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 Chareontawee 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 monodon (>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 heterosygosity excess compared to that based on the observed number of alleles. For Kerala population of M. rosenbergii the mean observed heteroszygosity (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 possesses higher genetic variability since they contains 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. This 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 asymptotic carriers (Otta et al., 1999; Nadala et al., 2000), which harbour latent virus.