0.0169	0.2273	0.7180
MRMB24	3	0.4062	0.3333	0.9201	0.6276	0.0286	0.3576	0.9193
MRMB25	6	0.1918	0.1667	0.8673	0.4002	0.0172	0.2245	0.7155
MRMC2	3	0.4943	0.3333	0.9445	0.6582	0.0302	0.3743	0.9441
MRMA8	3	0.3731	0.3333	0.9445	0.6442	0.0295	0.3600	0.9441
MRMA27	9	0.2044	0.1111	0.7476	0.2563	0.0061	0.1564	0.4616
MRMB1	4	0.2616	0.2500	0.9180	0.5469	0.0271	0.3082	0.8902
MRMB7	8	0.2314	0.1250	0.8200	0.3047	0.0100	0.1820	0.5751
MRMB10	3	0.3633	0.3333	0.9429	0.6341	0.0311	0.3633	0.9425
MRMB26	3	0.5352	0.3333	0.9232	0.6291	0.0289	0.3656	0.9224

Table 22: Heterozygosity statistics of Karnataka samples

Locus	Obs_Hom	Obs_Het	Exp_Hom	Exp_Het	Nei_Het	Ave_Het
MRMB11	0.4286	0.5714	0.5767	0.4233	0.4082	0.4082
MRMB13	0.2308	0.7692	0.5077	0.4923	0.4734	0.4734
MRMB15	0.3571	0.6429	0.5476	0.4524	0.4362	0.4362
MRMB16	0.0000	1.0000	0.4815	0.5185	0.5000	0.5000
MRMB24	0.0000	1.0000	0.4667	0.5333	0.5000	0.5000
MRMB25	0.5000	0.5000	0.6111	0.3889	0.3750	0.3750
MRMC2	0.3571	0.6429	0.5476	0.4524	0.4362	0.4362
MRMA8	0.5714	0.4286	0.6508	0.3492	0.3367	0.3367
MRMA27	0.3333	0.6667	0.2121	0.7879	0.7222	0.7222
MRMB1	0.0000	1.0000	0.4815	0.5185	0.5000	0.5000
MRMB7	0.0000	1.0000	0.1878	0.8122	0.7832	0.7832
MRMB10	0.4286	0.5714	0.5767	0.4233	0.4082	0.4082
MRMB26	0.3000	0.7000	0.5211	0.4789	0.4550	0.4550
Mean	0.2698	0.7302	0.4899	0.5101	0.4873	0.4873
St. Dev	0.2056	0.2056	0.1393	0.1393	0.1283	0.1283

Table 23. HWE probabilities for Karnataka samples

Locus	P-value
MRMB11	0.5053
MRMB13	0.0748
MRMB15	0.2209
MRMB16	0.0005*
MRMB24	0.0253*
MRMB25	0.5130
MRMC2	0.2209
MRMA8	1.0000
MRMA27	0.0442*
MRMB1	0.0005*
MRMB7	0.2728
MRMB10	0.5053
MRMB26	0.2198

Table 24. The Ewens-Watterson test for neutrality in Karnataka samples

Locus	k	Obs. F	Min. F	Max. F	Mean	SE	L95	U95
MRMB11	2	0.5918	0.5000	0.9311	0.7490	0.0231	0.5026	0.9311
MRMB13	2	0.5266	0.5000	0.9260	0.7432	0.0243	0.5030	0.9260
MRMB15	2	0.5638	0.5000	0.9311	0.7529	0.0237	0.5026	0.9311
MRMB16	2	0.5000	0.5000	0.9311	0.7490	0.0247	0.5026	0.9311
MRMB24	2	0.5000	0.5000	0.8828	0.7195	0.0202	0.5000	0.8828
MRMB25	2	0.6250	0.5000	0.9311	0.7447	0.0233	0.5026	0.9311
MRMC2	2	0.5638	0.5000	0.9311	0.7541	0.0246	0.5026	0.9311
MRMA8	2	0.6633	0.5000	0.9311	0.7527	0.0244	0.5026	0.9311
MRMA27	4	0.2778	0.2500	0.6250	0.3879	0.0075	0.2639	0.5833
MRMB1	2	0.5000	0.5000	0.9311	0.7500	0.0244	0.5026	0.9311
MRMB7	6	0.2168	0.1667	0.7066	0.3248	0.0088	0.2041	0.5765
MRMB10	2	0.5918	0.5000	0.9311	0.7580	0.0237	0.5026	0.9311
MRMB26	2	0.5450	0.5000	0.9050	0.7332	0.0214	0.5050	0.9050

Table 25. Summary of *F*-Statistics and gene flow for Karnataka population samples

Locus	F_{IS}	F_{IT}	F_{ST}
MRMB11	-0.4000	-0.4000	0.0000
MRMB13	-0.6250	-0.6250	0.0000
MRMB15	-0.4737	-0.4737	0.0000
MRMB16	-1.0000	-1.0000	0.0000
MRMB24	-1.0000	-1.0000	0.0000
MRMB25	-0.3333	-0.3333	0.0000
MRMC2	-0.4737	-0.4737	0.0000
MRMA8	-0.2727	-0.2727	0.0000
MRMA27	0.0769	0.0769	0.0000
MRMB1	-1.0000	-1.0000	0.0000
MRMB7	-0.2769	-0.2769	0.0000
MRMB10	-0.4000	-0.4000	0.0000
MRMB26	-0.5385	-0.5385	0.0000
<u>Mean</u>	-0.4987	-0.4987	0.0000

Table 26. Heterozygosity statistics of Kerala samples

Locus	Obs_Hom	Obs_Het	Exp_Hom	Exp_Het	Nei_Het	<u>Ave_Het</u>
MRMB11	0.1429	0.8571	0.3275	0.6725	0.6565	0.5323
MRMB13	1.0000	0.0000	1.0000	0.0000	0.0000	0.2367
MRMB15	0.2857	0.7143	0.5296	0.4704	0.4592	0.4477
MRMB16	0.3810	0.6190	0.3113	0.6887	0.6723	0.5862
MRMB24	0.6250	0.3750	0.4919	0.5081	0.4922	0.4961
MRMB25	0.0952	0.9048	0.2369	0.7631	0.7449	0.5599
MRMC2	0.2857	0.7143	0.5296	0.4704	0.4592	0.4477
MRMA8	0.1429	0.8571	0.4146	0.5854	0.5714	0.4541
MRMA27	0.5238	0.4762	0.2985	0.7015	0.6848	0.7035
MRMB1	0.0952	0.9048	0.4925	0.5075	0.4955	0.4977
MRMB7	0.0000	1.0000	0.2799	0.7201	0.7029	0.7431
MRMB10	0.2000	0.8000	0.5077	0.4923	0.4800	0.4441
MRMB26	1.0000	0.0000	0.5954	0.4046	0.3911	0.4231
<u>Mean</u>	0.3675	0.6325	0.4627	0.5373	0.5238	0.5055
St. Dev	0.3312	0.3312	0.1987	0.1987	0.1943	0.1285

Table 27. HWE probabilities for Kerala samples

Locus	P-value
MRMB11	0.1226
MRMB13	monomorphic
MRMB15	0.0201*
MRMB16	0.0003*
MRMB24	0.3477
MRMB25	0.0001*
MRMC2	0.0201*
MRMA8	0.0096*
MRMA27	0.0002*
MRMB1	0.0003*
MRMB7	0.0020*
MRMB10	0.0054*
MRMB26	0.0002*

Table 28. Null allele frequencies estimated for Kerala samples with MICROCHECKER software

Locus	Null Alleles	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
MRMB11	no	-0.1644	-0.1326	-0.1211	0
MRMB13	no	0	0	0	0.7868
MRMB15	no	-0.4655	-0.2174	-0.1748	0
MRMB16	no	0.0411	0.0413	0.0319	0.0319
MRMB24	no	0.1124	0.1351	0.0785	0.4564
MRMB25	no	-0.1065	-0.0969	-0.0916	0
MRMC2	no	-0.4655	-0.2174	-0.1748	0
MRMA8	no	-0.5002	-0.2000	-0.1818	0
MRMA27	YES	0.1472	0.1797	0.1238 *	0.1238
MRMB1	no	-0.6914	-0.2923	-0.2737	0
MRMB7	no	-0.2663	-0.1744	-0.1744	0
MRMB10	no	-0.5528	-0.2500	-0.2162	0.0709
MRMB26	YES	0.3727	1.0000	0.2812*	0.6189

Table 29. The Ewens-Watterson test for neutrality in Kerala samples

Locus	k	Obs. F	Min. F	Max. F	Mean	SE	L95	U95
MRMB11	3	0.3435	0.3333	0.9093	0.6204	0.0267	0.3549	0.9082
MRMB13	1	1.0000	1.0000	1.0000	x	x	x	x
MRMB15	2	0.5408	0.5000	0.9535	0.7703	0.0261	0.5011	0.9535
MRMB16	4	0.3277	0.2500	0.8673	0.5084	0.0230	0.2891	0.8220
MRMB24	2	0.5078	0.5000	0.9395	0.7611	0.0237	0.5020	0.9395
MRMB25	4	0.2551	0.2500	0.8673	0.5137	0.0217	0.2971	0.8220
MRMC2	2	0.5408	0.5000	0.9535	0.7719	0.0260	0.5011	0.9535
MRMA8	3	0.4286	0.3333	0.9093	0.6096	0.0264	0.3639	0.9082
MRMA27	5	0.3152	0.2000	0.8277	0.4231	0.0171	0.2438	0.7052
MRMB1	2	0.5045	0.5000	0.9535	0.7752	0.0262	0.5011	0.9535
MRMB7	4	0.2971	0.2500	0.8673	0.5020	0.0213	0.2846	0.8220
MRMB10	2	0.5200	0.5000	0.9513	0.7685	0.0256	0.5012	0.9513
MRMB26	2	0.6089	0.5000	0.9356	0.7558	0.0252	0.5022	0.9356

Table 30. Summary of F-Statistics and gene flow for Kerala samples

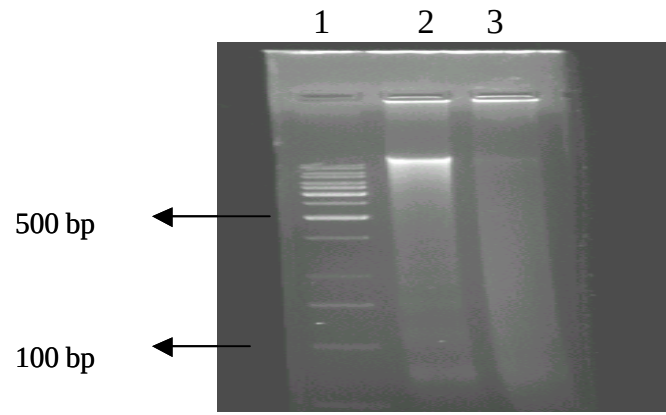
Locus	F_{IS}	F_{IT}	F_{ST}
MRMB11	-0.3057	-0.3057	0.0000
MRMB13	x	x	0.0000
MRMB15	-0.5556	-0.5556	0.0000
MRMB16	0.0793	0.0793	0.0000
MRMB24	0.2381	0.2381	0.0000
MRMB25	-0.2146	-0.2146	0.0000
MRMC2	-0.5556	-0.5556	0.0000
MRMA8	-0.5000	-0.5000	0.0000
MRMA27	0.3046	0.3046	0.0000
MRMB1	-0.8261	-0.8261	0.0000
MRMB7	-0.4226	-0.4226	0.0000
MRMB10	-0.6667	-0.6667	0.0000
MRMB26	1.0000	1.0000	0.0000
<u>Mean</u>	-0.2074	-0.2074	0.0000

Table 31. Asymptomatic carrier state detection of different viruses on *M. rosenbergii* samples

[illegible]

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Fig: 2. Restriction digestion of genomic DNA

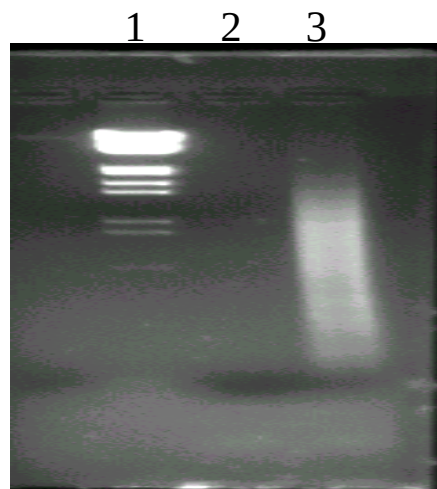


Lane 1: 1kb DNA ladder

Lane 2: Genomic DNA

Lane 3: *Sau3AI* digested genomic DNA

Fig: 3. PCR amplification of Adapter ligated DNA

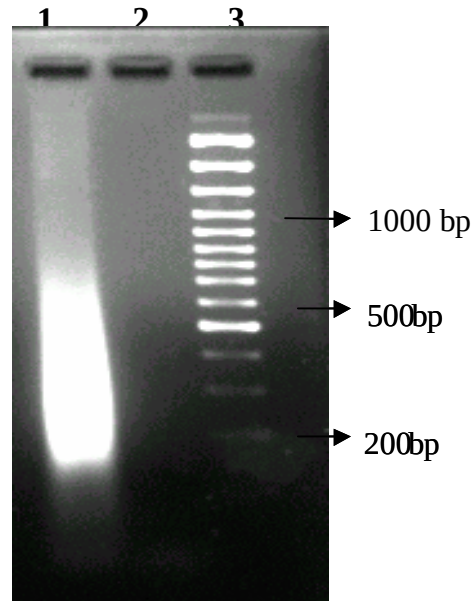


Lane1: 100 bp Molecular marker

Lane2: Negative control

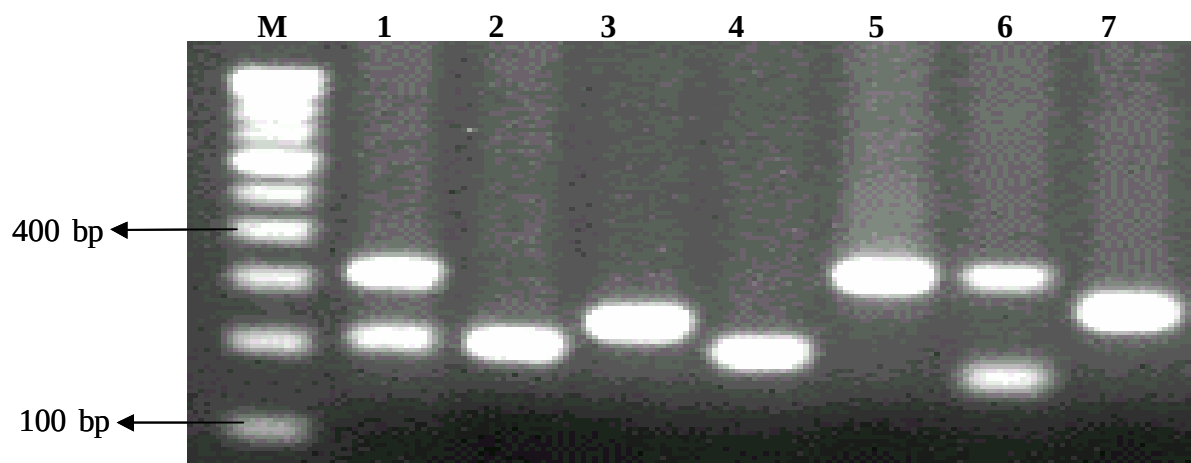
Lane3: Adapter ligated DNA

Fig: 4. Microsatellite capture PCR for (CA)₁₅



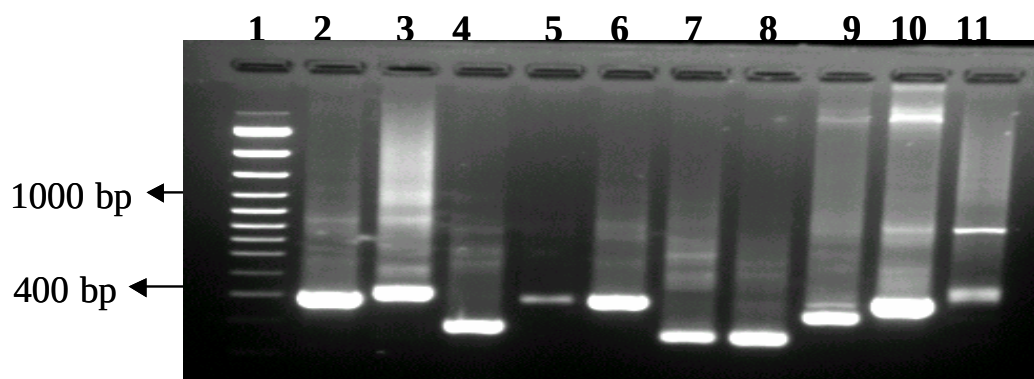
Lane1: (CA)₁₅
Lane2: Negative
Lane3: 1 kb DNA

Fig: 5. Screening PCR of recombinants



Lane M: 100 bp DNA ladder
Lane 1 – 7: PCR screening of clones
Lane 1 & 6 indicate double bands.

Fig: 6. M13 PCR based microsatellite size determination



Lane 1: 1kb DNA Ladder (Gene Ruler TM)

Lane 2: A3

Lane 3: A8

Lane 4: A10

Lane 5: A11

Lane 6: A12

Lane 7: A14

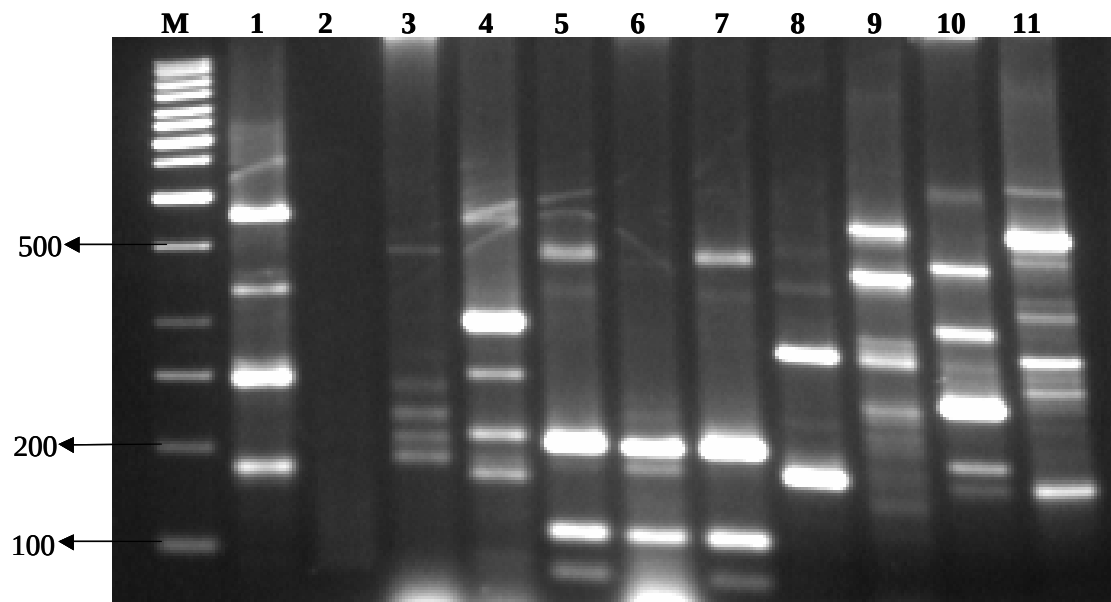
Lane 8: A15

Lane 9: A17

Lane 10: A27

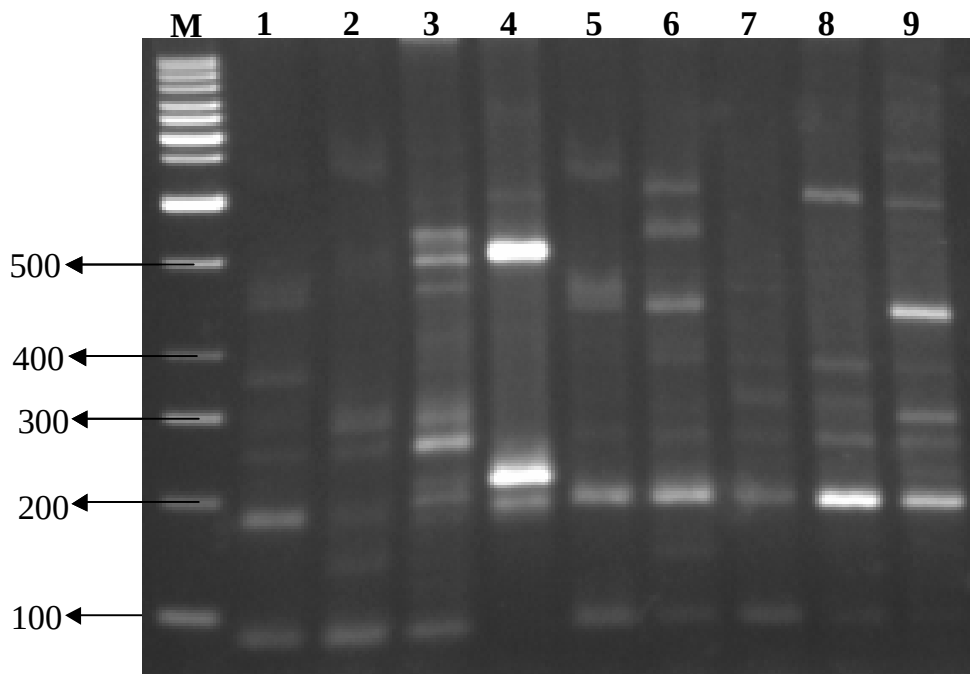
Lane 11: A30

Fig: 8. PCR amplification pattern of microsatellite locus MRMA 27



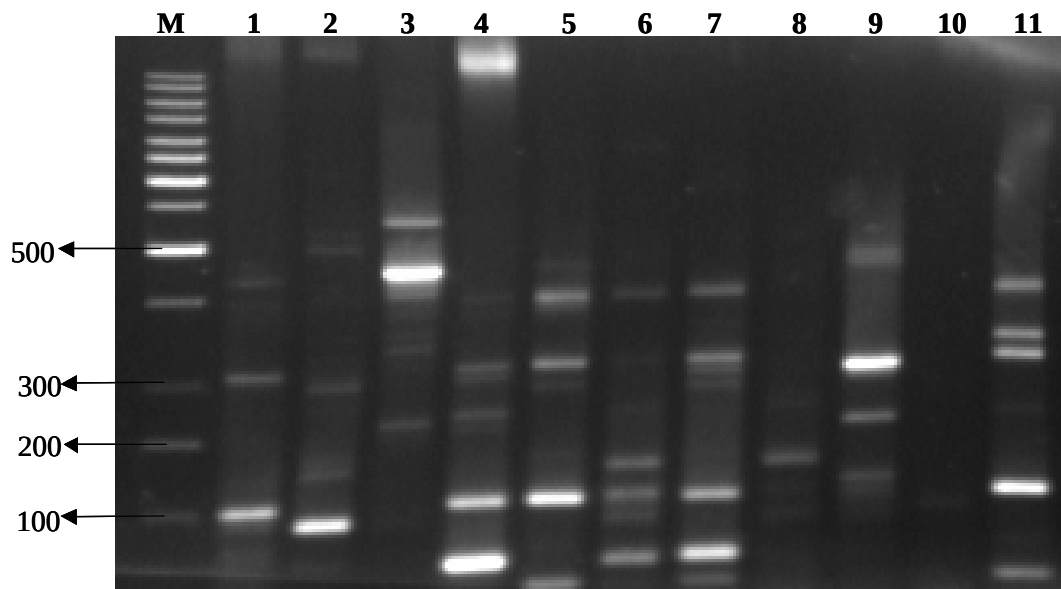
Lane M: 100 bp DNA ladder
Lane 1-11: Individual *M. rosenbergii* samples
Lane 2: Non-amplified sample

Fig: 9. PCR amplification pattern of microsatellite locus MRMB11



Lane M: 100 bp DNA ladder
Lane 1-9: Individual sample amplification

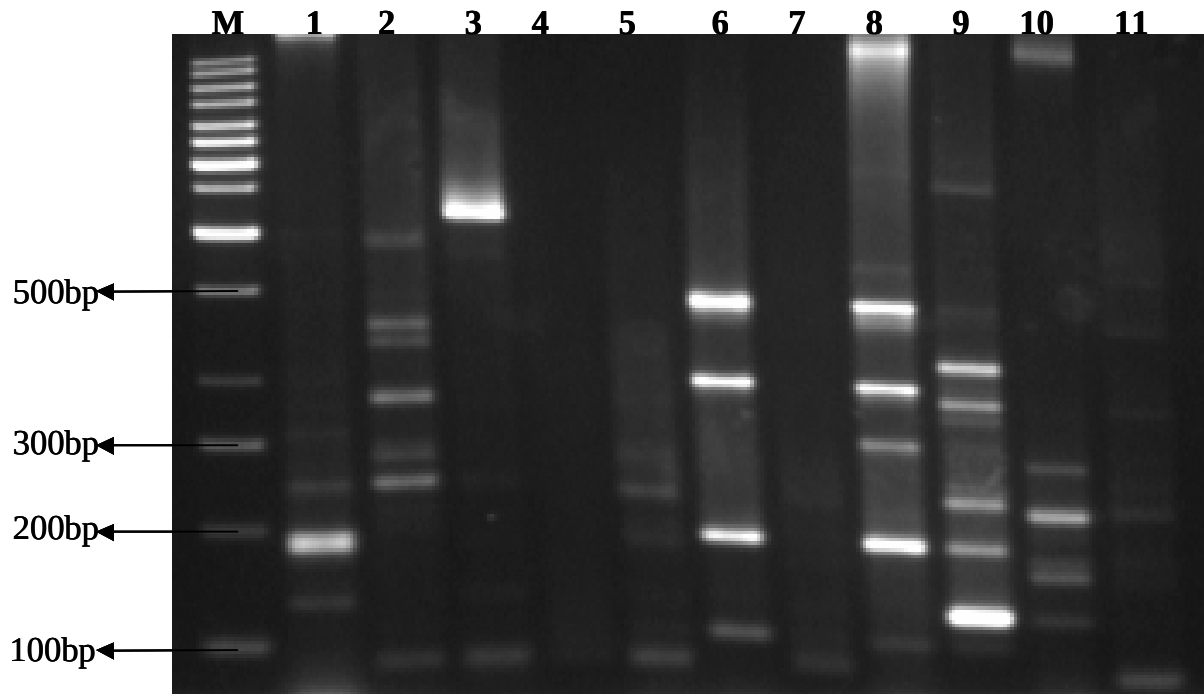
Fig: 10. PCR amplification pattern of the microsatellite locus MRMB10



Lane M: 100 bp DNA ladder

Lane 1-11: Individual sample amplification pattern

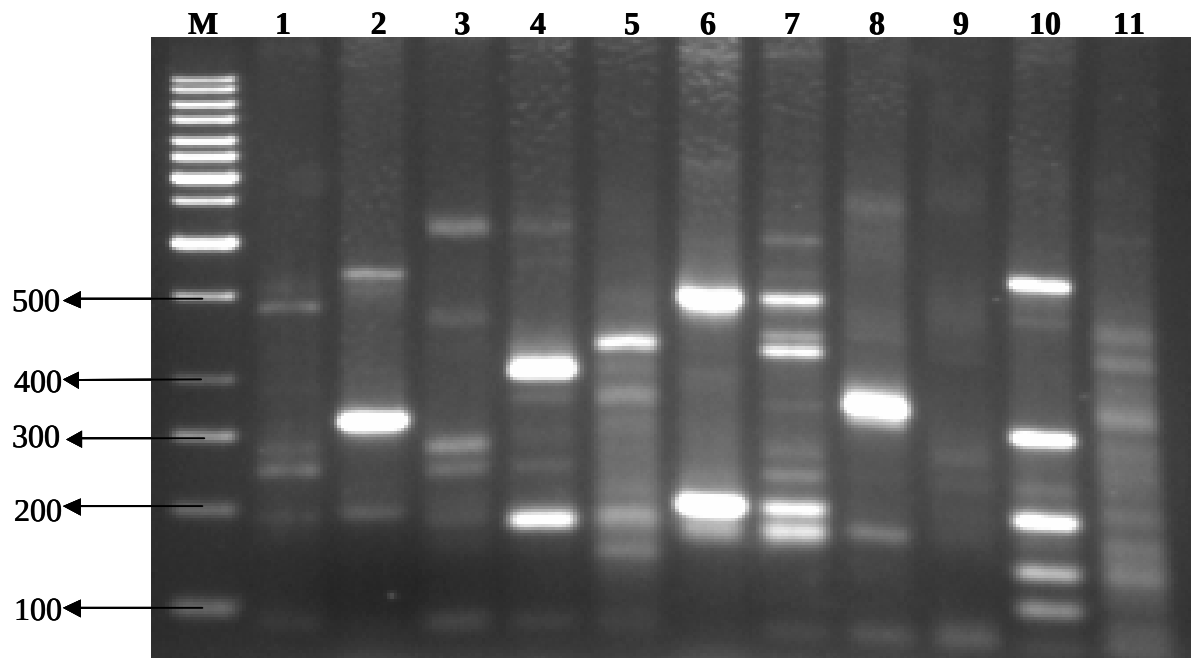
Fig:11. PCR amplification pattern of microsatellite locus MRMB7



Lane M: 100 bp DNA ladder

Lane 1-11: Individual sample PCR amplification pattern

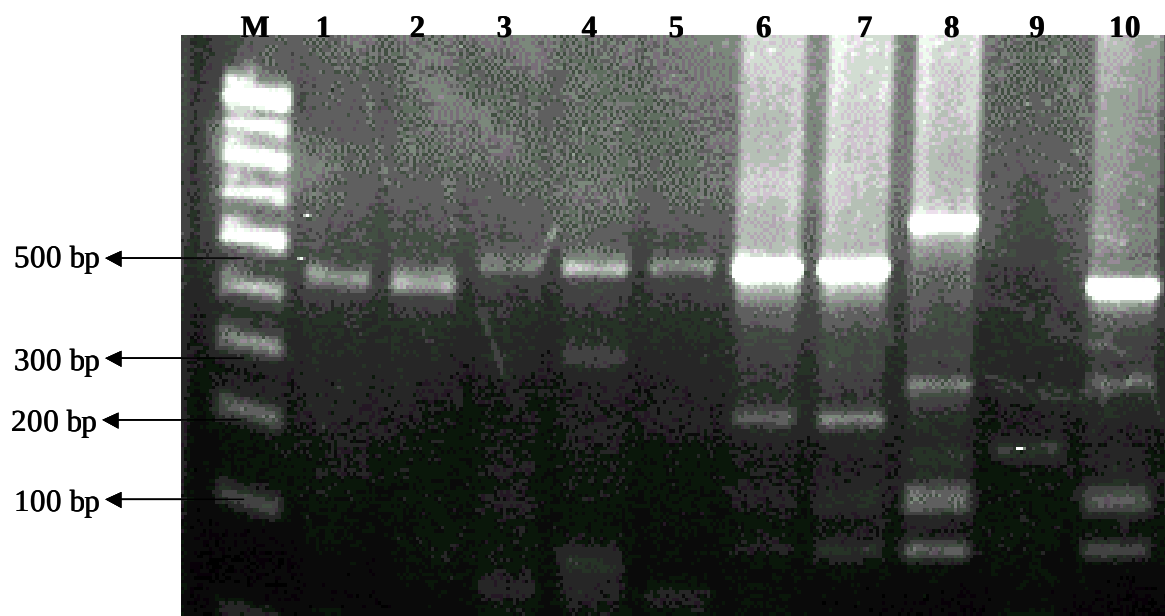
Fig:12. PCR amplification pattern of microsatellite locus MRMB11



LaneM : 100bp DNA ladder

Lane 1- 11: Individual *M. rosenbergii* samples

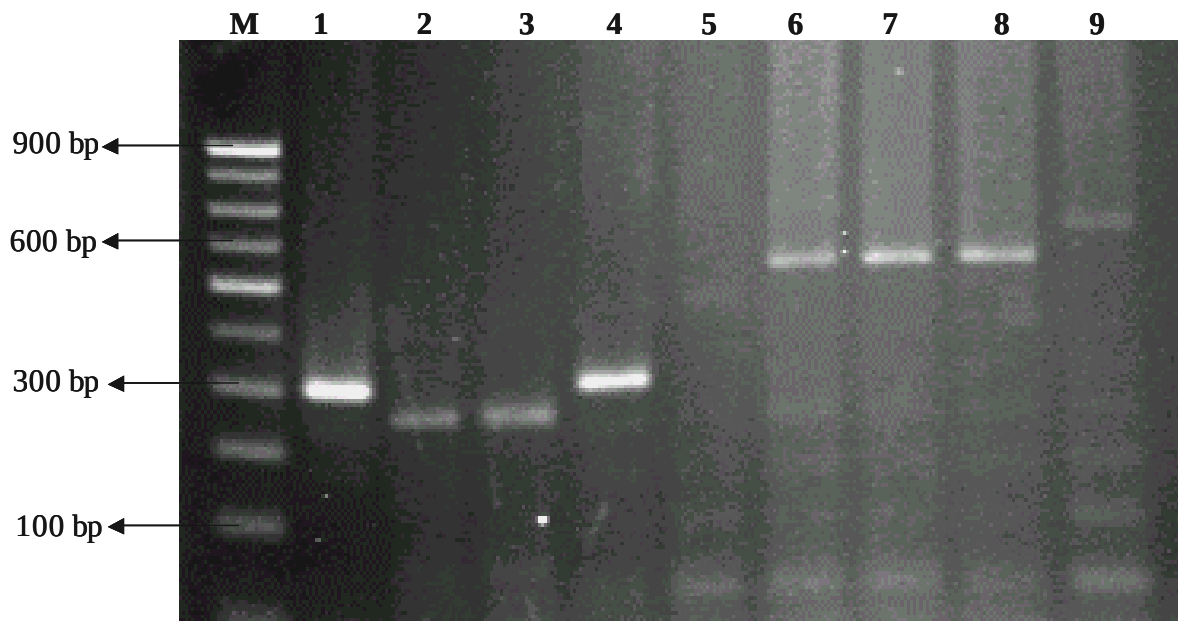
Fig: 13 . PCR amplification pattern of microsatellite locus MRMB15



Lane M: 100 bp DNA ladder

Lane 1-10: Individual *M. rosenbergii* samples

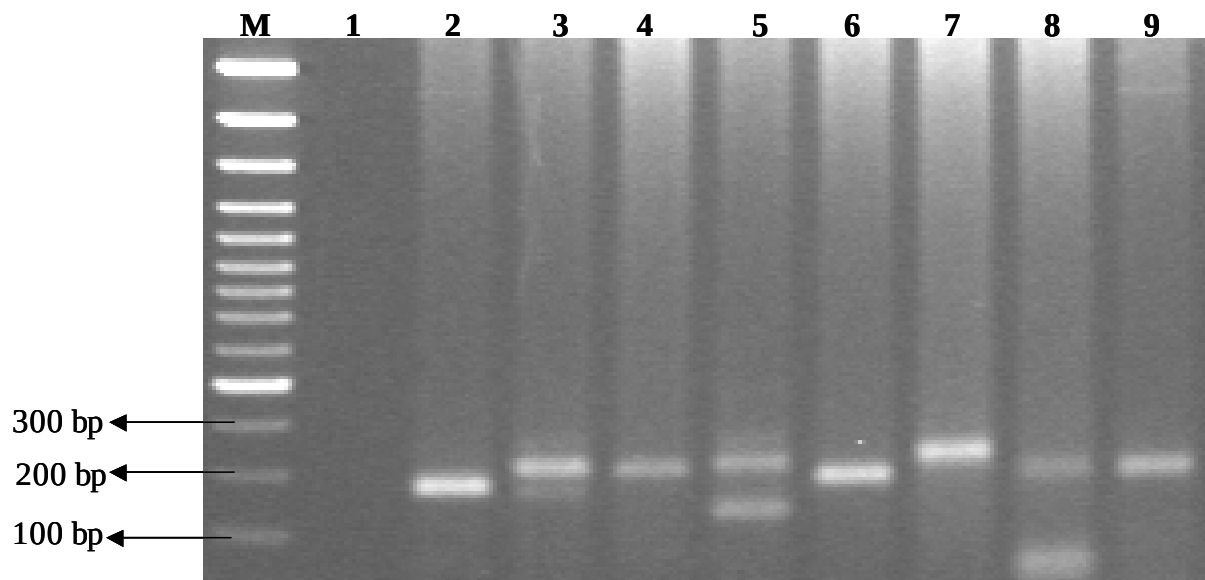
Fig: 14 . PCR amplification pattern of microsatellite locus MRMB16



Lane M: 100 bp DNA ladder

Lane 1-9: Individual *M. rosenbergii* samples

Fig: 15 . PCR amplification pattern of microsatellite locus MRMB25

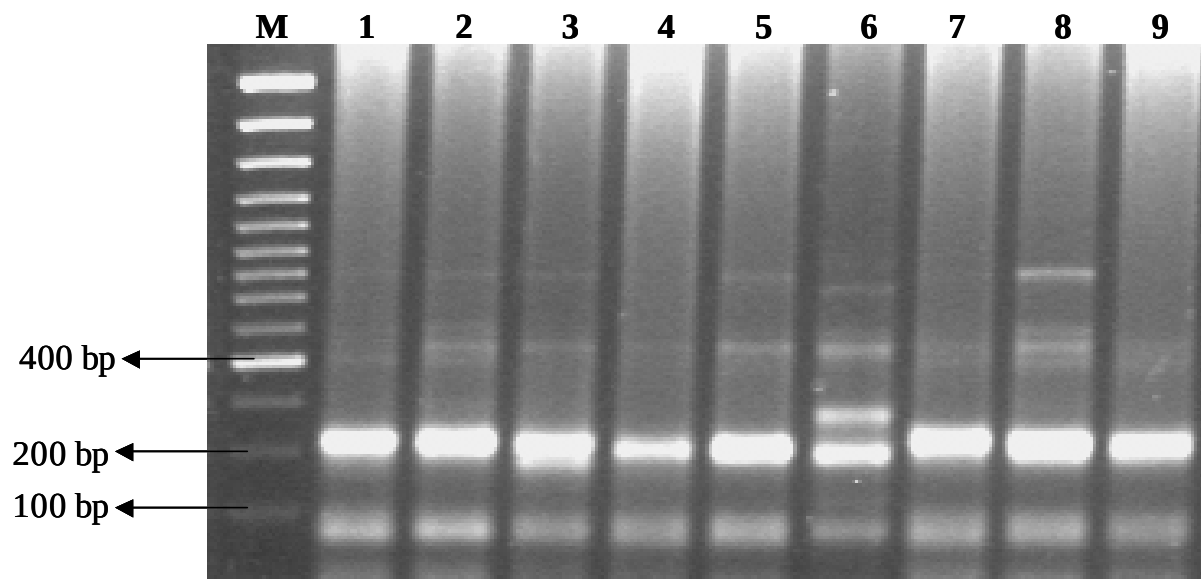


Lane M: 100 bp Plus DNA ladder

Lane 1: Negative control

Lane 2-9: Individual *M. rosenbergii* samples

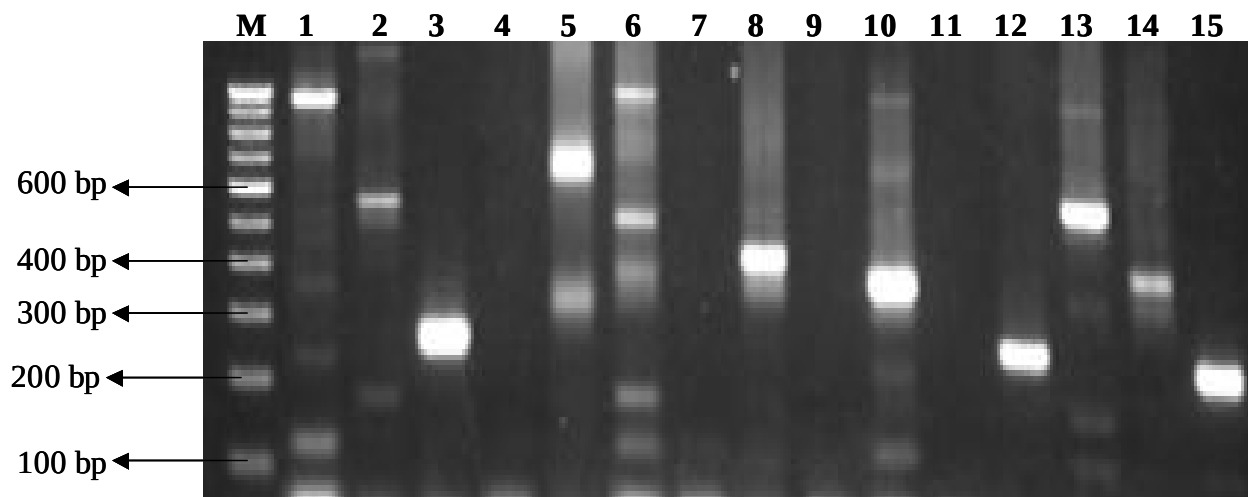
Fig: 16. PCR amplification pattern of microsatellite locus MRMB24



Lane M: 100 bp Plus DNA ladder

Lane 1-9: Individual *M. rosenbergii* samples

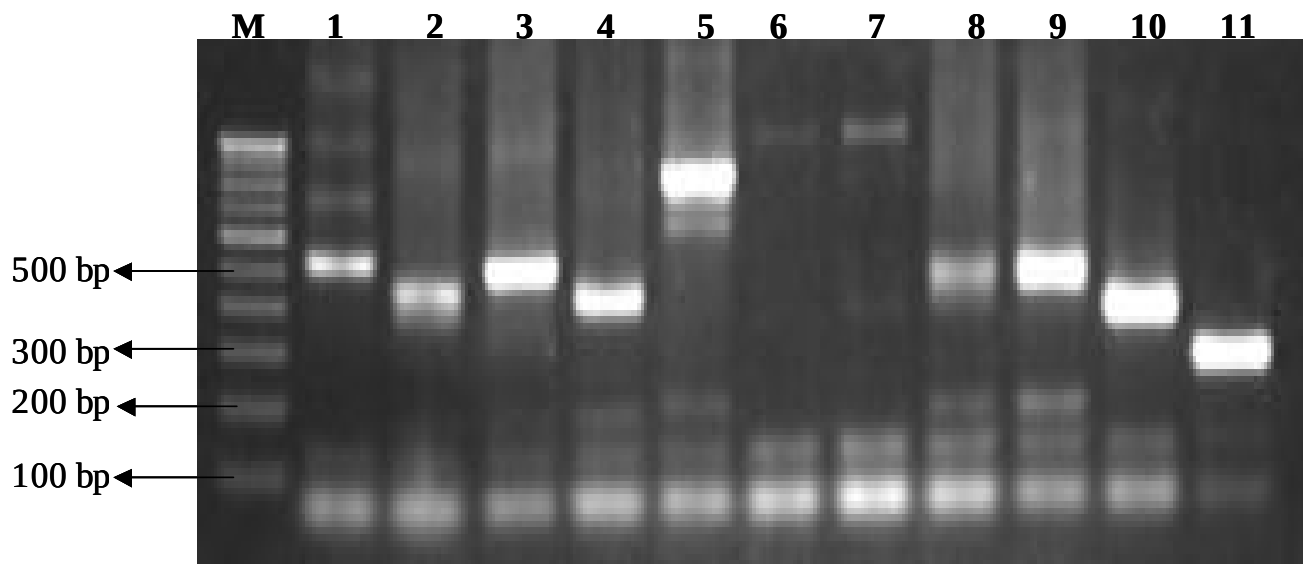
Fig:17 . PCR amplification pattern of microsatellite locus MRMB26



Lane M: 100 bp DNA ladder

Lane 1-15: Individual samples of *M. rosenbergii*

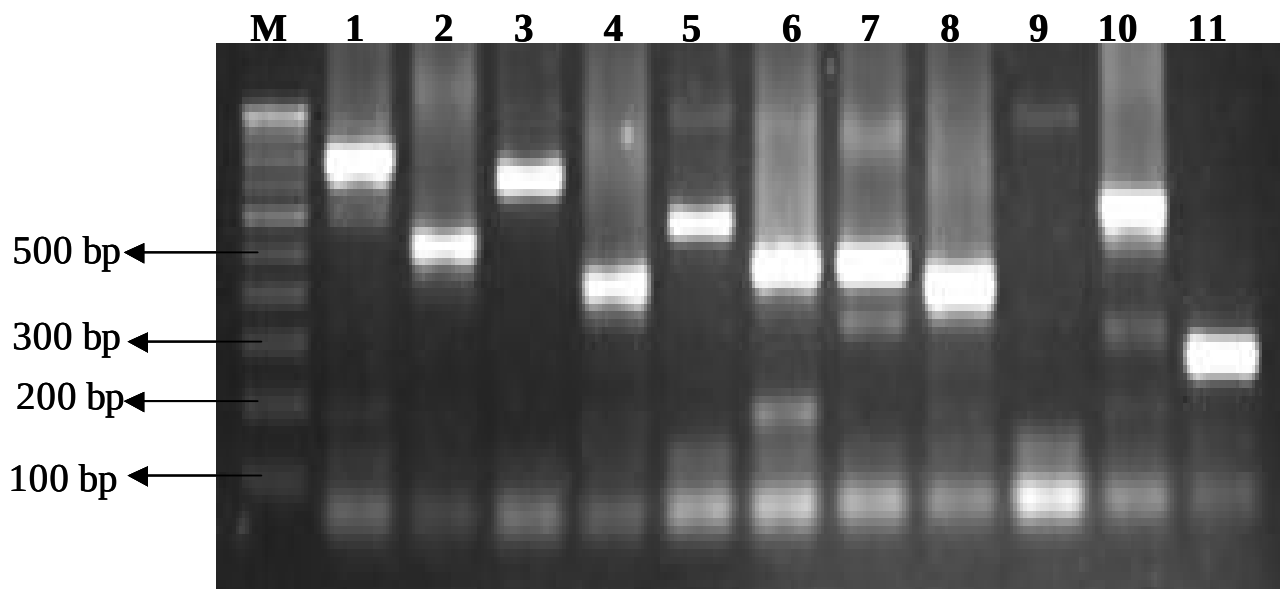
Fig. 18. PCR amplification pattern of microsatellite locus MRMB13



Lane M: 100 bp DNA ladder

Lane 1-11: Individual *M. rosenbergii* samples

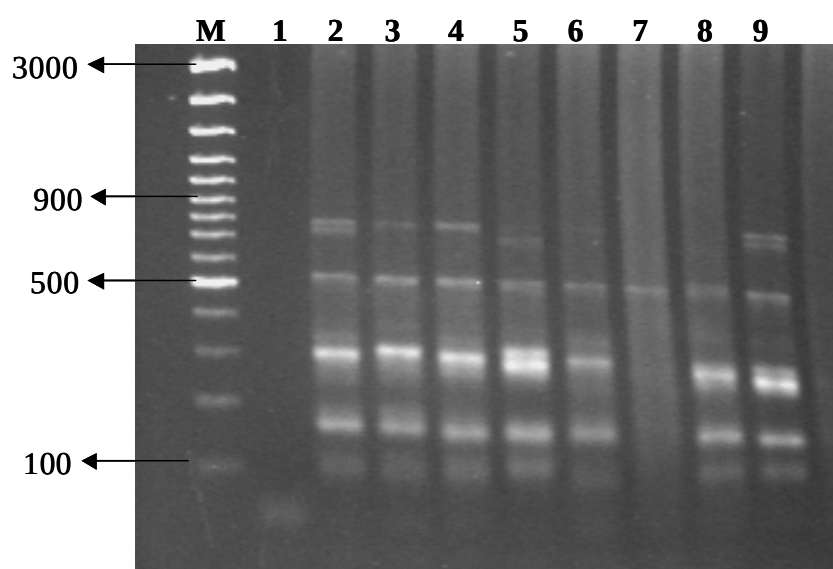
Fig. 19. PCR amplification pattern of microsatellite locus MRMC2



Lane M: 100 bp DNA ladder

Lane 1-11: Individual samples of *M. rosenbergii*

Fig: 7. PCR amplification pattern of microsatellite locus MRMA8



Lane M: 100bp plus DNA ladder

Lane 1 – 9: Individual samples