DISCUSSION

Technological advances in molecular biology and biochemistry have led to the development of a variety of genetic markers that can be used to address questions of relevance to the management and conservation of fish species. Genetic markers have been applied to three fisheries areas in particular - stock structure analysis, aquaculture and taxonomy/systematics (Ward and Grewe, 1994) - with varying degrees of success (Carvalho and Hauser, 1994). The detection of genetic variation among individuals is a requirement in all applications of genetic markers. Some applications will also require the partitioning of variation among groups of individuals (i.e., groups having different alleles or haplotype frequencies). Although some applications will place greater emphasis on genetic differences among groups (stock structure) (Carvalho and Hauser, 1994) and some will focus on differences among individuals within population (pedigree analysis), the detection of polymorphism remains the key. The most common use of genetic markers in fishery biology is to determine if samples from culture facilities or natural populations are genetically differentiated from each other (Ferguson and Danzmann, 1998). The detection of stock differentiation would imply that the source groups comprise different stocks (Carvalho and Hauser, 1994) and should be treated as separate management units (Moritz, 1994). In general, the objectives of the electrophoretic analysis of proteins and enzymes in different commercially important fish and shellfishes are to answer the basic fisheries management related questions such as (1) what is the level of the genetic variation in the species and its different populations? (2) whether the allelic frequencies in the sample populations are similar or different? (3) whether the observed or expected genotype frequencies are in Hardy-Weinberg equilibrium? and (4) if the populations are genetically homogenous or heterogeneous, then what are the implications of the findings with reference to their exploitation and conservation?
In the present study, the genetic characteristics of *Horabagrus brachysoma*, a catfish endemic to the Western Ghat region of Kerala and Karnataka were analysed for discriminating the natural populations by applying modern techniques viz., 1) electrophoretic analysis of tissue allozymes, 2) analysis of patterns of random amplified polymorphic DNA (RAPD) and 3) microsatellites using the primers developed from related species. The significance of the results of the study produced by these three independent methods is discussed below.

5.1. Allozymes

In population genetic studies based on interpretation of electrophoretically detectable banding patterns, the results and their logical conclusion depend upon the accuracy with which the observed banding patterns are interpreted. For this, repeatability and sharpness of bands are essential. In the present study, 14 allozymes (25 loci) gave sharp zones of enzyme activity with the conditions adopted, enabling proper interpretation of results thus discriminating 3 geographically isolated populations of *H. brachysoma*.

5.1.1. Polymorphic allozyme markers

In *H. brachysoma*, out of the 14 enzymes extensively studied, 12 enzymes were polymorphic (14 polymorphic loci) and they were used for the population genetic analysis of the species. Genetic variability has been quantified in populations and species of many freshwater teleosts, based on electrophoretically detectable polymorphic allozyme genes. Rognon *et al.* (1998) reported 16 enzyme systems out of which 13 polymorphic (23 loci) in *Clarias gariepinus*, *C. anguillaris* and *C. albopunctatus* to score both intra and inter-specific differences; while Agnese *et al.* (1997) studied 13 polymorphic loci comparing *Clarias gariepinus* and *C. anguillaris*. In different species of pangasiid catfish, Pouyaud *et al.* (2000) studied the 16 allozymes having 25 polymorphic loci from South-East Asia in *Pangasius* and *Helicophagus* species. Suzuki and Phan (1990a,b) used 10 enzymatic systems in 6 species of marine catfishes (Family: Ariidae) to study intra-specific variations and inter-specific relationships and they reported that six out of 17 loci were polymorphic.
In the population genetic analysis of *Barbus callensis*, Berrebi *et al.* (1995) reported 10 polymorphic allozyme markers and a polymorphic general protein. In *Cobitis* *spp*, Perdices *et al.* (1995) reported variations in 15 allozymes. In *Tenualosa ilisha*, Salini *et al.* (2004) used 3 polymorphic enzymes (5 loci) to detect genetic variation in Bangladesh populations. Lal *et al.* (2004a) reported polymorphism in 13 out of 26 scorable loci in *T. ilisha* population in River Ganges. Peres *et al.* (2002) studied 14 enzymatic systems out of which eight loci were polymorphic in *Hoplias malabaricus* in the upper Parana River Floodplain in Brazil. Appleyard and Mather (2002) reported 25 polymorphic allozyme loci out of 50, helpful to screen differences in two stocks of *Oreochromis niloticus*; red hybrid tilapia and *O. mossambicus*. Menezes (1993) reported 19 loci from 10 allozymes in oil sardine, *Sardinella longiceps* from the Western coast of India, but no polymorphic locus was detected by the 95% criterion. In Indian mackerel, *Rastrelliger kanagurta*, Menezes *et al.* (1993) reported only 3 polymorphic loci among the 11 loci studied from the coastal waters of Peninsular India and the Andaman Sea and suggested the number of polymorphic allozyme markers is generally less in marine finfish compared to that of freshwater species.

Migration, egg and larval dispersal through current and lack of population subdivision can be the reasons for the lack of genetic differentiation among the populations in marine teleosts (Grand *et al.*, 1987; Menezes *et al.*, 1993).

In all the above examples and in the present study, several polymorphic allozymes were common *viz*, AAT, EST, GLDH, G3PDH, GPI, LDH, PGM, SOD etc indicating their usefulness in delineating intra-specific differences. GAPDH, MDH, MEP, ODH, XDH etc are rarely used in stock structure studies in catfishes. In *H. brachysoma*, these allozymes were found to be helpful in estimating the degree of divergence. Unlike in human beings (Richardson *et al.*, 1986) G3PDH pattern did not exhibit sex-linked inheritance in *H. brachysoma*. Both male and female specimens exhibited homozygote and heterozygote patterns for this enzyme. However, the chromosomal mechanism of sex determination is yet to be studied in this species.
5.1.2. Amount of genetic variability and Hardy-Weinberg Equilibrium

The measurement of natural genetic variability is the first step in the study of population genetics, especially in the differentiation of genetically discrete stocks. The estimated values for average observed number of alleles (na), effective number of alleles (ne), percentage of polymorphic loci and above all, average heterozygosity (H) for the populations of a species are considered as indicators of the actual level of genetic variability in that species. Statistically significant differences in these values, particularly in the heterozygosities and allele frequencies between any two populations of the species are evidences of their reproductive isolation (unless they are not sympatric), in other words, the two populations belong to genetically different stocks which do not interbreed (Allendorf et al., 1987; Ayala and Keiger, 1980; Bye, 1983; Altukov, 1981).

Genetic diversity expressed in terms of mean of observed number of alleles (Na), is usually higher in species with wider geographic ranges, higher fecundity, greater longevity and larger population sizes (Nevo et al., 1984). The mean value of Na in H. brachysoma (2.3571) collected from 3 geographically distinct places exceeds that of many freshwater species like Tenualosa ilisha (Na = 1.49, Lal et al., 2004a) and Cirrhinus mrigala (Na = 1.31, Singh et al., 2004), but is comparable with those reported for 4 species of marine catfishes (family: Ariidae) from (Suzuki and Phan, 1990b) and coconut crab (Birgus latro) from the Vanuatu Archipelago in the Pacific Ocean (Lavery and Fieldder, 1993). Slightly lower values of Na were reported in other catfish species like Clarias gariepinus, C. anguillaris and C. albopunctatus (Rognon et al., 1998) and in pangasiid catfishes (Pouyaud et al., 2000). Appleyard and Mather (2002) also reported a lower value of Na for Oreochromis niloticus, O. mossambicus and their red hybrid (1.3475, 1.305 and 1.1665 respectively).

The mean value of polymorphic loci (P0.95) across populations was 0.56 (56%) in H. brachysoma. The value is greater than those in other catfishes like Clarias gariepinus (P0.95 = 48%) and C. anguillaris (P0.95 = 28%, Rognon et al. (1998), but lower than that of Pangasius species (P0.95 = 100%), reported by Pouyaud et al. (2000). In Oreochromis niloticus, O. mossambicus and the red hybrid of both species,
Appleyard and Mather, (2002) obtained 50% of the polymorphic loci with the criterion P0.05 which is comparable with that of *H. brachysoma*. The values of polymorphic loci exhibit a wide range, from 8-48% found in *Cobitis calderoni* and *C. marocean* (Berrebi *et al.*, 1995); 27% in Pacific herring (Grand and Utter, 1984); 50% in *Cyprinus carpio* (Kohlmann and Kersten, 1999); 28% in *Alphanius fuscatus* (Maltagliati, 1998); 37.5% in *Hoplias malabaricus* (Peres *et al.*, 2002) and 100% in *Tenualosa ilisha* (Salini *et al.*, 2004). In some marine species, lower values of polymorphic loci was reported (Menezes *et al.*, 1993; 1994; Begg *et al.*, 1998).

The best estimate of genetic variation in natural population is the mean observed heterozygosity (Hob) per locus (Allendorf and Utter, 1979). The values of Hob vary non-randomly between loci, populations and species. To avoid serious error in the estimation of Hob, a large number and wide range of allozyme loci should be examined (Allendorf and Utter, 1979). On the basis of 14 polymorphic loci, the mean observed heterozygosity (Hobs) per locus was 0.1724 for Meenachil, 0.1908 for Chalakkudy and 0.1704 for Nethravathi and the mean value for overall population was 0.1779. The Hobs value falls within the range reported for other catfishes like *Clarias gariepinus*, *C. anguillaris*, *C. albopunctatus* and *Heterobranchus longifilis* (Rognon *et al.*, 1998) and that of many *Pangasius* species, (Pouyaud *et al.*, 2000) and many other freshwater and marine species. Lower value of Hobs was reported by many authors in freshwater fishes (Berrebi *et al.*, 1995; Grand and Utter, 1984; Kohlmann and Kersten, 1999; Lal *et al.*, 2004a; Singh *et al.*, 2004; Salini *et al.*, 2004; Maltagliati, 1998; Penner *et al.*, 2002; Menezes *et al.*, 1993; Menezes, 1994; Begg *et al.*, 1998) and tiger prawn (*Penaeus monodon*) (Benzie *et al.*, 1992; 1993; Sugama *et al.*, 2002).

The observed heterozygosity (Hobs) values obtained in the present study in *H. brachysoma* are lower than that of the expected values (Hexp), indicating the deficiency of heterozygotes except in one or two loci in each population. All the loci except *G3PDH*, *G6PDH* and *SOD* in Meenachil; *EST-3* in Chalakkudy and *EST-2* and *PGM* in Nethravathi populations deviated significantly from Hardy-Weinberg equilibrium (HWE) after Bonferonni correction was applied. The Fis (inbreeding coefficient) figures were found to deviate significantly from zero in several loci in all
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three populations. Generally, where the loci did not conform to HW expectations a significant lack of heterozygotes was observed as evidenced from the positive $F_{is}$ values (Table-12).

Under Hardy-Weinberg Equilibrium allele frequencies are stable from one generation to the next. Deviations from the frequencies expected under HWE provide evidence that the assumptions of HWE are violated in natural populations of *H. brachysoma*. This could be due to non-random mating or effect of other evolutionary forces like selection/migration etc or reduction in effective breeding population. Mixing of non-native genetic stocks can also be one reason. *H. brachysoma* fetches a high price as ornamental and food fish and there has been a massive hunt for the species for aquarium trade over the last few years and its drastic decline in rivers was recorded in 1997 itself in the CAMP workshop (Anon, 1998) leading to it bearing an 'endangered' status as per latest IUCN categorization. Deficiency of heterozygotes and deviations from HWE in yellow catfish hence can be due to inbreeding, a situation caused by over-exploitation leading to decline of species in the wild. Other factors responsible for significant deviation from HW model may not hold true for *H. brachysoma* as samples were collected from geographically isolated river systems (minimum distance between Meenachil and Chalakkudy Rivers 140 KM and between Chalakkudy and Nethravathi 380 KM; these rivers flow westwards almost parallel, having no inter-connecting channels and open directly to the Arabian sea or Vembanad backwaters). Ranching and restocking of rivers with seeds of *H. brachysoma* has not been attempted so far, hence mixing of non-native genetic stocks can also be ruled out. Similar situation was reported in pearl oyster (Sapna, 1998), brown trout (Colihueque *et al.*, 2003) and coconut crab (Lavery and Fielder, 1993; Lavery *et al.*, 1996). Further analysis of the data using software such as "Bottleneck" only can determine whether populations of *H. brachysoma* has undergone any demographic bottleneck in recent times.

Appleyard and Mather (2002) attributed the lack of heterozygotes at some allozyme loci due to scoring difficulties especially at EST-1* (liver) and MEP* in tilapia, with cellulose acetate gel electrophoresis (CAGE). They reported that scoring of these two loci and aldehyde dehydrogenase (*ALDH-2* ) was difficult as allozyme
products of these loci exhibited complex and uninterpretable variations. However, in
the present study, using polyacrylamide electrophoresis (PAGE), the bands obtained
were always sharp (ALDH not tried) and scoring difficulties were not encountered.

No allozyme loci showed linkage disequilibrium (after Bonferroni correction)
in any of the 3 populations of *H. brachysoma*. It is therefore assumed that allelic
variation at allozyme loci could be independent as observed in many species of fishes
(Rognon *et al.*, 1998; Sapna, 1998; Pouyaud *et al.*, 2000; Cook *et al.*, 2000;
McGlashan and Hughes, 2000; Rebello, 2002; Appleyard and Mather 2002).

5.1.3. Private alleles

A locus at which complete differentiation exists between two populations can
be used to diagnose the population to which an individual belongs (Ayala, 1983).
Seven private alleles were obtained in two populations of *H. brachysoma*, i.e.,
Chalakkudy and Nethravathi, but in Meenachil population, there was no private allele
(Table-11). Many authors showed that the private alleles can be used to distinguish
stocks or to discriminate species. Agnese *et al.* (1997) reported 14 private alleles in 13
polymorphic allozyme loci in *C. gariepinus* and *C. anguillaris*. Rognon *et al.* (1998)
showed that private alleles were helpful in distinguishing clariid catfishes, *C.
gariepinus*, *C. anguillaris*, *C. albopunctatus* and *Heterobranchus logzfilis*. Pouyaud *et al.*
(2000) distinguished pangasiid species with 42 private alleles in 16 polymorphic
loci. Peres *et al.* (2002) reported the two private alleles, one in *GpDH-1* specific to
Parana River population and the other in *MDH-A₂* specific to the lagoon population in
*Hoplias malabaricus*. Salini *et al.* (2004) reported two private alleles (second allele of
*LDH-m* in the 8th population and third allele of *MDH-I* in the 5th population) in
*Tenualosa ilisha* in the Bangladesh region. The occurrence of 7 private alleles in 210
individuals of *H. brachysoma* as reported in the above mentioned species indicates
physical isolation and genetic differentiation and usefulness of these alleles in
identifying distinct populations of the species.
5.1.4. Population genetic structure and gene flow

Pair-wise comparisons between different riverine locations for allelic homogeneity in *H. brachysoma* yielded significant deviations at all loci in their frequencies after significant levels are adjusted for Bonferroni correction. This suggests partitioning of the breeding population, limitation in migration between different areas and existence of distinct stock structure among populations. The overall value (0.1537) of the coefficient of genetic differentiation ($F_{ST}$) among samples indicates that there is strong genetic differentiation into local populations in the species (Table-13). There was considerable heterogeneity between loci, with estimates of $F_{ST}$ ranging from 0.0042 to 0.7815 due to population differences. Similar values for $F_{ST}$ were reported in populations of *Clarias anguillaris* ($F_{ST} = 0.15$) by Rognon *et al.* (1998). The same authors also reported a lower $F_{ST}$ value (= 0.044) for populations of *Clarias gariepinus*. Peres *et al.* (2002) reported somewhat similar value that of *H. brachysoma* in *Hoplias malabaricus* ($F_{ST} = 0.140$); Suzuki and Phan (1990) in populations of four marine catfishes species from Brazil and Sapna (1998) populations of the pearl oyster, *Pinctada fucata* from India. In hilsa, *Tenualosa ilisha*, Salini *et al.* (2004) reported very low value of overall $F_{ST}$ (0.002) due to high rate of migration in the Bangladesh region. Appleyard and Mather (2002) reported high $F_{ST}$ values (0.501 to 0.598) in two species of *Oreochromis* (*O. niloticus* and *O. mossambicus*) indicating there was little evidence of introgression between species. A very high $F_{ST}$ value (0.814) was reported by Perdices *et al.* (1999) in the populations on the genus *Cobitis*. Coelho *et al.* (1995) reported the range of $F_{ST}$ values of 0.044 to 0.863 in *Leuciscus pyrenaicus* and *L. carolitertii*. Genetic relatedness of *H. brachysoma* populations derived using pair-wise $F_{ST}$ between populations differed significantly ($P < 0.0001$) from zero for all pairs of riverine locations indicating significant heterogeneity between populations. In the present study, the overall and pair-wise $F_{ST}$ values fell within the range reported for freshwater fishes. Overall and pair-wise $F_{ST}$ values indicated significant and large levels of genetic differentiation among populations of an Australian freshwater fish (*Craterocephalus stercusmuscarum*) using allozyme markers (McGlashan and Hughes, 2000) and stocks of freshwater prawn, *Macrobrachium australiense* between river catchments, Australia (Cook *et al.*, 2000).
The value of Nm (the average number of migrants per generation) derived from $F_{ST}$ based on the overall estimate of geneflow between populations was small in this study. The Nm value of 1.3760 indicates chances of restricted migration between populations and that genetic differentiation over time might have occurred due to genetic drift. Nm $>$ 4 suggests that the gene flow between the populations is adequate to counteract the effects of genetic drift in local populations (Kang and Chung, 1997). Many authors have reported a higher value of Nm in various fish and shellfish species showing no genetic differentiation of stocks (Verspoor et al., 1991; Benzie et al., 1982; Buonaccorgi et al., 1999). However, in species exhibiting significant genetic differences among populations, low values of Nm have been reported (in *Alphanicus fasciatus*, $N_{m} = 0.578$ (Mattagliati, 1998); and *Tor malabaricus* $N_{m} = 0.574$ (Silas et al., 2004)) as evident in the present study.

5.1.5. Genetic distance values

The genetic relationship among populations in *H. brachysoma* was determined using allozyme analysis. The genetic distance values ranged from 0.0299 to 0.0927, and the values were close to the average obtained by Shaklee et al. (1982) for conspecific populations of marine and freshwater fish ($D = 0.05$ and $I = 0.977$). In clariid catfishes, Nei's genetic distance at intraspecific level ranged from 0.008 to 0.29 in *Clarias gariepinus*; and 0.005 to 0.043 in *C. anguillaris* (Rognon et al., 1998). The mean genetic distance between the species was 0.207 $\pm$ 0.081 (Rognon et al., 1998).

Berrebi et al. (1995) reported a genetic distance ($D$) value of 0.379 between Moroccan and Tunisian populations of *Barbus barbus*. In 1984, Grand and Utter reported the average intra-populational genetic distance value of 0.039 in Pacific Herring (*Clupea pallasi*). Benzie et al. (1992) reported a very low value of genetic distance in populations of *Penaeus monodon* in Australia and it ranged 0.000 to 0.015. Based on the genetic distance, a UPGMA dendrogram was constructed for the *H. brachysoma* that showed 3 populations as 3 distinct groups with the Nethravathi stock farther from Chalakkudy and Meenachil groups.
In conclusion, the allozyme studies alone provide positive proof for the existence of genetically different stocks of *H. brachysoma* in the 3 rivers along the Western Ghats. Occurrence of distinct stocks of yellow catfish can be interpreted in two ways: 1) lack of gene flow between populations as a result of geographic isolation so that forces such as random genetic drift had operated to cause genetic divergence and 2) local genetic adaptations to different environmental conditions.

Piel and Nutt (2000) suggested that allozymes are not useful markers for population genetics, mainly because of low polymorphism levels, that decreased the ability to detect population structure and differentiation. Bye and Ponniah (1983) opined, as the allele frequencies involved only the conserved structured proteins that comprise approximately 1% of the total genome of an individual, allozymes were not always ideal to screen genetic divergence at intra-specific level. Allendorf *et al.* (1987) and Cagigas *et al.* (1999) pointed out, given the requirement of neutrality for a genetic marker, proving that any allozyme marker may not be affected by selective effects seems to be largely difficult and other markers such as mt DNA and microsatellites are better for population genetic studies. Ayala and Keiger (1980) opined that the success of detection of naturally existing discrete stocks of organisms using allozymes may depend on the screening of large number of loci so as to discover few loci that are polymorphic and heterogenic with reference to allele frequencies that can serve as potential genetic markers for genetic stock differentiation. However, there are many reports of significant stock differences detected using only allozymes in fishes and shellfishes (Utter, 1969; Ihsen *et al.*, 1981; Altukhov, 1981; Lester and Pante, 1992) and several papers on fish showing same pattern of genetic divergence when allozymes are used along with other genetic markers such as mtDNA, microsatellites RAPD and single copy nuclear DNA even though genetic variation within samples was lower for allozymes than for other molecular markers (McDonald *et al.*, 1996; Cagigas *et al*; 1999; Buonaccorsi *et al.*, 1999; McGlashan and Hughes, 2000; Cook *et al.*, 2000; Appleyard and Mather, 2002; Colihueque *et al.*, 2003). In the present study also, the pattern of genetic variability and divergence recorded within and between populations of yellow catfish using allozymes were same as that of RAPD and microsatellites. The broad overlap of
divergence levels from allozyme and molecular markers (RAPD and microsatellites) in this study suggests that all 3 sets of allelic frequency distributions represent neutral markers in *H. brachysoma*, as reported in above mentioned studies. Therefore, the present work on yellow catfish shows that the analysis of allozymes can still be an effective tool to evaluate genetic differentiation in fish, as long as proper screening methods are applied and sufficient number of polymorphic and heterogenic loci are used.

5.2. Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA (Williams *et al.*, 1990; Welsh and McClelland, 1990) is one of the common genetic marker, used for population genetic analysis, pedigree analysis and taxonomic discrimination of the species (Bardakci and Skibinski, 1994; Jayasankar and Dharmalingam, 1997; Khoo *et al.*, 2002; Klinbunga *et al.*, 2000a and 2000b; Appleyard and Mather, 2002; Callejas and Ochando, 2001 and 2002). Several authors have demonstrated that the RAPD-PCR method is a powerful tool in the assessment of discriminating differences at inter-population level in a wide range of organisms including fishes (Welsh and McClelland, 1990; Black *et al.*, 1992; Cenis *et al.*, 1993; Bardakci and Skibinski, 1994; Naish *et al.*, 1995). In the present study, RAPD markers were used for population structure analysis of *H. brachysoma* from three river systems.

5.2.1. Reproducibility of RAPD markers

Technical problems associated with application of the RAPD technique in the field of genetic population research have been reported by many authors (Hadrys *et al.*, 1992; Schierwater and Ender, 1993; Lynch and Milligam, 1994; Allegrucci *et al.*, 1995; Naish *et al.*, 1995). A disadvantage of this technique is reproducibility of the results (Liu *et al.*, 1999b; Dinesh *et al.*, 1995). RAPDs can generate unreliable products through PCR or the same pattern will not be obtained again even under identical screening conditions, unless the technique is well standardized. To get the reproducible results for RAPD, the quality and quantity of the template DNA used is a major key factor (Dinesh *et al.*, 1995).
To standardize the experimental conditions Mamuris et al. (1998) used two different DNA extraction methods, two different polymerases and two thermal cyclers. Taq polymerase purchased from different manufacturers produced similar results when applied on DNA from the same individual in the same thermal cycler. On the contrary, within the same laboratory, different polymerases as well as different thermal cycles having different temperature cycling profiles produced rather different banding patterns in all individuals screened. In addition, amplification of DNA obtained by different extraction protocols from the same individual showed slightly different banding patterns, at least after agarose gel electrophoresis (Mamuris et al., 1998). Thus, even if reproducibility of RAPD markers can be obtained in a single laboratory, this seems difficult for different laboratories, unless all conditions are identical. A possible implication of such differences is that qualitative comparisons of data produced by different laboratories, working on the same organism with identical primers would be meaningless, especially when the method is applied to assess specific markers between populations (Mamuris et al., 1998).

In the present study, RAPD analysis was carried out with DNA template extracted from several specimens from three different locations at different times. The DNA polymerase (Taq polymerase), buffer and dNTPs used were from the same source and PCR and electrophoresis were carried out at different intervals. The template DNA quantity (1 μl per single reaction mix) and concentration were kept uniform across samples. This resulted in high level of reproducibility and sharpness of RAPD profiles in *H. brachysoma* as reported by Ferguson et al. (1995) in *Salmo salar* and Ferguson and Danzmann (1998) in various fish species. The present study shows that under identical amplification conditions, RAPD profiles for any particular primer-template DNA concentration is highly reproducible over a wide range of template RAPD, as reported in seven other fish species by Dinesh et al. (1995).
5.2.2. Genetic variability in RAPD analysis

The RAPD method was applied to identify genetic similarity and diversity in yellow catfish *H. brachysoma* using 10 polymorphic Operon primers. The number of fragments generated per primer varied from six to 17. Similar number of fragments was reported in other fish species such as Korean catfish, *Silurus asotus* (Yoon and Kim, 2001) and tilapia (Bardakci and Skibinski, 1994; Appleyard and Mather, 2000). The size of DNA fragments amplified in *H. brachysoma* ranged from 150-3000 bp and this conforms with the range of fragment sizes observed in Korean catfish, *Silurus asotus* (100-1500 bp; Yoon and Kim, 2001); Brown trout, *Salmo trutta* (200-1000 bp; Cagigas *et al.*, 1999); different *Barbus* species (300-2000 bp; Callejas and Ochando, 2001); tilapia (250-2400; Dinesh *et al.*, 1996); seven *Epinephelus* species (100-2000 bp; Govindaraju and Jayasankar, 2004) and common carp, *Cyprinus carpio* (300-2200 bp; Dong and Zhou, 1998).

In *H. brachysoma*, 10 primers generated a total of 124 fragments, producing an average of 12.4 per primer. Among these fragments, 75 (60.48%) were found to be polymorphic as summarized in Table-19. In Meenachil population, a total 42 bands out of 112 amplicons (37.5%) were polymorphic in Chalakkudy 42 out of 109 (38.53%) and in Nethravathi 42 out of 111 (57.84%) were polymorphic. The percentage of polymorphism at intra-population level in *H. brachysoma* was relatively low, but in overall population the percentage was high (60.48%) compared to other species, Yoon and Kim (2001) reported a total of 652 and 692 bands from 5 primers in two populations (Kunsan and Yesan) of Korean catfish, *Silurus asotus* and among these 298 (45.7%) were polymorphic to Kunsan population and 282 (40.8%) were polymorphic to Kunsan population and 282 (40.8%) were polymorphic to Yesan population. Chong *et al.* (2000) reported 42 polymorphic RAPD markers in Malaysian river catfish, *Mystus nemurus*. Liu *et al.* (1998a) reported the production of 462 polymorphic bands, an average of 6.1 bands per primer in the channel catfish, *Ictalurus punctatus* and *I. furcatus*. Appleyard and Mather (2002) reported a total of 95 RAPD loci (13.6 loci/primer), of which, 37 were monomorphic and 58 were polymorphic among the stocks of *Oreochromis niloticus* and *O. mossambicus* and 17.24% for minke whales, *Balaenoptera acutorostrata* by Martinez and Pastene.
(1999). However, Liu et al. (1998a) reported a higher value for percentage polymorphic RAPD loci (61.05%) in the channel catfish, *Ictalurus punctatus* and *I. furcatus*. The higher percentage polymorphism scored with RAPD markers in the present study is probably due to preferential amplification of non-coding repetitive regions of the genome that may elude natural selection (Kazan et al., 1992; Callejas and Ochando, 2002).

The average gene diversity or average heterozygosity (H) in *H. brachysoma* ranged from 0.1053 (Chalakkudy population) to 0.1139 (Nethravathi population), with an average of 0.1097 (Table-20). Genetic polymorphism designated by the values of % P and H had the lowest values in each population in the species which could be due to its small population size and a higher level of inbreeding. The values of H and %P were lower than those reported for Guppy (*Poecilia reticulata*) populations and other fishes (Khoo et al., 2002; Chen, 1999). Many authors had estimated these parameters in a wide variety of organisms using a large variety of primers (Welsh et al., 1991; Smith et al., 1997; Cagigas et al., 1999; Bartish et al., 2000; Bernardi and Tally, 2000; Govindaraju and Jayasankar, 2004; McCormack et al., 2000; Lehmami et al., 2000; Kovacs et al., 2001; Callejas and Ochando, 1998, 2001 and 2002; Appleyard and Mather, 2000 and 2002).

### 5.2.3. The size and number of the RAPD-PCR products

The molecular weight of 124 RAPD-PCR fragments in *H. brachysoma* ranged from 150 to 3000 bp. Welsh et al. (1991) reported that the number and size of the fragments generated strictly depended upon the nucleotide sequence of the primer used upon the source of the template DNA, resulting in the genome-specific fingerprints of random DNA fragments. But, there was no correlation between the length of the primers and the number of amplified fragments generated in the present case as reported by Dong and Zhou (1998).

The number of amplified products may be related to the G+C content of the primer and template DNA sequence rather than to primer length (Caetano-Anolles et al., 1991). Dong and Zhou (1998) reported that primers with a higher G+C content
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generated more amplified products. The G+C content did not vary much in the primers selected for the present study, and hence the number of RAPD fragments also did not exhibit much variation with different Operon decamers.

5.2.4. Population specific RAPD markers

Using RAPD analysis, the present study observed eight population-specific bands in three natural populations of *H. brachysoma*. Among these, 6 specific markers were found in Nethravathi population and one each in Meenachil and Chalakkudy populations (Table-22). Population specific RAPD markers are reported also by Yoon and Kim (2001), in Korean catfish, *Silurus asotus*; Cagigas et al. (1999) in brown trout, *Salmo trutta*; Klinbunga et al. (2000b) in mud crabs; Govindaraju and Jaysankar, (2004) in seven species of groupers; and Barman et al. (2003) in Indian major carps. Kovacs et al. (2001) reported a special type of marker called 'SCAR' (sequence characterized amplified region) to distinguish male and female species of *Clarias gariepinus*. SCARs were also reported in tropical oyster (*Crassostrea belcheri*) in Thailand (Klinbunga et al., 2000a) to generate profiles at the intra-specific level. Such population-specific RAPD markers can be generated as genetic tags for *H. brachysoma* in the future that would be helpful in culture and selection programmes for the species.

5.2.5. Genetic differentiation and gene flow

A relatively high overall population genetic differentiation among river populations were obtained in *H. brachysoma* in the present study (*G_{ST} = 0.5060*). Gomes et al.(1998) reported a similar value of *Q_{ST}*, (an analogue of *G_{ST} or F_{ST}* (Excoffier et al., 1992)) i.e., 0.49 in the stock discrimination of four-wing flying fish, *Hirundichthys affinis*. However, Appleyard and Mather (2002) reported a much high value of *F_{DT}* (an analogue of *G_{ST} or F_{ST}* i.e., 0.652 to 0.670 for tilapia. But the *F_{ST}* value in population genetic studies of an asteroid with high dispersal capacity, *Acanthaster planci*, indicated low genetic differentiation between populations (*F_{ST} = 0.019 to 0.038*) as reported by Nash et al. (1988); Benzie and Stoddart (1992). Similarly, Silberman et al. (1994) suggested that a *Q_{ST}* value of 0.032 indicated little
evidence of genetic sub-division in the spiny lobster, *Panulirus argas*. D'Amato and Corach (1997) reported a very low $F_{ST}$ (0.0127) in freshwater crab, *Aegla jujuyana*. In *H. brachysoma*, the high $G_{ST}$ value suggests that there is little gene exchange between stocks at each site.

The overall gene flow ($Nm$) value was estimated as 0.4880 in the present study indicating restricted or no gene flow among populations of *H. brachysoma*. Similar value for $Nm$ was reported in several freshwater species (Khoo et al., 2002; Cagigas et al., 1999). But a relatively high (0.8-13.0) gene flow is reported in the populations of Brittle star, *Amphiura filiformis* (McCormack et al., 2000). The physical (geographical) barrier is the main reason to prevent the migration of populations and inter-breeding as in the case of *H. brachysoma* populations. Species with restricted gene flow generally show a greater tendency to differentiate into distinct populations (Govindaraju, 1989). The high $G_{ST}$ and low $Nm$ values in yellow catfish are justified by the above statement.

5.2.6. Genetic relationship between populations

Results of RAPD analysis indicate a more distant relationship between Meenachil and Nethravathi populations (genetic distance, $D = 0.2876$) of *H. brachysoma*. The populations between Meenachil and Chalakkudy are some what closely related ($D = 0.1347$). The genetic distance between Chalakkudy and Nethravathi population was 0.2113 (Table-24). The genetic distance values increased as the geographic distance increased. Klinbunga et al. (2000b) reported a similar value for genetic distance ($D = 0.171$ to 0.199) in the populations of mud crab, *Scylla serrata*. Similarly, D'Amato and Corach (1997) reported that the ‘$D$’ value ranges from 0.1755 to 0.215 in freshwater aromuran, *Aegla jujuyana*. Khoo et al. (2002) reported a similar result of genetic distance in guppy, *Poecilia reticulata*, population (0.085-0.249) and Gomes et al. (1998) in four-wing flying fish, *Hirundichthys affinis* (0.16 to 0.26). However, in red mullet, *Mullus barbatus*, a very low value of genetic distance ($D = 0.0024$ to 0.0366) was reported by Mamuris et al. (1998). Similarly, Saitoh (1998) reported a lower value of genetic distance ($D = 0.006$ to 0.018) in the populations of Pacific cod, *Gadus macrocephalus*, around Japan. In marine teleosts,
the genetic distance values appeared low compared to the freshwater counterparts (Govindaraju and Jayasankar, 2004).

Distinct clustering (dendrogram) of H. brachysoma (Fig-30) using RAPD analysis suggests that the populations are differentiated. The bootstrap values indicated the stocks have a robust cluster. Several authors have shown clear cut clustering in dendrograms based on RAPD estimates, demonstrating intra-specific variations in different species (Khoo et al., 2002; McCormack et al., 2000; Cagigas et al., 1999; Mamuris et al., 1998; Gomes et al., 1998); and inter-specific variations of same genus (Bardakci and Skibinski, 1994; Dinesh et al., 1996; Smith et al., 1996; Callejas and Ochando, 1998, 2001, 2002; Appleyard and Mather, 2002; Barman et al., 2003; Govindaraju and Jayasankar, 2004).

RAPD analysis is a rapid and convenient technique to generate useful information on stock structure of a species. Since the RAPD technique is less laborious compared to other fingerprinting methods; it produces results with low statistical error (Naish et al., 1995) and does not require prior knowledge of DNA sequences (Hadrys et al., 1992), it may be a promising method to estimate genetic affinities at nuclear level between populations of fish species. Consequently, depending on the level of identification required, a single primer or a combination of two can generate clear diagnostic profiles. The major drawbacks of RAPD markers that are dominant (i.e., it is not possible to determine if an individual in homozygote or heterozygote) at a locus and its reproducibility. Despite the apparent ease of the RAPD methodology, initial empirical optimizations for a given template primer combination can be time consuming. This is because of several parameters- such as quality of template DNA, components of amplification reaction, amplification conditions, primer sequence or the thermal cycler- which influence the quantity and size of the RAPD, and products generated have to be optimized (Micheli et al., 1994; Dinesh et al., 1995). Thus, one must be cautious about systematic conclusions based on RAPD analysis alone. On the other hand, the possible analysis with unlimited numbers of primers, each detecting variations at several region in the genome,
provides an advantage for RAPD analysis over other techniques (Appleyard and Mather, 2000).

The RAPD profiles in the present study displayed a high degree of polymorphism, which indicated a population structure for yellow catfish entirely consistent with that obtained from analysis of allozymes and microsatellites (in the coming pages) in the same fish. This confirms suitability of RAPD markers for discrimination of yellow catfish stocks. In brief, the study yielded highly reproducible RAPD fingerprints, which were used as reliable and useful tool for discrimination of population structure in *H. brachysoma* from three geographically separated river systems, viz. Meenachil, Chalakkudy and Nethravathi of the Western Ghat region.

5.3. Microsatellites

Several features of microsatellites render them invaluable for examining fish population structure. Microsatellites are co-dominant in nature and inherited in Mendelian fashion, revealing polymorphic amplification products helping in characterization of individuals in a population. High frequency of occurrence and uniformity of distribution within most eukaryotic genomes and high levels of variation have fostered a growing appreciation of their use in genome mapping, paternity and forensics (Gopalakrishnan and Mohindra, 2001). Because of their extremely high levels of polymorphism, they are widely used in stock structure studies in a number of species (Zardoya *et al*., 1996; O'Connell and Wright, 1997; Ferguson and Danzmann, 1998). In microsatellites the mutation rates are very high. The fast rates of microsatellite evolution are believed to be caused by replication slippage events (Zardoya *et al*., 1996). Two models of mutation generally proposed to account for variation at microsatellite loci are the stepwise mutation model (SMM) and the infinite allele mutation model (IAM) (Scribner *et al*., 1996). The SMM predicts mutation occurs through the gain or loss of a single repeat unit, e.g., GT. This means that some mutations will generate alleles already present in the population. In contrast, the IAM predicts that mutation can only lead to new allelic states and may involve any number of repeat units (Estoup *et al*., 1995; O'Connell *et al*., 1997).
Discussion

Many microsatellite loci despite their extremely fast rates of repeats evolution are quite conservative in their flanking regions and hence can persist for long evolutionary time spans much unchanged. Due to this, primers developed for a species from the flanking regions of a microsatellite locus can be used to amplify the same locus in other related species. Generally, the development of new species-specific microsatellite primers is expensive and time consuming, but the above mentioned alternative attractive option is cheap and fast. Primers developed for a species by this method have been successfully tested for cross-species amplification on its related species in several fish species (Zardoya et al., 1996; Scribner et al., 1996; Galbusera et al., 2000; Lal et al., 2004b; Gopalakrishnan et al., 2004a; Mohindra et al., 2004). It is possible to obtain a useful set of markers without developing specific primers for each study species (Galbusera et al., 2000).

In the present study, altogether 25 primer pairs developed for four fish species (resource species) belonging to the orders Siluriformes and Osteoglossiformes viz., *Pangasius hypophthalmus*, *Clarias macrocephalus*, *Clarias gariepinus* and *Scleropages formosus* were evaluated for cross-species amplification of microsatellite loci in *Horabagrus brachysoma*. Successful cross-priming was obtained with 8 primer pairs and all the 8 loci were polymorphic and ideal to be used as markers in stock identification studies. However, the optimum annealing temperature to get scorable band in *H. brachysoma* slightly differed from that reported for the respective primer pair in the resource species. Zardoya et al. (1996) and Galbusera et al. (2000) also reported necessity of optimization of PCR conditions for the study species in cross-amplification tests. Cross-species amplification of primers of the order Siluriformes and Osteoglossiformes in *H. brachysoma* shows the evidence of remarkable evolutionary conservation of microsatellite flanking regions (MFRs). Similar results are reported in other fishes (Mohindra et al., 2001a, b; 2002 a, b, c; Lal et al., 2002; Das and Barat, 2002 a, b, c; Gopalakrishnan et al., 2002; 2004a). Zardoya et al. (1996) also reported that homologous microsatellite locus could persist for about 300 million years in turtle and fish and their flanking regions are highly conserved. The successful cross-species amplification of primers of other species in *H. brachysoma* supports this view.
The present study demonstrated successful cross priming of microsatellite loci, between the fish species that are distant or not related. Certain sequences flanking the tandem repeats could be conserved between the different families of order Siluriformes as reported in other fishes by Scribner et al. (1996) and Zardoya et al. (1996). Interestingly, some microsatellite sequences from the primitive order Osteoglossiformes have also remained conserved in this species (order Siluriformes) of relatively later evolutionary origin. Schlotterer et al. (1991) also reported that homologous loci could be amplified from a diverse range of toothed (sub order Odontoceti) and baleen (sub order Mysticeti) whales with estimated divergence times of 35-40 million years. Similarly, microsatellites isolated in domestic dogs were used in studies of a variety of canid species (Gotelli et al., 1994; Roy et al., 1994). Moore et al. (1991) also found microsatellites were conserved across species as diverse as primates, artiodactyls and rodents. All these results indicate the highly conserved nature of some microsatellite flanking regions even across orders in different taxa and they can persist for long evolutionary time spans much unchanged. The use of heterologous PCR primers would significantly reduce the cost of developing similar set of markers for other siluriform species found in India.

Scribner et al. (1996) reported, the degree of homology of microsatellite primers has some degree of phylogenetic basis in that a greater number of primer pairs produce amplification products within the genus or family or order, compared with that of more distantly related taxa. In the present study also, even though 10 primer pairs from Osteoglossiformes were tried for cross-species amplification, only two primer pairs (D-33 and D-38) were found to be homologous in H. brachysoma. Galbusera et al. (1996) also noted remarkable differences in amplification success between microsatellite primer sets in genera of birds as in the present study. Differences between markers in evolutionary rates of change owing to heterogeneity in sequence organization and rates of mutation and fixation might explain this variation between primer sets (Galbusera et al., 1996). The remarkable conservation of loci of Siluriformes and Osteoglossiformes primers would be helpful even to document the evolution of microsatellites contained in these loci and to generate
Discussion

phylogenetic relationships across different species of these orders, in addition to their application as potential markers in stock identification of *H. brachysoma*.

5.3.1. Type and relative frequency of microsatellite arrays observed

In *H. brachysoma*, 13 amplified presumptive microsatellite loci were cloned and sequenced and among these, 8 loci were confirmed to contain microsatellites. 37.5% of the microsatellites sequenced were perfect and the figure falls within the range reported by Weber (1990) and Zardoya *et al.* (1996). The average length (= average number of repeats) of the microsatellite loci sequenced was 20.375 in *H. brachysoma*. This is slightly lower than previous estimates for other catfishes (Na-Nakorn *et al.*, 1999; Watanabe *et al.*, 2001; Krieg *et al.*, 1999) but higher than that of Malaysian bagrid *Mystus nemurus* (~ 12 repeats) (Usmani *et al.*, 2001).

The tandem repeats of 37.5% of the microsatellite loci observed in the present study are comparable to that of the resource species. The GT and CT repeats (*Phy01* and *Phy05* primers) of the resource species, *Pangasius hypophthalmus* are exactly similar in *H. brachysoma*, though the numbers of repeats varied. Similarly, the CA repeat of the microsatellite locus, D-33-2 (resource species, *Scleropages formosus*) was exactly same in *H. brachysoma*. But, the type of repeat motif in the resource species and *H. brachysoma* differed in loci *Phy07-1*, *Cma3*, *Cma4-2* and D-38-1. This can be due to the extremely fast rates of repeat evolution that may differ among loci, but keeping the highly conservative flanking regions unchanged, as reported by Zardoya *et al.* (1996) in cichlids and other perciform fishes. The study found GT and CA rich microsatellites abundant in *H. brachysoma* which is in conformity with the published reports (Na-Nakorn *et al.*, 1999; Krieg *et al.*, 1999; Neff and Gross, 2001; Watanabe *et al.*, 2001; Usmani *et al.*, 2001). The types of dinucleotide microsatellite arrays observed in *H. brachysoma* are similar to the ones from salmonids (O'Connell *et al.*, 1997; Estoup *et al.*, 1993; Sakamoto *et al.*, 1994; McConnell *et al.*, 1995). Generally, most of dinucleotide alleles are always visible as a ladder of bands rather than a single discrete product due to slipped-strand mispairing during PCR (Weber, 1990). This was not the case with the primers used in the present study, which always gave clear and discrete bands.
5.3.2. Genetic variability and Hardy-Weinberg Equilibrium

The number of alleles at different microsatellite loci in *H. brachysoma* varied from 3 to 7 with an average value of 5. Primers Phy 01 and *Cma3* exhibited maximum allele number (7) compared to other primers (three to five alleles). High microsatellite allele variation was recorded in Thai silver barb (*Puntius gonionotus*) in four microsatellite loci with average of 13.8 alleles per locus (Kamonrat, 1996); and in a number of marine fishes such as whiting (14-23 alleles/locus) (Rico et al., 1997); red sea bream (16-32 alleles/locus) (Takagi et al., 1999) and Atlantic cod (8-46 alleles/locus) (Bentzen et al., 1996). Relative low variation was observed among microsatellite loci of brown trout (5-6 alleles /locus) (Estoup et al., 1993), northern pike (3-5 alleles/locus) (Miller and Kapuscinski, 1996) and sea bass (4-11 alleles/locus) (Garcia De Leon et al., 1995). Neff and Gross (2001) reported mean number of alleles at different microsatellite loci of 27 species of marine and freshwater fin fishes as $13.7 \pm 9.1$ for an average allele length of $23.0 \pm 6.0$. They also reported a positive linear relationship between microsatellite length and number of alleles across all classes and within classes. Low values for mean number of alleles were recorded for many fish species such as African catfish (7.7; Galbusera et al., 1996); Atlantic salmon (6.0; McConnell et al., 1995); Chinook salmon (3.4; Angers et al., 1995) and northern pike (2.2; Miller and Kapuscinski, 1996) as in the present study. One reason for the low level of allele variation is probably the small sample size (Galburusa et al., 1996). Ruzzante (1998) suggested that a population size for microsatellite loci study be atleast 50 individuals per population and in *H. brachysoma*, 70 specimens were used for microsatellite analysis from each population. DeWoody and Avise (2000) and Neff and Gross (2001) showed that marine species have greater microsatellite allele variation as compared with freshwater species and that, this was consistent with the increased evolutionary effective population sizes of marine species. They also reported that much of the variation in polymorphism at microsatellite loci that exist between species and classes can be attributed to differences in population biology and life history and to a lesser extent to differences in natural selection pertaining to the function of the microsatellite loci. Fewer number of alleles in the microsatellite loci of *H. brachysoma* (which is
primarily a freshwater fish), can be due to differences in biology and life history traits compared to that of the marine species with higher number of microsatellite allele variation as suggested by Neff and Gross (2001) in other fishes.

In the present study, variations of allele sizes were quite low for Phy07-1, Cma4-2, D33-2 and D38-1 loci which might be due to their small number of repeat units and similar level of allele size variation is reported in selected freshwater teleosts and higher vertebrates (Carvalho and Hauser, 1994). The same trend of relationship was observed in Clarias macrocephalus (Na-Nakorn et al., 1999) and Thai silver barb (Kamonrat, 1996). However, Cma3, which has 18 repeat units, in the present study, exhibited much more allelic variation compared to Phy05, which had 25 repeat units.

The mean observed number of alleles at each locus (na = 5.00) in yellow catfish was higher than that observed by Watanabe et al. (2001) in other bagrid catfish, Pseudobagrus ichikawai (na = 4.75); in Mystus nemurus (na =3.2) (Usmani et al., 2003) and in siluroid catfish, Ictalurus punctatus (na = 3.9) (Tan et al., 1999). Volckaert et al. (1999) reported a lower value of mean observed number of alleles in Pangasius hypophthalmus. Similar results also reported by Han et al. (2000) in striped bass (Morone saxatilis) and Scribner et al. (1996) in Chinook salmon (Oncorhynchus tshawytscha) and many other teleosts (Reilly and Ward, 1998; McGowan and Reith, 1999; Supungul et al., 2000; Iyengar et al., 2000). However, a higher value of na was reported by Na-Nakorn et al. (1999) in Clarias macrocephalus (na = 12.0) and Volckaert et al. (1999) in Clarias batrachus (na = 5.8).

In H. brachysoma, the mean observed heterozygosity (H_{obs}) per locus per population was 0.4720 and the mean expected heterozygosity (H_{exp}) per locus per population was 0.6486. Usmani et al. (2003) in Mystus nemurus reported a similar value of mean observed heterozygosity (H_{obs} = 0.4986), however, the mean expected heterozygosity was lower than that of present study. In H. brachysoma, a significant overall deficiency of heterozygotes was revealed in all the populations with exception in some loci (D38-1 locus in Meenachil, Phy01, D33-2 and D38-1 loci in Chalakkudy and Phy01, Phy05, D33-2 and D38-1 in Nethravathi). In Clarias macrocephalus, Na-
Nakorn et al. (1999) reported the deficiency of heterozygotes (Hob = 0.67 and Hex = 0.76). But, Watanabe et al. (2001) and Usmani et al. (2003) reported the significant excess of heterozygotes in other bagrid catfishes, *Pseudobagrus ichikawai* (Hob = 0.54 and Hex = 0.56) and *Mystus nemurus* (Hob = 0.4986 and Hex = 0.4817) respectively and in silurid catfish, *Silurus glanis* (Hob = 0.677 and Hex = 0.608) Krieg et al. (1999). Small sample size can be a reason for inability to detect all the alleles in the population and heterozygote deficiency (Na-Nakorn et al., 1999). But the sample size of 70 for each population of *H. brachysoma* for microsatellite study is not small according to Ruzzante (1998), hence, this hypothesis is not convincing in the present case. Inbreeding and non-random mating would also result in heterozygote deficit (Donnelly et al., 1999). The positive value of FIS at almost all the loci indicated inbreeding in populations of *H. brachysoma*. Seven of the eight-microsatellite loci (except D38-1) showed significant deviations (P<0.05) from Hardy-Weinberg Equilibrium (HWE). Deviations from HWE is usually attributed to null alleles (Gopalakrishnan et al., 2004a; Garcia de Leon et al., 1995), selection (Garcia de Leon et al., 1995), or grouping of gene pools (Walhund effect) (Gibbs et al., 1997) or inbreeding or non-random mating (Beaumont and Hoare, 2003). Over-exploitation leading to drastic decline of the yellow catfish has been recorded in rivers of Kerala since 1997 and the species now categorized as endangered as per latest IUCN norms (Anon, 1998; Gopalakrishnan and Ponniah, 2000). Due to this, inbreeding can happen, which might result in deficiency of heterozygotes and deviation from HWE (Beaumont and Hoare, 2003). The microsatellite analysis agrees with the allozyme results of the present study. Similar situation was reported in other fishes that showed decline in catches due to over-exploitation (Rico et al., 1997; O'Connell et al., 1998; Beacham and Dempson, 1998; Scribner et al., 1997; Yue et al., 2000; Gopalakrishnan et al., 2004a).

There were no significant associations indicative of linkage disequilibrium between any pair wise combination of microsatellite alleles in *H. brachysoma* (after Bonferroni correction). It is therefore assumed that the allelic variation recorded at all the microsatellite loci could be independent as observed in many fishes (Na-Nakorn et al., 1999; Scribner et al., 1996; Usmani et al., 2003).
5.3.3. Null alleles

Presence of null alleles could be one of the possible factors responsible for the observed heterozygote deficiency. Null alleles are alleles that do not amplify during PCR because of mutation events changing the DNA sequence in one of the primer sites (mostly in 3'end), which causes the primer no longer to anneal to the template DNA during the PCR (Van Oosterhout et al., 2004, 2006). This may prevent certain alleles from being amplified efficiently by PCR (Paetkau and Strobeck, 1995). This results in either no PCR product, if null allele is homozygote or in false homozygote individuals, if the locus is a heterozygote. This will show apparent significant deviations from Hardy-Weinberg equilibrium and non-Mendelian inheritance of alleles (Donnelly et al., 1999). An excess of homozygote individuals as found in different populations of yellow catfish in the present study could be due to null alleles or by a real biological phenomenon. But, the analysis of data using MICRO-CHECKER indicated, occurrence of null alleles in all the 3 populations is very unlikely for the 7 primer pairs. This was supported by the absence of general excess of homozygotes over most of the allele size classes in MICRO-CHECKER analysis. In yellow catfish, significant departures from HWE were found within samples across loci rather than within loci and across most samples. Such a situation is not consistent with null alleles (Van Oosterhout et al., 2004). Also, there was no instance of non-amplifying samples in repeated trials with any of the primer pairs in *H. brachysoma*. Van Oosterhout et al. (2004) suggested that in such a situation, the overall homozygosity can be due to deviations from panmixia, inbreeding, short allele dominance, stuttering or large allele drop-outs. Short allele dominance occurs when excess of homozygotes is biased towards either extreme of the allele size - distribution and when there is a general homozygote excess and the allelic range exceeds 150 base pairs (Van Oosterhout et al., 2004). In the present study such conditions did not exist; hence, chances of short allele dominance could be ruled out. Stutter bands were practically absent in the present study, hence the possibility of changes in allele sizes due to stuttering can also be rules out. Large alleles (allelic size range exceeding 150 base pairs) normally do not amplify as efficient as small alleles, leading to large allele dropouts (Van Oosterhout et al., 2004). In the present investigation, all the amplified products were dinucleotide repeats and allele sizes ranged between 4-58 base pairs in
different loci and generally large alleles were not encountered. Hence, in the present study, the possible causes for excess of homozygosiy can be speculated as over-exploitation of the species over the years leading to reduction in catches ending with inbreeding as reported by CAMP (Anon., 1998) and as revealed from the investigator's constant interaction with the fishermen, local people and aquarium traders during the study period.

5.3.4. Stock-specific markers

Two microsatellite alleles (D33-2-192 and D38-1-310) found in Chalakkudy samples were not found in Meenachil and Nethravathi and hence they were treated as private alleles. The detection of significant alleles in Chalakkudy population is the evidence for no mixing of the gene pools between the populations. In Clarias macrocephalus, Na-Nakorn et al. (1999) reported twenty stock-specific markers in three loci in four populations in Thailand. Scribner et al. (1996) reported 22 stock specific alleles in three populations of Chinook salmon (Oncorhynchus tshawytscha) in Canada. Takagi et al. (1999) reported the stock specific markers in the populations of tuna species of the genus Thunnus. Coughlan et al. (1998) also reported the 5 stock specific alleles in the populations of turbot (Scophthalmus maximus) from Ireland and Norway. The stock specific microsatellite markers (private alleles) can be used as genetic tags for selection programs (Appleyard and Mather, 2000) and to distinguish the stocks for selective breeding programmes.

5.3.5. Genetic differentiation and Gene flow

Pair-wise comparison between different riverine locations for microsatellite allelic homogeneity in H. brachysoma yielded significant deviations at all loci in their frequencies after significant levels were adjusted for Bonferroni correction. The results are in agreement with that of allozyme markers in the present study and this suggests partitioning of breeding population, limitation in migration between different areas and existence of distinct stock structure among populations. The overall $F_{ST}$ value (0.1055) of microsatellite loci in H. brachysoma was significantly different from zero ($P < 0.0001$). This indicates a significant level of genetic differentiation among
the populations. The higher rates of mutation (and therefore polymorphism) of DNA markers result in greater power for population differentiation (Rousset and Raymond, 1995; Goudet et al., 1996). Levels of genetic differentiation demonstrated here for yellow catfish (overall $F_{ST} = 0.1055$) are comparable to those significant values seen in Pacific herring ($F_{ST} = 0.023$), Atlantic herring ($F_{ST} = 0.035$) and widespread anadromous fish like Atlantic salmon ($F_{ST} = 0.054$) (McConnell et al., 1997). The genetic relatedness of *H. brachysoma* populations derived from microsatellite loci, using pair-wise $F_{ST}$ between populations also differed significantly ($P<0.0001$) from zero for all the pairs of riverine locations indicating significant heterogeneity between populations. The allozyme markers used in the present study also gave the same trend.

The value of $N_\text{m}$ (1.5386) was derived from $F_{ST}$. The calculation of gene flow from $F_{ST}$ statistics assumes that migration occurs at random among all populations. In the present study, the $N_\text{m}$ value indicated chances of restricted migration between populations and that genetic differentiation over time might have occurred due to genetic drift. $N_\text{m} > 4$ suggests that the gene flow between the populations is adequate to counteract the effects of genetic drift in local populations (Donnelly et al., 1999; Kang and Chung, 1997). Many authors reported a higher value of $N_\text{m}$ in various fish and shellfish species (Taylor et al., 2001; in cichlids, $N_\text{m} = 3.36$) and in *Anopheles arabiensis* ($N_\text{m} = 3.37$, Donnelly et al., 1999). However, low values of $N_\text{m}$ have been reported in species exhibiting significant genetic differences and geographic isolation among populations as in the present study (Angers et al., 1995; Coughlan et al., 1998).

### 5.3.6. Genetic relationships among populations

The genetic relationships among populations would be explained largely through the geographic distance between sampling locations. The two populations, Meenachil and Chalakkudy always clustered more closely than the Nethravathi population as revealed in allozymes and RAPD analysis using the same samples. The Chalakkudy population was always intermediate in position between Meenachil and Nethravathi populations and their genetic distances calculated from microsatellite data.
agreed with geographic distance. The UPGMA dendrogram of *H. brachysoma* using microsatellite data also indicated similar topology as observed with allozymes and RAPD markers of this species.

'Homoplasy'—similarity of traits/genes for reasons other than co-ancestry (e.g. convergent evolution, parallelism, evolutionary reversals, horizontal gene transfer and gene duplication) - in molecular evolution has recently attracted the attention of population geneticists, as a consequence of the popularity of microsatellite markers. Homoplasy occurring at microsatellite is referred to as 'size homoplasy' (SH), i.e., electromorphs of microsatellites are identical by in state (i.e., have identical size), but are not necessarily identical by descent due to convergent mutation(s). It violates a basic assumption of the analysis of genetic markers - variance of similar phenotypes (e.g. base pair size) are assumed to derive from a common ancestry. Estoup *et al.* (2002) and Donnelly *et al.* (1999) reported homoplasy may affect $F_{ST}$ estimates of especially for markers with high mutation rates (microsatellites). Although a fraction of SH can be detected using analytical developments and computer simulations or through single strand confirmation polymorphism (SSCP) and sequencing; to evaluate empirically the potential effect of SH on population genetic analyses, an in-depth study with large number of loci, individuals and electromorphs (using SSCP/sequencing) is required. However, Estoup *et al.* (2002) in their review article made a major conclusion that SH does not represent a significant problem for many types of population genetic analyses and large amount of variability at microsatellite loci often compensates for their homoplasious evolution. Further studies will permit detection of homoplasious electromorphs and their effect on $F_{ST}$ and genetic relatedness among populations of *H. brachysoma*.

In conclusion, the analysis using novel hypervariable microsatellite loci in *Horabagrus brachysoma* revealed significant results: First, the potential use of heterologous PCR primers was explored and many of them appeared to be conserved in this bagrid (order: Siluriformes) catfish. Second, the utility of these markers for population genetic analyses was confirmed. All the eight amplified microsatellite loci were polymorphic and showed heterogeneity in allele frequency in yellow catfish populations between different river systems. Third, the study suggested that the three
natural populations of this species viz., Meenachil, Chalakkudy and Nethravathi that are divergent in their genetic characteristics can be identified through microsatellite loci. The information generated will be helpful to plan strategy for rehabilitation of declining stocks of *H. brachysoma* in these rivers. Finally, the results of the population screening using microsatellites agreed with those from allozyme and RAPD studies of the same populations, suggesting their wide utility for a variety of basic and applied research questions.

**5.4. Comparative analysis of results with three markers in *H. brachysoma***

Allozyme, RAPD and microsatellite markers could be considered as random indicators to discriminate the three populations of the yellow catfish, *H. brachysoma*. Therefore, it would be of interest to compare the results obtained from the application of these three approaches to the same individuals. To date only few studies have compared the results of allozymes with RAPD and microsatellites (Cagigas *et al.*, 1999; Colihuque *et al.*, 2003). All the three methods were successful in revealing a genetic heterogeneity between populations and producing stock-specific markers that could discriminate three populations. Although it was possible to gain a clear understanding of population structure using allozyme data alone, the use of more variable markers such as microsatellites and RAPDs could further confirm the analysis using allozymes. These DNA techniques involved the examination of putative non-coding genes thought to be neutral, which permits high rates of mutation and lead not only to different alleles at each locus but also to an increase in the amount of genetic variation (Cagigas *et al.*, 1999). The sampling for microsatellites and RAPD is usually non-lethal or minimum invasive unlike in allozyme that requires killing of specimens.

The percentage of polymorphism obtained using these three markers varied in *H. brachysoma*. Several factors contribute to the differences observed in the results produced by the three methods. Some are due to the dominant nature of RAPD. Therefore, gene frequency estimates or effective number of alleles calculated from RAPD data can vary from those obtained from co-dominant markers such as allozymes and microsatellites (Lynch and Milligan, 1994). In allozymes, only 56% of
loci were polymorphic, which was less compared with RAPD and microsatellites (75.08% and 100% respectively). This result can be explained by the fact that the mutation rate of allozymes is much lesser compared with that of the other two markers (Colihuque et al., 2003). Most of the allozymes are encoded by single copy regions of the genome, having a serious impact on important phenotypic characters and thus by being more easily subject to selective pressure (Mamuris et al., 1998). On the other hand, the RAPD technique, by its nature apart from single copy fractions, also amplifies DNA from highly repetitive regions (Williams et al., 1990) while microsatellite amplifies repetitive regions with help of specific primers. It is therefore probable that most of the RAPD and microsatellite markers are amplified products of less functional parts of the genome, which do not strongly respond to selection on the phenotypic level. Such DNA regions may accumulate more nucleotide mutations compared to those encoding allozymes. Thus, RAPD and microsatellite could detect more pronounced genetic polymorphism among geographically distant *H. brachysoma* samples than allozyme markers.

Compared with allozymes and RAPD, microsatellites exhibited a large number of alleles in *H. brachysoma* (40 alleles in 8 polymorphic microsatellite loci; 33 alleles in 14 polymorphic allozyme loci). In allozymes, some of the changes in DNA sequences are masked at protein level reducing the level of detectable allelic variation. Some changes in nucleotide sequence do not change the encoded polypeptide (silent substitutions) and some polypeptide changes do not alter the mobility of the proteins in an electrophoretic gel (synchronous substitutions), hence relatively low number of alleles (usually 2 to 3) are exhibited by most of the allozyme loci (Liu and Cordes, 2004). In RAPD, the primer can detect and amplify several regions in the whole genome of the species and the changes in one or two base pairs cannot be detected as separate locus/allele due to misinterpretation of the size of the bands in the gel (due to less resolution power of agarose gel). Furthermore, the substitution of the base pair does not change the size of the product. Whereas in microsatellites, the change in one or two base pairs can be detected as separate alleles in the gel (high percentage of polyacrylamide gel to resolve very small product was used in the present study) and addition, deletion or substitution of base pair may shuffle the tandem repeats. This is the main reason for occurrence of more alleles.
with microsatellite technique. The mean observed number alleles (Na) varied accordingly with both markers (5.00 and 2.3571 in microsatellites and allozymes respectively).

In the present study, the heterozygosity value was higher for microsatellites (H_{obs} = 0.4720) than the allozymes (H_{obs} = 0.1779). RAPD showed in between the value of average gene diversity or heterozygosity (H), i.e., 0.2222. All three markers expressed a deficiency of heterozygotes (except in one or two loci). Similar patterns of results using three markers was reported by Cagigas et al. (1999) in the populations of brown trout and using microsatellites and RAPD in common carp (Bartfai et al., 2003) and with allozymes and RAPD markers in red mullet (Mamuris et al., 1998).

The coefficient of genetic differentiation (F_{ST}) and gene flow (Nm) varied with each marker in H. brachysoma. The overall F_{ST} was high for RAPD (here G_{ST} = 0.5060) than allozymes (0.1537) and microsatellites (0.1055). This suggests that RAPD analysis has a greater resolving power than other markers. Smith et al. (1996) reported similar results in tarakihi (Nemadactylus macropterus) from New Zealand waters. Similar levels of F_{ST} levels from allozyme and microsatellite markers in this study suggests that both sets of allele frequency distributions represent neutral markers in yellow catfish. A similar concordance of polymorphic allozyme and molecular markers was observed in studies with brown trout (Cagigas et al., 1999); blue marlin (Buonaccorsi et al., 1999); red mullet (Mamuris et al., 1998) and chum salmon (Scribner et al., 1998). Genetic distance values between populations using this battery of markers showed similar pattern in H. brachysoma. Irrespective of the markers used, the topologies of the dendrogram also exhibited similar pattern of genetic divergence in the present study, indicating population structure of this species is entirely consistent with all the 3 markers. A similar pattern of UPGMA dendrogram using three markers was found in many organisms (Patwary et al., 1993; Cagigas et al., 1999; Von Soosten et al., 1998).

The three methods in the present study probably might have generated markers pertaining to different parts of yellow catfish genome. Similarity in genetic divergence values with all the 3 markers indicates the robustness of the techniques
applied; this reinforces reliability of interpretations and confirms existence of three genetically discrete stocks of yellow catfish. Although the three techniques could clearly discriminate the populations, microsatellite as a basic genetic tool overcome some of the disadvantages displayed by the other two. First, because specific primer development for a particular species can be both time-consuming and costly, primers developed in one species can be used to amplify homologous loci in closely related species (Scribner et al., 1996; Presa and Guyomard, 1996). Second, many microsatellite loci are thought to be neutral (Zardoya et al., 1996) but some allozyme loci may be influenced by selection pressure, allowing only a few alleles at each locus (Allendorf et al., 1987; Verspoor and Jordan, 1989; Mamuris et al., 1998).

Furthermore, because yellow catfish populations are under endangered category, killing specimens to collect liver and muscle for allozyme analysis becomes a significant inconvenience (fin clips and body slime may not give satisfactory results for all allozymes), which makes it advisable to adopt other techniques. Transportation of tissue samples from remote areas in liquid nitrogen (availability of liquid nitrogen in remote areas is often difficult in India) and their subsequent storage in -85°C freezer until further analysis are other disadvantages associated with allozyme analysis. The RAPD methodology also involves some disadvantages compared with microsatellites. The dominant character of RAPDs makes it impossible to distinguish between homozygote and heterozygote of a particular fragment, and the comparison of bands across different gels often makes data scoring more difficult. Although reproducibility both within and among laboratories has been proved for RAPD polymorphisms (Penner et al., 1993; Dinesh et al., 1995; also in the present study) some confusion still exists regarding its application in population genetics especially of endangered species (basic assumption in RAPD analysis is, the populations fit the Hardy-Weinberg equilibrium). The apparent disadvantages of the allozyme and RAPD techniques further enhance the utility of microsatellites for the analysis of population genetic problems. However, microsatellites are not free from short comings. Non-specific amplification, presence of stutter bands and very high level of polymorphism demanding large sample sizes (to adequately characterize the genetic variation both within and among populations, to ensure that apparent differences among populations are not due to sampling error) are often encountered with
microsatellites, complicating the genotyping and analysis. But in the present study, the number of alleles per locus was relatively less compared to other teleosts (Na-Nakorn et al., 1999). Also, the PCR conditions were optimized to overcome the problem of stutter bands and non-specific amplification in yellow catfish.

Finally, the present findings of genetic divergence levels with 3 marker types in *H. brachysoma* suggest that the populations of Meenachil, Chalakkudy and Nethravathi are not drawn from the same randomly mating gene pool. This observation and the identification of unique stock-specific markers (private alleles) are significant steps towards realizing the goal of stock-based management and conservation of yellow catfish resource in the Western Ghats. The result strengthens the observation made in CAMP workshop (Anon., 1998), regarding the need for conservation of this species and gives a signal that the populations exhibit signs of genetic bottleneck (as evidenced from the deficiency of heterozygote and deviation from Hardy-Weinberg Equilibrium). The study emphasizes the need for stock wise management of natural population of yellow catfish. The stock-wise propagation-assisted rehabilitation should involve brood stock of three rivers (Meenachil, Chalakkudy and Nethravathi) maintained separately. The hatchery-bred progeny will have to be released in three rivers without any chance of mixing of the stocks. The microsatellite markers and mtDNA analyses will further help in monitoring the rehabilitation programme.